Christine Dorresteyn Stevens
Linda E. Miller

Clinical Immunology and Serology A Laboratory Perspective

FOURTH EDITION

Clinical Immunology and Serology

A Laboratory Perspective

Fourth Edition

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A Laboratory Perspective

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F.A. Davis Company • Philadelphia

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1915 Arch Street
Philadelphia, PA 19103
www.fadavis.com

Senior Acquisitions Editor: Christa Fratantoro
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Library of Congress Cataloging-in-Publication Data

Names: Stevens, Christine Dorresteyn, author. | Miller, Linda E., author.
Title: Clinical immunology & serology : a laboratory perspective / Christine Dorresteyn Stevens and Linda E. Miller.
Other titles: Clinical immunology and serology
Description: Fourth edition. | Philadelphia : F.A. Davis Company, [2017] | Includes bibliographical references and index.
Identifiers: LCCN 2016023350 | ISBN 9780803644663
Subjects: | MESH: Immunity—physiology | Immune System Diseases—diagnosis | Immunologic Techniques | Immunologic Tests | Serologic Tests
Classification: LCC RB46.5 | NLM QW 540 | DDC 616.07/56—dc23 LC record available at https://lccn.loc.gov/2016023350

To my wonderful family: Eric, Kathy, Hannah, and Matthew, and Kevin, Melissa, Turner, and Avery for their love and encouragement.
— C.D.S.

To my wonderful family, for their love and support; to the Clinical Laboratory Science faculty and the Clinical Immunology laboratory staff at SUNY Upstate Medical University, in appreciation of their expertise and collegiality; and especially to my students, who have inspired me to share my passion for immunology over the years.
— L.E.M.

Preface

The fourth edition of *Clinical Immunology and Serology: A Laboratory Perspective* is built on the success of the first three editions. This text is tailored to meet the needs of clinical laboratory students on both the 2- and 4-year levels. It combines practical information about laboratory testing with a concise discussion of the theory behind the testing. For practicing laboratorians and other allied health professionals, the book may serve as a valuable reference about new developments in the field of immunology.

The organization of the chapters is based on the experience of many years of teaching immunology to clinical laboratory science students. The book is divided into four major sections: I. Nature of the Immune System; II. Basic Immunologic Procedures; III. Immune Disorders; and IV. Serological and Molecular Diagnosis of Infectious Disease. Sections build upon one another, and chapters relate previous material to new material by means of boxes titled Connections and Clinical Correlations. These new features help the students to recall information from previous chapters and to bridge theory with actual clinical diagnosis and testing. Information in the chapters is related to real world events in order to make it more interesting for the student and to show the important role that immunology plays in people's daily lives. New to this edition are the Study Guide Tables at the end of many of the chapters, which can be used as study tools by the students.

All chapters have been updated to include new information about the immune system as well as new treatments for immunologic diseases. With this edition comes added emphasis on the basic immune mechanisms. Three new chapters have been added: Innate Immunity (Chapter 3), Adaptive Immunity (Chapter 4), and Immunization and Vaccines (Chapter 25). These chapters have been added in response to comments from reviewers and readers, as well as the burgeoning information in these areas. The chapter on autoimmunity (Chapter 15) has been expanded to include some diseases that are increasing in importance. Additionally, Molecular Diagnostic Techniques (Chapter 12) and Tumor Immunology (Chapter 17) have been expanded to help bring readers up-to-date on new developments in the field. Information on quality assessment, regulatory issues, and quality management systems has been added to the chapter on laboratory safety. Perhaps the most exciting new change, however, is the addition of full color illustrations. Not only does this increase the visual appeal of the book, but full color is helpful to students in promoting a better understanding of principles and techniques discussed in the chapters.

The book remains a practical introduction to the field of clinical immunology that combines essential theoretical principles with serological techniques commonly used in the clinical laboratory. The theory is comprehensive but concise, and the emphasis is on direct application to the clinical laboratory. The text is readable and user-friendly, with learning outcomes, chapter outlines, and a glossary of all key terms. Each chapter is a complete learning module that contains theoretical principles, illustrations, definitions of relevant terminology, and questions and case studies that help to evaluate learning. For the instructor, there are many new online resources at Davis*Plus* to assist in course development. These resources include PowerPoint slides, suggested laboratory exercises, additional case studies, and a large bank of test questions that can be used for review or test preparation. Because the field of immunology is expanding so rapidly, the challenge in writing this book has been to ensure adequate coverage but to keep it on an introductory level. Every chapter has been revised to include current practices as of the time of writing. It is hoped that this book will kindle an interest in both students and laboratory professionals in this exciting and dynamic field.

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Acknowledgments

We are grateful for the assistance we received from a number of sources during the preparation of this fourth edition. I am delighted to have had the help of Linda Miller, my co-editor for this edition. Her input and sharing of ideas has been invaluable. Our chapter contributors, whose expertise enriched this book, are Thomas Alexander, Lela Buckingham, Marjorie Di Lorenzo, Uthayashanker Ezekiel, Ashley Frazer-Able, Candace Golightly, Marc Golightly, Jeannie Guglielmo, Rita Heuertz, Donald Lehman, John Schmitz, and James Vossler. A special thank you to Joe Cannon, who changed our ideas into workable full color illustrations for this edition. We would also like to thank the reviewers whose additional sets of eyes and thoughtful suggestions helped to strengthen the chapters.

Finally, we would like to acknowledge all the folks at F.A. Davis for their hard work in making this fourth edition a reality. To the Senior Acquisitions Editor for Health Professions, Christa Fratantoro, you were a gentle taskmaster who kept us going, and you were a wonderful listener and problem solver when we needed help. We also appreciate the efforts of Dean DeChambeau, our developmental editor, whose thoroughness and promptness were invaluable in keeping us on track. Thanks to George Lang and all the others behind the scenes who helped this book come to life.

Our immunology students—past, present, and future—are the reason for writing this book. We hope that this text will help make a very complex subject a little easier to understand.

A thank you to friends, especially Bayard, for their encouragement and interest.

Finally, to my two wonderful sons Eric and Kevin, their wives Kathy and Melissa, and my grandchildren, Hannah, Turner, Matthew, and Avery, you are my inspiration and my source of strength.

Contents

SECTION IV

1

Nature of the Immune System

1 Introduction to Immunity and the Immune System

Christine Dorresteyn Stevens, EdD, MT(ASCP)

LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Discuss how immunology as a science began with the study of immunity.
2. Describe what is meant by an attenuated vaccine.
3. Explain how the controversy over humoral versus cellular immunity contributed to expanding knowledge in the field of immunology.
4. Distinguish innate from adaptive immunity.
5. Describe the types of white blood cells (WBCs) capable of phagocytosis.
6. Explain the role of tissue cells in immunity.
7. Discuss how natural killer (NK) cells differ from T lymphocytes.
8. Identify the two primary lymphoid organs and discuss the main functions of each.
9. List four secondary lymphoid organs and discuss their overall importance to immunity.
10. Describe the function and architecture of a lymph node.
11. Compare a primary and a secondary follicle.
12. Explain the makeup of a cluster of differentiation.
13. Differentiate the roles of T cells and B cells in the immune response.

CHAPTER OUTLINE

You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Adaptive immunity
Antibodies
Antigens
Attenuation
Basophils
Bone marrow
Cell-mediated immunity
Chemotaxins
Clusters of differentiation (CD)
Cytokines
Dendritic cells
Diapedesis
Eosinophils
Germinal center
Humoral immunity
Immunity
Immunology
Innate (natural) immunity
Leukocytes
Lymph nodes
Lymphocyte
Macrophages
Mast cells
Memory cells
Monocytes
Natural killer (NK) cells
Neutrophil
Periarteriolar lymphoid sheath (PALS)
Phagocytosis
Plasma cells
Primary follicles
Primary lymphoid organs
Secondary follicles
Secondary lymphoid organs
Spleen
Thymocytes
Thymus

Although humans have been trying for many centuries to unravel the secrets of preventing disease, the field of immunology is a relatively new science. **Immunology** can be defined as the study of a host's reactions when foreign substances are introduced into the body. Such foreign substances that induce a host response are called **antigens.** Antigens are all around us in nature and they vary from substances such as pollen that may make us sneeze to serious bacterial pathogens such as *Staphylococcus aureus* or *Group A Streptococcus* that can cause life-threatening illnesses. The study of immunology has given us the ability to prevent diseases such as smallpox, polio, diphtheria, and measles through the development of vaccines. In addition, understanding how the immune system works has made successful organ transplantation possible and has given us new tools to treat diseases such as cancer and certain autoimmune diseases. Immunological techniques have affected testing in many areas of the clinical laboratory and allowed for such testing to be more precise and automated. Thus, the study of immunology is important to many areas of medicine. In this chapter, we will provide a brief look at the history of the field and then introduce the cells and tissues of the immune system to form a basis for understanding how the immune system works. In later chapters we will apply this knowledge to principles of testing for specific diseases.

Immunity and Immunization

Immunology as a science has its roots in the study of **immunity:** the condition of being resistant to infection. The first recorded attempts to deliberately induce immunity date back to the 1500s when the Chinese inhaled powder made from smallpox scabs in order to produce protection against this dreaded disease. The hypothesis was that if a healthy individual was exposed as a child or young adult the effects of the disease would be minimized. However, the early exposure did not always work. Further refinements did not occur until the late 1700s when an English country doctor by the name of Edward Jenner was able to successfully prevent infection with smallpox by injecting a more harmless substance—cowpox—from a disease affecting cows.[1] Details of the development of this first vaccine can be found in Chapter 25.

The next major development in disease prevention did not occur until almost a hundred years later when Louis Pasteur, often called the father of immunology, observed by chance that older bacterial cultures would not cause disease in chickens **(Fig. 1–1).**[2,3] Subsequent injections of more virulent organisms had no effect on the birds that had been previously exposed to the older cultures. In this manner, the first attenuated vaccine was discovered; this event can be considered the birth of immunology.[3] **Attenuation,** or change, means to make a pathogen less virulent; it takes place through heat, aging, or chemical means. Attenuation remains the basis for many of the immunizations that are used today. Pasteur applied this same principle of attenuation to the prevention of rabies in affected individuals.

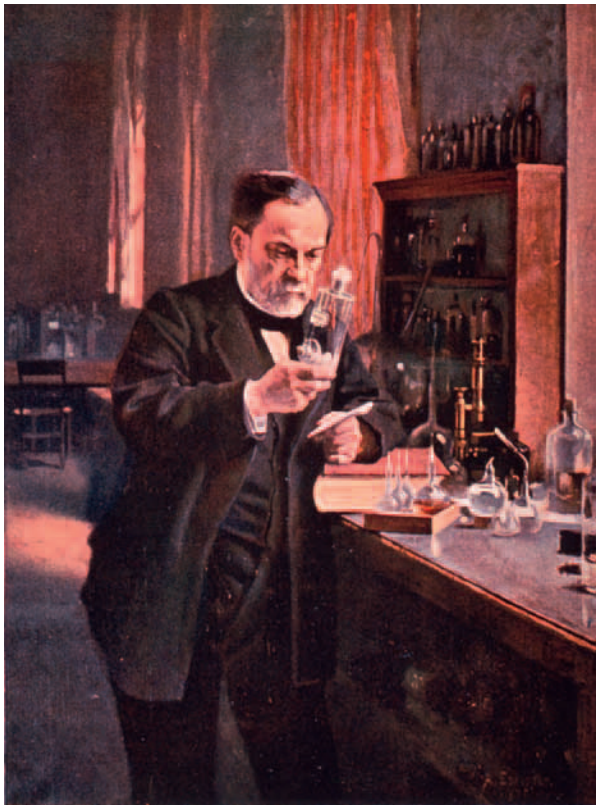


FIGURE 1–1 Louis Pasteur. *(Courtesy of the National Library of Medicine.)*

Innate Versus Adaptive Immunity

In the late 1800s, scientists turned to identifying the actual mechanisms that produce immunity in a host.[2] Elie Metchnikoff, a Russian scientist, observed under a microscope that foreign objects introduced into transparent starfish larvae became surrounded by motile amoeboid-like cells that attempted to destroy the penetrating objects. This process was later termed **phagocytosis,** meaning cells that eat cells.[2,4] He hypothesized that immunity to disease was based on the action of these scavenger cells and was a natural, or innate, host defense.[4]

Other researchers contended that noncellular elements in the blood were responsible for protection from microorganisms. Emil von Behring demonstrated that diphtheria and tetanus toxins, which are produced by specific microorganisms as they grow, could be neutralized by the noncellular portion of the blood of animals previously exposed to the microorganisms. The theory of **humoral immunity** was thus born and sparked a long-lasting dispute over the relative importance of cellular versus humoral immunity.

In 1903, an English physician named Almroth Wright linked the two theories by showing that the immune response involved both cellular and humoral elements. He observed that certain humoral, or circulating, factors called *opsonins* acted to coat bacteria so that they became more susceptible to ingestion by phagocytic cells.[2] These serum factors include specific proteins known as antibodies, as well as other factors called *acute-phase reactants* that increase nonspecifically in any infection. **Antibodies** are serum proteins produced by certain lymphocytes when exposed to a foreign substance and they react specifically with that foreign substance (see Chapter 5).

These discoveries showed that there were two major branches of immunity, currently referred to as innate immunity and adaptive immunity. **Innate,** or **natural immunity,** is the individual's ability to resist infection by means of normally present body functions. These are considered nonadaptive or nonspecific and are the same for all pathogens or foreign substances to which one is exposed. No prior exposure is required and the response lacks memory and specificity. Many of these mechanisms are subject to influence by such factors as nutrition, age, fatigue, stress, and genetic determinants. **Adaptive immunity,** in contrast, is a type of resistance that is characterized by specificity for each individual pathogen, or microbial agent, and the ability to remember a prior exposure. Memory and specificity result in an increased response to that pathogen upon repeated exposure, something that does not occur in innate immunity. Both systems are necessary to maintain good health. In fact, they operate in combination and are dependent upon one another for maximal effectiveness. Certain key cells are considered essential to both systems and they will be discussed next.

Cells of the Innate Immune System

Leukocytes in Peripheral Blood

White blood cells (WBCs), or **leukocytes,** in the peripheral blood play a key role in both innate and adaptive immunity. There are five principal types of leukocytes in peripheral blood: neutrophils, eosinophils, basophils, monocytes, and lymphocytes. The first four types are all part of innate immunity. Because lymphocytes are considered part of adaptive immunity, they will be considered in a separate section. Several cell lines that are found in the tissues, namely mast cells, macrophages, and dendritic cells, will also be discussed in this chapter because they all contribute to the process of immunity.

All blood cells arise from a type of cell called a hematopoietic stem cell (HSC). To form WBCs, the HSC gives rise to two distinct types of precursor cells: common myeloid precursors (CMP) and common lymphoid precursors (CLP). CMPs give rise to the WBCs that participate in phagocytosis, which are known as the myeloid line. Phagocytic cells are key to innate immunity, but they are also important in processing antigens for the adaptive response. Lymphocytes arise from CLPs and form the basis of the adaptive immune response. Mature lymphocytes are found in the tissues as well as in peripheral blood. Refer to **Figure 1–2** for a simplified scheme of blood cell development, known as *hematopoiesis*.

Neutrophils

The **neutrophil,** or polymorphonuclear neutrophilic (PMN) leukocyte, represents approximately 50% to 75% of the total peripheral WBCs in adults.[5] These are around 10 to 15 μm in diameter with a nucleus that has between two and five lobes **(Fig. 1–3).** Hence, they are often called segmented neutrophils, or "segs." They contain a large number of neutral staining granules when stained with Wright stain, two-thirds of which are specific granules; the remaining one-third are called azurophilic granules.[6] Azurophilic or primary granules contain antimicrobial products such as myeloperoxidase, lysozyme, elastase, proteinase-3, cathepsin G, and defensins, which are small proteins that have antibacterial activity.[5] Specific granules, also known as secondary granules, contain lysozyme, lactoferrin, collagenase, gelatinase, and respiratory burst components.[5,7] See Chapter 3 for a discussion of the oxidative burst, which takes place during phagocytosis. The main function of neutrophils is phagocytosis, resulting in the destruction of foreign particles.[6]

Normally, half of the total neutrophil population in peripheral blood is found in a marginating pool adhering to blood vessel walls, whereas the rest circulate freely for approximately 6 to 8 hours.[5] There is a continuous interchange, however, between the marginating and the circulating pools. Margination occurs to allow neutrophils to move from the circulating blood to the tissues through a process known as **diapedesis,** or movement through blood vessel walls. They are attracted to a specific area by chemotactic factors. **Chemotaxins** are chemical messengers that cause cells to migrate in a particular direction. Once in the tissues, neutrophils have a life span of up to several days. Normally, the influx of neutrophils from the bone marrow equals the output from the blood to the tissues to maintain a steady state. However, in the case of acute infection an increase of neutrophils in the circulating blood can occur almost immediately.[8]

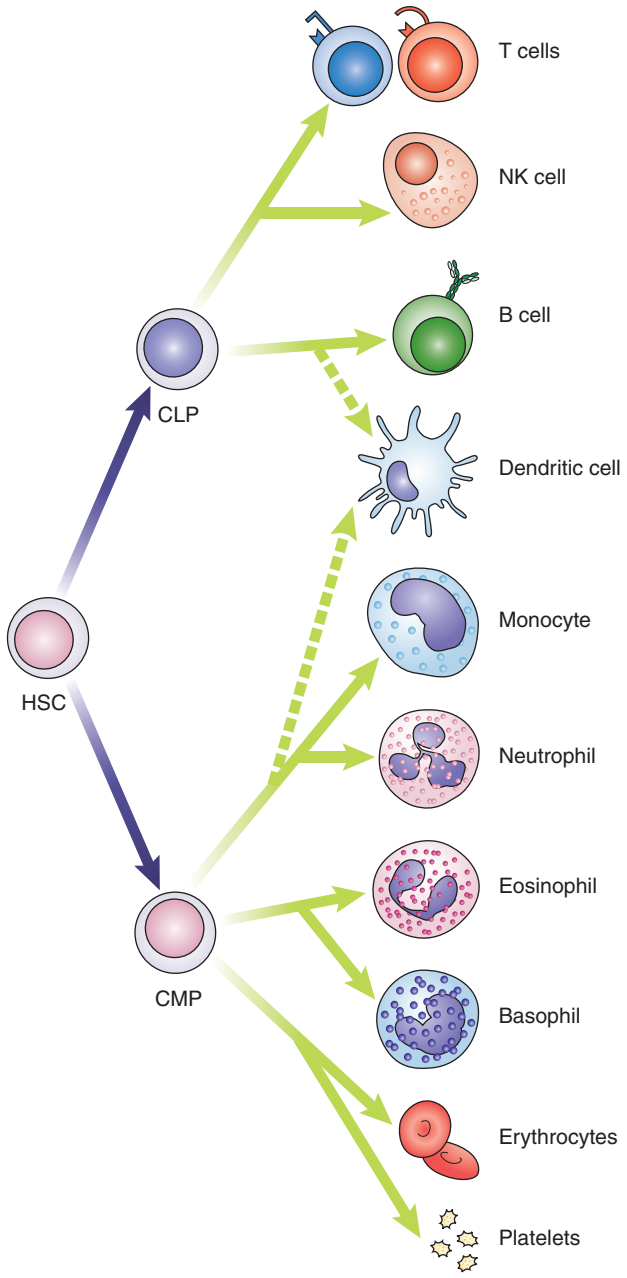


FIGURE 1–2 Simplified scheme of hematopoiesis. In the marrow, hematopoietic stem cells (HSC) give rise to two different lines—a common lymphoid precursor (CLP) and a common myeloid precursor (CMP). CLPs give rise to T/NK progenitors, which differentiate into T and NK cells, and to B-cell progenitors, which become B cells and dendritic cells. The CMP differentiates into neutrophils, monocytes/macrophages, eosinophils, basophils, erythrocytes, and platelets.

Eosinophils

Eosinophils are approximately 12 to 15 μm in diameter and normally make up between 1% and 3% of the circulating WBCs in a nonallergic person. Their number increases in an allergic reaction or in response to certain parasitic infections. The nucleus is usually bilobed or ellipsoidal and is often eccentrically located **(Fig. 1–4).** Eosinophils take up the acid eosin dye and the cytoplasm is filled with large orange to

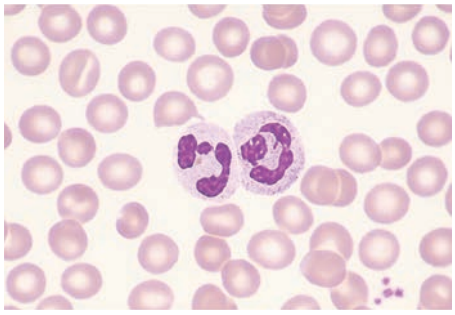


FIGURE 1–3 Neutrophils. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009. Fig. 1–4.)*

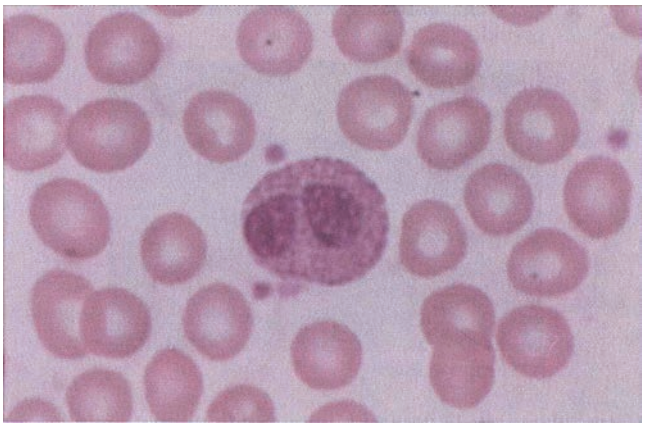


FIGURE 1–4 Eosinophil. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009. Fig. 1–6.)*

reddish-orange granules. Granules in eosinophils, which are spherical and evenly distributed throughout the cell, contain a large number of previously synthesized proteins including catalase, lysozyme, cytokines (chemical messengers), growth factors, and cationic proteins.[5,9]

Eosinophils are capable of phagocytosis but are much less efficient than neutrophils because they are present in smaller numbers and they lack digestive enzymes. Eosinophils are able to neutralize basophil and mast cell products. In addition, they can use cationic proteins to damage cell membranes and kill larger parasites that cannot be phagocytized. (See Chapter 22 for details.) However, the most important role of eosinophils is regulation of the immune response, including regulation of mast cell function.[5]

Basophils

Basophils are the least numerous of WBCs found in peripheral blood, representing less than 1% of all circulating WBCs. The smallest of the granulocytes, basophils are slightly larger than RBCs (between 10 to 15 μm in diameter) and contain coarse, densely staining deep-bluish-purple granules that often obscure the nucleus[5,9] **(Fig. 1–5).** Constituents of these granules include histamine, cytokines, growth factors, and a small amount of heparin, all of which have an important function in inducing and maintaining allergic reactions.[5,8] Histamine contracts smooth muscle and heparin is

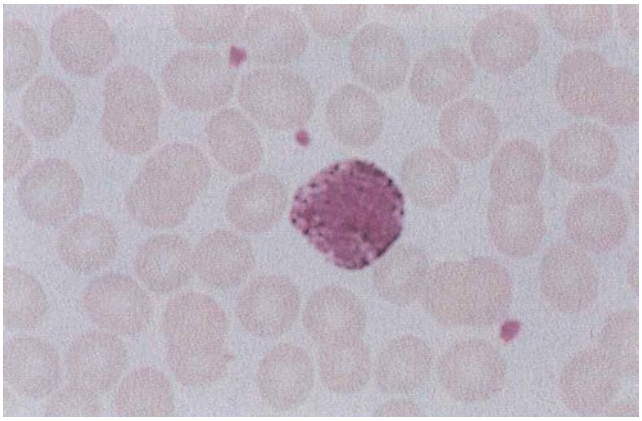


FIGURE 1–5 Basophil. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009. Fig. 1–7.)*

an anticoagulant. In addition, basophils regulate some T helper (Th) cell responses and stimulate B cells to produce the antibody IgE.[5,10] Basophils have a short life span of only a few hours in the bloodstream; they are then pulled out and destroyed by macrophages in the spleen.

Monocytes

Monocytes are the largest cells in the peripheral blood with a diameter that can vary from 12 to 22 μm (the average is 18 μm).[9] One distinguishing feature is an irregularly folded or horseshoe-shaped nucleus that occupies almost one-half of the entire cell's volume **(Fig. 1–6).** The abundant cytoplasm stains a dull grayish blue and has a ground-glass appearance because of the presence of fine dustlike granules. These granules are actually of two types. The first type contains peroxidase, acid phosphatase, and arylsulfatase, indicating that these granules are similar to the lysosomes of neutrophils.[8] The second type of granule may contain β-glucuronidase, lysozyme, and lipase, but no alkaline phosphatase. Digestive vacuoles may also be observed in the cytoplasm. Monocytes make up between 4% and 10% of total circulating WBCs; however, they do not remain in the circulation for long. They stay in peripheral blood for up to 30 hours; they then migrate to the tissues and become known as macrophages.[5]

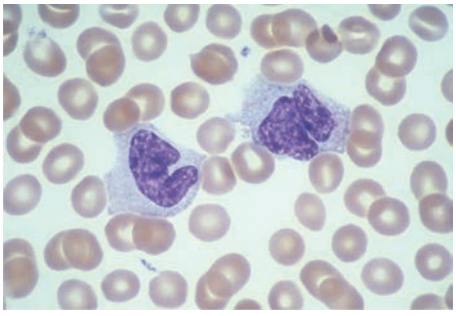


FIGURE 1–6 Two monocytes. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009. Fig. 1–13.)*

Tissue Cells

Macrophages

All **macrophages** arise from monocytes, which can be thought of as macrophage precursors because additional differentiation and cell division takes place in the tissues. The transition from monocyte to macrophage in the tissues is characterized by progressive cellular enlargement to between 25 and 80 μm.[8] Unlike monocytes, macrophages contain no peroxidase.[8] Tissue distribution appears to be a random phenomenon.

Macrophages have specific names according to their particular tissue location. Macrophages in the lung are alveolar macrophages; in the liver, Kupffer cells; in the brain, microglial cells; in the bone, osteoclasts; and in connective tissue, histiocytes. Macrophages may not be as efficient as neutrophils in phagocytosis because their motility is slow compared with that of the neutrophils. Some macrophages progress through the tissues by means of amoeboid action, whereas others are immobile. However, their life span appears to be in the range of months rather than days.

Macrophages play an important role in initiating and regulating both innate and adaptive immune responses. Their innate immune functions include microbial killing, anti-tumor activity, intracellular parasite eradication, phagocytosis, and secretion of cell mediators. Killing activity is enhanced when macrophages become "activated" by contact with microorganisms or with chemical messengers called **cytokines,** which are released by T lymphocytes during the immune response. (See Chapter 6 for a complete discussion of cytokines.) Macrophages play a major role in the adaptive immune response by presenting antigens to T and B cells.

Mast Cells

Tissue **mast cells** resemble basophils, but they come from a different lineage. Mast cells are distributed throughout the body in a wide variety of tissues such as skin, connective tissue, and the mucosal epithelial tissue of the respiratory, genitourinary, and digestive tracts.[5] Mast cells are larger than basophils with a small round nucleus and more granules **(Fig. 1–7).** Unlike basophils, they have a long life span of between 9 and 18 months.[11] The

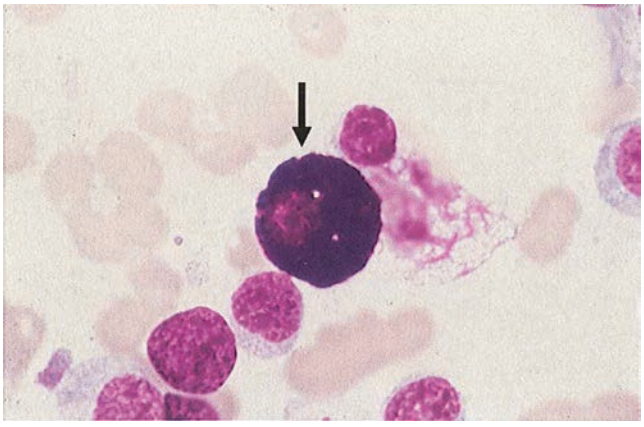


FIGURE 1–7 Mast cell. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009. Fig. 1–44.)*

enzyme content of the granules in mast cells helps to distinguish them from basophils because they contain acid phosphatase, alkaline phosphatase, and protease, as well as histamine.[5,7,8] Mast cells play a role in allergic reactions, but they can also function as antigen-presenting cells (APCs). They can both enhance and suppress the adaptive immune response.

Dendritic Cells

Dendritic cells are so named because they are covered with long membranous extensions that make them resemble nerve cell dendrites. They were discovered by Steinman and Cohn in 1973.[7] Progenitors in the bone marrow give rise to dendritic cell precursors that travel to lymphoid as well as nonlymphoid tissue.[12] They are classified according to their tissue location in a similar manner to macrophages. After capturing an antigen in the tissue by phagocytosis or endocytosis, dendritic cells present the antigen to T lymphocytes to initiate the adaptive immune response in a similar way as macrophages. Dendritic cells, however, are considered the most effective APC in the body, as well as the most potent phagocytic cell.[13,14]

Cells of the Adaptive Immune System

The key cell involved in the adaptive immune response is the **lymphocyte.** Lymphocytes represent between 20% and 40% of the circulating WBCs. The typical small lymphocyte is similar in size to RBCs (7–10 μm in diameter) and has a large rounded nucleus that may be somewhat indented. The nuclear chromatin is dense and tends to stain a deep blue **(Fig. 1–8).**[9] Cytoplasm is sparse, containing few organelles and no specific granules, and consists of a narrow ring surrounding the nucleus.[6] The cytoplasm stains a lighter blue. These cells are unique because they arise from an HSC and then are further differentiated in the primary lymphoid organs, namely the bone marrow and the thymus. Lymphocytes can be divided into three major populations—T cells, B cells, and natural killer (NK) cells—based on specific functions and the proteins on their cell surfaces. In the peripheral blood of adults, approximately 10% to 20% of lymphocytes are B cells, 61% to 80% are T cells, and 10% to 15% are NK cells.[13]

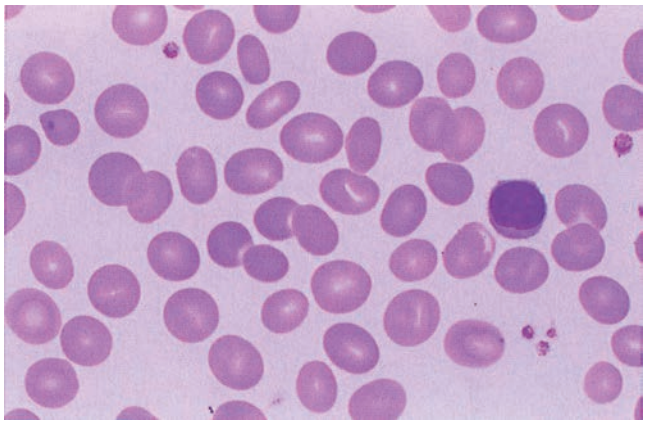


FIGURE 1–8 Typical lymphocyte found in peripheral blood. *(From Harr R.* Clinical Laboratory Science Review. *4th ed. Philadelphia, PA: F.A. Davis; 2013. Color Plate 31.)*

The three types of cells are difficult to distinguish visually. In the laboratory, proteins, or antigens, on cell surfaces can be used to identify each lymphocyte subpopulation. In order to standardize the nomenclature, scientists set up the Human Leukocyte Differentiation Antigens Workshops to relate research findings.[15] Panels of antibodies from different laboratories were used for analysis and antibodies reacting similarly with standard cell lines were said to define **clusters of differentiation (CD).** As each antigen, or CD, was found, it was assigned a number. The list of CD designations currently numbers more than 500.[16] **Table 1–1** lists some of the most important CD numbers used to identify lymphocytes.

B Cells

B cells are derived from a lymphoid precursor that differentiates to become either a T cell, B cell, or NK cell depending on exposure to different cytokines. B cells remain in the environment provided by bone marrow stromal cells. B-cell precursors go through a developmental process that prepares them for their role in antibody production and, at the same time, restricts the types of antigens to which any one cell can respond. The end result is a B lymphocyte programmed to produce a unique antibody molecule. B cells can be recognized by the presence of membrane-bound antibodies of two types, namely immunoglobulin M (IgM) and immunoglobulin (IgD). Other surface proteins that appear on the B cell include CD19, CD21, and class II major histocompatibility complex (MHC) molecules (see Chapter 2).[10]

T Cells

T cells are so named because they differentiate in the thymus. Lymphocyte precursors called **thymocytes** enter the thymus from the bone marrow through the bloodstream. As they mature, the T cells express unique surface markers that allow them to recognize foreign antigens bound to cell membrane proteins called MHC molecules. The role of T cells is to produce cytokines that contribute to immunity by stimulating B cells to produce antibodies, assisting in killing tumor cells or infected target cells, and helping to regulate both the innate and adaptive immune response. The process is known as **cell-mediated immunity.**

Three main subtypes of T cells can be distinguished according to their unique functions: helper, cytolytic, and regulatory T cells. The subtypes can be identified by the presence of the CD3 marker on their cell surface, and either CD4, or CD8. T cells bearing the CD4 receptor are mainly either helper or regulatory cells, whereas the CD8-positive (CD8+) population consists of cytotoxic T cells. The ratio of CD4+ to CD8+ cells is approximately 2:1 in peripheral blood.

Natural Killer (NK) Cells

A small percentage of lymphocytes do not express the markers of either T cells or B cells. They are named **natural killer**

Table 1–1 Surface Markers on T, B, and NK Cells

ANTIGEN	MOL WT (KD)	CELL TYPE	FUNCTION
CD3	20–28	Thymocytes, T cells	Found on all T cells; associated with T-cell antigen receptor
CD4	55	T helper cells, monocytes, macrophages	Identifies T helper cells; also found on most T regulatory cells
CD8	60–76	Thymocyte subsets, cytotoxic T cells	Identifies cytotoxic T cells
CD16	50–80	Macrophages, NK cells, neutrophils	Low affinity Fc receptor for antibody; mediates phagocytosis
CD19	>120	B cells, follicular dendritic cells	Part of B-cell coreceptor; regulates B-cell development and activation
CD21	145	B cells, follicular dendritic cells	Receptor for complement component C3d; part of B-cell coreceptor with CD19
CD 56	175–220	NK cells, subsets of T cells	Not known

(NK) cells because they have the ability to kill target cells without prior exposure to them. NK cells do not require the thymus for development but appear to mature in the bone marrow itself.[17,18] NK cells are generally larger than T cells and B cells at approximately 15 μm in diameter and contain kidney-shaped nuclei with condensed chromatin and prominent nucleoli. Described as large granular lymphocytes, NK cells make up 10% to 15% of the circulating lymphoid pool and are found mainly in the liver, spleen, and peripheral blood.[5,10]

There are no surface markers that are unique to NK cells, but they express a specific combination of antigens that can be used for identification. Two such antigens are CD16 and CD56. CD16 is a receptor for the nonspecific end of antibodies. (See Chapter 5 for more details.) Because of the presence of CD16, NK cells are able to make contact with and then lyse any cell coated with antibodies.[10] NK cells are also capable of recognizing any foreign cell and represent the first line of defense against virally infected cells and tumor cells.[19]

Although NK cells have traditionally been considered part of the innate immune system because they can respond to a variety of antigens, it appears that they also have the capability to develop memory to specific antigens in a similar manner to T cells.[19] Normally, NK cells have a half-life of 7 to 10 days, but new evidence suggests that they are able to survive for a longer time because they can generate highly specific memory cells.[19,20] Thus, they play an important role as a transitional cell bridging the innate and the adaptive immune response against pathogens.[17]

Organs of the Immune System

Just as the cells of the immune system have diverse functions, so, too, do key organs that are involved in the development of the immune response. The bone marrow and thymus are considered the **primary lymphoid organs** where maturation of B lymphocytes and T lymphocytes takes place, respectively. The secondary organs provide a location where contact with foreign antigens can occur **(Fig. 1–9). Secondary lymphoid organs** include the spleen, lymph nodes, and various types of mucosal-associated lymphoid tissues (MALT). The primary and secondary organs are differentiated according to their function in both adaptive and innate immunity.

Primary Lymphoid Organs

Bone Marrow

Bone marrow is considered one of the largest tissues in the body and it fills the core of all long flat bones. It is the main source of hematopoietic stem cells, which develop into erythrocytes, granulocytes, monocytes, platelets, and lymphocytes. Each of these lines has specific precursors that originate from the pleuripotential stem cells.

Some lymphocyte precursors remain in the marrow to mature and become NK and B cells. B cells received their name because they were originally found to mature in birds in an organ called the bursa of Fabricius, which is similar to the appendix in humans. After searching for such an organ in humans, it was discovered that B-cell maturation takes place within the bone marrow itself. Thus, the naming of these cells was appropriate. Other lymphocyte precursors go to the thymus and develop into T cells, so named because of where they mature.[7] Immature T cells appear in the fetus as early as 8 weeks in the gestational period.[21] Thus, differentiation of lymphocytes appears to take place very early in fetal development and is essential to acquisition of immunocompetence by the time the infant is born.

Thymus

T cells develop their identifying characteristics in the **thymus,** which is a small, flat, bilobed organ found in the thorax, or chest cavity, right below the thyroid gland and overlying the heart. In humans, the thymus reaches a weight of 30 to 40 g by puberty and then gradually shrinks in size.[22] It was first thought that the thymus produces enough virgin T lymphocytes early in life to seed the entire immune system, making the organ unnecessary later on. However, it now appears that

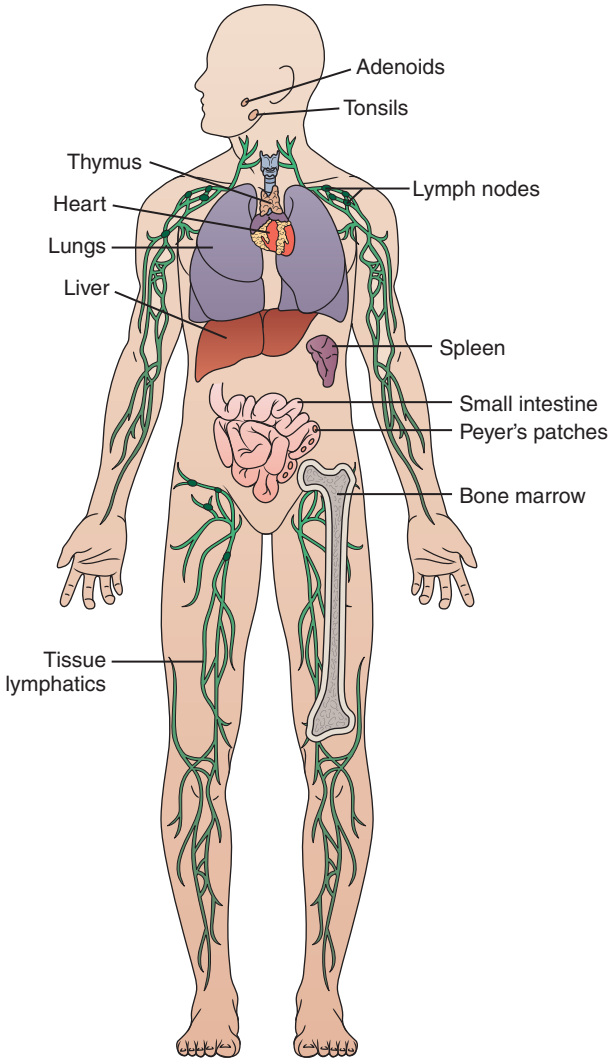


FIGURE 1–9 Sites of lymphoreticular tissue. Primary organs include the bone marrow and the thymus. Secondary organs are distributed throughout the body and include the spleen, lymph nodes, and mucosal-associated lymphoid tissue (MALT). The spleen filters antigens in the blood, whereas the lymphatic system filters fluid from the tissues.

although the thymus diminishes in size as humans age, it is still capable of producing T lymphocytes, although at a diminished rate.[22,23]

Each lobe of the thymus is divided into smaller lobules filled with epithelial cells that play a central role in the differentiation process. Maturation of T cells takes place over a 3-week period as cells filter through the thymic cortex to the medulla. Different surface antigens are expressed as T cells mature. In this manner, a repertoire of T cells is created to protect the body from foreign invaders. Mature T lymphocytes are then released from the medulla.

Secondary Lymphoid Organs

Once lymphocytes mature in the primary organs, they are released and make their way to secondary lymphoid organs, which include the spleen, lymph nodes, cutaneous-associated lymphoid tissue (CALT), and MALT in the respiratory, gastrointestinal, and urogenital tracts. It is within these secondary organs that the main contact with foreign antigens takes place. Lymphocyte circulation between the secondary organs is complex and is regulated by different cell surface adhesion molecules and by cytokines.

Each lymphocyte spends most of its life span in solid tissue, entering the circulation only periodically to go from one secondary organ to another. Lymphocytes in these organs travel through the tissue and return to the bloodstream by way of the thoracic duct. The thoracic duct is the largest lymphatic vessel in the body. It collects most of the body's lymph fluid and empties it into the left subclavian vein. The majority of circulating lymphocytes are T cells.[5] Continuous recirculation increases the likelihood of a T lymphocyte coming into contact with the specific antigen with which it can react.

Lymphocytes are segregated within the secondary organs according to their particular functions. T lymphocytes are effector cells that serve a regulatory role, whereas B lymphocytes produce antibodies. It is in the secondary organs that contact with foreign antigens is most likely to take place.

Lymphopoiesis, or multiplication of lymphocytes, occurs in the secondary lymphoid tissue and is strictly dependent on antigenic stimulation. Formation of lymphocytes in the bone marrow, however, is antigen-independent, meaning that lymphocytes are constantly being produced without the presence of specific antigens. Most naïve or resting lymphocytes die within a few days after leaving the primary lymphoid organs unless activated by the presence of a specific foreign antigen. Antigen activation gives rise to long-lived memory cells and shorter-lived effector cells that are responsible for the generation of the immune response.

Spleen

The **spleen,** the largest secondary lymphoid organ, has a length of approximately 12 cm and weighs 150 g in the adult. It is located in the upper-left quadrant of the abdomen just below the diaphragm and is surrounded by a thin connective tissue capsule. The organ can be characterized as a large discriminating filter as it removes old and damaged cells and foreign antigens from the blood.

Splenic tissue can be divided into two main types: red pulp and white pulp. The red pulp makes up more than one-half of the total volume and its function is to destroy old red blood cells (RBCs). Blood flows from the arterioles into the red pulp and then exits by way of the splenic vein. The white pulp comprises approximately 20% of the total weight of the spleen and contains the lymphoid tissue, which is arranged around arterioles in a **periarteriolar lymphoid sheath (PALS) (Fig. 1–10).** This sheath contains mainly T cells. Attached to the sheath are **primary follicles,** which contain B cells that are not yet stimulated by antigens. Surrounding the PALS is a marginal zone containing dendritic cells that trap antigens. Lymphocytes enter and leave this area by means of the many capillary branches that connect to the arterioles. The spleen receives a blood volume of approximately 350 mL/minute, which allows lymphocytes and macrophages to constantly survey for infectious agents or other foreign matter.[22]

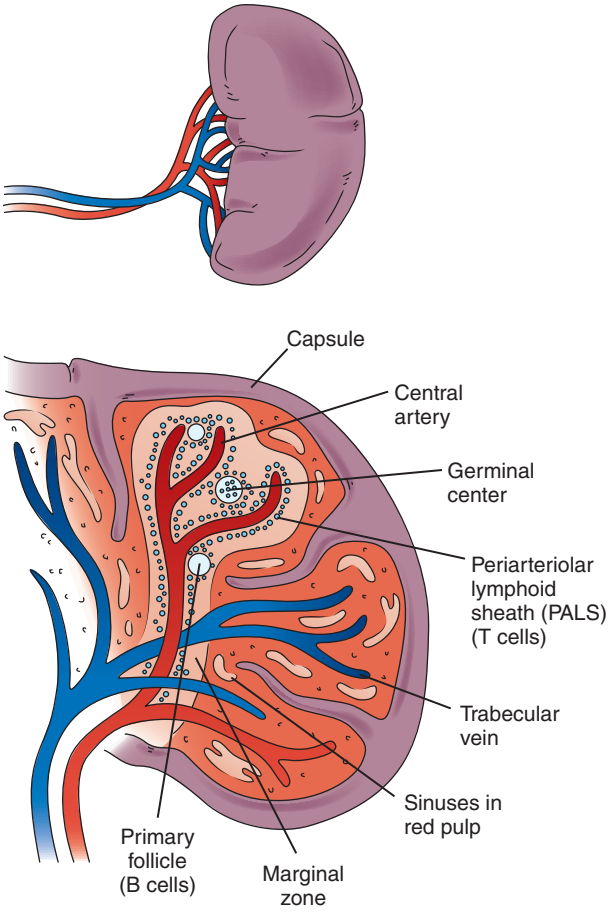


FIGURE 1–10 Cross-section of the spleen showing organization of the lymphoid tissue. T cells surround arterioles in the PALS. B cells are just beyond in follicles. When stimulated by antigens, the B cells form germinal centers. All of the lymphoid tissue is referred to as the *white pulp.*

Lymph Nodes

Lymph nodes serve as central collecting points for lymph fluid from adjacent tissues. Lymph fluid is a filtrate of the blood and arises from passage of water and low-molecular-weight solutes out of blood vessel walls and into the interstitial spaces between cells. Some of this interstitial fluid returns to the bloodstream through venules, but a portion flows through the tissues and is eventually collected in thin-walled vessels known as *lymphatic vessels.* Lymph nodes are located along lymphatic ducts and are especially numerous near joints and where the arms and legs join the body (see Fig. 1–9).

Filtration of interstitial fluid from around cells in the tissues is an important function of these organs because it allows contact between lymphocytes and foreign antigens from the tissues to take place. Whereas the spleen helps to protect us from foreign antigens in the blood, the lymph nodes provide the ideal environment for contact with foreign antigens that have penetrated into the tissues. The lymph fluid flows slowly through spaces called *sinuses,* which are lined with macrophages, creating an ideal location where phagocytosis can take place. The node tissue is organized into an outer cortex, a paracortex, and an inner medulla **(Fig. 1–11).**

Lymphocytes and any foreign antigens present enter nodes via afferent lymphatic vessels. Numerous lymphocytes also enter the nodes from the bloodstream by means of specialized venules called *high endothelial venules,* which are located in the paracortical areas of the node tissues.[24] The outermost layer, the cortex, contains macrophages and aggregations of B cells in primary follicles similar to those found in the spleen. These are the mature, resting B cells that have not yet been exposed to antigens. Specialized cells called *follicular dendritic cells* are also located here. These cells exhibit a large number of receptors for antibodies and help to capture antigens to present to T and B cells.

Secondary follicles consist of antigen-stimulated proliferating B cells. The interior of a secondary follicle is known as the **germinal center** because it is here that transformation of the B cells takes place. When exposed to an antigen, **plasma cells (Fig. 1–12),** which actively secrete antibodies, and **memory cells,** which are just a step away from forming plasma cells, are formed. Thus, the lymph nodes provide an ideal environment for the generation of B-cell memory.

T lymphocytes are mainly localized in the paracortex, the region between the follicles and the medulla. T lymphocytes are in close proximity to APCs called *interdigitating cells.* The medulla is less densely populated than the cortex but contains some T cells (in addition to B cells), macrophages, and numerous plasma cells.

Particulate antigens are removed from the fluid as it travels across the node from cortex to medulla. Fluid and lymphocytes exit by way of the efferent lymph vessels. Such vessels form a larger duct that eventually connects with the thoracic duct and the venous system. As a result, lymphocytes are able to recirculate continuously between lymph nodes and the peripheral blood.

If contact with an antigen takes place, lymphocyte traffic shuts down. Lymphocytes able to respond to a particular antigen proliferate in the node. Accumulation of lymphocytes and other cells causes the lymph nodes to become enlarged, a condition known as *lymphadenopathy.* As lymphocyte traffic resumes, recirculation of expanded numbers of lymphocytes occurs.

Other Secondary Organs

Additional areas of lymphoid tissue include the mucosal-associated tissue known as MALT. MALT is found in the gastrointestinal, respiratory, and urogenital tracts. Some examples include the tonsils; appendix; and Peyer's patches, a specialized type of MALT located at the lower ileum of the intestinal tract. These mucosal surfaces represent some of the main ports of entry for foreign antigens, and thus, numerous macrophages and lymphocytes are localized here.

The skin is considered the largest organ in the body and the epidermis contains a number of intraepidermal lymphocytes. Most of these are T cells, which are uniquely positioned to combat any antigens that enter through the skin. In addition, monocytes, macrophages, and dendritic cells are found here. The collective term for these cells is the cutaneous-associated lymphoid tissue, or CALT.

All of these secondary organs function as potential sites for contact with foreign antigens and they increase the probability

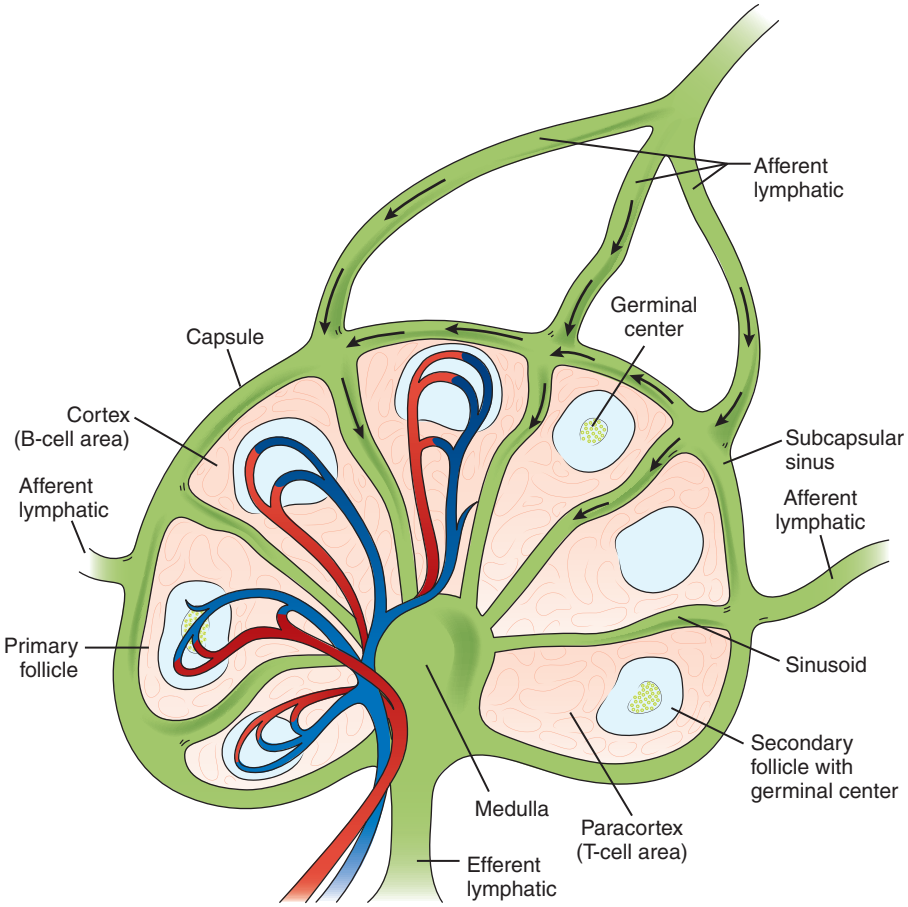


FIGURE 1–11 Structure of a lymph node. A lymph node is surrounded by a tough outer capsule. Right underneath is the subcapsular sinus, where lymph fluid drains from afferent lymphatic vessels. The outer cortex contains collections of B cells in primary follicles. When stimulated by antigens, secondary follicles are formed. T cells are found in the paracortical area. Fluid drains slowly through sinusoids to the medullary region and out the efferent lymphatic vessel to the thoracic duct.

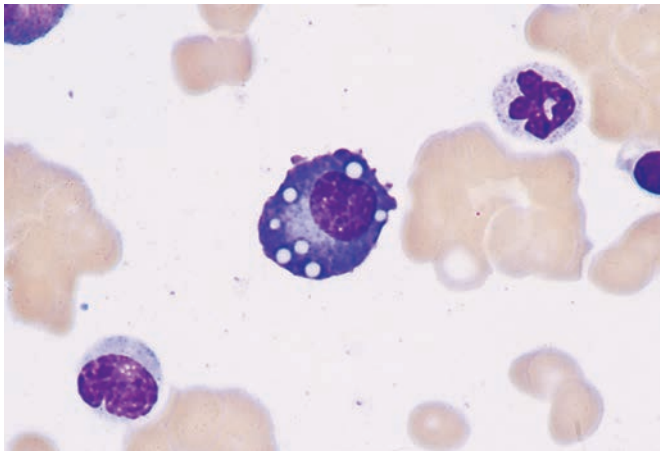


FIGURE 1–12 A typical plasma cell. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis 2009. Fig. 1–47.)*

of an immune response. Within each of these secondary organs, T and B cells are segregated and perform specialized functions. B cells differentiate into memory cells and plasma cells and are responsible for humoral immunity or antibody formation. T cells play a role in cell-mediated immunity; as such, they produce sensitized lymphocytes that secrete cytokines. Both cell-mediated immunity and humoral immunity are part of the overall adaptive immune response.

SUMMARY

- Immunology has its roots in the study of immunity—the condition of being resistant to disease.
- Jenner performed the first vaccination against smallpox by using cowpox.
- Louis Pasteur is considered the father of immunology for his use of attenuated vaccines.
- Metchnikoff was the first to observe phagocytosis—meaning cells that eat cells.
- Immunity has two branches. Innate immunity is the ability of the body to resist infection through means of normally present nonspecific body functions. Adaptive immunity is characterized by specificity, memory, and dependence upon lymphocytes.
- All blood cells arise in the bone marrow from hematopoietic stem cells.
- The five principal types of leukocytes are neutrophils, eosinophils, basophils, monocytes, and lymphocytes.
- Tissue cells involved in immunity include mast cells, dendritic cells, and macrophages that arise from monocytes.
- Cells that are involved in the innate immune response and are actively phagocytic include neutrophils, monocytes, macrophages, and dendritic cells.

- Lymphocytes are the key cells involved in the adaptive immune response.
- CD stands for clusters of differentiation, which are types of proteins found on cell surfaces that can be used for identification of specific cells and cell stages.
- B cells are a type of lymphocyte that develop in the bone marrow and are capable of secreting antibody when mature. They can be identified by the presence of CD19 and surface antibody.
- T cells acquire their specificity in the thymus and consist of two subtypes: CD4+, which are mainly helper or regulatory T cells, and CD8+, which are cytotoxic T cells.
- Natural killer (NK) cells are types of lymphocyte that arise from a lymphocyte precursor but do not develop in the thymus. They can kill virally infected or cancerous target cells without previous exposure to them.
- The bone marrow and the thymus are considered primary lymphoid organs. B cells remain in the bone marrow to mature, whereas the thymus is where T cells develop their specific characteristics.
- Secondary lymphoid organs include the spleen, lymph nodes, mucosal-associated lymphoid tissue (MALT), and cutaneous-associated lymphoid tissue (CALT).

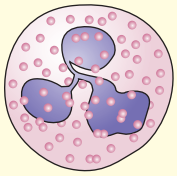
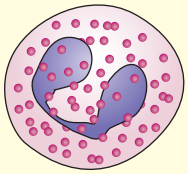
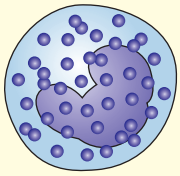
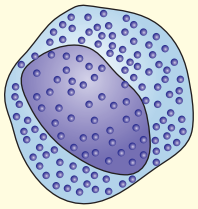
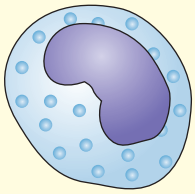
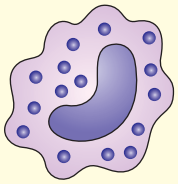
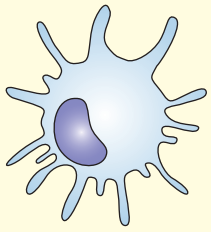
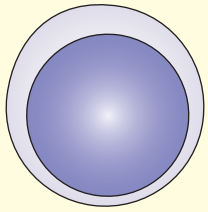
Study Guide: Comparison of T, B, and NK Cells

T CELLS	B CELLS	NK CELLS
Develop in the thymus	Develop in the bone marrow	Develop in the bone marrow
Found in lymph nodes, thoracic duct fluid 60–80% of circulating lymphocyte pool in blood	Found in bone marrow, spleen, lymph nodes, 10–15% of circulating lymphocyte pool in blood	Found in spleen, liver 5–15% of circulating lymphocyte pool in blood
Adaptive immunity: end products of activation are cytokines	Adaptive immunity: end product of activation is antibody	Innate immunity: lysis of virally infected cells and tumor cells; production of cytokines
Antigens include CD2, CD3, CD4, or CD8	Antigens include CD19, CD20, CD21 surface antibody	Antigens include CD16, CD56

Study Guide: Primary and Secondary Lymphoid Organs

LYMPHOID ORGAN CATEGORY	ORGANS INVOLVED	FUNCTION
Primary	Bone marrow	Produces hematopoietic stem cells; maturation of B and NK cells
	Thymus	Maturation of T cells
Secondary	Spleen	Filters blood
	Lymph nodes	Places where contact between T cells, antigens, and B cells occur
	Mucosal associated lymphoid tissue (MALT)	
	Cutaneous-associated lymphoid tissue (CALT)	

Study Guide: Cells of the Immune System

CELL TYPE	WHERE FOUND	FUNCTION	
Neutrophil	50–75% of circulating WBCs, also in tissue	First responder to infection, phagocytosis	
Eosinophil	1–3% of circulating WBCs	Kill parasites, neutralize basophil and mast cell products, regulate mast cells	
Basophil	< 1% of circulating WBCs	Induce and maintain allergic reactions, stimulate production of IgE	
Mast cell	Found in skin, connective tissue, mucosal epithelium	Antigen presentation to T and B cells; enhancement and suppression of the adaptive immune response	
Monocyte	4–10% of circulating WBCs	Phagocytosis; migrate to tissues to become macrophages	
Macrophage	In lungs, liver, brain, bone, connective tissue, other tissue	Phagocytosis; kill intracellular parasites; tumoricidal activity; antigen presentation to T and B cells	
Dendritic cell	In skin, mucous membranes, heart, lungs, liver, kidney, other tissue	Most potent phagocytic cell; most effective at antigen presentation	
Lymphocyte	20–40% of circulating WBCs; also found in lymph nodes, spleen, other secondary lymphoid organs	Subtypes are T cells, B cells, and NK cells; T cells produce cytokines, B cells produce antibody in adaptive immune response, and NK cells are involved in innate immunity	

CASE STUDIES

1. A 13-year-old girl had her ears pierced at a small jewelry store in a mall. Although she was instructed to clean the area around the earrings with alcohol, she forgot for the first 2 days. On the third day she noticed that the area around one earlobe was red and slightly swollen.

Questions

a. Which branch of the immune system is likely the cause of the symptoms?
b. What type of cell would you expect to see in the tissue?

2. You and a friend are discussing the relative merits of immunizations. Your friend says that he doesn't want to get a tetanus booster shot because he has a good immune system and his natural defenses will take care of any possible infection. You have just been studying this subject in your immunology class.

Question

a. What argument could you make to convince him that a tetanus booster is a good idea?

REVIEW QUESTIONS

1. Which of the following can be attributed to Pasteur?
   a. Discovery of opsonins
   b. Observation of phagocytosis
   c. First attenuated vaccines
   d. Theory of humoral immunity

2. Which WBC is capable of further differentiation in tissues?
   a. Neutrophil
   b. Eosinophil
   c. Basophil
   d. Monocyte

3. The cells that Metchnikoff first observed are associated with which phenomenon?
   a. Innate immunity
   b. Adaptive immunity
   c. Humoral immunity
   d. Specific immunity

4. Where are all undifferentiated lymphocytes made?
   a. Bone marrow
   b. Spleen
   c. Thymus
   d. Lymph nodes

5. Which of the following statements is true of NK cells?
   a. They rely upon memory for antigen recognition.
   b. They have the same CD groups as B cells.
   c. They are found mainly in lymph nodes.
   d. They kill target cells without prior exposure to them.

6. Which cell is the most potent phagocytic cell in the tissue?
   a. Neutrophil
   b. Dendritic cell
   c. Eosinophil
   d. Basophil

7. The ability of an individual to resist infection by means of normally present body functions is called
   a. innate immunity.
   b. humoral immunity.
   c. adaptive immunity.
   d. cross-immunity.

8. A cell characterized by a nucleus with two to five lobes, a diameter of 10 to 15 μm, and a large number of neutral staining granules is identified as a(n)
   a. eosinophil.
   b. monocyte.
   c. basophil.
   d. neutrophil.

9. Which of the following is a primary lymphoid organ?
   a. Lymph node
   b. Spleen
   c. Thymus
   d. MALT

10. What type of cells would be found in a primary follicle?
    a. Unstimulated B cells
    b. Germinal centers
    c. Plasma cells
    d. Memory cells

11. Which of the following is a distinguishing feature of B cells?
    a. Act as helper cells
    b. Presence of surface antibody
    c. Able to kill target cells without prior exposure
    d. Active in phagocytosis

12. Where do lymphocytes mainly come in contact with antigens?
    a. Secondary lymphoid organs
    b. Bloodstream
    c. Bone marrow
    d. Thymus

13. Which of the following is found on the T cell subset known as helpers?

a. CD19
b. CD4
c. CD8
d. CD56

14. Which of the following statements best characterizes adaptive immunity?

a. Relies on normally present body functions
b. Response is similar for each exposure
c. Specificity for each individual pathogen
d. Involves only cellular immunity

15. The main function of T cells in the immune response is to

a. produce cytokines that regulate both innate and adaptive immunity.
b. produce antibodies.
c. participate actively in phagocytosis.
d. respond to target cells without prior exposure.

16. Which of the following is a part of humoral immunity?

a. Cells involved in phagocytosis
b. Neutralization of toxins by serum
c. Macrophages and mast cells in the tissue
d. T and B cells in lymph nodes

17. Immunity can be defined as

a. the study of medicines used to treat diseases.
b. a specific population at risk for a disease.
c. the condition of being resistant to disease.
d. the study of the noncellular portion of the blood.

18. A blood cell that has reddish staining granules and is able to kill large parasites describes

a. basophils.
b. monocytes.
c. neutrophils.
d. eosinophils.

19. Which of the following statements best describes a lymph node?

a. It is considered a primary lymphoid organ.
b. It removes old RBCs.
c. It collects fluid from the tissues.
d. It is where B cells mature.

20. Antigenic groups identified by different sets of antibodies reacting in a similar manner to certain standard cell lines best describes

a. cytokines.
b. clusters of differentiation (CD).
c. neutrophilic granules.
d. opsonins.

2 Nature of Antigens and the Major Histocompatibility Complex

Christine Dorresteyn Stevens, EdD, MT(ASCP)

LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Define and characterize the nature of immunogens.
2. Differentiate an immunogen from an antigen.
3. Discuss several biological properties of individuals that influence the nature of the immune response.
4. Describe four important characteristics of immunogens that affect the ability to stimulate a host response.
5. Identify the characteristics of a hapten.
6. Describe how an epitope relates to an immunogen.
7. Discuss the role of adjuvants.
8. Differentiate heterophile antigens from alloantigens and autoantigens.
9. Explain what a haplotype is in regard to inheritance of major histocompatibility complex (MHC) antigens.
10. Describe the differences in the structure of class I and class II molecules.
11. Compare the transport of antigen to cellular surfaces by class I and class II molecules.
12. Describe the role of transporters associated with antigen processing (TAP) in selecting peptides for binding to class I molecules.
13. Discuss the differences in the source and types of antigen processed by class I and class II molecules.
14. Explain the clinical significance of the class I and class II molecules.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Adjuvant
Alleles
Alloantigens
Antigen
Antigen presentation
Autoantigens
Class I MHC (HLA) molecules
Class II MHC (HLA) molecules
Conformational epitope
Epitope
Haplotype
Haptens
Heteroantigens
Heterophile antigens
Immunogenicity
Immunogens
Invariant chain (Ii)
Linear epitopes
Major histocompatibility complex (MHC)
Transporters associated with antigen processing (TAP1 and TAP2)

Whereas the innate immune system responds nonspecifically to certain patterns found on pathogens, the adaptive immune system is characterized by specific recognition of individual pathogens. Lymphocytes are the key cells that are responsible for the specificity, diversity, and memory that characterize adaptive immunity. The immune response of lymphocytes is triggered by materials called **immunogens,** which are macromolecules capable of triggering an adaptive immune response by inducing the formation of antibodies or sensitized T cells in an immunocompetent host. Immunogens can then specifically react with such antibodies or sensitized T cells. The term **antigen** refers to a substance that reacts with an antibody or sensitized T cells but may not be able to evoke an immune response in the first place. Thus, all immunogens are antigens, but the converse is not true. However, many times the terms are used synonymously and the distinction between them is not made. In discussing serological reactions or particular names of substances such as blood groups, the term *antigen* is still more commonly used; hence, both terms are used in this chapter.

One of the most exciting areas of immunological research focuses on how and why we respond to particular immunogens. This response is actually caused by a combination of factors: unique biological properties of the individual, the nature of the immunogen itself, genetic coding of **major histocompatibility complex (MHC)** molecules that must combine with an immunogen before T cells are able to respond, and immunogen processing and presentation. This chapter focuses on all these areas and discusses future clinical implications of some recent findings.

Factors Influencing the Immune Response

Biological properties of the individual that influence the nature of the immune response include several factors such as age, overall health, dose, route of inoculation, and genetic capacity. In general, older individuals are more likely to have a decreased response to antigenic stimulation. At the other end of the age scale, neonates do not fully respond to immunogens because their immune systems are not completely developed. Overall health plays a role because individuals who are malnourished, fatigued, or stressed are less likely to mount a successful immune response.

A significant quantity of an immunogen must be present in order for an adaptive immune response to take place. The larger quantity required for the response allows the innate immune response to take care of small amounts of pathogens and leave the adaptive response for pathogens that are present in large numbers. Generally, the larger the amount of an immunogen one is exposed to, the greater the immune response. However, very large amounts can result in T- and B-cell tolerance, a phenomenon that is not well understood.

There are many ways that we come in contact with immunogens in nature. How we are exposed to them and where they get into our bodies determines the actual amount of immunogen needed to generate an immune response. Such routes include intravenous (into a vein), intradermal (into the skin), subcutaneous (beneath the skin), and oral contact. The route where the immunogen enters the body also determines which cell populations will be involved in the response.[1] For example, if an immunogen enters the body via an intravenous route, as might occur with a deep puncture wound, the immunogen goes directly to the spleen, where a response is mounted. On the other hand, if an immunogen enters subcutaneously, such as through a cut or scratch, local lymph nodes are involved.

Finally, a genetic predisposition may be involved that allows individuals to respond to particular immunogens. This predisposition is linked to the MHC and to the receptors generated during T- and B-lymphocyte development. The MHC is a system of genes that code for cell-surface molecules that play an important role in antigen recognition. Further details are found in a later section in this chapter.

Traits of Immunogens

In general, **immunogenicity—**the ability of an immunogen to stimulate a host response—depends on the following characteristics: (1) macromolecular size, (2) foreignness, (3) chemical composition and molecular complexity, and (4) the ability to be processed and presented with MHC molecules.[1] Usually, an immunogen must have a molecular weight of at least 10,000 to be recognized by the immune system and the most active immunogens typically have a molecular weight of over 100,000 daltons.[1] However, there are exceptions because a few substances with a molecular weight of lower than 1,000 have been known to induce an immune response. For the most part, the rule of thumb is that the greater the molecular weight, the more potent the molecule is as an immunogen.

Another characteristic that all immunogens share is foreignness. The immune system is normally able to distinguish

between self and nonself; those substances recognized as nonself are immunogenic. This ability is acquired as lymphocytes mature in the primary lymphoid organs. Any lymphocyte capable of reacting with self-antigen is normally eliminated. Typically, the more distant taxonomically the source of the immunogen is from the host, the more successful it is as a stimulus. For example, plant protein is a better immunogen for an animal than is material from a related animal. Occasionally, however, autoantibodies, or antibodies to self-antigens, exist. This is the exception rather than the rule; this phenomenon is discussed in Chapter 15.

Immunogenicity is also determined by a substance's chemical composition and molecular complexity. Proteins and polysaccharides are the most effective immunogens. Proteins are powerful immunogens because they are made up of a variety of units known as *amino acids*. The particular sequential arrangement of amino acids, the primary structure, determines the secondary structure, which is the relative orientation of amino acids within the chain. Chains are usually arranged in an alpha helix or a beta pleated sheet. The tertiary structure embodies the spatial or three-dimensional orientation of the entire molecule and is based on folding of particular regions of the molecule; the quaternary structure is based on the association of two or more chains into a single polymeric unit **(Fig. 2–1).** Because of the variations in subunits, proteins may have an enormous variety of three-dimensional shapes. In contrast, synthetic polymers such as nylon or Teflon are made up of a few simple repeating units with no bending or folding within the molecule, which means these materials are nonimmunogenic. For this reason, they are used in making artificial heart valves, elbow replacements, and other medical appliances.

Carbohydrates are less immunogenic than protein because they are smaller than proteins and have a limited number of sugars available to create their structures. As immunogens, carbohydrates most often occur in the form of glycolipids or glycoproteins. Many of the blood group antigens are composed of such carbohydrate complexes. For example, the A, B, and H blood group antigens are glycolipids and the Rh and Lewis antigens are glycoproteins.[2,3] Other carbohydrates that are important immunogens are the capsular polysaccharides of bacteria such as *Streptococcus pneumoniae*. Pure nucleic acids and lipids are not immunogenic by themselves, although a response can be generated when they are attached to a suitable carrier molecule.[3] This is the case for autoantibodies to DNA that are formed in systemic lupus erythematosus (SLE). These autoantibodies are actually stimulated by a DNA-protein complex rather than by DNA itself.

Finally, for a substance to elicit an immune response it must be subject to antigen processing, which involves enzymatic digestion to create small peptides or pieces that can be complexed to MHC molecules to present to responsive lymphocytes. If a macromolecule cannot be degraded and presented with MHC molecules, then it would be a poor immunogen. The particular MHC molecules produced also determine responsiveness to individual antigens. Each individual inherits the ability to produce a certain limited repertoire of MHC molecules, discussed in the section on the genes coding for MHC molecules.

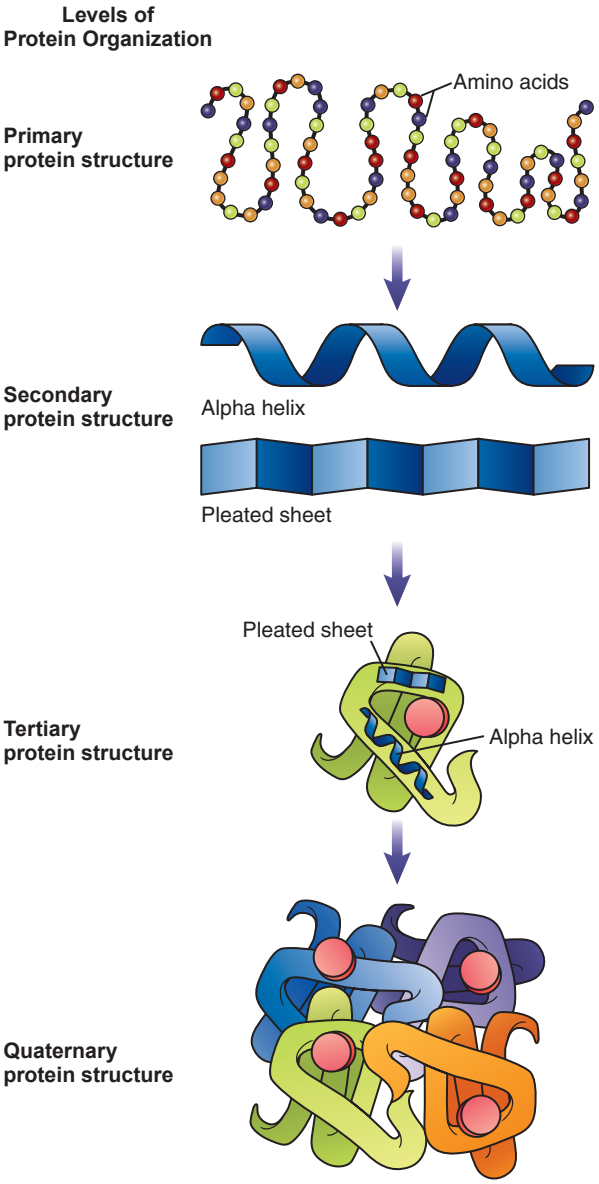


FIGURE 2–1 Levels of protein organization.

Epitopes

Although an immunogen must have a molecular weight of at least 10,000, only a small part of the immunogen is actually recognized in the immune response. This key portion of the immunogen is known as the *determinant site* or **epitope.** Epitopes are molecular shapes or configurations that are recognized by B or T cells. There is evidence that for proteins, epitopes recognized by B cells may consist of as few as 6 to 15 amino acids.[4] Large molecules may have numerous epitopes and each one may be capable of triggering specific antibody production or a T-cell response. Epitopes may be repeating copies or they may have differing specificities. They may also be sequential or **linear epitopes** (i.e., amino acids following one another on a single chain) or they may be conformational. A **conformational epitope** results from the folding of one chain or multiple chains, bringing certain

Connections

The Florida Panther

Polymorphism of the MHC genes in a species is thought to serve as a protection against infectious diseases because diverse genes allow for a response to a wide variety of antigens. The fate of the Florida panther is a good example of what happens when there is a lack of genetic diversity in a particular population **(Fig. 2–2).** In the early 1990s, only about 20 to 25 adult panthers remained in Florida because of a combination of factors, including destruction of their habitat and inbreeding. The latter severely decreased the genetic pool of the population. It has been postulated that the panthers became increasingly susceptible to viral diseases because of the limited polymorphism of the MHC antigens. Conservationists decided to increase the strength of the gene pool by moving eight females from the Texas population to Florida.

Now, a decade and a half after introducing new females into the population, the Florida panthers have exhibited a marked improvement in health and fitness. The increased diversity of the MHC genes allowed for a response to diverse pathogens. The Florida panthers are able to withstand disease better because of the introduction of these new genes into the population.

FIGURE 2–2 The Florida panther. *(John Hollingsworth/U.S. Fish and Wildlife Service.)*

amino acids from different segments of a linear sequence or sequences into close proximity with each other so they can be recognized together **(Fig. 2–3).**

Epitopes recognized by B cells may differ from those recognized by T cells.[1] Surface antibody on B cells may react with both

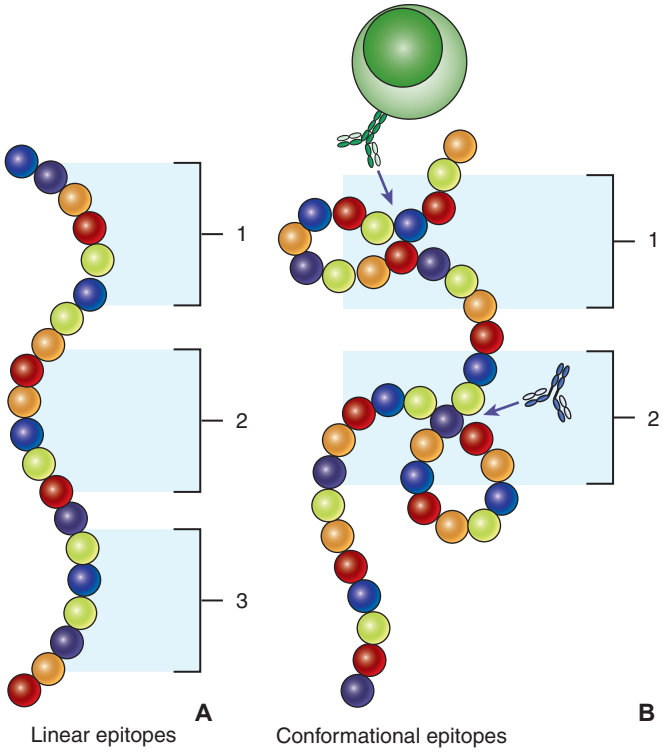


FIGURE 2–3 Linear versus conformational epitopes. (A) Linear epitopes consist of sequential amino acids on a single polypeptide chain. There may be several different types on one chain. (B) Conformational epitopes result from the folding of a polypeptide chain or chains, and nonsequential amino acids are brought into close proximity.

linear and conformational epitopes present on the surface of an immunogen. Anything that is capable of cross-linking surface immunoglobulin molecules is able to trigger B-cell activation. The immunogen does not necessarily have to be degraded first. However, for T cells to be able to recognize an immunogen it must first be degraded into small peptides by an antigen-presenting cell (APC). Then the peptides form a complex with MHC proteins and are carried to the surface of the APC. (See *Role of Class I and II Molecules in the Immune Response* later in this chapter.)

Haptens

Some substances are too small to be recognized, but if they are combined with larger molecules they are then able to stimulate a response. **Haptens** are nonimmunogenic materials that, when combined with a carrier, create new antigenic determinants. Thus, by themselves haptens are antigens but not immunogens. Once antibody production is initiated, the hapten is capable of reaction with antibody even when the hapten is not complexed to a carrier molecule. However, precipitation or agglutination reactions will not occur because a hapten has a single determinant site and cannot form the cross-links with more than one antibody molecule that are necessary for precipitation or agglutination **(Fig. 2–4).**

Haptens may be artificially joined to carrier molecules in a laboratory setting or this may occur naturally within a host and

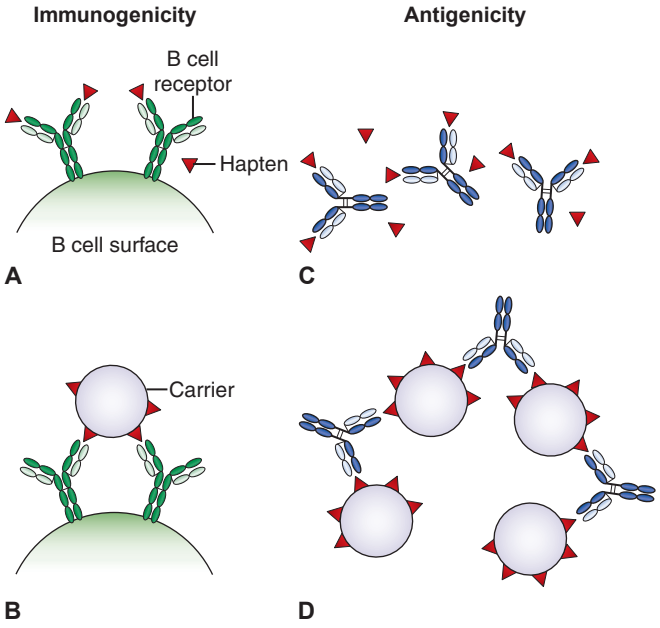


FIGURE 2–4 Characteristics of haptens. (A) Haptens bind B-cell receptors, but the receptors remain independent: no activation (not immunogenic). (B) Hapten/carrier complex cross-links receptors: B cells are activated and begin producing antibodies (immunogenic). (C) Although haptens can bind to the antibody binding sites (antigenic), all of the antibody-hapten complexes remain independent: The reaction cannot be visualized. (D) When bound to carriers, the haptens contribute to the formation of an interconnected lattice, which is the basis for the precipitation and agglutination reactions.

set off an immune response. An example of the latter is an allergic reaction to poison ivy. Poison ivy *(Rhus radicans)* contains chemical substances called *catechols*, which are haptens. Once in contact with the skin, these can couple with tissue proteins to form the immunogens that give rise to contact dermatitis. Another example of haptens coupling with normal proteins in the body to provoke an immune response occurs with certain drug-protein conjugates. The best known example of this occurs with penicillin, which can result in a life-threatening allergic response.

Karl Landsteiner, an Austrian scientist perhaps best known for his discovery of the ABO blood groups, conducted the most famous study of haptens. In his book *The Specificity of Serological Reactions,* published in 1917, he detailed the results of an exhaustive study of haptens that has contributed greatly to our knowledge of antigen–antibody reactions. Landsteiner immunized rabbits with haptens attached to a carrier molecule and then tested the serum to measure how the antibodies produced reacted with different haptens. He discovered that antibodies not only recognize chemical features such as polarity, hydrophobicity, and ionic charge, but the overall three-dimensional configuration is also important.[1] The spatial orientation and the chemical complementarity are responsible for the lock-and-key relationship that allows for tight binding between antibody and epitope **(Fig. 2–5).** Today, it is known that many therapeutic drugs and hormones can function as haptens.[2]

Adjuvants

The power of immunogens to generate an immune response can be increased through the use of adjuvants. An **adjuvant** is a substance administered with an immunogen that increases the immune response in order to provide immunity to a particular disease. Addition of an adjuvant to a substance used for an immunization helps to make the immunization more effective. Adjuvants actually work by targeting APCs, which are key to the adaptive immune response.[5] Substances used as adjuvants protect immunogens from degradation and allow a longer response time that attracts a large number of immune system cells to the injection site, which helps to

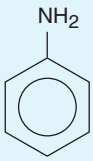
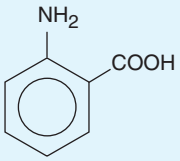
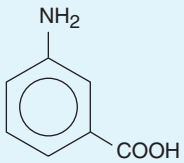
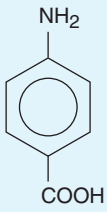
	Reactivity with			
				
Antiserum against	Aminobenzene (aniline)	*o*-Aminobenzoic acid	*m*-Aminobenzoic acid	*p*-Aminobenzoic acid
Aminobenzene	+ + +	0	0	0
o-Aminobenzoic acid	0	+ + +	0	0
m-Aminobenzoic acid	0	0	+ + + +	0
p-Aminobenzoic acid	0	0	0	+ + +/+ + + +

FIGURE 2–5 Landsteiner's study of the specificity of haptens. Spatial orientation of small groups is recognized, because antibodies made against aminobenzene coupled with a carrier will not react with other similar haptens. The same is true for antiserum to *o*-aminobenzoic acid, *m*-aminobenzoic acid, and *p*-aminobenzoic acid. Antibody to a carboxyl group in one location would not react with a hapten, which has the carboxyl group in a different location. *(From Landsteiner K.* The Specificity of Serological Reactions. *Revised Edition. New York, NY: Dover Press; 1962.)*

boost the strength of the response.[5,6] Aluminum salts are the only ones currently approved for clinical use in the United States and are used to complex with the immunogen to increase its size and to prevent a rapid escape from the tissues.[6] It must be injected into the muscle to work. The hepatitis B vaccination is an example of using this type of adjuvant.

Adjuvants are used to accelerate the immune response and increase the duration of protection, thus reducing the need for booster immunizations.[6] In addition to successfully enhancing the immune response, they must meet the criterion of causing minimal toxicity to humans, which is why more substances are not approved for use at this time.

Relationship of Antigens to the Host

Antigens can be placed in broad categories according to their relationship to the host. **Autoantigens** are those antigens that belong to the host. These do not evoke an immune response under normal circumstances. However, if an immune response does occur to autoantigens, it may result in an autoimmune disease. Refer to Chapter 15 for a discussion of some of the most well-known autoimmune diseases. **Alloantigens** are from other members of the host's species and are capable of eliciting an immune response. They are important to consider in tissue transplantation and in blood transfusions. **Heteroantigens** are from other species, such as other animals, plants, or microorganisms.

Heterophile antigens are heteroantigens that exist in unrelated plants or animals but are either identical or closely related in structure so that antibody to one will cross-react with antigen of the other. An example of this is the human blood group A and B antigens, which are related to bacterial polysaccharides.[3] It is believed that anti-A antibody, which is normally found in individuals with blood types other than A (e.g., type B and type O), is originally formed after exposure to pneumococci or other similar bacteria. Naturally occurring anti-B antibody is formed after exposure to a similar bacterial cell wall product. The presence of naturally occurring antibodies is an important consideration in selecting the correct blood type for transfusion purposes.

Normally in serological reactions, the ideal is to use a reaction that is completely specific, but the fact that cross-reactivity exists can be helpful for certain diagnostic purposes. Indeed, the first test for infectious mononucleosis (IM) was based on a heterophile antibody reaction. During the early states of IM, a heterophile antibody is formed, stimulated by an unknown antigen. This antibody was found to react with sheep red blood cells (SRBCs), which formed the basis of the Paul-Bunnell screening test for mononucleosis (see Chapter 23). This procedure was a useful screening test when the causative agent of IM had not yet been identified. Current rapid screening tests for IM are based on the principle of detection of heterophile antibody.

Major Histocompatibility Complex

For years, scientists searched to identify possible immune response genes that would account for differences in how individuals respond to particular immunogens. Evidence now indicates that the genetic capability to mount an immune response is linked to a group of molecules originally referred to as human leukocyte antigens (HLA). The French scientist Dausset gave them this name because they were first defined by discovering an antibody response to circulating white blood cells (WBCs).[7] These molecules are now known as MHC molecules because they determine whether transplanted tissue is histocompatible and thus accepted or recognized as foreign and rejected. MHC molecules are actually found on all nucleated cells in the body and they play a pivotal role in the development of both humoral and cellular immunity. Although MHC molecules themselves can function as antigens when transplanted from one individual to another, their main function is to bring antigen in the body to the surface of cells for recognition by T cells. T-cell activation will occur only when antigen is combined with MHC molecules on the surface of other cells. The genes that encode these cell-surface molecules are the system of genes known as the MHC.

Genes Coding for MHC Molecules (HLA Antigens)

The MHC system is the most polymorphic system found in humans.[7,8] MHC genes code for proteins that play a pivotal role in immune recognition and it is thought that this polymorphism is essential to our survival because it allows for an immune response to diverse immunogens.[7] Genes coding for the MHC molecules in humans are found on the short arm of chromosome 6 and are divided into three categories or classes. Class I genes are found at three different locations or loci, termed A, B, and C. Class II genes are situated in the D region, and there are several different loci, known as DR, DQ, and DP. For class I molecules, there is only one gene coding for each particular molecule. Class II molecules, in contrast, have one gene that codes for the α chain and one or more genes that code for the β chain. The area of class III genes lies between the class I and class II regions on chromosome 6. Class III genes code for the C4A, C4B, C2, and B complement proteins, as well as cytokines such as tumor necrosis factor (TNF) **(Fig. 2–6).** The class I and II molecules are involved in antigen recognition; in this role, they influence the repertoire of antigens to which T cells can respond. In contrast, class III molecules are secreted proteins that have an immune function, but they are not expressed on cell surfaces, as are class I and II. Class III molecules also have a completely different structure compared with the other two classes.

At each of these loci, or locations, there is the possibility of multiple alleles. **Alleles** are alternate forms of a gene that code for slightly different varieties of the same product. The MHC system is described as polymorphic because there are so many possible alleles at each location. There are more than 2,013 different alleles of HLA-A, over 2,605 alleles of HLA-B, and 1,551 alleles of HLA-C that have been identified at this time.[1,8]

The probability that any two individuals will express the same MHC molecules is very low. One individual inherits two copies of chromosome 6; thus, there is a possibility of

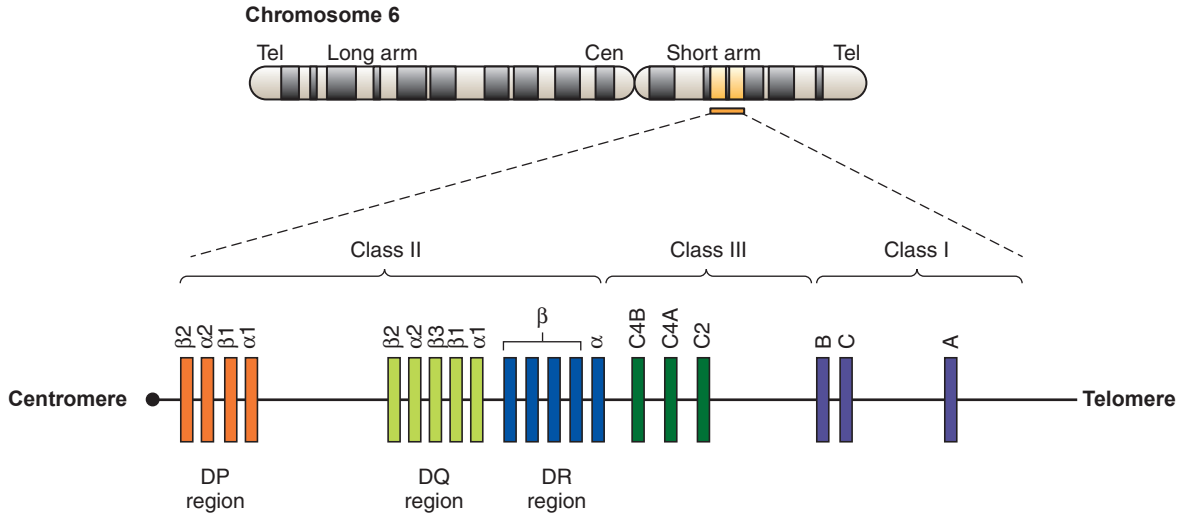


FIGURE 2–6 The major histocompatibility complex. Location of the class I, II, and III genes on chromosome 6. Class I consists of loci A, B, and C, whereas class II has at least three loci: DR, DQ, and DP.

two different alleles for each gene on the chromosome unless that person is homozygous (has the same alleles) at a given location. These genes are described as *codominant,* meaning that all alleles that an individual inherits code for products that are expressed on cells. Because the MHC genes are closely linked, they are inherited together as a package called a **haplotype.** Thus, each inherited chromosomal region consists of a package of genes for A, B, C, DR, DP, and DQ. The full genotype would consist of two of each gene at a particular locus (see Chapter 16).

One haplotype is inherited from each parent. Because there are numerous alleles or variant forms at each locus, an individual's MHC type is about as unique as a fingerprint. Because of the tremendous diversity of alleles, more than 1.7 billion different class I haplotypes alone are possible in the human population.[1] The frequency of particular HLA alleles differs significantly among different ethnic populations and can sometimes be used to trace one's ancestry or ethnic origins.[7]

Traditionally, HLA nomenclature had been defined serologically through the use of a battery of antibodies. Advances in DNA analysis have made identification of actual genes possible. The nomenclature has become correspondingly more complex. For instance, the notation HLA DRB1*1301 indicates the actual gene involved in coding for the β chain of an HLA DR1 antigen is the number 13 and the 01 is a specific subtype. The uniqueness of the HLA antigens creates a major problem in matching organ donors to recipients because these antigens are highly immunogenic. However, in cases of disputed paternity, polymorphisms can be used as a helpful identification tool.

Structure of Class I and II MHC Molecules

Each of the MHC genes codes for a protein molecule that appears on cell surfaces. All the proteins of a particular class share structural similarities and are found on the same types of cells **(Fig. 2–7).** Whereas **class I MHC (HLA) molecules** are expressed on all nucleated cells, **class II MHC (HLA) molecules** are found primarily on APCs. Their differing structures, as explained in the text that follows, are tailored to their specific functions in the adaptive immune response.

Structure of Class I MHC Molecules

Although class I MHC molecules are expressed on all nucleated cells, they differ in the level of expression. They are highest on lymphocytes and myeloid cells and low or undetected on liver hepatocytes, neural cells, muscle cells, and sperm.[4,7] This may explain why HLA matching is not done in the case of liver transplants. Additionally, HLA-C antigens are expressed at a much lower level than HLA-A and HLA-B antigens, so the latter two are the most important to match for transplantation.[7]

Each class I antigen is a glycoprotein dimer made up of two noncovalently linked polypeptide chains. The α chain has a molecular weight of 44,000.[4,7,8] A lighter chain associated with it, called a β_2–microglobulin, has a molecular weight of 12,000 and is encoded by a single gene on chromosome 15 that is not polymorphic.[8] This means that every class I molecule contains the same β_2–microglobulin. The α chain is folded into three domains—α1, α2, and α3—and it is inserted into the cell membrane via a transmembrane segment that is hydrophobic.[4,7] The three external domains consist of about 90 amino acids each; the transmembrane domain has about 25 hydrophobic amino acids along with a short stretch of about 5 hydrophilic amino acids, as well as an anchor of 30 amino acids.[1]

β_2–microglobulin does not penetrate the cell membrane, but it is essential for proper folding of the α chain. X-ray crystallographic studies indicate that the α1 and α2 domains each form an alpha helix and that these serve as the walls of a deep groove at the top of the molecule that functions as

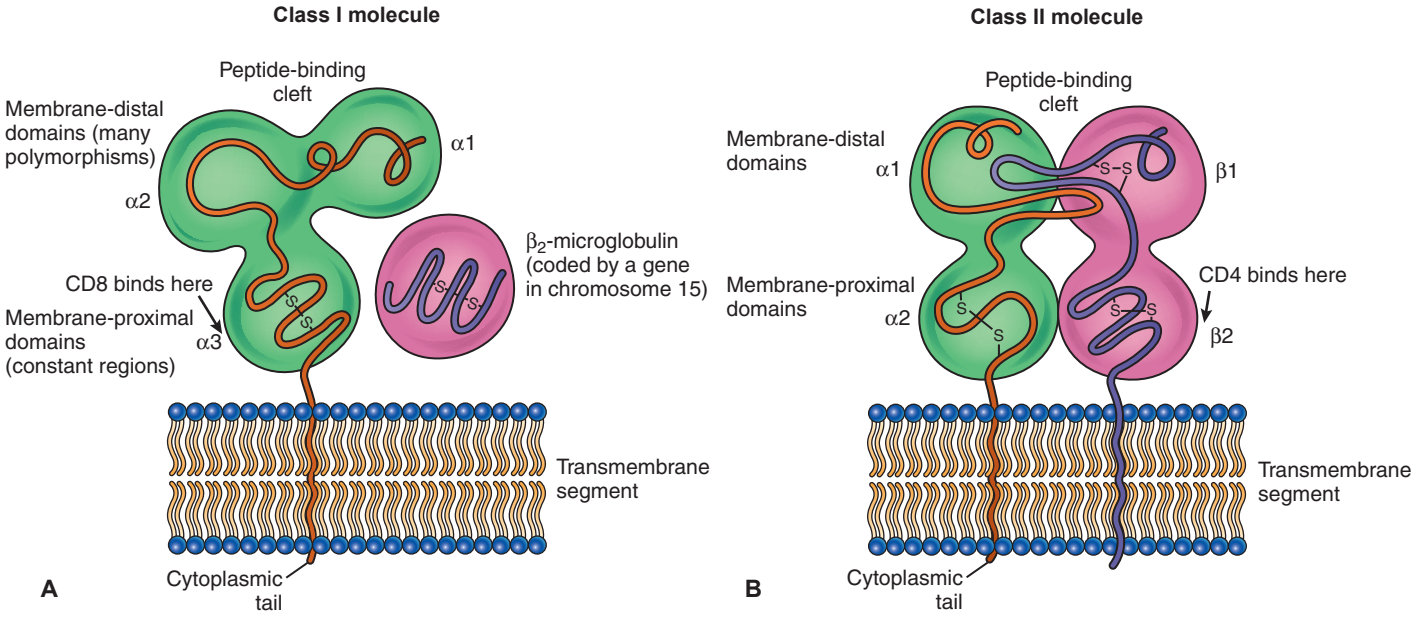


FIGURE 2–7 Structure of (A) class I and (B) class II MHC products. Class I MHC molecules consist of an α chain with three domains and a shorter chain, β_2–microglobulin, common to all class I molecules. Class II MHC molecules consist of an α chain and a β chain, each of which has two domains.

the peptide-binding site in antigen recognition.[4,7] This binding site is able to hold peptides that are between 8 and 11 amino acids long. Most of the polymorphism resides in the α1 and α2 regions, whereas the α3 and β2 regions are relatively constant.[4,7] The α3 region reacts with CD8 on cytotoxic T cells. Binding of peptides to class I and class II molecules is not as specific as the binding of peptides to T-cell receptors (TCRs) because numerous different peptides can bind to a particular MHC molecule.[1]

Another group of molecules called the nonclassical class I antigens are designated E, F, and G. This group of molecules, except for G, are not expressed on cell surfaces and do not function in antigen recognition but may play other roles in the immune response. G antigens are expressed on fetal trophoblast cells during the first trimester of pregnancy, where they come in direct contact with maternal tissue. There the G antigens are thought to help ensure tolerance for the fetus by protecting placental tissue from the action of NK cells. The G antigens bind to NK inhibitory receptors and turn off the NK cytotoxic response.[6] (See Chapter 3 for the action of NK cells.) E molecules play a similar role. The function of the F antigens is unknown at this time.[8]

Structure of Class II MHC Molecules

Class II MHC molecules are found on the APCs that include B lymphocytes, monocytes, macrophages, dendritic cells, and thymic epithelium[4,7] Because dendritic cells are the most effective APCs, they have the highest levels of class II molecules on their surface.[1]

The major class II molecules—DP, DQ, and DR—consist of two noncovalently bound polypeptide chains that are encoded by separate genes in the MHC complex. These molecules are called heterodimers because they contain two different chains. DR is expressed at the highest level because it accounts for about one-half of all the class II molecules on a particular cell.[7] The DRβ gene is the most highly polymorphic; close to 2000 different alleles are known at this time.[9] DP molecules are found in the shortest supply.[7]

Both the α chain, with a molecular weight of 34,000, and the β chain, with a molecular weight of 29,000, are anchored to the cell membrane.[4,8] Each chain has two domains and the α1 and β1 domains come together to form the peptide-binding site, similar to the one found on class I molecules[7] (see Fig. 2–7). However, both ends of the peptide-binding cleft are open and thus allow class II molecules to capture longer peptides than class I molecules. The α1 domains and the β2 domains are highly conserved in a similar manner to the class I molecules. At least three other class II genes have been described—DM, DN, and DO, the so-called nonclassical class II genes. Products of these genes play a regulatory role in antigen processing.[7]

DM helps to load peptides onto class II molecules, whereas DO modulates antigen binding. The function of DN is not known at this time.[10]

Role of Class I and II Molecules in the Immune Response

The main role of the class I and II MHC molecules is in **antigen presentation,** the process by which degraded peptides within cells are transported to the plasma membrane where T cells can then recognize them. T cells can only "see" and respond to antigens when they are combined with MHC molecules.

Whereas one individual can express only a small number of MHC molecules, each molecule can present a large number of different antigenic peptides to T cells.[1] This allows the body to respond to many different antigens, which is important for survival of the species. It is thought that the two main classes of these molecules have evolved to deal with two types of infectious agents: those that attack cells from the outside (such as bacteria) and those that attack from the inside (viruses and other intracellular pathogens). Class I molecules mainly present peptides synthesized within the cell to CD8 (cytotoxic) T cells, whereas class II molecules present exogenous antigen to CD4 (helper) T cells. Exogenous proteins presented by class II molecules are those taken into the cell from the outside and degraded.[11,12] Class I molecules are thus the watchdogs of viral, tumor, and certain parasitic antigens that are synthesized within the cell, whereas class II molecules help to mount an immune response to bacterial infections or other pathogens found outside cells.[11,13] In either case, for a T-cell response to be triggered, peptides must be available in adequate supply for MHC molecules to bind, they must be able to be bound effectively, and they must be recognized by a TCR.[2] Some viruses, such as herpes simplex and adenovirus, have managed to block the immune response by interfering with one or more processes involved in antigen presentation.[2] These viruses are able to maintain a lifelong presence in the host.

The difference in functioning of the two molecules is tied to the mechanisms by which processed antigen is transported to the surface. Both types of molecules, however, must be capable of presenting an enormous array of different antigenic peptides to T cells. The chemistry of the MHC antigens controls what sorts of peptides fit in the binding pockets. These two pathways are discussed here.

Class I MHC-Peptide Interaction

Class I molecules are synthesized in the rough endoplasmic reticulum and for a time they remain anchored in the endoplasmic reticulum membrane. It is here that these molecules bind peptides. This is known as the endogenous pathway of antigen presentation because antigens that bind to class I proteins are actually synthesized in the same cell as the class I molecules.[14,15] In fact, binding of the newly synthesized proteins helps to stabilize the association of the α chain of class I with the β_2–microglobulin.[10] However, before binding with antigen occurs, newly synthesized α chains freely bind a molecule called *calnexin.* This 88-kd molecule is membrane-bound in the endoplasmic reticulum and keeps the α chain in a partially folded state while it awaits binding to β_2–microglobulin.[11,14] Another molecule, ERp57, binds to this complex also. When β_2–microglobulin binds, calnexin and ERp57 are released and two other chaperone molecules—calreticulin and tapasin—associate with the complex and help to stabilize it for peptide binding[14,16] **(Fig. 2–8).**

Peptides that bind with the class I molecules are approximately 8 to 11 amino acids in length and are derived from partial digestion of proteins synthesized in the cytoplasm. These intracellular peptides may include viral, tumor, or even bacterial antigens.[8] Other peptides that may also bind are from newly made cellular proteins that fail to fold correctly and hence are defective.

Digestion of these intracellular proteins is carried out by proteases that reside in large cytoplasmic complexes called *proteasomes.*[1,11] Proteasomes are packets of enzymes formed into a cylindrical shape through which peptides pass and are cleaved. Peptides must be unfolded before entering the cylindrical chamber of the proteasome; they are then cleaved into the proper size for delivery to class I molecules. Once cleaved, the peptides must then be pumped from the cytoplasm to the lumen of the endoplasmic reticulum by specialized transporter proteins.[13] Two proteins, **transporters associated with antigen processing (TAP1 and TAP2),** are responsible for the adenosine triphosphate-dependent transport of peptides suitable for binding to class I molecules.[1,14,16,17] TAP1 and TAP2 are most efficient at transporting peptides that are between 8 to 16 amino acids in size.[13,17] Tapasin brings the TAP transporters into close proximity to the newly formed MHC molecules and mediates interaction with them so that peptides can be loaded onto the class I molecules.[11,13] Once the α chain has bound the peptide, the class I MHC peptide complex is rapidly transported to the cell surface[8] (see Fig. 2–8).

Of the thousands of peptides that may be processed in this manner, only a small fraction of them (1% or less) actually induce a T-cell response.[13] Binding is based on interaction of only two or three amino acid residues with the class I binding groove. Different class I molecules will have slightly different binding affinities and these small differences determine to which particular antigens one individual will respond.

It is estimated that a single cell may express between 100,000 and 200,000 copies of each class I molecule, so many different peptides can be captured and expressed in this manner.[8] As few as 10 to 100 identical antigen class I MHC complexes can induce a cytotoxic response.[13] In healthy cells, most of these class I MHC complexes contain self-peptides that are ignored by the T cells, whereas in diseased cells peptides are derived from viral proteins or proteins associated with cancerous states. Display of hundreds of class I molecules complexed to antigen allows CD8+ T cells to continuously check cell surfaces for the presence of nonself antigen. If it recognizes an antigen as being foreign, the CD8+ T cell produces cytokines that cause lysis of the entire cell **(Fig. 2–9).**

Class II MHC–Peptide Interaction

Class II MHC molecules participate in the exogenous pathway of antigen presentation. This means that antigen is taken into the cell from the outside by means of either phagocytosis or endocytosis, processes by which cells ingest extracellular molecules by enclosing them in a small portion of the plasma membrane. Dendritic cells, the most potent activators of T cells, are excellent at capturing and digesting exogenous antigens such as bacteria. Hydrolytic enzymes within the endosomes digest antigen into peptides of 13 to 18 amino acids in length.[1]

Class II molecules are synthesized in the endoplasmic reticulum and associate with a protein called the **invariant chain (Ii).** Because the open structure of class II molecules would permit

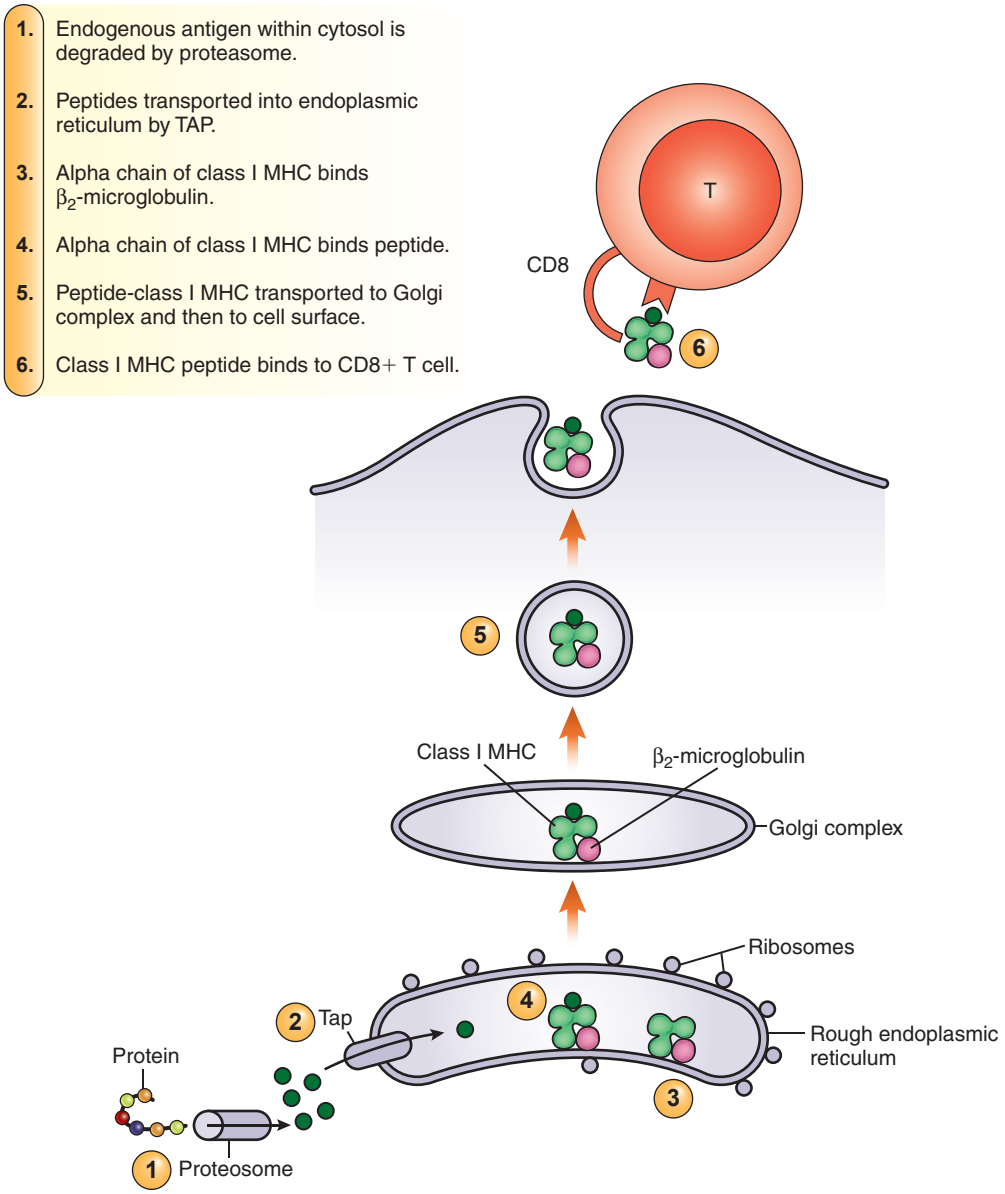


FIGURE 2–8 Antigen-processing pathway for endogenous antigens.

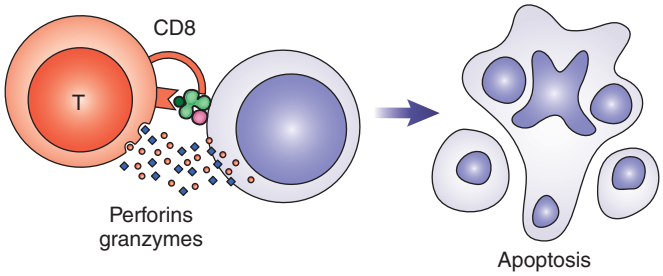


FIGURE 2–9 The CD8+ T cell recognizes antigen in association with class I MHC. If the antigen is recognized as being foreign, cytokines are released, causing destruction of the target cell.

binding of segments of endogenous peptides within the ER, Ii serves to protect the binding site.[8,15] This chain is a 31-kd protein that is made in excess so that enough is available to bind with all class II molecules shortly after they are synthesized. Ii may be responsible for helping to bring α and β chains together in the ER lumen and then moving them out through the Golgi complex to the endocytic vesicles where digested antigen is found.[15] Unlike class I molecules, class II molecules must be transported from the endoplasmic reticulum (ER) to an endosomal compartment where they can then bind peptides[17] **(Fig. 2–10)**.

Once transported to an endosomal compartment, class II molecules encounter peptides derived from endocytosed, exogenous proteins. The invariant chain is gradually degraded by a protease, leaving just a small fragment called *class II invariant chain peptide* (CLIP) attached to the peptide-binding cleft.[1,15] CLIP is then exchanged for exogenous peptides. Selective binding of peptides is favored by the low pH of the endosomal compartment.[14] HLA-DM molecules help to mediate the reaction by removing the CLIP fragment and helping to load peptides into the binding groove.[1,15] Generally, peptides of approximately 13 to 18 amino acid residues can bind because the groove is open on both ends, unlike

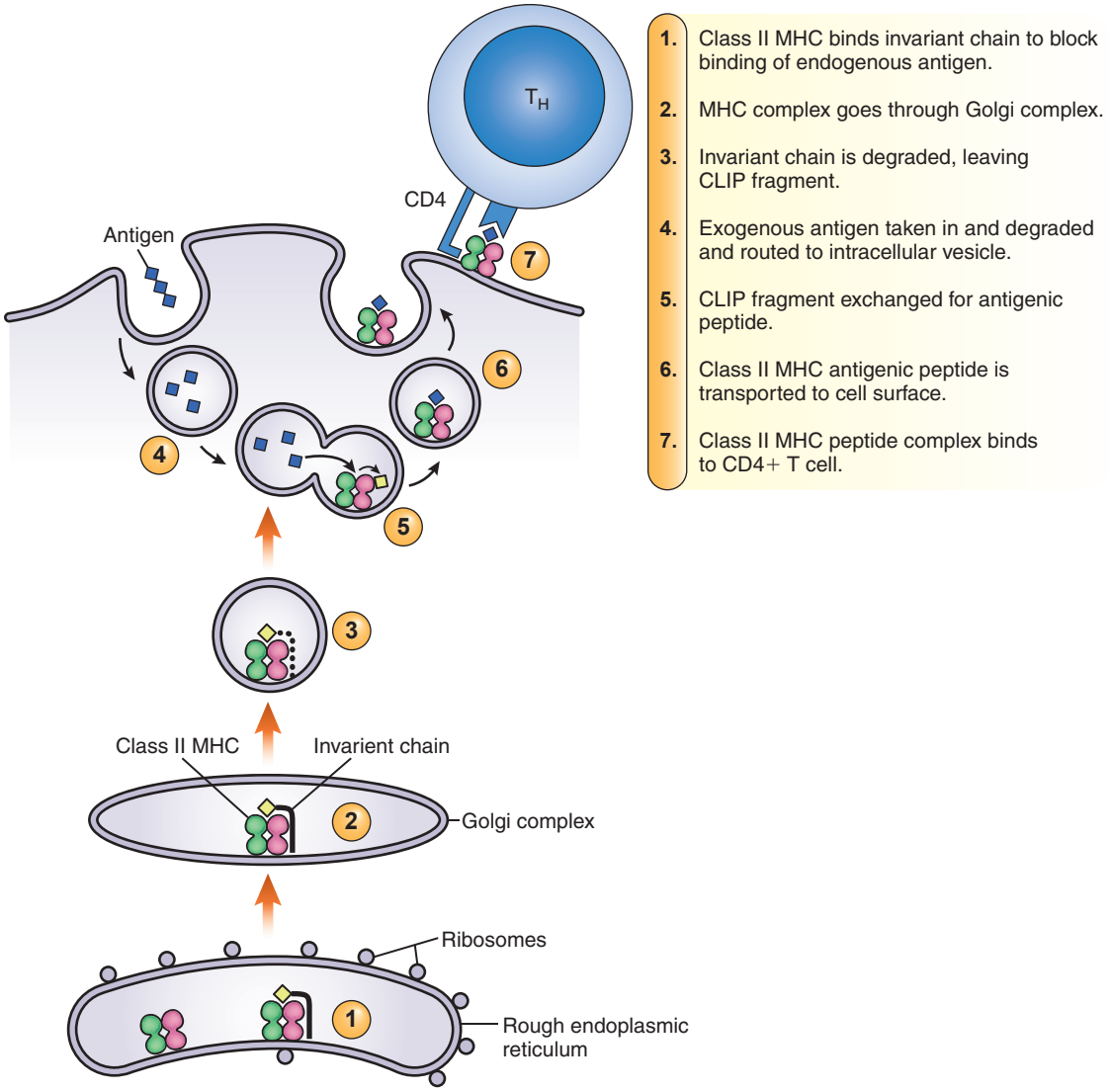


FIGURE 2–10 Antigen-processing pathway for exogenous antigen. The binding site of class II MHC molecules is first occupied by an invariant chain (Ii). This is degraded and exchanged for short exogenous peptides in an endosomal compartment. The exogenous peptide class II MHC complex is then transported to the cell surface.

class I molecules, which have a closed end.[12,18,19] Within a central core of the bound peptide, 7 to 10 residues provide the major contact points.[1]

Hydrogen bonding takes place along the length of the captured peptide; this is in contrast to class I molecules, which only bond at the amino and carboxy-terminal ends.[19,20] There are also several pockets in the class II proteins that easily accommodate amino acid side chains. This gives class II proteins more flexibility in the types of peptides that can be bound.[19,20] Once binding has occurred, the class II protein-peptide complex is stabilized and is transported to the cell surface (see Fig. 2–10). On the cell surface, class II molecules are responsible for forming a trimolecular complex that occurs between antigen, class II molecule, and an appropriate TCR. If binding occurs with a TCR on a CD4+ T cell, the T helper (Th) cell recruits and triggers a B-cell response, resulting in antibody formation **(Fig. 2–11).**

Clinical Significance of MHC

Testing for MHC antigens has typically been carried out before tissue transplant procedures because both class I and class II molecules can induce a response that leads to graft rejection. Testing methodology is transitioning from serological principles to molecular methods because they are more accurate. The role of the laboratory in transplantation is presented in Chapter 16. MHC antigens also appear to play a role in the development of autoimmune diseases. Inheritance of certain HLA antigens appears to predispose a person to certain autoimmune diseases. The closest link known is

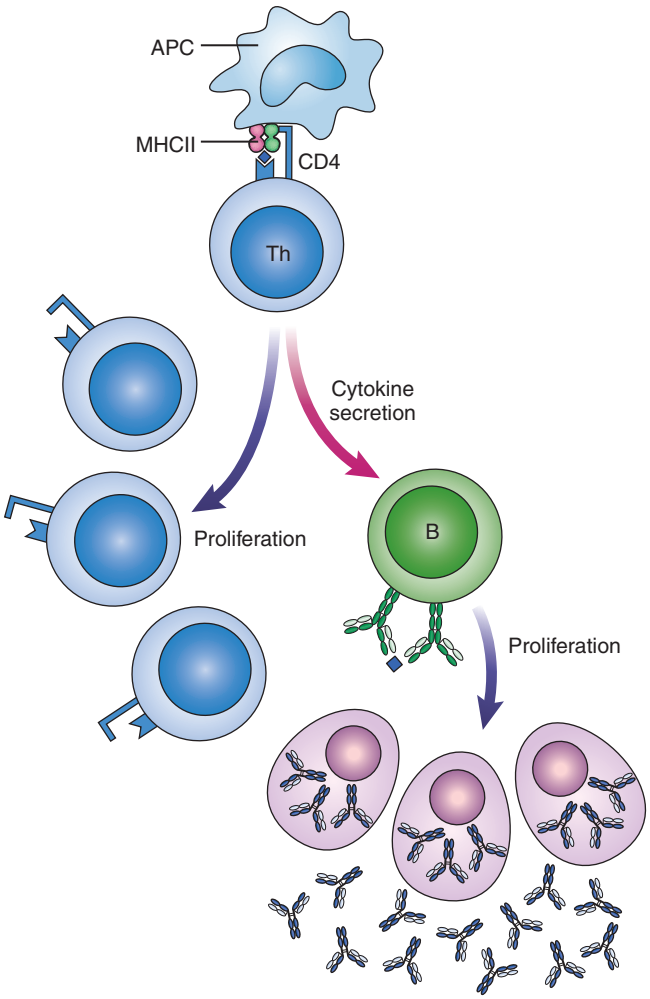


FIGURE 2–11 CD4+ T cells recognize exogenous antigen on phagocytic antigen-presenting cells along with class II MHC. CD4+ helper T cells are stimulated by contact with antigen and clonal expansion takes place. These cells secrete cytokines that cause an antigen-activated B cell to proliferate and produce plasma cells, which make antibody.

between inheritance of HLA B27 and the disease called ankylosing spondylitis, a progressive chronic inflammatory disorder affecting the vertebrae of the spine.[21] See **Table 2–1** for other links between HLA antigens and diseases. This topic is discussed more fully in Chapter 15.

However, the evidence that both class I and class II molecules play a major role in antigen presentation has more far-reaching consequences. They essentially determine the types of peptides to which an individual can mount an immune response. Although the MHC molecules typically have a broad binding capacity, small biochemical differences in these proteins are responsible for differences seen in the ability to react to a specific antigen. It is possible that nonresponders to a particular vaccine such as hepatitis B do not have the genetic capacity to respond. On the other hand, the presence of a particular MHC protein may confer additional protection, as the example of HLA B8 and increased resistance to HIV infection shows.[1] Therefore, it will be important to know an individual's MHC type for numerous reasons.

Much of the recent research has focused on the types of peptides that can be bound by particular MHC molecules.[19,22,23] Future developments may include tailoring vaccines to certain groups of such molecules. As more is learned about antigen processing, researchers can specifically develop vaccines containing certain amino acid sequences that serve as immunodominant epitopes. These vaccines may avoid the risk associated with using live organisms. Additionally, if an individual suffers from allergies, knowing a person's MHC type may also help predict the types of allergens to which he or she may respond.[24] Certain drug hypersensitivities have also been linked to particular HLA alleles and knowledge of one's HLA genes might prevent severe reactions to these drugs.[25] Another area for future research is the development of possible tumor vaccines based on an individual's MHC type.[17] It is likely that knowledge of the MHC molecules will affect many areas of patient care in the future.

Table 2–1 Association of HLA Alleles and Disease

DISEASE	SYMPTOMS	HLA ALLELE	STRENGTH OF ASSOCIATION
Ankylosing spondylitis	Inflammation of the vertebrae of the spine	B27	+++
Celiac disease	Diarrhea, weight loss, intolerance to gluten	DQ2 DQ8	+++ +
Rheumatoid arthritis	Inflammation of multiple joints	DR4	+
Type 1 diabetes	Increase in blood glucose because of destruction of insulin-producing cells	DQ8 DQ2	++ +

+++ = very strong association, ++ = strong association, + = clear association, – = negative association. (From Margulies DH, Natarajan K, Rossjohn J, and McCluskey J. Major histocompatibility complex (MHC) molecules: structure, function, and genetics. In: Paul WE ed. Fundamental Immunology. *6th ed. Philadelphia, PA: Wolters Kluwer, Lippincott Williams & Wilkins; 2012; Ch 21:487–523.)*

SUMMARY

- Immunogens are macromolecules that elicit formation of immunoglobulins or sensitized cells in an immunocompetent host.
- The term antigen is sometimes used to denote a substance that does not elicit a host response but reacts with antibody once it has been formed.
- Immunogenicity is influenced by factors such as age, health, route of inoculation, and genetic capacity.
- Most immunogens have a molecular weight of at least 100,000, molecular complexity, and are foreign to the host. Although immunogens are fairly large molecules, the immune response is keyed to only small portions of these molecules, or epitopes, and very small differences in these epitopes can be detected by the immune system.
- Haptens are nonimmunogenic substances that must be combined with a carrier in order to provoke an immune response. They are too small to initiate a response by themselves.
- Once an antibody response is generated, haptens are capable of reacting with that antibody, but precipitation or agglutination will not occur.
- Adjuvants are substances that can be mixed with antigen to enhance the immune response. Most adjuvants work by keeping the antigen in the area and by increasing the number of cells involved in the immune response.
- Antigens can be characterized by their relationship to the host. Autoantigens are those that belong to the host; alloantigens are from the same species as the host but are not identical to the host; heteroantigens are from other species.
- Heterophile antigens exist in unrelated species, but their structure is so similar that antibody formed to one will cross-react with antigen from a different species.
- The major histocompatibility complex (MHC) encodes class I and class II molecules, which play a major role in antigen presentation to T cells.
- The MHC has so many different alleles that it is considered the most polymorphic system found in humans.
- Class I and class II molecules bind peptides within cells and transport them to the plasma membrane, where the peptides can be recognized by T cells.
- Class I MHC molecules are found on all nucleated cells; these molecules associate with foreign antigens, such as viral proteins, synthesized within a host cell. This is known as the endogenous pathway for antigen presentation.
- Class II molecules are only found on B cells, monocytes, macrophages, dendritic cells, and thymic epithelium. These molecules associate with foreign antigens taken into the cell from the outside, in the pathway known as exogenous antigen presentation.
- Class I MHC molecules consist of an α chain encoded by the MHC complex, as well as a second lighter chain called β_2–microglobulin, encoded by a gene on chromosome 15.
- Class II MHC molecules have an α and a β chain, both of which are encoded by genes in the MHC complex.
- Class I molecules present antigen to CD8+ T cells, triggering a cytotoxic reaction.
- Class II molecules present antigen to CD4+ T cells, which are helper cells involved in antibody production.

Study Guide: A Comparison of Class I and Class II MHC Molecules

	CLASS I MHC MOLECULES	CLASS II MHC MOLECULES
Cellular Distribution	All nucleated cells	B cells, monocytes, macrophages, dendritic cells, thymic epithelial cells
Structure	One α chain and β_2–microglobulin	An α chain and a β chain
Classes	A, B, C	DP, DQ, DR
Size of Peptides Bound	8 to 11 amino acids	13 to 18 amino acids
Nature of Peptide Binding Cleft	Closed at both ends	Open at both ends
Interaction with T Cells	Presents endogenous antigen to CD8+ T cells	Presents exogenous antigen to CD4+ T cells

CASE STUDY

A 15-year-old boy needs to have a kidney transplant because of the effects of severe diabetes. His family members consist of his father, mother, and two sisters. All of them are willing to donate a kidney so that he can come off dialysis. He is also on a list for a cadaver kidney. His physician suggests that the family be tested first for the best HLA match.

Questions

a. How many alleles are shared by mother and son? Father and son?
b. What are the chances that one of the sisters would be an exact match?
c. Is there a possibility that a cadaver kidney might be a better match than any of the family members'?

REVIEW QUESTIONS

1. All of the following are characteristics of an effective immunogen *except*
   a. internal complexity.
   b. large molecular weight.
   c. the presence of numerous epitopes.
   d. found on host cells.

2. Which of the following best describes a hapten?
   a. Cannot react with antibody
   b. Antigenic only when coupled to a carrier
   c. Has multiple determinant sites
   d. A large chemically complex molecule

3. Which would be the most effective immunogen?
   a. Protein with a molecular weight of 200,000
   b. Nylon polymer with a molecular weight of 250,000
   c. Polysaccharide with a molecular weight of 220,000
   d. Protein with a molecular weight of 175,000

4. Which of the following individuals would likely respond most strongly to a bacterial infection?
   a. An adult who is 75 years of age
   b. A malnourished 40-year-old
   c. A weightlifter who is 35 years old
   d. A newborn baby

5. Which best describes an epitope?
   a. A peptide that must be at least 10,000 MW
   b. An area of an immunogen recognized only by T cells
   c. A segment of sequential amino acids only
   d. A key portion of the immunogen

6. Adjuvants act by which of the following methods?
   a. Protects antigen from being degraded
   b. Facilitates rapid escape from the tissues
   c. Limits the area of the immune response
   d. Decreases number of APCs

7. A heterophile antigen is one that
   a. is a self-antigen.
   b. exists in unrelated plants or animals.
   c. has been used previously to stimulate antibody response.
   d. is from the same species but is different from the host.

8. Which of the following is true of class II MHC (HLA) antigens?
   a. They are found on B cells and macrophages.
   b. They are found on all nucleated cells.
   c. They all originate at one locus.
   d. They are coded for on chromosome 9.

9. Class II MHC molecules are recognized by which of the following?
   a. CD4+ T cells
   b. CD8+ T cells
   c. Natural killer cells
   d. Neutrophils

10. Which of the following best describes the role of TAP?
    a. They bind to class II molecules to help block the antigen-binding site.
    b. They bind to class I proteins in proteasomes.
    c. They transport peptides into the lumen of the endoplasmic reticulum.
    d. They help cleave peptides for transport to endosomes.

11. What is the purpose of the invariant chain in antigen processing associated with class II MHC molecules?
    a. Helps transport peptides to the binding site
    b. Blocks binding of endogenous peptides
    c. Binds to CD8+ T cells
    d. Cleaves peptides into the proper size for binding

12. An individual is recovering from a bacterial infection and tests positive for antibodies to a protein normally found in the cytoplasm of this bacterium. Which of the following statements is true of this situation?

a. Class I molecules have presented bacterial antigen to CD8+ T cells.
b. Class I molecules have presented bacterial antigen to CD4+ T cells.
c. Class II molecules have presented bacterial antigen to CD4+ T cells.
d. B cells have recognized bacterial antigen without help from T cells.

13. In relation to a human, alloantigens would need to be considered in which of the following events?

a. Transplantation of a kidney from one individual to another
b. Vaccination with the polysaccharide coat of a bacterial cell
c. Oral administration of a live but heat-killed virus particle
d. Grafting skin from one area of the body to another

14. Which is characteristic of class I MHC molecules?

a. Consists of one α and one β chain
b. Binds peptides made within the cell
c. Able to bind whole proteins
d. Coded for by DR, DP, and DQ genes

15. Class I MHC antigens E and G serve which function?

a. Enhance the response by macrophages
b. Transport antigen for recognition by CD4+ T cells
c. Bind to A, B, and C antigens to protect the binding site
d. Protect fetal tissue from destruction by NK cells

16. Which best explains the difference between immunogens and antigens?

a. Only antigens are large enough to be recognized by T cells.
b. Only immunogens can react with antibody.
c. Only immunogens can trigger an immune response.
d. Only antigens are recognized as foreign.

17. When a child inherits one set of six HLA genes together from one parent, this is called a(n)

a. genotype.
b. haplotype.
c. phenotype.
d. allotype.

18. HLA molecules A, B, and C belong to which MHC class?

a. Class I
b. Class II
c. Class III
d. Class IV

3 Innate Immunity

Christine Dorresteyn Stevens, EdD, MT(ASCP)

LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Differentiate between the external and internal defense systems.
2. Give examples of several external defense mechanisms.
3. Describe how normal flora act as a defense against pathogens.
4. Explain what a pathogen-associated molecular pattern (PAMP) is and give some examples.
5. Discuss the role of pathogen recognition receptors (PRRs) in both the innate and adaptive immune responses.
6. Describe the function of Toll-like receptors (TLRs).
7. Discuss the role of acute-phase reactants in the innate immune response.
8. Explain how each of the following acute-phase reactants contributes to innate immunity: C-reactive protein (CRP), serum amyloid A, complement, alpha$_1$-antitrypsin, haptoglobin, fibrinogen, and ceruloplasmin.
9. Determine the significance of abnormal levels of acute-phase reactants.
10. Describe the process of inflammation.
11. List the steps in the process of phagocytosis.
12. Discuss the intracellular mechanism for destruction of foreign particles during the process of phagocytosis.
13. Explain the importance of phagocytosis in both innate and adaptive immunity.
14. Explain how natural killer (NK) cells recognize target cells.
15. Describe two methods that NK cells use to kill target cells.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Acute-phase reactants
Alpha$_1$-antitrypsin (AAT)
Antibody-dependent cell cytotoxicity (ADCC)
Ceruloplasmin
Chemotaxis
Complement
C-reactive protein (CRP)
Diapedesis
External defense system
Fibrinogen
Haptoglobin
Inflammation
Innate immunity
Internal defense system
Opsonins
Oxidative burst
Pathogen-associated molecular patterns (PAMPs)
Pathogen recognition receptors (PRRs)
Phagocytosis
Phagolysosome
Phagosome
Serum amyloid A (SAA)
Toll-like receptor (TLR)

Humans are protected by two systems of immunity—innate and adaptive—as discussed in Chapter 1. **Innate immunity** consists of the defenses against infection that are ready for immediate action when a host is attacked by a pathogen. If a pathogen manages to evade these defenses, there is a coordinated series of interactions between various cells and molecules to destroy any invading pathogens before disease can occur.[1] These defenses are considered nonadaptive or nonspecific; regardless of the infectious agent to which the body is exposed, innate immunity produces the same response. Components of innate immunity can be thought of as the first responders because they react immediately to infectious agents. Adaptive immunity, in contrast, is a more tailored response. It takes a longer time to be activated, but it is more specific and longer lasting. The two systems, however, are highly interactive and interdependent; innate immunity actually sets the stage for the more specific and longer lasting adaptive immune response.

The innate immune system is composed of two parts: the external defense system and the internal defense system. The external defense system consists of anatomical barriers designed to keep microorganisms from entering the body. If these defenses are overcome, then the internal defense system is triggered within minutes and clears invaders as quickly as possible. Internal defenses include cellular responses that recognize specific molecular components of pathogens. Both of these systems work together to promote phagocytosis. The process of **phagocytosis,** as defined in Chapter 1, is the engulfment and destruction of foreign cells or particulates by leukocytes, macrophages, and other cells. The process of inflammation brings cells and humoral factors to the injured area. If the healing process is begun and resolved as quickly as possible, the tissues are less likely to be damaged. The innate immune system is so efficient that most pathogens are destroyed before they ever encounter cells that are part of the adaptive immune response.

External Defense System

The **external defense system** is composed of physical, chemical, and biological barriers that function together to prevent most infectious agents from entering the body **(Fig. 3–1).** First and foremost are the unbroken skin and the mucosal membrane surfaces. The outer layer of the skin, the epidermis, contains several layers of tightly packed epithelial cells. These cells are coated with a protein called keratin, making the skin impermeable to

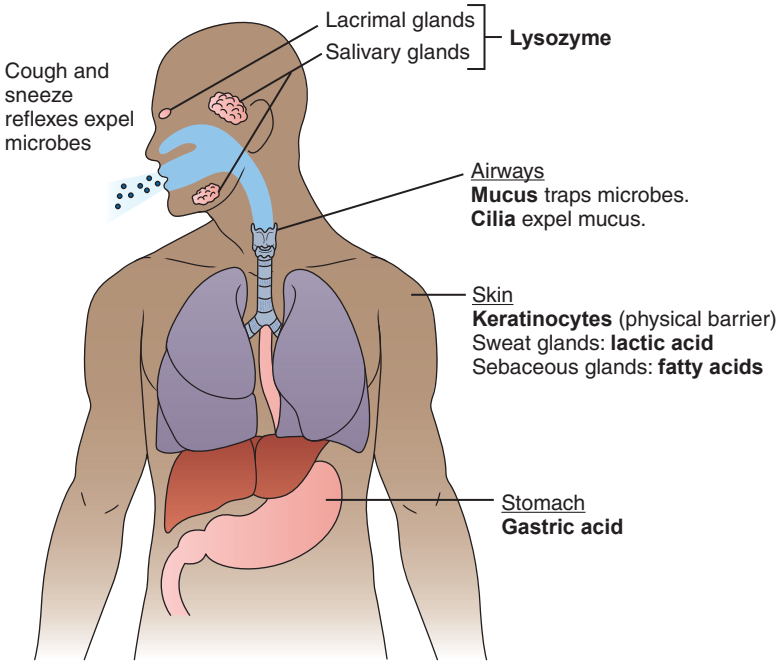


FIGURE 3–1 The external defense system.

most infectious agents. The outer skin layer is renewed every few days to keep it intact.[1] The dermis is a thicker layer just underneath the epidermis that is composed of connective tissue with blood vessels, hair follicles, sebaceous glands, sweat glands, and white blood cells (WBCs) including macrophages, dendritic cells, and mast cells. To understand how important a role the skin plays, one has only to consider how vulnerable victims of severe burns are to infection.

Not only does the skin serve as a major structural barrier, but the presence of several secretions on it discourages the growth of microorganisms. Lactic acid in sweat, for instance, and fatty acids from sebaceous glands maintain the skin at a pH of approximately 5.6. This acidic pH keeps most microorganisms from growing. In addition, human skin cells produce psoriasin, a small protein that has antibacterial effects, especially against gram-negative organisms such as *Escherichia coli.*

Additionally, each of the various organ systems in the body has its own unique mechanisms. In the respiratory tract, mucous secretions block the adherence of bacteria to epithelial cells. These secretions contain small proteins called surfactants that are produced by the epithelial cells and bind to microorganisms to help move pathogens out. The motion of the cilia that line the nasopharyngeal passages clears away almost 90% of the deposited material. The simple acts of coughing and sneezing also help to move pathogens out of the respiratory tract. The flushing action of urine, plus its slight acidity, helps to remove many potential pathogens from the genitourinary tract. Lactic acid production in the female genital tract keeps the vagina at a pH of about 5, which is another means of preventing invasion of pathogens. In the digestive tract, the stomach's hydrochloric acid keeps the pH as low as 1. We take in many microorganisms with food and drink and the low pH serves to halt microbial growth. Lysozyme—an enzyme found in many bodily secretions such as tears and saliva—attacks the cell walls of microorganisms, especially those that are gram-positive.

In many locations of the human body, the presence of normal flora (also called microbiota) helps to keep pathogens from establishing themselves in these areas. Normal flora is the mix of bacteria that are normally found at specific body sites and do not typically cause disease. Resident microorganisms in the gut, for example, may produce *colicins,* a type of protein that binds to the negatively charged surface of certain bacteria and kills them by penetrating the membrane.[2] The significance of the presence of normal flora is readily demonstrated by looking at the side effects of antimicrobial therapy. For example, women who take an antibiotic for a urinary tract infection (UTI) frequently develop a yeast infection because of the presence of *Candida albicans.* In this case, antimicrobial therapy wipes out not only the pathogenic bacteria but also the normal flora that would ordinarily compete with such opportunists that are usually present in very small numbers.

Internal Defense System

If microorganisms do penetrate the barriers of the external defense system, the innate immune system has additional mechanisms to destroy foreign invaders. The **internal defense system** is composed of both cells and soluble factors that have specific and essential functions. Cells that are capable of phagocytosis play a major role (see Chapter 1). Phagocytic cells engulf and destroy most of the foreign cells or particles that enter the body; this is the most important function of the internal defense system. Phagocytosis is enhanced by specific receptors on cells that capture invaders through identification of unique microbial substances. In addition, soluble factors called *acute-phase reactants* act by several different methods to either facilitate contact between microbes and phagocytic cells or mop up and recycle important proteins after the process of phagocytosis has taken place. Both cellular receptors and soluble factors are described in more detail here.

Pathogen Recognition Receptors

The internal defense system is designed to recognize molecules that are unique to infectious organisms.[3] Macrophages and dendritic cells constituting between 10% and 15% of the total cellular population in the tissues are the most important cells involved in pathogen recognition.[2] They are able to distinguish pathogens from normally present molecules in the body by means of receptors known as **pathogen recognition receptors (PRRs),** which are also found on neutrophils, eosinophils, monocytes, mast cells, T cells, and epithelial cells.[3-6] These receptors are encoded by the host's genomic DNA and act as sensors for extracellular infection. PRRs thus play a pivotal role as a second line of defense if microorganisms penetrate the external barriers.[6] Once these receptors bind to a pathogen, phagocytic cells become activated and are better able to engulf and eliminate any microorganisms.[2,6] Activated cells proceed to secrete proinflammatory cytokines and chemokines, chemical messengers that make capillaries more permeable and recruit additional phagocytic cell types to the area of infection. In addition, cytokines and chemokines also trigger the adaptive immune response.

PRRs are able to distinguish self from nonself by recognizing substances, known as **pathogen-associated molecular patterns (PAMPs),** that are only found in microorganisms. Some examples include peptidoglycan in gram-positive bacteria, lipoproteins in gram-negative bacteria, zymosan in yeast, and flagellin in bacteria with flagellae.[2]

Charles Janeway's discovery of the first receptor in humans, the **Toll-like receptor (TLR),** had a major impact on the understanding of innate immunity.[7] Toll is a protein originally discovered in the fruit fly *Drosophila,* where it plays an important role in antifungal immunity in the adult fly. Very similar molecules were found on human leukocytes and some other cell types. The highest concentration of these TLRs occurs on monocytes, macrophages, and neutrophils[5] **(Fig. 3–2).**

Ten different TLRs have been identified in humans; some are found on cell surfaces, whereas others are found in the cytoplasm.[2,5] **(Table 3–1).** TLR1, TLR2, TLR4, TLR5, and TLR6 are found on cell surfaces, whereas TLR3, TLR7, TLR8, and TLR9 are found in the endosomal compartment of a cell.[4] Each of these receptors recognizes a different microbial product. For example, TLR2 recognizes teichoic acid and peptidoglycan found in gram-positive bacteria; TLR4 recognizes

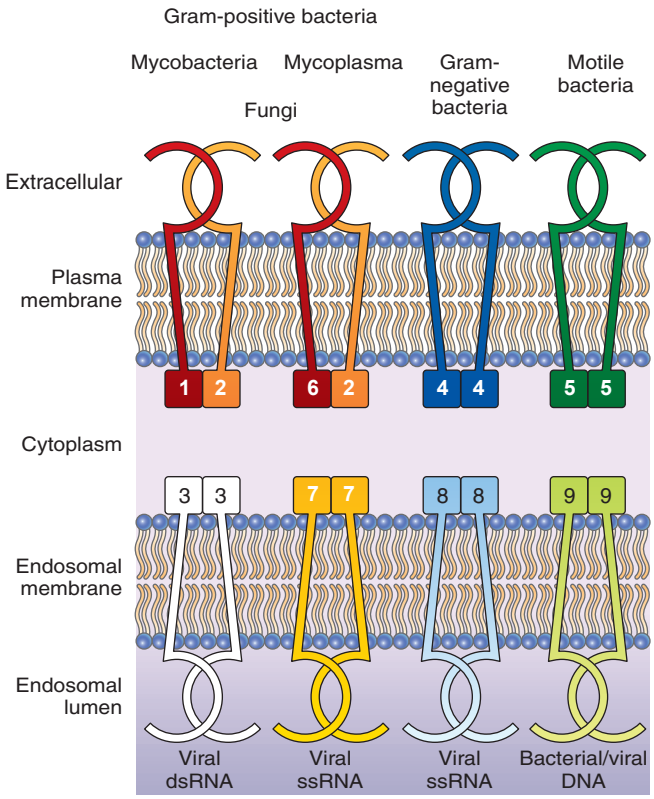


FIGURE 3–2 Toll-like receptors on a WBC membrane. Each of the 10 different TLRs recognizes a different pathogenic product. TLRs found on the cell surface tend to form dimers to increase chances of binding to a foreign substance.

lipopolysaccharide, which is found in gram-negative bacteria; and TLR5 recognizes bacterial flagellin **(Fig. 3–3).** The function of TLR10 is not yet known.

TLRs are membrane-spanning glycoproteins that share a common structural element called leucine-rich repeats (LRRs).[5] Once TLRs bind to their particular substances, host immune responses are rapidly activated by production of cytokines and chemokines. Neutrophils are recruited to the area because of increased capillary permeability; in addition, macrophages and dendritic cells become more efficient because of increased expression of adhesion molecules on their cell surfaces. These processes enhance phagocytosis and destroy most pathogens that humans are exposed to before disease sets in.

In addition to TLRs, there are several other families of receptors that activate innate immune responses. One such family is the C-type lectin receptor (CLR). CLRs are plasma membrane receptors found on monocytes, macrophages, dendritic cells, neutrophils, B cells, and T-cell subsets. These receptors bind to mannan and β-glucans found in fungal cell walls.[5] Although the initial signaling pathway differs from TLRs, the end result is the same—production of cytokines and chemokines to eliminate microbes.

Other families of receptors that recognize pathogens include retinoic acid-inducible gene-I-like receptors (RLRs) and nucleotide-binding oligomerization domain receptors (NOD). The RLR family recognizes RNA from RNA viruses in the cytoplasm of infected cells and induces inflammatory cytokines and type I interferons. Type I interferons inhibit viral replication and induce apoptosis (cell death) in infected cells. NOD receptors bind peptidoglycans found in bacterial cell walls and also help to protect against intracellular protozoan parasites.[1,5] Mutations in NOD receptors may result in Crohn's disease, a painful inflammatory disease of the bowel.[1]

Acute-Phase Reactants

In addition to the cells and receptors that enhance the destruction of pathogens, the internal defense system also consists of soluble factors called acute-phase reactants that contribute to the innate immune response. **Acute-phase reactants** are normal serum constituents that increase rapidly because of infection, injury, or trauma to the tissues. Many act by binding to

Table 3–1 The 10 Toll-Like Receptors

RECEPTOR	SUBSTANCE RECOGNIZED	TARGET MICROORGANISM
TLR Receptors Found on Cell Surfaces		
TLR1	Lipopeptides	Mycobacteria
TLR2	Peptidoglycan, lipoproteins, zymosan	Gram-positive bacteria, mycobacteria, yeasts
TLR4	Lipopolysaccharide, fusion proteins, mannan	Gram-negative bacteria, RSV fungi
TLR5	Flagellin	Bacteria with flagellae
TLR6	Lipopeptides, lipoteichoic acid, zymosan	Mycobacteria, gram-positive bacteria, yeasts
TLR Receptors Found in Endosomal Compartments		
TLR3	Double-stranded RNA	RNA viruses
TLR7	Single-stranded RNA	RNA viruses
TLR8	Single-stranded RNA	RNA viruses
TLR9	Double-stranded DNA	DNA viruses, bacterial DNA
TLR10	Unknown	Unknown

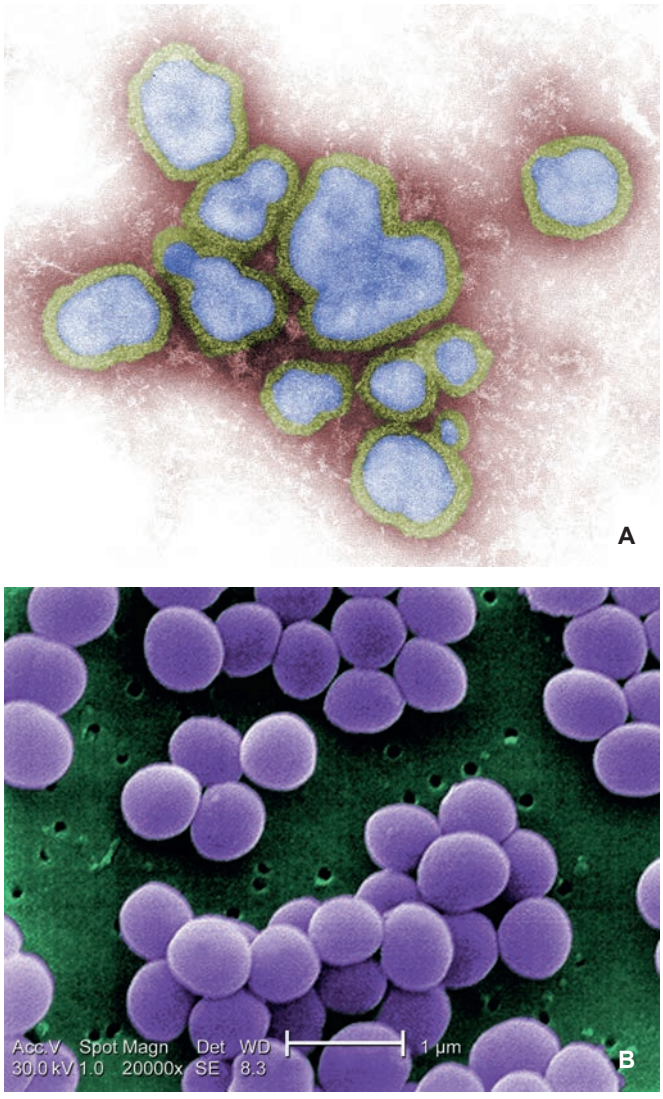


FIGURE 3–3 A. Digitally colored and negative stained transmission electron micrograph of the influenza A virus, an RNA virus that TLR3, TLR7, and TLR8 recognize as foreign. B. Scanning electron micrograph of *Staphylococcus aureus;* gram-positive bacteria that are recognized by the TLR2 receptor. *(A. Courtesy of the CDC/FA Murphy, Public Health Image Library. B. Courtesy of the CDC/Matthew Arduino, Public Health Image Library, DRPH.)*

microorganisms and promoting adherence, the first step in phagocytosis. Others help to limit destruction caused by the release of proteolytic enzymes from WBCs as the process of phagocytosis takes place. Some of the most important ones are C-reactive protein, serum amyloid A, complement components, alpha$_1$-antitrypsin, haptoglobin, fibrinogen, and ceruloplasmin.[8] They are produced primarily by hepatocytes (liver parenchymal cells) within 12 to 24 hours in response to an increase in cytokines (see Chapter 6 for a complete discussion of cytokines). The particular cytokines involved are interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), all of which are produced by monocytes and macrophages at the sites of inflammation.[1,9] **Table 3–2** summarizes characteristics of the main acute-phase reactants.

C-Reactive Protein

C-reactive protein (CRP) is a trace constituent of serum originally thought to be an antibody to the C-polysaccharide of pneumococci. It was discovered by Tillet and Francis in 1930 when they observed that serum from patients with *Streptococcus pneumoniae* infection precipitated with a soluble extract of the bacteria.[10] Now CRP is known to have a more generalized role in innate immunity.

The molecular weight of CRP is between 118,000 and 144,000 daltons[8] and has a structure that consists of five identical subunits held together by noncovalent bonds. It is a member of the family known as the pentraxins, all of which are proteins with five subunits. CRP acts somewhat like an antibody because it is capable of opsonization (the coating of foreign particles), agglutination, precipitation, and activation of complement by the classical pathway. However, binding is calcium-dependent and nonspecific. The main substrate is phosphocholine, a common constituent of microbial membranes. It also binds to small ribonuclear proteins; phospholipids; peptidoglycan; and other constituents of bacteria, fungi, and parasites.[11] In addition, CRP promotes phagocytosis by binding to specific receptors found on monocytes, macrophages, and neutrophils. Thus, CRP can be thought of as a primitive, nonspecific form of an antibody molecule that is able to act as a defense against microorganisms or foreign cells until specific antibodies can be produced.

Table 3–2 Characteristics of Acute-Phase Reactants

PROTEIN	RESPONSE TIME (HR)	NORMAL CONCENTRATION (MG/DL)	INCREASE	FUNCTION
C-reactive protein	4–6	0.5	1000X	Opsonization, complement activation
Serum amyloid A	24	5	1000X	Activates monocytes and macrophages
Alpha$_1$-antitrypsin	24	200–400	2–5X	Protease inhibitor
Fibrinogen	24	200–400	2–5X	Clot formation
Haptoglobin	24	40–290	2–10X	Binds hemoglobin
Ceruloplasmin	48–72	20–40	2X	Binds copper and oxidizes iron
Complement C3	48–72	60–140	2x	Opsonization, lysis

CRP is a relatively stable serum protein with a half-life of about 18 hours.[10,11] It increases rapidly within 4 to 6 hours following infection, surgery, or other trauma to the body. Levels increase dramatically as much as a hundredfold to a thousandfold, reaching a peak value within 48 hours.[10,11] CRP also declines rapidly with cessation of the stimuli.[10] Elevated levels are found in conditions such as bacterial infections, rheumatic fever, viral infections, malignant diseases, tuberculosis, and after a heart attack. The median CRP value for an individual increases with age, reflecting an increase in subclinical inflammatory conditions.[12]

Because the levels rise and then decline so rapidly, CRP is the most widely used indicator of acute inflammation. Although CRP is a nonspecific indicator of disease or trauma, monitoring of its levels can be useful clinically to follow a disease process and observe the response to treatment of inflammation and infection.[11] It is a nonsurgical means of following the course of malignancy and organ transplantation because a rise in the level may mean a return of the malignancy or, in the case of transplantation, the beginning of organ rejection. CRP levels can also be used to monitor the progression or remission of autoimmune diseases. Assays for CRP are sensitive, reproducible, and relatively inexpensive.[10]

CRP has lately received attention as a risk marker for cardiovascular disease. In accord with the finding that atherosclerosis (coronary artery disease) may be the result of a chronic inflammatory process,[13] an increased level of CRP has been shown to be a significant risk factor for myocardial infarction, ischemic stroke, and peripheral vascular disease in men and women who have no previous history of cardiovascular disease.[14–19] When inflammation is chronic, increased amounts of CRP react with endothelial cells lining vessel walls and predispose these walls to vasoconstriction, platelet activation, thrombosis (clot formation), and vascular inflammation.[10] Monitoring of CRP, therefore, is now an established clinical tool to evaluate subtle chronic systemic inflammation and predict cardiovascular or cerebrovascular disease.[8,14] The ability to monitor CRP is significant because cardiovascular disease is a major cause of mortality in the world today.[8,14,15]

The Centers for Disease Control and Prevention (CDC) has recommended that a CRP concentration of less than 1 mg/L is associated with a low risk for cardiovascular disease; 1 to 3 mg/L is associated with an average risk; and greater than 3 mg/L is associated with a high risk.[20,21] Normal levels in adults range from approximately 0.47 to 1.34 mg/L.[8] A mean for people with no coronary artery disease is 0.87 mg/L.[14] Thus, monitoring CRP may be an important preventative measure in determining the potential risk of heart attack or stroke. High-sensitivity CRP testing has the necessary lower level of detection of 0.01 mg/L, which enables measurement of much smaller increases than the traditional latex agglutination screening test.[11]

CRP is easily destroyed by heating serum to 56°C for 30 minutes. The destruction of CRP is often necessary in the laboratory because it interferes with some testing for the presence of antibodies.

Serum Amyloid A

Serum amyloid A (SAA) is the other major protein besides CRP whose concentration can increase almost a thousandfold in response to infection or injury. It is an apolipoprotein that is synthesized in the liver and has a molecular weight of 11,685 daltons. Normal circulating levels are approximately 5 to 8 ug/mL.[22] In plasma, SAA has a high affinity for HDL cholesterol and is transported by HDL to the site of infection. SAA appears to act as a chemical messenger, similar to a cytokine, and it activates monocytes and macrophages to then produce products that increase inflammation.[22] It has been found to increase significantly more in bacterial infections than in viral infections.[22] Levels reach a peak between 24 to 48 hours after an acute infection.[23] SAA can also be increased because of chronic inflammation, atherosclerosis, and cancer.[23] Because SAA has been found in atherosclerotic lesions, it is now thought to contribute to localized inflammation in coronary artery disease.[9] Elevated levels may predict a worse outcome for the patient.[9]

Complement

Complement refers to a series of serum proteins that are normally present and whose overall function is mediation of inflammation. Nine such proteins are activated by bound antibodies in a sequence known as the *classical cascade;* an additional number are involved in the alternate pathway that is triggered by the presence of microorganisms. The major functions of complement are opsonization, chemotaxis, and lysis of cells. Complement is discussed more fully in Chapter 7.

Alpha$_1$-Antitrypsin

Alpha$_1$-antitrypsin (AAT) is a 52-kD protein that is primarily synthesized in the liver. It is the major component of the alpha band when serum is electrophoresed. Although the name implies that it acts against trypsin, it is a general plasma inhibitor of proteases released from leukocytes.[8] Elastase, one such protease, is an enzyme secreted by neutrophils during inflammation that can degrade elastin and collagen. In chronic pulmonary inflammation, elastase activity damages lung tissue. Thus, AAT acts to "mop up" or counteract the effects of neutrophil invasion during an inflammatory response. It also regulates expression of proinflammatory cytokines such as TNF-α, interleukin-1β, and interleukin-6, mentioned previously. As a result, activation of monocytes and neutrophils is inhibited, limiting the harmful side effects of inflammation.[24]

AAT deficiency can result in premature emphysema, especially in individuals who smoke or who have frequent exposure to noxious chemicals.[8] In such a deficiency, uninhibited proteases remain in the lower respiratory tract, leading to destruction of parenchymal cells in the lungs and to development of emphysema or idiopathic pulmonary fibrosis. It has been estimated that as many as 100,000 Americans suffer from this deficiency, although many of them are undiagnosed.[25,26] There are at least 75 alleles of the gene coding for AAT and 17 of these are associated with low production of the enzyme.[8] One particular variant gene for AAT is responsible for a complete lack of production of the enzyme; individuals who inherit this gene are at risk of developing liver disease and emphysema.[22]

Homozygous inheritance of this particular gene may lead to development of cirrhosis, hepatitis, or hepatoma in early childhood. The only treatment is a liver transplant.[8]

AAT can also react with any serine protease, such as proteases generated by the triggering of the complement cascade or fibrinolysis. Once bound to AAT, the protease is completely inactivated and is subsequently removed from the area of tissue damage.

Haptoglobin

Haptoglobin is an alpha$_2$-globulin with a molecular weight of 100,000 daltons. It binds irreversibly to free hemoglobin released by intravascular hemolysis. Haptoglobin thus acts as an antioxidant to provide protection against oxidative damage mediated by free hemoglobin.[27,28] Once bound, the complex is cleared rapidly by macrophages in the liver.[29,30] A two- to tenfold increase in haptoglobin can be seen following inflammation, stress, or tissue necrosis.[8] Early in the inflammatory response, however, haptoglobin levels may drop because of intravascular hemolysis, consequently masking the protein's behavior as an acute-phase reactant.[28] Thus, plasma levels must be interpreted in light of other acute-phase reactants. Normal plasma concentrations range from 40 to 290 mg/dL.[8]

Fibrinogen

Fibrinogen is an acute-phase protein involved in the coagulation pathway. A small portion is cleaved by thrombin to form fibrils that make up a fibrin clot.[8] The molecule is a dimer with a molecular weight of 340,000 daltons. Normal levels range from 200 to 400 mg/dL.[8,31] The clot increases the strength of a wound and stimulates endothelial cell adhesion and proliferation, which are critical to the healing process. Formation of a clot creates a barrier that helps prevent the spread of microorganisms further into the body. Fibrinogen makes blood more viscous and serves to promote aggregation of red blood cells (RBCs) and platelets. Increased levels may contribute to an increased risk for developing coronary artery disease.[8,15,18,31]

Ceruloplasmin

Ceruloplasmin consists of a single polypeptide chain with a molecular weight of 132,000 daltons.[8] It is the principal copper-transporting protein in human plasma, binding more than 70% of the copper found in plasma by attaching six cupric ions per molecule.[32]Additionally, ceruloplasmin acts as an enzyme, converting the toxic ferrous ion (Fe^{2+}) to the nontoxic ferric form (Fe^{3+}).[32] The normal plasma concentration for adults is 20 to 40 mg/dL.

A depletion of ceruloplasmin is found in Wilson's disease, an autosomal recessive genetic disorder characterized by a massive increase of copper in the tissues. Normally, circulating copper is absorbed out by the liver and either combined with ceruloplasmin and returned to the plasma or excreted into the bile duct. In Wilson's disease, copper accumulates in the liver and subsequently in other tissues such as the brain, corneas, kidneys, and bones.[8,33] Treatment is either long-term chelation therapy to remove the copper or a liver transplant.[8]

Inflammation

When pathogens breach the outer barriers of innate immunity, both cellular and humoral mechanisms are involved in a complex, highly orchestrated process known as inflammation. **Inflammation** can be defined as the body's overall reaction to injury or invasion by an infectious agent. Each individual reactant plays a role in initiating, amplifying, or sustaining the reaction and a delicate balance must be maintained for the process to be speedily resolved. The four cardinal signs or clinical symptoms of inflammation are redness (erythema), swelling (edema), heat, and pain. Major events that occur rapidly after tissue injury are:

1. Increased blood supply to the affected area. Dilation of the blood vessels caused by the release of chemical mediators such as histamine from injured mast cells brings additional blood flow to the affected area, resulting in redness and heat.
2. Increased capillary permeability caused by contraction of the endothelial cells lining the vessels. The increased permeability of the vessels allows fluids in the plasma to leak into the tissues, resulting in the swelling and pain associated with inflammation.
3. Migration of WBCs, mainly neutrophils, from the capillaries to the surrounding tissue in a process called **diapedesis.** As the endothelial cells of the vessels contract, neutrophils move through the endothelial cells of the vessel and out into the tissues. Soluble mediators, which include acute-phase reactants, chemokines, and cytokines, act as chemoattractants to initiate and control the response. Neutrophils are mobilized within 30 to 60 minutes after the injury and their emigration may last 24 to 48 hours.
4. Migration of macrophages to the injured area.[27] Migration of macrophages and dendritic cells from surrounding tissue occurs several hours later and peaks at 16 to 48 hours.
5. Acute-phase reactants stimulate phagocytosis of microorganisms. Macrophages, neutrophils, and dendritic cells all attempt to clear the area through phagocytosis; in most cases, the healing process is completed with a return of normal tissue structure **(Fig. 3–4).**

The *acute* inflammatory response acts to combat the early stages of infection and also begins a process that repairs tissue damage. However, when the inflammatory process becomes prolonged, it is said to be *chronic.* The failure to remove microorganisms or injured tissue may result in continued tissue damage and loss of function.

Phagocytosis

The main purpose of the inflammatory response is to attract cells to the site of infection and remove foreign cells or pathogens by means of phagocytosis. Although the acute-phase reactants enhance the process of phagocytosis, it is the cellular elements of the internal defense system that play the major role. The cells that are most active in phagocytosis are

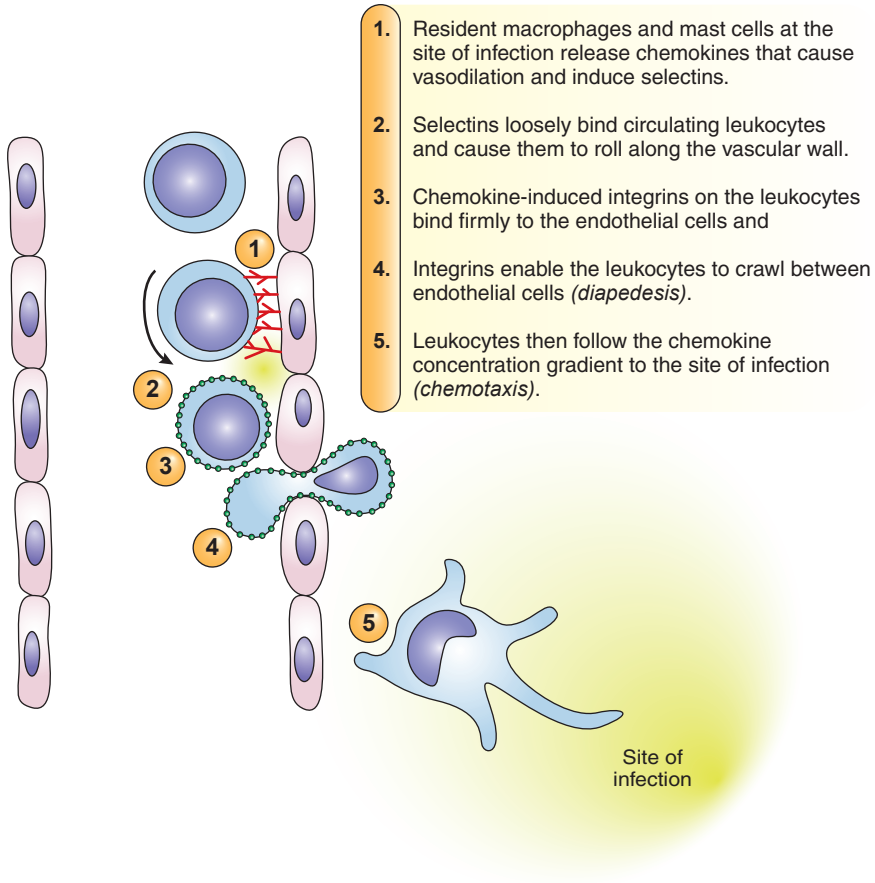


FIGURE 3–4 Local inflammatory events.

neutrophils, monocytes, macrophages, and dendritic cells, as discussed in Chapter 1.

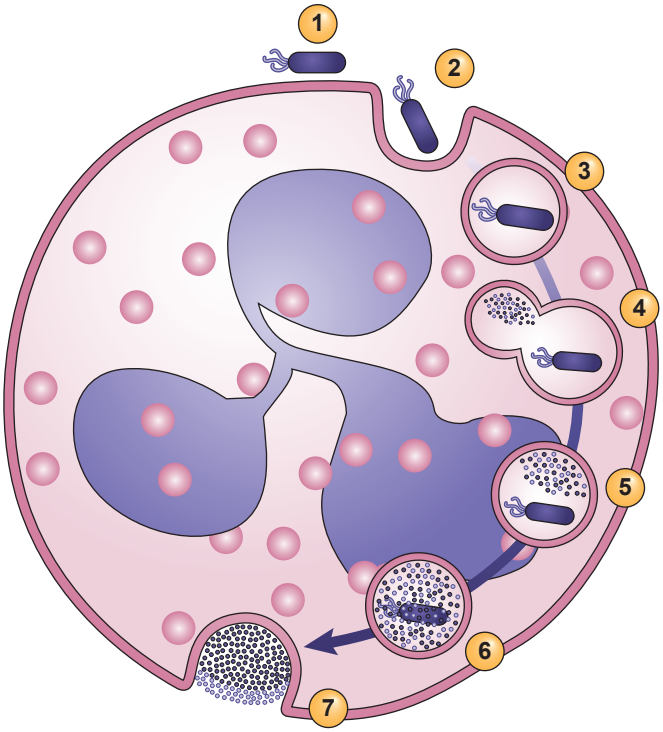
Once the WBCs are attracted to the area, the actual process of phagocytosis consists of seven main steps **(Fig. 3–5):**

1. Physical contact between the WBC and the foreign cell
2. Outflowing of the cytoplasm to surround the microorganism
3. Formation of a phagosome
4. Fusion with lysosomal granules with the phagosome
5. Formation of the phagolysosome with release of lysosomal contents
6. Digestion of microorganisms by hydrolytic enzymes
7. Release of debris to the outside by exocytosis

Physical contact occurs as neutrophils roll along in the bloodstream in a random pattern until they encounter the site of injury or infection.[34] They adhere to receptors on the endothelial cell wall of the blood vessels and penetrate through to the tissue by means of diapedesis. This adhering process is aided by **chemotaxis,** whereby cells are attracted to the site of inflammation by chemical substances such as soluble bacterial factors or acute-phase reactants including complement components and CRP. Macrophages and dendritic cells already reside in the tissues. Receptors on neutrophils, macrophages, and dendritic cells bind to certain molecular patterns on a foreign particle surface as discussed previously. This binding process is enhanced by **opsonins,** a term derived from the Greek word meaning "to prepare for eating." Opsonins are serum proteins that attach to a foreign cell or pathogen and help prepare it for phagocytosis. CRP, complement components, and antibodies are all important opsonins. Opsonins may act by neutralizing the surface charge on the foreign particle, making it easier for the cells to approach one another. In addition to receptors for pathogens themselves, phagocytic cells also have receptors for immunoglobulins and complement components, which aid in contact and in initiating ingestion.

Once contact with surface receptors occurs, phagocytic cells secrete chemoattractants such as cytokines and chemokines; these recruit additional cells to the site of infection. Neutrophils are followed by monocytes, after which macrophages and dendritic cells arrive at the site.[1] Macrophages and dendritic cells are not only able to ingest whole microorganisms, but they can also clean up injured or dead host cells.

After attachment to a foreign cell or pathogen has occurred, the cell membrane invaginates and pseudopodia (outflowing of cytoplasm) surround the pathogen. The pseudopodia fuse to completely enclose the pathogen, forming a structure known as a **phagosome.** The phagosome is moved toward the center of the cell. Lysosomal granules quickly migrate to the phagosome and fusion between granules and the phagosome occurs. At this point, the fused elements are known as a **phagolysosome.** The granules contain lysozyme, myeloperoxidase, and other proteolytic enzymes. The contents of the granules are



1. Adherence: physical contact between the phagocytic cell and the microorganism occurs, aided by opsonins.
2. Engulfment: outflowing of cytoplasm to surround the microorganism.
3. Formation of phagosome: microorganism is completely surrounded by a part of the cell membrane.
4. Granule contact: lysosomal granules contact and fuse with the phagosome.
5. Formation of the phagolysosome: contents of the lysosome are emptied into this membrane-bound space.
6. Digestion of the microorganism by hydrolytic enzymes.
7. Excretion: contents of phagolysosome are expelled to the outside by exocytosis.

FIGURE 3–5 Steps involved in phagocytosis.

released into the phagolysosome and digestion occurs. Any undigested material is excreted from the cells by exocytosis. Heavily opsonized particles are taken up in as little as 20 seconds and killing is almost immediate.[2,35]

The elimination of pathogens actually occurs by two different processes: an oxygen-dependent pathway and an oxygen-independent pathway. In the oxygen-dependent process, an increase in oxygen consumption, known as the **oxidative burst,** occurs within the cell as the pseudopodia enclose the particle within a vacuole. This mechanism generates considerable energy via oxidative metabolism. The hexose monophosphate shunt is used to change nicotinamide adenine dinucleotide phosphate (NADP) to its reduced form by adding a hydrogen. Electrons then pass from NADPH to oxygen in the presence of NADPH oxidase, a membrane-bound enzyme that is only activated through conformational change triggered by microbes themselves.[36] A radical known as O_2^- (superoxide) is then formed. Superoxide is highly toxic but can be rapidly converted to even more lethal products. By adding hydrogen ions, the enzyme superoxide dismutase (SOD) converts superoxide to hydrogen peroxide or the hydroxyl radical OH.

Hydrogen peroxide has long been considered an important bactericidal agent and is more stable than any of the free radicals. Its antimicrobial effect is further enhanced by the formation of hypochlorite ions through the action of the enzyme myeloperoxidase in the presence of chloride ions. Hypochlorite is a powerful oxidizing agent and is highly toxic for microorganisms. It is the main component of household bleach used to disinfect surfaces **(Fig. 3–6).**

NADPH oxidase also plays a major role in the oxygen-independent pathway. NADPH oxidase depolarizes the membrane when fusion with the phagosome occurs, allowing hydrogen and potassium ions to enter the vacuole. This alters the pH, which in turn activates proteases that contribute to microbial elimination. Some of these lytic enzymes include small cationic proteins called *defensins*. When defensins are released from lysosomal granules, they are able to cleave segments of bacterial cell walls without the benefit of oxygen. Defensins kill a wide spectrum of organisms, including both gram-positive and gram-negative bacteria, many fungi, and some viruses. Cathepsin G is another example of a protein that is able to damage bacterial cell membranes.[2,34] Chapter 1 lists some of the contents of granules in neutrophils.

The importance of NADPH oxidase in the elimination of microbes is demonstrated by the fact that a lack of it may lead to an increased susceptibility to infection. Patients with chronic granulomatous disease have a gene mutation that causes a defect in NADPH oxidase, resulting in an inability to kill bacteria during the process of phagocytosis. Individuals with this disease suffer from recurring, severe bacterial infections (see Chapter 19).[34]

Following phagocytosis, macrophages and dendritic cells process peptides from pathogens for presentation to T cells. T cells then interact with B cells to produce antibodies (see Chapter 5 for details). Because T cells are not able to respond to intact pathogens, phagocytosis is a crucial link between the innate and the adaptive immune systems.

Action of Natural Killer Cells

Another important cellular defense that is part of innate immunity is the action of natural killer (NK) cells. Although phagocytosis is important in eliminating infectious agents, NK cells represent the first line of defense against cells that are

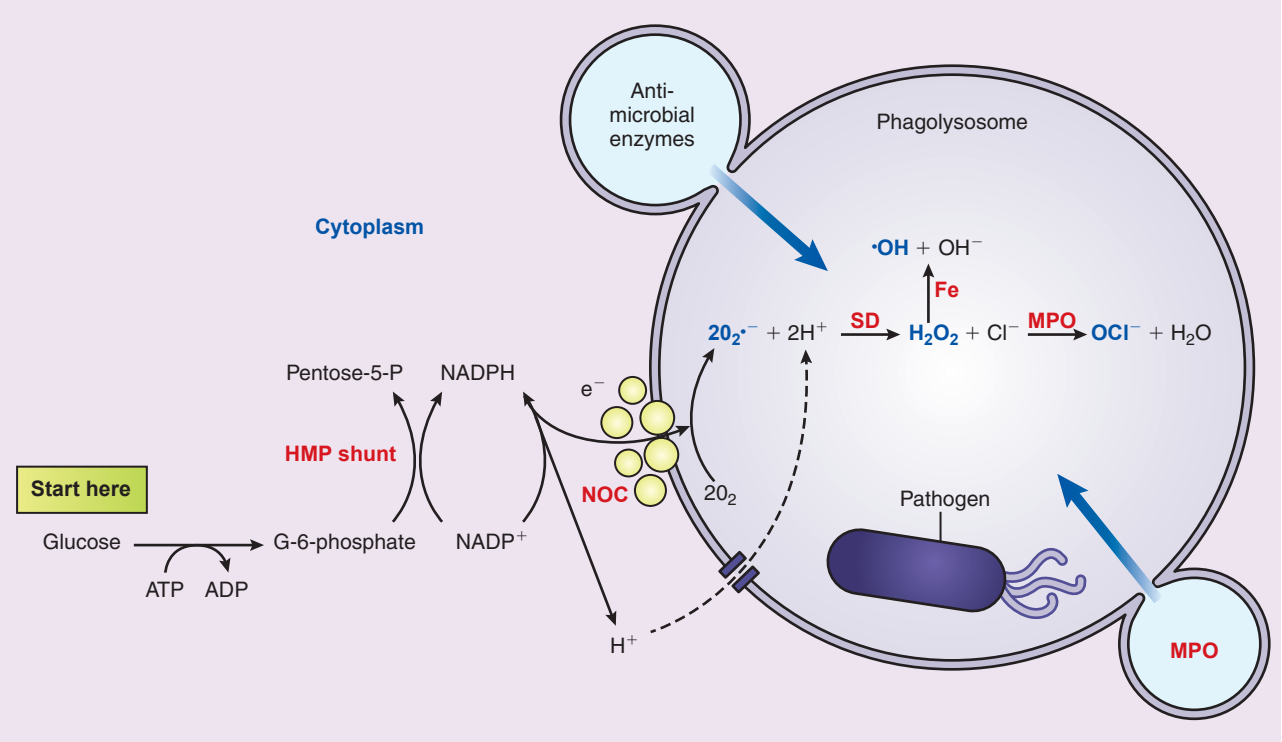


FIGURE 3–6 Creation of oxygen radicals in the phagocytic cell. The hexose monophosphate (HMP) shunt reduces NADP to NADPH. NADPH reduces oxygen to superoxide ($2O_2^{\bullet -}$) when the NADPH oxidase complex (NOC) is assembled in the membrane of the phagolysosome. Superoxide dismutase (SOD) catalyzes the conversion of superoxide to hydrogen peroxide (H_2O_2). Myeloperoxidase (MPO) catalyzes formation of hypochlorite (OCl^-), a very powerful oxidizing agent. Hydroxyl radicals (•OH), which are also powerful oxidizing agents, may also be formed if iron ions are present.

virally infected, cells infected with other intracellular pathogens, and tumor cells.[37–39] NK cells have the ability to recognize any damaged cell and to eliminate such target cells without prior exposure to them. The fact that they lack specificity in their response is essential to their function as early defenders against pathogens.[37,38] By quickly engaging infected target cells, NK cells give the immune system time to activate the adaptive response of specific T and B cells.

NK cell activity is stimulated by exposure to cytokines such as interleukin-12, interferon-α, and interferon-β. Because these cytokines rise rapidly during a viral infection, NK cells are able to respond early during an infection and their activity peaks in about 3 days, well before antibody production or a cytotoxic T-cell response. They localize in the tissues in areas where inflammation is occurring and where dendritic cells are found.[37] Once activated, NK cells themselves become major producers of cytokines such as interferon-gamma (IFN-γ) and TNF-α that help to recruit T cells.[37,39] In addition, NK cells release various colony stimulating factors that act on developing granulocytes and macrophages. Actions of NK cells, therefore, have a major influence on both innate and adaptive immunity.

Mechanism of Cytotoxicity

For years it had been a mystery how NK cells tell the difference between normal and abnormal cells. However, the mechanism is now beginning to be understood. NK cells are constantly monitoring potential target cells through two main classes of binding receptors on NK cells: inhibitory receptors, which deliver inhibitory signals, and activating receptors, which deliver signals to activate the cytotoxic mechanisms.[37] It appears that there is a balance between activating and inhibitory signals that enables NK cells to distinguish healthy cells from infected or cancerous ones.

The inhibitory signal is based on recognition of class I major histocompatibility complex (MHC) proteins, which are expressed on all healthy cells (see Chapter 2 for details). If NK cells react with class I MHC proteins, then inhibition of natural killing occurs. Examples of this type of inhibitory receptor include killer cell immunoglobulin-like receptors (KIRs)[37] and CD94/NKG2A receptors, both of which bind class I MHC molecules.

Diseased and cancerous cells tend to lose their ability to produce MHC proteins. NK cells are thus triggered by a lack of MHC antigens, sometimes referred to as recognition of "missing self."[37,39] This lack of inhibition appears to be combined with an activating signal switched on by the presence of proteins produced by cells under stress, namely those cells that are infected or cancerous.[39]

Examples of activating receptors that bind stress proteins are CD16 and NKG2D.[37] If an inhibitory signal is not received when binding to activating receptors occurs, then NK cells release substances called *perforins* and *granzymes* **(Fig. 3–7).** These substances are released into the space between the NK cell and the target cell. Perforins are proteins that form channels (pores) in the target cell membrane.[40] Granzymes are packets of enzymes that may enter through the channels and mediate cell lysis. The elimination of target cells can occur in as little

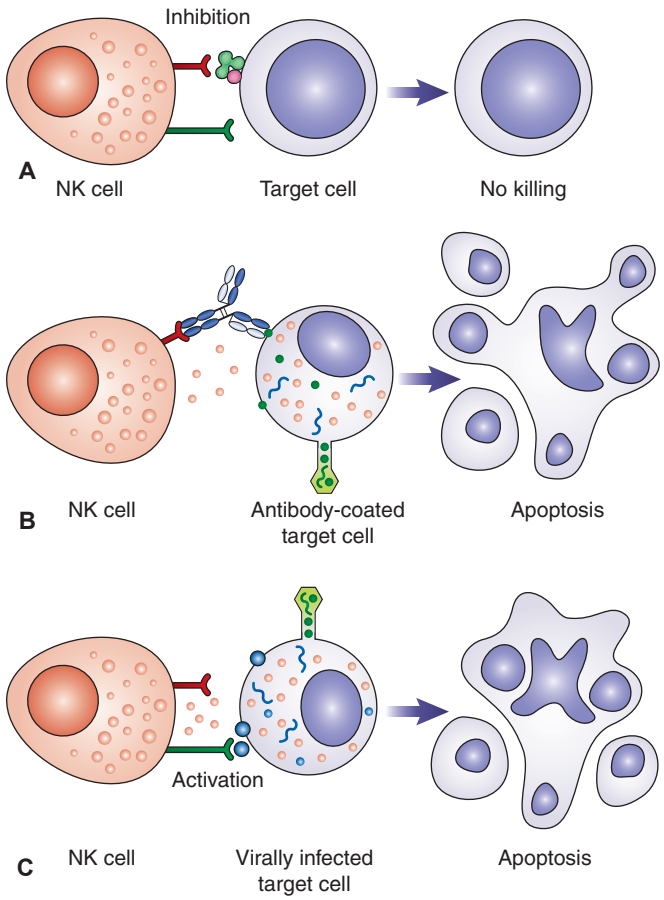


FIGURE 3–7 Actions of NK cells. NK cells are constantly surveying cells. (A) If class I MHC protein is present and there are no foreign or stress proteins, then an inhibitory signal is sent to the NK cell, no killing occurs, and the normal cell is released. (B) Alternatively, infected cells may express foreign proteins on their surface that are recognized by antibody. NK cells express CD16 receptors that bind the immobilized antibody and activate the release of perforins and granzymes *(antibody-dependent cell-mediated cytotoxicity)*. (C) If an activating receptor is engaged by a foreign or stress protein and class I MHC is altered or missing ("missing self"), then no inhibitory signal is given, granzymes and perforins are released, and the infected or diseased cell is eliminated by apoptosis.

as 30 to 60 minutes.[2] Thus, depending on the signals, the NK cell either proceeds to activate cell destruction or detaches and moves on to search for another target cell.

Antibody-Dependent Cell Cytotoxicity

A second method of destroying target cells is also available to NK cells. They recognize and lyse antibody-coated cells through a process called **antibody-dependent cell cytotoxicity (ADCC).** Binding occurs through the CD16 receptor for the Fc portion of immunoglobulin G (IgG). Any target cell coated with IgG can be bound and destroyed. This method is not unique to NK cells, as monocytes, macrophages, and neutrophils also exhibit such a receptor and act in a similar manner. Nonetheless, the overall importance of NK cells as a defense mechanism is demonstrated by the fact that patients who lack these cells have recurring, serious viral infections and an increased incidence of tumors.[41]

SUMMARY

- Innate immunity encompasses all the body's normally present defense mechanisms for resisting disease. It is characterized by lack of specificity, no need for a prior exposure, and a similar response with each exposure.
- External defenses are structural barriers such as skin, mucous membranes, cilia, and secretions such as lactic acid and lysozyme that keep microorganisms from entering the body.
- Internal defenses include both cells capable of phagocytosis and acute-phase reactants that enhance the process of phagocytosis. Cells that are most active in phagocytosis include neutrophils, monocytes, macrophages, and dendritic cells.
- Pathogen recognition receptors (PRRs) are molecules on host cells that recognize substances found only on pathogens. They are found on neutrophils, monocytes, eosinophils, mast cells, and dendritic cells. Once receptors bind a pathogen, phagocytosis can take place.
- Pathogen-associated molecular patterns (PAMPS) are molecules found only on pathogens, which allow host cells to distinguish them from self. They are recognized by the PRRs.
- Acute-phase reactants are serum proteins that increase rapidly in response to infection or injury and include C-reactive protein (CRP), serum amyloid A, complement components, alpha$_1$-antitrypsin, haptoglobin, fibrinogen, and ceruloplasmin.
- CRP is the most widely monitored acute-phase reactant; it increases 100 to 1000 times in response to infection or trauma, acts as an opsonin, and is able to fix complement.
- All acute-phase reactants increase the likelihood of phagocytosis of pathogens and help healing occur.
- The first step in phagocytosis is physical contact between the phagocytic cell and the foreign particle.
- Chemotaxis is the process that attracts cells to the area of infection.
- Cytoplasm flows around the foreign particle to form a phagosome. Fusion of the phagosome with lysosomal granules creates a phagolysosome. Inside this structure, enzymes such as lysozyme and myeloperoxidase are released and the foreign particle is digested.
- Creation of hypochlorite and hydroxyl ions, which damage protein irreversibly, occur in the oxygen-dependent phase of phagocytosis.
- Phagocytosis must occur before the specific immune response can be initiated, so this process is essential to both innate and adaptive immunity.
- Inflammation is the body's response to injury or invasion by a pathogen. It is characterized by increased blood supply to the affected area, increased capillary permeability, migration of neutrophils to the surrounding tissue, and migration of macrophages to the injured area.

- Natural killer (NK) cells are able to kill target cells that are infected with a virus or other intracellular pathogen. They also recognize malignant cells.
- The action of NK cells does not require prior exposure and is nonspecific. They recognize a lack of class I MHC protein found on normal cells. This capability is called recognition of missing self.
- NK cells bind to and kill any antibody-coated target cells.
- NK cells represent an important link between the innate and adaptive immune systems.

Study Guide: Mechanisms of Innate Immunity

TYPE OF DEFENSE	EXAMPLE	FUNCTION
External	Skin and mucous membranes	Biological barriers
	Lactic acid	Keeps down growth of microorganisms
	Cilia	Move pathogens out of respiratory tract
	Stomach acid	Low pH keeps pathogens from growing
	Urine	Flushes out pathogens from the body
	Lysozyme	Attacks cell walls of pathogens
	Normal flora	Compete with pathogens Produce antimicrobial peptides
Internal	Cells	Participate in phagocytosis NK cells destroy target cells using granzymes and perforins
	Pathogen recognition receptors (e.g., Toll-like receptors)	Help phagocytic cells recognize pathogens
	Acute-phase reactants	Recruit WBCs for phagocytosis Coat pathogens to enhance phagocytosis Mop up debris

CASE STUDIES

1. A 45-year-old male named Rick went to his physician for an annual checkup. Although he was slightly overweight, his laboratory results indicated that both his total cholesterol and his HDL cholesterol were within normal limits. His fibrinogen level was 450 mg/dL and his CRP level was 3.5 mg/dL. His physical examination was perfectly normal. The physician cautioned Rick that he might be at risk for a future heart attack and he counseled him to be sure to exercise and eat a healthy, low-fat diet. Rick's wife told him that as long as his cholesterol level was normal, he didn't have anything to worry about.

   Question

   a. Who is correct? Explain your answer.

2. A 20-year-old female college student went to the infirmary with symptoms of malaise, fatigue, sore throat, and a slight fever. A complete blood count (CBC) was performed and both the RBC and WBC count were within normal limits. A normal WBC count ruled out the possibility of a bacterial infection. A rapid strep test was performed, which was negative. A slide agglutination test for infectious mononucleosis was indeterminate (neither positive or negative), whereas a slide agglutination test for CRP was positive. Results of a semiquantitative CRP determination indicated an increased level of approximately 20 mg/dL. The student was advised to return in a few days for a repeat mono test.

   Questions

   a. What conditions might cause a rise in CRP?
   b. Would an increase in CRP be consistent with the possibility of infectious mononucleosis?

REVIEW QUESTIONS

1. The term for enhancement of phagocytosis by coating of foreign particles with serum proteins is
   - **a.** opsonization.
   - **b.** agglutination.
   - **c.** solubilization.
   - **d.** chemotaxis.

2. Which of the following plays an important role as an external defense mechanism?
   - **a.** Phagocytosis
   - **b.** C-reactive protein
   - **c.** Lysozyme
   - **d.** Complement

3. The process of inflammation is characterized by all of the following *except*
   - **a.** increased blood supply to the area.
   - **b.** migration of WBCs.
   - **c.** decreased capillary permeability.
   - **d.** appearance of acute-phase reactants.

4. Skin, lactic acid secretions, stomach acidity, and the motion of cilia represent which type of immunity?
   - **a.** Innate
   - **b.** Cross
   - **c.** Adaptive
   - **d.** Auto

5. The structure formed by the fusion of engulfed material and enzymatic granules within the phagocytic cell is called a
   - **a.** phagosome.
   - **b.** lysosome.
   - **c.** vacuole.
   - **d.** phagolysosome

6. The presence of human microbiota (normal flora) acts as a defense mechanism by which of the following methods?
   - **a.** Maintaining an acid environment
   - **b.** Competing with potential pathogens
   - **c.** Keeping phagocytes in the area
   - **d.** Coating mucosal surfaces

7. Measurement of CRP levels can be used for all of the following *except*
   - **a.** monitoring drug therapy with anti-inflammatory agents.
   - **b.** tracking the progress of an organ transplant.
   - **c.** diagnosis of a specific bacterial infection.
   - **d.** determining active phases of rheumatoid arthritis.

8. Pathogen recognition receptors act by
   - **a.** recognizing molecules common to both host cells and pathogens.
   - **b.** recognizing molecules that are unique to pathogens.
   - **c.** helping to spread infection because they are found on pathogens.
   - **d.** all recognizing the same pathogens.

9. Which of the following are characteristics of acute-phase reactants?
   - **a.** Rapid increase following infection
   - **b.** Enhancement of phagocytosis
   - **c.** Nonspecific indicators of inflammation
   - **d.** All of the above

10. Which is the most significant agent formed in the phagolysosome for the elimination of microorganisms?
    - **a.** Proteolytic enzymes
    - **b.** Hydrogen ions
    - **c.** Hypochlorite ions
    - **d.** Superoxides

11. Which acute-phase reactant helps to prevent formation of peroxides and free radicals that may damage tissues?
    - **a.** Haptoglobin
    - **b.** Fibrinogen
    - **c.** Ceruloplasmin
    - **d.** Serum amyloid A

12. Which statement best describes Toll-like receptors (TLRs)?
    - **a.** They protect adult flies from infection.
    - **b.** They are found on all host cells.
    - **c.** They only play a role in adaptive immunity.
    - **d.** They enhance phagocytosis.

13. The action of CRP can be distinguished from that of an antibody because
    - **a.** CRP acts before the antibody appears.
    - **b.** only the antibody triggers the complement cascade.
    - **c.** binding of the antibody is calcium-dependent.
    - **d.** only CRP acts as an opsonin.

14. How does innate immunity differ from adaptive immunity?
    - **a.** Innate immunity requires prior exposure to a pathogen.
    - **b.** Innate immunity depends upon normally present body functions.
    - **c.** Innate immunity develops later than adaptive immunity.
    - **d.** Innate immunity is more specific than adaptive immunity.

15. A 40-year-old male who is a smoker develops symptoms of premature emphysema. The symptoms may be caused by a deficiency of which of the following acute-phase reactants?

a. Haptoglobin
b. Alpha$_1$-antitrypsin
c. Fibrinogen
d. Ceruloplasmin

16. Which statement best describes NK cells?

a. Their response against pathogens is very specific.
b. They only react when an abundance of MHC antigens is present.
c. They react when both an inhibitory and activating signal is triggered.
d. They are able to kill target cells without previous exposure to them.

4 Adaptive Immunity

Christine Dorresteyn Stevens, EdD, MT(ASCP)

LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Compare and contrast adaptive immunity and innate immunity.
2. Discuss the role of the thymus in T-cell maturation.
3. Describe the CD3 receptor for antigen on a T cell.
4. Explain how positive and negative selection contribute to the development of immunocompetent T cells.
5. List and describe five different subsets of T cells that bear the CD4 marker.
6. Describe maturation of a B cell from the pro-B cell to a plasma cell.
7. Contrast the antigen-independent and antigen-dependent phases of B-cell development.
8. Explain how cytotoxic T cells recognize and kill target cells.
9. Discuss the role of class I MHC and class II MHC molecules in the presentation of antigens to T cells.
10. Differentiate T-dependent antigens from T-independent antigens on the basis of how each activates B cells.
11. Discuss how T helper (Th) cells stimulate B cells to transform into plasma cells.
12. Explain the importance of both T and B memory cells to the adaptive immune response.
13. Apply knowledge of T- and B-cell function to immunologically based disease states.
14. Describe current testing used to identify T and B cells.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Adaptive immunity
Allelic exclusion
Antigen-dependent phase
Antigen-independent phase
Cell flow cytometry
Cell-mediated immunity
Central tolerance
Chemokines
Clonal deletion
Cytotoxic T cells
Double-negative (DN) thymocytes
Double-positive (DP) thymocytes
Humoral immunity
Immature B cells
MHC restriction
Negative selection
Plasma cell
Positive selection
Pre-B cells
Pro-B cells
Surrogate light chain
T-dependent antigens
T helper (Th) cells
T-independent antigens
T regulatory (Treg) cells
Thymocytes
Variable regions

As you learned in Chapter 3, innate immunity is based on external defenses that keep pathogens from entering the body. If these external defenses are breached, there are internal defenses, including phagocytic cells found in the blood and tissues, that respond similarly to any pathogens they encounter and attempt to destroy them. **Adaptive immunity,** in contrast, is a type of resistance characterized by:

- Specificity for each individual pathogen or microbial agent
- The ability to remember a prior exposure
- An increased response to that pathogen upon repeated exposure

Adaptive immunity is a more tailored response. It takes a longer time to be activated, but it is more specific and longer lasting.

The key cell involved in the adaptive immune response is the lymphocyte. The two main types of lymphocytes are T cells and B cells. T lymphocytes mature in the thymus and serve a regulatory role by providing help to B cells in responding to antigens as well as by killing virally infected target cells. B lymphocytes mature in the bone marrow and differentiate into plasma cells that produce antibodies. Immunologic memory is based on clonal selection, expansion, and differentiation of antigen-specific T and B cells.[1] The result is an ability to respond with greater speed and intensity to a re-encounter with the same pathogen, thus protecting the host from reinfection.[1]

B and T cells go through an elaborate maturation process in which this specificity is developed while possible self-reactive cells are destroyed. Each stage is marked by well-orchestrated signaling mechanisms that help to develop specific receptors for antigens while at the same time only selecting cells that will be helpful and not harmful to the host. During this process, creation of a wide variety of antigen-specific receptors must occur, enough to recognize any harmful antigens to which we may be exposed.

Differentiation of lymphocytes appears to take place very early in fetal development and is essential to the acquisition of immunocompetence by the time the infant is born. Progenitors of T and B cells appear in the fetal liver as early as 8 weeks of pregnancy.[2] Later in fetal development, production of lymphocyte progenitors shifts to the bone marrow, which becomes the primary producer of hematopoietic cells at birth. This chapter will discuss developmental stages of both T and B cells and will then relate this to their function in the adaptive immune response.

T-Cell Differentiation

Between 60% to 80% of circulating lymphocytes in the peripheral blood are T cells **(Fig. 4–1)** that become differentiated in the thymus. Lymphocyte precursors enter the thymus from the bone marrow. Within the lobules of the thymus are two main zones, the outer cortex and the inner medulla. Early precursors enter the thymus at the cortico-medullary junction and migrate to the outer cortex. Migration occurs in waves and is driven by chemical messengers called **chemokines.**[3,4] Chemokines are a large family of cytokines that have the ability to recruit specific cells to a particular site. Once in the thymus, precursors that are committed to becoming T cells are known as **thymocytes.**

As thymocytes travel through the thymus, there is an orderly rearrangement of the genes coding for the antigen receptor. At the same time, distinct surface markers appear during specific stages of development. Maturation is an elaborate process that takes place over a 3-week period as cells filter through the cortex to the medulla. Thymic stromal cells include epithelial cells, macrophages, fibroblasts, and dendritic cells, all of which play a role in T-cell development.[5] Interaction with stromal cells under the influence of cytokines, especially interleukin-7 (IL-7), is critical for growth and differentiation.[6] A significant selection process occurs as maturation takes place because an estimated

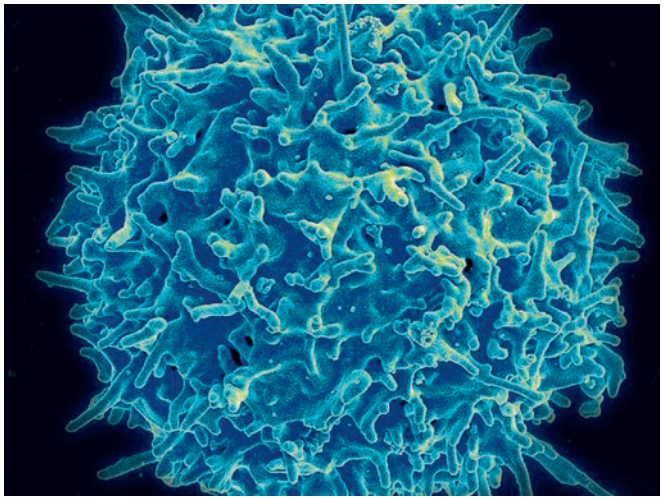


FIGURE 4–1 Scanning electron micrograph of a typical T cell. *(Courtesy of National Institute of Allergy and Infectious Diseases [(NIAID].)*

97% of the cortical cells die intrathymically before becoming mature T cells.[7] This selection process includes both positive and negative selection; each is discussed in more detail in the next section.

Double-Negative Stage

Early thymocytes lack CD4 and CD8 markers, which are important to their later function; hence, they are known as **double-negative (DN) thymocytes (Fig. 4–2).** These large DN thymocytes actively proliferate in the outer cortex under the influence of IL-7.

Rearrangement of the genes that code for the antigen receptor known as the T-cell receptor (TCR) begins at this stage[3,7] This random gene rearrangement is what builds in the diversity that allows T cells to respond to the myriads of different antigens that the body might encounter in a lifetime. The TCR consists of two specific chains, called alpha (α) and beta (β) chains, which both contain **variable regions** that recognize specific antigens **(Fig. 4–3).** These two chains occur in a complex with

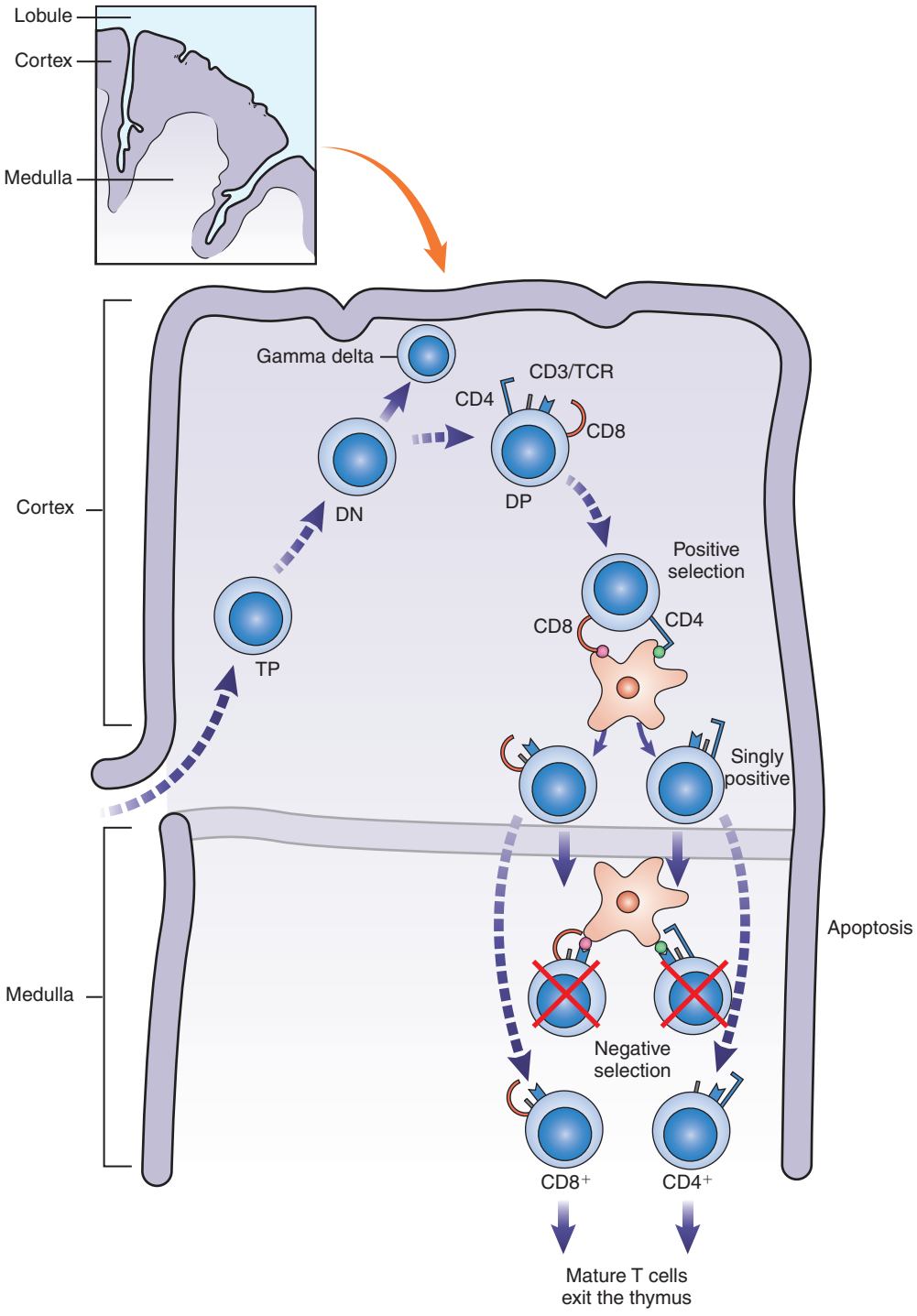


FIGURE 4–2 T-cell maturation in the thymus. T-lymphocyte precursors (TP) enter the thymus at the corticomedullary junction. They migrate upward in the cortex and begin development of the T-cell receptor. A small percentage of precursors develop gamma-delta chains, whereas the majority develop alpha-beta chains and become double-positive (DP) (both CD4 and CD8 are present). Positive and negative selection takes place through the CD3/T-cell receptor for antigen. If positively selected, the T cell becomes single-positive (SP); that is, either CD4+ or CD8+. Further interactions with macrophages or dendritic cells take place to weed out any T cells able to respond to self-antigen. Surviving CD4+ and CD8+ cells exit the thymus to the peripheral blood.

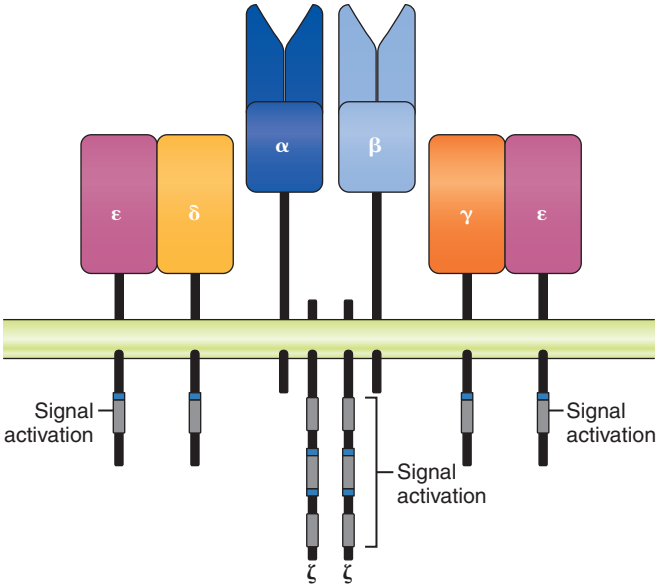


FIGURE 4–3 The CD3:T-cell receptor complex. The TCR that recognizes antigen consists of two chains, α and β, which have constant and variable regions. Four other types of chains are collectively known as CD3. These are ε, γ, δ, and ζ. They take part in signaling to the interior of the cell when antigen binding occurs. Note that the γ and δ chains found here differ from those found on CD4–/CD8– T cells.

six other chains that are common to all T cells. The combination of the eight chains is known as the CD3/TCR complex. The six chains of the nonspecific CD3 portion of the complex assist in signaling when an antigen binds to the T cells.[3,7,8] These chains occur in three pairs: delta-epsilon (δ–ε), gamma-epsilon (γ–ε), and a tau-tau (ζ–ζ) chain that is in the cytoplasm of the cell.[8]

The α and β chains of the TCR are coded for by the selection of certain gene segments and deletion of others in a random fashion.[3] Rearrangement of the β chain coded for on chromosome 7 occurs first; then, the α chain coded for on chromosome 14 is rearranged afterward.[3,7,8] Three different gene segments—V, D, and J—are rearranged and combined with a constant region to code for the β chain; in contrast, there are only two gene segments combined with a constant region for the β chain. The appearance of a functional α chain on the cell surface sends a signal to suppress any further β chain gene rearrangements. The selection of an allele on one chromosome only is known as **allelic exclusion.** The combination of the β chain with the rest of CD3 forms the pre-TRC receptor. The appearance of the β chain also triggers the thymocyte to become CD4–positive (CD4+) and CD8–positive (CD8+).[3,4]

Early on, some thymocytes, representing 10% or less of the total number, rearrange and express two other chains—gamma (γ) and delta (δ)—when there is not a productive rearrangement of DNA coding for a β chain.[3,7] These cells proceed down a different developmental pathway and they typically remain negative for both CD4 and CD8. However, as mature T cells, they appear to represent the dominant T-cell population in the skin, intestinal epithelium, and pulmonary epithelium. Their tasks include wound healing and protection of the epithelium.[3,7] They are capable of recognizing antigens without being presented by major histocompatibility complex (MHC) proteins, so they may represent an important bridge between innate and adaptive immunity.

Double-Positive Stage

At this second stage, when thymocytes express both CD4 and CD8 antigens, they are called **double-positive (DP) thymocytes.** Young DP thymocytes begin to rearrange the genes coding for the α chain.[1] When the CD3-αβ receptor complex (TCR) is complete and expressed on the cell surface, a **positive selection** process takes place that allows only DP cells with functional TCR receptors to survive. T cells must recognize foreign antigen in association with class I or class II MHC molecules **(Fig. 4–4).** When thymocytes bind to self-MHC antigens in the cortex by means of the newly formed TCR receptors, an enzyme cascade involving a group of enzymes called kinases is activated. Enzyme activity causes changes in cell shape and motility that lead to increased cell survival.[3] The selection of thymocytes that will only interact with the MHC antigens found on host cells is known as **MHC restriction.** Any thymocytes that have either a very low or a very high affinity for self-MHC antigens die by apoptosis.[5] This weeding out is important, because functioning T cells must be able to recognize foreign antigen along with MHC molecules.

A second selection process, known as **negative selection,** takes place among the surviving DP T cells. This second selection process takes place in the corticomedullary region and the medulla of the thymus as medullary epithelial cells express a wide variety of self-antigens[3,7] **(Fig. 4–5).** Strong reactions with self-peptides other than MHC antigens triggers apopotosis.[7] The process of elimination of clones of T cells that would be capable of an autoimmune response is called **clonal deletion.** This selection process is very rigorous as evidenced by the fact that only 1% to 3% of the DP thymocytes in the cortex survive.[4]

Mature T Cells

Survivors of selection exhibit only one type of marker, either CD4 or CD8. It is not certain why one marker is downregulated, but it may depend on which MHC protein the cell interacts with, how strongly they react, and to which cytokines they are exposed.[7] CD4+ T cells recognize antigen along with class II MHC protein, whereas CD8+ T cells interact with antigen and class I MHC proteins. The two separate mature T-cell populations created differ greatly in function. T cells bearing the CD4 receptor are mainly **T helper (Th) cells,** whereas the CD8+ population consists of **cytotoxic T cells.** Approximately two-thirds of peripheral T cells express CD4 antigen, whereas the remaining one-third express CD8 antigen.

Several subsets of Th cells exist, of which the most prominent are termed Th1 and Th2 cells. All Th cells have a different role to play in the immune response **(Fig. 4–6).** Th1 cells produce interferon gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-β (TNF-β), which protect cells against intracellular pathogens by activating cytotoxic lymphocytes and macrophages.[1] Th2 cells produce a variety of interleukins,

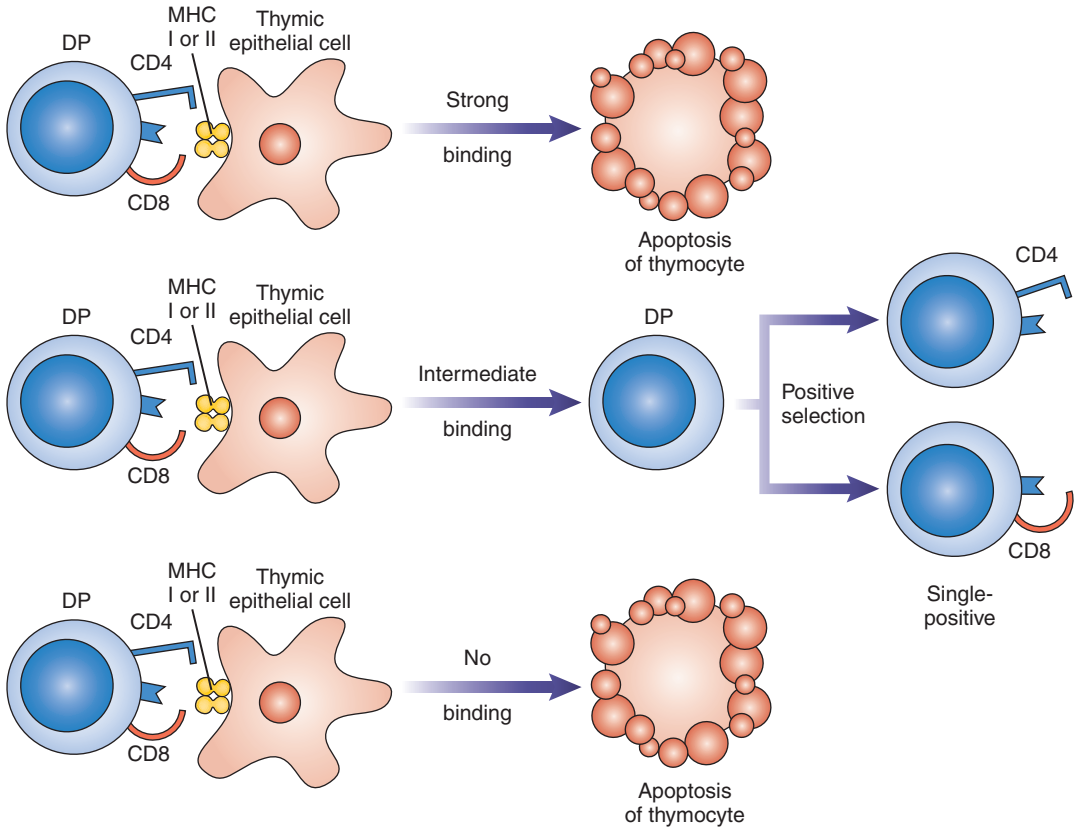


FIGURE 4–4 Positive selection of thymocytes in the cortex. Double-positive (CD4+ and CD8+) thymocytes interact with thymic epithelial cells. If very strong bonding occurs, cells are eliminated by apoptosis. If very weak or no bonding occurs, cells are also eliminated.

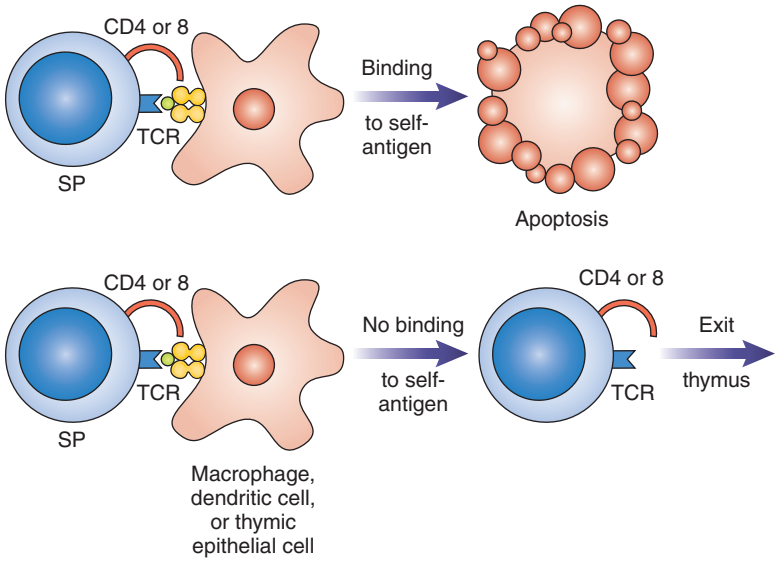


FIGURE 4–5 Negative selection of thymocytes in the medulla. When self-antigen is presented by a macrophage, dendritic cell, or thymic epithelial cell to a thymocyte, if the T-cell receptor (TCR) binds, the thymocyte is eliminated by apoptosis.

including IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. The essential role of the Th2 cells is to help B cells produce antibodies against extracellular pathogens and to generally regulate B-cell activity.[1]

An additional T-cell subpopulation, called **T regulatory (Treg) cells,** possess the CD4 antigen as well as CD25.[3] These cells comprise approximately 5% of all CD4+ T cells.[3,9,10] Tregs play an important role in suppressing the immune response to self-antigens. They inhibit proliferation of other T-cell populations by secreting inhibitory cytokines and the response is antigen-specific.[1]

Two other T-cell subpopulations have been identified. These cells are called Th9 and Th17, based on the type of cytokines they produce. Th9 cells produce interleukin-9 (IL-9) and appear

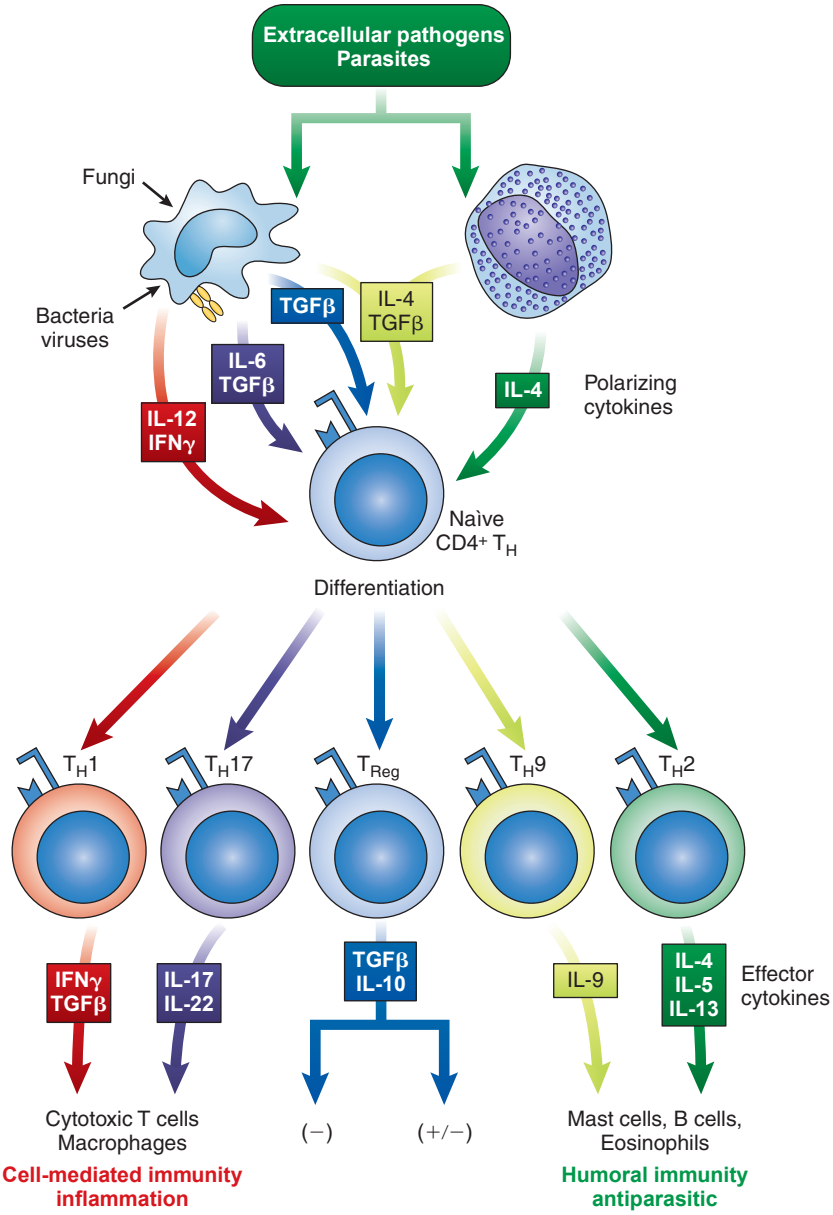


FIGURE 4–6 T helper (Th) subsets. Depending upon the type of pathogen encountered, antigen-presenting cells (APCs) secrete a specific combination of polarizing cytokines that direct naïve CD4+ Th cells to further differentiate into one of five subsets: Th1, Th2, Treg, Th9, or Th17. These specialized T cells release different types of cytokines to coordinate an appropriate immune response against the pathogen.

to have a proinflammatory effect. They may play a role at epithelial surfaces by warding off fungi and extracellular bacteria.[9] However, in the process, they stimulate growth of hematopoietic cells, especially mast cells; as such, they may promote autoimmune inflammation.[11] Th17 cells produce interleukin-17 (IL-17) and interleukin-22 (IL-22).[12] Both of these cytokines can increase inflammation and joint destruction. They have been associated with autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease.[11]

All single-positive T cells spend approximately 12 days in the medulla.[4] Additional proliferation of these carefully screened T cells occurs; they are then released from the thymus to seed peripheral lymphoid organs and recirculate through the bloodstream and peripheral organs approximately once every 12 to 24 hours.[13] Recirculation is important for ensuring that T cells make contact with antigen. Resting T cells have a life span of up to several years in these peripheral organs.

When antigen recognition occurs in the secondary lymphoid tissue, T lymphocytes are activated and differentiate into functionally active small lymphocytes that produce cytokines. Activities of specific cytokines include assisting B cells in commencing antibody production, eliminating tumor and other target cells, rejecting grafts, stimulating hematopoiesis in the bone marrow, and initiating delayed hypersensitivity allergic reactions. This type of immune response is known as **cell-mediated immunity**. These activities are discussed more fully in *The Role of T Cells in the Adaptive Immune Response* later.

Stages in B-Cell Differentiation

Pro-B Cells

B cells are derived from a hematopoietic stem cell that develops into an early lymphocyte progenitor in the bone marrow. Unlike

T cells, which leave the bone marrow and travel to the thymus to mature, B cells remain and mature in the bone marrow itself. Bone marrow stromal cells form special niches where stem cells and B-cell precursors reside.[14] The niches keep B-cell precursors localized in order to receive signals for differentiation.[15] B-cell precursors go through a developmental process that prepares them for their role in antibody production and, at the same time, restricts the types of antigens to which any one cell can respond. This process can be divided into three distinct phases:

- Development of mature immunocompetent B cells
- Activation of B cells by antigen
- Differentiation of activated B cells into plasma cells, which produce antibodies

The first phase of B-cell development in the bone marrow, which results in mature B cells that have not yet been exposed to antigen, is known as the **antigen-independent phase.** This phase can be divided according to formation of several distinct subpopulations: **pro-B cells** (progenitor B cells), **pre-B cells** (precursor B cells), **immature B cells,** and mature B cells.[16] **Figure 4–7** depicts the changes that occur as B cells mature from the pro-B stage to become memory cells or plasma cells.

At the earliest developmental stage, B-cell progenitors require direct contact with bone marrow stromal cells.[15] Several transcription, or growth, factors are necessary to differentiate common lymphoid precursors into pro-B cells. Some of these factors are E2A, EBF (early B-cell factor), interferon regulatory factor (IFR8), and paired box protein 5 (PAX5).[14,15,17] In addition, a cytokine called interleukin-7 (IL-7) is also necessary at this early developmental stage. All of these factors are produced in the microenvironment of the bone marrow.

During this maturation process, the first step in the pro-B phase is the rearrangement of genes that code for the heavy and light chains of an antibody molecule. Although portions of each chain are identical for every antibody molecule, it is the so-called variable regions that make each antibody molecule specific for a certain antigen or group of antigens. Rearrangement of the DNA by cutting out certain regions is similar to the process that occurs in T cells, where antigen specificity is built into the α and β chains

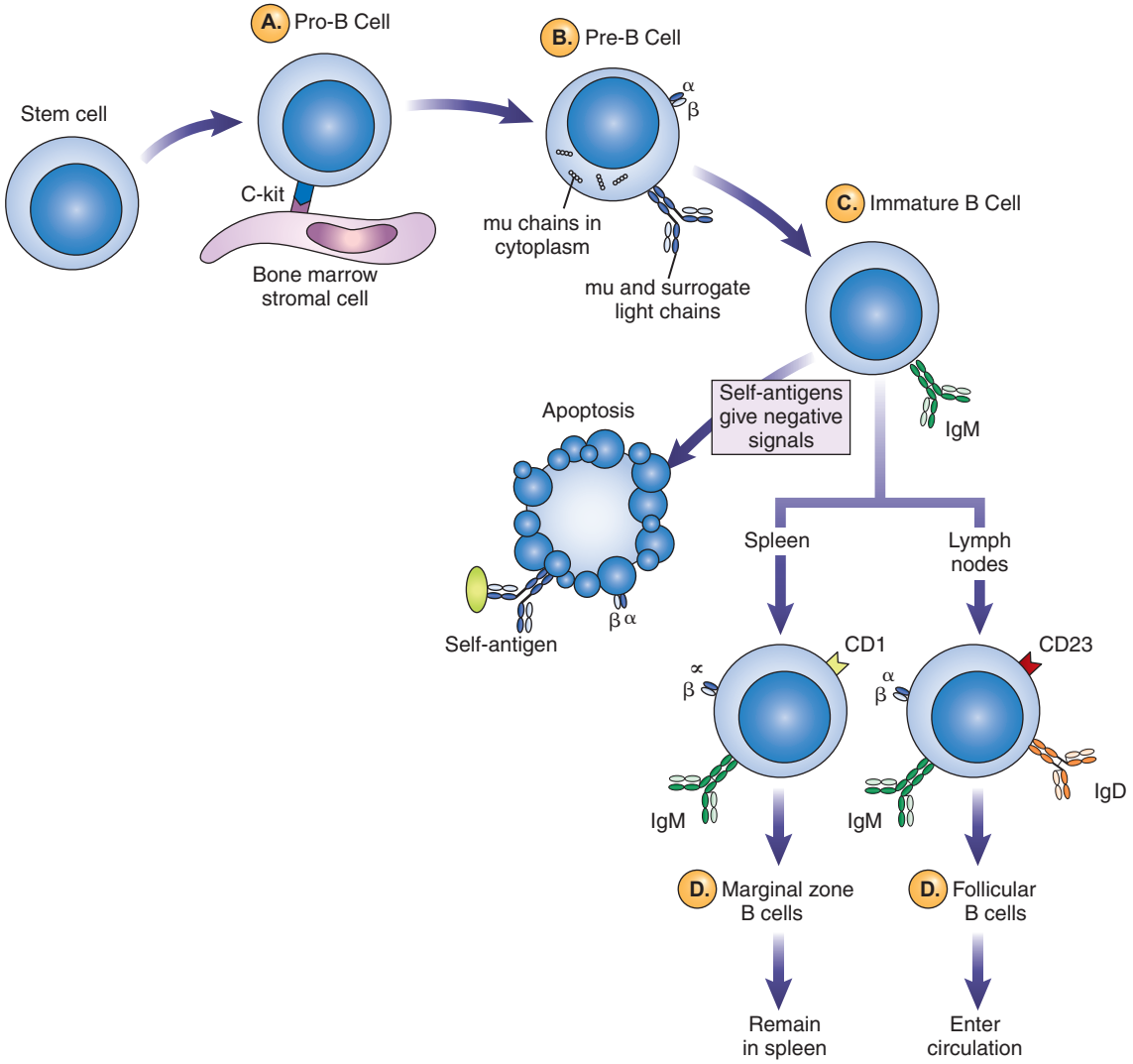


FIGURE 4–7 B-cell development in the bone marrow. Selected markers are shown for the various stages in the differentiation of B cells. Stages up to the formation of mature B cells occur in the bone marrow. (A) Pro-B cell. (B) Pre-B cell. (C) Immature B cell. (D) Mature B cell.

of the TCR for antigen. Heavy chains of antibody molecules are coded for on chromosome 14 and light chains are coded for on chromosomes 2 and 22.

Gene rearrangement of the DNA that codes for antibody production occurs in a strict developmental sequence. Rearrangement of genes on chromosome 14, which code for the heavy-chain part of the antibody molecule, takes place first in a random fashion (see Chapter 5 for details). C-Kit, a receptor on the pro-B cell, interacts with a cell surface molecule called *stem cell factor* found on stromal cells.[15,16] This interaction triggers the activation process. The DNA is cleaved randomly at certain possible recombination sites and the enzyme terminal deoxyribonucleotidyl transferase (TdT) helps to join the pieces back together by incorporating additional nucleotides in the joining areas.[2,15]

Differentiation of pro-B cells into pre-B cells occurs upon successful rearrangement of heavy-chain genes on one of the number 14 chromosomes.[17,18] If rearrangement of genes on the first chromosome 14 is not successful, then rearrangement of genes on the second chromosome 14 occurs. If neither rearrangement is successful, development of the cell is halted. Only pro-B cells that successfully rearrange one set of heavy-chain genes go on to become pre-B cells.

Pre-B Cells

Once heavy-chain genes are rearranged, then these genes are transcribed to make the protein that will be part of an antibody molecule. When synthesis of the heavy-chain part of the antibody molecule occurs, the pre-B stage begins.[15] The first heavy chains synthesized are the μ chains, which belong to the class of immunoglobulins called immunoglobulin M (IgM). The μ chains accumulate in the cytoplasm.[15] Pre-B cells may also express μ chains on the cell surface, accompanied by an unusual light chain molecule called a **surrogate light chain.**[15] Surrogate light chains consist of two short polypeptide chains that are noncovalently associated with each other, along with two shorter chains, Ig-α and Ig-β, which are signal-transducing subunits[14] (see Fig. 4–7B). The combination of the two heavy chains along with Ig-α, Ig-β and the surrogate light chains form the pre-B cell receptor (pre-BCR). It appears that only pre-B cells expressing the μ heavy chains in association with surrogate light chains survive and proceed to further differentiation.[14,15] Signaling through the pre-B receptors formed stimulates a burst of clonal expansion. If, however, gene rearrangement does not work, then B-cell development is halted and cells are destroyed by apoptosis.[15]

Immature B Cells

Immature B cells are distinguished by the appearance of complete IgM antibody molecules on the cell surface [2,16] (see Fig. 4–7C). This indicates that rearrangement of the genetic sequence coding for light chains on either chromosome 2 or 22 has taken place by this time. Completion of light chain rearrangement commits a cell to produce an antibody molecule with specificity for a particular antigen or group of related antigens. IgM molecules thus serve as the receptor for antigen. Variable regions, which occur on both the light and heavy chains, determine the specificity for antigen. Preexisting diversity of receptors for antigen is a hallmark of the adaptive immune system. It means that the capability to respond to a specific antigen is built in before a B cell ever encounters an antigen. Once surface immunoglobulins appear, μ chains are no longer detectable in the cytoplasm.

Other surface proteins that appear on the immature B cell include CD21, CD40, and class II MHC molecules. CD21 acts as a receptor for a breakdown product of the complement component C3, known as C3d (see Chapter 6 for details on complement). Presence of the CD21 receptor enhances the likelihood of contact between B cells and antigens because antigens frequently become coated with complement fragments during the immune response. CD40 and class II MHC are important for interaction of B cells with T cells.

At this stage, there is evidence that self-antigens give a negative signal to immature B cells.[14,15] Immature B cells that tightly bind self-antigens through cross-linking of surface IgM molecules receive a signal to halt development, resulting in arrested maturation and cell death.[15] Thus, many B cells capable of producing antibody to self-antigens are deleted from the marrow by the process of apoptosis. It is estimated that more than 90% of B cells die in this manner without leaving the bone marrow.[15] The elimination of B cells that bear self-reactive receptors is known as **central tolerance.** Immature B cells that survive this selection process leave the bone marrow and proceed to the spleen, where they become mature B cells.

Mature B Cells

In the spleen, immature B cells develop into mature cells known as either *marginal zone B cells* or *follicular B cells.*[2] Marginal B cells remain in the spleen in order to respond quickly to any blood-borne pathogens they may come into contact with.[2] Follicular B cells migrate to lymph nodes and other secondary organs. Unlike marginal B cells that remain in the spleen, follicular B cells are constantly recirculating throughout the secondary lymphoid organs. In addition to IgM, all mature B cells exhibit immunoglobulin D (IgD), another class of antibody molecule, on their surface (see Fig. 4–7D). Both IgM and IgD have the same specificity for a particular antigen or group of antigens. These surface immunoglobulins provide the primary activating signal to B cells when contact with antigen takes place.[19,20] IgD is not required for B-cell function, but it may prolong the life span of mature B cells in the periphery. Unless contact with antigen occurs, the life span of a mature B cell is typically only a few days.[2] If, however, a B cell is stimulated by antigen, it undergoes transformation to a blast stage that eventually forms memory cells and antibody-secreting plasma cells **(Fig. 4–8).** This process is known as the **antigen-dependent phase** of B-cell development. The production of antibodies by plasma cells is called **humoral immunity.**

Plasma Cells

Plasma cells are spherical or ellipsoidal cells between 10 and 20 μm in size that are characterized by the presence of

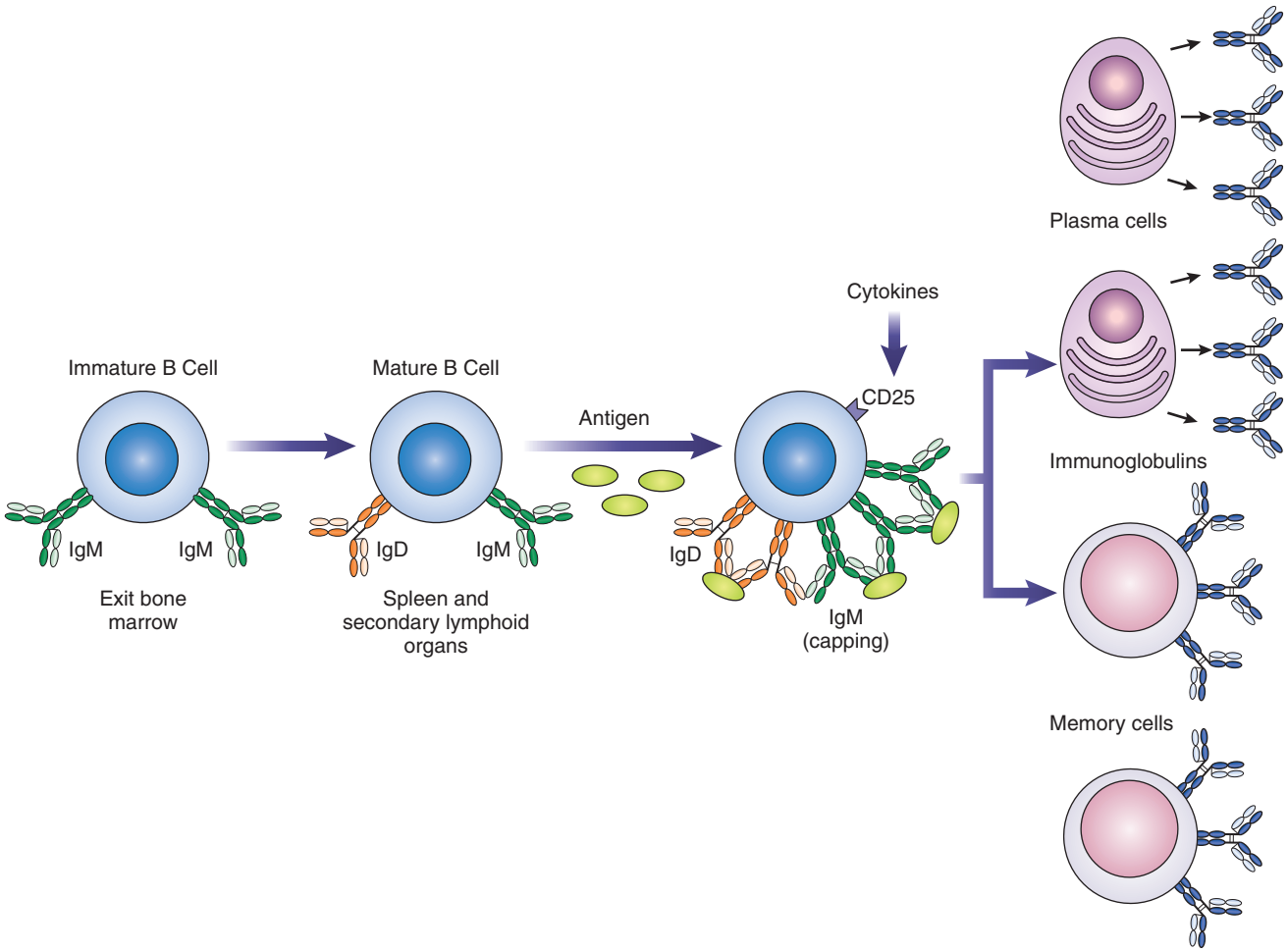


FIGURE 4–8 B-cell activation in peripheral lymph nodes. B cells capture specific antigen by means of immunoglobulin receptors. The activity of cytokines produced by Th cells produces transformation of naïve B cells into antibody-producing plasma cells and memory cells.

abundant cytoplasmic immunoglobulin and little to no surface immunoglobulin[21] (**Fig. 4–9**). The nucleus is eccentric or oval with heavily clumped chromatin that stains darkly. An abundant endoplasmic reticulum and a clear well-defined Golgi zone are present in the cytoplasm. Plasma cells represent the most fully differentiated lymphocyte and their main function is antibody production. They are not normally found in the blood; rather. they are located in germinal centers in the peripheral lymphoid organs or they reside in the bone marrow. In the bone marrow, plasma cells can survive in niches surrounded by stromal cells. Stromal cells provide chemical stimulation by cytokines, which allow plasma cells to be long-lived and continually produce antibodies.[1] Plasma cells found elsewhere are nondividing and, after several days of antibody production, they die without further proliferation.[17]

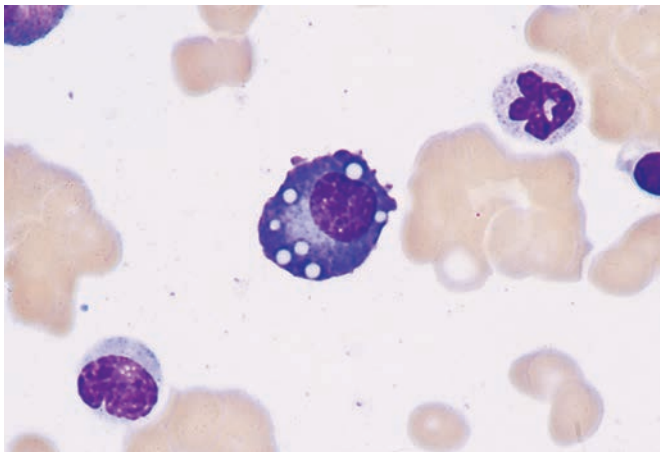


FIGURE 4–9 A typical plasma cell. *(From Harmening DM.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009.)*

The Role of T Cells in the Adaptive Immune Response

Interaction between T cells and antigen-presenting cells (APCs) is the initiating event in the adaptive immune response.[9] APCs such as macrophages and dendritic cells are first activated during the innate immune response through their pattern recognition receptors (see Chapter 3). Cells displaying processed antigen on the outside along with either class I or class II MHC proteins are transported to the T-cell zones of the lymph node or spleen. As you recall from Chapter 2, class I

MHC presents antigen that is from intracellular pathogens such as viruses to CD8+ T cells, whereas class II MHC presents antigen from extracellular pathogens such as bacteria processed by a phagocytic cell to CD4+ T cells.

Each mature T cell has a unique receptor for antigen (TCR). T cells circulate continuously through the bloodstream, lymph nodes, and secondary lymphoid tissue searching for antigen. It is estimated that each naïve or unstimulated T cell circulates from the lymph nodes to the blood and back again within 12 to 24 hours.[9] Only about 1 in 10^5 naïve T cells are specific for any given antigen, so recirculating increases the chances that a T cell will make contact with the appropriate antigen.[9]

As discussed in the section *T-Cell Differentiation* earlier, T cells have either CD4 or CD8 on their cell surface. CD4+ T cells are known as Th cells and CD8+ T cells are called cytotoxic T cells (Tc). Each of these two main types of T cells has a different role to play in the adaptive immune response. The function of the Th cells will be discussed first.

Action of T Helper Cells

The first activating signal to induce transformation of a T cell occurs when a CD4+ T cell encounters an antigen along with a class II MHC molecule and binds by using its antigen receptor. CD4 acts as a co-receptor to stabilize binding. A second signal is provided by the binding of CD28 on the T cell with CD80 and CD86 found on APCs.[19] Cytokines, chemical messengers, provide an additional signal (see Chapter 6).

Within 1 to 2 days after antigen recognition has occurred, T lymphocytes are transformed into large activated blast cells that are characterized by polyribosome-filled cytoplasm. They then begin cell division and generate progeny with an antigen receptor that is identical to that of the parent T cell. These functionally active small lymphocytes produce cytokines. The two main subsets of Th cells, Th1 and Th2, secrete different types of cytokines and affect different classes of cells. The Th1 subset secretes IL-2, interferon-gamma (IFN-γ) and TNF-β, which are responsible for the activation of cytotoxic T lymphocytes and macrophages. Th2 cells secrete interleukins that regulate B-cell activity. Most antigens encountered in the body are so-called **T-dependent antigens,** meaning that T-cell help is required in order for B cells to respond to antigen **(Fig. 4–10).**

In addition to effector cells that immediately secrete interleukins, T memory cells are also generated. T memory cells arise early in the course of an immune response, but the actual cells of origin are not yet known. They may arise independently from effector cells or they may arise as soon as the original T cell is stimulated.[9] T memory cells have a higher affinity for antigen than unstimulated T cells and are capable of immediate cytokine production when they reencounter the initiating antigen.[22] T memory cells are able to proliferate sooner than naïve T cells, express a broader array of cytokines, and appear to persist for years.[22]

Action of Cytotoxic T Cells

T cells that bear the CD8 marker have a different role to play than that of the Th cells. Once activated by antigen in the lymph nodes or spleen, cytotoxic T cells leave the secondary lymphoid tissue and circulate to sites of infection. Here they bind and kill infected cells by triggering apoptosis. This action differs from that of NK cells because it is antigen-specific. Cytotoxic T cells recognize antigen in association with class I MHC complexes, which were described in Chapter 2. Because all nucleated host cells express class I antigens on their surfaces, cytotoxic T cells act as a primary defense against intracellular pathogens such as viruses, as well as other altered host cells such as tumor cells that exhibit new antigens. The cytotoxic T cells bind to altered host cells by using the TCR receptor for antigen and by CD8, which recognizes class I MHC molecules **(Fig. 4–11).**

Naïve CD8+ T cells require 5 to 8 days after antigen activation to differentiate into cytotoxic lymphocytes. During this time, they proliferate to increase the numbers that can respond to the same antigen. After differentiation, they migrate to the affected tissue to find and destroy target cells.[19]

Cytotoxic T cells can kill target cells in two major ways. Once antigen recognition occurs, the cytotoxic T cells either release the contents of granules that damage the cell or they bind to the host cell and, using intracellular signaling, induce apoptosis. In either case, the target cell is induced to undergo apoptosis, usually within 30 minutes of contact[19,23] (see Fig. 4–11).

Granules within cytotoxic T cells contain two different types of toxins: granzymes and perforins. Granzymes are a class of enzymes called serine proteases and perforins are pore-forming proteins that insert themselves into the target cell membrane. Once a cytotoxic T cell has bound to a target cell, the granules move within the T cell to the site where the target cell has bound. Granules fuse with the T-cell membrane and the perforins and granzymes are released into the space between the target cell and the T cell. Perforins insert themselves into the target cell membrane and polymerize, forming pores in the membrane. Granzymes enter through the pores. Once the granzymes are in the cytoplasm of the target cell, a cascade of events is initiated that fragment the target cell DNA into small pieces.[23,24] Granzymes themselves do not directly break down DNA , but they activate a nuclease that destroys the target cell DNA as well as any viral DNA that may be contained inside it. Granzymes can also activate enzymes in the target cell that disrupt the cell's mitochondria.[24] The end result in either case is apoptosis or cell death.

The Role of B Cells in the Adaptive Immune Response

Antigen-dependent activation of B cells takes place in the primary follicles of peripheral lymphoid tissue. Follicular dendritic cells present antigen to B cells and thus play a key role in the immune response.[1,2]

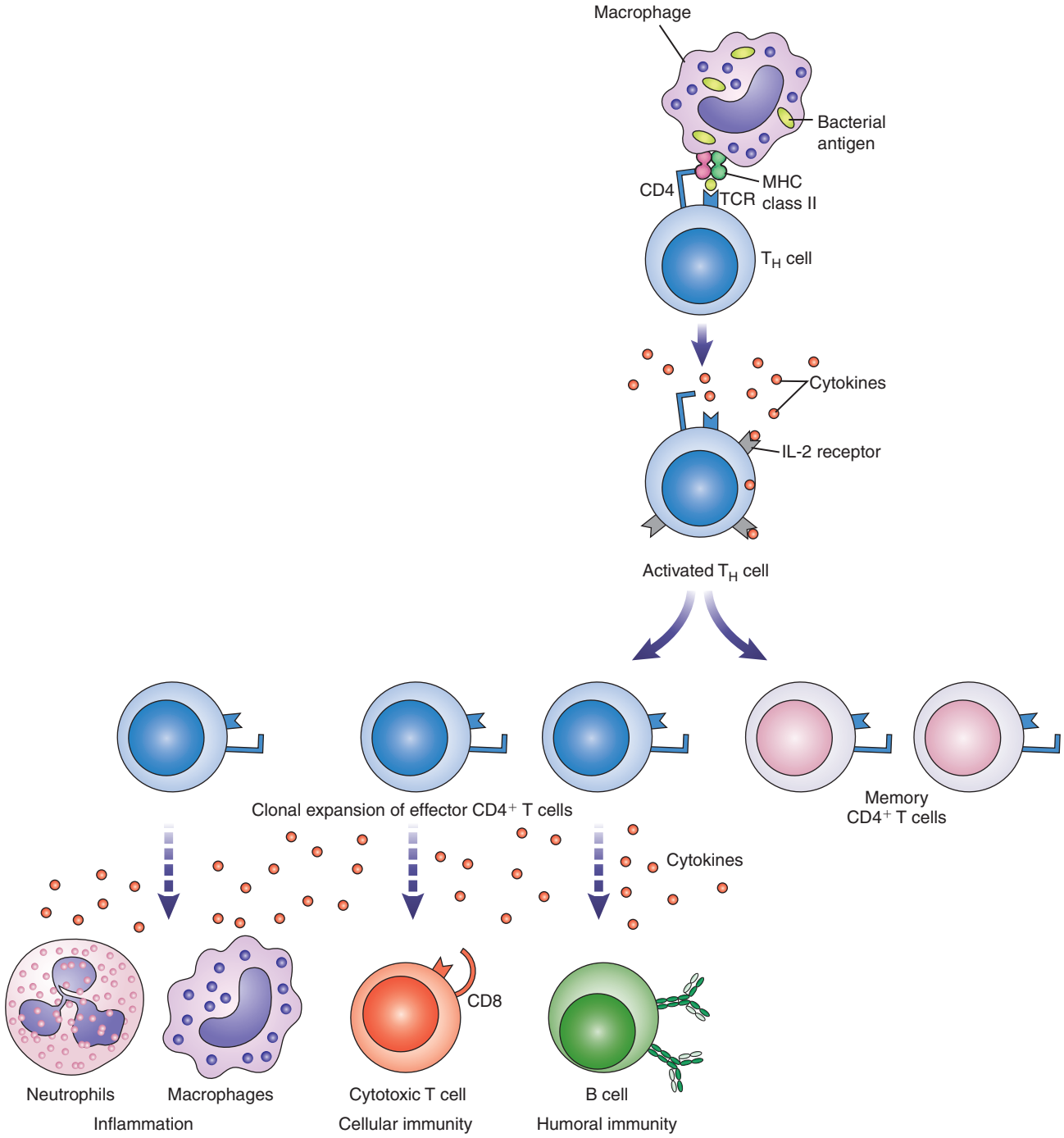


FIGURE 4–10 Activation of Th cells. Exposure to antigen presented by macrophages causes production of CD25 receptors for interleukin-2 (IL-2). IL-2 causes sensitized CD4+ T cells to secrete cytokines, resulting in CD4+ effector cells that have various functions. Some CD4+ cells secrete interleukins that recruit macrophages and neutrophils, whereas others activate CD8+ T cells to increase cytotoxicity against virally infected cells. Activated Th cells also enhance antibody production by B cells.

Response to T-Dependent Antigens

When B cells respond to T-dependent antigens, the B cells require two signals to be activated. The first occurs when antigen binds to membrane immunoglobulin receptors on the B-cell surface and cross-links them. Cross-linking leads to activation of intracellular signaling pathways that allow stimulated B cells to interact with T cells.[16] The second signal is provided by Th cells themselves, which bind to the B cell both through its antigen receptor and through CD40 on the B cell and CD40L on the activated Th cell.[13,16] The bound T cell then delivers cytokines and other signals to fully activate the B cell **(Fig. 4–12)**.

Connections

Mutation of CD40L Gene Causes Immunodeficiency

When the delicate balance between T- and B-cell interaction is disrupted, immune deficiencies may result. An X-linked mutation causing a CD40L deficiency on Th cells was found to affect certain immunoglobulin classes. Normal or increased levels of IgM are present, but B cells are unable to switch to producing other antibody classes having a higher affinity for the initiating antigen. IgG and IgA levels are decreased, leading to an increased susceptibility to infection in individuals with this mutation. See Chapter 17 for further discussion on immunodeficiency diseases.

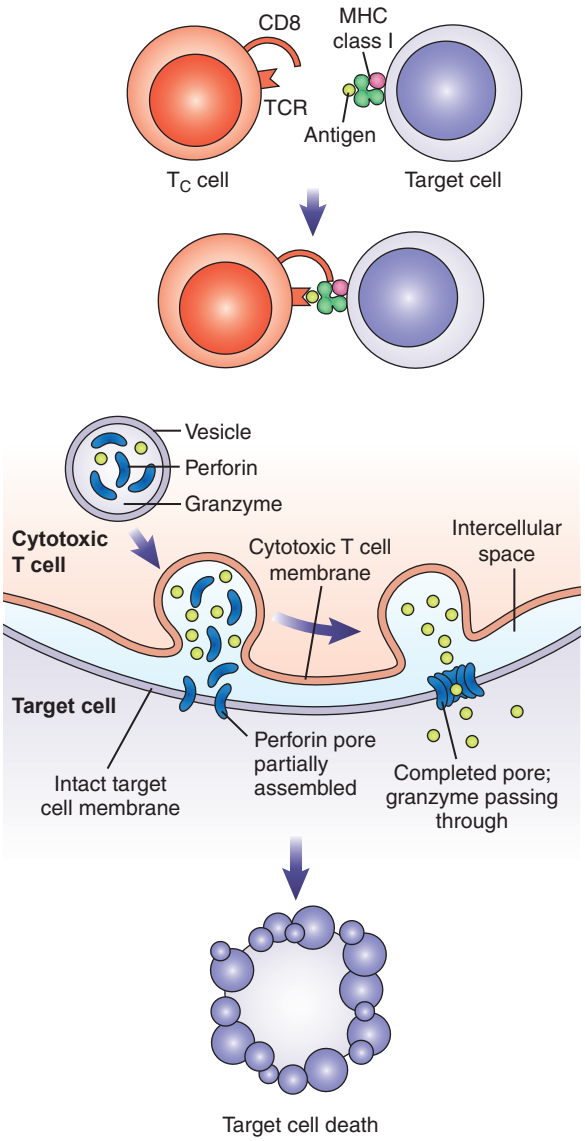


FIGURE 4–11 Activation of cytotoxic T cells. The CD8+ T cell recognizes foreign antigen along with class I MHC. When binding occurs, granules move toward the point of contact with the target cell. Granules fuse with the membrane and release perforin. Perforin inserts itself into the target cell membrane and polymerizes to form a pore. Contents of the granules are released, triggering apoptosis of the target cell.

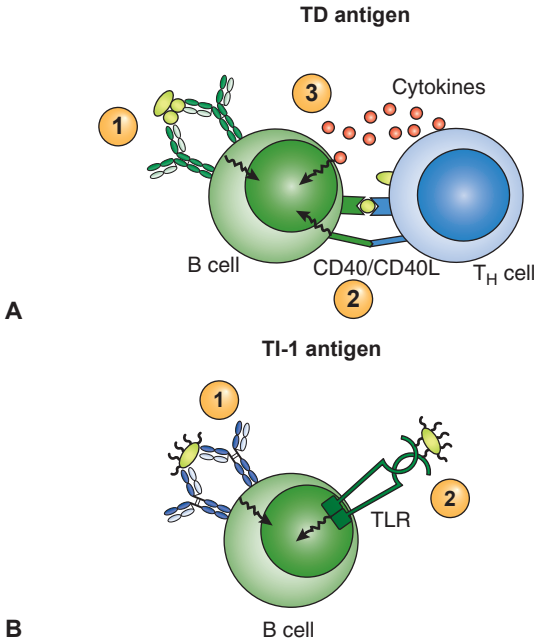


FIGURE 4–12 Activating signals for T-dependent and T-independent antigens. (A) T-dependent antigens bind to immunoglobulin receptors on B cells. The antigen is processed and delivered to CD4+ T cells. The Th cell binds by means of its CD3-TCR complex and delivers further activating signals through binding of CD40 on the B cell to the CD40L receptor on the Th cell. Cytokines are released from the T cell, which enhance B-cell transformation to plasma cells. (B) T-independent antigens can bind to B cells through immunoglobulin receptors and trigger B-cell transformation directly. Several antigen receptors must be cross-linked in order to activate a B cell directly. Antigens can also be bound to B cells' innate immune receptors such as TLRs.

Activated B cells exhibit identifying markers that include CD25, which is found on both activated T and B cells and which acts as a receptor for IL-2, a growth factor produced by T cells. Additional receptors that appear at this time are specific for other growth factors produced by T cells. Once a B cell has become activated, it will replicate. All the daughter cells will have the same receptor as the parent cell for the initiating antigen. The daughter cells of proliferating B cells either migrate into the T-cell zone in lymph nodes and differentiate into plasma cells that secrete IgM in about 4 days or they enter B-cell follicles to form germinal centers.[1,13]

Within the follicles, however, B cells undergo further differentiation under the influence of follicular Th cells. The CD40 on B cells must interact with CD40L on Th cells in order for germinal center formation to occur.[16] Here, both plasma cells and memory cells are formed **(Fig. 4–13).** Memory cells are progeny of B cells that have been exposed to antigen; they are characterized by a long life span and a rapid response to second exposure to the triggering antigen.[25,26] They are similar in appearance to unstimulated B cells, but they remain in an activated state for months or years, ready to respond to the initial antigen. CD27 is used as a marker to identify memory cells because they are similar in appearance to mature B cells.[2] It is

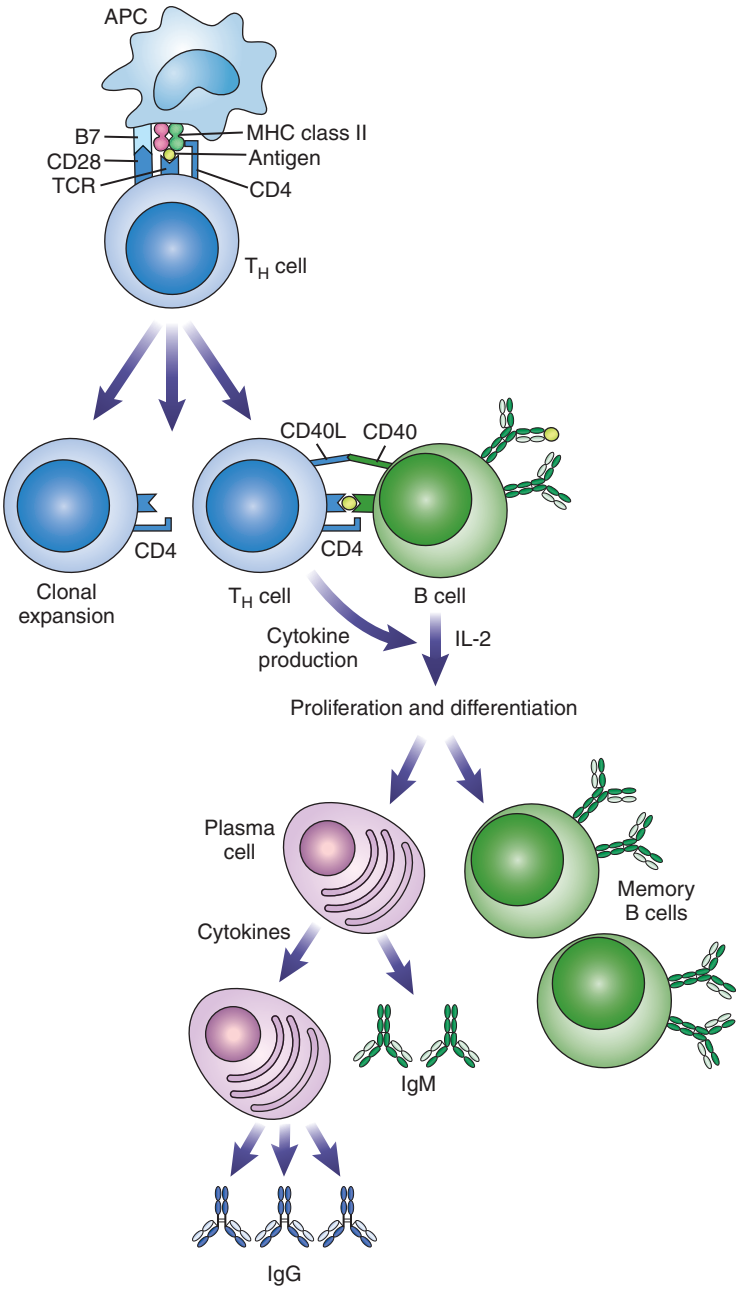


FIGURE 4–13 T- and B-cell cooperation in the immune response. CD4+ T cells recognize exogenous antigen on a macrophage along with class II MHC. Binding between CD28 and B7 enhances interaction between the cells. Th cells go through clonal expansion and produce cytokines, including interleukin-2 (IL-2). B cells capable of responding to the same antigen present antigen to Th cells through the class II MHC receptor. The TCR binds antigen and CD4 binds to class II MHC. CD40L bind to CD40, enhancing the reaction. Cytokine production by the T cell causes B cells to proliferate and produce plasma cells, which secrete antibody.

this built-in memory that distinguishes the adaptive immune response from innate immunity.

Gene rearrangement in B cells in germinal centers allows for expression of different classes of immunoglobulins. Cytokines acting on B cells determine the class of antibody that will be expressed. The variable regions remain the same, so the antigen specificity does not change. Rearrangement only occurs in the constant region, which determines the particular class of antibody that will be expressed. These altered B cells with rearranged DNA then differentiate into plasma cells capable of making antibody other than IgM. Most class switching results in IgG-producing plasma cells. Therefore, the antibody found in the greatest concentration in the serum is IgG. (See Chapter 5 for a complete discussion on types of immunoglobulins.)

Once formed, plasma cells leave the lymph nodes and circulate in the peripheral tissues. They secrete antibodies and have a relatively short life span. Some plasma cells migrate to the bone marrow, as mentioned previously, where they provide long-lasting memory. Others locate to the gastrointestinal tract, where they secrete an antibody type called IgA. The IgA serves as a protection against organisms that may reach the gut.[13]

Response to T-Independent Antigens

Some antigens are able to elicit antibody formation in the absence of T cells. These antigens are called **T-independent antigens.** Such antigens are able to interact with multiple immunoglobulin receptors on a B cell to cross-link them and induce proliferation and antibody production[2,17] **(see Fig. 4-12B).** Examples of these include plant lectins, polymerized proteins with repeating molecular patterns, and lipopolysaccharides found in bacterial

cell walls.[17,25] Typically, these antigens produce IgM only because the induction of memory cells does not occur to any great extent.[25]

Laboratory Identification of Lymphocytes

Identification of lymphocytes as either T cells or B cells may be useful in diagnosis of any of the following states: malignancies such as leukemias and lymphomas; immunodeficiency diseases involving either T or B cells or both; and acquired immunodeficiency disease (AIDS). In some immunodeficiency diseases such as X-linked hypogammaglobulinemia, B cells are frequently absent, whereas in severe combined immunodeficiency disease (SCID) both T and B cells are either absent or present in very low numbers. Because the human immunodeficiency virus (HIV) infects and progressively kills CD4+ T cells, assays for CD4+ T cells are useful in evaluating the stage of infection. Laboratory analysis usually involves distinguishing the following lymphocyte subsets: T cytotoxic cells (CD8), Th cells (CD4), and B cells. The gold standard for testing is cell flow cytometry.[27]

Cell flow cytometry is an automated system for identifying cells based on the scattering of light as cells in a stream of fluid flow in single file by a laser beam. Flow cytometry is able to segregate lymphocytes into subsets using a technique that relies on labeled monoclonal antibodies against specific surface antigens. Monoclonal antibodies are highly specific antibodies. Some of the more common antigens tested for include CD2, CD3, CD4, and CD8 on T cells and CD19, CD20, CD22, and surface immunoglobulin on B cells. Fluorescent antibodies are used to screen for subpopulations, such as B cells, Th cells, and T cytotoxic cells. Each antibody has a different fluorescent tag. The principles of flow cytometry are discussed more fully in Chapter 12.

Recently, some point-of-care testing has been developed using either fluorescent or antibody-labeled beads. These technologies measure the CD4 count and report it as a percentage of the total T-cell count. Although not as accurate as flow cytometry, it can be used in areas of the world where there is limited access to laboratories with sophisticated equipment.[28,29]

SUMMARY

- All undifferentiated lymphocytes arise in the bone marrow from hematopoietic stem cells. They mature in the primary lymphoid organs and are the key cell involved in the adaptive immune response.
- Adaptive immunity is characterized by specificity, the ability to remember a prior exposure to an antigen, and an increased response upon re-exposure to that same antigen.
- B cells mature in the bone marrow itself, whereas T cells acquire their specificity in the thymus.
- B cells can be recognized by the presence of surface antigens, or CDs, that are detected by monoclonal antibodies. B-cell markers include CD19, class II MHC proteins, and surface immunoglobulins.
- Surface immunoglobulins on B cells are receptors for antigen. Their specificity is built in as the B cell matures.
- Antigen-independent development of B cells occurs in the bone marrow, whereas the antigen-dependent phase takes place in the secondary lymphoid organs.
- Class II MHC proteins allow B cells to interact with T helper cells in the production of antibodies.
- When contact with a specific antigen occurs, B cells differentiate into plasma cells that produce antibodies and memory cells that are able to respond more quickly the next time the same antigen is encountered.
- Production of antibodies is known as humoral immunity.
- T cells are distinguished by the presence of CD3, CD2, and either CD4 or CD8.
- Cells that express CD4 belong to a T-cell subset that includes helper/inducer cells.
- CD8+ T cells are cytotoxic cells that are able to destroy cancer cells or virally infected host cells by producing perforins and granzymes.
- CD3/TCR is the T-cell receptor for antigen. The major portion of it is common to all T cells, but two chains—alpha and beta—contain variable regions that can bind to only certain antigens.
- Positive selection of immature T cells is based on interaction with the unique MHC antigens of the host.
- Negative selection in T-cell maturation is based on interaction with self-antigens of the host. If a T cell recognizes self-antigens, then it is destroyed by apoptosis.
- T cells are responsible for cell-mediated immunity, which involves production of cytokines that serve as regulatory factors for the immune response.
- Laboratory determination of individual lymphocyte populations is essential in diagnosis of such conditions as lymphomas, immunodeficiency diseases, unexplained infections, or acquired immune diseases such as AIDS.
- Lymphocytes are identified using monoclonal antibodies directed against specific surface antigens. They are enumerated through the use of cell flow cytometry, which categorizes cells on the basis of light scattering.

Study Guide: Comparison of T and B Cells

T CELLS	B CELLS
Develop in the thymus	Develop in the bone marrow
Found in blood (60–80% of circulating lymphocytes), thoracic duct fluid, lymph nodes	Found in bone marrow, spleen, lymph nodes
Identified by rosette formation with SRBCs	Identified by surface immunoglobulin
End products of activation are cytokines	End product of activation is antibody
Antigens include CD2, CD3, CD4, CD8	Antigens include CD19, CD20, CD21, CD40, class II MHC
Located in paracortical region of lymph nodes	Located in cortical region of lymph nodes

SRBC = Sheep red blood cells

CASE STUDIES

1. A 2-year-old boy is sent for immunologic testing because of recurring respiratory infections, including several bouts of pneumonia. The results show decreased immunoglobulin levels, especially of IgG. Although his WBC count was within the normal range, the lymphocyte count was low. Flow cytometry was performed to determine the levels of different classes of lymphocytes. The result showed a decrease in CD4+ cells. The CD19+ lymphocyte population was normal.

   Questions

   a. How can these findings be interpreted?
   b. How can this account for his recurring infections?

2. You and a friend of yours in your immunology class are discussing how the body is able to fight infection. Your friend states that as long as you have a good innate immune system and you can make antibodies, then a decrease in CD8+ T cells is not really important.

   Question

   a. How do you answer your friend?

REVIEW QUESTIONS

1. Which MHC molecule is necessary for antigen recognition by CD4+ T cells?
   a. Class I
   b. Class II
   c. Class III
   d. No MHC molecule is necessary.

2. Which would be characteristic of a T-independent antigen?
   a. The IgG antibody is produced exclusively.
   b. A large number of memory cells are produced.
   c. Antigens bind only one receptor on B cells.
   d. It consists of a limited number of repeating determinants.

3. Humoral immunity refers to which of the following?
   a. Production of antibody by plasma cells
   b. Production of cytokines by T cells
   c. Elimination of virally infected cells by cytotoxic cells
   d. Downregulation of the immune response

4. Where does antigen-independent maturation of B lymphocytes take place?
   a. Bone marrow
   b. Thymus
   c. Spleen
   d. Lymph nodes

5. In the thymus, positive selection of immature T cells is based upon recognition of which of the following?
   a. Self-antigens
   b. Stress proteins
   c. MHC antigens
   d. μ chains

6. Which of these are found on a mature B cell?
   a. IgG and IgD
   b. IgM and IgD
   c. Alpha and beta chains
   d. CD3

7. How do cytotoxic T cells kill target cells?
   a. They produce antibodies that bind to the cell.
   b. They engulf the cell by phagocytosis.
   c. They stop protein synthesis in the target cell.
   d. They produce granzymes that stimulate apoptosis.

8. Which of the following can be attributed to antigen-stimulated T cells?
   a. Humoral response
   b. Plasma cells
   c. Cytokines
   d. Antibody

9. Which is a distinguishing feature of a pre-B cell?
   a. μ chains in the cytoplasm
   b. Complete IgM on the surface
   c. Presence of CD21 antigen
   d. Presence of CD25 antigen

10. When does genetic rearrangement for coding of antibody light chains take place during B-cell development?
    a. Before the pre-B cell stage
    b. As the cell becomes an immature B cell
    c. Not until the cell becomes a mature B cell
    d. When the B cell becomes a plasma cell

11. Which of the following antigens are found on the T-cell subset known as helper/inducers?
    a. CD3
    b. CD4
    c. CD8
    d. CD11

12. Where does the major portion of antibody production occur?
    a. Peripheral blood
    b. Bone marrow
    c. Thymus
    d. Lymph nodes

13. Which of the following would represent a double-negative thymocyte?
    a. CD2–CD3+CD4–CD8+
    b. CD2–CD3–CD4+CD8–
    c. CD2+CD3+CD4–CD8–
    d. CD2–CD3–CD4+CD8–

14. Which of the following best describes the T-cell receptor for antigen?
    a. It consists of IgM and IgD molecules.
    b. It is the same for all T cells.
    c. It is present in the double-negative stage.
    d. Alpha and beta chains are unique for each antigen.

15. A cell flow cytometry pattern belonging to a 3-year-old patient showed the following: normal CD4+ T-cell count, normal CD19+ B-cell count, low CD8+ T-cell count. Which type of immunity would be affected?
    a. Production of antibody
    b. Formation of plasma cells
    c. Elimination of virally infected cells
    d. Downregulation of the immune response

16. Which of the following is a unique characteristic of adaptive immunity?
    a. Ability to fight infection
    b. Ability to remember a prior exposure to a pathogen
    c. A similar response to all pathogens encountered
    d. Process of phagocytosis to destroy a pathogen

17. Clonal deletion of T cells as they mature is important in which of the following processes?
    a. Elimination of autoimmune responses
    b. Positive selection of CD3/TCR receptors
    c. Allelic exclusion of chromosomes
    d. Elimination of cells unable to bind to MHC antigens

18. Where do germinal centers occur?
    a. In the thymus
    b. In the bone marrow
    c. In peripheral blood
    d. In lymph nodes

5 Antibody Structure and Function

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Describe the structure of a typical immunoglobulin.
2. Identify the electrophoretic fraction of serum that contains the majority of immunoglobulins.
3. Differentiate between isotypes, allotypes, and idiotypes.
4. Characterize the five immunoglobulin types found in humans.
5. Differentiate between light and heavy chains of immunoglobulins.
6. Describe experimental evidence for the structure of IgG.
7. Discuss how the IgG subclasses differ in functional capability.
8. Explain how the structure of IgM differs from that of IgG.
9. Relate differences in structure of the five immunoglobulin classes to their function.
10. Describe the secretory component of IgA.
11. Discuss how IgD differs from other immunoglobulin types.
12. Identify the types of cells that IgE binds to in allergic reactions.
13. Compare the primary and secondary responses to antigen.
14. Describe how recent knowledge about immunoglobulin genes supports the clonal selection hypothesis.
15. Discuss the process of monoclonal antibody production.
16. Relate the influence of monoclonal antibodies to current laboratory testing practices.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Allelic exclusion
Allotypes
Antibody
Bence Jones proteins
Class switching
Clonal selection hypothesis
Constant region
Domains and neutrophils
$F(ab')_2$
Fab fragments
Fc fragment
Heavy (H) chains
Hinge region
Hybridoma
Idiotype
Immunoglobulin
Isotype
Joining (J) chain
Kappa (κ) chains
Lambda (λ) chains
Light (L) chains
Monoclonal antibodies
Primary response
Secondary response
Secretory component (SC)
Tetrapeptide
Variable region

When B lymphocytes are stimulated by antigen and undergo differentiation, the end product is an **antibody,** also known as an immunoglobulin. **Immunoglobulins,** glycoproteins found in the serum portion of the blood, constitute approximately 20% of plasma proteins in healthy individuals. All immunoglobulins are composed of 86% to 98% polypeptide and 2% to 14% carbohydrate.[1] Some of the early experiments attempting to determine the structure of immunoglobulins involved serum protein electrophoresis. In electrophoresis, serum is placed on an agarose gel and an electrical current is applied to separate out the proteins. If electrophoresis is carried out at a pH of 8.6, most serum proteins can be separated out on the basis of size and charge. Five distinct bands are obtained in this manner. Immunoglobulins are the slowest moving proteins and appear primarily in the gamma (γ) band (**Fig. 5–1**). It was discovered that the gamma band contained most of the antibody activity. Hence, an early name for antibody was gamma globulin. Serum protein electrophoresis is used today as a screening tool for some clinical diseases involving over- or underproduction of antibodies, so it will be discussed in later chapters in relation to specific diseases.

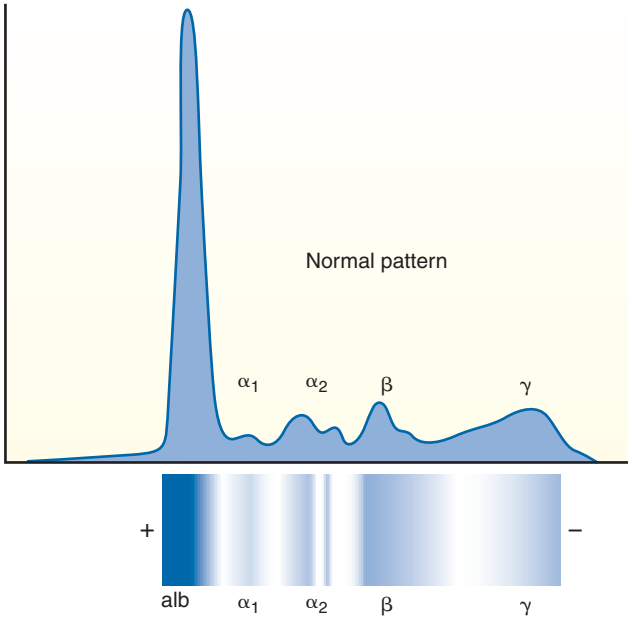


FIGURE 5–1 Serum electrophoresis. Serum is subjected to an electrical charge, and proteins are separated out on the basis of size and charge. Antibodies are found in the gamma region because they are the slowest proteins and have a charge that is close to neutral. Hence, they do not move far from the origin.

Immunoglobulins are considered to be the main humoral element of the adaptive immune response. They play an essential role in antigen recognition and in biological activities related to the immune response such as opsonization and complement activation. Immunoglobulins are divided into five major classes on the basis of a part of the molecule called the heavy chain. These classes are designated as IgG, IgM, IgA, IgD, and IgE (with *Ig* being the abbreviation for immunoglobulin). The heavy chains are γ, μ, α, δ, and ε, respectively. Although each class has distinct properties, all immunoglobulin molecules share many common features and their basic structure is similar.

The immunoglobulin structure is also similar to other molecules belonging to the immunoglobulin superfamily, a group of glycoproteins that share a common ancestral gene. This gene originally coded for 110 amino acids, but it has been duplicated and mutated over time. However, each type of immunoglobulin is made up of a number of regions called **domains,** which consist of approximately 110 amino acids each. Although the different immunoglobulin classes may have differing numbers of domains, the three-dimensional structure of each is essentially the same.

This chapter presents the nature of this generalized structure and discusses the characteristics of each immunoglobulin type. Specific functions for each of the classes are examined in relation to structural differences. Knowing the type of immunoglobulin produced is important in the diagnosis of many infectious diseases, allergies, and autoimmune diseases. There are different laboratory tests for particular antibodies based on the immunoglobulin class. This is especially important in blood banking because some antibodies are more harmful than others when considering giving blood to a patient. Thus, knowing the particular type of antibody produced is extremely helpful in clinical diagnosis.

Tetrapeptide Structure of Immunoglobulins

All immunoglobulin molecules are made up of a basic four-chain **tetrapeptide** unit that consists of two large chains called **heavy** or **H chains** and two smaller chains called **light** or **L chains.** Each chain has a single variable region and one or more constant regions. The variable region is unique to

each specific antibody. These chains are held together by noncovalent forces and disulfide interchain bridges. The basic structure of immunoglobulins was first discovered in the 1950s and 1960s because of the efforts of two men: Gerald Edelman, working at the Rockefeller Institute in the United States, and Rodney Porter, working at Oxford University in England. They chose to work with immunoglobulin G (IgG), the most abundant of all the antibodies. For their contributions, these men shared the Nobel Prize in physiology and medicine in 1972.

Edelman's work centered on using the analytic ultracentrifuge, a very high speed centrifuge, to separate out immunoglobulins on the basis of molecular weight.[2] He found that intact IgG molecules had a sedimentation coefficient of 7 S (the Svedberg unit [S] indicates the sedimentation rate in an analytical ultracentrifuge). Larger molecules will travel farther and thus have a larger sedimentation coefficient. On obtaining a purified preparation of IgG, Edelman used 7 M urea to unfold the molecule. Once unfolded, the exposed sulfhydryl bonds could be broken by a reducing agent such as mercaptoethanol. After such treatment, the material was subjected again to ultracentrifugation, and two separate fractions, one at 3.5 S and one at 2.2 S, were obtained.

The 3.5 S fraction, with a molecular weight of approximately 50,000, was designated the H chain; the 2.2 S fraction, with a molecular weight of 22,000, was named the L chain.[3] These two pieces occurred in equal amounts, indicating that the formula for IgG had to be H_2L_2. This is the generalized formula for all immunoglobulins **(Fig. 5–2).** In any one immunoglobulin molecule, the two H chains are identical as are the two L chains.

Porter's work was based on the use of the proteolytic enzyme papain, which was used to cleave IgG into three pieces of about equal size, each having a sedimentation coefficient of 3.5 S and representing a molecular weight of approximately 45,000 to 50,000 d.[3,4] Carboxymethyl cellulose ion exchange chromatography separated this material into two types of fragments, one of which spontaneously crystallized at 4°C. This fragment, known as the **Fc fragment** (for "fragment crystallizable"), had no antigen-binding ability and is now known to represent the carboxy-terminal halves of two H chains that are held together by S–S bonding.[3] The Fc fragment is important in effector functions of immunoglobulin molecules, which include opsonization and complement fixation.

The remaining two identical fragments were found to have antigen-binding capacity and were named **Fab fragments** (fragment antigen binding). Because precipitation would not occur if Fab fragments were allowed to react with antigen, it was guessed that each fragment represented one antigen-binding site and that two such fragments were present in an intact antibody molecule. Such a molecule would be able to form a cross-linked complex with antigen and the complex would precipitate. Each Fab fragment thus consists of one L chain and one-half of an H chain held together by disulfide bonding[4,5] (see Fig. 5–2).

Alfred Nisonoff used pepsin to obtain additional evidence for the structure of immunoglobulins.[3] This proteolytic enzyme was found to cleave IgG at the carboxy-terminal side of the

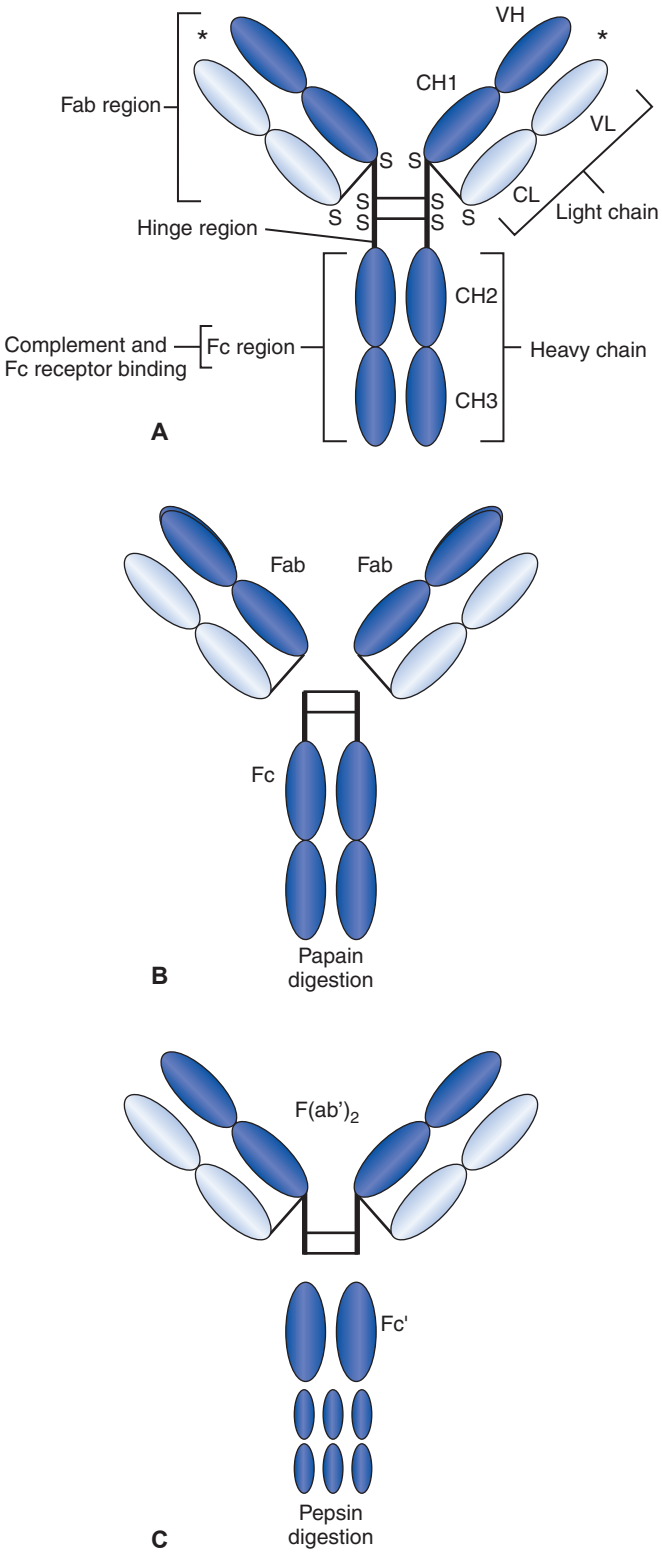


FIGURE 5–2 Basic structure of an immunoglobulin molecule, as discovered by Gerald Edelman. (A) Immunoglobulin G is made up of two H chains (50,000 MW each) and two L chains (22,000 MW each), held together by disulfide bonds. Intrachain disulfide bonds create looped regions or domains. The amino-terminal end of each chain is a variable region, whereas the carboxy-terminal end consists of one or more constant regions. (B) Papain digestion yields two Fab fragments and an Fc portion. (C) Pepsin digestion yields an $F(ab')_2$ fragment with all the antibody activity, as well as an Fc′ portion.

interchain disulfide bonds, yielding one single fragment with a molecular weight of 100,000 d and all the antigen-binding ability, known as **$F(ab')_2$.** An additional fragment called Fc' was similar to Fc except that it disintegrated into several smaller pieces.

The combined work of the three researchers provided a basic picture of the four-chain unit of the immunoglobulin molecule, which indicated that each L chain was bonded to an H chain by means of an S–S bond and the H chains were joined to each other by one or more S–S bonds (see Fig. 5–2). The exact number of disulfide bonds differs among antibody classes and subclasses.

Once the overall structure of immunoglobulins was discovered, scientists wanted to look more closely at the makeup of the individual chains. In order to perform amino acid analysis of individual immunoglobulin chains, it was necessary to have a source for pure antibody molecules. Immunologic reactions in both animals and humans normally produce a mixture of antibodies, making it difficult to isolate a single antibody type. This proved to be an obstacle to early research on the nature of immunoglobulins.

The Nature of Light Chains

The difficulty in obtaining a significant amount of a specific immunoglobulin for amino acid analysis was overcome by the chance discovery that **Bence Jones proteins,** found in the urine of patients with multiple myeloma, were in fact L chains that were being secreted by the malignant plasma cells.[3] Dr. Henry Bence Jones discovered the proteins in 1845 when he noted their peculiar behavior: When heated to 60°C, they precipitate from urine, but on further heating to 80°C, they redissolve. These characteristics made it possible to isolate the L chains and obtain the amino acid sequence.

Analysis of several Bence Jones proteins revealed that there were two main types of L chains, which were designated **kappa (κ) chains** and **lambda (λ) chains.** Each contained between 200 and 220 amino acids; from position number 111 onward (the amino terminus is position number 1), it was discovered that each type had essentially the same sequence. This region was called the **constant region** and the amino-terminal end was called the **variable region.** Thus, all κ L chains have an almost identical carboxy-terminal end; the same is true of λ chains. Sixty percent of L chains are κ chains because they are coded for first in DNA transcription of genes coding for antibody molecules.[3] The difference between the κ and λ chains lies in the amino acid substitutions at a few locations along the chain. There are no functional differences between the two types. Both κ and λ L chains are found in all five classes of immunoglobulins, but only one type is present in a given molecule.

Heavy-Chain Sequencing

Heavy-chain sequencing demonstrates the presence of domains similar to those in the L chains—that is, variable and constant regions. The first approximately 110 amino acids at the amino-terminal end constitute the variable domain; the remaining amino acids can typically be divided up into three or more constant regions with very similar sequences, designated C_H1, C_H2, and C_H3. Constant regions of the H chain are unique to each class and give each immunoglobulin type its name. Hence, IgG has an γ H chain, IgM a μ chain, IgA an α chain, IgD a δ chain, and IgE an ε chain. Each of these represents an **isotype,** a unique amino acid sequence that is common to all immunoglobulin molecules of a given class in a given species. Minor variations of these sequences that are present in some individuals but not others are known as **allotypes (Fig. 5–3).** Allotypes occur in the four IgG subclasses, in one IgA subclass, and in the κ L chain.[3] These genetic markers are found in the constant region and are inherited in simple Mendelian fashion. Some of the best-known examples of allotypes are variations of the γ chain known as G1m3 and G1m17.

The variable portions of each chain are unique to a specific antibody molecule, and they constitute what is known as the **idiotype** of the molecule. The amino-terminal ends of both L and H chains contain these regions, which are essential to the formation of the antigen-binding site. Together they serve as the antigen-recognition unit.

Hinge Region

The segment of H chain located between the C_H1 and C_H2 regions is known as the **hinge region.** It has a high content of proline and hydrophobic residues; the high proline content allows for flexibility.[3] This ability to bend lets the two antigen-binding sites operate independently and engage in an angular motion relative to each other and to the Fc stem. Thus, two Fab arms can cover a good bit of territory.[5] Such flexibility also assists in effector functions including initiation of the complement cascade (see Chapter 7 for details) and binding to cells with specific receptors for the Fc portion of the molecule.[5] Gamma, delta, and alpha chains all have a hinge region, but mu and epsilon chains do not. However, the C_H2 domains of these latter two chains are paired in such a way as to confer flexibility to the Fab arms.[3]

In addition to the four polypeptide chains, all types of immunoglobulins contain a carbohydrate portion, which is localized between the C_H2 domains of the two H chains. Functions of the carbohydrate include (1) increasing the solubility of immunoglobulin, (2) providing protection against degradation, and (3) enhancing functional activity of the Fc domains. This

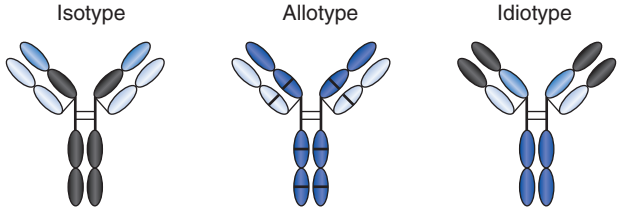


FIGURE 5–3 Antibody variations (shown in black). (A) Isotype—the H chain that is unique to each immunoglobulin class. (B) Allotype—genetic variations in the constant regions. (C) Idiotype—variations in variable regions that give individual antibody molecules specificity.

latter function may be the most important because glycosylation appears to be critical for recognition by Fc receptors that are found on phagocytic cells.[5]

Three-Dimensional Structure of Antibodies

The basic four-chain structure of all immunoglobulin molecules does not actually exist as a straight Y shape but is in fact folded into compact globular subunits based on the formation of balloon-shaped loops at each of the domains.[6] Intrachain disulfide bonds stabilize these globular regions. Within each of these regions or domains, the polypeptide chain is folded back and forth on itself to form what is called a β-pleated sheet. The folded domains of the H chains line up with those of the L chains to produce a cylindrical structure called an *immunoglobulin fold* **(Fig. 5–4).**[6] Antigen is captured within the fold by binding to a small number of amino acids at strategic locations on each chain known as *hypervariable regions.*

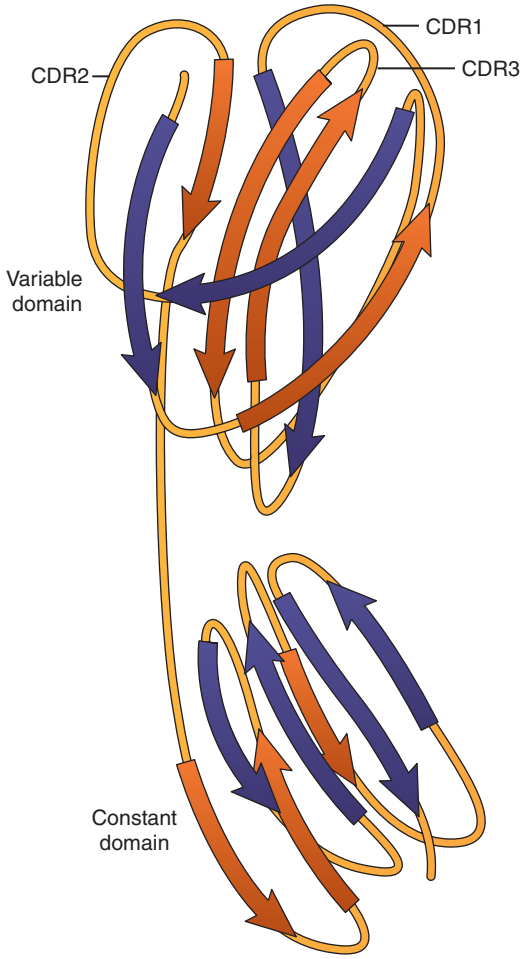


FIGURE 5–4 Three-dimensional structure of an L chain. In this ribbon diagram tracing the polypeptide backbone, β strands (polypeptide chains) are shown as wide ribbons, with other regions as narrow strings. Each of the two globular domains consists of a barrel-shaped assembly of seven to nine antiparallel β strands (polypeptide chains). The three hypervariable regions (CDR1, CDR2, and CDR3) are flexible loops that project outward from the amino-terminal end of the V_L domain.

Three small hypervariable regions consisting of approximately 30 amino acid residues are found within the variable regions of both H and L chains. Each of these regions, called *complementarity-determining regions* (CDRs), is between 9 and 12 residues long.[3] They occur as loops in the folds of the variable regions of both L and H chains. The antigen-binding site is actually determined by the apposition of the six hypervariable loops, three from each chain (see Fig. 5–4). Antigen binds in the middle of the CDRs, with at least four of the CDRs involved in the binding.[3] Thus, a small number of amino acids can create an immense diversity of antigen-binding sites. Properties of individual antibody classes are considered in the following sections.

Immunoglobulin G (IgG)

IgG is the predominant immunoglobulin in humans, comprising approximately 70% to 75% of the total serum immunoglobulins. As seen in **Table 5–1,** IgG has the longest half-life of any immunoglobulin class, approximately 23 days, which may help to account for its predominance in serum. There are four major subclasses with the following distribution: IgG1, 66%; IgG2, 23%; IgG3, 7%; and IgG4, 4%.[6] These subclasses differ mainly in the number and position of the disulfide bridges between the γ chains, as seen in **Figure 5–5.** Variability in the hinge region affects the ability to reach for antigen and the ability to initiate important biological functions such as complement activation.[1,7] IgG3 has the largest hinge region and the largest number of interchain disulfide bonds; therefore, it is the most efficient at binding complement, followed by IgG1.[1] IgG2 and IgG4 have shorter hinge segments, which tend to make them poor mediators of complement activation.[1,7] All subclasses have the ability to cross the placenta except IgG2.

Major functions of IgG include the following:

- Providing immunity for the newborn because IgG is the only antibody that can cross the placenta
- Fixing complement
- Coating antigen for enhanced phagocytosis (opsonization)
- Neutralizing toxins and viruses
- Participating in agglutination and precipitation reactions

All subclasses are able to participate in the secondary immune response, an enhanced and quicker response to antigen, although their appearance depends on the triggering antigen. IgG1and IgG3 are induced in response to protein antigens, whereas IgG2 and IgG4 are associated with polysaccharide antigens.[7]

Macrophages, monocytes, and neutrophils have receptors on their surfaces that are specific for the Fc region of IgG. This enhances contact between antigen and phagocytic cells and generally increases the efficiency of phagocytosis. IgG1 and IgG3 are particularly good at initiating phagocytosis, because they bind most strongly to Fc receptors.[7]

IgG has a high diffusion coefficient that allows it to enter extravascular spaces more readily than other immunoglobulin

Table 5–1 Properties of Immunoglobulins

	IgG	IgM	IgA	IgD	IgE
Molecular weight	150,000	900,000	160,000 monomer	180,000	190,000
Sedimentation coefficient	7 S	19 S	7 S	7 S	8 S
Serum half-life (days)	23	6	5	1–3	2–3
Serum concentration (mg/dL)	800–1600	120–150	70–350	1–3	0.005
Percent of total immunoglobulin	70–75	10	10–15	<1	0.02
H chain	γ	μ	α	δ	ε
H chain subclasses	γ1, γ2, γ3, γ4	None	α1, α2	None	None
H chain molecular weight	50,000–60,000	70,000	55,000–60,000	62,000	70,000–75,000
Constant domains (H chain)	3	4	3	3	4
Carbohydrate content (weight percent)-	2–3	12	7–11	9–14	12
Electrophoretic migration	γ2–α1	γ1–β12	γ2–β2	γ1	γ1
Complement fixation	Yes	Yes	No	No	No
Crosses placenta	Yes	No	No	No	No

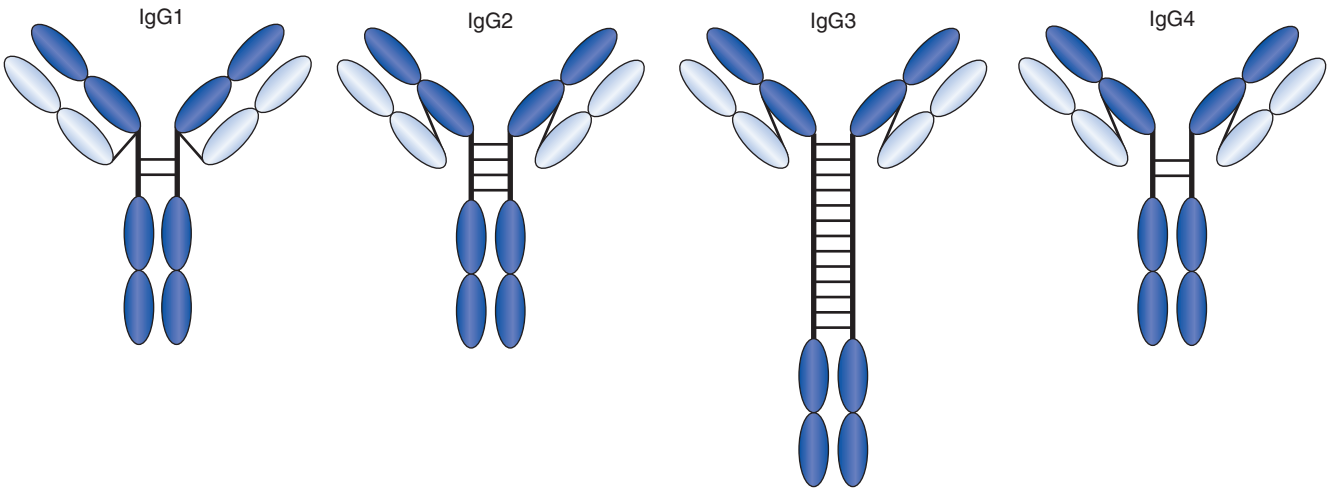


FIGURE 5–5 The four IgG subclasses are IgG1, IgG2, IgG3, and IgG4. These differ in the number and linkages of the disulfide bonds.

types. In fact, it is distributed almost equally between the intravascular and extravascular spaces.[1] Thus, it plays a major role in neutralizing toxins and viruses.

Agglutination and precipitation reactions take place in vitro, although it is not known how significant a role these reactions play in vivo. IgG is better at precipitation reactions than at agglutination because precipitation involves small soluble particles, which are more easily brought together by the relatively small IgG molecule. Agglutination is the clumping together of larger particles such as red blood cells (RBCs); because it is a larger molecule, IgM is much more efficient at this than IgG.

Immunoglobulin M (IgM)

IgM is known as a *macroglobulin* because it has a sedimentation rate of 19 S, which represents a molecular weight of approximately 900,000.[6] The half-life of IgM is about 6 days, much shorter than that of IgG (see Table 5–1). It accounts for between 5% and 10% of all serum immunoglobulins.

If IgM is treated with mercaptoethanol, it dissociates into five 7 S units, each having a molecular weight of 190,000 and a four-chain structure that resembles IgG. The molecular weight of the H or μ chain is approximately 70,000. It consists of about 576 amino acids and includes one more constant

domain than is found on the γ chain. The pentamer form is found in serum, whereas the monomer form occurs on the surface of B cells.[1,5]

The five monomeric units are held together by a **J** or **joining chain,** which is a glycoprotein made in plasma cells that contains several cysteine residues.[6] These serve as linkage points for disulfide bonds between two adjacent monomers. Linkage occurs at the carboxy-terminal end of two of the μ chains, and it appears that the J chain may initiate polymerization by stabilizing Fc sulfhydryl groups so that cross-linking can occur.[5] The J chain also facilitates secretion at mucosal surfaces.[7] The molecular weight of the J chain is approximately 15,000. One J chain is present per pentamer.

IgM thus configured assumes a starlike shape **(Fig. 5–6)** with 10 functional binding sites. The Fab arms can bend out of the plane to bind two or more separate antigens or multivalent antigens.[7] The high valency of IgM antibodies helps to overcome the fact that they tend to have a low affinity for antigen.

Because of its large size, IgM is found mainly in the intravascular pool and not in other body fluids or tissues. It cannot cross the placenta. IgM is known as the primary response antibody; it is the first to appear after antigenic stimulation and the first to appear in the maturing infant. It is synthesized only as long as antigen remains present because there are no memory cells for IgM. Thus, IgM can be used to diagnose an acute infection, as its presence indicates a primary exposure to antigen.[7]

Figure 5–7 depicts the difference between the primary response, which is predominantly IgM, and the secondary response, which is mainly IgG. The **primary response** is characterized by a long lag phase, a slow increase in antibody, and a short-lived response. The second or anamnestic response is distinguished by a shortened lag period, a much more rapid rise in antibody, and higher serum levels for a longer period of time. The **secondary response** is the result of the larger number of antigen-specific memory T and B cells generated during the primary response.

The functions of IgM include (1) complement fixation, (2) agglutination, (3) opsonization, and (4) toxin neutralization. IgM is the most efficient of all immunoglobulins at triggering the classical complement pathway (see Chapter 7) because a single molecule can initiate the reaction as a result of its multiple binding sites. This probably represents the most important function of IgM. The larger number of binding sites also makes IgM more efficient at agglutination reactions, especially with multivalent antigens. Thus, IgM forms a potent defense against many bacterial diseases.

IgM also serves as a surface receptor for antigen. Mu chains first appear in the cytoplasm of the pre-B cell. When they associate with the early surrogate L chains, rearrangement of the genes controlling L chain synthesis is begun.[5,8] Later, as L chains are synthesized, IgM monomers are formed and become inserted into the plasma membrane. The presence of membrane IgM classifies lymphocytes as immature B cells. (See Chapter 4 for a complete discussion of B-cell development.)

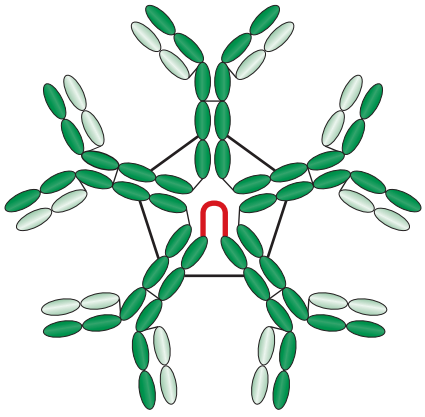


FIGURE 5–6 The pentameric structure of IgM is linked by a J chain (shown in red). Each arm can bend out of the plane to capture antigen.

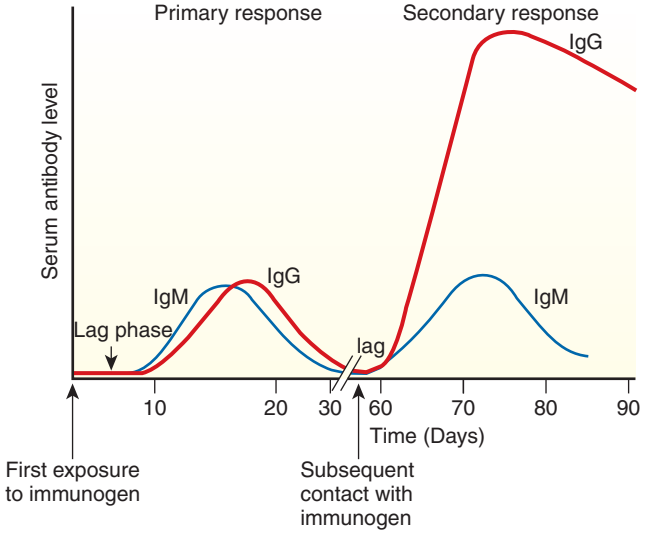


FIGURE 5–7 A comparison of the primary and secondary responses to an immunogen. The primary response is characterized by a long lag phase, whereas the secondary or anamnestic response has a shortened lag phase and a much more rapid increase in antibody titer, mainly IgG.

Immunoglobulin A (IgA)

In the serum, IgA represents 10% to 15% of all circulating immunoglobulin. It appears as a monomer with a molecular weight of approximately 160,000, has a sedimentation coefficient of 7 S, and migrates between the β and γ regions on electrophoresis. The H chain, called the α chain, has a molecular weight between 55,000 and 60,000 and consists of about 472 amino acids. These amino acids comprise one variable and three constant regions. There are two subclasses, designated IgA1 and IgA2. They differ in content by 22 amino acids, 13 of which are located in the hinge region and are deleted in IgA2. The lack of this region appears to make IgA2 more resistant to some bacterial proteinases that are able to cleave IgA1.[7,9] Hence, IgA2 is the predominant form in secretions at mucosal surfaces, whereas IgA1 is mainly found in serum. The major role of serum IgA is as an

anti-inflammatory agent.[10] Serum IgA appears to downregulate IgG-mediated phagocytosis, chemotaxis, bactericidal activity, and cytokine release.

IgA2 is found as a dimer along the respiratory, urogenital, and intestinal mucosa; it also appears in breast milk, colostrum, saliva, tears, and sweat.[7,9,11] Because mucosal surfaces are a major point of entry for pathogens, IgA2 serves to keep antigens from penetrating farther into the body.

The IgA dimer consists of two monomers held together by a J chain that has a molecular weight of about 15,000. The J chain is essential for the polymerization and secretion of IgA.[7] Secretory IgA is synthesized in plasma cells found mainly in mucosal-associated lymphoid tissue and is released in dimeric form. IgA is synthesized at a much greater rate than that of IgG—approximately 3 grams per day in the average adult—but because it is mainly in secretory form, the serum concentration is much lower.[9,10,12]

A **secretory component (SC),** which has a molecular weight of about 70,000, is later attached to the Fc region around the hinge portion of the α chains.[3,6,9,13] This protein, consisting of five immunoglobulin-like domains, is derived from epithelial cells found in close proximity to the plasma cells.[1,3] As **Figure 5–8** indicates, SC precursor, with a molecular weight of 100,000, is actually found on the surface of epithelial cells and serves as a specific receptor for IgA. Plasma cells that secrete IgA are attracted to subepithelial tissue, where IgA can bind as soon as it is released from the plasma cells.[3,12,14] This homing, or seeking out the subepithelial tissue by activated lymphocytes, depends on a high level of certain adhesion molecules that allow binding to epithelial cells.[9] Once binding takes place, IgA and SC precursor are taken inside the cell and then released to the opposite surface by a process known as *transcytosis.* The vesicle carrying IgA and the SC receptor fuses with the membrane on the cell's opposite side; a small fragment of SC is then cleaved to liberate the IgA dimer with the remaining SC.[9,12] The SC may thus act to facilitate transport of IgA to mucosal surfaces.[12] It also makes the dimer more resistant to enzymatic digestion by masking sites that would be susceptible to protease cleavage.[3,6]

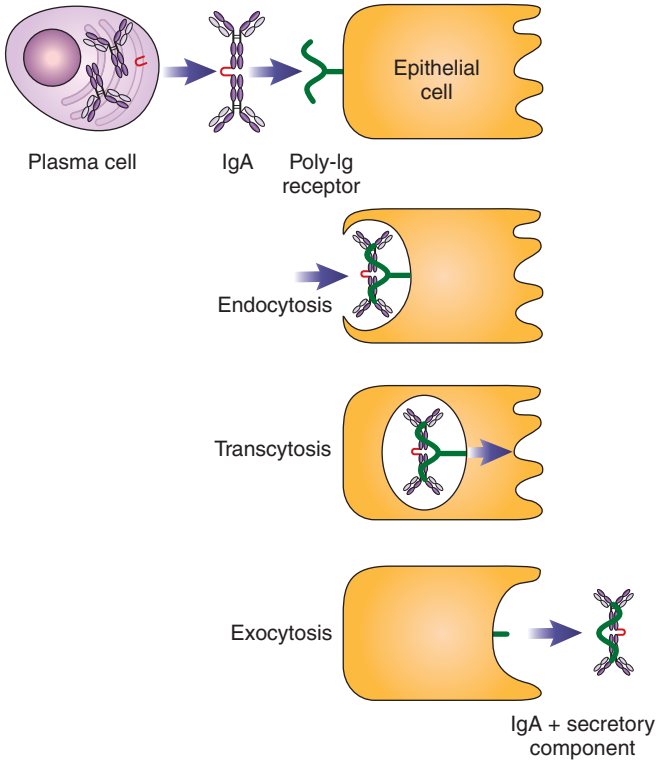


FIGURE 5–8 Formation of secretory IgA. IgA is secreted as a dimer from plasma cells and is captured by specific receptors on epithelial cells. The receptor is actually an SC, which binds to IgA and exits the cell along with it.

The main function of secretory IgA is to patrol mucosal surfaces and act as a first line of defense. It plays an important role in neutralizing toxins produced by microorganisms and helps to prevent bacterial and viral adherence to mucosal surfaces.[1,13] Complexes of IgA and antigen are easily trapped in mucus and then eliminated by the ciliated epithelial cells of the respiratory or intestinal tract. This prevents pathogens from colonizing the mucosal epithelium.[11] Because IgA is found in breast milk, breastfeeding helps to maintain the health of newborns by passively transferring antibodies and greatly decreasing infant death from both respiratory and gastrointestinal infections.[12]

It appears that IgA is not capable of fixing complement by the classical pathway, although aggregation of immune complexes may trigger the alternate complement pathway[9,14] (see Chapter 7). Lack of complement activation may actually assist in clearing antigen without triggering an inflammatory response, thus minimizing tissue damage.[11,13]

Additionally, neutrophils, monocytes, and macrophages possess specific receptors for IgA. Binding to these sites triggers a respiratory burst and degranulation.[7,11] This occurs for both serum and secretory IgA, indicating that they are capable of acting as opsonins. The success of oral immunizations such as the Sabin vaccine, which induces IgA almost exclusively, demonstrates the effectiveness of IgA's protective role on mucosal surfaces.

Immunoglobulin D (IgD)

IgD was not discovered until 1965, when it was found in a patient with multiple myeloma, a cancer of the plasma cells. It is extremely scarce in the serum, representing less than 0.001% of total immunoglobulins. It is synthesized at a low level and has a half-life of only 1 to 3 days. The molecule has a molecular weight of approximately 180,000 and migrates as a fast γ protein. The delta (δ) H chain has a molecular weight of 62,000 and appears to have an extended hinge region consisting of 58 amino acids.[1]

Most of the IgD is found on the surface of immunocompetent but unstimulated B lymphocytes. It is the second type of immunoglobulin to appear (IgM being the first) and it may play a role in B-cell activation, although its function is not completely understood.[7] The high level of surface expression and its intrinsic flexibility make it an ideal early responder to

antigen.[5,6] Those cells bearing only IgM receptors appear incapable of an IgG response, whereas those with both IgM and IgD receptors are capable of responding to T-cell help and switching to synthesis of IgG, IgA, or IgE.[5] Thus, IgD may play a role in regulating B-cell maturation and differentiation.[1]

Because of its unusually long hinge region, IgD is more susceptible to proteolysis than other immunoglobulins. This may be the main reason for its short half-life. In the secreted form in the serum, IgD does not appear to serve a protective function because it does not bind complement, it does not bind to neutrophils or macrophages, and it does not cross the placenta.[1]

Immunoglobulin E (IgE)

IgE is best known for its very low concentration in serum and the fact that it has the ability to activate mast cells and basophils. It is the least abundant immunoglobulin in the serum, accounting for only 0.0005% of total serum immunoglobulins.[1] The molecular weight of IgE is approximately 190,000, making it an 8 S molecule, and it has a carbohydrate content of 12%.[15] The epsilon (ε) or H chain is composed of around 550 amino acids that are distributed over one variable and four constant domains. A single disulfide bond joins each ε chain to an L chain and two disulfide bonds link the H chains to one another.

IgE is the most heat-labile of all immunoglobulins; heating to 56°C for between 30 minutes and 3 hours results in conformational changes and loss of ability to bind to target cells. IgE does not participate in typical immunoglobulin reactions such as complement fixation, agglutination, or opsonization. Additionally, it is incapable of crossing the placenta. Instead, shortly after synthesis it attaches to basophils, Langerhans cells, eosinophils, and tissue mast cells by means of specific surface proteins, termed *high-affinity Fc ε RI receptors,* which are found exclusively on these cells.[3,5] The molecule binds at the C_H3 domain on the Fc region,[16] leaving the antigen-binding sites free to interact with specific antigen **(Fig. 5–9).** Plasma cells that produce IgE are located primarily in the lungs and in the skin.[5]

Mast cells are also found mainly in the skin and in the lining of the respiratory and alimentary tracts. One such cell may have several hundred thousand receptors, each capable of binding an IgE molecule. When two adjacent IgE molecules on a mast cell bind specific antigen, a cascade of cellular events is initiated that results in degranulation of the mast cells with release of vasoactive amines such as histamine and heparin. Release of these mediators induces what is known as a type I immediate hypersensitivity or allergic reaction (see Chapter 14). Typical reactions include hay fever, asthma, vomiting and diarrhea, hives, and life-threatening anaphylactic shock.[5] Recently developed anti-IgE antibody that targets free IgE has been used as therapy for allergies and asthma.[7]

IgE appears to be a nuisance antibody; however, it may serve a protective role by triggering an acute inflammatory reaction that recruits neutrophils and eosinophils to the area to help destroy invading antigens that have penetrated IgA defenses.[14] Eosinophils, especially, play a major part in the destruction of large antigens such as parasitic worms that cannot be easily phagocytized (see Chapter 22 for details).

Theories to Explain Antibody Diversity

It is estimated that humans can make at least 10^6 to 10^9 different antibody molecules.[17] Attempts to explain how this many possible combinations with exquisite specificity for a particular antigen can occur began long before the actual structure of immunoglobulins was discovered. The central issue was whether an antigen selected lymphocytes with the inherent capability of producing specific antibody to it or whether the presence of antigen added a new specificity to a generalized type of antibody.

Ehrlich's Side-Chain Theory

One of the first theories to be formulated was that of Paul Ehrlich in the early 1900s, termed the *side-chain theory.* Ehrlich postulated that certain cells had specific surface receptors for antigen that were present before contact with antigen occurred. Once antigen was introduced, it would select the cell with the proper receptors, combination would take place, and then receptors would break off and enter the circulation as antibody

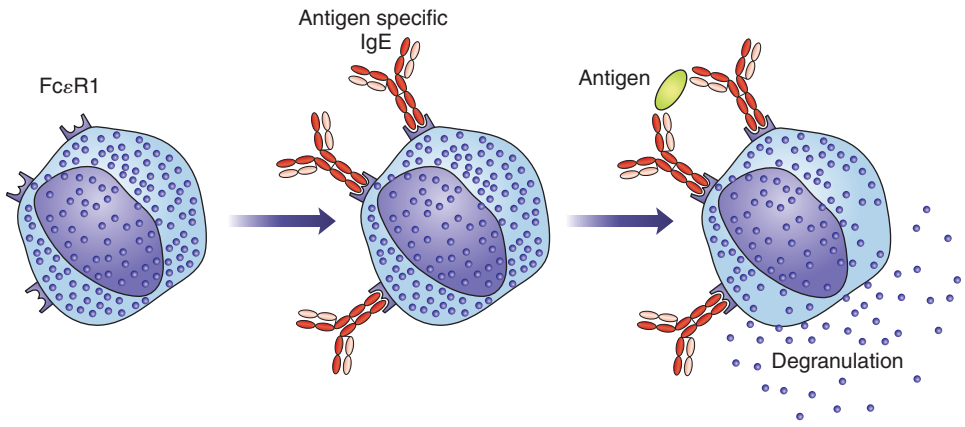


FIGURE 5–9 Action of IgE on mast cells. (A) IgE binds to specific ε receptors on mast cells. (B) When antigen bridges two nearby IgE molecules, the membrane is disturbed and degranulation results. Chemical mediators are released.

molecules. New receptors would form in place of those broken off, after which this process could be repeated. Although this represented a rather simplistic explanation for antibody synthesis, two key premises emerged: first, the lock-and-key concept of the fit of antibody for antigen, and second, the idea that an antigen selected cells with the built-in capacity to respond to it. Although this theory did not explain the kinetics of the immune response or the idea of immunologic memory, it laid the foundation for further hypotheses.

Clonal Selection Hypothesis

In the 1950s, Niels Jerne and Macfarlane Burnet independently supported the idea of a clonal selection process for antibody formation.[18,19,20] The key premise of the **clonal selection hypothesis** is that individual lymphocytes are genetically preprogrammed to produce one type of immunoglobulin and that a specific antigen finds or selects those particular cells capable of responding to it, causing them to proliferate. The receptors Ehrlich originally postulated are the surface immunoglobulins IgM and IgD, which are found on unstimulated B lymphocytes. Repeated contact with antigen would continually increase a specific lymphocyte pool. Such a model provides an explanation for the kinetics of the immune response.

The main drawback to the clonal selection hypothesis was consideration of the genetic basis for the diversity of antibody molecules. If separate genes were present to code for antibody to every possible antigen, an overwhelming amount of DNA would be needed. In 1965, Dreyer and Bennett proposed a solution to this dilemma by suggesting that the constant and variable portions of immunoglobulin chains are actually coded for by separate genes.[21] There could be a small number coding for the constant region and a larger number coding for the variable region. This would considerably simplify the task of coding for such variability. This notion implied that although all lymphocytes start out with identical genetic germline DNA, diversity is created by a series of recombination events that occur as the B cell matures. Scientific evidence now indicates that this is exactly what happens, as explained in the following discussion.

Genes Coding for Immunoglobulins

Tonegawa's pioneering experiments with DNA revealed that chromosomes contain no intact immunoglobulin genes, only building blocks from which genes can be assembled. This confirmed the hypothesis of Dreyer and Bennett.[17] Tonegawa was awarded the Nobel Prize in 1987 for this monumental discovery. Human immunoglobulin genes are found in three unlinked clusters: H chain genes are located on chromosome 14, κ chain genes are on chromosome 2, and λ chain genes are on chromosome 22. Within each of these clusters, a selection process occurs. The genes cannot be transcribed and translated into functional antibody molecules until this rearrangement, assisted by special recombinase enzymes, takes place. Gene rearrangement involves a cutting and splicing process that gets rid of much of the intervening DNA, resulting in a functional gene that codes for a specific antibody. Once this rearrangement occurs, it permanently changes the DNA of the particular lymphocyte.

Rearrangement of Heavy-Chain Genes

The selection process begins with rearrangement of the genes for the H chains. All H chains are derived from a single region on the long arm of chromosome 14. The genes that code for the variable region are divided into three groups—V_H, D, and J. There are at least 39 V_H (variable) genes, approximately 27 functional D (diversity) genes, and 6 J (joining) genes.[6,7,22] In addition, there is a set of genes (C) that codes for the constant region. This set includes one gene for each H chain isotype. They are located in the following order: Cμ, Cδ, Cγ3, Cγ1, Cα1, Cγ2, Cγ4, Cε, and Cα2, as shown in **Figure 5–10.** Only one of these constant regions is selected at any one time. For synthesis of the entire H chain, a choice is made from each of the sections so as to include one V_H gene, one D gene, one J gene, and one constant region. During the process of B-cell maturation, the pieces are spliced together to commit that B lymphocyte to making antibody of a single specificity.

Joining of these segments occurs in two steps: First, at the DNA level, one D and one J are randomly chosen and are joined by means of a recombinase enzyme with deletion of the intervening DNA (see Fig. 5–10). Next, a V gene is joined to the DJ complex, resulting in a rearranged V(D)J gene. The VJD gene combination codes for the entire variable region of the H chain. This rearrangement occurs early in B-cell development in pro-B cells.[22–24] (See Chapter 4 for additional details.) The recombinase enzymes RAG-1 and RAG-2, which are distinctive markers of this stage, are essential for initiating this process. The recombinase enzymes recognize specific target sequences called recombination signal sequences that flank all immunoglobulin gene segments.[21] However, joining of the V, J, and D segments doesn't always occur at a fixed position, so each sequence can vary by a small number of nucleotides. This variation is called junctional diversity, which is a major source of additional diversity.[7,17] If a successful rearrangement of DNA on one chromosome 14 occurs, then the genes on the second chromosome are not rearranged; this phenomenon is known as **allelic exclusion.** If the first rearrangement is nonproductive, then rearrangement of the second set of genes on the other chromosome 14 occurs.

The variable and constant regions are joined at the ribonucleic acid (RNA) level, thus conserving the DNA of the constant regions and allowing for a later phenomenon called **class switching,** whereby daughter plasma cells can produce antibody of another type. During transcription and synthesis of messenger ribonucleic acid (mRNA), a constant region is spliced to the VDJ complex.[25] Because Cμ is the region closest to the J region, μ H chains are the first to be synthesized; these are the markers of the pre-B lymphocytes. The Cδ region, which lies closest to the Cμ region, is often transcribed along with Cμ. The presence of DNA for both the Cμ and Cδ regions

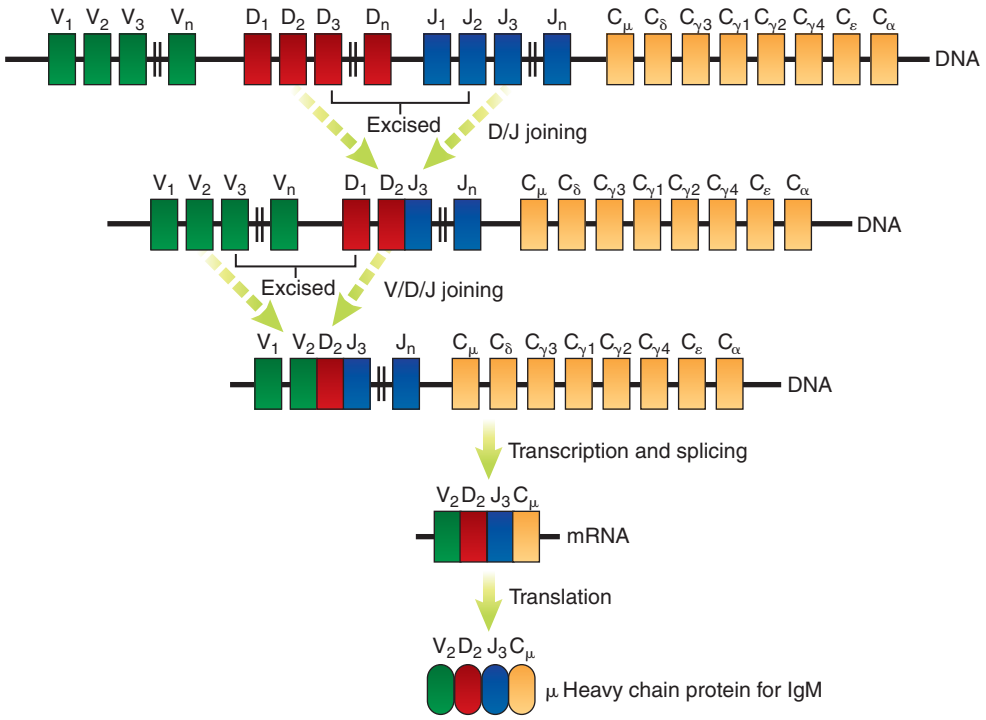


FIGURE 5–10 Coding for immunoglobulin H chains. Four separate regions on chromosome 14 code for H chains. DJ regions are spliced first, and then this segment is joined to a variable region. When RNA synthesis occurs, one constant region is attached to the VDJ combination. μ H chains are made first, but the cell retains its capacity to produce immunoglobulin of another class.

allows for RNA for IgD and IgM to be transcribed at the same time. Thus, a B cell could express IgD and IgM with the same variable domain on its surface at the same time. The process of switching to other immunoglobulin classes occurs later as a result of the looping out and deletion of other constant regions. This allows the same VDJ region to be coupled with a different C region to produce antibody of a different class (i.e., IgA, IgG, or IgE) but with the identical specificity for antigen.[7,24] Contact with T cells and with cytokines provides the signal for switching to take place.

Light Chain Rearrangement

Because L chain rearrangement occurs only after μ chains appear, μ-chain synthesis represents a pivotal step in the process. L chains exhibit a similar genetic rearrangement, except they lack a D region. Recombination of segments on chromosome 2, coding for κ chains, occurs before that on chromosome 22, which codes for λ chains.[7,22] The process of VJ joining is accomplished by cutting out intervening DNA. This results in V_κ and J_κ segments becoming permanently joined to one another on the rearranged chromosome. Transcription begins at one end of the V_κ segment and proceeds through the J_κ and C_κ segments. Unrearranged J segments are removed during RNA splicing, which occurs in the translation **(Fig. 5–11)**.

A productive rearrangement of the κ genes with subsequent protein production keeps the other chromosome 2 from rearranging and shuts down any recombination of the λ-chain locus on chromosome 22. Only if a nonfunctioning gene product arises from κ rearrangement does λ chain synthesis occur. The λ locus contains approximately 30 to 36 V_λ, 7 J_λ, and four functional C_λ segments.[7] If functional H and L chains are not produced by these rearrangements, then the particular B cell dies by apoptosis.

L chains are then joined with μ chains to form a complete IgM antibody, which first appears in immature B cells. Once IgM and IgD are present on the surface membrane, the B lymphocyte is fully mature and capable of responding to antigen (see Chapter 4). The large variety of V, J, D, and C combinations

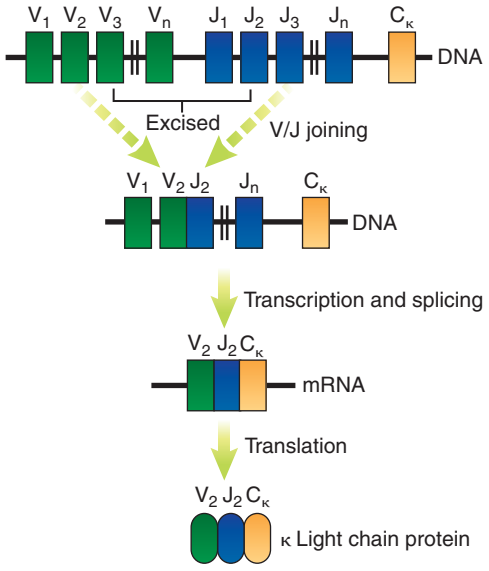


FIGURE 5–11 Assembly and expression of the κ L chain locus. A DNA rearrangement fuses one V segment to one J segment. The VJ segment is then transcribed along with a unique C region to form mature κ mRNA. Unarranged J segments are removed during RNA splicing.

for each type of chain, plus the different possibilities for L and H chain combination, make for more than enough configurations to allow us to respond to any antigen in the environment.

Monoclonal Antibody

The knowledge that B cells are genetically preprogrammed to synthesize very specific antibody has been used in developing **monoclonal antibodies** for diagnostic testing. Monoclonal antibodies are so called because they are derived from a single parent antibody-producing cell that has reproduced many times, thus forming a clone. Every cell in the clone is just like every other cell; the antibody produced by each cell is exactly the same as that of every other cell. This differs from the normal response to an antigen, which is heterogeneous, because even a purified antigen has multiple epitopes that stimulate a variety of B cells. In 1975, Georges Kohler and Cesar Milstein discovered a technique to produce antibody arising from a single B cell, which has revolutionized serological testing. They were awarded the Nobel Prize in 1984 for their pioneering research.

Hybridomas

Kohler and Milstein's technique fuses an activated B cell with a myeloma cell that can be grown indefinitely in the laboratory. This fusion of two different types of cells is called a **hybridoma.** Myeloma cells are cancerous plasma cells. Normally, plasma cells produce antibody, so a particular cell line that is not capable of producing antibody is chosen. In addition, this cell line has a deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) that makes it incapable of synthesizing nucleotides from hypoxanthine and thymidine, which are needed for DNA synthesis. The fact that these myeloma cells cannot make their own DNA means that they will die out unless they are fused to a plasma cell that has the enzymes necessary to synthesize DNA. This deficiency keeps the myeloma cells from reproducing on their own.

Hybridoma Production

The production of hybridomas begins by immunizing a mouse with a certain antigen. After a time, the mouse's spleen cells are harvested. Spleen cells are combined with myeloma cells in the presence of polyethylene glycol (PEG), a surfactant. The PEG brings about fusion of plasma cells with myeloma cells, producing a hybridoma. Only a small percentage of cells actually fuse, and some of these are like cells—that is, two myeloma cells or two spleen cells. After fusion, all cells are placed in culture using a medium containing hypoxanthine, aminopterin, and thymidine (HAT). This culture medium separates out the hybridoma cells by allowing them to grow selectively and not allowing fused myeloma cells or fused spleen cells to survive. Myeloma cells are normally able to grow indefinitely in tissue culture, but in this case they cannot because both pathways for the synthesis of nucleotides are blocked. One pathway, the salvage pathway, which builds DNA from degradation of old nucleic acids, is blocked because the myeloma cell line employed is deficient in the required enzymes HGPRT and thymidine kinase.[26,27] The other pathway, the de novo pathway, which makes DNA from new nucleotides, is blocked by the presence of aminopterin. Consequently, the myeloma cells die out. Normal B cells cannot be maintained continuously in cell culture, so these die out as well. This leaves only the fused hybridoma cells, which have the ability (acquired from the myeloma cell) to reproduce indefinitely in culture and the ability (acquired from the normal B cell) to synthesize nucleotides by the HGPRT and thymidine kinase pathway **(Fig. 5–12).**

Selection of Specific Antibody-Producing Clones

The remaining hybridoma cells are diluted out and placed in microtiter wells, where they are allowed to grow. Each well, containing one clone, is then screened for the presence of the desired antibody by removing the supernatant. Once identified, a hybridoma is capable of being maintained in cell culture indefinitely and produces a permanent and uniform supply of monoclonal antibody that reacts with a single epitope.[26,27]

Clinical Applications

Monoclonal antibodies were initially used for in vitro diagnostic testing. A familiar example is pregnancy testing, which uses antibody specific for the β chain of human chorionic gonadotropin, thereby eliminating many false-positive reactions. Other examples include detection of tumor antigens and measurement of hormone levels.

Recently, however, there has been an emphasis on the use of monoclonal antibodies as therapeutic agents. Because mouse monoclonal antibodies are highly immunogenic for humans, several techniques have been used to "humanize" monoclonal antibodies to prevent reactions to the mouse antibodies. One of these techniques grafts the antibody-combining site from antibodies grown in mice onto the rest of a human immunoglobulin.[28] Another technique injects human DNA that codes for antibody molecules into bacteriophage. Bacteriophage are viruses that only grow inside bacteria. Bacteriophage with human genes are used to infect *Escherichia coli;* as *E coli* colonies grow, they produce human antibodies.[27,28]

One of the major breakthroughs in the treatment of cancer involves the use of monoclonal antibodies. There are now a number of such antibodies that have been approved for cancer treatment. In the case of metastatic breast cancer, trastuzumab (Herceptin), an antibody directed against HER-2 protein, has been helpful in slowing the disease's progress.[28,29] HER-2 protein is present in large numbers on tumor cells and regulates growth. Another example is rituximab (Rituxan), the first monoclonal antibody to be approved by the FDA for the treatment of malignancies.[30] Rituximab targets the B-cell marker CD20, leading to depletion of peripheral B cells. The antibody is used to treat non-Hodgkin lymphoma and other B-cell malignancies. Other monoclonal antibodies approved by the FDA include cetuximab (Erbitux) to treat colorectal cancer and head and neck cancers and bevacizumab (Avastin) to treat colorectal, non-small lung, and breast cancers.[26,28]

In some therapies, monoclonal antibodies are linked to cytotoxic agents, such as radioactive substances and other drugs that are delivered directly to cancerous cells. These monoclonal

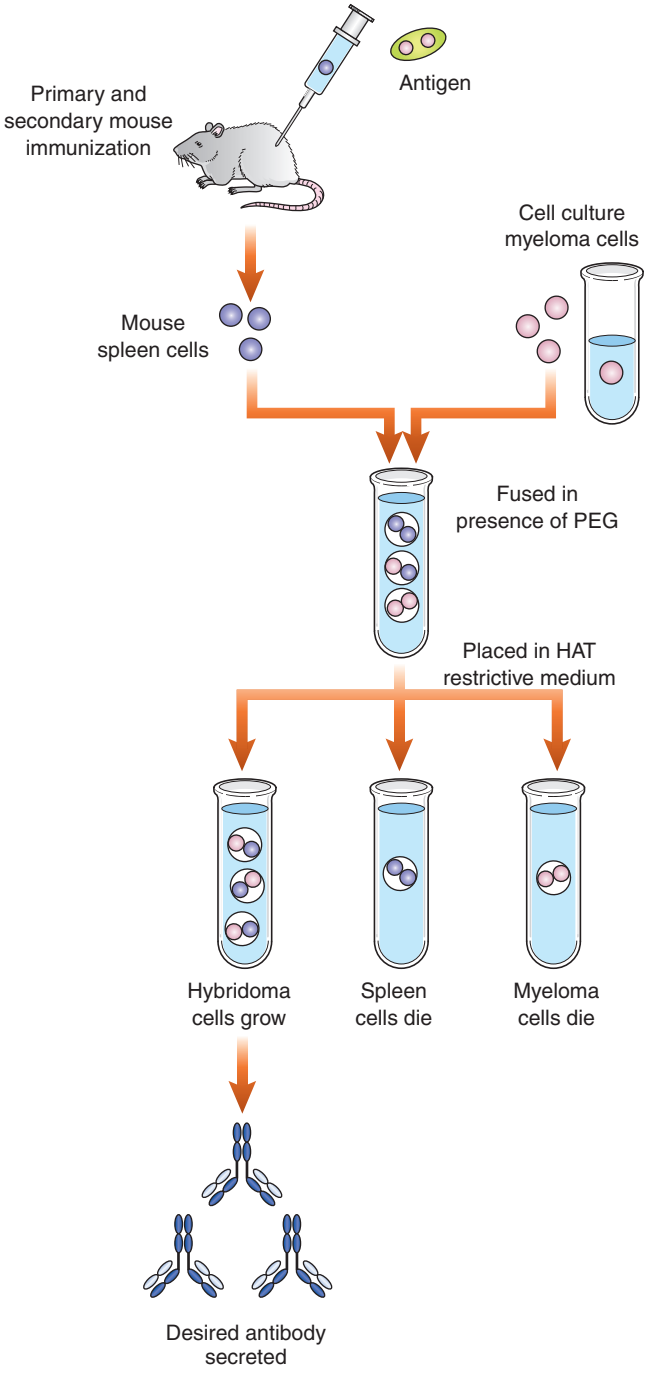


FIGURE 5–12 Formation of a hybridoma in monoclonal antibody production. A mouse is immunized and spleen cells are *removed*. These cells are fused with nonsecreting myeloma cells and then plated in a restrictive medium. Only the hybridoma cells will grow in this medium where they synthesize and secrete a monoclonal immunoglobulin specific for a single determinant on an antigen.

antibodies are called antibody-drug conjugates. Drugs in this category include ibritumomab tiuxetan (Zevalin) for cancerous B lymphocytes and tositumomab (Bexxar) to treat some non-Hodgkin lymphomas that no longer respond to rituximab.[26,31]

Monoclonal antibodies are also useful in the treatment of autoimmune diseases. One of the biggest success stories is in the treatment of two such diseases: rheumatoid arthritis (RA) and Crohn's disease (a progressive inflammatory colitis). Both of these diseases have been treated with a monoclonal antibody called infliximab that blocks the action of tumor necrosis factor-alpha (TNF-α.[29,32,33] Another TNF blocker, adalimumab (Humira), has also proven effective in decreasing symptoms of these two diseases.[29] Rituximab is also used in the treatment of several autoimmune diseases, including RA and systemic lupus erythematosis.[34] (See Chapter 15 for a discussion of autoimmune diseases.)

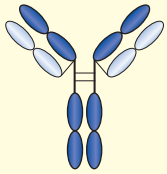
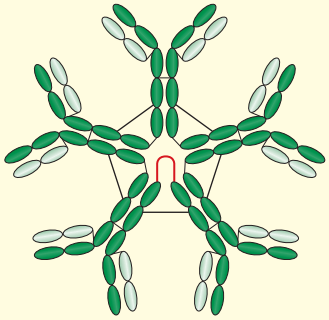
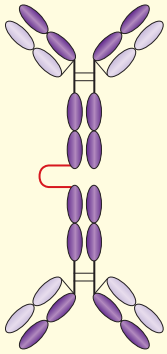
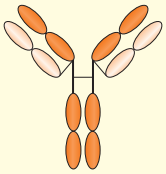
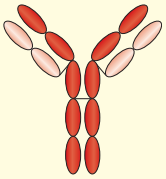
Use of monoclonal antibodies in several areas of medicine continues to grow. This represents a developing area of research in pharmacology; as it rapidly expands, it is likely to change future treatment options for numerous diseases. (See Chapter 25 for additional information about the use of monoclonal antibodies.)

SUMMARY

- The basic structural unit for all immunoglobulins is a tetrapeptide composed of two L and two H chains joined together by disulfide bonds.
- The five classes of antibodies are IgM, IgG, IgA, IgD, and IgE. IgG, IgD, and IgE exist as monomers. IgA has a dimeric form, whereas IgM is a pentamer whose subunits are held together by a J chain.
- Kappa and lambda (L chains) are found in all types of immunoglobulins, but the H chains differ for each immunoglobulin class.
- Each immunoglobulin molecule has constant and variable regions. The variable region is at the amino-terminal end, called the Fab fragment; this determines the specificity of that molecule for a particular antigen.
- The constant region, located at the carboxy-terminal end of the molecule and named the Fc fragment, is responsible for binding to effector cells such as neutrophils, basophils, eosinophils, and mast cells.
- The five different types of heavy chains are called isotypes.
- Minor variations in a particular type of heavy chain are called allotypes.
- The variable portion of the light and heavy chains unique to a particular immunoglobulin molecule is known as the idiotype.
- IgG is relatively small and easily penetrates into tissues, whereas IgM is much larger and excels at complement fixation.
- IgA has an SC that protects it from enzymatic digestion while it patrols mucosal surfaces.
- An extended hinge region gives IgD an advantage as a surface receptor for antigen.
- IgE binds to mast cells to initiate a local inflammatory reaction.
- The primary response to an antigen takes 5 to 7 days before antibody can be detected.
- The primary response consists of approximately equal amounts of IgM and IgG.

- The secondary response to antigen occurs in a shorter time; the amount of IgM is similar to that of the primary response, whereas IgG may be up to one hundred times greater than that of the primary response.
- Ehrlich's side-chain theory is based on the antigen selecting the correctly programmed B lymphocyte.
- The clonal selection hypothesis postulated that lymphocytes are generally pre-endowed to respond to one antigen or a group of antigens.
- Several genes code for a particular immunoglobulin; through a random selection process, these individual segments are joined to make antibody of a single specificity.
- Monoclonal antibodies are made when a cancerous cell or myeloma is fused with an antibody-producing cell to form a hybridoma.
- Hybridomas formed by fusion of one of each cell type (e.g., myeloma and B cell) are identified by using HAT, a selective medium.
- Monoclonal antibodies are used both in diagnosis and treatment of disease.

Study Guide: The Five Classes of Immunoglobulins

IgG	IgM	IgA	IgD	IgE
				
Most abundant in serum	Primary response antibody	Monomer and dimer	Present on B cells	Binds to mast cells
Able to cross placenta	Pentamer with 10 antibody-combining sites	Protects mucosal surfaces	Role in B-cell activation	Triggers allergic response
Increases with second exposure	Indicates acute infection	Has secretory component	Identifies mature B cells	Role in response to parasites

CASE STUDIES

1. A 15-year-old male exhibited symptoms of fever, fatigue, nausea, and sore throat. He went to his primary care physician who ordered a rapid strep test and a test for infectious mononucleosis to be performed in the office. The rapid strep test result was negative, but the test result for infectious mononucleosis was faintly positive. The patient mentioned that he thought he had mononucleosis about 2 years earlier, but it was never officially diagnosed. His serum was sent to a reference laboratory to test with specific Epstein-Barr viral antigens. The results indicated the presence of IgM only.

Questions

a. Is this a new or reactivated case of mononucleosis? Explain your answer.
b. How do the results relate to the difference between the primary and a secondary response to exposure to the same antigen?

2. A 10-year-old female experienced one cold after another in the springtime. She had missed several days of school and her mother was greatly concerned. The mother took her daughter to the pediatrician, worried that her daughter might be immunocompromised because she couldn't seem to fight off infections. A blood sample was obtained and sent to a reference laboratory for a determination of antibody levels, including an IgE level. The patient's IgM, IgG, and IgA levels were all normal for her age, but the IgE level was greatly increased.

Questions

a. What does the increase in IgE signify?
b. Should there be a concern about the patient being immunocompromised?

REVIEW QUESTIONS

1. Which of the following is characteristic of variable domains of immunoglobulins?
   - **a.** They occur on both the H and L chains.
   - **b.** They represent the complement-binding site.
   - **c.** They are at the carboxy-terminal ends of the molecules.
   - **d.** They are found only on H chains.

2. All of the following are true of IgM *except* that it
   - **a.** can cross the placenta.
   - **b.** fixes complement.
   - **c.** has a J chain.
   - **d.** is a primary response antibody.

3. How does the structure of IgE differ from that of IgG?
   - **a.** IgG has a secretory component and IgE does not.
   - **b.** IgE has one more constant region than IgG.
   - **c.** IgG has more antigen-binding sites than IgE.
   - **d.** IgG has more light chains than IgE.

4. How many antigen-binding sites does a typical IgM molecule have?
   - **a.** 2
   - **b.** 4
   - **c.** 6
   - **d.** 10

5. Bence Jones proteins are identical to which of the following?
   - **a.** H chains
   - **b.** L chains
   - **c.** IgM molecules
   - **d.** IgG molecules

6. A Fab fragment consists of
   - **a.** two H chains.
   - **b.** two L chains.
   - **c.** one L chain and one-half of an H chain.
   - **d.** one L chain and an entire H chain.

7. Which antibody best protects mucosal surfaces?
   - **a.** IgA
   - **b.** IgG
   - **c.** IgD
   - **d.** IgM

8. Which of the following pairs represents two different immunoglobulin allotypes?
   - **a.** IgM and IgG
   - **b.** IgM1 and IgM2
   - **c.** Anti-human IgM and anti-human IgG
   - **d.** IgG1m3 and IgG1m17

9. The structure of a typical immunoglobulin consists of which of the following?
   - **a.** 2L and 2H chains
   - **b.** 4L and 2H chains
   - **c.** 4L and 4H chains
   - **d.** 2L and 4 H chains

10. Which of the following are L chains of antibody molecules?
    - **a.** Kappa
    - **b.** Gamma
    - **c.** Mu
    - **d.** Alpha

11. If the results of serum protein electrophoresis show a significant decrease in the gamma band, which of the following is a likely possibility?
    - **a.** Normal response to active infection
    - **b.** Multiple myeloma
    - **c.** Immunodeficiency disorder
    - **d.** Monoclonal gammopathy

12. The subclasses of IgG differ mainly in
    - **a.** the type of L chain.
    - **b.** the arrangement of disulfide bonds.
    - **c.** the ability to act as opsonins.
    - **d.** molecular weight.

13. Which best describes the role of the secretory component of IgA?
    - **a.** A transport mechanism across endothelial cells
    - **b.** A means of joining two IgA monomers together
    - **c.** An aid to trapping antigen
    - **d.** Enhancement of complement fixation by the classical pathway

14. Which represents the main function of IgD?
    - **a.** Protection of the mucous membranes
    - **b.** Removal of antigens by complement fixation
    - **c.** Enhancing proliferation of B cells
    - **d.** Destruction of parasitic worms

15. Which antibody is best at agglutination and complement fixation?
    - **a.** IgA
    - **b.** IgG
    - **c.** IgD
    - **d.** IgM

16. Which of the following can be attributed to the clonal selection hypothesis of antibody formation?

a. Plasma cells make generalized antibody.
b. B cells are preprogrammed for specific antibody synthesis.
c. Proteins can alter their shape to conform to antigen.
d. Cell receptors break off and become circulating antibody.

17. All of the following are true of IgE *except* that it

a. fails to fix complement.
b. is heat stable.
c. attaches to tissue mast cells.
d. is found in the serum of allergic persons.

18. Which best describes coding for immunoglobulin molecules?

a. All genes are located on the same chromosome.
b. L chain rearrangement occurs before H chain rearrangement.
c. Four different regions are involved in coding of H chains.
d. Lambda rearrangement occurs before kappa rearrangement.

19. What is the purpose of HAT medium in the preparation of monoclonal antibody?

a. Fusion of the two cell types
b. Restricting the growth of myeloma cells
c. Restricting the growth of spleen cells
d. Restricting antibody production to the IgM class

20. Papain digestion of an IgG molecule results in which of the following?

a. 2 Fab' and 1 Fc' fragment
b. $F(ab')_2$ and 1 Fc' fragment
c. 2 Fab and 2 Fc fragments
d. 2 Fab and 1 Fc fragment

21. Which antibody provides protection to the growing fetus because it is able to cross the placenta?

a. IgG
b. IgA
c. IgM
d. IgD

22. Which best characterizes the secondary response?

a. Equal amounts of IgM and IgG are produced.
b. There is an increase in IgM only.
c. There is a large increase in IgG but not IgM.
d. The lag phase is the same as in the primary response.

6 Cytokines

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Define cytokine.
2. Define and describe the term *cytokine storm* and relate its medical importance.
3. Distinguish between autocrine, paracrine, and endocrine effects of cytokines.
4. Define *pleiotropy* as it relates to cytokine activities.
5. Explain the functions of interleukin-1 (IL-1) in mediating the immune response.
6. Explain the effects of tumor necrosis factor (TNF).
7. Discuss how interleukin-6 (IL-6) affects inflammation and other activities of the immune system.
8. Determine the role of chemokines in chemotaxis of white blood cells (WBCs).
9. Compare the functions of type 1 and type 2 interferons (IFN).
10. Describe the actions of interleukin-2 (IL-2) on target cells.
11. Discuss the biological roles of the hematopoietic growth factors.
12. Discuss cytokines involved in differentiation of T helper (Th) cell subpopulations: Th1, Th2, Th17, and T regulatory.
13. Describe the biological role of colony stimulating factors.
14. Describe the current types of anticytokine therapies.
15. Describe clinical assays for cytokines.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Adaptive T regulatory 1 (Tr1) cells
Antagonism
Autocrine
Cascade induction
Chemokines
Colony stimulating factors (CSF)
Cytokines
Endocrine
Endogenous pyrogen
Erythropoietin (EPO)
Granulocyte colony stimulating factor (G-CSF)
Granulocyte-macrophage colony stimulating factor (GM-CSF)
Hypercytokinemia
Integrins
Interferons (IFN)
Interleukins (IL)
Macrophage colony stimulating factors (M-CSF)
Paracrine
Pleiotropy
Redundancy
Synergistic
T helper 1 cells (Th1)
T helper 2 cells (Th2)
T helper 17 cells (Th17)
T regulatory (Treg) cells
Transforming growth factor-β (TGF-β)
Tumor necrosis factors (TNF)

Introduction to Cytokines

The cells of the immune system are spread throughout the body and need a communication system to coordinate an immune response. **Cytokines** are the chemical messengers that regulate the immune system, orchestrating both innate immunity and the adaptive response to infection. They are small proteins produced by several different types of cells that influence the hematopoietic and immune systems through activation of cell-bound receptors.[1] Cytokines are induced in response to the binding of stimuli, such as bacterial lipopolysaccharides, flagellin, or other bacterial products, to specific cell receptors or through the recognition of foreign antigens by host lymphocytes. The effects of cytokines in vivo include regulation of growth, differentiation, and gene expression by many different cell types, including leukocytes. These effects are achieved through both **autocrine** stimulation (i.e., affecting the same cell that secreted it) and **paracrine** (i.e., affecting a target cell in close proximity) activities. Occasionally, cytokines will also exert **endocrine** (i.e., systemic) activities **(Fig. 6–1).** Thus, individual cytokines do not act alone but in conjunction with many other cytokines that are induced during the process of immune activation. The resulting network of cytokine expression regulates leukocyte activity and leads to elimination of the infection.

Initially, cytokines were named based on their activities and types of cells from which they were first isolated. For example, cytokines released from lymphocytes were called lymphokines, cytokines released from monocytes and macrophages were called monokines, and cytokines secreted by leukocytes that mainly act on other leukocytes were called interleukins. Now, cytokines are grouped into families, which include tumor necrosis factor (TNF), interferon (IFN), chemokine, transforming growth factor (TGF), and colony stimulating factor (CSF). There are also the interleukins (IL), which currently number from IL-1 to IL-38.[2] The major functions of these groups will be discussed in this chapter.

Cytokines were originally thought to act solely on cells of the immune system, but it soon became apparent that many also act on cells outside the immune system. Functionally, cytokines may exhibit pleiotrophy, redundancy, synergy, antagonism, and cascade induction. **Pleiotropy** means that a single cytokine can have many different actions. When different cytokines activate some of the same pathways and genes, it is called **redundancy.** The redundancy may be explained by the fact that many cytokines share receptor subunits. For instance, IL-6 and IL-11 use the gp130 subunit as part of their receptors.[3] Thus, some cytokines may have overlapping effects and may alter the activity of many of the same genes.

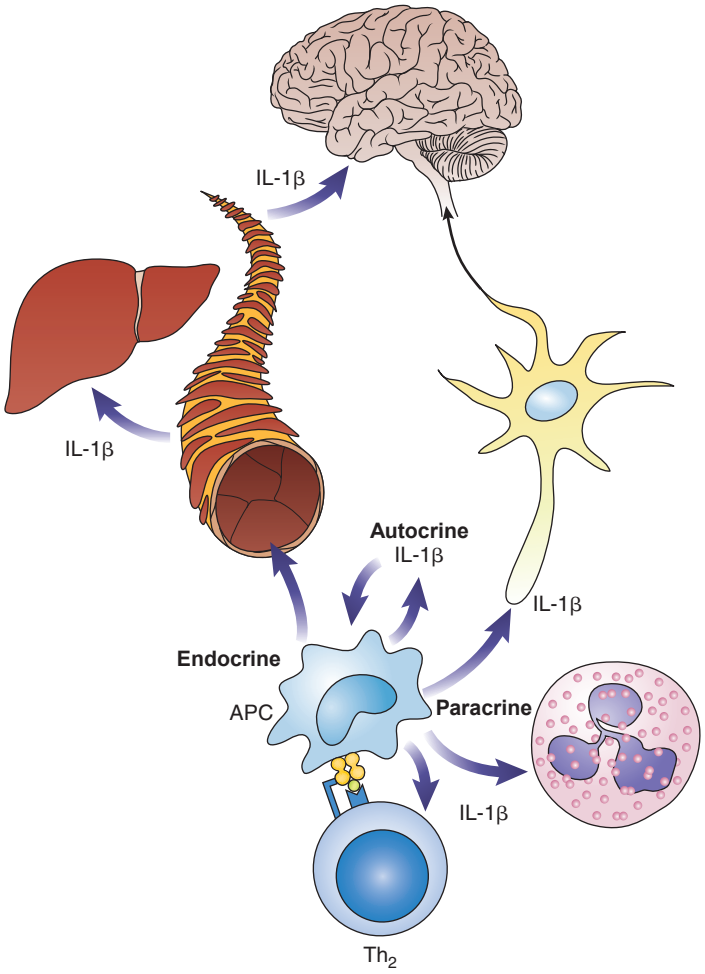

FIGURE 6–1 Range of cytokine actions. Autocrine: Cytokine acts on the cell that secreted it (e.g., IL-1β increases activation of APC). Paracrine: Cytokine acts on nearby cells (e.g., IL-1β stimulates Th cells, activates neutrophils, depolarizes neurons). Endocrine: Cytokine travels through blood vessels to distant cells (e.g., IL-1β stimulates acute-phase protein synthesis in the liver, as well as fever induction in the hypothalamus).

Cytokines often act in networks; if the effects complement and enhance each other, these are called **synergistic** interactions

(Fig. 6–2). In certain instances, one cytokine may counteract the action of another cytokine, causing **antagonism** as an action (see Fig 6–2B). **Cascade induction** may also occur, in which a cytokine secreted by a specific type of cell can activate target cells to produce additional cytokines (see Fig. 6–2C). For example, activated T helper (Th) cells secrete IFN-gamma, which in turn activates macrophages to secrete IL-12, which then activates Th cells to produce other cytokines.

A cytokine cascade produces a spectrum of activities that lead to the rapid generation of both innate and adaptive immune responses. In fact, the ability or inability to generate certain cytokine patterns often determines the outcome and clinical course of

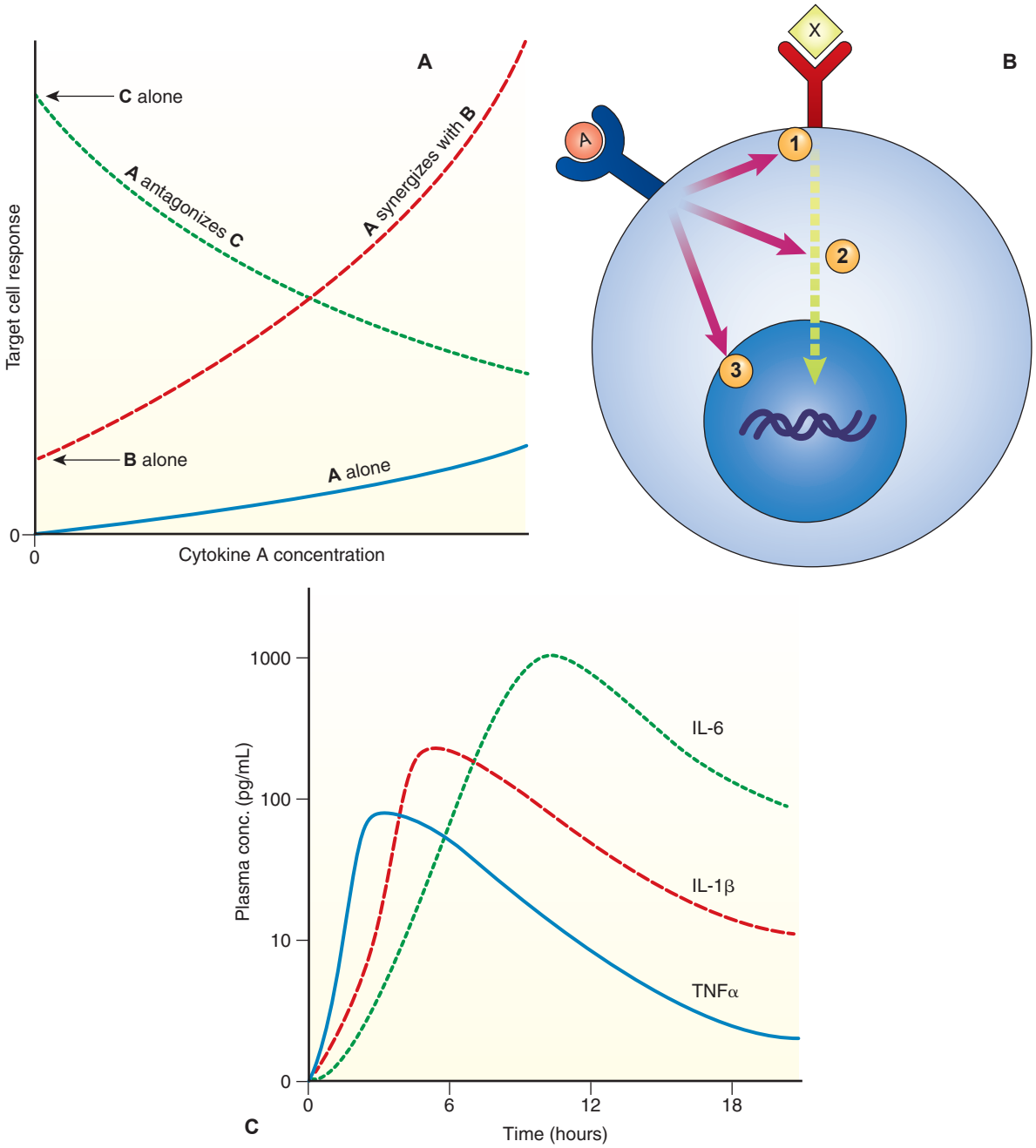

FIGURE 6–2 (A) Cytokine characteristics of synergy, antagonism, and cascade induction. Synergy: Neither cytokine A nor B induce a strong response individually; however, when combined, the net response is much greater than the sum of the individual responses. Antagonism: individually, cytokine C induces a strong response and cytokine A induces a weak (but positive) response. When combined, cytokine A diminishes the action of cytokine C. In this illustration, the concentrations of cytokines B and C are held constant, whereas cytokine A increases along the horizontal axis. (B) Synergistic and antagonistic interactions may be the result of cytokine A (1) altering the expression or function of the receptor for cytokine X, (2) altering the activity of a key enzyme in the signaling pathway for cytokine X, or (3) altering the stability and/or translation of the mRNA induced by cytokine X. (C) Cytokines can induce release of other cytokines in an amplifying cascade. For example, following intravenous injection of a bacterial toxin, TNF-α is first released by monocytes and macrophages. Both the toxin and TNF-α induce subsequent release of IL-1β. Then all three induce IL-6 release from a wide variety of cell types.

infection. In extreme circumstances, massive overproduction and dysregulation of cytokines produce hyperstimulation of the immune response or **hypercytokinemia,** a condition commonly referred to as cytokine storm. Cytokine storms may lead to shock, multiorgan failure, or even death, thus contributing to pathogenesis.[4,5] Select pathogens have been known to induce cytokine storms. For example, pathogenic viruses (e.g., influenza A) and bacteria (e.g., *Francisella tularensis*) disrupt the delicate balance of a suitable inflammatory response, tipping it from beneficial to destructive by causing the release of large amounts of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Indeed, cytokine storms were considered a main cause of death in the 1918 Spanish flu pandemic. Historical documents that describe the symptoms suggest that the massive fatalities likely resulted from the production of extremely high levels of cytokines.[1] Hypotension, fever, and edema follow infection and eventually lead to organ dysfunction and death. It is evident that the inflammatory response requires regulation to prevent the damaging systemic inflammation of a cytokine storm. Anti-inflammatory cytokines can resolve the inflammation and associated collateral damage to host cells.[6] **Table 6–1** displays major proinflammatory and anti-inflammatory cytokines.

The increasing clinical usage of cytokines, cytokine antagonists, and cytokine receptor antagonists in conditions such as rheumatoid arthritis (RA), asthma, and Crohn's disease drives demand for cytokine assays in the clinical laboratory. In addition, the clinical laboratory plays an important role in assessing treatment modalities, effectiveness, and potential gene-replacement therapies for numerous immunodeficiency syndromes and leukemias caused by defects in cytokines or their receptors/signal transduction circuits.[7]

The following sections will discuss cytokines according to their participation in either the innate immune response or the adaptive immune response. The major cytokines and their functions are summarized in the Study Guides at the end of this chapter.

Cytokines in the Innate Immune Response

Cytokines involved in the innate immune response are responsible for many of the physical symptoms attributed to inflammation, such as fever, swelling, pain, and cellular infiltrates into damaged tissues. The innate immune response is nonspecific but occurs within hours of first contact with microorganisms (see Chapter 1). It may play a crucial part in recovery from infection. The main function of the innate immune response is to recruit effector cells to the area. Cytokines involved in triggering this response are interleukin-1 (IL-1), TNF-α, interleukin-6 (IL-6), chemokines, transforming growth factor-β, and interferons α and (β. The function of each is discussed here.

Table 6–1 Examples of Major Proinflammatory and Anti-Inflammatory Cytokines

MAJOR PROINFLAMMATORY CYTOKINES	MAJOR ANTI-INFLAMMATORY CYTOKINES
TNF-α	TGF-β
IL-1	IL-10
IL-6	IL-13
IFN-γ	IL-35

Interleukin-1 (IL-1)

The IL-1 family consists of IL-1α, IL-1β, and IL-1RA (IL-1 receptor antagonist).[8] IL-1α and IL-1β are proinflammatory cytokines produced by monocytes, macrophages, and dendritic cells early on in the immune response. IL-1 production may be induced by the presence of microbial pathogens, bacterial lipopolysaccharides, or other cytokines. IL-1α and IL-1β exhibit the same activities in many test systems and share about 25% sequence homology.[8] However, IL-1α remains within the cells that produce it and is rarely found outside these cells. IL-1α can be released after cell death and can help attract inflammatory cells to areas where cells and tissues are being killed or damaged.

IL-1β is responsible for most of the systemic activity attributed to IL-1, including fever, activation of phagocytes, and production of acute-phase proteins. It is cleaved intracellularly to an active form that is then secreted by monocytes. IL-1 acts as an **endogenous pyrogen** (induces fever) in the acute-phase response through its actions on the hypothalamus.[9] The hypothalamus functions as the thermostat for the human body; IL-1 sets the thermostat at a higher level. Elevated body temperatures may serve to inhibit growth of pathogenic bacteria and viruses and also increase lymphocyte activity. Additionally, IL-1 induces the production of vascular cell-adhesion molecules as well as chemokines and IL-6. These chemokines and cell-adhesion molecules attract and assist leukocytes to enter the inflamed area through a process known as *diapedesis,* which is the passage of leukocytes through the walls of the blood vessels into the tissues (see Chapter 1). IL-1 also induces the production of CSFs in the bone marrow, thereby increasing the available number of phagocytic cells that can respond to the damaged tissues.[10]

IL-1RA, which is also produced by monocytes and macrophages, is the best characterized cytokine inhibitor. IL-1RA acts as an antagonist to IL-1 by blocking the IL-1 receptor, which helps to regulate the physiological response to IL-1 and turn off the response when no longer needed.

Tumor Necrosis Factors

Tumor necrosis factors (TNF) were first isolated from lymphocytes and macrophages and were so named because they induced lysis in tumor cells. TNF-α is the most prominent member of the TNF superfamily, which consists of at least 19 different peptides that have diverse biological functions.[11] TNF-α exists in both membrane-bound and soluble forms and causes vasodilation and increased vasopermeability. The main trigger for TNF-α production is the presence of lipopolysaccharide, which is found in gram-negative bacteria.

TNF-α secreted by activated monocytes and macrophages can activate T cells through its ability to induce expression of class II MHC molecules, vascular adhesion molecules, and chemokines in a manner similar to IL-1. These actions enhance antigen presentation and activate T cells to respond to the pathogen that triggered the initial inflammatory response. However, when secreted at higher levels, TNF can have deleterious systemic effects, leading to septic shock. This condition results from large amounts of TNF secreted in response to gram-negative bacterial infections, causing a decrease in blood pressure, reduced tissue perfusion, and disseminated intravascular coagulation. The latter may lead to uncontrolled bleeding.

Clinical Correlation

The Role of Cytokine Storm in Ebola Virus Infection

The Ebola virus causes one of the most serious and fatal diseases known to humans **(Fig. 6–3).** This single-stranded RNA virus begins its attack on the body within 2 to 21 days after exposure. Early symptoms include severe headache, fever, muscle pain, fatigue, diarrhea, vomiting, abdominal pain, and unexplained bruising or hemorrhaging. It is extremely infectious and can spread quickly through a population.

As the immune system tries to halt the spread of the virus, a profuse release of proinflammatory cytokines occurs, often called a cytokine storm. It is actually the overabundance of cytokines that is responsible for the devastating effects on the body. TNF-α, in particular, causes the blood vessels to become more permeable, resulting in dangerously low blood pressure. As the platelet count drops, excessive bleeding occurs from every orifice in the body. Death typically results in 1 to 2 weeks after infection.

The cytokine storm is a profound example of the importance of regulation of the immune system. If left unchecked, the cytokines that normally help to overcome infection can have a deleterious effect.

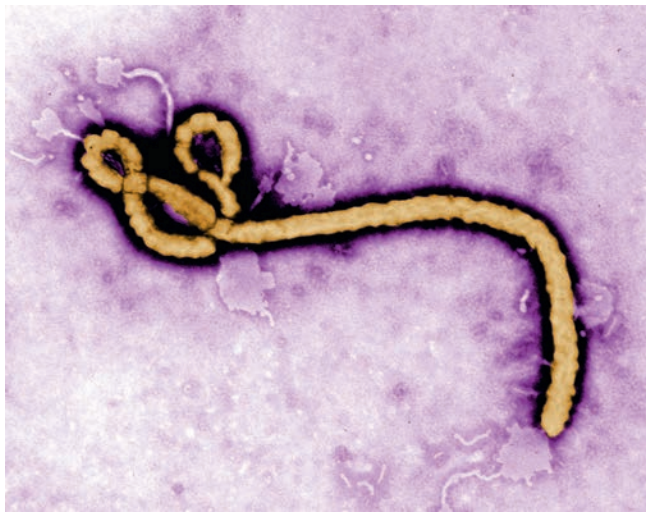

FIGURE 6–3 False-colored micrograph of the Ebola virus virion. *(Courtesy of the CDC/Frederick A. Murphy, Public Health Image Library.)*

TNFR1 (TNF receptor 1) is constitutively expressed on most tissues and binds soluble TNF-α. It is the primary mediator of TNF-α signal transduction in most cell types. TNFR2 is usually expressed in epithelial cells and cells of the immune system and is activated by the membrane-bound form of TNF-α. Overall, TNF-α activity is at least partially regulated by soluble forms of both TNF receptors. These receptors act to bind excess TNF-α and, combined with the short half-life of the soluble form, serve to limit the cytokine's signaling activity.

TNF-α as well as IL-1 are present in rheumatoid synovial fluids and synovial membranes of patients with rheumatoid arthritis (RA). Studies with anti-TNF-α and anti-IL-1 demonstrate that TNF-α is the central mediator of pathological processes in RA and other inflammatory illnesses such as Crohn's disease, ulcerative colitis, and juvenile arthritis.[12]

Given the profound therapeutic potential of TNF-α inhibition, it has been suggested that different chronic inflammatory diseases may share common pathophysiology and that several different cytokine-targeted therapies may emerge as feasible tools to disrupt the cytokine network, leading to TNF-α activation.

Interleukin-6

IL-6 is a single protein produced by both lymphoid and non-lymphoid cell types. It is part of the cytokine cascade released in response to lipopolysaccharide and plays an important role in acute-phase reactions. IL-6 is expressed by a variety of normal and transformed cells, including T cells, B cells, monocytes and macrophages, vascular endothelial cells, and various tumor cells. IL-1 primarily triggers its secretion.

This pleiotropic cytokine affects inflammation, acute-phase reactions, immunoglobulin synthesis, and the activation states of B cells and T cells. IL-6 stimulates B cells to proliferate and differentiate into plasma cells and induces CD4+ T cells to produce greater quantities of both pro- and anti-inflammatory cytokines.[3]

Only one IL-6 receptor has been identified that consists of IL-6Rα (the IL-6-specific receptor) and gp130 (the common signal-transducing receptor subunit used by several cytokines). Binding of IL-6 to the IL-6Rα induces dimerization of gp130 with the α-subunit **(Fig. 6–4).** Homodimerization following IL-6 binding causes conformational changes in gp130 that expose tyrosine residues in the intracellular portion of the molecule. Through a series of phosphorylation reactions, genes for acute-phase proteins such as C-reactive protein (CRP), the third component of complement (C3), and fibrinogen are activated, as is interferon regulatory factor-1 (IRF-1). B- and T-cell genes are turned on in the same manner.

Studies have demonstrated that chronic psychosocial stress increases the risk of atherosclerotic cardiovascular disease, which may involve many mediators and pathways. The hypothesis has been formulated that stress promotes atherogenesis by activation of vascular inflammation via elevated circulating proinflammatory cytokine levels (e.g., TNF-α, IL-6). Although it is clear that circulating cytokine levels serve as reliable biomarkers of systemic inflammation, further studies are needed to better define the role of cytokines in mediating inflammatory reactions.[13]

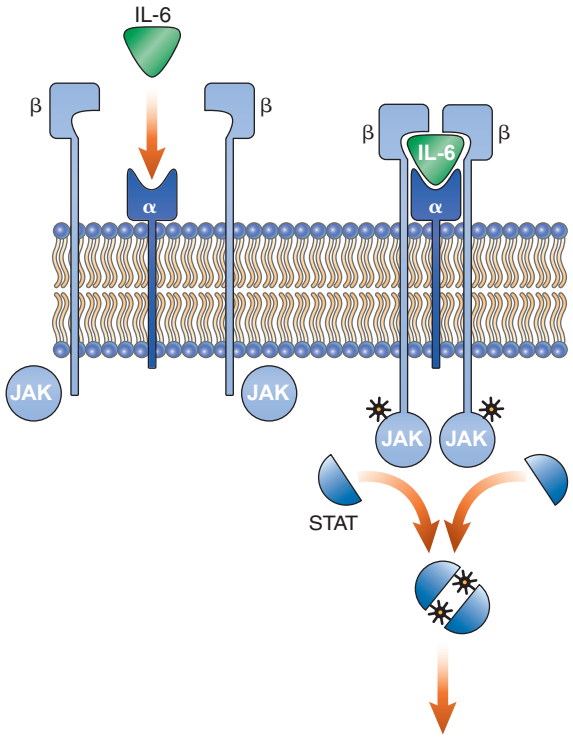

FIGURE 6–4 IL-6 binding to the IL-6R subunit recruits two gp130 subunits to the receptor complex. Signal transduction takes place through tyrosine phosphorylation of the gp130 subunits by one of the Janus kinases (J).

Chemokines

Chemokines are a family of cytokines that enhance motility and promote migration of many types of white blood cells (WBCs) toward the chemokine source via a process known as chemotaxis.[14] Most of the chemotactic activity of leukocytes is regulated by the activities of chemokines, including the response to infectious diseases, autoimmune inflammation, cancer, and the homing of lymphocytes to all the lymphoid tissues. The chemokines are classified into four families based on the position of N-terminal cysteine residues. The first group—the alpha, or CXC, chemokines—contains a single amino acid between the first and second cysteines. The second group—the beta, or CC, chemokines—has adjacent cysteine residues. The third group—the C chemokines—lacks one of the cysteines. CX3C, the last major group, has three amino acids between the cysteines. Chemokines play key roles in the initiation and development of inflammatory responses in numerous disease processes. Currently, over 40 chemokines and 20 chemokine receptors have been identified: select chemokines and receptors are displayed **(Table 6–2).**

Both TNF-α and IL-6 are among the many cytokines that induce chemokine production in the inflammatory response. Combined with cell adhesion molecules, the chemokines facilitate the extravasation of leukocytes into the tissues. Leukocytes rolling on capillary endothelial cells activate their chemokine receptors in the presence of chemokines. **Integrins,** or cell adhesion molecules on leukocytes, are then activated, leading to firm adhesion to the endothelial cells. Shared expression of chemokine receptors among different types of leukocytes allows for the co-localization of multiple cell types to the damaged tissue and helps to broaden the response to tissue damage. The gradient of chemokine concentration enables the leukocytes to migrate between the endothelial cells into the tissue in the direction of increasing chemokine concentration.

Table 6–2 Select Chemokines and Their Receptors

CHEMOKINE GROUP	CHEMOKINE NAMES	CHEMOKINE RECEPTORS
CC Chemokines	CCL2	CCR2
	CCL3	CCR1 CCR5
	CCL4	CCR5
	CCL5	CCR1 CCR3 CCR5
	CCL11	CCR3
	CCL19	CCR7
	CCL20	CCR6
	CCL25	CCR9
CXC Chemokines	CXCL1 CXCL2 CXCL3 CXCL5 CXCL7	CXCR2
	CXCL8	CXCR1 CXCR2
	CXCL9 CXCL10 CXCL11	CXCR3-A CXCR3-B
	CXCL12	CXCR4

The spectrum of chemokines and cytokines expressed in the inflammatory response determines the types of cells that respond and the genes that are turned on in response to the stimuli. The types of cell surface receptors expressed by leukocytes are often developmentally regulated—for example, immature T cells possess only the chemokine receptors related to lymphoid tissue homing. Only mature T cells express the receptors that allow them to participate in an ongoing immune reaction.

HIV uses the chemokine receptors CXCR4 and CCR5 as co-receptors for infection of CD4+ T lymphocytes and macrophages.[15] Individuals with certain polymorphisms in these chemokine receptors are long-term nonprogressors. They remain asymptomatic with normal CD4+ T-cell counts and immune function as well as low or undetectable viral loads. The altered protein sequences of the receptors block or diminish the virus's ability to enter the cells and thereby increase the infected individual's chances of survival. The CCR5-δ32 polymorphism is a 32 bp deletion in the CCR5 gene and is the most important of the host resistance factors. Homozygous individuals are protected

from HIV infection, whereas heterozygous persons exhibit longer periods between HIV infection and AIDS development.

Transforming Growth Factor-β

The **transforming growth factor-β (TGF-β)** superfamily is composed of three isoforms: TGF-β1, TGF-β2, and TGF-β3. TGF-β was originally characterized as a factor that induced growth arrest in tumor cells. Later, it was identified as a factor that induces antiproliferative activity in a wide variety of cell types. Active TGF-β is primarily a regulator of cell growth, differentiation, apoptosis, migration, and the inflammatory response. Thus, it acts as a control to help downregulate the inflammatory response when no longer needed.

In the immune response, TGF-β functions as both an activator and an inhibitor of proliferation, depending on the developmental stage of the affected cells.[16] TGF-β regulates the expression of CD8 in CD4–CD8– thymocytes and acts as an autocrine inhibitory factor for immature thymocytes. It inhibits the activation of macrophages and the growth of many different somatic cell types and functions as an anti-inflammatory factor for mature T cells. TGF-β blocks the production of IL-12 and strongly inhibits the induction of IFN-gamma. In addition, the production of TGF-β by T helper 2 (Th2) cells is now recognized as an important factor in the establishment of oral tolerance to bacteria normally found in the mouth. In activated B cells, TGF-β typically inhibits proliferation and may function as an autocrine regulator to limit the expansion of activated cells.

Interferon-α and Interferon-β

Interferons (IFN) were originally so named because they interfere with viral replication. However, it is the type I IFNs consisting of IFN-α and IFN-β that function primarily in this manner. These IFNs are produced by dendritic cells and induce production of proteins and pathways that directly interfere with viral replication and cell division. In most cases, this helps limit the infection to one relatively small area of the body. Type I IFN activates natural killer (NK) cells and enhances the expression of class I MHC proteins, thus increasing the recognition and killing of virus-infected cells.

The type I IFNs are also active against certain malignancies and other inflammatory processes. For instance, IFN-β is efficacious in treating multiple sclerosis, although the exact mechanism of action remains unclear.[17] A clue has been gained by recent genome-wide association studies that discovered a relationship between IFN-β response and ion receptor channel proteins; however, the role of these proteins is not elucidated.[18] IFN-α has been used to treat hepatitis C and Kaposi's sarcoma, as well as certain leukemias and lymphomas.[19]

Cytokines in the Adaptive Immune Response

Cytokines involved in the innate immune response are produced by many different cell types. Cytokines function mainly to increase acute-phase reactants and to recruit WBCs to the area of infection. In contrast, cytokines involved in the adaptive immune response are mainly secreted by T cells, especially Th cells, and affect T- and B-cell function more directly. There are three main subclasses of Th cells: **T helper 1 (Th1), T helper 2 (Th2),** and **T regulatory (Treg) cells.** Each has a specific function and produces a different set of cytokines. **T helper 17 (Th17) cells** are a fourth subset of Th cells. Because Th17 cells affect both the innate and adaptive immune response, these will be discussed separately.

Once the T-cell receptor (TCR) captures antigen, clonal expansion of those particular CD4+ Th cells occurs.[20] Differentiation into Th1, Th2, or Treg cell lineages is influenced by the spectrum of cytokines expressed in the initial response **(Fig. 6–5).**[21] The Th1 lineage is driven by the expression of IL-12 by dendritic cells and is primarily responsible for cell-mediated immunity, whereas Th2 cells drive antibody-mediated immunity and are developmentally

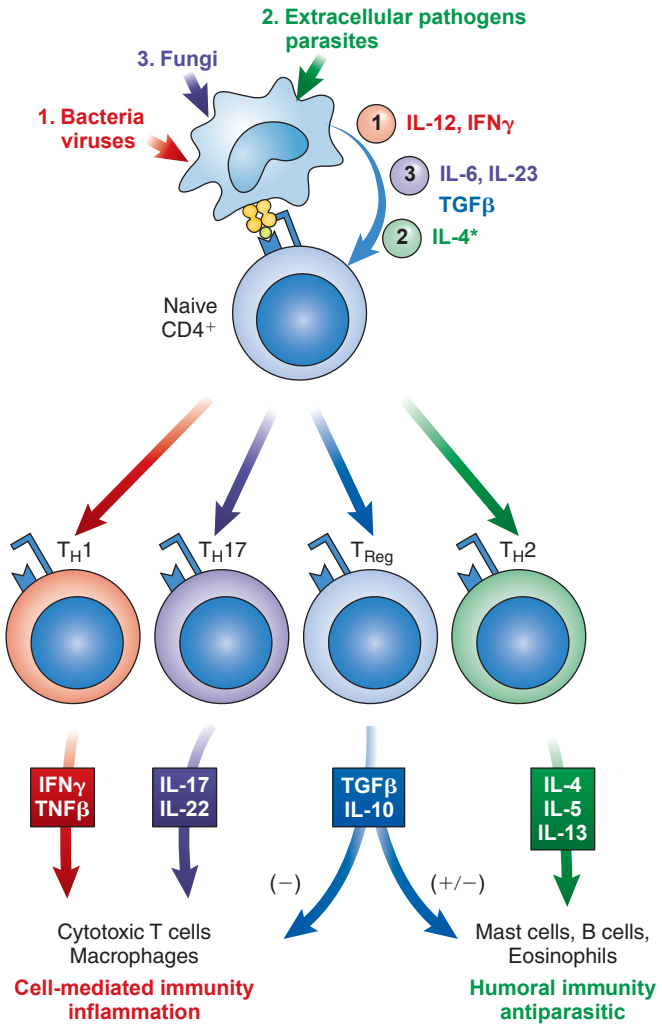

FIGURE 6–5 Subpopulations of T cells. Depending upon the pathogen encountered, antigen-presenting cells (APCs) secrete a specific combination of polarizing cytokines that direct naïve T helper (Th) cells to differentiate into specialized subsets. Each T-cell subpopulation exerts different types of immune functions. The release of effector cytokines coordinates an appropriate immune response against the pathogen.

regulated by IL-4. Treg cells are derived from naïve T cells in response to TGF-β. Tregs help to regulate the activities of Th1 and Th2 cells.

Th1 Cytokines

Dendritic cells in damaged tissues produce IL-12 in response to certain stimuli such as mycobacteria, intracellular bacteria, and viruses. It is also produced by macrophages and B cells and has multiple effects on both T cells and NK cells. IL-12 binds to its receptor on naïve T cells and causes the expression of a new set of genes, including those that determine maturation into the Th1 lineage. Activation of Th1 cells induces high-level expression of IFN-gamma.[22] IL-12 also increases the cytolytic ability of NK cells; therefore, it serves as an important link between the innate and adaptive immune responses by enhancing defenses against intracellular pathogens.

Interferon-γ (IFN-γ)

IFN-γ, the principal molecule produced by Th1 cells, affects the RNA expression levels of more than 200 genes.[23] A major function of IFN-γ is stimulation of antigen presentation by class I MHC and class II MHC molecules. Increased expression of class I and II MHC molecules on antigen-presenting cells (APCs) increases the likelihood of antigen capture and involvement of additional lymphocytes. IFN-γ is also the most potent activator of macrophages and boosts their tumoricidal activity.[24] In this capacity, it stimulates the phagocytic and cytotoxic abilities of these cells, creating activated or "super" macrophages. In addition, IFN-γ is involved in regulation and activation of CD4+ Th1 cells, CD8+ cytotoxic lymphocytes, and NK cells. Thus, IFN-γ influences the immune response in a number of key ways.

Interleukin-2 (IL-2)

Th1 cells also secrete IL-2 in addition to IFN-γ. IL-2, also known as the T-cell growth factor, drives the growth and differentiation of both T and B cells and induces lytic activity in NK cells. IL-2 and IFN-γ induce the development of Th1 cells, which, in turn, induces macrophage activation and delayed type hypersensitivity. Th1 cells stimulate the production of IgG1 and IgG3 opsonizing and complement fixing antibodies by antigen-activated B cells. These isotypes assist in cell-mediated immune responses driven by Th1 cells.

IL-2 alone can activate proliferation of Th2 cells and helps to generate IgG1- and IgE-producing cells. Clonal expansion of activated Th cells is a necessary part of mounting an adequate immune response to any immunologic challenge. The cytokine network that develops will continue to regulate T-cell growth and differentiation until the challenge is gone and the response subsides.

Transcription of the gene for IL-2 and IL-2R begins within 1 hour of binding to the TCR. The functional IL-2R consists of α, β, and γ subunits or just β and γ subunits. The β and γ subunits increase the affinity of the receptor for IL-2 and are responsible for most of the signal transduction through the receptor. The γ chain is also shared by the receptors for IL-4, IL-7, IL-9, IL-15, and IL-21.[25] The importance of the γ chain is demonstrated in individuals who have mutations in this chain. These persons have X-linked severe combined immunodeficiency syndrome and lack functional T and B cells.[26]

Th2 Cytokines

As mentioned previously, Th2 cells are primarily responsible for antibody-mediated immunity. Among the cytokines produced are IL-4 and IL-10. Both of these cytokines are important regulators of the immune response but have opposite effects.

Interleukin-4 (IL-4)

IL-4 is one of the key cytokines regulating Th2 immune activities and helps drive antibody responses in a variety of diseases.[26] The IL-4 receptor is expressed on lymphocytes and on numerous nonhematopoietic cell types. IL-4 activity on naïve T cells turns on the genes that generate Th2 cells and turns off the genes that promote Th1 cells.

Th2 cells are responsible for regulating many aspects of the immune response, including those related to allergies, autoimmune diseases, and parasites. IL-4 induces production of MHC-I, IL-4, IL-5, IL-13, and the costimulatory molecules CD80 and CD86. IL-4 also stimulates the production of IgG2a and IgE and, along with IL-5, drives the differentiation and activation of eosinophils in both allergic immune responses and response to parasitic infections.[26] IL-13 is a cytokine with many of the same properties as IL-4; both cytokines induce worm expulsion and favor IgE-class switching. IL-13, however, differs from IL-4 because it also plays an anti-inflammatory role by inhibiting activation and cytokine secretion by monocytes.

Interleukin-10 (IL-10)

In contrast to the cytokines previously discussed, IL-10 has primarily inhibitory effects on the immune system. It is produced by monocytes, macrophages, CD8+ T cells, and Th2 CD4+ T cells and has anti-inflammatory and suppressive effects on Th1 cells. It also inhibits antigen presentation by macrophages and dendritic cells. In addition, one of the major effects of IL-10 is the inhibition of IFN-gamma production via the suppression of IL-12 synthesis by accessory cells and the promotion of a Th2 cytokine pattern.[27] Thus, IL-10 serves as an antagonist to IFN-gamma—it is a downregulator of the immune response.

Cytokines Associated With T Regulatory Cells

The third major subclass of CD4+ T cells are the T regulatory (Treg) cells. Tregs are CD4+ CD25+ T cells that are selected in the thymus.[28] An additional population of Tregs, called induced Tregs (iTregs), can develop from mature T cells in the periphery.[26] Tregs play a key role in establishing peripheral tolerance to a wide variety of self-antigens, allergens, tumor antigens, transplant antigens, and infectious agents. CD4+ CD25+ Tregs affect T-cell activity primarily through the actions of TGF-β. TGF-β induces expression of Foxp3, a transcription factor that causes Treg cells to suppress the activity of other

T cells.[29] Tregs may be found in transplanted tissue and help to establish tolerance to the graft by the host immune system through the alteration of antigen presentation.

Tregs are also responsible for inducing IL-10 and TGF-β expression in **adaptive T regulatory 1 (Tr1) cells** in the peripheral circulation. Tr1 cells are CD4+ T cells that are induced from antigen-activated naïve T cells in the presence of IL-10. They exert their suppressive activities on both Th1 and Th2 cells by producing more IL-10, TGF-β, or IL-35. T-cell suppression occurs through IL-10 inhibition of proinflammatory cytokines and inhibition of costimulatory molecule expression on APCs. TGF-β downregulates the function of APCs and blocks proliferation and cytokine production by CD4+ T cells. IL-35 is a recently identified cytokine that has immunosuppressive effects on Th1, Th2, and Th17 cells while promoting growth of Tregs.[20] All of these activities lead to downregulation of the immune response and the prevention of chronic inflammation.

Both Tregs and Tr1 cells operate through a network of cytokines to establish peripheral tolerance to certain antigens; therefore, they play a key role in limiting autoimmunity. The relative importance of each phenotype depends largely on the antigen involved, the context of antigen presentation, and the biology of the tissue.

Th17 Cytokines in Innate and Adaptive Immune Responses

The Th17 subset secretes the IL-17 family of cytokines and plays critical roles in both innate and adaptive immune responses. Key cytokines that differentiate T cells to maintain them as Th17 cells are TGF-β and IL-6.[30] Interleukin-23, produced by macrophages and dendritic cells, also plays a role in finalizing the commitment to Th17 cells.[31]

IL-17A, the first IL-17 identified, is the most studied IL-17 cytokine. Other IL-17 family members include IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. Most of the IL-17 cytokine family members are potent proinflammatory cytokines and induce expression of TNF-α, IL-1β, and IL-6 in epithelial, endothelial, keratinocyte, fibroblast, and macrophage cells.

Th17 cells play an important role in host defense against bacterial and fungal infections at mucosal surfaces. Upon encounter with bacteria or fungus, APCs secrete cytokines, which differentiate Th17 subsets of cells. Th17 cells invade the infected area and secrete IL-17 cytokines necessary for continuous recruitment of neutrophils.[31] Th17 cells in the local tissue may be important for long-term maintenance of antimicrobial response during chronic bacterial infections. Mucosal surfaces, in response to IL-17 cytokine stimulation, secrete antimicrobial peptides. IL-17A and IL-17F induce epithelial cells, endothelial cells, and fibroblasts to produce CXC ligand 8 (CXCL-8), which is crucial for recruitment of neutrophils to the site of inflammation. IL-17A and IL-17F also act together with granulocyte-macrophage CSF to produce CXCL-8 in macrophages, which also signals neutrophils to the site.[31]

The fine regulation of APC cytokines (IL-23 or IL-12) and Th17 development may also be important for antimicrobial defense. Dysregulation of Th17 cell subsets and secreted cytokines has been implicated in pathogenesis of multiple inflammatory diseases and several autoimmune conditions, including RA, MS, inflammatory bowel disease (IBD), and psoriasis.[32,33]

IL-17 can produce proinflammatory mediators from myeloid and synovial fibroblasts and perpetuate the inflammatory process in RA.[33] Increased numbers of Th17 cells and IL-17A have also been observed in asthmatic and allergic patients.[33] IL-17A directly induces IgE production by B cells[33]; higher amounts of IL-17A and IL-17F in patient lungs have been associated with more severe asthma.[34] Interestingly, removal of Th17 cells from peripheral blood mononuclear cells of allergic asthma patients has led to decreased levels of IgE.[33]

Hematopoietic Growth Factors

A number of cytokines produced during innate and adaptive immune responses stimulate the proliferation and differentiation of bone marrow progenitor cells. Thus, the responses that require a supply of leukocytes produce mediators to provide those cells. The primary mediators of hematopoiesis are called **colony stimulating factors (CSFs)** because they stimulate the formation of colonies of cells in the bone marrow. The CSFs include IL-3, **erythropoietin (EPO), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF),** and **granulocyte-macrophage colony stimulating factor (GM-CSF).**[20,35–37] In response to inflammatory cytokines such as IL-1, the different CSFs act on bone marrow cells at different developmental stages and promote specific colony formation for the various cell lineages. IL-3 is a multilineage CSF that induces bone marrow stem cells to form T and B cells. In conjunction with IL-3, the CSFs direct immature bone marrow stem cells to develop into red blood cells (RBCs), platelets, and the various types of WBCs **(Fig. 6–6).** IL-3 acts on bone marrow stem cells to begin the differentiation cycle; the activity of IL-3 alone drives the stem cells into the lymphocyte differentiation pathway.

GM-CSF acts to drive differentiation toward other WBC types. If M-CSF is activated, the cells become macrophages. M-CSF also increases phagocytosis, chemotaxis, and additional cytokine production in monocytes and macrophages. If G-CSF is activated, the cells become neutrophils. G-CSF enhances the function of mature neutrophils and affects the survival, proliferation, and differentiation of all cell types in the neutrophil lineage. It decreases IFN-gamma production, increases IL-4 production in T cells, and mobilizes multipotential stem cells from the bone marrow. These stem cells are used to repair damaged tissues and create new vasculature to reconstruct the tissues following an infection. However, IL-3 in conjunction with GM-CSF drives the development of basophils and mast cells, whereas the addition of IL-5 to IL-3 and GM-CSF drives the cells to develop into eosinophils. The net effect is an increase in WBCs to respond to the ongoing inflammatory processes.

EPO regulates RBC production in the bone marrow but is primarily produced in the kidneys. EPO-α is the form licensed for clinical use by the FDA. EPO-α is often prescribed to improve

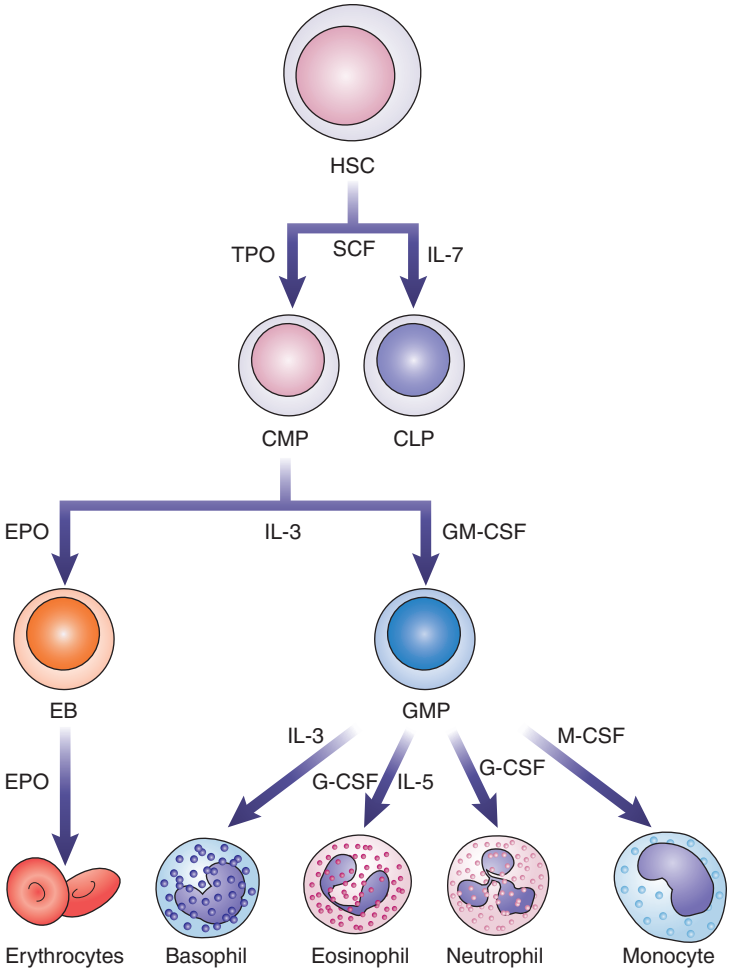

FIGURE 6–6 Influence of colony stimulating factors on growth and differentiation of blood cells. Growth of hematopoietic stem cells (HSC) requires stem cell factor (SCF) with differentiation determined by IL-7 or thrombopoietin (TPO). Growth of common myeloid progenitors (CMP) depends upon IL-3. Differentiation is driven by granulocyte-macrophage colony stimulating factor (GM-CSF) or erythropoietin (EPO). Common granulocyte/monocyte precursors (GMP) differentiate into granulocytes in response to granulocyte-CSF. Further specificity is provided by IL-3 or IL-5. Macrophage-CSF (M-CSF) promotes development of monocytes. CLP = common lymphoid progenitor, EB = erythroblast.

RBC counts for individuals with anemia and for those with cancer who have undergone radiation and chemotherapy. RBC proliferation induced by EPO improves oxygenation of the tissues and eventually switches off EPO production. The normal serum EPO values range from 5 to 28 U/L but must be interpreted in relation to the hematocrit because levels can increase up to a thousandfold during anemia.

Cytokine and Anticytokine Therapies

Cytokine-inhibiting biologics disrupt the interaction between cytokines and their cognate receptors. Initial studies used murine monoclonal antibodies that were able to function only for short periods of time before the host mounted an immune response against them. Recombinant DNA techniques have allowed for the production of humanized monoclonal antibodies that are much less immunogenic and that function as cytokine antagonists. An example is infliximab (Remicade), a chimeric antibody containing human constant regions and murine antigen-specific arms that bind human TNF-α.[38] Infliximab is a valuable treatment option for patients with Crohn's disease or RA because it blocks activity of TNF-α in RA and Crohn's disease with rapid onset of therapeutic action after administration.

Another approach to anticytokine therapy is the development of a class of hybrid proteins containing cytokine receptor binding sites attached to immunoglobulin constant regions. Etanercept (Enbrel), for example, is a member of this class that has been approved for use in humans.[39] Enbrel consists of the extracellular domains of the type 2 TNF receptor fused to the heavy-chain constant region of IgG1. The fusion protein can bind TNF-α and block its activity. It has a 4.8-day half-life in serum. Etanercept remains an important cost-effective treatment option in patients with RA, ankylosing spondylitis, psoriatic arthritis or psoriasis, and pediatric arthritis and psoriasis. Etanercept effectively reduces signs and symptoms, disease activity, and disability and improves health-related quality of life, with these benefits sustained during long-term treatment.

The blockade of IL-17 function has also been a therapeutic target. Researchers are currently developing an IL-17 blocking antibody to prevent IL-17 mediated pathogenesis, such as in psoriasis.[40] RA patients treated with the IL-17A blocking antibody known as ixekizumab (formerly LY2439821 from Eli Lilly) in clinical trials showed significantly improved signs and symptoms with no significant safety concern.[41] Blockade of IL-17 receptors is another approach under therapeutic assessment for specific subgroups of asthma patients.[42] An additional cytokine receiving attention is IL-23, which enhances the differentiation of Th17 lymphocytes. Blocking of this interleukin has

become another focus of research in the treatment of asthma and may have an application for other autoimmune diseases.[43]

Preclinical studies, such as those supporting the central role of IL-17 in models of inflammatory bowel disease, have not only reinforced the concept of common pathophysiology of several autoimmune diseases such as RA, but have also raised hopes that blockade of new cytokines may show even superior efficacy over TNF-α inhibition. These lines of evidence suggest that there is a shared cytokine framework that defines highly conserved mechanisms of inflammation in certain human diseases and indicates multiple vulnerable nodes.[12] Neutralization of one of these nodes may therefore suffice to disrupt the inflammatory process in a large variety of human inflammatory diseases.

Clinical Assays for Cytokines

Clinical evaluation of the cytokine profile in the patient could be of prognostic and diagnostic value to the physician when treating autoimmune diseases such as the ones mentioned in the previous sections. Several cytokine assay formats are available for basic and clinical research use, including ELISpot assays, multiplexed ELISAs, and microbead assays.

The ELISpot assay employs the enzyme-linked immunosorbent assay (ELISA) technique on in vitro-activated peripheral WBCs. In the ELISpot process, either a monoclonal or polyclonal antibody specific for the chosen cytokine is precoated onto a microplate. Antigen-stimulated, mitogen-stimulated (positive control), or saline-stimulated (negative control) WBCs are pipetted into the wells and the microplate is placed into a humidified CO_2 incubator at 37°C for a specified period of time. During the incubation period, the immobilized antibody in the immediate vicinity of the secreting cells binds the secreted cytokine. After any cells and unbound substances are washed away, a biotinylated polyclonal antibody specific for the chosen cytokine is added to the wells. Following a wash to remove any unbound biotinylated antibody, alkaline-phosphatase conjugated to streptavidin is added. Unbound enzyme is subsequently washed away and a substrate solution is added. A colored precipitate forms and appears as spots at the sites of cytokine localization, with each spot representing an individual cytokine-secreting cell. The spots can be counted with a stereomicroscope or with an automated ELISpot reader.

Multiplexed ELISAs use several detector antibodies bound to individual microwells or antibody microarrays and allow for simultaneous detection of several cytokines from serum or plasma.[44] Current formulations allow for the detection of 12 to 25 pro- and anti-inflammatory cytokines in one reaction. In the microarray format, each well on the slide contains a microarray of spotted antibodies, with "spots" for each of the cytokines plus additional "spots" for positive and negative controls. The replicate spots allow for acquisition of reliable quantitative data from a single sample.

New microbead assays allow for the simultaneous detection of multiple cytokines in a single tube.[45] Each bead type has its own fluorescent wavelength, which, when combined with the fluorescent secondary antibody bound to a specific cytokine, allows for the detection of up to 100 different analytes in one tube. The use of a multiplexed bead array enables simultaneous measurement of a multitude of biomarkers; these include acute-phase reactants such as CRP; proinflammatory cytokines; Thl/Th2 distinguishing cytokines such as IFN-gamma, IL-2, IL-4, IL-5, and IL-10; other nonspecific acting cytokines; and CSFs.

One drawback of protein-based technologies is the short half-life of certain cytokines. This can be overcome by looking at RNA expression in cells using reverse transcription polymerase chain reaction (PCR). The PCR product is made using a fluorescent-labeled primer and can be hybridized to either solid-phase or liquid microarrays. Solid-phase arrays have up to 40,000 spots containing specific ssDNA oligonucleotides representing individual genes. Clinically useful arrays generally have substantially fewer genes represented. Fluorescence of a spot indicates that the gene was expressed in the cell and that the cell was producing the cytokine.

The liquid arrays use the same beads as the antibody microbead arrays discussed earlier. However, instead of antibodies, these beads have oligonucleotides on their surfaces and allow up to 100 different cDNAs to be identified. The combination of the bead fluorescence and the fluorescence of the labeled cDNA produces an emission spectrum that identifies the cytokine gene that was expressed in the cells.

Real time PCR has emerged as a means of detecting cytokine response at the level of gene expression.[46] This type of testing is much faster than traditional PCR. See Chapter 12 for a discussion of molecular techniques.

SUMMARY

- Cytokines are small, soluble proteins secreted by white blood cells and a variety of other cells. They act as chemical messengers to regulate the immune system.
- Cytokines are induced in response to specific stimuli such as bacterial lipopolysaccharides, flagellin, or other bacterial products.
- The effects of cytokines in vivo include regulation of growth, differentiation, and gene expression by many different cell types.
- If a cytokine has an autocrine effect, it affects the same cell that secreted it.
- A cytokine can have a paracrine effect when it affects a target cell in close proximity.
- Occasionally, cytokines will exert systemic or endocrine activities.
- Individual cytokines do not act alone but in conjunction with many other cytokines that are induced during the process of immune activation.
- The combined cytokines produce a spectrum of activities that lead to the rapid generation of innate and adaptive immune responses.
- Massive overproduction is called a "cytokine storm" that leads to shock, multi-organ failure, or even death, thus contributing to pathogenesis.

- The pleiotropic nature of cytokine activity relates to the ability of a cytokine to affect many different types of cells.
- If several different cytokines activate some of the same cells, it is termed redundancy.
- The major cytokines involved in the initial stages of the inflammatory response are IL-1, IL-6, TNF-α, and the chemokines. These cytokines are responsible for many of the physical symptoms attributed to inflammation such as fever, swelling, pain, and cellular infiltrates into damaged tissues.
- Naïve T cells can differentiate into Th1, Th2, or Treg cell lineages, with the help of cytokines involved in the adaptive immune response.
- The Th1 lineage is driven by the expression of IL-12 by dendritic cells and is primarily responsible for cell-mediated immunity.
- Th2 cells drive antibody-mediated immunity and are regulated by IL-4.
- Treg cells are derived from naïve T cells in response to IL-10 and TGF-β and help to regulate the activities of Th1 and Th2 cells.
- Th17 cells produce IL-17, a proinflammatory cytokine that induces expression of TNF-α, IL- 1β, and IL-6. Forms of IL-17 also recruit neutrophils to an infected area.
- Colony stimulating factors (CSFs) are responsible for inducing differentiation and growth of all WBC types.
- Anticytokine therapies are aimed at disrupting the interaction between cytokines and their specific receptors in diseases such as rheumatoid arthritis and Crohn's disease.
- Cytokine assay formats available for basic and clinical research use include ELISpot assays, multiplexed ELISAs, and microbead assays.
- ELISpot assays are enzyme-linked immunosorbent assays on in vitro activated peripheral WBCs that detect one cytokine at a time.
- Multiplexed ELISAs and microbead assays can detect several cytokines in serum at a time.

Study Guide: Cytokines Associated With Innate Immunity

CYTOKINE	SECRETED BY	ACTIONS
Interleukin-1β (IL-1β)	Monocytes, macrophages, dendritic cells	Inflammation, fever, initiation of the acute-phase response
Tumor necrosis factor-α (TNF-α)	Monocytes, macrophages, neutrophils, NK cells, activated T cells	Inflammation, initiation of the acute-phase response, death of tumor cells
Interleukin-6 (IL-6)	Monocytes, macrophages, endothelial cells, Th2 cells	Initiation of the acute-phase response, activation of B and T cells
Transforming growth factor-β (TGF-β)	T cells, macrophages, other cell types	Inhibition of both T- and B-cell proliferation, induction of IgA, inhibition of macrophages
Interferon-α (IFN-α) Interferon-β (IFN-β)	Macrophages, dendritic cells, virally infected cells	Protects cells against viruses, increases class I MHC expression, activates NK cells

Study Guide: Cytokines Associated With Adaptive Immunity

CYTOKINE	SECRETED BY	ACTIONS
Interleukin-2 (IL-2)	T cells	Growth and proliferation of T and B cells NK activation and proliferation
Interleukin-4 (IL-4)	Th2 cells, mast cells	Promotion of Th2 differentiation, stimulation of B cells to switch to IgE production
Interleukin-5 (IL-5)	Th2 cells	Eosinophil generation and activation, B-cell differentiation
Interleukin-10 (IL-10)	Th2 cells, monocytes, macrophages	Suppression of Th2 cells, inhibition of antigen presentation, inhibition of interferon-gamma
Interferon-γ (IFN-γ)	Th1 cells, CD8+ T cells, NK cells	Activation of macrophages, increased expression of class I and II MHC molecules, increased antigen presentation

CASE STUDY

A 55-year-old woman being treated for acute lymphocytic leukemia (ALL) was not responding well to chemotherapy. Her physicians felt that increasing the dosage of her chemotherapy drugs was necessary to eliminate the cancer. However, laboratory results showed the patient was severely neutropenic (477 neutrophils/mL) as a result of the drugs. The medical team could not risk further lowering of the neutrophil count because of the increased risk of infection. Therefore, it was necessary to treat the patient for neutropenia in order to continue with chemotherapy. The patient was also enrolled in a research study designed to look at cytokine expression in ALL patients with neutropenia. The study used a liquid bead array that included the CSFs and the cytokines typically seen in the innate immune response and in Th1 and Th2 responses.

Questions

a. What colony stimulating factor should the physicians prescribe to overcome the neutropenia?
b. What are some of the cytokines that might be detected in a Th1 type response?
c. What are some of the cytokines that might be detected in a Th2 type response?

REVIEW QUESTIONS

1. The ability of a single cytokine to alter the expression of several genes is called
   a. redundancy.
   b. pleiotropy.
   c. autocrine stimulation.
   d. endocrine effect.

2. Which of the following effects can be attributed to IL-1?
   a. Mediation of the innate immune response
   b. Differentiation of stem cells
   c. Halted growth of virally infected cells
   d. Stimulation of mast cells

3. Which of the following precursors are target cells for IL-3?
   a. Myeloid precursors
   b. Lymphoid precursors
   c. Erythroid precursors
   d. All of the above

4. A lack of IL-4 may result in which of the following effects?
   a. Inability to fight off viral infections
   b. Increased risk of tumors
   c. Lack of IgM
   d. Decreased eosinophil count

5. Which of the following cytokines is also known as the T-cell growth factor?
   a. IFN-γ
   b. IL-12
   c. IL-2
   d. IL-10

6. Which of the following represents an autocrine effect of IL-2?
   a. Increased IL-2 receptor expression by the Th cell producing it
   b. Macrophages signaled to the area of antigen stimulation
   c. Proliferation of antigen-stimulated B cells
   d. Increased synthesis of acute-phase proteins throughout the body

7. IFN-α and IFN-β differ in which way from IFN-gamma?
   a. IFN-α and IFN-β are called immune interferons, and IFN-gamma is not.
   b. IFN-α and IFN-β primarily activate macrophages, whereas IFN-gamma halts viral activity.
   c. IFN-α and IFN-β are made primarily by activated T cells, whereas IFN-gamma is made by fibroblasts.
   d. IFN-α and IFN-β inhibit cell proliferation, whereas IFN-gamma stimulates antigen presentation by class II MHC molecules.

8. A patient in septic shock caused by a gram-negative bacterial infection exhibits the following symptoms: high fever, very low blood pressure, and disseminated intravascular coagulation. Which cytokine is the most likely contributor to these symptoms?
   a. IL-2
   b. TNF
   c. IL-12
   d. IL-7

9. IL-10 acts as an antagonist to what cytokine?

a. IL-4
b. TNF-α
c. IFN-gamma
d. TGF-β

10. Which would be the best assay to measure a specific cytokine?

a. Blast formation
b. T-cell proliferation
c. Measurement of leukocyte chemotaxis
d. ELISA testing

11. Selective destruction of Th cells by the human immunodeficiency virus contributes to immune suppression by which means?

a. Decrease in IL-1
b. Decrease in IL-2
c. Decrease in IL-8
d. Decrease in IL-10

12. Why might a colony stimulating factor be given to a cancer patient?

a. Stimulate activity of NK cells
b. Increase production of certain types of leukocytes
c. Decrease the production of TNF
d. Increase production of mast cells

13. Which of the following would result from a lack of TNF?

a. Decreased ability to fight gram-negative bacterial infections
b. Increased expression of class II MHC molecules
c. Decreased survival of cancer cells
d. Increased risk of septic shock

14. Which cytokine acts to promote differentiation of T cells to the Th1 subclass?

a. IL-4
b. IFN-α
c. IL-12
d. IL-10

15. What is the major function of T regulatory cells?

a. Suppression of the immune response by producing TNF
b. Suppression of the immune response by inducing IL-10
c. Proliferation of the immune response by producing IL-2
d. Proliferation of the immune response by inducing IL-4

16. Th17 cells affect the innate immune response by inducing production of which cytokines?

a. IFN-γ and IL-2
b. IL-4 and IL-10
c. IL-2 and IL-4
d. TNF-α and IL-6

7 Complement System

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Describe the roles of the complement system.
2. Differentiate between the classical, alternative, and lectin pathways and indicate proteins and activators involved in each.
3. Discuss the formation of the three principal units of the classical pathway: recognition, activation, and membrane attack units.
4. Describe how initiation of the lectin pathway occurs.
5. Explain how C3 plays a key role in all pathways.
6. Describe regulators of the complement system and their role in the complement system.
7. Discuss the complement-related kidney disorders and applicable complement testing.
8. Relate biological manifestations of complement activation to generation of specific complement products.
9. Describe the deficiencies of complement components and the diseases they cause.
10. Differentiate tests for functional activity of complement from measurement of individual complement components.
11. Analyze laboratory findings and indicate disease implications in relation to complement abnormalities.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Activation unit
Alternative pathway
Anaphylatoxin
Bystander lysis
C1 inhibitor (C1-INH)
C3 glomerulopathies (C3G)
C4-binding protein (C4BP)
Classical pathway
Complement receptor type 1 (CR1)
Decay-accelerating factor (DAF)
Factor H
Factor I
Hemolytic titration (CH50) assay
Hemolytic uremic syndrome (HUS)
Hereditary angioedema (HAE)
Immune adherence
Lectin pathway
Mannose-binding lectin (MBL)
Membrane attack complex (MAC)
Membrane cofactor protein (MCP)
Paroxysmal nocturnal hemoglobinuria (PNH)
Properdin
Recognition unit
S protein

As described in Chapter 3, complement is a complex series of more than 30 proteins that play a major part in amplifying the inflammatory response to destroy and clear foreign antigens. These soluble and cell-bound proteins interact in a very specific way and have powerful abilities. They can lyse foreign cells, opsonize and tag the invaders for clearance, and direct the adaptive immune system to the site of infection.[1] Complement activation is also proinflammatory in its ability to increase vascular permeability, recruit monocytes and neutrophils to the area of antigen concentration, and trigger secretion of immunoregulatory molecules that amplify the immune response.[2] In their proinflammatory role, complement proteins serve as an important link between innate and adaptive immunity.

Complement has important "housekeeping" roles as well. Complement recognizes cellular debris such as apoptotic cells and immune complexes, tagging them for removal by innate immune cells.[3] Because of its potential for far reaching effects, complement activation needs to be carefully regulated. Chronic activation can lead to inflammation and tissue damage to the host. Any deficiencies to the complement system can result in an increased susceptibility to infection or the accumulation of immune complexes resulting in possible autoimmune disorders.[4] However, numerous proteins act as controls or regulators of the system. These controls, as well as the major proteins involved in activation, will be discussed in detail.

Pathways of the Complement System

The complement system can be activated in three different ways. The first pathway described, the **classical pathway,** involves nine proteins that are triggered primarily by antigen–antibody combination. Pillemer and colleagues discovered an antibody-independent pathway in the 1950s that plays a major role as a natural defense system.[5] This second pathway, the **alternative pathway,** was originally called the *properdin system* because the protein properdin was thought to be the main initiator of this pathway. Now it is known that properdin's major function is to stabilize a key enzyme complex formed along the pathway and that the other forms of activation are more prominent. The third pathway, likely the most ancient of the three, is the **lectin pathway,** another antibody-independent means of activating complement proteins. Its prototypic constituent, **mannose-** (or mannan-) **binding lectin (MBL),** adheres to mannose found mainly in the cell walls or outer coating of bacteria, viruses, yeast, and protozoa. Although each of these pathways will be considered separately, activation seldom involves only one pathway.

Most plasma complement proteins are synthesized in the liver with the exception of C1 components; these are mainly produced by intestinal epithelial cells and Factor D, which is made in adipose tissue.[1,6] Other cells, such as monocytes and macrophages, are additional sources of early complement components, including C1, C2, C3, and C4.[6,7] Most of these proteins are inactive precursors, or zymogens, which are converted to active enzymes in a very precise order. **Table 7–1** lists the characteristics of the main complement proteins.

The Classical Pathway

The classical pathway, the first activation cascade described, is the main antibody-directed mechanism for triggering complement activation. However, not all immunoglobulins are able to activate this pathway. The immunoglobulin classes that can activate the classical pathway include IgM, IgG1, IgG2, and IgG3, but not IgG4, IgA, or IgE. IgM is the most efficient of the activating immunoglobulins because it has multiple binding sites; thus, it takes only one molecule attached to two adjacent antigenic determinants to initiate the cascade. Two IgG molecules must attach to antigen within 30 to 40 nm of each other before complement can bind; it may take at least 1,000 IgG molecules to ensure that there are two close enough to initiate such binding.[1,8] Some epitopes, notably the Rh group,

Connections

Jules Bordet

Jules Bordet was awarded the Nobel Prize in 1919 for his role in elucidating the nature of complement. Complement was originally recognized in the 1890s; Paul Ehrlich coined the term *complement* because the substance complements the action of antibody in destroying microorganisms.[1] Present as a substance in normal nonimmune serum, complement is part of the innate immune system. Complement is considered an acute phase reactant because levels rise during an infection. It also has a unique property of being easily inactivated (i.e., heat-labile) by heating serum to 56°C for 30 minutes. Refer to Chapter 3 for a further description of the innate immune system.

Table 7–1 Proteins of the Complement System

SERUM PROTEIN	MOLECULAR WEIGHT (KD)	CONCENTRATION (μG/ML)	FUNCTION
Classical Pathway			
C1q	410	150	Binds to Fc region of IgM and IgG
C1r	85	50	Activates C1s
C1s	85	50	Cleaves C4 and C2
C4	205	300–600	Part of C3 convertase (C4b)
C2	102	25	Binds to C4b—forms C3 convertase
C3	190	1,200	Key intermediate in all pathways
C5	190	80	Initiates membrane attack complex
C6	110	45	Binds to C5b in MAC
C7	100	90	Binds to C5bC6 in MAC
C8	150	55	Starts pore formation on membrane
C9	70	60	Polymerizes to cause cell lysis
Alternative Pathway			
Factor B	93	200	Binds to C3b to form C3 convertase
Factor D	24	2	Cleaves Factor B
Properdin	55	15–25	Stabilizes C3bBb–C3 convertase
MBL Pathway			
MBL	200–600	0.0002–10	Binds to mannose
MASP-1	93	1.5–12	Unknown
MASP-2	76	Unknown	Cleaves C4 and C2

Fc = Fragment crystallizable; Ig = immunoglobulin; MAC = membrane attack complex; MBL = mannose-binding lectin; MASP = MBL-associated serine protease.

are too far apart on the cell for this to occur; therefore, they are unable to fix complement. Within the IgG group, IgG3 is the most effective, followed by IgG1 and then IgG2.[8]

In addition to antibodies, a few substances can bind complement directly to initiate the classical cascade. These include C-reactive protein (CRP), several viruses, mycoplasmas, some protozoa, and certain gram-negative bacteria such as *Escherichia coli*.[8] However, most infectious agents can directly activate only the alternative or lectin pathways.

Complement activation can be divided into three main stages, each of which is dependent on the grouping of certain reactants as a unit. The first stage involves the **recognition unit,** which in the case of the classical pathway is C1. Once C1 is fixed, the next components activated are C4, C2, and C3, known collectively as the **activation unit** of the classical pathway (and the lectin pathway). C5 through C9 comprise the **membrane attack complex (MAC);** this last unit completes the lysis of foreign particles. Each of these is discussed in detail in the following sections. **Figure 7–1** depicts a simplified scheme of the entire pathway.

The Recognition Unit

The first complement component of the classical pathway to bind is C1, a molecular complex of 740,000 d. It consists of three subunits—C1q, C1r, and C1s—which require the presence of calcium to maintain structure.[1,8] The complex is made up of one C1q subunit and two each of the C1r and C1s subunits **(Fig. 7–2).** Although the C1q unit is the part that binds to antibody molecules, the C1r and C1s subunits generate enzyme activity to begin the cascade.

C1q has a molecular weight of 410,000 and is composed of six strands that form six globular heads with a collagen-like tail portion. This structure has been likened to a bouquet of tulips with six blossoms extending outward (see Fig. 7–2). As long as calcium is present in the serum, C1r and C1s remain associated with C1q.

C1q "recognizes" the fragment crystallizable (Fc) region of two adjacent antibody molecules, but at least two of the globular heads of C1q must be bound to initiate the classical pathway. C1r and C1s are serine protease proenzymes, also called zymogens. As binding of C1q occurs, both are converted into active enzymes. Autoactivation of C1r results from a conformational change that takes place as C1q is bound. Once activated, C1r cleaves a thioester bond on C1s which, in turn, activates it. Activated C1r is extremely specific because its only known substrate is C1s. Likewise, C1s has a limited specificity, with its only substrates being C4 and C2. Once C1s is activated the recognition stage ends.

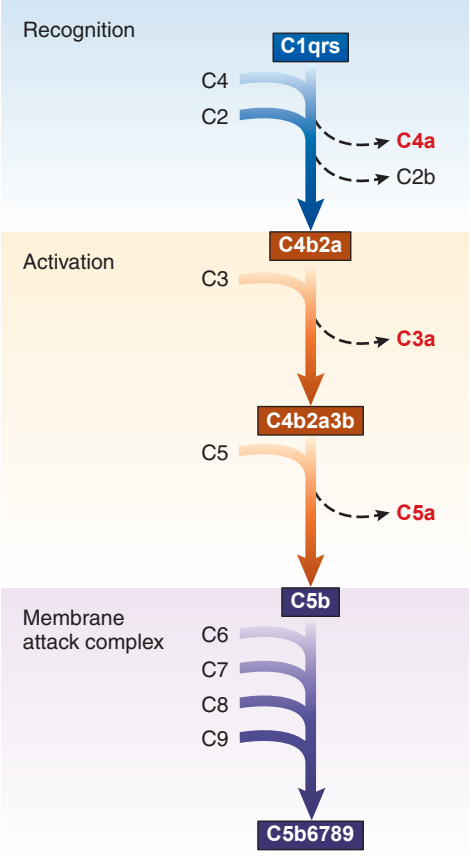


FIGURE 7–1 The classical complement cascade. C1qrs is the recognition unit that binds to the FC portion of two antibody molecules. C1s is activated and cleaves C4 and C2 to form C4b2a, also known as C3 convertase. C3 convertase cleaves C3 to form C4b2a3b, known as C5 convertase. The combination of C4b2a3b is the activation unit. C5 convertase cleaves C5. C5b attracts C6, C7, C8, and C9, which bind together, forming the membrane attack complex (MAC). C9 polymerizes to cause lysis of the target cell.

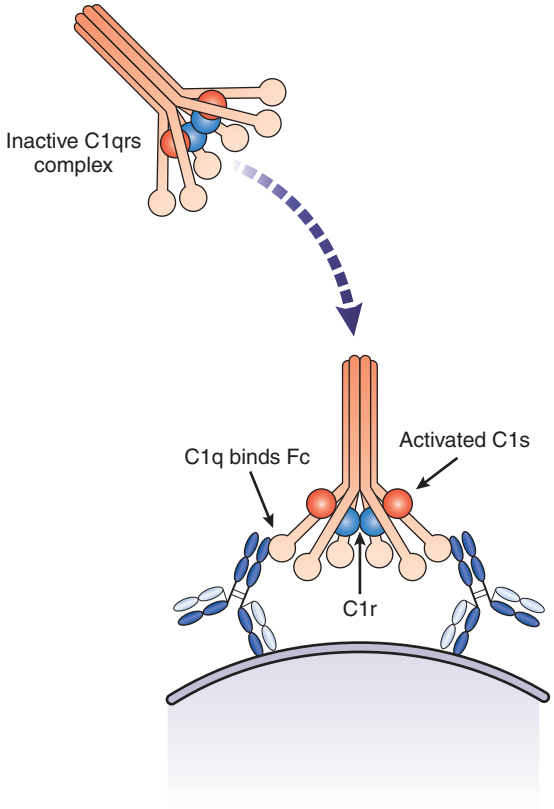


FIGURE 7–2 Structure of C1qrs. When two or more globular heads of C1q attach to bound immunoglobulin molecules, the collagen-like stalks change their configuration. The resulting shape change causes C1r to become a serine protease, which cleaves a small fragment of C1s, uncovering the C1s protease, whose only targets are C4 and C2.

The Activation Unit

Phase two, the formation of the activation unit, begins when C1s cleaves C4 and ends with the production of the enzyme C5 convertase **(Fig. 7–3).** C4 is the second most abundant complement protein, with a serum concentration of approximately 600 μg/mL.[8] C1s cleaves C4 to split off a 77-amino acid fragment called C4a. In the process, it opens a thioester-containing active site on the remaining part, C4b. C4b must bind to protein or carbohydrate within a few seconds or it will react with water molecules to form iC4b, which is rapidly degraded. Thus, C4b binds mainly to antigen in clusters that are within a 40-nm radius of C1. This represents the first amplification step in the cascade because for every C1 attached approximately 30 molecules of C4 are split and attached.[1]

C2 is the next component to be activated. Complement proteins were named as they were isolated before the sequence of activation was known—hence the irregularity in the numbering system. The C2 gene is closely associated with the gene for Factor B (alternative pathway) on chromosome 6 in the major histocompatibility complex (MHC). Each serves a similar purpose in its particular pathway.[8]

When combined with C4b in the presence of magnesium ions, C2 is cleaved by C1s to form C2a (which has a molecular weight of 70,000) and C2b (which has a molecular weight of 34,000) (see Fig. 7–3A). This is the only case for the designation "a" to be given to the cleavage piece with enzyme activity, though there are discussions to make the nomenclature of C2 match that of the other components of complement with the "a" fragment being the smaller fragment. The short life of these reactive species serves as a mechanism of control, keeping the reaction localized.

The combination of C4b and C2a is known as C3 convertase (see Fig. 7–3B). This is written as $\overline{\text{C4b2a}}$ to indicate that the complex is an active enzyme. This complex is not very stable. The half-life is estimated to be between 15 seconds and 3 minutes, so C3 must be bound quickly. If binding does occur, C3 is cleaved into two parts, C3a and C3b.

C3, the major and central constituent of the complement system, is present in the plasma at a concentration of 1 mg/mL to 1.5 mg/mL.[8] It serves as the pivotal point for all three pathways. The cleavage of C3 to C3b represents the most significant step in the entire process of complement activation.[9] The molecule has a molecular weight of 190,000 and consists of

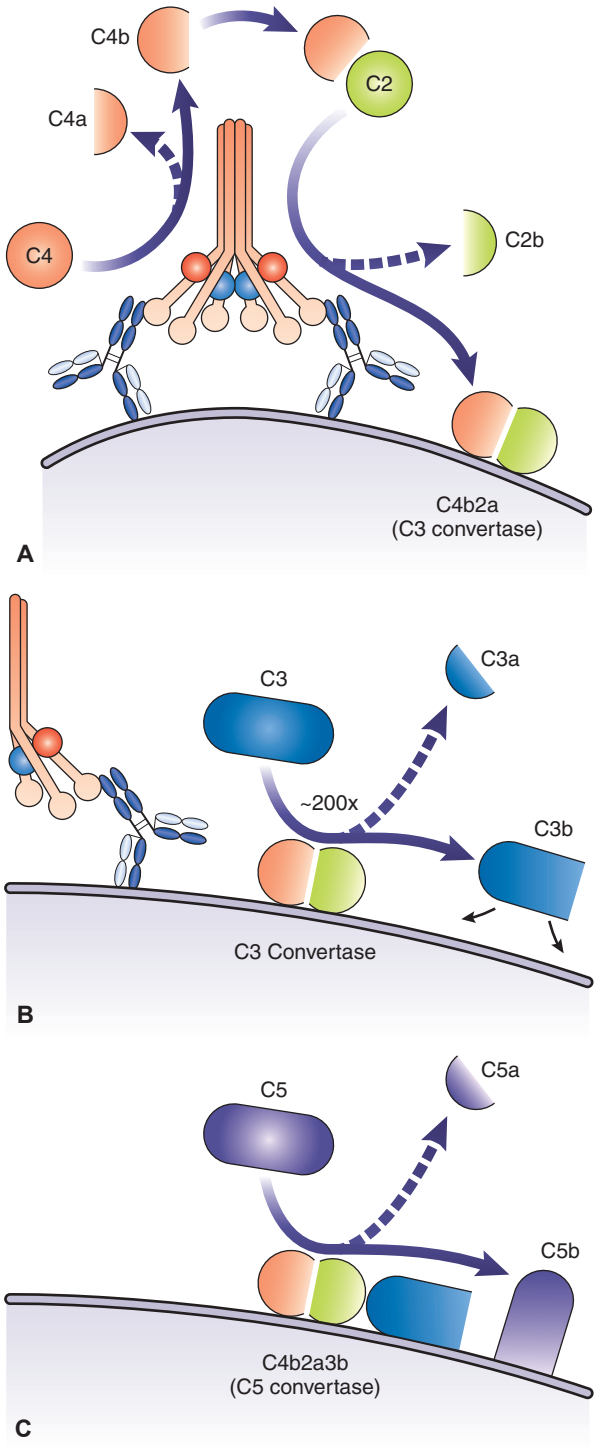


FIGURE 7–3 Formation of the activation unit. (A) Activated C1qrs cleaves C4 and C2, with the larger pieces, C4b and C2a, binding to the target cell surface and forming the enzyme C3 convertase. (B) Each C3 convertase cleaves ~200 C3 molecules into C3a and C3b. C3b is a powerful opsonin that binds to the target in many places. (C) Some C3b associates with C4b2a, forming C4b2a3b, also known as the C5 convertase. This convertase cleaves C5 into the anaphylatoxin C5a and C5b, which binds to the target cell.

two polypeptide chains, alpha (α) and beta (β). The α chain contains a highly reactive thioester group. When C3a is removed by cleavage of a single bond in the α chain, the thioester is exposed; the remaining piece, C3b, is then capable of binding to hydroxyl groups on carbohydrates and proteins in the immediate vicinity.[2,5,9] C3b is estimated to have a half-life of 60 microseconds if not bound to antigen. Therefore, only a small percentage of cleaved C3 molecules bind to antigen; most are hydrolyzed by water molecules and decay in the fluid phase.[8,10]

The cleavage of C3 represents a second and major amplification process because about 200 molecules are split for every molecule of $\overline{C4b2a}$.[10] In addition to being required for the formation of the MAC, C3b also serves as a powerful opsonin. Macrophages have specific receptors for it (discussed later in the chapter) and make a major contribution to the process of phagocytosis. A large number of molecules are needed for this to occur; hence, the need for amplification.

If C3b is bound within 40 nm of the $\overline{C4b2a}$, this creates a new enzyme known as C5 convertase. **Figure 7–3C** depicts this last step in the formation of the activation unit. The cleaving of C5 with deposition of C5b at another site on the cell membrane constitutes the beginning of the MAC.

The Membrane Attack Complex (MAC)

C5 consists of two polypeptide chains, α and β, which are linked by disulfide bonds to form a molecule with a molecular weight of about 190,000. C5 convertase, consisting of $\overline{C4b2b3b}$, splits off a 74-amino acid piece known as C5a that is released into circulation, whereas C5b attaches to the cell membrane, forming the beginning of the MAC. The splitting of C5 and the cleavage of C3 represent the most significant biological consequences of the complement system as explained in the section on biological manifestations of complement activation. However, C5b is extremely labile and rapidly inactivated unless binding to C6 occurs.[1]

Once C6 is bound to C5b, subsequent binding involves C7, C8, and C9. None of these proteins has enzymatic activity; they are all present in much smaller amounts in serum than the preceding components. C6 and C7 each have molecular weights of approximately 110,000 and have similar physical and chemical properties. C8 is made up of three dissimilar chains joined by disulfide bonds and has a total molecular weight of about 150,000.[6] C9 is a single polypeptide chain with a molecular weight of 70,000. The carboxy-terminal end is hydrophobic, whereas the amino-terminal end is hydrophilic. The hydrophobic part serves to anchor the MAC within the target membrane. Formation of the membrane attack unit is pictured in **Figure 7–4**. The complex of C5b-C6-C7-C8 and C9 is known as C5b-9 or MAC. If the complex is soluble in circulation, it is known as sC5b-9. Measurement of the level of sC5b-9 is an indicator of the amount of terminal pathway activation that is occurring. When formed, the MAC presents a pore of 70 to 100Å that allows ions to pass in and out of the membrane.[1,10] Destruction of target cells actually occurs through an influx of water and a corresponding loss of electrolytes. The presence of C9

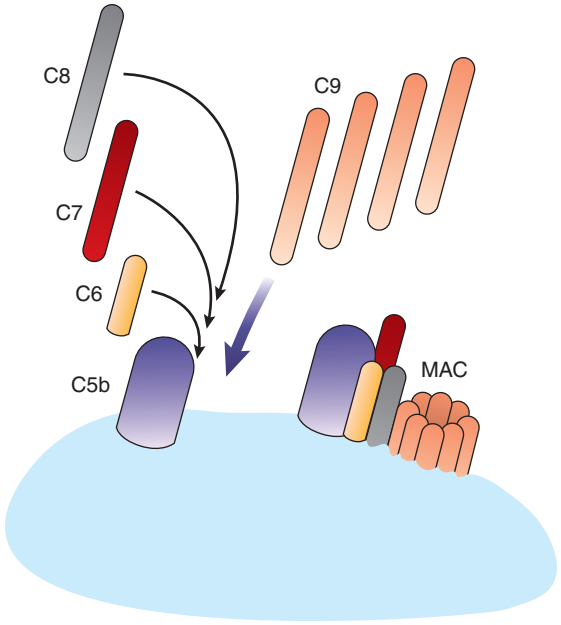


FIGURE 7–4 Formation of the membrane attack unit. C5b binds to the target cell, whereas C6 and C7 attach to it. C8 binds to these associated molecules and begins (along with C7) to penetrate the cell membrane. Multiple C9 molecules bind to C5b678 and polymerize to form a transmembrane channel, the membrane attack complex, which causes lysis of the cell.

greatly speeds this lysis. However, sufficient perturbation of the membrane can occur in the absence of C9 so that deficiencies in C9 appear largely benign.

The Lectin Pathway

The lectin pathway represents another means of activating complement. Instead of activation through antibody binding, the lectin pathway is activated by recognition of surface moieties that are found on pathogens.[11] This pathway provides an additional link between the innate and acquired immune response because it involves nonspecific recognition of carbohydrates that are common constituents of microbial cell walls and that are distinct from those found on human cell surfaces.[12,13] Although this pathway is the most recently described of the three activation pathways of complement, it is probably the most ancient. The lectin pathway molecules are structurally similar to those of the classical; the classical and lectin pathways even share the components C4 and C2. Once C4 and C2 are cleaved, the rest of the pathway is identical to the classical pathway. The role C1q serves in the classical pathway is filled by three classes of recognition molecules in the lectin pathway: lectins, ficolins, and CL-K1.[11] The structure of all three classes of recognition molecules is similar to that of C1q because they are all classed as collectins. One key lectin, called mannose-binding, or mannan-binding, lectin (MBL), binds to mannose or related sugars in a calcium-dependent manner to initiate this pathway.[14] These sugars are found in glycoproteins or carbohydrates of a wide variety of microorganisms such as bacteria, yeasts, viruses, and some parasites. MBL is considered an acute phase protein because it is produced in the liver and is normally present in the serum but increases during an initial inflammatory response.[13] The enzymatic role played by C1r and C1s in the classical pathway is played in the lectin pathway by serine proteases called MBL-associated serine proteases (MASPs). There are currently three MASPs identified, labeled MASP-1, MASP-2, and MASP-3. The lectin pathway plays an important role as a defense mechanism in infancy, during the interval between the loss of maternal antibody and the acquisition of a full-fledged antibody response to pathogens.[15]

The Alternative Pathway

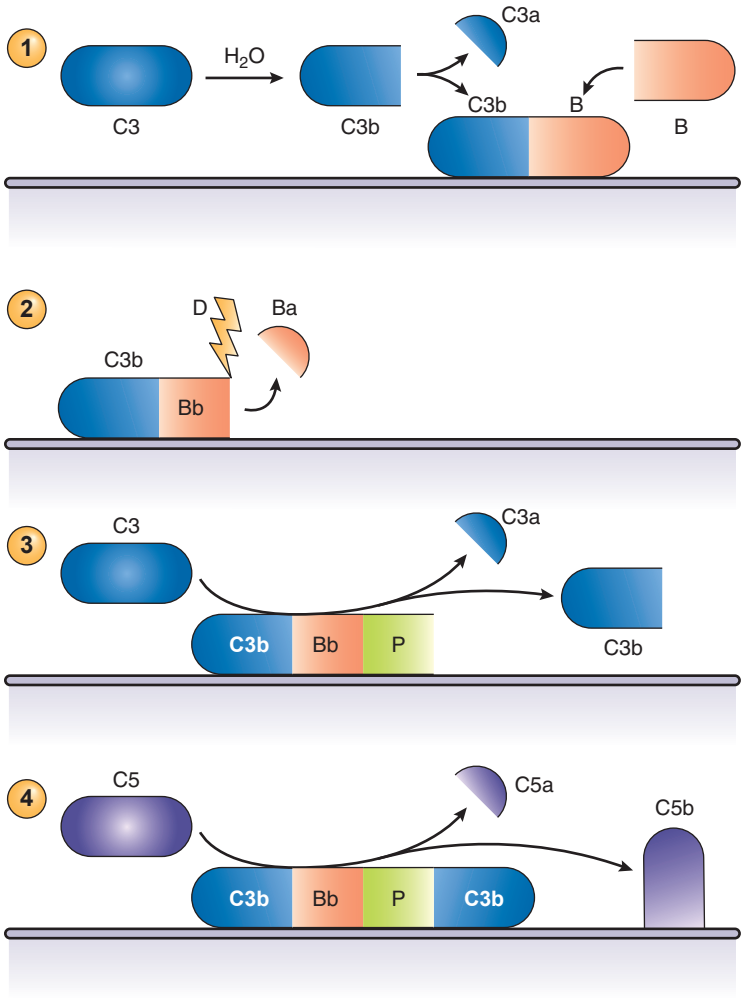
First described by Pillemer and his associates in the early 1950s, the alternative pathway was originally named for the protein **properdin,** a constituent of normal serum with a concentration of approximately 5 to 15 μg/mL.[5] Although the alternative pathway can be activated on its own, it appears that it functions mainly as an amplification loop for activation started from the classical or lectin pathways. Although properdin has been confirmed to bind and initiate activation, the primary function of properdin is to stabilize the C3 convertase formed from activation of other factors. In addition to properdin, the serum proteins Factor B and Factor D are unique to this pathway. C3 is a key component of this pathway as well as the two other pathways. The alternative pathway is summarized in **Figure 7–5.**

Triggering substances for the alternative pathway include bacterial cell walls, especially those containing lipopolysaccharide, fungal cell walls, yeast, viruses, virally infected cells, tumor cell lines, and some parasites, especially trypanosomes.[1] All of these can serve as sites for binding the complex $\overline{\text{C3bBb}}$, one of the end products of this pathway. The conversion of C3 is the first step in this pathway.

Native C3 is not stable in plasma. Water is able to hydrolyze a thioester bond, thus spontaneously activating a small number of these molecules.[16] C3b, sometimes called iC3, is formed by this spontaneous hydrolysis, from activation, or from the classical or lectin pathways. It acts as the seed of activation of the alternative pathway. The C3b binds to Factor B, which has a molecular weight of 93,000 and is fairly abundant in the serum, at a level of 200 μg/ mL.[8,17] Once bound to C3b, Factor B can be cleaved by Factor D. The role of Factor B is thus analogous to that of C2 in the classical pathway because it forms an integral part of a C3 convertase.

Factor D is a plasma protein that goes through a conformational change when it binds to Factor B.[17,18] It is a serine protease with a molecular weight of 24,000; its only substrate is bound Factor B. The concentration of Factor D in the plasma is the lowest of all the complement proteins, approximately 2 μg/mL.[10] It cleaves Factor B into two pieces: Ba (with a molecular weight of 33,000) and Bb (with a molecular weight of approximately 60,000). Bb remains attached to C3b, forming the initial C3 convertase of the alternative pathway. Bb is rapidly inactivated unless it becomes bound to a site on one of the triggering cellular antigens.

As the alternative pathway convertase, $\overline{\text{C3bBb}}$ is then capable of cleaving additional C3 into C3a and C3b. Some C3b attaches



1. C3 is hydrolyzed by water to produce C3b, which binds Factor B and together they attach to target cell surface.
2. B is cleaved by Factor D into the fragments Ba and Bb. Bb combines with C3b to form C3bBb, an enzyme with C3 convertase activity.
3. More C3 is cleaved, forming more C3bBb. This enzyme is stabilized by properdin, and it continues to cleave additional C3.
4. If a molecule of C3 remains attached to the C3bBbP enzyme, the convertase now has the capability to cleave C5. The C5 convertase thus consists of C3bBbP3b. After C5 is cleaved, the pathway is identical to the classical pathway.

FIGURE 7–5 The alternative pathway.

to cellular surfaces and acts as a binding site for more Factor B, resulting in an amplification loop; activation initiated by the classical or lectin pathways is amplified to levels of biological consequence. All C3 present in plasma would be rapidly converted by this method were it not for the fact that the enzyme C3bBb is extremely unstable unless properdin binds to the complex. Binding of properdin increases the half-life of C3bBb from 90 seconds to several minutes.[8,17] In this manner, optimal rates of alternative pathway activation are achieved.[14]

C3bBb can also cleave C5, but it is much more efficient at cleaving C3.[19] If, however, some of the C3b produced remains bound to the C3 convertase, the enzyme is altered to form C3bBb3bP, which has a high affinity for C5 and exhibits C5 convertase activity.[16,19] C5 is cleaved to produce C5b, the first part of the membrane attack unit. From this point on the alternative, lectin, and classical pathways are identical. **Figure 7–6** shows the convergence of all three pathways.

System Controls

Activation of complement could cause tissue damage and have devastating systemic effects if it were allowed to proceed uncontrolled. To ensure that infectious agents and not self-antigens are destroyed and that the reaction remains localized, several plasma proteins act as system regulators. In addition, there are specific receptors on certain cells that also exert a controlling influence on the activation process. In fact, approximately one-half of the complement components serve as controls for critical steps in the activation process. Because activation of C3 is the pivotal step in all pathways, the majority of the control proteins are aimed at halting accumulation of C3b. However, there are controls at all crucial steps along the way. Regulators will be discussed according to their order of appearance in each of the three pathways. A brief summary of these is found in **Table 7–2.**

Regulation of the Classical and Lectin Pathways

C1 inhibitor (C1-INH) inhibits activation at the first stages of both the classical and lectin pathways. Its main role is to inactivate C1 by binding to the active sites of C1r and C1s. Clr and Cls become instantly and irreversibly dissociated from C1q.[6,10] C1q remains bound to antibody, but all enzymatic activity ceases. C1-INH[10] also inactivates MASP-2 binding to the MBL-MASP complex, thus halting the lectin pathway.[12,18] C1-INH is a

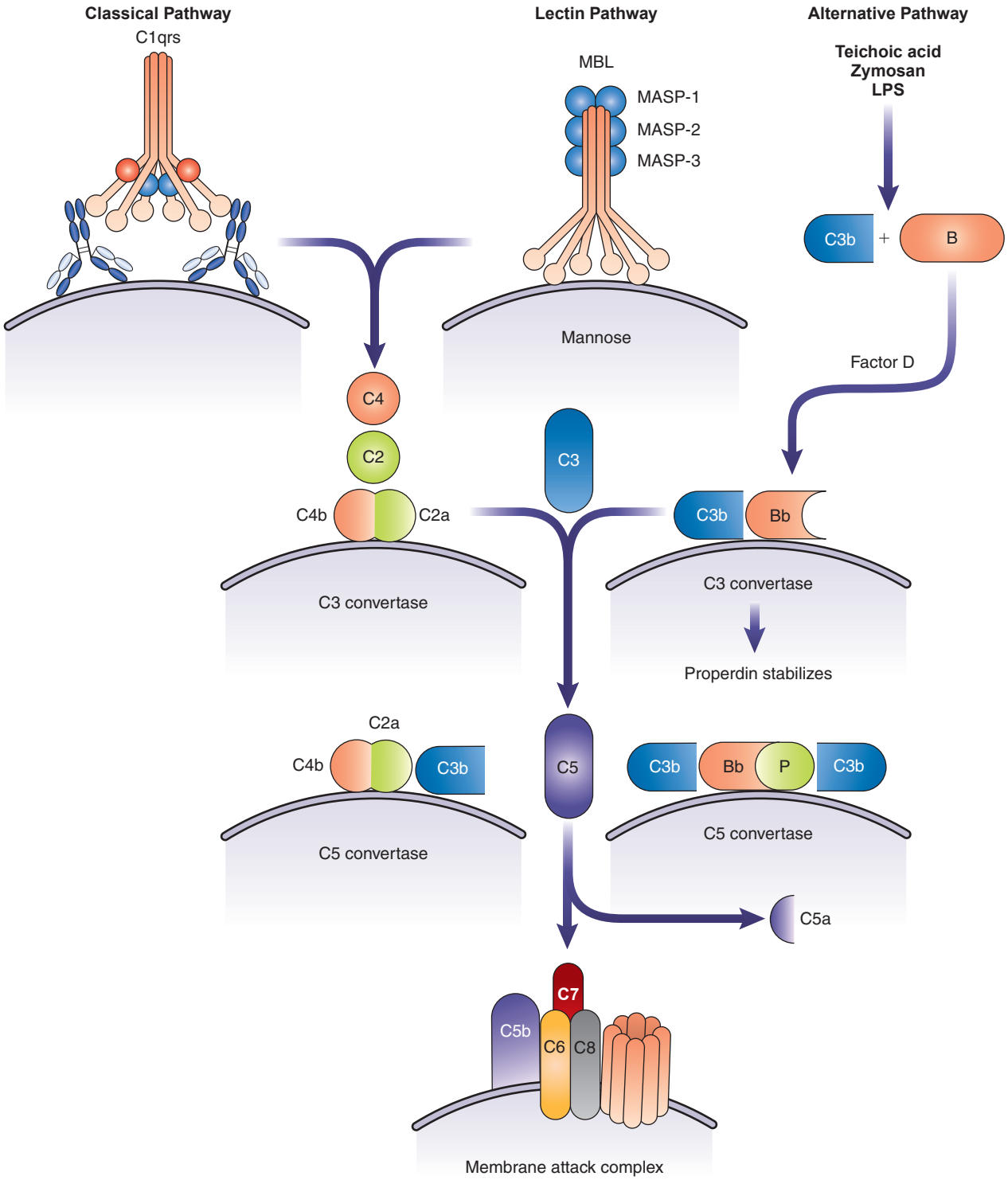


FIGURE 7–6 Convergence of the classical, alternative, and lectin pathways. The binding of C1qrs to two antibody molecules activates the classical pathway, whereas the alternative pathway is started by hydrolysis of C3. The lectin pathway is triggered by binding of MBP to mannose on bacterial cell walls. MASP-1, MASP-2, and MASP-3 bind to form an activated C1-like complex. MASP-2 cleaves C2 and C4 and proceeds like the classical pathway. Factor B and Factor D operate in the alternative pathway. Although C3 convertase is formed differently in each pathway, C3 is a key component in each one. The C5 convertase in the alternative pathway consists of C3bBb3bP. In the classical and lectin pathways, C5 convertase is made up of C4b2a3b. After C5 is cleaved, the pathway is common to all.

Table 7–2 Plasma Complement Regulators

SERUM PROTEIN	MOLECULAR WEIGHT (KD)	CONCENTRATION (MG/ML)	FUNCTION
C1 inhibitor (C1-INH)	105	240	Dissociates C1r and C1s from C1q
Factor I	88	35	Cleaves C3b and C4b
Factor H	150	300–450	Cofactor with I to inactivate C3b; prevents binding of B to C3b
C4-binding protein (C4BP)	520	250	Acts as a cofactor with I to inactivate C4b
S protein (vitronectin)	84	500	Prevents attachment of the C5b67 complex to cell membranes

glycoprotein with a molecular weight of 105,000. Like most of the other complement proteins, it is mainly synthesized in the liver; however, monocytes also may be involved to some extent in its manufacture.

Further formation of C3 convertase in the classical and lectin pathways is inhibited by four main regulators: soluble **C4-binding protein (C4BP)** and three cell-bound receptors, **complement receptor type 1 (CR1), membrane cofactor protein (MCP),** and **decay-accelerating factor (DAF).**[1,18] All of these act in concert with **Factor I,** a serine protease that inactivates C3b and C4b when bound to one of these regulators. C4BP is abundant in the plasma and has a molecular weight of about 520,000. It is capable of combining with either fluid-phase or bound C4b; therefore, C4b cannot bind to C2 and is made available for degradation by Factor I. If C4BP attaches to cell-bound C4b, it can dissociate it from C4b2a complexes, causing the cessation of the classical pathway.

CR1, also known as CD35, is a large polymorphic glycoprotein with a molecular weight between 165,000 and 280,000.[7] It is found mainly on peripheral blood cells, including neutrophils, monocytes, macrophages, erythrocytes, eosinophils, B lymphocytes, some T lymphocytes, and follicular dendritic cells.[3] It binds C3b and C4b but has the greatest affinity for C3b.[6,20] Once bound to CR1, both C4b and C3b can then be degraded by Factor I.

A main function of CR1 is as a receptor on platelets and red blood cells (RBCs), which helps to mediate transport of C3b-coated immune complexes to the liver and spleen.[7,20] It is there that fixed tissue macrophages strip the immune complexes from the RBCs, process the complexes, and return the RBCs intact to circulation. The ability of cells to bind complement-coated particles is referred to as **immune adherence.**

MCP, or CD46, has a molecular weight between 50,000 and 70,000 and is found on virtually all epithelial and endothelial cells except erythrocytes.[7] MCP is the most efficient cofactor for Factor I-mediated cleavage of C3b. It can serve as a cofactor for cleavage of C4b, but it is not as effective as C4BP. MCP also helps to control the alternative pathway because binding of Factor B to C3b is inhibited.

DAF or CD55, a 70,000 d membrane glycoprotein, is the third main receptor and has a wide tissue distribution. It is found on peripheral blood cells, on endothelial cells and fibroblasts, and on numerous types of epithelial cells.[6–8] DAF is capable of dissociating both classical and alternative pathway C3 convertases. It can bind to both C3b and C4b in a manner similar to CR1.[4] It does not prevent initial binding of either C2 or Factor B to the cell but can rapidly dissociate both from their binding sites, thus preventing the assembly of an active C3 convertase.

The carboxy-terminal portion of DAF is covalently attached to a glycophospholipid anchor that is inserted into the outer layer of the membrane lipid bilayer. This arrangement allows DAF mobility within the membrane so it can reach C3 convertase sites that are not immediately adjacent to it **(Fig. 7–7).**[2] The presence of DAF on host cells protects them from **bystander lysis.** It is one of the main mechanisms used in discrimination of self from nonself because foreign cells do not possess this substance. However, it does not permanently modify C3b or C4b; they are capable of re-forming elsewhere as active convertases.

Regulation of the Alternative Pathway

The principal soluble regulator of the alternative pathway is **Factor H,** which has a molecular weight of 160,000.[18] It acts by binding to C3b, preventing the binding of Factor B. C3b in the fluid phase has a hundredfold greater affinity for Factor H than for Factor B, but on cell surfaces C3b preferentially binds to Factor B. Factor H also accelerates the dissociation of the C3bBb complex on cell surfaces. When Factor H binds to C3bBb, Bb becomes displaced. In this manner, C3 convertase activity is curtailed in plasma and on cell surfaces.

Additionally, Factor H acts as a cofactor that allows Factor I to break down C3b. It appears that only those molecules with tightly bound Factor H acquire high-affinity binding sites for Factor I.[21] When Factor I binds, a conformational change takes place that allows it to cleave C3b.[21] On cellular surfaces, C3b is cleaved into C3f, which is released into the plasma, and iC3b, which remains attached but is no longer an active enzyme. iC3b is further broken down to C3c and C3dg by Factor I in conjunction with another cofactor: the CR1 receptor **(Fig. 7–8).**[10] With this key role in complement regulation, it should not be surprising that Factor H has recently been shown to play a role in a variety of disorders (discussed in the text that follows).

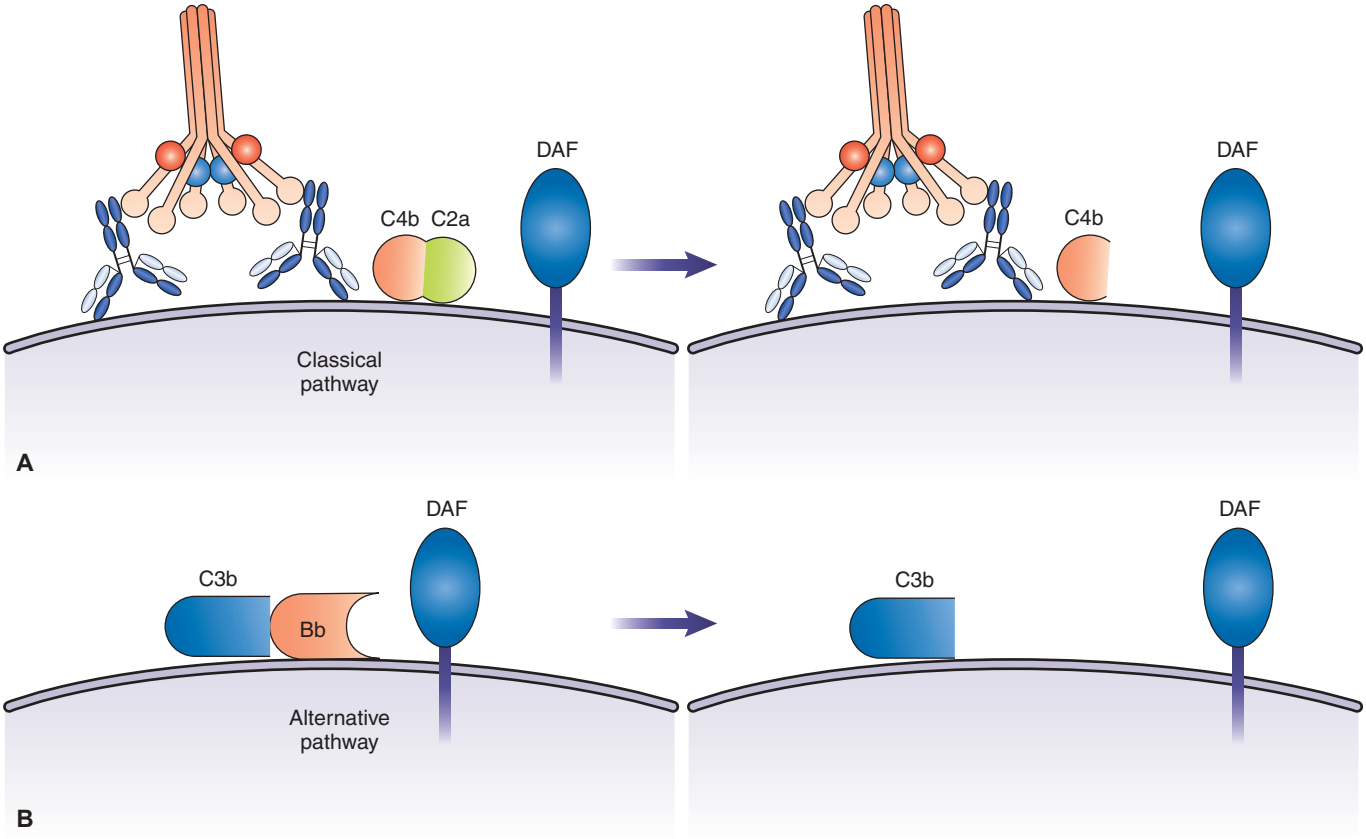


FIGURE 7–7 A. In the classical pathway, DAF dissociates C2a from C4b. B. Inhibitory effects of DAF. In the alternative pathway, when C3b binds to cell surfaces that have DAF present, DAF helps dissociate Bb from binding to C3b.

Regulation of Terminal Components

S protein is a soluble control protein that acts at a deeper level of complement activation. Also known as *vitronectin,* S protein interacts with the C5b-7 complex as it forms in the fluid phase and prevents it from binding to cell membranes.[8] Binding of C8 and C9 still proceeds, but polymerization of C9 does not occur; therefore, the complex is unable to insert itself into the cell membrane or to produce lysis.[4]

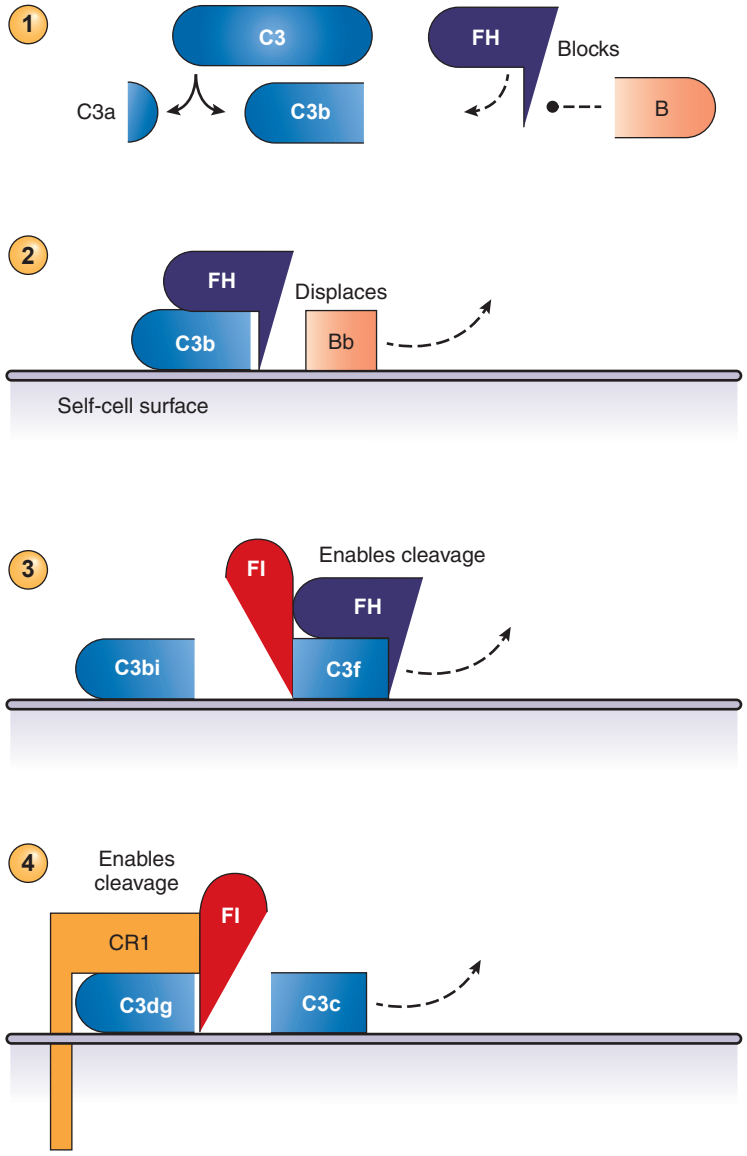
A receptor, known by various terms, including membrane inhibitor of reactive lysis (MIRL) or CD59, also acts to block formation of the MAC. MIRL is widely distributed on the cell membranes of all circulating blood cells, including RBCs, and on endothelial, epithelial, and many other types of cells.[8,10] **Table 7–3** lists the receptors and indicates the types of cells on which they are found.

Complement Receptors and Their Biological Roles

Some complement receptors found on host cells amplify and enhance the immune response by augmenting phagocytosis and stimulating accessory cells rather than acting as regulators (see Table 7–3). CR1 has been discussed in the previous section. A second receptor, CR2 (or CD21), is found mainly on B lymphocytes and follicular dendritic cells.[22] Ligands for CR2 include degradation products of C3b, such as C3dg, C3d, and iC3b. In addition, the Epstein-Barr virus gains entry to B cells by binding to this receptor. CR2 is present only on mature B cells and is lost when conversion to plasma cells occurs. CR2 plays an important role as part of the B-cell co-receptor for antigen. Acting in concert with CD19, it binds complement-coated antigen and cross-links it to membrane immunoglobulin to activate B cells. In this manner, immune complexes are more effective at enhancing B-cell differentiation and production of memory cells than is antigen by itself.[2,22]

Another receptor, CR3 (CD11b/CD18), found on monocytes, macrophages, neutrophils, and natural killer (NK) cells, specifically binds particles opsonized with iC3b, a C3b degradation product.[7] It does this in a calcium-dependent manner. The CR3 receptor plays a key role in mediating phagocytosis of particles coated with these complement fragments **(Fig. 7–9).** These proteins trigger surface adhesion and increased activity of phagocytic cells.[2] Patients whose white blood cells (WBCs) lack these receptors fail to exhibit functions such as chemotaxis, surface adherence, and aggregation. Deficiencies in phagocytosis are also noted. These individuals have an impaired capacity to bind iC3b-coated particles and are subject to recurrent infections.

The CR4 (CD11c/CD18) receptor is very similar to CR3 in that it also binds iC3b fragments in a calcium-dependent fashion. CR4 proteins are found on neutrophils, monocytes, tissue macrophages, activated T cells, dendritic cells, NK cells, and



1. Factor H (FH) competes with factor B (B) for binding to spontaneously (hydrolytically) activated C3b.
2. Factor H dissociates any C3bBb complexes that form on self-cell surfaces.
3. Factor H is a cofactor with factor I (FI), enabling cleavage of C3b. The resuting C3bi loses enzymatic activity, but is still an opsonin.
4. CR1 is a cofactor with FI, enabling cleavage of C3bi. The resulting C3dg is an opsonin and a cofactor in B cell stimulation.

FIGURE 7–8 Complement controls. CR1 receptor acts as a cofactor in the inactivation of C3b. Factor I cleaves C3b to form C3dg and C3c. C3dg is not an effective opsonin and is not capable of further participation in the complement cascade.

Table 7–3 Receptors on Cell Membranes for Complement Components

RECEPTOR	LIGAND	CELL TYPE	FUNCTION
CR1 (CD35)	C3b, iC3b, C4b	RBCs, neutrophils, monocytes, macrophages, eosinophils, B and T cells, follicular dendritic cells	Cofactor for Factor I; mediates transport of immune complexes
CR2 (CD21)	C3dg, C3d, iC3b	B cells, follicular dendritic cells, epithelial cells	B-cell co-receptor for antigen with CD19
CR3 (CD11b/CD18)	iC3b, C3d, C3b	Monocytes, macrophages, neutrophils, NK cells	Adhesion and increased activity of phagocytic cells
CR4 (CD11c/CD18)	iC3b, C3b	Monocytes, macrophages, neutrophils, NK cells, activated T and B cells, dendritic cells	Adhesion and increased activity of phagocytic cells

Continued

Table 7–3 Receptors on Cell Membranes for Complement Components—cont'd

RECEPTOR	LIGAND	CELL TYPE	FUNCTION
DAF (CD55)	C3b, C4b	RBCs, neutrophils, platelets, monocytes, endothelial cells, fibroblasts, T cells, B cells, epithelial cells	Dissociates C2b or Bb from binding sites, thus preventing formation of C3 convertase
MIRL (CD59)	C8	RBCs, neutrophils, platelets, monocytes, endothelial cells, epithelial cells	Prevents insertion of C9 into cell membrane
MCP (CD46)	C3b, C4b	Neutrophils, monocytes, macrophages, platelets, T cells, B cells, endothelial cells	Cofactor for Factor I cleavage of C3b and C4b

RBC = red blood cell; NK = natural killer.

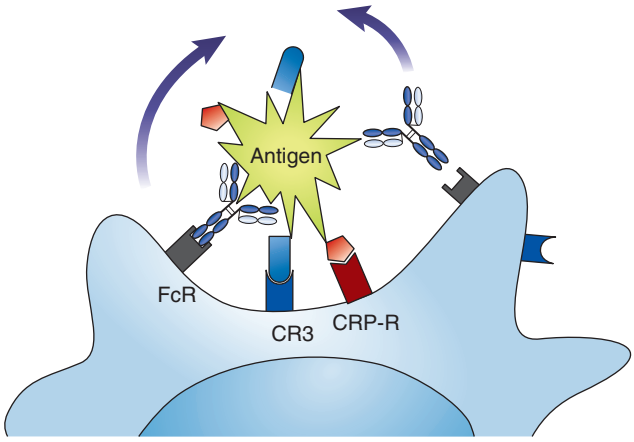


FIGURE 7–9 Role of C3b in opsonization. C3b, antibody, and C-reactive protein are all important opsonins that coat antigens and accelerate phagocytosis. Receptors for C3b (CR3), antibody (FcR), and C-reactive protein (CRP-R) on the phagocytic cell membrane bind the opsonins and pull the membrane around to envelop the antigen.

activated B cells.[2,7] Neutrophils and monocytes, however, possess smaller amounts of CR4 than of CR3. Their function appears to be similar to that of CR3 and they may assist neutrophil adhesion to the endothelium during inflammation.

Receptors specific for C1q are found on neutrophils, monocytes, macrophages, B cells, platelets, and endothelial cells.[7,23] These receptors, known as collectin receptors, bind the collagen portion of C1q and generally enhance the binding of C1q to FC receptors. Interacting only with bound C1q, the receptors appear to increase phagocytic cells' uptake of immune complexes opsonized with C1q. On neutrophils, they may also act to enhance the respiratory burst triggered by IgG binding to FC receptors.

Biological Manifestations of Complement Activation

Activation of complement is a very effective means of amplifying the inflammatory response to destroy and clear foreign antigens. The cycle does not always have to proceed to lysis for this to be accomplished; hence, some of the initiating proteins are much more plentiful than proteins that form the MAC. Complement proteins also serve as a means of linking innate and adaptive immunity. They act as opsonins to facilitate recognition and subsequent destruction by phagocytic cells; in addition, they play a major role in the uptake and presentation of antigens so a specific immune response can occur. They also facilitate B-cell activation. Recent work demonstrates that complement is necessary for maintaining immunologic memory.[24] Effector molecules generated earlier in the cascade play a major role in all these areas. Such molecules can be classified into three main categories: anaphylatoxins, chemotaxins, and opsonins.

An **anaphylatoxin** is a small peptide that causes increased vascular permeability, contraction of smooth muscle, and release of histamine from basophils and mast cells. Proteins that play such a part are C3a and C5a. Both of these have molecular weights between 9000 and 11,000 and are formed as cleavage products from larger complement components. Of these molecules, C5a is the most potent; it is at least 200 times more powerful than C3a.[25]

C3a and C5a attach to specific receptors on neutrophils, basophils, mast cells, eosinophils, smooth muscle cells, and vascular endothelium.[24,25] C3a attaches to the C3a receptor (C3aR), whereas C5a attaches to the C5a receptor (C5aR). When binding occurs on basophils and mast cells, histamine is released, increasing vascular permeability and causing contraction of smooth muscles. C5a causes neutrophils to release hydrolytic enzymes, oxygen radicals, and prostaglandins, which aid in the destruction of foreign antigens.[25]

C5a also serves as a chemotaxin for neutrophils, basophils, eosinophils, mast cells, monocytes, and dendritic cells. In this manner, these cells are directed to the source of antigen concentration. Because of increased vascular permeability, neutrophils migrate from blood vessels to the tissues and tend to aggregate.

Binding of C5a to monocytes causes them to undergo an oxidative burst that includes increased production of hydrolytic enzymes, neutrophil chemotactic factor, platelet-activating factors, interleukin-1 (IL-1), and toxic oxygen metabolites.[13,19] IL-1 is a protein that enhances T-cell activation. The activation may produce fever and lead to an increase in acute-phase reactants, both of which are characteristic of an inflammatory response.

C3a and C5a are rapidly inactivated by an enzyme in the plasma called carboxypeptidase N to localize and control their

effects. C3a is cleaved in seconds, whereas conversion of C5a occurs more slowly.

The last major effect of complement-derived peptides is opsonization. C4b, C3b, iC3b, and C3dg, which accumulate on cell membranes as complement activation proceeds, bind to specific receptors on erythrocytes, neutrophils, monocytes, and macrophages as previously discussed. This binding facilitates phagocytosis and clearance of foreign substances or cellular debris, which is one of the key functions of the complement system. In addition, attachment of C3 products to an antigen has been found to enhance the B-cell response.

Complement and Disease States

Although complement acts as a powerful weapon to combat infection by amplifying phagocytosis, in some cases it can actually contribute to tissue damage or death. Complement can be harmful if

- It is activated systemically on a large scale as in gram-negative septicemia
- It is activated by tissue necrosis such as myocardial infarction
- Lysis of red blood cells occurs

In the case of septicemia caused by a gram-negative organism, large quantities of C3a and C5a are generated, leading to neutrophil aggregation and clotting. Damage to the tiny pulmonary capillaries and interstitial pulmonary edema may result.[2]

Tissue injury following obstruction of the blood supply, such as occurs in a myocardial infarction or heart attack, can cause complement activation and deposition of MACs on cell surfaces. Receptors for C3a and C5a have been found in coronary plaques, indicating that complement components may increase the damage to heart tissue.[25,26]

Lysis may be another end result of complement activation. Hemolytic diseases such as cold autoimmune hemolytic anemia are characterized by the presence of an autoantibody that binds at low temperatures. When these cells warm up, complement fixation results in lysis. (See Chapter 15 for a more complete discussion of complement-mediated autoimmune diseases.)

Complement Deficiencies

Major Pathway Components

Although excess activation of the complement system can result in disease states, lack of individual components also has a deleterious effect. Hereditary deficiency of any complement protein, with the exception of C9, usually manifests itself in increased susceptibility to infection and delayed clearance of immune complexes. Most of these conditions are inherited on an autosomal recessive gene and are quite rare, occurring in 0.03% of the general population.[26] A lack of C2, the most common deficiency, is found in 1 in 20,000 individuals.[4,27–29] Recent evidence indicates that atherosclerosis may be related to a C2 deficiency.[28] C2-deficient individuals may also be more prone to recurrent streptococcal and staphylococcal infections.[30] Because the Factor B locus is nearby, C2-deficient persons are often reported to have decreases in Factor B also. Other types of complement deficiencies are less common.

A second deficiency that occurs with some frequency is that of MBL. Deficiencies and polymorphisms in MBL occur in about 30% of the population. The health consequences of such variation in MBL levels remain unclear, however. Lack of MBL has been associated with pneumonia, sepsis, and meningococcal disease in infants.[13,15,31,32] Low MBL has also been associated with the risk of some cancers, infection during chemotherapy, and certain autoimmune disorders such as systemic lupus erythematosus (SLE), but these connections are not yet well defined.[33]

The most serious deficiency is that of C3 because it is the key mediator in all pathways. C3 deficiencies are, however, extremely rare.[28] Individuals with a C3 deficiency are prone to developing severe, recurrent life-threatening infections with encapsulated bacteria such as *Streptococcus pneumoniae* and may also be subject to immune complex diseases.[16] Such complexes can lodge in the kidney and result in glomerulonephritis.[26,34]

It appears that a deficiency of any of the terminal components of the complement cascade (C5–C8) causes increased susceptibility to systemic *Neisseria* infections, including meningococcal meningitis and disseminated gonorrheal disease.[10,28] **Table 7–4** lists the complement components and the disease states associated with the absence of each individual factor.

Table 7–4 Deficiencies of Complement Components

DEFICIENT COMPONENT	ASSOCIATED DISEASE
C1 (q, r, or s)	Lupuslike syndrome; recurrent infections
C2	Lupuslike syndrome; recurrent infections; atherosclerosis
C3	Severe recurrent infections; glomerulonephritis
C4	Lupuslike syndrome
C5–C8	*Neisseria* infections
C9	No known disease association
C1-INH	Hereditary angioedema
DAF	Paroxysmal nocturnal hemoglobinuria
MIRL	Paroxysmal nocturnal hemoglobinuria
Factor H or Factor I	Recurrent pyogenic infections
MBL	Pneumococcal diseases, sepsis, *Neisseria* infections
Properdin	*Neisseria* infections
MASP-2	Pneumococcal diseases

C1-INH = C1 inhibitor; DAF = decay-accelerating factor; MASP-2 = mannose-associated serine protease; MBL = mannose-binding lectin; MIRL = membrane inhibitor of reactive lysis.

Regulatory Factor Components

A prime example of a disease caused by a missing or defective regulatory component is **paroxysmal nocturnal hemoglobinuria (PNH).** Individuals with this disease have RBCs that are deficient in DAF. Hence, the RBCs are subject to lysis by means of the bystander effect once the complement system has been triggered. These individuals appear to have a deficiency in the glycophospholipid anchor of the DAF molecule that prevents its insertion into the cell membrane.[10,22] When C3b is deposited on erythrocytes through activation of either pathway, the result is complement-mediated intravascular and extravascular hemolysis, resulting in a chronic hemolytic anemia.

Some studies indicate that a DAF deficiency is associated with a lack of CD59 (MIRL) and both are implicated in PNH.[32] CD59 has the same glycophospholipid anchor found in DAF; therefore, the gene deficiency affects both molecules. As mentioned previously, CD59 prevents insertion of C9 into the cell membrane by binding to the C5b-8 complex, inhibiting formation of transmembrane channels.[25] Both DAF and CD59 are important in protecting RBCs against bystander lysis.

Another complement deficiency disorder that has recently received considerable attention is **hereditary angioedema (HAE).** HAE involves recurrent attacks of swelling that affect the extremities, the skin, the gastrointestinal tract, and other mucosal surfaces. This disease is caused by a deficiency or lack of C1-INH, which occurs with a population frequency of 2 in 10,000.[32] Although C1-INH was named for its role in controlling complement, it is the function of C1-INH in controlling the contact pathway of the coagulation system that is critical in this disease. C1-INH is a serpin (serine protease inhibitor) that controls many of the serine proteases on contact. The lack of C1-INH results in localized swelling that can be either subcutaneous or found within the bowel or upper-respiratory tract.[35] Normally, this spontaneously subsides in 48 to 72 hours, but if the edema occurs in the area of the oropharynx, life-threatening upper-airway obstruction may develop.[26] These attacks can be quite debilitating even when they are not life threatening, so there is a need for proper diagnosis for these patients.

HAE is separated into two types, type I and type II. Type I is characterized by a decrease in the C1-INH protein; type II has normal levels of C1-INH, but the function is decreased. The genetic cause of either type is an autosomal dominant gene that codes for either a dysfunctional or an inactive protein. In addition to the hereditary forms of the disorder, there are acquired forms that result from either consumption of C1-INH or from autoantibodies blocking the function of C1-INH. To differentiate the acquired and hereditary forms, measurement of C1q can be helpful; C1q will be low in the acquired forms, but not in the hereditary forms. Measurement of C4 can also be a very helpful screen for HAE, particularly during an attack, because patients who do not exhibit a drop in C4 at that time are rare.[4,35]

In addition to these well-described diseases of complement, there are a growing number of disorders that are being connected to deficiencies, polymorphisms, or autoantibodies involving one or multiple complement components. Key among these is a set of rare kidney disorders associated with complement. **Hemolytic uremic syndrome (HUS)** is the most common cause of renal failure in children and is characterized by hemolytic anemia, low platelet count, and acute renal failure.[36] The primary cause of HUS is a Shiga toxin related to infection that is associated with acute diarrhea. The atypical form of HUS (aHUS) occurs because of complement dysregulation caused by genetic polymorphisms. The atypical HUS has a less acute onset and may not be associated with diarrhea; otherwise, the clinical presentation is similar. The genetic mutations associated with aHUS include those of Factor H, MCP, Factor I, Factor B, and thrombomodulin, as well as autoantibodies to Factor H and Factor I.[36]

Complement has also been implicated in **C3 glomerulopathies (C3G),** which are diseases involving the glomeruli of the kidneys. Recently several forms of rare glomerulonephritis (GN) were reclassified based on immunofluorescence and the presence or absence of C3 and immunoglobulins. For the C3 glomerulopathies, it is only C3 that is found in the deposits on the kidney. Analysis of these patients has shown that 71% to 100% of these patients have mutation in a complement protein, specifically C3, Factor B, Factor H, or Factor I. Other patients have an acquired autoantibody. These autoantibodies are known as C3 nephritic factors (C3NeF).

A C3NeF is an antibody that binds the C3-convertase from the alternative pathway, C3bBb, holding it together and making it impervious to the normal control mechanisms. In this way, a C3NeF leads to uncontrolled cleavage of C3 with concomitant uncontrolled deposition of C3 on the kidneys. C3G caused by C3NeF is clinically indistinguishable from the hereditary form of the disorder.[37] It is only with laboratory measurement of the presence or absence of a C3NeF that the nature of the disorder can be differentiated. In addition, an investigation of a possible complement deficiency can be complicated by depletion of complement components because of consumption through activation. For both the autoantibodies and activation-related consumption, laboratory testing is the key way to tell the acquired forms from the hereditary forms of complement disorders.

Laboratory Detection of Complement Abnormalities

Determining the levels of complement components can be useful in diagnosing disease. Hereditary deficiencies can be identified and much can be learned about inflammatory or autoimmune states by following the consumption of complement proteins. Techniques to determine complement abnormalities generally fall into two categories: (1) measurement of components as antigens in serum and (2) measurement of functional activity.[8] Many assays that are unavailable in routine clinical laboratories are available in specialized laboratories. Some of the more common assays will be discussed in the text that follows.

Immunologic Assays of Individual Components

The methods most frequently used to measure individual components include radial immunodiffusion (RID) and nephelometry.[26,34] C3 and C4 levels are routinely measured in most clinical laboratories by nephelometry or by turbidity measurements that are often automated. Both of these types of measurements and the methods used for the other components rely on the precipitation of antigen (the complement component being measured) and antibody. Nephelometry measures concentration according to the amount of light scattered by a solution containing a reagent antibody and a measured patient sample (refer to Chapter 10 for more details on nephelometry). Generally, the more antigen–antibody complexes that are present, the more a beam of light will scatter as it passes through the solution. Such systems have a high degree of accuracy; results are available quickly and processing is easy because of the use of automation. However, they involve expensive equipment.

Components for which there are standardized reagents include C1q, C4, C3, C5, Factor B, Factor H, and Factor I. RID uses agarose gel into which specific antibody is incorporated. Serum serves as the antigen and is placed in wells that are cut in the gel. Diffusion of the antigen from the well occurs in a circular pattern **(Fig. 7–10).** The radius of the resulting circle can be related to antigen concentration (see Chapter 10 for further details on RID). This is a sensitive technique when performed correctly, but at least 24 hours are needed before test results are available.

None of the assays for individual components are able to distinguish whether the molecules are functionally active. Thus, although the preceding techniques give quantitative results and are relatively easy to perform, test results must be interpreted carefully. Nephelometry and RID are both sensitive tests.[32] Enzyme-linked immunosorbent assay (ELISA) methods are available for the measurement of the inhibitor C1-INH.

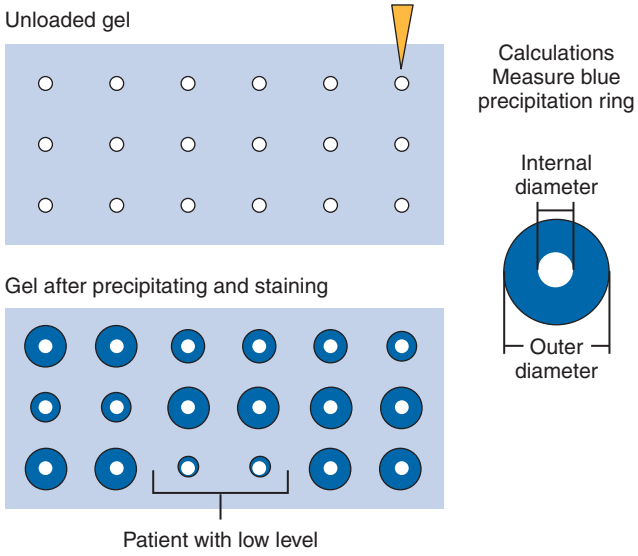


FIGURE 7–10 Radioimmunodiffusion for measurement of complement component C5.

Assays for the Classical Pathway

Assays that measure lysis, the end point of complement activation, are functional tests that are frequently run in conjunction with testing of individual components. The **hemolytic titration (CH50) assay** is most commonly used for this purpose.[26,32] This assay measures the amount of patient serum required to lyse 50% of a standardized concentration of antibody-sensitized sheep erythrocytes. Because all proteins from C1 to C9 are necessary for this to occur, absence of any one component will result in an abnormal CH50, essentially reducing this number to zero.

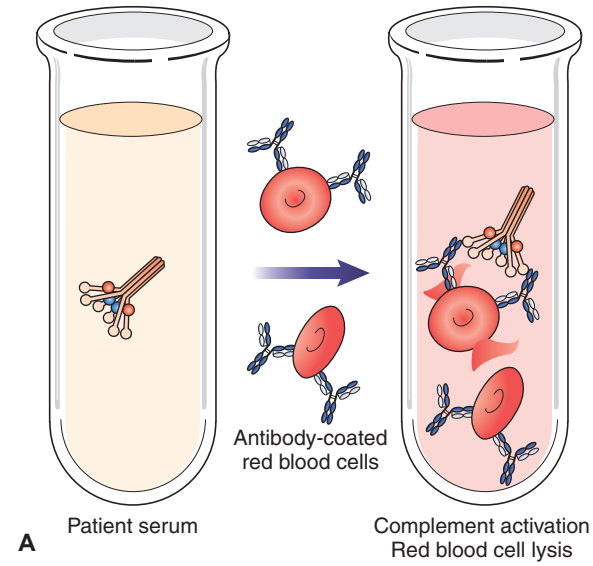
The titer is expressed in CH50 units, which is the reciprocal of the dilution that is able to lyse 50% of the sensitized cells.[32,38] The 50% point is used because this is when the change in lytic activity per unit change in complement is at maximum **(Fig. 7–11).** Most laboratories need to establish their own normal values.

An additional CH50 test has also been developed based on lysis of liposomes that release an enzyme when lysed. This lysis can be read on an analyzer and is more accurate than traditional CH50 testing.[25] However, lytic assays in general are complicated to perform and lack sensitivity. Individual laboratories must establish their own normal values. When the results are abnormal, the reasons cannot be determined. Such procedures are useful in establishing functional activity or lack thereof. Additional testing for individual components should be performed to follow up on any abnormality.

Lytic activity can also be measured by radial hemolysis in agarose plates. Rabbit RBCs that have been sensitized with antibody are implanted in agarose and patient serum is added to wells punched in the gel. Lysis appears as a clear zone around each well; if complement standards are run, the size of the zone can be related to complement concentration.[26,32,39] Solid-phase IgM attached to the walls of microtiter plates is used to initiate complement activation. Anti-human antibody to C9 conjugated to alkaline phosphatase is the indicator of complement activation. When a substrate is added, if any C9 is present and the antibody conjugate has attached, a color change will be evident. (Refer to Chapter 11 for a complete discussion of the principle of ELISA techniques.) This type of testing, which is very sensitive, is probably the best screen for complement abnormalities.[25] The same method can detect split products that result from complement activation. These products include C4a, C4d, C3a, iC3b, C5a, and the soluble form of the MAC sC5b-9, all of which are generated only if complement activation has occurred.

Alternative Pathway Assays

Alternative pathway activation can be measured by several different means. An AH50 can be performed in the same manner as the CH50, except magnesium chloride and ethylene glycol tetraacetic acid (EGTA) are added to the buffer and calcium is left out.[32] This buffer chelates calcium, which blocks classical pathway activation. Rabbit RBCs are used as the indicator because they provide an ideal surface for alternative pathway activation.



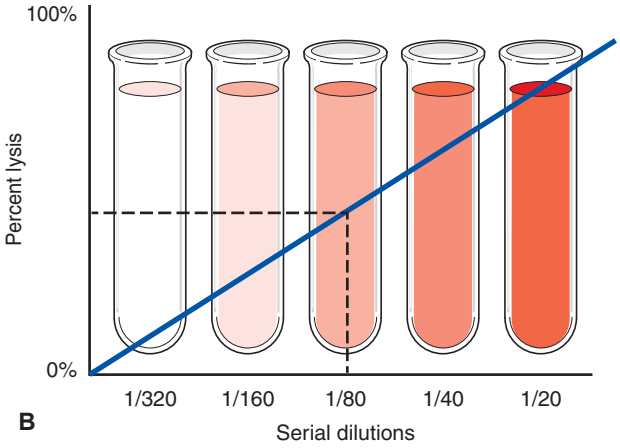


FIGURE 7–11 CH50 testing. (A) Antibody-coated RBCs are added to patient serum. This activates the complement in the sample and the RBCs are lysed. Although only C1qrs is shown for simplicity, this test requires C1 through C9 to be present in order for lysis to occur. The degree of lysis indicates the functional capacity of the complete classical pathway. (B) CH50 methodology.

An additional means of testing for alternative pathway function is via ELISA. One such test can detect C3bBbP or C3bP complexes in very small quantities. Microtiter wells are typically coated with bacterial polysaccharide to trigger activation of the alternative pathway.

One test system has been developed that can determine the activity of all three pathways.[32,39] Strips used for the classical pathway are coated with IgM, strips for the alternative pathway are coated with lipopolysaccharide, and strips for the MBL pathway are coated with mannose. Such testing is easy to perform and is not dependent upon the use of animal erythrocytes, which may be hard to obtain. Deficiencies can be detected using the combined test results.

Interpretation of Laboratory Findings

Decreased levels of complement components or activity may be caused by decreased production, consumption, or in vitro consumption. The third condition must be ruled out before either of the other two is considered. Specimen handling is extremely important. Blood should be collected in a clot tube with no serum separator.[39] The tube should be spun down and the serum should be frozen or placed on dry ice if it is not tested within 1 to 2 hours. If a specimen has been inadequately refrigerated, been subjected to multiple freeze–thaws, or been in prolonged storage, the results may be invalid and the test needs to be repeated with a fresh specimen. Control serum should also be included with each batch of test sera.

If a complement deficiency is suspected, it is possible to narrow down the possible candidate components with a CH50 and an AH50 assay. If the CH50 is low but the AH50 is normal, the components unique to the classical pathway should be investigated. If the CH50 is normal but the AH50 is low, the alternative pathway components need to be investigated. If both the CH50 and AH50 are low, suspicion should be on the components of the terminal pathway because that pathway is shared by both the classical and alternative pathways. Although this analysis will be true for most patients, it is also possible, if there is sufficient activation of complement through any one pathway, that the activation could consume enough components to lower the function of the other pathways. As previously stated, complement activation is rarely limited to just one pathway. Such consumption can result from loss of a control protein, presence of an autoantibody, an ongoing infection, or other activation circumstances.

Once a CH50 and AH50 hemolytic assay have been performed, it is appropriate to move to testing the levels or function of the components as directed by the relative results of the CH50 and AH50. For many of the components, an antigen level is sufficient to determine where the deficiency lies; however, there are a few instances in which measurement of the function would be more informative. For C8, it is necessary to measure function because it is a three-subunit protein. The loss of one of the three subunits would not put the level out of normal range, but it would render the remaining two subunits nonfunctional. C2 type II deficiency results from a genetic mutation in C2 that renders the protein nonfunctional but does not decrease the level of expression, which is also true for C1-INH in type II HAE.

A typical screening test for complement abnormalities usually includes determination of the following: C3 and C4, as well as hemolytic content. Testing for products of complement activation such as C3a, C4a, C5a, and Ba (as well as breakdown products including iC3b and C4d) can also be performed as a means of monitoring inflammatory processes such as rheumatoid arthritis and SLE. **Table 7–5** presents some of the possible

Table 7–5 Diagnosis of Complement Abnormalities

IMPAIRED FUNCTION OR DEFICIENCY	CLASSICAL PATHWAY	LECTIN PATHWAY	ALTERNATIVE PATHWAY
C1q, C1r, C1s	Low	Normal	Normal
C4, C2	Low	Low	Normal
MBL, MASP2	Normal	Low	Normal
B, D, P	Normal	Normal	Low
C3, C5, C6, C7, C8, C9	Low	Low	Low
C1-INH	Low	Low	Low
Factor H and I	Low	Low	Low
Improperly handled sera	Low	Low	Low

Adapted from Seelen MA, et al. An enzyme-linked immunosorbent assay-based method for functional analysis of the three pathways of the complement system. In: Detrick B, Hamilton RG, and Folds JD, eds. Manual of Molecular and Clinical Laboratory Immunology. *7th ed. Washington, DC: ASM Press; 2006:124.*

screening results from ELISA testing and correlates these with deficiencies of individual factors. An understanding of these patterns may be helpful in differentiating hereditary deficiencies from activational states that consume available complement components. Additional testing would be necessary, however, to actually pinpoint hereditary deficiencies.

SUMMARY

- The complement system is a series of more than 30 soluble and cell-bound proteins that interact with both the innate and adaptive immune systems to enhance host defenses against infection.
- Activities of complement include lysis of foreign or damaged cells, opsonization, increase in vascular permeability, and attraction of monocytes and macrophages to areas where needed.
- The classical complement pathway is triggered by IgG or IgM binding to the surface of pathogens. Nine major proteins are involved in this pathway.
- Three distinct units are involved in the classical pathway. They are the recognition unit consisting of C1qrs; the activation unit consisting of C2, C4, and C3; and the MAC, consisting of C5, C6, C7, C8, and C9.
- The lectin pathway is activated by carbohydrates present in microbial cell walls and serves as an important link between the innate and adaptive immune responses. Molecules distinct to the lectin pathway include mannose-binding lectin (MBL), MASP-1, MASP-2, and MASP-3.
- The alternative pathway is triggered by bacterial and fungal cell walls, yeast, viruses, tumor cells, and certain parasites. Factors unique to the alternative pathway include Factor B, Factor D, and properdin.
- The MAC is common to all three pathways.
- Plasma protein regulators of the complement system play an extremely important role because if uncontrolled, complement activation could have devastating systemic effects.
- Soluble regulators include C1 inhibitor (C1-INH), C4-binding protein (C4BP), Factor H, Factor I, and S protein.
- Examples of cell-bound regulators are complement receptor type 1 (CR1), membrane cofactor protein (MCP), and decay-accelerating factor (DAF).
- Specific complement receptors found on host cells amplify the immune response by enhancing phagocytosis and stimulating other accessory cells. Some of these receptors include CR1, CR2, CR3, CR4, and collectin receptors.
- Effector molecules generated during complement activation play a major role in recognition and presentation of antigens, activation of B cells, and maintenance of immunologic memory. They are classified as anaphylatoxins, chemotaxins, and opsonins.
- Anaphylatoxins increase vascular permeability, whereas chemotaxins attract phagocytic cells to a specific area and opsonins coat damaged or foreign cells to enhance phagocytosis.
- Deficiencies of complement components can place an individual at risk for certain infections. Missing or deficient regulators are the cause of diseases such as paroxysmal nocturnal hemoglobinuria and hereditary angioedema.
- Laboratory assays for individual complement components include radial immunodiffusion and nephelometry.
- The hemolytic titration or CH50 assay is a measure of lysis, the end point of complement activation in the classical pathway. The AH50 assay is a similar test for measuring the activity of the alternative pathway.

CASE STUDIES

1. A 3-year-old child has a history of serious infections and is currently hospitalized with meningitis. The doctor suspects that he may have a complement deficiency and orders testing. The following results are obtained: decreased CH50, decreased AH50, and normal C4 and C3 levels.

Questions

a. What do the results indicate about the possible pathway(s) affected?
b. Which component(s) are likely to be lacking?
c. What sort of additional follow-up would be recommended?

2. A 25-year-old female appeared at the local hospital's emergency department with symptoms of abdominal pain as well as severe vomiting and swelling of the legs and hands. She stated that she has had these symptoms on several previous occasions. After ruling out appendicitis, the physician ordered a battery of tests, including some for abnormalities of complement components. The following results were obtained: red and white blood cell count normal, total serum protein normal, CH50 decreased, alternative pathway function normal, C3 level normal, and C4 and C2 levels decreased.

Questions

a. What symptoms led physicians to consider a possible complement abnormality?
b. What are possible reasons for a decrease in both C4 and C2?
c. What other testing would confirm your suspicions?

REVIEW QUESTIONS

1. The classical complement pathway is activated by
   a. most viruses.
   b. antigen–antibody complexes.
   c. fungal cell walls.
   d. mannose in bacterial cell walls.

2. Which of the following is characteristic of complement components?
   a. Normally present in serum
   b. Mainly synthesized by B cells
   c. Present as active enzymes
   d. Heat stable

3. All of the following are true of the recognition unit *except*
   a. it consists of C1q, C1r, and C1s.
   b. the subunits require calcium for binding together.
   c. binding occurs at the FC region of antibody molecules.
   d. C1q becomes an active esterase.

4. Which of the following is referred to as C3 convertase?
   a. C1qrs
   b. C3bD
   c. C3bBb
   d. C4b5a

5. Mannose-binding protein in the lectin pathway is most similar to which classical pathway component?
   a. C3
   b. C1rs
   c. C1q
   d. C4

6. Which of the following describes the role of properdin in the alternative pathway?
   a. Stabilization of C3/C5 convertase
   b. Conversion of B to Bb
   c. Inhibition of C3 convertase formation
   d. Binding and cleavage of Factor B

7. Which best characterizes the membrane attack complex (MAC)?
   a. Each pathway uses different factors to form it.
   b. C5 through C9 are not added in any particular order.
   c. One MAC unit is sufficient to lyse any type of cell.
   d. C9 polymerizes to form the transmembrane channel.

8. All of the following represent functions of the complement system *except*
   a. decreased clearance of antigen–antibody complexes.
   b. lysis of foreign cells.
   c. increase in vascular permeability.
   d. migration of neutrophils to the tissues.

9. Which of the following is true of the amplification loop in complement activation?
   a. It is only found in the alternative pathway.
   b. The membrane attack unit is amplified.
   c. C3b is the product that is increased.
   d. Increasing amounts of C1qrs are produced.

10. Factor H acts by competing with which of the following for the same binding site?

a. Factor B
b. Factor D
c. C3B
d. Factor I

11. A lack of CR1 receptors on RBCs would result in which of the following?

a. Decreased binding of C3b to RBCs
b. Decreased clearance of immune complexes by the spleen
c. Decreased breakdown of C1qrs
d. Decreased binding of Factor H

12. Which best describes the role of CR2 on cell membranes?

a. Binds C1qrs to inactivate it
b. Acts as co-receptor on B cells for antigen
c. Increases clearance of immune complexes
d. Binds particles opsonized with C3b

13. Which of the following best characterizes hemolytic uremic syndrome?

a. It is a rare cause of renal failure in children.
b. It can be associated with deficiencies in Factor H.
c. The major cause is lack of DAF on RBCs.
d. It is associated with antibody-to-C3 convertase.

14. The CH50 test measures which of the following?

a. Patient serum required to lyse 50% of sensitized sheep RBCs
b. Functioning of both the classical and alternative pathways
c. Genetic deficiencies of any of the complement components
d. Functioning of the lectin pathway only

15. Which of the following would be most effective in preventing bystander lysis of RBCs?

a. C1-INH
b. Factor B
c. DAF
d. Factor H

16. A decreased CH50 level and a normal AH50 level indicate which deficiency?

a. Decrease in components in the lectin pathway only
b. Decrease in components in the alternative pathway only
c. Decrease in components of both classical and alternative pathways
d. Decrease in components of the classical pathway only

17. Which best describes the role of an anaphylatoxin?

a. Coats cells to increase phagocytosis
b. Attracts WBCs to the area of antigen concentration
c. Increases production of interleukin-1
d. Increases permeability of blood vessels

18. Which best describes the role of Factor H?

a. Acts with DAF to break down C3b
b. Prevents binding of Factor B to C3b
c. Binds to the C5C6C7 complex
d. Binds to C1q to shut down the classical pathway

19. A lack of C1-INH might result in which of the following conditions?

a. Paroxysmal nocturnal hemoglobinuria
b. Hemolytic uremic syndrome
c. Hereditary angioedema
d. Increased bacterial infections

20. Which would be most effective in measuring an individual complement component?

a. CH50 assay
b. Radial immunodiffusion
c. AH50 assay
d. Lytic assay with liposome

II

Basic Immunologic Procedures

8 Safety and Quality Management

Marjorie Schaub Di Lorenzo, MT(ASCP)SH

LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. List the six components of the chain of infection and the safety precautions that break the chain.
2. Correctly perform hand hygiene procedures following CDC guidelines.
3. Describe the types of personal protective equipment used by laboratory personnel.
4. Differentiate between universal precautions, body substance isolation, and standard precautions.
5. State the acceptable methods for disposal of biological waste and sharp objects in the laboratory.
6. Discuss the federal regulations and guidelines for preparing and shipping patient samples from the laboratory.
7. Explain the components of the Occupational Exposure to Bloodborne Pathogens Compliance Directive.
8. Describe safety precautions utilized when handling chemicals.
9. Discuss the components of Chemical Hygiene Plans and Safety Data Sheets.
10. State and interpret the components of the National Fire Protection Association hazardous material labeling system.
11. Describe precautions that laboratory personnel should take with regard to radioactive, electrical, fire, and physical hazards.
12. Explain the RACE and PASS actions to be taken when a fire is discovered.
13. Recognize standard hazard warning symbols.
14. Define the preexamination (preanalytical), examination (analytical), and postexamination (postanalytical) components of quality management.
15. Distinguish between the components of internal quality control, external quality control, electronic quality control, and external quality assessment (proficiency testing).
16. Discuss the roles of the Clinical Laboratory Improvement Amendments (CLIA), Clinical and Laboratory Standards Institute (CLSI), the The Joint Commission (TJC), and the College of American Pathologists (CAP) in the regulation of health care.
17. State and describe the 12 quality essentials used in a quality management system.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

18. Describe the purpose of quality indicators.
19. List the six areas of the Lean system and describe how it can benefit the laboratory.
20. State the purpose of the Six Sigma methodology in a management system.

KEY TERMS

Accuracy
Biohazardous material
Chain of infection
Chemical Hygiene Plan
Clinical Laboratory Improvement Amendments (CLIA)
Clinical and Laboratory Standards Institute (CLSI)
Coefficient of variation (CV)
Control mean
Delta check
Examination variables
External quality assessment (EQA)
Infection control
The Joint Commission (TJC)
Lean system
Occupational Safety and Health Administration (OSHA)
Personal protective equipment (PPE)
Postexamination variables
Postexposure prophylaxis (PEP)
Precision
Preexamination variables
Proficiency testing
Quality assessment (QA)
Quality control (QC)
Quality indicators
Quality management (QM)
Quality management system (QMS)
Quality system essentials (QSEs)
Reliability
Safety data sheet (SDS)
Shift
Six Sigma
Standard deviation (SD)
Standard of care
Standard precautions (SP)
Trend
Turnaround time (TAT)
Variable

Laboratory Hazards

The clinical laboratory contains a wide variety of safety hazards, many capable of producing serious injury or life-threatening disease. To work safely in this environment, clinical laboratorians must learn what hazards exist and the basic safety precautions associated with them. They must apply the basic rules of common sense required for everyday safety. Some hazards are unique to the health-care environment and others are encountered routinely throughout life **(Table 8–1).** It is essential that laboratory personnel know where all safety equipment is located and be trained in all aspects of its use on a yearly basis.

Biological Hazards

In the immunology laboratory, the most significant hazard exists in obtaining and testing patient specimens. Understanding how microorganisms are transmitted **(chain of infection)** is necessary to prevent infection. The chain of infection requires a continuous link between six elements: an infectious agent, a reservoir, a portal of exit, a means of transmission, a portal of entry, and a susceptible source.[1]

Infectious agents consist of bacteria, fungi, parasites, and viruses. A reservoir is a place where the infectious agent can live and multiply, such as a contaminated clinical specimen or an infected patient. Humans and animals (hosts) or contaminated inanimate objects (fomites) that contain blood, urine, or other body fluids make ideal reservoirs. The infectious agent leaves the reservoir through a portal of exit such as through the nose, mouth, and mucous membranes, as well as in blood or other body fluids, and is transmitted to a susceptible source to continue the chain of infection.

Means of transmission include direct contact (the unprotected host touches the patient, specimen, or a contaminated object); droplet (the host inhales infected aerosol droplets from a patient or specimen); airborne (the host inhales dried aerosol

Table 8–1 Types of Safety Hazards

TYPE	SOURCE	POSSIBLE INJURY
Biological	Infectious agents	Bacterial, fungal, viral, or parasitic infections
Sharp	Needles, lancets, and broken glass	Cuts, punctures, or bloodborne pathogen exposure
Chemical	Preservatives and reagents	Exposure to toxic, carcinogenic, or caustic agents
Radioactive	Equipment and radioisotopes	Damage to a fetus or generalized overexposure to radiation
Electrical	Ungrounded or wet equipment and frayed cords	Burns or shock
Fire or explosive	Open flames and organic chemicals	Burns or dismemberment
Physical	Wet floors, heavy boxes, and patients	Falls, sprains, or strains

particles circulating on the air currents or dust particles); vehicle (the host ingests contaminated food or water); or by a vector (from an animal or mosquito bite). The infectious agent now must enter a new reservoir through a portal of entry, which can be the same as the portal of exit and include mucous membranes of the nose, mouth, and eyes; breaks in the skin; and open wounds to complete the chain of infection. A susceptible source can be another patient, health-care personnel, or visitors. Patients receiving chemotherapy, the elderly, and immunocompromised patients are susceptible hosts. The immune system is still developing in newborns and infants and begins to weaken as people age, making these groups of patients more susceptible to infection. The immune system also is depressed by stress, fatigue, and lack of proper nutrition, which contribute to the susceptibility of patients and health-care personnel.[1] Once the chain of infection is complete, the infected host then becomes another source able to transmit the microorganisms to others.[1]

The most likely source of infection in serological testing is through contact with patient specimens; the main concern is exposure to viruses such as the hepatitis viruses and human immunodeficiency virus (HIV). Therefore, safety precautions are designed to protect health-care workers from exposure to potentially harmful infectious agents. The ultimate goal of biological safety is to prevent completion of the chain by preventing transmission. The **infection control** team develops procedures to control and monitor infections occurring within health-care facilities. **Figure 8–1** contains the universal symbol for **biohazardous material** and illustrates the chain of infection and how it can be broken by following safety practices.[1]

Preventing the transmission of microorganisms from infected sources to susceptible hosts is critical in controlling the spread of infection. Procedures used to prevent microorganism transmission include hand hygiene, wearing **personal protective equipment (PPE),** isolating highly infective or highly susceptible patients, and properly disposing of contaminated materials. Strict adherence to guidelines published by the Centers for Disease Control and Prevention (CDC) and the **Occupational Safety and Health Administration (OSHA)** is essential.[2]

Hand Hygiene

Hand contact represents the number one method of infection transmission. Hands should always be sanitized before patient contact, after gloves are removed, before leaving the work area, whenever the hands have been knowingly contaminated, before going to designated break areas, and before and after using bathroom facilities. Hand hygiene includes both hand washing and using alcohol-based antiseptic cleansers. Alcohol-based cleansers are not recommended after contact with spore-forming bacteria, including *Clostridium difficile* and *Bacillus* sp.

The CDC's guidelines for the correct hand washing technique are pictured in **Figure 8–2.**[3] If using alcohol-based cleansers, apply the cleanser to the palm of one hand. Rub your hands together and over the entire cleansing area, including between the fingers and thumbs. Continue rubbing until the alcohol dries.

Personal Protective Equipment

PPE used by laboratorians includes gloves, gowns or laboratory coats, masks, goggles, face shields, and Plexiglas countertop shields. Gloves are worn to protect the health-care worker's hands from contamination by patient body substances and to protect the patient from possible microorganisms on the health-care worker's hands. However, wearing gloves is not a substitute for hand sanitizing. Hands must always be sanitized when gloves are removed. A variety of gloves are available, including sterile and nonsterile, powdered and unpowdered, and latex and nonlatex.

Allergy to latex is decreasing among health-care workers due to the availabliity of other types of gloves. However, laboratorians should be alert for symptoms of reactions associated with latex contact, including irritant contact dermatitis that produces patches of dry, itchy irritation on the hands; delayed hypersensitivity reactions resembling poison ivy that appear 24 to 48 hours following exposure; and true immediate hypersensitivity reactions often characterized by facial flushing and respiratory difficulty (see Chapter 14). Hand sanitizing immediately after removal of gloves and avoiding powdered gloves may aid in preventing the development of latex allergy. Any signs of a latex reaction should be reported to a supervisor because a true latex allergy can be life threatening.[4]

In the immunology laboratory, fluid-resistant laboratory coats with wrist cuffs are worn at all times to protect skin and clothing from contamination by patient specimens. They must be completely buttoned with gloves pulled over the cuffs. Both gloves and laboratory coats should be changed as soon as possible if they become visibly soiled and must be removed when leaving the laboratory.

The mucous membranes of the eyes, nose, and mouth must be protected from specimen splashes and aerosols. A variety of protective equipment is available, including goggles, full-face plastic shields, and Plexiglas countertop shields **(Fig. 8–3).** Particular care should be taken to avoid splashes and aerosols when removing container tops and when transferring and centrifuging specimens. Never centrifuge specimens in uncapped tubes or in uncovered centrifuges. When specimens are received in containers with contaminated exteriors, the exterior of the container must be disinfected; if necessary, a new specimen may be requested.

Standard Precautions

The CDC developed **standard precautions (SP)** by combining recommendations of *universal precautions* (UP) and *body substance isolation* (BSI) procedures. Under UP, all patients were assumed to be potential carriers of bloodborne pathogens. The BSI modified UP by requiring that gloves be worn when encountering blood or any other body substance. The CDC continually modifies SP as changes occur in the health-care environment. SP assumes every person in the health-care setting is potentially infected or colonized by an organism that could be transmitted. SP applies to all blood and body fluids, mucous membranes, and nonintact skin and stresses hand washing.[5]

Standard precautions that apply directly to the laboratory are as follows:

- Hand hygiene—Hand hygiene includes both hand washing and the use of alcohol-based antiseptic cleansers. Sanitize hands after touching blood, body fluids, secretions, excretions, and contaminated items, whether or not gloves are

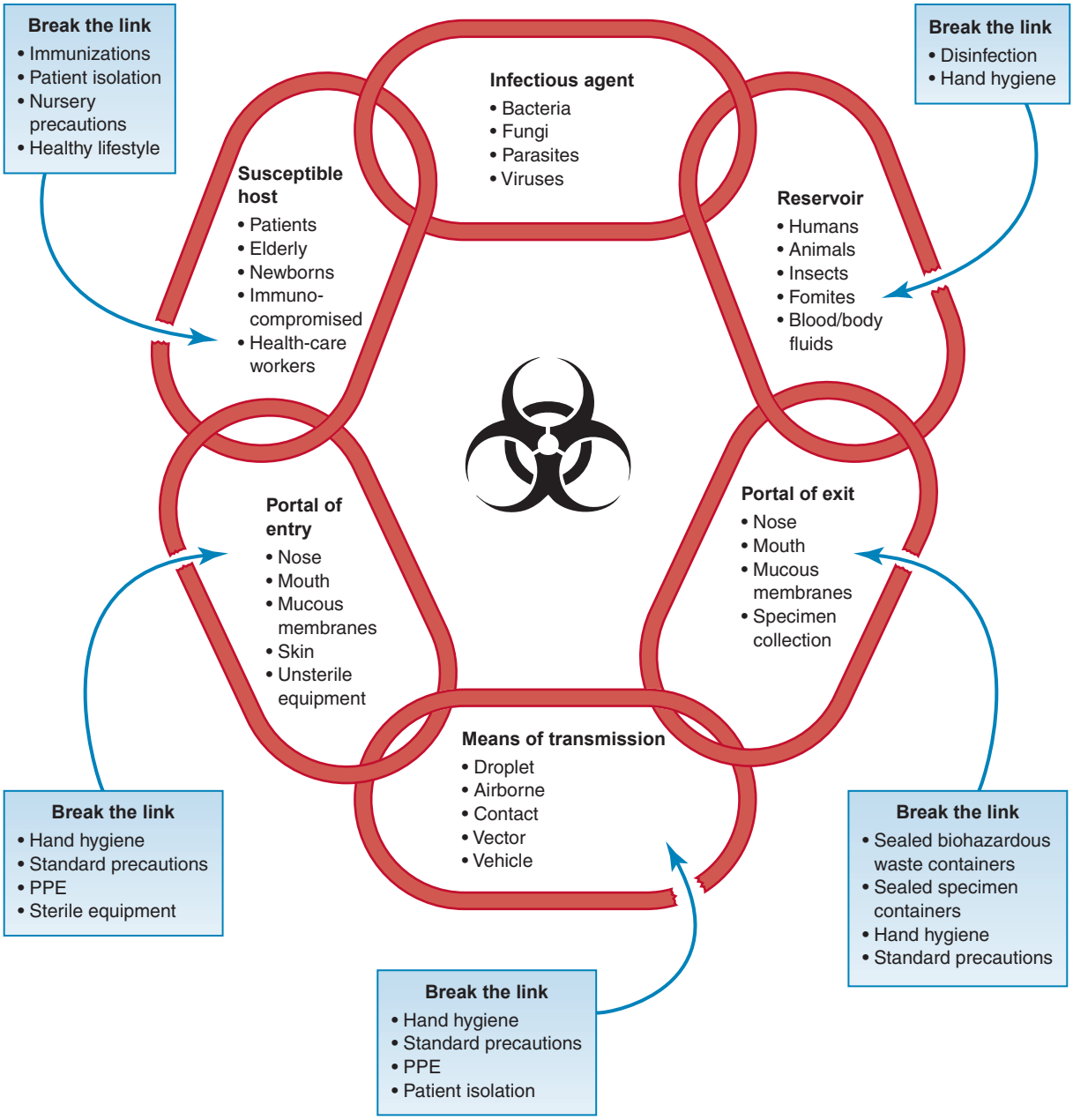


FIGURE 8–1 Chain of infection and safety practices related to the biohazard symbol. *(From Strasinger SK, DiLorenzo MS.* The Phlebotomy Textbook. *3rd ed. Philadelphia, PA: F.A. Davis, Philadelphia; 2011, with permission.)*

worn. Sanitize hands immediately after gloves are removed, between patient contacts, and when otherwise indicated to avoid transfer of microorganisms to other environments.

- Gloves—Wear gloves (clean, nonsterile gloves are adequate) when touching blood, body fluids, secretions, excretions, and contaminated items. Remove gloves promptly after use, before touching noncontaminated items and environmental surfaces, and before going to another patient. Sanitize hands immediately to avoid transfer of microorganisms to other patients or environments.
- Mask, nose, and eye protection—Wear a mask and eye protection or a face shield to protect mucous membranes of the eyes, nose, and mouth during procedures and patient-care activities that are likely to generate splashes or sprays of blood, body fluids, secretions, and excretions.
- Gown—Wear a gown (a clean, nonsterile gown is adequate) to protect skin and to prevent soiling of clothing during procedures that are likely to generate splashes of blood, body fluids, secretions, or excretions. Select a gown that is appropriate for the activity and amount of fluid likely to be encountered (e.g., fluid-resistant in the laboratory). Remove a soiled gown before leaving the laboratory environment and sanitize hands to avoid transfer of microorganisms to other environments.
- Respiratory hygiene and cough etiquette—Educate health-care personnel, patients, and visitors to contain

1. Wet hands with warm water. Do not allow any part of the body to touch the sink.

2. Apply soap.

3. Rub to form a lather, create friction, and loosen debris. Thoroughly clean between the fingers and under the fingernails for at least 20 seconds; include thumbs and wrists in the cleaning.

4. Rinse hands in a downward position to prevent recontamination of hands and wrists.

5. Dry hands with paper towel.

6. Turn off faucets with a clean paper towel to prevent contamination.

FIGURE 8–2 Hand hygiene. *(From Strasinger SK, DiLorenzo MS.* Urinalysis and Body Fluids. *6th ed. Philadelphia, PA: F.A. Davis; 2014, with permission.)*

respiratory secretions to prevent droplet and fomite transmission of respiratory pathogens. Offer masks to coughing patients, distance symptomatic patients from others, and practice good hand hygiene to prevent the transmission of respiratory pathogens.

- Needles—Never recap used needles or otherwise manipulate them using both hands; in addition, never use any technique that involves directing the point of a needle toward any part of the body; rather, use self-sheathing needles or a mechanical device designed to conceal the needle. Do not remove unsheathed needles from disposable syringes by hand; use a mechanical device. Do not bend, break, or otherwise manipulate used needles by hand. Place used disposable syringes and needles, scalpel blades, and other sharp items in appropriate puncture-resistant containers. Place reusable syringes and needles in a puncture-resistant

FIGURE 8–3 Personal protective equipment using a plastic shield. *(From Strasinger SK, DiLorenzo MS.* Urinalysis and Body Fluids. *6th ed. Philadelphia, PA: F.A. Davis; 2014, with permission.)*

container for transport to the reprocessing area. (See *Sharps Hazards* later for additional information.)

Occupational Exposure to Bloodborne Pathogens

The federal government has enacted regulations to protect health-care workers from exposure to bloodborne pathogens. These regulations are monitored and enforced by OSHA. The Occupational Exposure to Bloodborne Pathogens Standard[6] requires all employers to have a written Bloodborne Pathogen Exposure Control Plan and to provide necessary protection, free of charge, for employees. A later compliance directive called Enforcement Procedures for the Occupational Exposure to Bloodborne Pathogens Standard placed more emphasis on using engineering controls to prevent accidental exposure to bloodborne pathogens.[7] The components of the current Bloodborne Pathogens Exposure Control Plan that is required of all institutions are shown in the **In the Laboratory: Components of the OSHA Bloodborne Pathogen Exposure Control Plan** box. Each health-care institution is responsible for designing and implementing its own exposure control plan.[8]

Any accidental exposure to blood through needlestick, mucous membranes, or nonintact skin must be reported to a supervisor and a confidential medical examination must be immediately started. Evaluation of the incident must begin right away to ensure appropriate **postexposure prophylaxis (PEP)** is initiated within 24 hours. Needlesticks are the most frequently encountered exposure and place the laboratorian in danger of contracting HIV, HBV, and hepatitis C virus (HCV). The CDC has recommended procedures to prevent these (see **In the Laboratory: Postexposure Prophylaxis**).

Biological Waste Disposal

All biological waste, except urine, must be placed in appropriate containers labeled with the biohazard symbol. This waste includes not only specimens but also the materials with which the specimens come in contact. Any supplies contaminated with blood and body fluids must also be disposed of in containers clearly marked with the biohazard symbol or with red or yellow color coding. These supplies include alcohol pads, gauze, bandages, disposable tourniquets, gloves, masks, gowns, and plastic tubes and pipettes. Disposal of needles and other sharp objects is discussed in the next section.

Contaminated nondisposable equipment, blood spills, and blood and body fluid processing areas must be disinfected. The most commonly used disinfectant is a 1:10 dilution of sodium hypochlorite (household bleach) prepared daily and stored in a plastic, not a glass, bottle. The bleach should be allowed to air-dry on the contaminated area before being wiped off.[9]

Transporting Patient Specimens

If a laboratory accepts specimens from other health-care institutions, then it is important to know the regulations for packaging, transporting, and receiving these specimens. The U.S. Department of Transportation (DOT), the International Air Transport Association (IATA), and the United Nations (UN) have stringent regulations that must be followed if a laboratory is going to be involved in transporting or receiving patient specimens from another institution.[10,11]

In the Laboratory

Components of the OSHA Bloodborne Pathogen Exposure Control Plan

Engineering Controls

1. Providing sharps disposal containers and needles with safety devices
2. Requiring discarding of needles with the safety device activated and the holder attached
3. Labeling all biohazardous materials and containers

Work Practice Controls

4. Requiring all employees to practice standard precautions
5. Prohibiting eating, drinking, smoking, and applying cosmetics in the work area
6. Establishing a daily work surface disinfection protocol

Personal Protective Equipment

7. Providing laboratory coats, gowns, face shields, and gloves to employees and laundry facilities for nondisposable protective clothing

Medical

8. Providing immunization for the hepatitis B virus free of charge
9. Providing medical follow-up to employees who have been accidentally exposed to bloodborne pathogens

Documentation

10. Documenting annual training of employees in safety standards
11. Documenting evaluations and implementation of safer needle devices
12. Involving employees in the selection and evaluation of new devices and maintaining a list of those employees and the evaluations
13. Maintaining a sharps injury log including the type and brand of safety device, location and description of the incident, and confidential employee follow-up

From Strasinger SK, DiLorenzo MA. *The Phlebotomy Textbook.* 3rd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.

In the Laboratory

Postexposure Prophylaxis

1. Draw a baseline blood sample from the employee and test it for HBV, HCV, and HIV.
2. If possible, identify the source patient, collect a blood sample, and test it for HBV, HCV, and HIV. Patients must usually give informed consent for these tests and they do not become part of the patient's record. In some states, a physician's order or court order can replace patient consent because a needlestick is considered a significant exposure.
3. Testing must be completed within 24 hours for maximum benefit from PEP.

Source patient tests positive for HIV:

1. Employee is counseled about receiving PEP using zidovudine (ZDV) and one or two additional anti-HIV medications.
2. Medications are started within 24 hours.
3. Employee is retested at intervals of 6 weeks, 12 weeks, and 6 months.
4. Additional evaluation and counseling is needed if the source patient is unidentified or untested.

Source patient tests positive for HBV:

1. Unvaccinated employees can be given hepatitis B immune globulin (HBIG) and HBV vaccine.
2. Vaccinated employees are tested for immunity and receive PEP, if necessary.

Source patient tests positive for HCV:

1. No PEP is available.
2. Employee is monitored for early detection of HCV infection and treated appropriately.

Any exposed employee should be counseled to report any symptoms related to viral infection that occur within 12 weeks of the exposure.

From Strasinger SK, DiLorenzo MA. *The Phlebotomy Textbook.* 3rd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.

DOT and IATA Specimen Transport. Under DOT and IATA regulations, all diagnostic specimens require triple packaging **(Fig. 8–4).** This includes the following:

- The primary container (glass, metal, or plastic) must be watertight with a positive (screw-on) cap.
- The primary container must be wrapped with enough absorbent material to be capable of absorbing all of its contents. Multiple specimens must be wrapped individually before placing them in the leakproof secondary container.
- The secondary container must be placed in a sturdy outer container made of corrugated fiberboard, wood, metal, or rigid plastic. An itemized list of contents in a sealed plastic bag is also placed in the outer container. Ice packs are placed between the secondary and the outer container. Additional measures must be taken when using ice and dry ice.

Courier-Delivered Specimen Transport. Specimens transported by a hospital courier among clinics, physicians' offices, and the hospital laboratory are exempt from most DOT rules, unless they are suspected of containing an infectious substance. If specimens may contain an infectious substance, then all DOT rules apply. The transport vehicle should be used exclusively for transport of specimens and should be equipped to secure the transport containers.[12] Minimum shipping standards for this type of transportation include[13]

- Leakproof, watertight specimen containers
- Tightly capped tubes placed in a rack to maintain an upright position
- Leakproof inner packaging surrounded by enough absorbent material to completely absorb all the liquid present
- A leakproof plastic or metal transport box with a secure, tight-fitting cover
- Properly labeled transport boxes accompanied by specimen data and identification forms

Specimens picked up by a courier that are to be shipped to an out-of-the-area laboratory, such as a reference laboratory, must follow DOT regulations. Many of these laboratories supply shipping containers to their clients.

Sharps Hazards

Sharp objects in the laboratory, including needles, lancets, and broken glassware, present a serious biological hazard for possible exposure to bloodborne pathogens caused by accidental puncture. Although bloodborne pathogens are also transmitted through contact with mucous membranes and nonintact skin, a needle or lancet used to collect blood has the capability to produce a very significant exposure to bloodborne pathogens. It is essential that safety precautions be followed at all times when sharp hazards are present.

The number one personal safety rule when handling needles is to *never* recap a needle. Many safety devices are available for needle disposal that provide a variety of safeguards. These include needle holders that become a sheath, needles that automatically resheath or become blunt, and needles with attached sheaths. All sharps must be disposed of in puncture-resistant, leakproof containers labeled with the biohazard symbol **(Fig. 8–5).** Containers should be located in close proximity to the work area and must always be replaced when the safe capacity mark is reached. Never use any technique that involves directing the point of a needle toward any part of the body.

The Needlestick Safety and Prevention Act was signed into law in 2001.[14] In June 2002, OSHA issued a revision to the Bloodborne Pathogens Standard compliance directive mentioned previously.[15] In the revised directive, the agency requires that all blood holders with needles attached be immediately discarded into a sharps container after the device's safety feature is activated. The rationale for the new directive was based on the exposure of workers to the unprotected stopper-puncturing end of evacuated tube needles, the increased needle manipulation required to remove it from the holder, and the possible worker exposure from the use of contaminated holders.

Chemical Hazards

Serological testing may involve use of chemical reagents that must be handled in a safe manner to avoid injury. The general rules for safe handling of chemicals include taking precautions to avoid getting chemicals on the body, clothes, and work

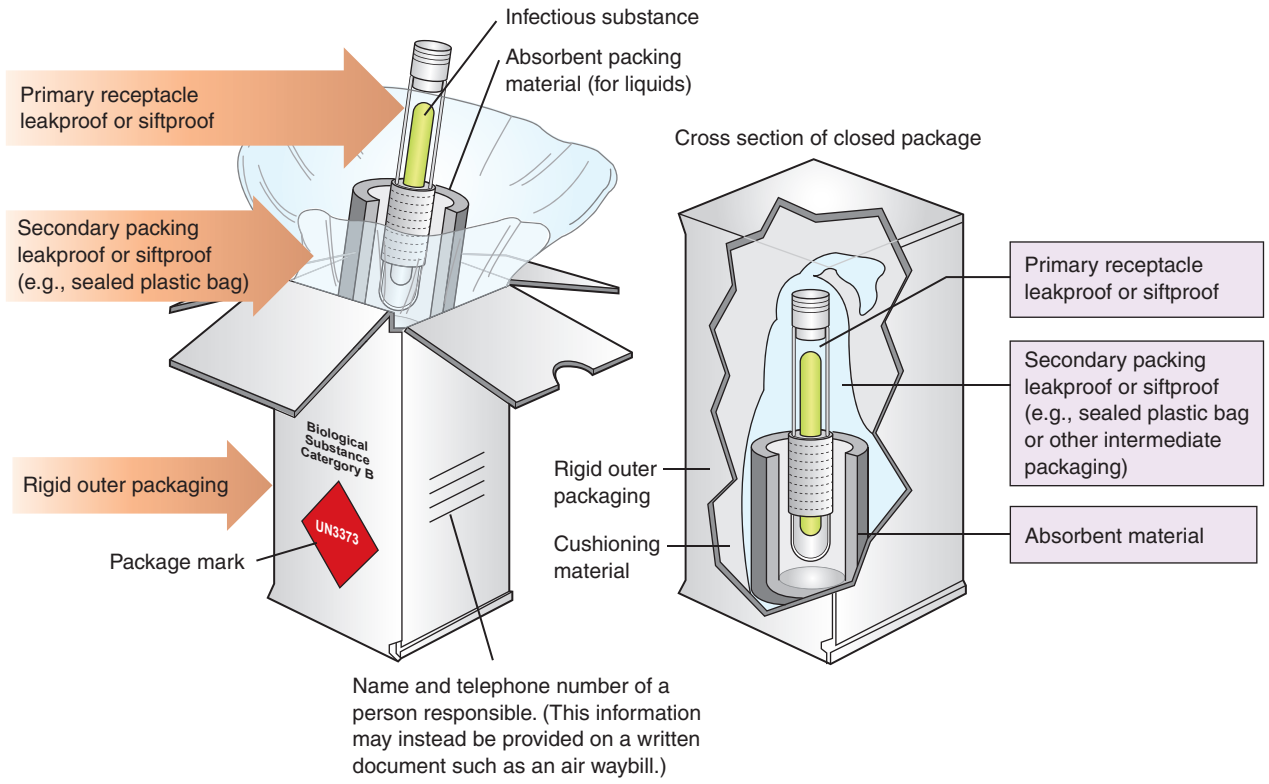


FIGURE 8–4 Packing and labeling of category B infectious substances. If multiple fragile primary receptacles are placed in a single secondary package, they must be either individually wrapped or separated to prevent contact. *(From U.S. Department of Transportation. Pipeline and Hazardous Materials Safety Administration.)*

FIGURE 8–5 Examples of puncture–resistant containers. *(From Strasinger SK, DiLorenzo MS.* The Phlebotomy Textbook. *3rd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

area; wearing PPE such as safety goggles when pouring chemicals; observing strict labeling practices; and following instructions carefully. Preparing reagents under a fume hood is also a recommended safety precaution. Chemicals should never be mixed together unless specific instructions are followed; in addition, they must be added in the order specified. This is particularly important when combining acid and water because acid should always be added to water, rather than adding water to acid, to avoid the possibility of sudden splashing.

When skin or eye contact occurs, the best first aid is to immediately flush the area with water for at least 15 minutes and then seek medical attention. Laboratorians must know the location of the emergency shower and eyewash station in the laboratory. Do not try to neutralize chemicals spilled on the skin.

Safety Data Sheets (SDS)

All chemicals and reagents containing hazardous ingredients in a concentration greater than 1% are required by OSHA to have a **safety data sheet (SDS)** on file in the work area. By law, vendors must provide these sheets to purchasers; however, it is the responsibility of the facility to obtain and keep them available to employees. A SDS contains information on physical and chemical characteristics, fire, explosion reactivity, health hazards, primary routes of entry, exposure limits and carcinogenic potential, precautions for safe handling, spill cleanup, and emergency first aid. Containers of chemicals that pose a high risk must be labeled with a chemical hazard symbol representing the possible hazard, such as flammable, poisonous, corrosive, and so on. State and federal regulations should be consulted for the disposal of chemicals. Containers of chemicals that pose a high risk must be labeled with a chemical hazard symbol representing the possible hazard, such as flammable, poison, or corrosive **(Fig. 8–6).**

Chemical Hygiene Plan

OSHA requires that all facilities that use hazardous chemicals have a written **Chemical Hygiene Plan** available to employees.[16,17] The purpose of the plan is to detail the following:

- Appropriate work practices
- Standard operating procedures
- Personal protective equipment

FIGURE 8–6 Chemical hazard symbols. *(From Strasinger SK, DiLorenzo MS.* The Phlebotomy Textbook. *3rd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

- Engineering controls, such as fume hoods and safety cabinets for flammables
- Employee training requirements
- Medical consultation guidelines

Each facility must appoint a chemical hygiene officer who is responsible for implementing and documenting compliance with the plan.

Chemical Waste Disposal

Any hazardous chemical waste should be disposed of per current EPA regulations. Most reagents used in the laboratory come with a SDS, mentioned previously. The SDS gives specific information for disposal of particular chemicals. All chemicals used should be disposed of by following SDS directions. Many kits used in immunologic testing often contain sodium azide as a preservative, which can be disposed of by flushing down the drain with plenty of water. Using large amounts of water helps to avoid buildup in plumbing.

Radioactive Hazards

Laboratorians can be exposed to radioactivity in the clinical laboratory when performing procedures using radioisotopes, such as radioimmunoassay. The amount of radioactivity present in most medical situations is very small and represents little danger. However, the effects of radiation are related to the length of exposure and are cumulative. Exposure to radiation is dependent on the combination of time, distance, and shielding. Persons working in a radioactive environment are required to wear measuring devices to determine the amount of radiation they are accumulating.

Laboratorians should be familiar with the radioactive symbol shown in **Figure 8–7.** This symbol must be displayed on the doors of all areas where radioactive material is present. Exposure to radiation during pregnancy presents a danger to the fetus; personnel who are or who think they may be pregnant should avoid areas with this symbol.

Medical Radioactive Waste Disposal

Disposal of medical radioactive waste is regulated by the Nuclear Regulatory Commission (NRC) and is also subject to local regulations. Such waste must be separated from other waste materials in the laboratory and placed in containers marked with the radioactive symbol. Disposal varies with the type of material (solid, liquid, or volatile chemical) and depends upon the amount of radioactivity present. Very few immunology laboratories use radioactivity in testing anymore, mainly because of the problem of disposing of waste. If radioactivity is used, the laboratory typically contracts with a waste disposal service that picks up the radioactive waste material.

FIGURE 8–7 Radioactive symbol. *(From Strasinger SK, DiLorenzo MS.* The Phlebotomy Textbook. *3rd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

Electrical Hazards

The laboratory setting contains electrical equipment with which laboratorians have frequent contact. The same general rules of electrical safety observed outside the workplace apply in the laboratory, such as checking for frayed cords or overloaded circuits. Laboratorians also have frequent contact with water, fluids, and chemical agents; therefore, the danger of water or fluid coming in contact with equipment is greater in the laboratory setting. Equipment should not be operated with wet hands. Designated hospital personnel closely monitor electrical equipment. However, laboratory personnel should be observant for any dangerous conditions and report them to the appropriate persons.

When an accident involving electrical shock occurs, the electrical source must be removed immediately without touching the person or the equipment involved. Persons responding to the accident must avoid transferring the current to themselves by turning off the circuit breaker before unplugging the equipment or moving the equipment using a nonconductive glass or wood object. The victim should receive immediate medical assistance following discontinuation of the electricity. Cardiopulmonary resuscitation (CPR) may be necessary.

Fire and Explosive Hazards

Clinical laboratory work involves the use of potentially volatile or explosive chemicals that require special procedures for handling and storage. Flammable chemicals should be stored in safety cabinets and explosion–proof refrigerators. Cylinders of compressed gas should be located away from heat and securely fastened to a stationary device to prevent accidental tipping.

The Joint Commission (TJC), an independent body that certifies and accredits health-care organizations in the United States, requires that all health-care facilities post evacuation routes and detailed plans to follow in the event of a fire. Laboratory personnel should be familiar with these routes. When a fire is discovered, all employees are expected to take the actions described by the acronym RACE:

Rescue—rescue anyone in immediate danger
Alarm—activate the institutional fire alarm system
Contain—close all doors to potentially affected areas
Extinguish or Evacuate—attempt to extinguish the fire if possible, or evacuate, closing the door

Fire blankets should be present in the laboratory. Persons whose clothes are on fire should be wrapped in the blanket to smother the flames. The acronym PASS can be used to remember the steps in operating a fire extinguisher:

1. **P**ull pin
2. **A**im at the base of the fire
3. **S**queeze handles
4. **S**weep nozzle side to side

The Standard System for the Identification of the Fire Hazard of Materials, NFPA 704, is a symbol system used to inform firefighters of the hazards they may encounter when fighting a fire in a particular area. The color-coded areas contain information relating to health hazards, flammability, reactivity, use of water, and personal protection. These symbols are placed on doors, cabinets, and reagent bottles. An example of the hazardous material symbol and information is shown in **Figure 8–8.**

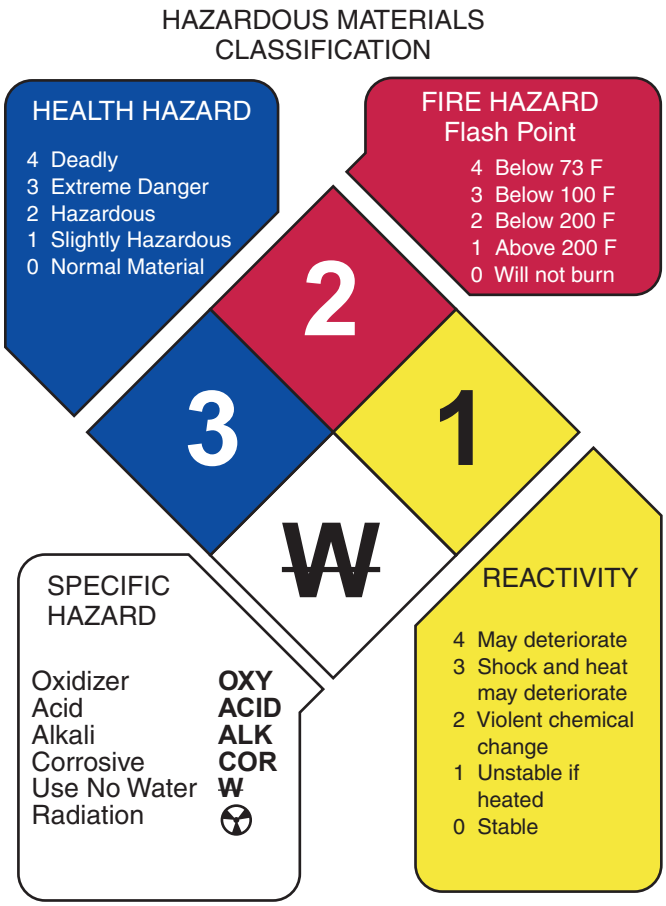


FIGURE 8–8 NFPA hazardous material symbol and classification. *(From Strasinger SK, DiLorenzo MS.* The Phlebotomy Textbook. *3rd ed. Philadelphia, PA: F.A. Davis; 2014.)*

Physical Hazards

Physical hazards are not unique to the laboratory; routine precautions observed outside the workplace apply. Maintaining a clean and organized work area is essential for minimizing the hazards. General precautions to consider include not running in rooms and hallways, watching for wet floors, bending the knees when lifting heavy objects, keeping long hair pulled back, and avoiding dangling jewelry. Closed-toed shoes that provide maximum support are essential for safety and comfort.

Quality Management

The term **quality management (QM)** refers to the overall process of guaranteeing quality patient care. As it relates to the clinical laboratory, QM is the continual monitoring of the entire test process from test ordering and specimen collection through reporting and interpreting results. Written policies and documented actions as they pertain to the patient, the laboratory, ancillary personnel, and the health-care provider are required. In addition, written remedial actions mandating the steps to take when any part of the system fails is essential to a QA program.

The **Clinical Laboratory Improvements Amendments (CLIA)** are regulations that specify required components for QA to include patient test management assessment, quality control (QC) assessment, proficiency testing assessment, comparison of test results, relationship of patient information to patient test results, patient confidentiality, specimen identification and integrity, personnel competency, personnel qualifications and evaluations, communication protocols, complaint investigations, QA review with staff, and maintenance of QA records for 2 years.[18]

Documentation of QA procedures is required by all laboratory accreditation agencies, including TJC, College of American Pathologists (CAP), American Association of Blood Banks (AABB), American Osteopathic Association (AOA), American Society of Histocompatibility and Immunogenetics (ASHI), and the Commission on Laboratory Assessment (COLA); it is also required for Medicare and Medicaid reimbursement. Guidelines published by CAP and the **Clinical and Laboratory Standards Institute (CLSI)** provide very complete instructions for documentation and are used as a reference for the ensuing discussion of the specific areas of immunology QA.[1] Documentation in the form of a procedure manual is required in all laboratories; this format is used as the basis for the following discussion.

Procedure Manual

A procedure manual (paper or digital) containing all the procedures performed in the immunology section of the laboratory

must be available for reference in the working area and must comply with the CLSI guidelines. For each test performed, the procedure manual provides

- The principle or purpose of the test
- Clinical significance
- Patient identification and preparation
- Specimen type
- Method of collection
- Specimen labeling
- Specimen preservation
- Conditions of transport and storage before testing
- Specimen acceptability and criteria for rejection
- Reagents
- Standards and controls acceptability and expiration policy
- Instrument calibration and maintenance protocols and schedules
- Step-by-step procedure
- Calculations
- Frequency and tolerance limits for controls and corrective actions
- Reference values and critical values
- Interpretation of results
- Common interferences
- Specific procedure notes
- Limitations of the method
- Method validation
- Confirmatory testing
- Recording of results
- References
- Effective date
- Author
- Review schedule[19]

Current package inserts for all test kits used should be reviewed and included in the manual. The laboratory must also have a documented procedure for the correction of erroneous results.[19]

The printed procedural manuals and electronic procedural manuals are subjected to proper document control. Only authorized persons may make changes; these are dated and signed (manually or digitally). The manuals must undergo periodic review; documentation (i.e., proof) of the review is included in the manual.

Evaluating procedures and adopting new methodologies is an ongoing process in the clinical laboratory. Whenever changes are made, the written procedure in the manual should be reviewed, referenced, and signed by a person with designated authority, such as the laboratory director or section supervisor, and personnel should be notified of the changes. An annual review of all procedures by the designated authority must also be documented.

The procedure manual provides the basis for all testing in the immunology laboratory. Quality care in testing relies on strictly following procedures as written. Documentation includes every step from specimen collection to the reporting of results. A well-documented QM program ensures quality test results and patient care.

Preexamination Variables

In a clinical laboratory, a QM program encompasses **preexamination variables** (e.g., specimen collection, handling, and storage), **examination variables** (e.g., reagent and test performance, instrument calibration and maintenance, personnel requirements, and technical competence), **postexamination variables** (e.g., reporting of results and interpretations), and documentation that the program is being meticulously followed.

A **variable** is defined as anything that can be changed or altered. Identification of variables throughout the testing process provides the basis for development of procedures and policies within the immunology department that are located in the procedure manual.

Preexamination variables occur before the actual testing of the specimen and include test requests, patient preparation, timing, specimen collection, handling, and storage. Healthcare personnel outside the immunology department control many of these factors, such as ordering tests and collecting specimens; however, communication between departments and adequate training on the correct procedures for ordering a test, collecting a specimen, and transporting the specimen improves the **turnaround time (TAT)** of results, avoids duplication of test orders, and ensures a high-quality specimen. TAT is defined as the amount of time required between the point at which a test is ordered by the health-care provider and the results are reported to the health-care provider. The laboratory can monitor the TATs for both stat and routine tests to determine areas in the process that need improvement.

Specimen Collection and Documentation

Specific guidelines for specimen collection and handling should be stated at the beginning of each procedure listed in the manual. In addition to following the guidelines for specimen collection for each specific procedure, requisition forms and electronic entry forms should be used to document the type of specimen to be collected and the time and date of collection. The form should have space for documenting (1) the patient's first and last name, (2) the patient's gender, (3) the patient's age or date of birth, (4) the name of the person requesting the test, (5) the name of the person to contact with critical results, (6) the name of the test ordered, (7) any special handling requirements, (8) the time and date of specimen collection, (9) the time the specimen was delivered to the laboratory, and (10) any additional information pertinent to laboratory interpretation.[20] Information regarding patient preparation (e.g., fasting or elimination of interfering medications) and the type and volume of specimen required must be included in the specific procedure.

The criteria for specimen rejection for both physical characteristics and labeling errors must be present. If a specimen is rejected, the criteria for rejecting that specimen must be documented and available to the health-care provider and nursing staff. Laboratory personnel must determine the suitability of a specimen and document any problems and corrective actions taken using an internal laboratory quality improvement form (see **In the Laboratory: An Example of an Internal Laboratory Quality Improvement Form**). This report enables the

In the Laboratory

An Example of an Internal Laboratory Quality Improvement Form

Quality Improvement Follow-Up Report
CONFIDENTIAL

Instructions: Section I should be completed by the individual identifying the event.

Date of report: _____ Reported by: _____
Date of incident: _____ Date/time of discovery: _____
Patient MR#: _____ Patient accession #: _____

Section I. Summary of Incident
Describe what happened: _____
_____
_____
What immediate corrective action was taken? _____
_____
_____

Provide the ORIGINAL to team leader or technical specialist within 24 hours of incident discovery.
Date: _____
To: _____

Forwarded for follow-up:
Date: _____
To: _____

Section II. Management Investigation: *Tracking #:* _____
Instructions: Section II should be completed by laboratory management within 72 hours.
Check the appropriate problem category.

- ☐ Unacceptable patient samples (Caused by hemolysis, QNS, or contamination)
- ☐ Equipment-related event
- ☐ Standard operating procedure deviation
- ☐ Communication problem or complaint
- ☐ Accident
- ☐ Wrong tube type
- ☐ Misidentified sample
- ☐ Wrong location
- ☐ Other (explain)

Explain answers: _____
_____
_____
Preventive or corrective action recommendations: _____
_____
_____

Technical specialist or team leader: _____ Date: _____
Medical director review: _____ Date: _____
Quality assurance review: _____ Date: _____
FDA reportable: Yes or no _____ Date reported: _____

Adapted from Danville Regional Medical Center Laboratory, Danville, VA, with permission.

laboratory director to capture the information to determine the root cause and develop a preventive or corrective action plan. Laboratory information systems have the capability to electronically generate these forms for review. An acceptable specimen requires verification of the patient's identification information on the requisition form and the tube label, proper collection and processing procedures, and timely transport to the laboratory.

Examination Variables

The examination variables are the processes that directly affect the testing of specimens. They include reagents, instrumentation and equipment, testing procedure, QC, preventive maintenance (PM), access to procedure manuals, and the competency of personnel performing the tests.

Reagents

The name and chemical formula of each reagent used, any necessary instructions for preparation or company source of prepared materials, storage requirements, and procedures for reagent QC are all found in the procedure manual. The type of water used for preparing reagents and controls must be specified. Distilled or deionized water or clinical laboratory reagent water (CLRW) must be available. A bold-type statement of any safety or health precautions associated with reagents should be present.

All reagents must be properly labeled with the date of preparation or opening, purchase and received date, expiration date, and appropriate safety information. Reagents should be checked against two levels of commercial control solutions on each shift, or at a minimum once a day and whenever a new reagent is opened. Results of all reagent checks are properly recorded.

Instrumentation and Equipment

The procedure manual must clearly provide instructions regarding the operation, performance, frequency of calibration, and limitations of the instrumentation and equipment. The procedures to follow when limitations or linearity are exceeded, such as dilution procedures, must be included in the manual, as well as instructions detailing the appropriate recording procedures.

Two levels of commercial controls must be run and recorded. Evidence of corrective action for any failed QC tests must be documented. No patient's testing may be performed until QC is acceptable. A routine PM schedule for instruments and equipment should be prepared as mandated by the TJC or CAP guidelines and records kept of all routine and nonroutine maintenance performed.

Deionized water used for reagent preparation is quality controlled by checking its pH and purity meter resistance on a weekly basis, as well as the bacterial count on a monthly schedule. All results must be recorded on the appropriate forms.

Testing Procedures

Detailed and concise testing instructions are written in a step-by-step manner. Instructions should begin with specimen preparation, such as time and speed of centrifugation, and include types of glassware needed, time limitations and stability of specimens and reagents, calculation formulas and a sample calculation, health and safety precautions, and procedures. Additional procedure information including reasons for special precautions, sources of error and interfering substances, helpful hints, clinical situations that influence the test, alternative procedures, and acceptable TATs for stat tests are listed under the title of Procedure Notes following the step-by-step procedure.

Reference sources should be listed. The manufacturer's package inserts may be included but cannot replace the written procedure. The laboratory director must sign and date new procedures and all modifications of procedures before they are used.[21]

Quality Control

Quality control (QC) refers to the materials, procedures, and techniques that monitor the **accuracy, precision,** and **reliability**

of a laboratory test. QC procedures are performed to ensure that acceptable standards are met during the process of patient testing. Specific QC information regarding the type of control specimen, preparation and handling, frequency of use, tolerance levels, and methods of recording should be included in the step-by-step instructions in the procedures manual for each test.

QC is performed at scheduled times, such as at the beginning of each shift or before testing patient samples, and it must always be performed if reagents are changed, an instrument malfunction has occurred, or if test results are questioned by the health-care provider. Control results must be recorded in a paper or electronic log. Patient test results may not be reported until the QC is verified.

External Controls. External controls are used to verify the accuracy (ability to obtain the expected result) and precision (ability to obtain the same result on the same specimen) of a test. The control material is exposed to the same conditions as the patient samples. Reliability is the ability to maintain both precision and accuracy. Analysis of two levels of control material is required. One of these is a high level control and the other is a low level control. The concentration of controls should be at medically significant levels and should be as much like the human specimen as possible. Documentation of QC includes dating and initialing the material when it is first opened and recording the manufacturer's lot number and the expiration date each time a control is run and the test result is obtained. Food and Drug Administration (FDA) standards require that control material test negative for HIV and HBV. External controls are tested and interpreted in the laboratory by the same person performing the patient testing.

The control data are evaluated before releasing patient results. Data obtained from repeated measurements have a Gaussian distribution or spread in the values that indicate the ability to repeat the analysis and obtain the same value. The laboratory, after repeated testing, establishes the value for each analyte and calculates the **control mean** (the average of all data points) and the **standard deviation (SD)** (a measurement statistic that describes the average distance each data point in a normal distribution is from the mean). The **coefficient of variation (CV)** is the SD expressed as a percentage of the mean. The CV indicates whether the distribution of values about the mean is in a narrow versus broad range and should be less than 5%. Confidence intervals are the limits between which the specified proportion or percentage of results will lie. Control ranges are determined by setting confidence limits that are within ±2 SD or ±3 SD of the mean, which indicates that 95.5% to 99.7% of the values are expected to be within that range.

Values are plotted on Levey-Jennings control charts to visually monitor control values. Immediate decisions about patient results are based on the ability of control values to remain within a preestablished limit. Changes in accuracy of results are indicated by either a **trend,** a gradual changing in the mean in one direction that may be caused by a gradual deterioration of reagents or deterioration of instrument performance, or a **shift,** an abrupt change in the mean that may be caused by a malfunction of the instrument or a new lot number of reagents **(Fig. 8–9).** Changes in precision are shown by a large amount of scatter about the mean and an uneven distribution above and below the mean that are most often caused by errors in technique.

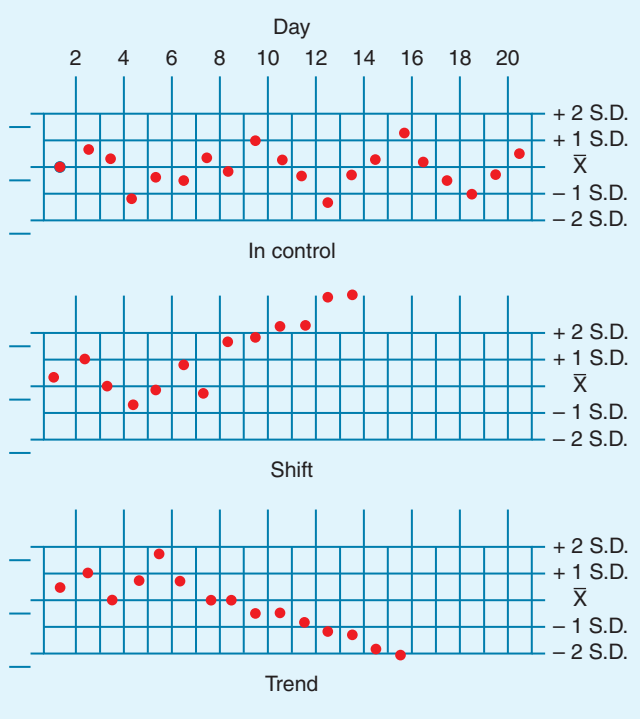


FIGURE 8–9 Levey–Jennings charts showing in–control, shift, and trend results. *(From Strasinger SK, DiLorenzo MS.* Urinalysis and Body Fluids. *6th ed. Philadelphia, PA: F.A. Davis; 2014.)*

When control values are outside the tolerance limits, corrective action—including the use of new reagents, or controls, and the verification of lot numbers and expiration dates—must be taken and documented. A protocol for corrective action is shown in **Figure 8–10.** A designated supervisor reviews all of the QC results.

Some laboratories may participate in a commercial QC program run by the manufacturer of the QC material. The results from the same lot of QC material are returned to the manufacturer for statistical analysis and comparison with other laboratories using the same methodology.

Internal Controls. Internal controls, also called procedural controls, consists of internal monitoring systems built into the test system. Internal controls monitor the sufficient addition of a patient specimen or reagent, the instrument's and reagent's interaction, and, for lateral flow test methods, whether the sample migrated through the test strip properly.[22]

Electronic Controls. Electronic controls use a mechanical or electrical device in place of a liquid QC specimen. This type of QC can be an internal or an external component inserted into a point-of-care (POC) instrument. Electronic controls verify the functional ability of a testing device, but it does not verify the integrity of the testing supplies. Many test systems use a combination of external and internal controls to verify that the entire test system is working properly.

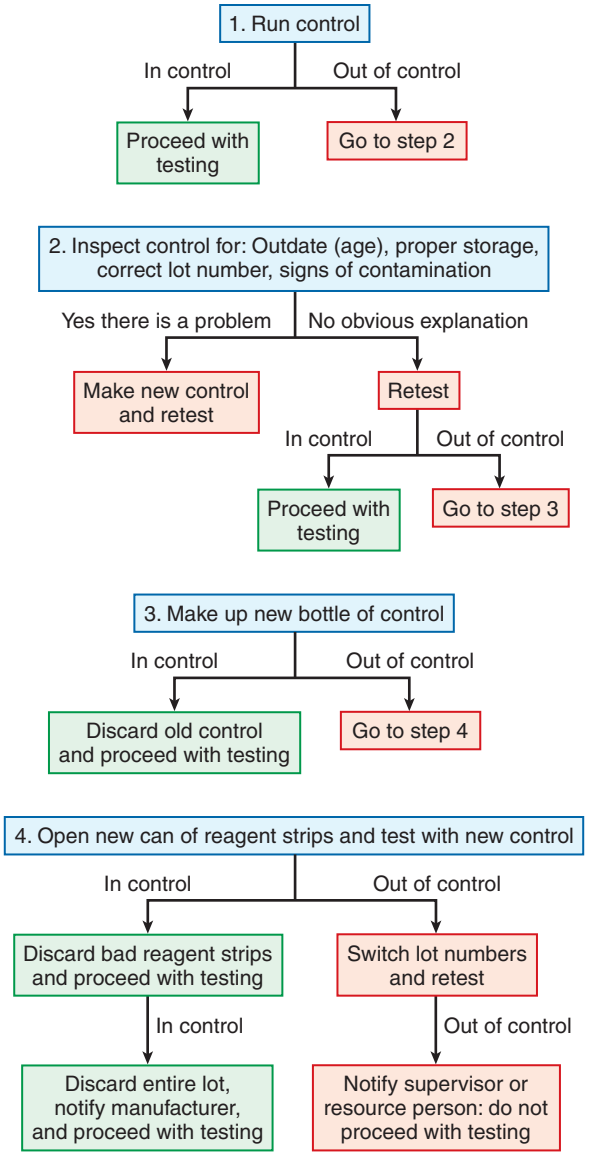


FIGURE 8–10 Procedure for "out of control" results. *(Adapted from Schweitzer SC, Schumann JL, Schumann GB. Quality assurance guidelines for the urinalysis laboratory.* J Med Technol. *1986; 3(11):567–572.*

Proficiency Testing (External Quality Assessment). **Proficiency testing**, or **external quality assessment (EQA)**, is the testing of unknown samples received from an outside agency. It provides unbiased validation of the accuracy, and thus quality, of patient test results. Several commercial vendors, such as the CAP, provide proficiency testing. Laboratories subscribing to these programs receive lyophilized or ready-to-use specimens. The results are returned to the proficiency testing vendors, where they are statistically analyzed with those from all participating laboratories. The laboratory director receives a report from the vendor, which enables the director to evaluate the laboratory's accuracy and compare it with other laboratories using the same method of analysis. The director ensures that the laboratory takes action to correct unacceptable results.[16] The CLIA mandate comparison testing for laboratory accreditation.[23]

Personnel and Facilities

QC is only as good as the personnel performing and monitoring it. Personnel assessment includes education and training, continuing education, competency assessment, and performance appraisals. Each new employee must have documentation of training during his or her orientation to the laboratory. This documentation is a checklist of procedures and must include the date and initials of the person doing the training as well as the employee being trained. Up-to-date reference materials and atlases should be readily available and documentation of continuing education must be maintained.[22]

An adequate, uncluttered, safe working area is also essential for both quality work and personnel morale. Standard precautions for handling body fluids must be followed at all times.

Postexamination Variables

Postexamination variables are processes that affect the reporting of results and correct interpretation of data.

Reporting Results

Standardized reporting methods minimize health-care provider confusion when interpreting results. The forms for reporting results should be designed so that they present the information in a logical sequence and provide adequate space for writing. A standardized reporting format and, when applicable, reference ranges should be included with each procedure in the procedure manual.

Electronic transmission is now the most common method for reporting results. Many automated instruments have the capability for the laboratorian to transmit results directly from the instrument to the designated health-care provider. It is essential that the laboratorian carefully review results before transmittal. Results may also be manually entered into the laboratory computer system and then transmitted to the health-care providers.

Documentation of the reporting of results is essential and required by accrediting agencies. In addition, permanent records of all reported results must be available. A method to verify the actual reporting of results also must be available and used by all employees.

The telephone is frequently used to transmit results of stat tests and critical values. Personnel on hospital units and from health-care providers often call requesting additional results. When telephoning results, confirm that the results are being reported to the appropriate person. The time of the call and the name of the person receiving the results must be documented according to the facility's policy. The Joint Commission Patient Safety Goals require that when verbally reporting test results the information must be repeated by the person receiving the information and documented by the person giving the report. Written procedures should be available for the reporting of critical values.

Result Errors

Errors may be discovered in the laboratory through a QM procedure known as the **delta check** that compares a patient's test results

with the previous results. Variation outside the established parameters alerts laboratory personnel to the possibility of an error that occurred during the testing procedure or in patient identification. Autoverification is often programmed into many laboratory analyzers.[20]

Erroneous results must be corrected in a timely manner to assure that the patient does not receive treatment based on incorrect results. Errors can occur in patient identification, specimen labeling, or result transcription. The patient's record should be corrected as soon as the error is detected. However, the original result must not be erased in the event that the health-care provider treated the patient based on the erroneous results. Appropriate documentation of erroneous results should follow institutional protocol. The manual must contain a written procedure for reporting, reviewing, and correcting errors.

The **In the Laboratory: Summary of Quality Management Errors** box summarizes QM errors in each phase of laboratory testing.

Interpreting Results

The specificity and the sensitivity for each test should be included in the procedure manual for correct interpretation of results. Sensitivity and specificity vary among manufacturers. All known interfering substances should be listed for evaluation of patient test data.

In the Laboratory

Summary of Quality Management Errors

Preexamination
- Patient misidentification
- Wrong test ordered
- Incorrect specimen type collected
- Insufficient specimen volume
- Delayed transport of specimen to the laboratory
- Inadequate processing of specimen
- Delayed separation of serum or plasma from cells
- Incorrect storage of specimen

Examination
- Sample misidentification
- Erroneous instrument calibration
- Reagent deterioration
- Poor testing technique
- Instrument malfunction
- Interfering substances present
- Misinterpretation of quality control data

Postexamination
- Patient misidentification
- Poor handwriting
- Transcription error
- Poor quality of instrument printer
- Failure to send report
- Failure to call critical values
- Inability to identify interfering substances

From Strasinger SK, DiLorenzo MA. *The Phlebotomy Textbook.* 3rd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.

Regulatory Issues

QM is regulated throughout the total testing system. The health-care regulation systems include both governmental and public agencies. All agencies have the same goal, which is to provide safe and effective health care.

Clinical Laboratory Improvement Amendments (CLIA)

The CLIA is a governmental regulatory agency administered by the Centers for Medicare and Medicaid Services (CMS) and the Food and Drug Administration (FDA). CLIA stipulates that all laboratories that perform testing on human specimens for the purposes of diagnosis, treatment, monitoring, or screening must be licensed and obtain a certificate from the CMS. Laboratories with CLIA certification are inspected to document compliance with the regulations. The inspections may be performed by CMS personnel or an accrediting agency recognized by CMS such as the CAP, the TJC, or the Commission on Laboratory Assessment (COLA).

CLIA classifies laboratory tests into three categories: waived, provider-performed microscopy procedures (PPMP), and nonwaived testing (See **In the Laboratory: CLIA Test Classifications**). Nonwaived testing is separated into the categories of moderate and high complexity with regard to requirements for personnel performing the tests. Laboratories must obtain the correct certification for the level of testing complexity performed. For each category, there is a description of the educational level necessary for personnel who

In the Laboratory

CLIA Test Classifications

Waived Testing
- Tests considered easy to perform by following the manufacturer's instructions that have little risk of error. No special training or education is required.
- Example: Urine pregnancy test

Provider-Performed Microscopy Procedures (PPMP)
- Microscopy tests performed by a physician, midlevel practitioner, or a dentist.
- Example: Microscopic urinalysis

Nonwaived Tests
- Moderate complexity tests

  Tests that require documentation of training in test principles, instrument calibration, periodic proficiency testing, and on-site inspections.

  Example: Automated complete blood count (CBC)
- High complexity tests

  Tests that require sophisticated instrumentation and a high degree of interpretation.
- Proficiency testing and on-site inspections are required.

  Example: Urine culture and susceptibility

From Strasinger SK, DiLorenzo MA. *The Phlebotomy Textbook.* 3rd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.

may perform the test, as well as the type of quality assurance procedures that must be in place.

Clinical and Laboratory Standards Institute (CLSI)

The CLSI is a nonprofit organization that publishes recommendations by nationally recognized experts for the performance of laboratory testing. CLSI standards are considered the standard of care for laboratory procedures. The **standard of care** is the attention, caution, and prudence that a reasonable person in the same circumstances would exercise. In a legal situation, the CLSI standards would be considered the standard of care that should have been met.

The Joint Commission (TJC)

TJC is an independent, not-for-profit organization that accredits and certifies more than 15,000 health-care organizations and programs in the United States. The mission of TJC is to continuously improve the safety and quality of care provided to the public through the provision of health-care accreditation and related services that support performance improvement in health-care organizations.

TJC has recently published the Joint Commission Patient Safety Goals. It is essential that health-care organizations adhere to these goals to maintain their accreditation. Goals pertaining to the laboratory are:

Goal 1: Improving the accuracy of patient identification
Goal 2: Improving the effectiveness of communication among health-care givers
Goal 7: Reduce the risk of health care-associated infections (HAIs)
Goal 13: Encourage patients' active involvement in their own care as a patient safety strategy

College of American Pathologists (CAP)

The CAP is an organization of board-certified pathologists that advocates high-quality and cost-effective medical care. CAP provides laboratory accreditation and proficiency testing for laboratories.

For accreditation purposes, CAP-trained pathologists and laboratory managers and technologists perform on-site laboratory inspections on a biennial basis. Inspectors examine the laboratory's records and QC of procedures for the preceding 2 years. Also examined are the qualifications of the laboratory staff including continuing education attendance, the laboratory's equipment, facilities, safety program, and laboratory management. CAP accreditation is accepted by both the CMS and the TJC and fulfills Medicare and Medicaid requirements.

As previously described, laboratories that subscribe to this proficiency program receive periodic samples to analyze and return their results to the CAP. The laboratory receives a report on how its results compared with other laboratories performing the procedures in a similar manner. Failure to perform satisfactorily on a proficiency test can result in a laboratory losing its CLIA certificate to perform the failed test.

Quality Management Systems

A **quality management system (QMS)** incorporates many of the objectives of total quality management and continuous quality improvement to ensure quality results, staff competence, and efficiency within an organization. In addition, QMS also utilizes the concepts of the International Organization for Standardization (ISO 151189) and the Lean and Six sigma methods. The requirements of TJC and the CAP accreditation organizations are included in QMS.

A QMS is designed to coordinate activities to direct and control an organization with regard to quality and the reduction of medical errors. The first step in a laboratory QMS is to determine the pathway of workflow through the laboratory as discussed previously under the preexamination, examination, and postexamination phases of testing. In each area of the pathway, all the processes and procedures that occur are determined and analyzed so everyone knows what they are supposed to do, how they are supposed to do it, and when they are supposed to do it.

Quality System Essentials

Quality system essentials (**QSEs)** form the basis of a QMS. The 12 QSEs contain the management information needed for a laboratory to perform quality work **(Table 8–2).** They were developed by the former National Committee for Clinical Laboratory Standards and the current CLSI and include the methods to meet the requirements of regulatory, accreditation, and standard setting organizations. **Quality indicators** are the measurements developed by each laboratory to determine if the quality system essentials are being met. They may include such items as appropriateness of the testing, correct patient identification, timely reporting of laboratory results, and correct proficiency testing results.

The Lean System

The **Lean system** originated with the automobile manufacturing industry in Japan. Its concepts have been adopted by many American industries, including the health-care industry. Lean utilizes a tool called "6S," which stands for: sort, straighten, scrub, safety, standardize, and sustain. The focus is on the elimination of waste to allow a facility to do more with less and at the same time increase customer and employee satisfaction. In the health-care environment, the ability to decrease costs while providing quality health care is of primary importance.

Six Sigma

Six Sigma is a statistical modification of the original Plan-Do-Check-Act (PDCA) method adopted by the TJC as a guideline for health-care organizations. The primary goal of Six Sigma is to reduce variables and decrease errors to a level of 3.4 defects per 1 million opportunities. Attaining this goal indicates that the laboratory is addressing factors critical to customer satisfaction and quality care.

Table 8–2 The 12 Laboratory Quality System Essentials

QUALITY SYSTEM ESSENTIALS	PROCESSES AND PROCEDURES
The Laboratory QSEs	
1. Organization	Personnel roles, responsibilities, and reporting relationships Quality planning and risk assessment Allocation of personnel and material resources Review and assessment of meeting goals
2. Facilities and safety	Space designed for efficiency Adequate storage space Required safety precautions and equipment availability Housekeeping Safety training
3. Personnel	Qualifications Current job descriptions Orientation of new employees Competency assessment Continuing education
4. Equipment	Selection criteria Space needed and special instrument requirements Ongoing preventive maintenance Service and repair records
5. Purchasing and inventory	Inventory of initial materials and reagents Service contracts Availability of reagents, supplies, and service
The Work System QSEs	
6. Process control	Identification of all laboratory processes Procedure manuals and instructions for tasks Test method verification Verification that manufacturer specifications are in procedure manuals Quality control and statistics
7. Documents and records	Availability of all process and procedure documents Periodic review of all process and procedure documents Access to quality control records Monitoring of record storage and retention
8. Information management	Availability of patient records Security of patient records Methods for providing patient information Processes to prevent Medicare and Medicaid fraud
The Measurement QSEs	
9. Occurrence management and nonconforming event management	Identification and reporting of all events Remedial actions taken Plans to eliminate future events Initiation of changes
10. Assessments: external and internal	Obtaining external licensing and accreditation Participation in external proficiency testing Periodic on-site auditing by accrediting agencies Development of quality indicators for each phase of testing
11. Customer service	Feedback from customers including patients, patients' families, and health-care providers Feedback from employees Feedback from offsite referral laboratories and health-care providers
12. Process improvement	Monitoring of the above QSEs results Determination of the root cause of problems Utilize the Lean system tools Utilize Six Sigma methodology

The Six Sigma methodology is represented by the acronym DMAIC:

Define goals and current processes
Measure current processes and collect data
Analyze the data for cause-and-effect information
Improve the process using the data collected
Control the correction of concerns displayed in the data

By instituting quality improvement methodologies, a healthcare institution can develop a structured standardized format to systematically assess and document the quality of services to the customer.

SUMMARY

- Transmission of biological hazards that are encountered when testing patient specimens requires a chain of infection, which consists of an infectious agent, reservoir, portal of exit, mode of transmission, portal of entry, and a susceptible host.
- Hand hygiene and wearing PPE are essential actions to prevent transmission of infectious organisms. Standard precautions should be followed at all times.
- Specimens, except urine, and contaminated supplies must be disposed of in a biohazard container.
- All sharps, including needles and holders, must be disposed of in puncture-proof containers. Recapping of needles is prohibited.
- The Occupational Exposure to Bloodborne Pathogens Standards are a means of providing protection from accidental exposure to bloodborne pathogens through the use of engineering controls, work practice controls, and use of PPE.
- When transporting biological specimens, Department of Transportation and International Air Transit Association regulations must be followed. They include placing specimens in screw cap containers, wrapping them in absorbent material, and placing them in a sturdy leakproof container.
- Follow specific directions when mixing chemicals and always add acid to water, rather than water to acid.
- When chemical contact with the skin or eyes occurs, immediately flush the area with water for 15 minutes.
- A SDS and a Chemical Hygiene Plan must be available to employees. Dispose of chemicals per EPA guidelines.
- Dispose of radioactive material following NRC guidelines.
- Be observant for frayed cords, overloaded circuits, and improperly grounded equipment. Avoid working with electrical equipment when you, or the equipment, is wet.
- Follow routine safety protocols and maintain a clean, organized work area to avoid physical hazards.
- The acronym RACE outlines the steps to follow when a fire is discovered: (R) rescue anyone in danger, (A) activate the fire alarm, (C) contain the fire, (E) extinguish the fire if possible or evacuate, closing the door.
- Quality management is the overall process of guaranteeing quality throughout the entire testing system.
- Quality control involves performing individual procedures using acceptable standards and control material at various medically significant levels.
- Documentation includes a procedure manual, policies to control and monitor procedure variables, and records of competency assessment and continuing education.
- Preexamination variables occur before sample testing. Examination variables occur during the specimen testing. Postexamination variables occur during interpretation and reporting of test results.
- Agencies regulating the laboratory include:
  - CLIA—provides requirements for persons performing waived, provider-performed microscopy, moderate-complexity, and high-complexity testing
  - TJC—provides accreditation and certification of healthcare organizations
  - CAP—provides laboratory accreditation and provision of proficiency testing
  - CLSI—develops written standards and guidelines for sample collection, handling and processing, and laboratory testing and reporting
- The 12 quality essentials provide the management documentation needed to demonstrate quality work. Quality indicators are developed to monitor each phase of testing.
- The Lean system utilizes the "6S" tools (sort, straighten, scrub, safety, standardize, and sustain) to enhance efficiency and proficiency.
- The goal of the statistical Six Sigma method is to reduce variables and decrease errors to a level of 3.4 defects per 1 million opportunities.

CASE STUDIES

1. The immunology supervisor who has been working for the last 20 years in a small rural hospital is training a new employee. A dilution of a patient's serum must be made to run a particular test. The supervisor is having difficulty using a serological pipette so she removes one glove. In uncapping the serum tube, a small amount of serum splashes onto the workbench. She cleans this up with a paper towel, which she discards in the regular paper trash. She also spills a small amount onto her disposable lab coat. She tells the new employee that, because it is such a small amount, she isn't going to worry about it and continues on to pipette the specimen. She then replaces the glove onto her ungloved hand and says that, because it is almost break time, she will wait to wash her hands until then.

   Questions

   a. Please identify all the safety violations involved.

2. As the supervisor of the immunology section, you encounter the following situations. Explain whether you would accept them or take corrective action.

   Questions

   a. You are told that only the supervisor performs the CAP proficiency survey.
   b. QC is not performed daily on the Centaur instrument.
   c. The Streptozyme test reporting procedure has been recently revised.
   d. Opened, unlabeled commercial quality control bottles are in the refrigerator.

REVIEW QUESTIONS

1. A technologist who observes a red rash on her hands after removing her gloves
   a. should apply antimicrobial lotion to the hands.
   b. may be washing the hands too frequently.
   c. may have developed a latex allergy.
   d. should not create friction when washing the hands.

2. In the chain of infection, a contaminated work area would serve as which of the following?
   a. Reservoir
   b. Means of transmission
   c. Portal of entry
   d. Portal of exit

3. The only biological waste that does not have to be discarded in a container with a biohazard symbol is
   a. urine.
   b. serum.
   c. feces.
   d. serum tubes.

4. Patient specimens transported by the Department of Transportation must be labeled as a
   a. diagnostic specimen.
   b. clinical specimen.
   c. biological specimen, category b.
   d. laboratory specimen.

5. A technician places tightly capped noninfectious serum tubes in a rack and places the rack and the specimen data in a labeled leakproof metal courier box. Is there anything wrong with this scenario?
   a. Yes, DOT requirements are not met.
   b. No, the tubes are placed in a rack.
   c. Yes, absorbent material is missing.
   d. No, the box contains the specimen data.

6. The Occupational Exposure to Bloodborne Pathogens Standard developed by OSHA requires employers to provide all of the following *except*
   a. hepatitis B immunization.
   b. safety training.
   c. hepatitis C immunization.
   d. laundry facilities for nondisposable lab coats.

7. An employee who receives an accidental needlestick should immediately
   a. apply sodium hypochlorite to the area.
   b. notify a supervisor.
   c. receive HIV prophylaxis.
   d. receive a hepatitis B booster shot.

8. The first thing to do when acid is spilled on the skin is to
   a. notify a supervisor.
   b. neutralize the area with a base.
   c. apply burn ointment.
   d. flush the area with water.

9. When combining acid and water,
   a. acid is added to water.
   b. water is added to acid.
   c. water is slowly added to acid.
   d. both solutions are combined simultaneously.

10. To determine the chemical characteristics of sodium azide, an employee would consult the
   a. Chemical Hygiene Plan.
   b. Merck manual.
   c. SDS.
   d. NRC guidelines.

11. A technician who is pregnant should avoid working with
   a. organic chemicals.
   b. radioisotopes.
   c. HIV-positive serum.
   d. needles and lancets.

12. Which of the following laboratory regulatory agencies classifies laboratory tests by their complexity?
   a. OSHA
   b. CAP
   c. TJC
   d. CMS

13. Which of the following organizations publishes guidelines that are considered the standard of care for laboratory procedures?
   a. CLIA
   b. CLSI
   c. TJC
   d. CAP

14. Quality managment refers to
   a. performance of two levels of testing controls.
   b. reliable control results.
   c. increased productivity.
   d. quality of specimens and patient care.

15. When external quality control is run, what information must be documented?
   a. The lot number
   b. Expiration date of the control
   c. The test results
   d. All of the above

16. What steps are taken when the results of the quality control testing are outside of the stated confidence limits?
   a. Check the expiration date of the control material
   b. Run a new control
   c. Open a new control bottle
   d. All of the above

17. When a new bottle of QC material is opened, what information is placed on the label?
   a. The time the bottle was opened
   b. The supervisor's initials
   c. The lot number
   d. The date and the laboratory worker's initials

18. What is the primary goal of TQM?
   a. Precise test results
   b. Increased laboratory productivity
   c. Improved patient outcomes
   d. Reproducible test results

19. Would a control sample that has accidentally become diluted produce a trend or a shift in the Levey-Jennings plot?
   a. Trend
   b. Shift

Fill in the Blank

20. Indicate whether each of the following would be considered a (1) preexamination, (2) examination, or (3) postexamination variable by placing the appropriate number in the space.
   _____ Reagent expiration date
   _____ Rejection of a hemolyzed specimen
   _____ Construction of a Levey-Jennings chart
   _____ Telephoning a critical result to the nurse
   _____ Calibrating the centrifuge
   _____ Pipetting the diluent

9 Principles of Serological Testing

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Describe how whole blood is processed in order to obtain serum for serological testing.
2. Explain the difference between a volumetric and a graduated pipette.
3. Define the following: serial dilution, solute, diluent, compound dilution.
4. Describe how an accurate measurement is made using a serological pipette that is marked TD.
5. Calculate the final dilution of a sample, given the initial dilution and all subsequent dilutions.
6. Explain how an antibody titer is determined.
7. Calculate the amount of diluent needed to prepare a specific dilution of a serum specimen.
8. Determine how to make a specific percent solution from a concentrate.
9. Differentiate sensitivity and specificity as it relates to serological testing.
10. Discuss how positive and negative predictive values determine the chance that an individual has a truly positive or truly negative test result.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Blowout pipette
Diluent
Graduated pipettes
Micropipette
Negative predictive value
Positive predictive value
Sensitivity
Serial dilution
Serological pipette
Serology
Serum
Solute
Specificity
Titer
Volumetric pipettes

Serology is the study of the fluid components in the blood, especially antibodies. **Serum,** the liquid portion of the blood, minus the coagulation factors is the most frequently encountered specimen in immunologic testing. Knowledge of specimen preparation and dilutions is essential to understanding all serological testing in the clinical laboratory.

Blood Specimen Preparation and Measuring

Blood is collected aseptically by venipuncture into a clean, dry, sterile tube. Care must be taken to avoid hemolysis as this may produce a false-positive test. The blood specimen is allowed to clot at room temperature or at 4°C, depending upon the protocol for the specific procedure. It is then centrifuged, after which serum should be promptly separated into another tube without transferring any cellular elements. Fresh serum that has not been inactivated by heating is usually recommended for testing. For some testing, however, complement must be inactivated because it interferes with test results. In this case, the serum is heated to 56°C for 30 minutes to destroy any complement present. In either circumstance, if testing cannot be performed immediately, serum may be stored between 2°C and 8°C for up to 72 hours. If there is any additional delay in testing, the serum should be frozen at –20°C or below.

Pipettes are commonly used to measure either serum for testing or liquid for making reagents and dilutions. The pipettes are calibrated to transfer or deliver specific volumes as marked on their surfaces. They can be categorized as either volumetric or graduated.

Volumetric pipettes are marked and calibrated to deliver only one volume of the specified liquid **(Fig. 9–1).**[1] The design enables the user to dispense the exact measure of liquid with a small drop left behind. Pipettes are usually labeled TD, meaning *to deliver.* The volumetric pipettes have an oval bulb in the center and a tapered dispensing end. Volumetric pipettes are used with some sort of suctioning device such as a rubber bulb. The bulb is squeezed to draw the measured amount of liquid into the pipette. Excess fluid is wiped off the outside of the pipette and then the pipette is held vertically with the tip against the surface of a container. The suction is released and the liquid is allowed to flow by gravity into the container.

Graduated pipettes have markings that allow for varying amounts of liquid to be measured **(Fig. 9–2).** A graduated or measuring pipette has marks all along its length. If it is a **serological pipette,** the marks go all the way down to the tip. Some serological pipettes have a frosted band around the opening; this type is called a **blowout pipette.** These pipettes may be labeled TC, meaning *to contain*. If they are filled to the end, the last drop of liquid must be forced out using a pipetting bulb or other device to deliver an accurate volume.[1] Alternatively, a specified amount of liquid can be measured from point to point in the pipette. For example, if 0.3 mL is measured using a 1-mL TC pipette, the liquid can be dispensed by going from 0.0 to the 0.3 mL mark, rather than filling the pipette to the 0.7 mL mark and dispensing down to the end.

Generally, a measuring pipette is held in a vertical position when drawing up a liquid **(Fig. 9–3).** The bottom of the meniscus should be level with the calibration line on the pipette. This is sighted at eye level (see Fig. 9–3B). In practice, measuring pipettes are more typically used to prepare reagents rather than for measurement of patient specimens and controls. Often, an automatic pipette filler is used with measuring pipettes to increase the accuracy **(Fig. 9–4).** For patient specimens and control calibrators, micropipettes are much more accurate.[2]

Micropipettes deliver volumes in the microliter (μL) range and can be used when very small volumes are needed. Micropipettes are mechanical pipettes that draw up and then release a certain volume by depressing a plunger **(Fig. 9–5).** A one-time use disposable tip is used for each different specimen. Some micropipettes are adjustable and can hold a small range

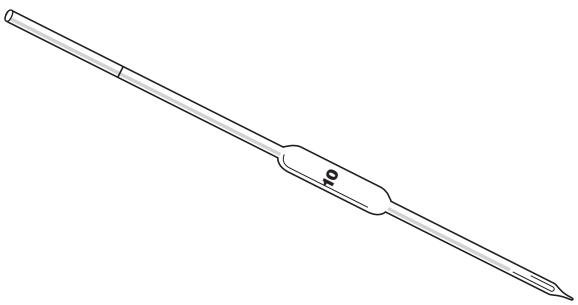


FIGURE 9–1 Volumetric pipette.

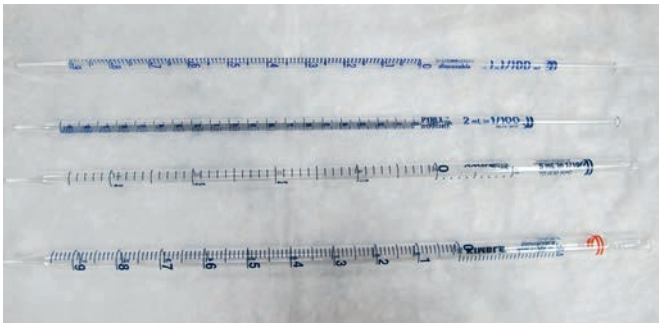


FIGURE 9–2 Four different sizes of graduated pipettes.

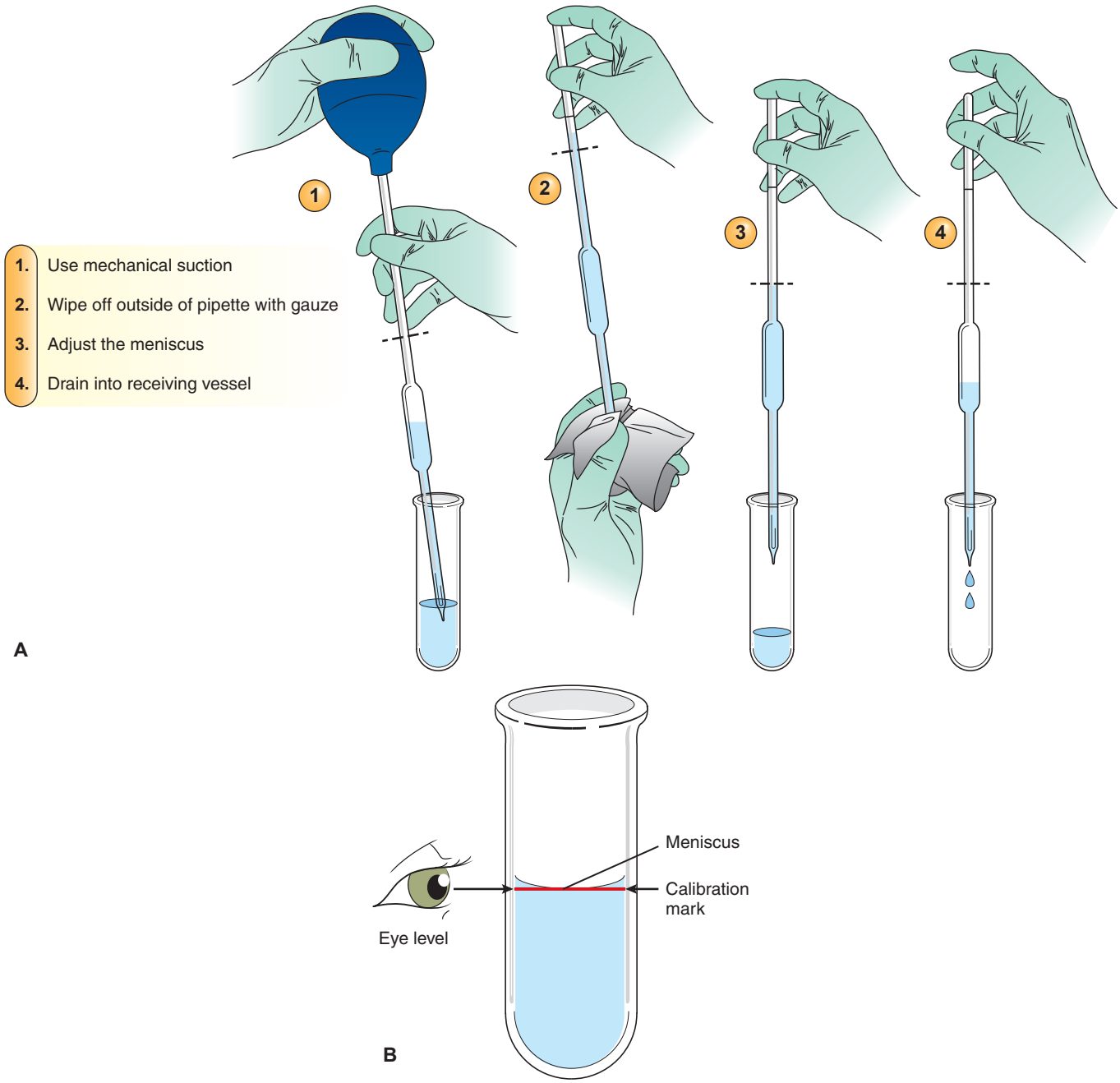


FIGURE 9–3 (A) Steps in accurately measuring a liquid with a serological pipette. (B) How to read a meniscus.

of volumes, whereas others only hold a fixed volume. Micropipettes are easier to use than pipettes with safety bulbs, as well as more accurate.

Dilutions

In the clinical laboratory, it is often necessary to make a less concentrated solution from a reagent such as an acid or a buffer in order to use the reagent in a particular procedure. In this case, either water or saline is added to the concentrate to make the reagent the proper strength for testing. For many serology tests, it is the serum that is concentrated; it may be necessary to dilute it with saline in order for a visible reaction to occur. If the relative proportions of antigen and antibody present are not similar, the reaction cannot be detected. When too much antibody is present, an end point may not be reached. In this case, the serum that contains antibody must be diluted. Therefore, knowledge of dilutions is essential to understanding all serological testing.

Simple Dilutions

A dilution involves two entities: the **solute,** which is the material being diluted, and the **diluent,** which is the medium making up the rest of the solution. The relationship between these two is a ratio that can also be expressed as a fraction. For example, a 1:20 dilution can be set up as a fraction,

FIGURE 9–4 A pipette filler for use with serological pipettes ranging in size from 1 to 100 mL.

FIGURE 9–5 Three different sizes of micropipettes.

1/20. This dilution implies 1 part of solute and 19 parts of diluent. The number on the bottom of the fraction is the total volume, reached by adding the volumes of the solute and diluent together.

$$1/\text{Dilution} = \text{Amount of Solute}/\text{Total Volume}$$

To create a certain volume of a specified dilution, it is helpful to know how to manipulate this relationship. An algebraic equation can be set up to find the total volume, the amount of solute, or the amount of diluent needed to make a dilution. Consider the following example:

> 2 mL of a 1:20 dilution is needed to run a specific serological test. How much serum and how much diluent are needed to make this dilution?

The equation is set up using the fraction for the dilution, indicating the relationship between the total volume and the solute, or the amount of serum needed:

$$1/20 = x/2 \text{ mL}$$

Note that the 20 represents the total number of parts in the solution and that 2 mL is the total volume desired.

Cross-multiplying to solve this equation for x gives 0.1 mL for the amount of serum needed to make this dilution. The amount of diluent is obtained by subtracting 0.1 mL from 2.0 mL to give 1.9 mL of diluent. To check the answer, set up a proportion between the amount of solute over the total volume. This should equal the dilution desired.

$$0.1 \text{ mL}/(1.9 \text{ mL diluent} + 0.1 \text{ mL serum}) = 1/20$$

Thus, the correct answer has been obtained.

If, on the other hand, one knows the amount of serum to be used, a problem can be set up in the following manner:

> A 1:5 dilution of patient serum is necessary to run a serological test. There is 0.1 mL of serum that can be used. What amount of diluent is necessary to make this dilution using all of the serum?

A slightly different formula can be used to solve this problem:

$$1/\text{Dilution} - 1 = \text{Amount of Solute}/\text{Amount of Diluent}$$

$$¼ = 0.1 \text{ mL}/x$$

$$x = 0.4 \text{ mL of diluent}$$

Note that the final volume is obtained by adding 0.1 mL of solute to the 0.4 mL of diluent. Dividing the volume of the solute by the total volume of 0.5 mL yields the desired 1:5 ratio.

Depending on the unknown being solved for, either of these formulas can be used. To calculate the total volume, the total dilution factor must be used. If, however, the amount of diluent is to be calculated, the formula using dilution–1 can be used.

Consider a further example:

> Instructions that come with a buffer indicate that it must be mixed with 19 parts of water for use in a serological test. The volume of the buffer concentrate is 50 mL. How would we find out the amount of water to add and what would be the final dilution factor?

The equation would be set up as follows:

$$1/\text{Dilution} - 1 = 50 \text{ mL}/\text{ Amount of Diluent}$$

$$1/19 = 50/x$$

$$x = 50 \times 19$$

$$x = 950 \text{ mL of diluent}$$

First, we multiply 50 mL by the 19 parts to give us 950 mL, which is the amount of water that needs to be added to the buffer concentrate. To give the final volume, we add the 50 mL of concentrate to the 950 mL of water to get 1,000 mL total.

Sometimes it is necessary to dilute a concentrate to a specific percentage. A percentage is simply a different way of expressing a dilution. For instance, a 10% solution can also be expressed as 1/10. In dealing with volumes, the relationship

is volume/volume. It can also be expressed as weight/volume if a solid is to be dissolved in a liquid. Let's take the example of diluting glacial acetic acid to make a 10% solution for a testing procedure. A volume of 500 mL of a 10% solution of acetic acid is needed. How much glacial acetic acid is needed and how much diluent should be used?

We know the final volume and we know the percentage, so then we are solving for the amount of the concentrated acetic acid needed.

$$1/10 = x/500 \text{ mL}$$

Cross-multiplying and solving for x gives us 50 mL of glacial acetic acid needed. Then the amount of diluent is the total volume minus the concentrate, or 500 mL – 50 mL = 450 mL of diluent to make up the 10% solution. Additional problems which give more practice with dilution calculations can be found in the Review Questions section at the end of this chapter.

Compound Dilutions

The previous examples represent simple dilutions. Occasionally in the laboratory it is necessary to make a very large dilution; if so, it is more accurate and less costly to do this in several steps rather than all at once. Such a process is known as a compound dilution. The same approach is used, but the dilution occurs in several stages. For example, if a 1:500 dilution is necessary, it would take 49.9 mL of diluent to accomplish this in one step with 0.1 mL of serum. If only a small amount of solution is needed to run the test, this is wasteful; furthermore, inaccuracy may occur if the solution is not properly mixed. Therefore, it is helpful to make several smaller dilutions.

To calculate a compound dilution problem, the first step is to plan the number and sizes of simple dilutions necessary to reach the desired end point. To use the preceding example, a 1:500 dilution can be achieved by making a 1:5 dilution of the original serum, a 1:10 dilution from the first dilution, and another 1:10 dilution. This can be shown as follows:

Serum:

1:5 dilution	**1:10 dilution**	**1:10 dilution**
0.1 mL serum	0.1 mL of 1:5 dilution	0.1 mL of 1:10 dilution
0.4 mL diluent	0.9 mL diluent	0.9 mL diluent

Multiplying 5 × 10 × 10 equals 500, or the total dilution. Each of the simple dilutions is calculated individually by doing mental arithmetic or by using the formula given for simple dilutions. In this example, the 1:500 dilution was made using very little diluent in a series of test tubes, rather than having to use a larger volume in a flask. The volumes were kept small enough so that mixing could take place easily. The final volume of 1.0 mL is all that is necessary to perform a test.

If, in each step of the dilution, the dilution factor is exactly the same, this is known as a **serial dilution.** Serial dilutions are often used to obtain a **titer,** or indicator of an antibody's strength. A series of test tubes is set up with exactly the same amount of diluent in each **(Fig. 9–6).** The most common serial dilution is a doubling dilution in which the amount of serum is cut in half with each dilution. For example, six test tubes can be set up with 0.2 mL of diluent in each. If 0.2 mL of serum is added to the first tube, this becomes a 1:2 dilution.

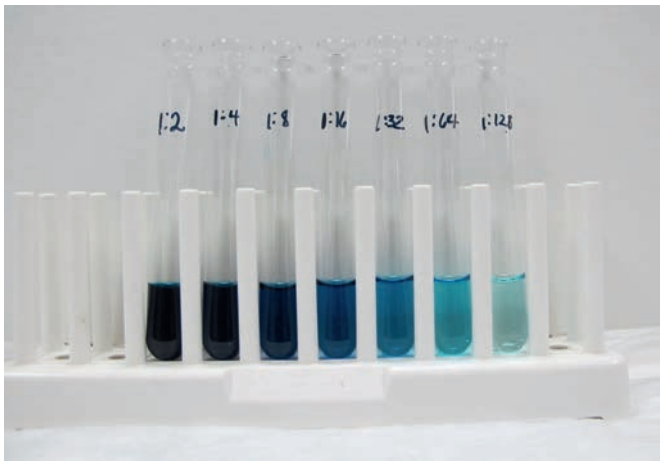


FIGURE 9–6 Serial dilution. Each tube contains the same amount of diluent and a blue dye. Each time a dilution is made, the amount of dye is cut in half in each successive tube. The final volume of each tube should be exactly the same. The color becomes visibly lighter with each dilution.

$$0.2 \text{ mL Serum}/(0.2 \text{ mL Serum} + 0.2 \text{ mL Diluent}) = 0.2 \text{ mL}/0.4 \text{ mL} = 1/2$$

When 0.2 mL of the 1:2 dilution is added to 0.2 mL of diluent, a 1:4 dilution is obtained. The final dilution is obtained by counting the number of tubes and setting up a multiplication series in which the original dilution factor is raised to a power equal to the number of tubes. In this example, if the first tube contains a 1:2 dilution, the dilution in tube number six is

$$½ \times ½ \times ½ \times ½ \times ½ \times ½ = 1/64$$

If, in this instance, an end point was reached at tube number five, the actual titer would be 1:32. To avoid confusion, this is customarily written as the reciprocal of the dilution—that is, 32. The titer is the last tube in which a positive reaction is visible. The tubes are read all the way to the end of the dilution; then, after finding the first negative tube, the positive tube before it is reported out as the titer.

Serial dilutions do not always have to be doubling dilutions. Consider the following set of test tube dilutions:

$$1:5 \rightarrow 1:25 \rightarrow 1:125 \rightarrow 1:625 \rightarrow 1:3125$$

For each successive tube, the dilution is increased by a factor of 5, so this would indeed be considered a serial dilution. Having the ability to work with simple and compound dilutions and interpret serial dilutions is a necessary skill for laboratory work.

Test Parameters

A question sometimes arises as to whether or not the patient actually has the condition or disease when results are

obtained in a laboratory test. In order for the clinician to make such a judgment, it is important to know the sensitivity and specificity of a particular test. The **sensitivity** can be defined as the proportion of people who have a specific disease or condition and who have a positive test.[3] It is described by the following ratio:

$$\text{Sensitivity (\%)} = \frac{\text{True Positives}}{\text{True Positives and False Negatives}} \times 100$$

The sensitivity thus indicates how small an amount can be measured and still produce a positive test result. The **specificity** refers to the proportion of people who do not have the disease or condition and who have a negative test.[3] It can be expressed as the following:

$$\text{Specificity (\%)} = \frac{\text{True Negatives}}{\text{True Negatives and False Positives}} \times 100$$

Let's take an example of test results to calculate the sensitivity and specificity. A certain new laboratory test was used with a particular population. The results were as follows: 200 patients were tested and there were 160 true positives, 20 true negatives, 8 false positives, and 12 false negatives. The sensitivity is calculated by taking the true positives and dividing by the true positives added to the false negatives:

$$\text{Sensitivity (\%)} = 160/(160 + 12) \times 100$$
$$\text{Sensitivity} = 93\%$$

To calculate the specificity, the following formula is used:

$$\text{Specificity (\%)} = 20/(20 + 8) \times 100$$
$$\text{Specificity} = 71.4\%$$

Thus, this particular test is highly sensitive, but it is not very specific. A test that is highly specific means that it measures only the substance that it is designed to measure and does not measure any interfering substances. If a test is both highly sensitive and highly specific, a positive result indicates that the patient likely has the disease or condition.

Sensitivity and specificity are characteristics of the test itself. However, in order for a clinician to determine whether a person with a positive test actually has the disease, it is important to look at how often the disease occurs in the particular population. The **positive predictive value** is the probability that a person with a positive screening test actually has the disease. The **negative predictive value** is the probability that a person with a negative screening test does not have the disease. Let's see how this is calculated with some actual test results. The positive predictive value is determined by dividing the true positive results by the true positive and the false positive results added together. Let's use the example with the sensitivity of 93% calculated earlier.

$$\text{Positive Predictive Value (\%)} = \frac{\text{True Positives}}{\text{True Positives and False Positives}} \times 100$$

In the example given earlier, out of 200 patients, 160 were truly positive and 8 were falsely positive. Using these values in the equation,

$$\text{Positive Predictive Value (\%)} = \frac{160}{160 + 8} \times 100$$
$$\text{Positive predictive value (\%)} = 95\%$$

Thus, in this case, if an individual tests positive for a certain disease, it is highly likely that the individual has the disease.

Now let's look at negative predictive value for the same test results.

$$\text{Negative Predictive Value (\%)} = \frac{\text{True Negatives}}{\text{True Negatives + False Negatives}} \times 100$$

$$\text{Negative Predictive Value (\%)} = \frac{20}{20 + 12} \times 100$$
$$\text{Negative predictive value (\%)} = 62.5\%$$

In this case, because there are a number of false-negative results, the negative predictive value is not as high as the positive predictive value. These results would help the clinician make a decision about an individual patient based on his or her test results and the prevalence of the disease in that particular population.

SUMMARY

- Serum for serological testing is obtained by allowing a sterile tube to clot at either room temperature or 4°C and then carefully removing the serum from the clot after centrifugation has taken place.
- Volumetric pipettes hold a specified amount of liquid and are calibrated to deliver (TD) that exact amount.
- Serological pipettes are calibrated all the way to the bottom of the pipette and must be blown out to deliver the exact amount of liquid required.
- A dilution is the addition of a liquid to make a weaker solution of either a reagent or a patient specimen. In order for a visible end point to occur in antigen–antibody reactions, often a dilution needs to be made.
- Patient serum, the solute, is made weaker by adding diluent so that the antibody present is not as concentrated. The relationship between the serum and the total volume can be expressed as a ratio, 1:20, or as a fraction—for example, 1/ 20.
- When several dilutions are made in which the dilution factor is the same in each case, this is called a serial dilution.
- Serial dilutions are used to determine the titer, or strength, of an antibody. The last tube in which a visible reaction is seen is considered the end point.
- Sensitivity is defined as the proportion of people who have a specific disease or condition and have a positive test for that disease or condition.
- Specificity refers to the proportion of people who do not have the disease or condition and who have a negative test for that disease or condition.

- If a test is highly sensitive and highly specific, it is a good indicator that a patient has the disease or condition if positive results are obtained.
  - The positive predictive value is the likelihood that a person with a positive screening test actually has the disease.
  - The negative predictive value is the probability that a person with a negative screening test does not have the disease.
  - Positive and negative predictive values help the clinician to determine whether a positive or a negative test is likely to be a true result based on a specific test population.

CASE STUDY

The serology supervisor who has been working for the last 20 years in a small rural hospital is training a new employee. A dilution of a patient's serum must be made to run a particular test. The supervisor is showing the new employee how to pipette the serum specimen. The amount needed is 0.1 mL. Using a serological pipette, she draws up the patient specimen to the 0.9 mL mark. She then lets it drain out. There is a tiny bit left in the pipette, but she explains to the new person that this is close enough. She then adds 1.9 mL of diluent to the tube with the serum. She needs a 1:40 dilution of the serum to run the test.

Questions

a. Explain any mistakes the supervisor may have made during her demonstration.
b. Was the dilution correct? If necessary, correct the dilution.

REVIEW QUESTIONS

1. If serum is not tested immediately, how should it be treated?

a. It can be left at room temperature for 24 hours.
b. It can be stored in the refrigerator for up to 72 hours.
c. It can be stored in the refrigerator for up to 48 hours.
d. It needs to be frozen immediately.

2. A 1:750 dilution of serum is needed to perform a serological test. Which of the following series of dilutions would be correct to use in this situation?

a. 1:5, 1:15, 1:10
b. 1:5, 1:10, 1:5
c. 1:15, 1:10, 1:3
d. 1:15, 1:3, 1:5

3. How much diluent needs to be added to 0.2 mL of serum to make a 1:20 dilution?

a. 19.8 mL
b. 4.0 mL
c. 3.8 mL
d. 10.0 mL

4. If glacial acetic acid needs to be diluted with water to make a 10% solution, what does the glacial acetic acid represent?

a. Solute
b. Diluent
c. Titer
d. Serial dilution

5. A pipette that has markings all the way down to its tip is called a

a. volumetric pipette.
b. serial pipette.
c. graduated pipette.
d. micropipette.

6. A serological test requires 5 mL of a 1:50 dilution. How much serum is required to make this dilution?

a. 0.5 mL
b. 0.01 mL
c. 1.0 mL
d. 0.1 mL

7. If 0.02 mL of serum is diluted with 0.08 mL of diluent, what dilution of serum does this represent?
   a. 1:4
   b. 1:5
   c. 1:10
   d. 1:20

8. A tube containing a 1:40 dilution is accidently dropped. A 1:2 dilution of the specimen is still available. A volume of 4 mL is needed to run the test. How much of the 1:2 dilution is needed to remake 4 mL of a 1:40 dilution?
   a. 0.2 mL
   b. 0.4 mL
   c. 0.5 mL
   d. 1.0 mL

9. If 0.4 mL of serum is mixed with 15.6 mL of diluent, what dilution of serum does this represent?
   a. 1:4
   b. 1:40
   c. 2:70
   d. 1:80

10. How much diluent needs to be added to 0.1 mL of serum to make a 1:15 dilution?
    a. 1.4 mL
    b. 1.5 mL
    c. 5.0 mL
    d. 15 mL

11. Which of the following choices would be considered a serial dilution?
    a. 1:5, 1:15, 1:20
    b. 1:2, 1:10, 1:25
    c. 1:15, 1:30, 1:40
    d. 1:5, 1:15, 1:45

12. The following dilutions were set up to titer an antibody. The following results were obtained: 1:4 +, 1:8 +, 1:16 +, 1:32 +, 1:64 –. How should the titer be reported out?
    a. 4
    b. 16
    c. 32
    d. 64

13. If a serological test is positive for an individual who does not have a particular disease, the result was caused by a problem with
    a. sensitivity.
    b. specificity.
    c. accuracy.
    d. poor pipetting.

14. Which of the following would be the correct way to make a 5% solution of hydrochloric acid from concentrated hydrochloric acid?
    a. 0.5 mL of acid and 9.5 mL of water
    b. 0.5 mL of acid and 95 mL of water
    c. 0.1 mL of acid and 9.9 mL of water
    d. 0.1 mL of acid and 4.9 mL of water

15. What is the final dilution of serum obtained from the following serial dilutions: 1:4, 1:4, 1:4, 1:4, 1:4, 1:4?
    a. 1:24
    b. 1:256
    c. 1:1,024
    d. 1:4,096

16. A new laboratory assay gave the following results: number of patients tested = 100; number of true positives = 54, number of true negatives = 42; number of false positives = 2; number of false negatives = 2. What is the specificity of this assay in whole numbers?
    a. 75%
    b. 85%
    c. 95%
    d. 98%

17. What is the sensitivity of the assay in Question 16?
    a. 84%
    b. 90%
    c. 92%
    d. 96%

18. A screening test gave the following results: number of patients tested = 150; number of true positives = 50; number of true negatives = 85; number of false positives = 5; number of false negatives = 10. What is the positive predictive value rounded off to a whole number for a patient whose test is positive?
    a. 91%
    b. 83%
    c. 89%
    d. 56%

10 Precipitation and Agglutination Reactions

Christine Dorresteyn Stevens, EdD, MT(ASCP)

LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Discuss affinity and avidity and their influence on antigen–antibody reactions.
2. Describe how the law of mass action relates to antigen–antibody binding.
3. Distinguish between precipitation and agglutination.
4. Explain how the zone of equivalence is related to the lattice hypothesis.
5. Differentiate between turbidimetry and nephelometry and discuss the role of each in measurement of precipitation reactions.
6. Compare single diffusion to double diffusion.
7. Summarize the principle of the end-point method of radial immunodiffusion.
8. Determine the relationship between two antigens by looking at the pattern of precipitation resulting from Ouchterlony immunodiffusion.
9. Describe immunofixation electrophoresis and explain how it differs from passive diffusion.
10. Recognize how immunoglobulin M (IgM) and immunoglobulin G (IgG) differ in their ability to participate in agglutination reactions.
11. Describe physiological conditions that can be altered to enhance agglutination.
12. Define and give an example of each of the following:
    - **a.** Direct agglutination
    - **b.** Passive agglutination
    - **c.** Reverse passive agglutination
    - **d.** Agglutination inhibition
    - **e.** Hemagglutination inhibition
13. Describe the principle of measurement used in particle-counting immunoassay (PACIA).
14. Identify conditions that must be met for optimal results in agglutination testing.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Affinity
Agglutination
Agglutination inhibition
Agglutinins
Avidity
Direct agglutination
Electrophoresis
End-point method
Hemagglutination
Hemagglutination inhibition
Immunofixation electrophoresis
Lattice
Law of mass action
Nephelometry
Ouchterlony double diffusion
Particle-counting immunoassay (PACIA)
Passive agglutination
Passive immunodiffusion
Postzone phenomenon
Precipitation
Prozone phenomenon
Radial immunodiffusion (RID)
Rate nephelometry
Reverse passive agglutination
Sensitization
Turbidimetry
Zone of equivalence

The combination of an antigen with a specific antibody plays an important role in the laboratory in diagnosing many different diseases. Immunoassays have been developed to detect either antigen or antibody and vary from easily performed manual tests to highly complex automated assays. The first such assays were based on the principles of precipitation or agglutination. **Precipitation** involves combining soluble antigen with soluble antibody to produce insoluble complexes that are visible. **Agglutination** is the process by which particulate antigens such as cells aggregate to form larger complexes when a specific antibody is present. Precipitation and agglutination are considered unlabeled assays because a marker label is not needed to detect the reaction. Labeled assays, which were developed much later, will be considered in Chapter 11.

Precipitation was first noted in 1897 by Kraus, who found that culture filtrates of enteric bacteria would precipitate when they were mixed with specific antibodies. For such reactions to occur, both the antigen and antibody must have multiple binding sites for one another and the relative concentration of each must be equal. Binding characteristics of antibodies, called affinity and avidity, also play a major role.

Antigen–Antibody Binding

The primary union of binding sites on an antibody with specific epitopes on an antigen depends on two characteristics of antibody known as affinity and avidity. Such characteristics are important because they relate to the sensitivity and specificity of testing in the clinical laboratory.

Affinity

Affinity is the initial force of attraction that exists between a single Fab site on an antibody molecule and a single epitope or determinant site on the corresponding antigen.[1,2] As the epitope and binding site come into close proximity to each other, they are held together by rather weak bonds occurring only over a short distance of approximately 1×10^{-7} mm.[1]

The strength of attraction depends on the specificity of antibody for a particular antigen. One antibody molecule may initially attract numerous different antigens, but it is the epitope's shape and the way it fits together with the binding sites on an antibody molecule that determines whether the bonding will be stable. Antibodies are capable of reacting with antigens resembling the original antigen that induced antibody production, which is known as cross-reactivity. The more the cross-reacting antigen resembles the original antigen, the stronger the bond will be between the antigen and the binding site. However, if the epitope and the binding site have a perfect lock-and-key fit, as is the case with the original antigen, the affinity will be maximal **(Fig. 10–1).** When the affinity is higher, the assay reaction is more sensitive because more antigen–antibody complexes will be formed and visualized more easily.

Avidity

Avidity represents the overall strength of antigen–antibody binding and is the sum of the affinities of all the individual antibody–antigen combining sites.[1,3] Avidity refers to the strength with which a multivalent antibody binds a multivalent antigen and is a measure of the overall stability of an antigen–antibody complex.[2] In other words, once binding has occurred, it is the force that keeps the molecules together. A high avidity can actually compensate for a low affinity. Different classes of antibodies actually differ in avidities. The more bonds that form between antigen and antibody, the higher the avidity is. IgM, for instance, has a higher avidity than IgG because IgM has the potential to bind 10 different antigens **(Fig. 10–2).** Both affinity and avidity contribute to the stability of the antigen–antibody complexes,

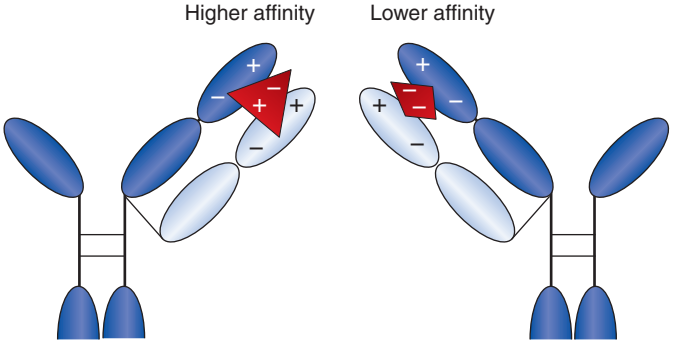


FIGURE 10–1 Affinity is determined by the three-dimensional fit and molecular attractions between one antigenic determinant and one antibody-binding site. The antigenic determinant on the left has a better fit and charge distribution than the epitope on the right and hence will have a higher affinity.

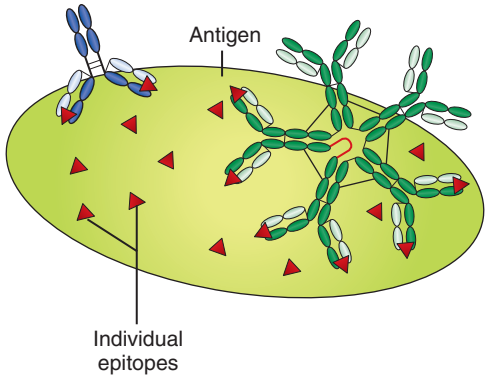


FIGURE 10–2 Avidity is the sum of the forces binding multivalent antigens to multivalent antibodies. In a comparison between IgG and IgM, IgM has the most potential binding sites for antigen and thus the higher avidity. Note that the monomers in IgM can swing up or down in order to bind more effectively.

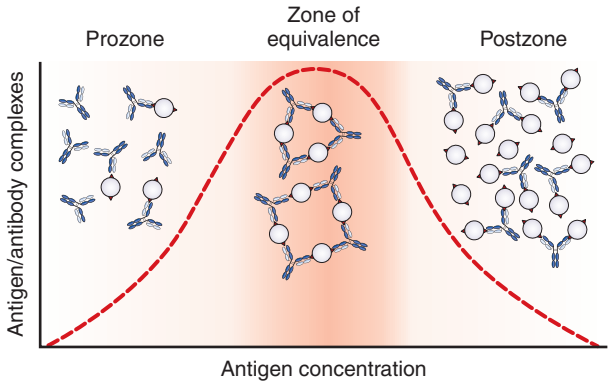


FIGURE 10–3 Precipitin curve. The precipitin curve shows how the amount of precipitation varies with varying antigen concentrations when the amount of antibody is kept constant. Excess antibody is called the *prozone* and excess antigen concentration is called the *postzone*.

which is essential to detecting the presence of an unknown, whether it is antigen or antibody.

Law of Mass Action

All antigen–antibody binding is reversible and is governed by the **law of mass action.** This law states that free reactants are in equilibrium with bound reactants.[3] The equilibrium constant K represents the difference in the rates of the forward and reverse reactions according to the following equation:

$$K = [AgAb]/[Ab][Ag]$$

where [AgAb] = concentration of the antigen–antibody complex (mol/L)
[Ab] = concentration of free antibody (mol/L)
[Ag] = concentration of free antigen (mol/L)

The value of K depends on the strength of binding between antibody and antigen. As the strength of binding, or avidity, increases, the tendency of the antigen–antibody complexes to dissociate decreases, which increases the value of K. When the value of K is higher, the amount of antigen–antibody complex is larger and the assay reaction is more visible or easily detectable. The ideal conditions in the clinical laboratory would be to have an antibody with a high affinity, or initial force of attraction, and a high avidity, or strength of binding. The higher the values are for both of these and the more antigen–antibody complexes that are formed, the more sensitive the test.

Precipitation Curve

In addition to the affinity and avidity of the antibody involved, precipitation depends on the relative proportions of antigen and antibody present. Optimum precipitation occurs in the zone of equivalence.

Zone of Equivalence

In the **zone of equivalence,** the number of multivalent sites of antigen and antibody are approximately equal. In this zone, precipitation is the result of random, reversible reactions whereby each antibody binds to more than one antigen and vice versa, forming a stable network or **lattice.** The lattice hypothesis, as formulated by Marrack, is based on the assumptions that each antibody molecule must have at least two binding sites and the antigen must be multivalent. As they combine, this arrangement results in a multimolecular lattice that increases in size until it precipitates out of solution.[4]

As illustrated by the precipitin curve shown in **Figure 10–3,** when the same amount of soluble antigen is added to increasing dilutions of antibody, the amount of precipitation increases up to the zone of equivalence. When the amount of antigen overwhelms the number of antibody-combining sites present, precipitation begins to decline because fewer lattice networks are formed.

Prozone and Postzone

As can be seen on the precipitin curve, precipitation declines on either side of the equivalence zone because of an excess of either antigen or antibody. In the case of antibody excess, the **prozone phenomenon** occurs, in which antigen combines with only one or two antibody molecules and no cross-linkages are formed. In the prozone, usually only one site on an antibody molecule is used and many free antibody molecules remain in solution.

At the other side of the zone, where there is antigen excess, the **postzone phenomenon** occurs in which small aggregates are surrounded by excess antigen. Again, no lattice network is formed.[3] In this case, every available antibody site is bound to a single antigen and no cross-links are formed. Thus, for precipitation reactions to be detectable, they must be carried out in the zone of equivalence.

The prozone and postzone phenomena must be considered in the clinical setting because negative reactions occur in both. A false-negative reaction may take place in the prozone because of high antibody concentration. If it is suspected that the reaction is a false negative, diluting out antibody and performing the test again may produce a positive result. In the postzone,

excess antigen may obscure the presence of a small amount of antibody. Typically, such a test is repeated with an additional patient specimen taken about a week later. The extra time would allow for the further production of antibody. If the repeated test is negative, it is unlikely that the patient has that particular antibody.

Measurement of Precipitation by Light Scattering

Precipitation is one of the simplest methods of detecting antigen–antibody reactions because most antigens are multivalent and thus capable of forming aggregates in the presence of the corresponding antibody. When antigen and antibody solutions are mixed, the antigen cross-links with numerous antibody molecules and the lattice networks become so large that they precipitate out of solution.[5] Precipitates in fluids can be measured by means of turbidimetry or nephelometry.

Turbidimetry is a measure of the turbidity or cloudiness of a solution. A detection device is placed in direct line with an incident light, collecting the light after it has passed through the solution. This device measures the reduction in light intensity caused by reflection, absorption, or scatter.[6] Scattering occurs when a beam of light passes through a solution and encounters molecules in its path.[6] Light then bounces off the molecules and travels in all directions. The amount of scatter is proportional to the size, shape, and concentration of molecules present in solution. It is recorded in absorbance units, a measure of the ratio of incident light to that of transmitted light. The measurements are made using a spectrophotometer or an automated clinical chemistry analyzer.

Nephelometry measures the light that is scattered at a particular angle from the incident beam as it passes through a suspension[6] **(Fig. 10–4).** The amount of light scattered is an index of the solution's concentration. If a solution has excess antibody, adding increasing amounts of antigen results in an increase in antigen–antibody complexes and thus an increase in light scattering. The relationship between antigen concentration, as indicated by antigen–antibody complex formation, and the amount of light scattering will form a straight line if plotted on a graph.[4] Light scatter may be directly extrapolated by a computer to give actual concentrations in milligrams per deciliter (mg/dL) or international units per milliliter (IU/mL), based on established values of standards. Nephelometers typically measure light scatter at angles ranging from 10 degrees to about 90 degrees. If a laser beam is used, light deflected only a few degrees from the original path can be measured. Although the sensitivity of turbidity has increased, nephelometry is more sensitive, with a lower limit of detection of 1 to 10 mg/L for serum proteins.[3]

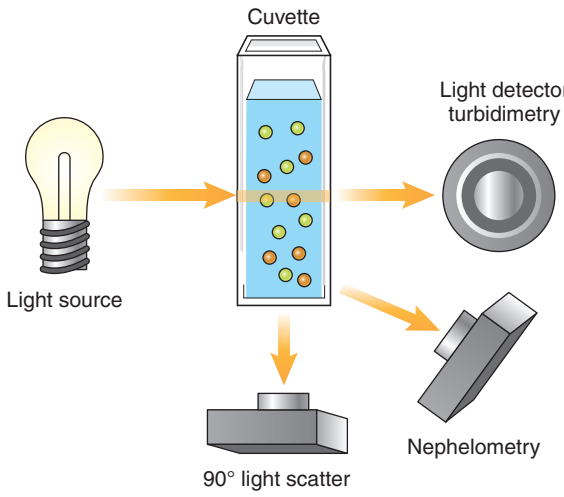


FIGURE 10–4 Principles of nephelometry. The light detection device is at an angle to the incident light, in contrast to turbidity, which measures light rays passing directly through the solution.

Nephelometry can be used to detect either antigen or antibody, but typically it is run with antibody as the reagent and patient antigen as the unknown. Many automated instruments use a technique called **rate nephelometry** for the measurement of serum proteins. In this instance, the rate of scattering increase is measured immediately after the reagent antibody is added. This rate change is directly related to antigen concentration if the concentration of antibody is kept constant.[4] Quantification of immunoglobulins such as IgG, IgA, IgM, and IgE, as well as kappa and lambda light chains, is mainly done by rate nephelometry because other methods are more labor intensive.[4] Other serum proteins quantified by this method include complement components, C-reactive protein, haptoglobin, and ceruloplasmin.[4] Nephelometry provides accurate and precise quantitation of serum proteins, and because of automation the cost per test is typically lower than other methods. Additionally, very small samples can be analyzed.

Passive Immunodiffusion Techniques

The precipitation of antigen–antibody complexes can also be determined in a support medium such as a gel. Agarose, a purified high-molecular-weight complex polysaccharide derived from seaweed, is used for this purpose. When antigen and antibody diffuse toward one another in a gel matrix, visible lines of precipitation will form.[5] Agarose helps stabilize the diffusion process and allow visualization of the precipitin bands.

Antigen and antibody are added to wells in the gel and antigen–antibody combination occurs by means of diffusion. When no electrical current is used to speed up this process, it is known as **passive immunodiffusion.** The rate of diffusion is affected by the size of the particles, the temperature, the gel viscosity, and the amount of hydration. Immunodiffusion reactions can be classified according to the number of reactants diffusing and the direction of diffusion.

Radial Immunodiffusion

A single-diffusion technique, called **radial immunodiffusion (RID),** has been used in the clinical laboratory. In this technique, antibody is uniformly distributed in the support gel and antigen is applied to a well cut into the gel. As the antigen diffuses out from the well, antigen– antibody combination occurs

in changing proportions until the zone of equivalence is reached and a stable lattice network is formed in the gel. The area of the ring obtained is a measure of antigen concentration that can be compared with a standard curve obtained by using antigens of known concentration.[3] **Figure 10–5** depicts some typical results.

One technique for the measurement of radial immunodiffusion was developed by Mancini and is known as the **end-point method**. In this technique, antigen is allowed to diffuse to completion; when equivalence is reached, there is no further change in the ring diameter.[7] Equivalence occurs between 24 and 72 hours. The square of the diameter is then directly proportional to the concentration of the antigen. A graph is obtained by plotting concentrations of standards on the x axis versus the diameter squared on the y axis, creating a smooth curve to fit the points. The major drawback to this method is the time it takes to obtain results. Another method, the kinetic or Fahey method, uses ring diameter readings taken at about 19 hours before equivalence is reached.[8] The diameter is then proportional to the log of the concentration and a graph is plotted using semi-log paper. The diameter is plotted on the x axis and the concentration is on the y axis, which automatically gives a log value. Many kits also contain a chart provided by the manufacturer that indicates concentration relative to the ring diameter.

The precision of the assay is directly related to accurate measurement of samples and standards. Sources of error include overfilling or underfilling the wells, nicking the side of the wells when filling, spilling sample outside the wells, improper incubation time and temperature, and incorrect measurement. Radial immunodiffusion has been used to measure IgG and IgA subclasses as well as complement components. Immunodiffusion is simple to perform and requires no instrumentation, but it has largely been replaced by more sensitive methods such as nephelometry and enzyme-linked immunoassays.

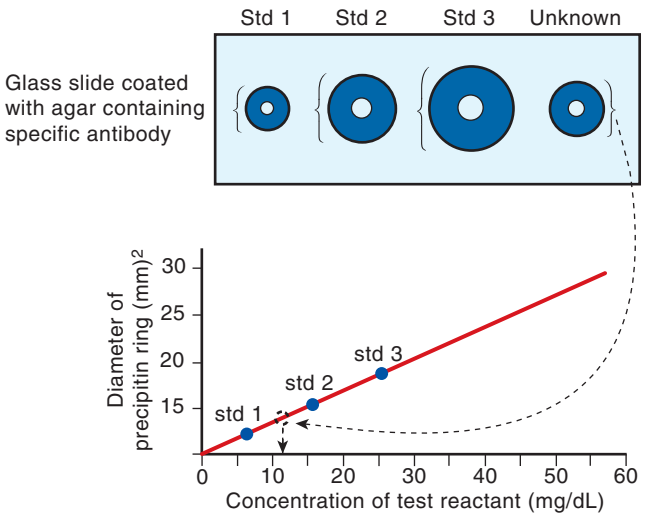


FIGURE 10–5 Radial immunodiffusion. The amount of precipitate formed is in proportion to the antigen present in the sample. In the Mancini end-point method, concentration is in proportion to the diameter squared.

Ouchterlony Double Diffusion

One of the older, classic immunochemical techniques is **Ouchterlony double diffusion.** In this technique, both antigen and antibody diffuse independently through a semisolid medium in two dimensions, horizontally and vertically. Wells are cut in a gel and reactants are added to the wells. Most Ouchterlony plates are set up with a central well surrounded by four to six equidistant outer wells. Antibody that is multispecific is placed in the central well and different antigens are placed in the surrounding wells to determine if the antigens share identical epitopes. Diffusion takes place radially from the wells. After an incubation period of between 12 and 48 hours in a moist chamber, precipitin lines form where the moving front of antigen meets that of antibody and the point of equivalence is reached. The density of the lines reflects the amount of immune complex formed.[5]

The position of the precipitin bands between wells allows for the antigens to be compared with one another. Several patterns are possible: (1) Fusion of the lines at their junction to form an arc represents serological identity or the presence of a common epitope, (2) a pattern of crossed lines demonstrates two separate reactions and indicates that the compared antigens share no common epitopes, and (3) fusion of two lines with a spur indicates partial identity. In this last case, the two antigens share a common epitope, but some antibody molecules are not captured by antigen and travel through the initial precipitin line to combine with additional epitopes found in the more complex antigen. Therefore, the spur always points to the simpler antigen[9] **(Fig. 10–6).** Although of more limited use because it is labor intensive and requires experience to read, Ouchterlony double diffusion is still used to identify fungal antigens such as *Aspergillus, Blastomyces, Coccidioides,* and *Candida.*[9]

Electrophoretic Techniques

Diffusion can be combined with electrophoresis to speed up or sharpen the results. **Electrophoresis** separates molecules according to differences in their electric charge when they are placed in an electric field. A direct current is forced through the gel, causing antigen, antibody, or both to migrate. As diffusion takes place, distinct precipitin bands are formed.

Immunoelectrophoresis is a double-diffusion technique that incorporates electrophoresis to enhance results. Typically, the source of antigen is serum, which is electrophoresed to separate out the main proteins. A trough is then cut in the gel parallel to the line of separation. Antiserum is placed in the trough and the gel is incubated for 18 to 24 hours. Double diffusion occurs at right angles to the electrophoretic separation and precipitin lines develop where specific antigen–antibody combination takes place. Interpretation is difficult; therefore, it has largely been replaced by immunofixation electrophoresis, which gives faster results and is easier to interpret.[3]

Immunofixation electrophoresis, as first described by Alper and Johnson,[10] is similar to immunoelectrophoresis except that after electrophoresis takes place, antiserum is

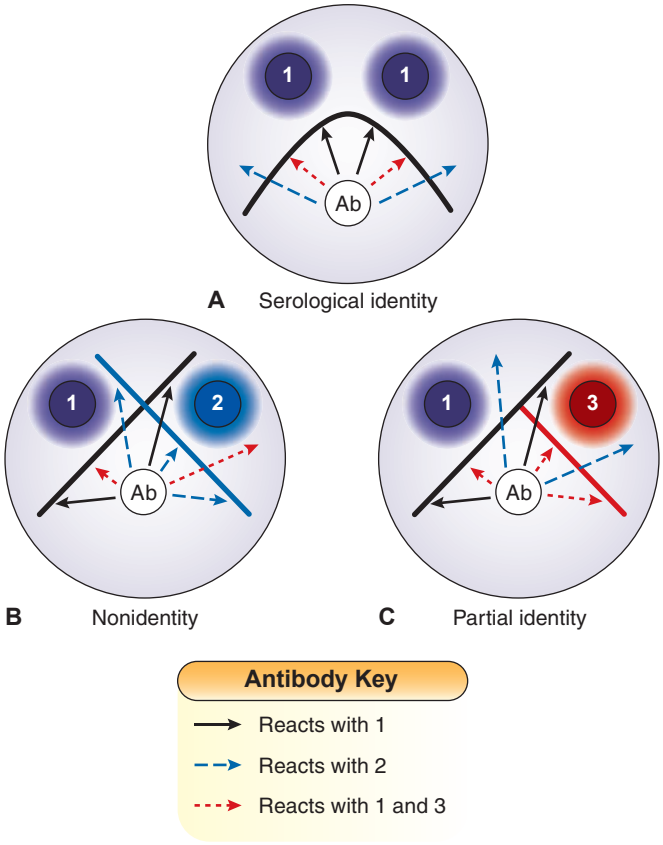


FIGURE 10–6 Ouchterlony diffusion patterns. An antibody mixture is placed in the central well. Unknown antigens are placed in the outside wells. The antibodies and antigens all diffuse radially out of the wells. (A) Serological identity. If the antigens are identical, they will react with the same antibody and the precipitate line forms a continuous arc. (B) Nonidentity. If the antigens share no identical determinants, they will react with different antibodies and two crossed lines are formed. (C) If antigen 3 has a determinant in common with antigen 1, one of the antibodies reacts with both antigens. Another antibody that reacts with different determinants on antigen 1 (absent on antigen 3) passes through one precipitation line and forms the spur on the other line. The spur formed always points to the simpler antigen with fewer antigenic determinants.

applied directly to the gel's surface rather than placed in a trough. Agarose or cellulose acetate can be used for this purpose. Immunodiffusion takes place in a shorter time and results in a higher resolution than when antibody diffuses from a trough. Because diffusion is only across the thickness of the gel, approximately 1 mm, the reaction usually takes place in less than 1 hour.

Most often, an antibody of known specificity is used to determine whether patient antigen is present. The unknown antigen is placed on the gel, electrophoretic separation takes place, and then the reagent antibody is applied. Immunoprecipitates form only where specific antigen–antibody combination has taken place and the complexes have become trapped in the gel. The gel is washed to remove any nonprecipitating proteins and can then be stained for easier visibility. Typically, patient serum is applied to six lanes of the gel; after electrophoresis, five lanes are overlaid with one each of the following antibodies: antibody to gamma, alpha, or mu heavy chains and to kappa or lambda light chains.[3] The sixth lane is overlaid with antibody to all serum proteins and serves as the reference lane. Reactions in each of the five lanes are compared with the reference lane. Hypogammaglobulinemias, which are characterized by low antibody production, will exhibit faintly staining bands, whereas polyclonal hypergammaglobulinemias (overproduction of antibody) show darkly staining bands in the gamma region. The presence of monoclonal antibody, such as is found in certain malignancies of the immune system, will result in dark and narrow bands in specific lanes[11] **(Fig. 10–7).**

Immunofixation electrophoresis is especially useful in demonstrating those antigens present in serum, urine, or spinal fluid in low concentrations.[11,12] This method has great versatility and is easy to perform at a relatively low cost, but it requires more manual manipulation than other methods.[12]

Comparison of Precipitation Techniques

Each type of precipitation technique has its own distinct advantages and disadvantages. Some techniques are technically more demanding, whereas others are more automated. Each type of precipitation testing has particular applications for which it is best suited. **Table 10–1** presents a comparison of the precipitation techniques discussed in this chapter.

Principles of Agglutination Reactions

Whereas precipitation reactions involve soluble antigens, agglutination is the visible aggregation of particles caused by combination with specific antibody. Antibodies that produce such reactions are often called **agglutinins.** Because this reaction takes place on the surface of the particle, antigen must be exposed and able to bind with antibody. Types of particles participating in such reactions include erythrocytes, bacterial cells, and inert carriers such as latex particles. Each particle must

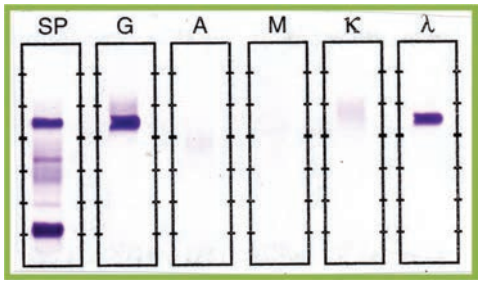


FIGURE 10–7 Immunofixation electrophoresis. A complex antigen mixture such as serum proteins is separated by electrophoresis. An antiserum template is aligned over the gel. Then protein fixative and monospecific antisera, IgG, IgA, IgM, κ, and λ are applied to the gel. After incubating for 30 minutes, the gel is stained and examined for the presence of paraproteins. Precipitates form where specific antigen–antibody combination has taken place. In this case, the patient has an IgG monoclonal antibody with λ chains. *(Courtesy of Helena Laboratories, Beaumont, TX.)*

Table 10–1 Comparison of Precipitation Techniques

TECHNIQUE	APPLICATION	SENSITIVITY (μG AB/ML)	PRINCIPLE
Nephelometry	Immunoglobulins, complement, C-reactive protein, other serum proteins	1–10	Light that is scattered at an angle is measured, indicating the amount of antigen or antibody present.
Radial immunodiffusion	Immunoglobulins, complement	10–50	Antigen diffuses out into gel that is infused with antibody. Measurement of the radius indicates concentration of antigen.
Ouchterlony double diffusion	Complex antigens such as fungal antigens	20–200	Both antigen and antibody diffuse out from wells in a gel. Lines of precipitate formed indicate the relationship of antigens.
Immunoelectrophoresis	Differentiation of serum proteins	20–200	Electrophoresis of serum followed by diffusion of antibody from wells.
Immunofixation electrophoresis	Over- or underproduction of antibody	Variable	Electrophoresis of serum followed by direct application of antibody to the gel.

have multiple antigenic or determinant sites, which are cross-linked to sites on other particles through the formation of antibody bridges.[4]

In 1896, Gruber and Durham published the first report about the ability of antibody to clump cells, based on observations of agglutination of bacterial cells by serum.[13] This finding gave rise to the use of serology as a tool in the diagnosis of disease and also led to the discovery of the ABO blood groups. Widal and Sicard developed one of the earliest diagnostic tests in 1896 for the detection of antibodies occurring in typhoid fever, brucellosis, and tularemia. Agglutination reactions now have a wide variety of applications in the detection of both antigens and antibodies. Such testing is simple to perform and the end points can easily be read visually.

Agglutination, like precipitation, is a two-step process that results in the formation of a stable lattice network. The first reaction, called **sensitization**, involves antigen–antibody combination through single antigenic determinants on the particle and is rapid and reversible.[14] The second step, or lattice formation, is the formation of cross-links that form the visible aggregates. This represents the stabilization of antigen–antibody complexes with the binding together of multiple antigenic determinants[14] **(Fig. 10–8).**

Sensitization is affected by the nature of the antigens on the agglutinating particles. If epitopes are sparse or if they are obscured by other surface molecules, they are less likely to interact with antibody. Additionally, red blood cells (RBCs) and bacterial cells have a slight negative surface charge; because like charges tend to repel one another, it is sometimes difficult to bring such cells together into a lattice formation.[4]

The class of immunoglobulin is also important; IgM with a potential valence of 10 is over 700 times more efficient in agglutination than is IgG with a valence of 2.[4] (See Fig. 10–2 for a comparison of IgG versus IgM.) Antibodies of the IgG class often cannot bridge the distance between particles because their small size and restricted flexibility at the hinge region may

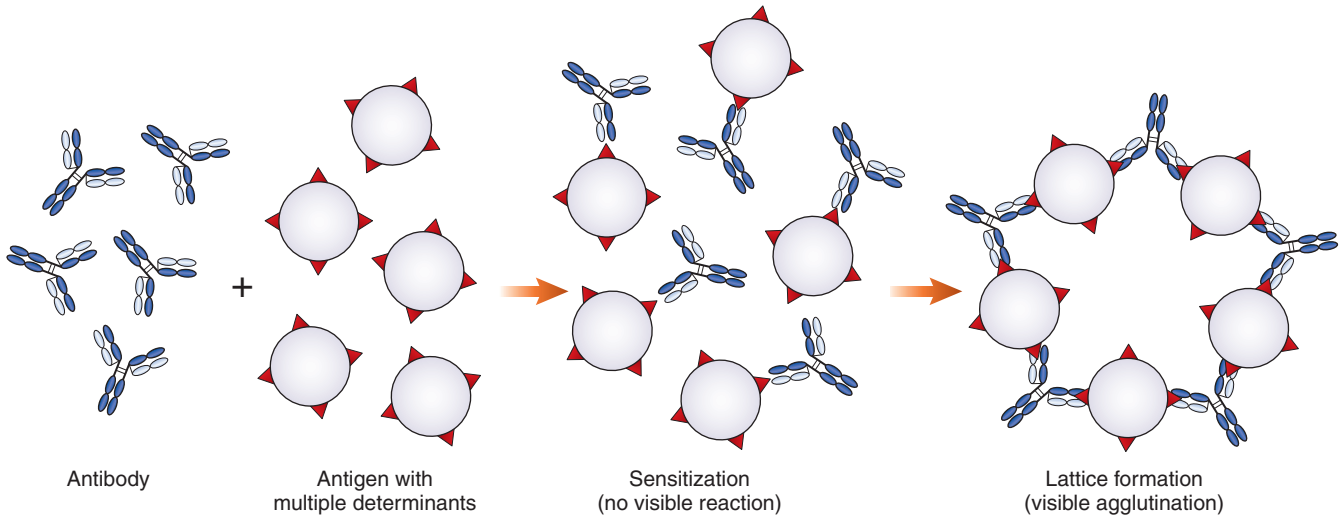


FIGURE 10–8 Phases of agglutination. Sensitization: Antigen and antibody unite through antigenic determinant sites. Lattice formation: Rearrangement of antigen and antibody bonds to form a stable lattice.

prohibit multivalent binding.[14] IgM antibodies, on the other hand, are strong agglutinins because of their larger size.

Achieving visible reactions with IgG often requires the use of enhancement techniques that vary physicochemical conditions such as the ionic strength of the solution, the pH, and the temperature. Antibodies belonging to the IgG class agglutinate best at 30°C to 37°C, whereas IgM antibodies react best at temperatures between 4°C and 27°C. Because naturally occurring antibodies against the ABO blood groups belong to the IgM class, these reactions are best run at room temperature. Antibodies to other human blood groups usually belong to the IgG class; reactions involving these must be run at 37°C. These latter reactions are the most important to consider in selecting compatible blood for a transfusion because these are the ones that will actually occur in the body.

In addition to temperature considerations, detection of IgG antibodies often requires the use of a second antibody, anti-human immunoglobulin, to visualize a reaction. Anti-human immunoglobulin is also known as Coombs reagent and is used frequently in blood bank testing. Coombs reagent will attach to the Fc portion of IgG and help to bridge the gap between RBCs so a visible agglutination reaction will occur. **Figure 10–9** demonstrates how Coombs reagent works.

Types of Agglutination Reactions

Agglutination reactions are easy to carry out, require no complicated equipment, and can be performed as needed in the laboratory without having to batch specimens. Batching specimens is done if a test is expensive or complicated; in this case, a large number are run at one time, which may result in a time delay. Many kits are available for standard testing, so reagent preparation is minimal. Agglutination reactions are a frequently employed serological test; they can be used to identify either antigen or antibody. Typically, most agglutination tests are qualitative, simply indicating the absence or presence of antigen or antibody, but dilutions can be made to obtain semiquantitative results. Many variations exist that can be categorized according to the type of particle used in the reaction and whether antigen or antibody is attached to it.

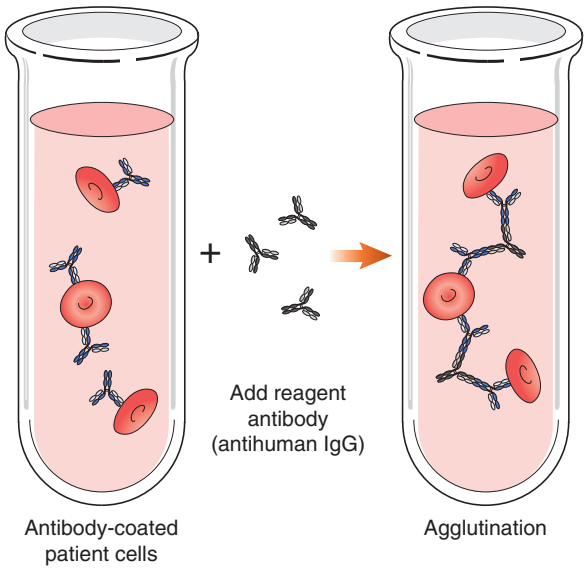


FIGURE 10–9 Coombs reagent. Coombs reagent is anti-human immunoglobulin used to enhance agglutination reactions by attaching to the Fc portion of IgG found on antibody-coated RBCs. Coombs reagent helps to bridge the gap between RBCs so a visible agglutination reaction will occur.

Direct Agglutination

Direct agglutination occurs when antigens are found naturally on a particle. One of the best examples of direct agglutination testing involves known bacterial antigens used to test for the presence of unknown antibodies in the patient. Typically, patient serum is diluted into a series of tubes or wells on a slide and reacted with bacterial antigens specific for the suspected disease. Detection of antibodies is primarily used in diagnosis of diseases for which the bacterial agents are extremely difficult to cultivate. One such example is the Widal test, a rapid screening test used to help determine the possibility of typhoid fever. A significant finding is a fourfold increase in antibody titer over time when paired dilutions of serum samples are tested with any of these antigens. Although more specific tests are now available, the Widal test is still considered useful in diagnosing typhoid fever in developing countries and remains in use in many areas throughout the world.[15]

If an agglutination reaction involves RBCs, then it is called **hemagglutination.** The best example of this occurs in ABO blood group typing of human RBCs, one of the world's most frequently used immunoassays.[5] Patient RBCs mixed with antisera of the IgM type can be used to determine the presence or absence of the A and B antigens; this reaction is usually performed at room temperature without the need for any enhancement techniques. Group A RBCs will agglutinate with anti-A antibody and Group B RBCs will agglutinate with anti-B antibody. This type of agglutination reaction is simple to perform, is relatively sensitive, and is easy to read **(Fig. 10–10).**

A titer that yields semiquantitative results can be performed in test tubes or microtiter plates by making serial dilutions of the antibody. The reciprocal of the last dilution still exhibiting a visible reaction is the titer, indicating the antibody's strength. Interpretation of the test is done on the basis of the cell sedimentation pattern. If there is a dark red, smooth button at the bottom of the microtiter well, the result is negative. A positive result will have cells that are spread across the well's bottom, usually in a jagged pattern with an irregular edge. Test tubes also can be centrifuged and then shaken to see if the cell button can be evenly resuspended. If it is resuspended with no visible clumping, then the result is negative. Positive reactions can be graded to indicate the strength of the reaction **(Fig. 10–11).**

Passive Agglutination

Passive, or indirect, **agglutination** employs particles that are coated with antigens not normally found on their surfaces. A variety of particles, including erythrocytes, latex, and gelatin, are used for passive agglutination.[4] The use of synthetic beads or particles provides the advantages of consistency and uniformity.

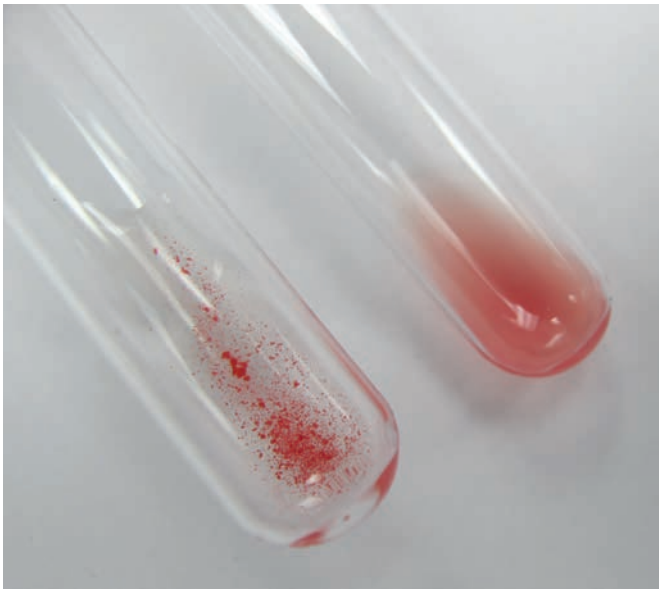


FIGURE 10–10 Red blood cell agglutination. The tube on the left is a positive test for RBC agglutination, whereas the tube on the right is a negative test showing that the RBCs have remained in a smooth suspension.

Reactions are easy to read visually and give quick results. Many antigens, especially polysaccharides, adsorb to RBCs spontaneously, so they are also relatively easy to manipulate. Particle sizes vary from 7 μm for RBCs down to 0.8 μm for fine latex particles.[16]

In 1955, Singer and Plotz found by happenstance that IgG was naturally adsorbed to the surface of polystyrene latex particles. Latex particles are inexpensive, are relatively stable, and are not subject to cross-reactivity with other antibodies. A large number of antibody molecules can be bound to the surface of latex particles, so the number of antigen-binding sites is large.[16] Additionally, the large particle size facilitates reading of the test.

Latex agglutination tests have been used to detect rheumatoid factor, antibodies to Group A *Streptococcus* antigens, and antibodies to viruses such as rotavirus, cytomegalovirus, rubella, and varicella-zoster.[4] Hemagglutination kits are available for detection of antibodies to hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) I and II, to cite just a few examples.[2]

Because many of these kits are designed to detect IgM antibody and there is always the risk of nonspecific agglutination caused by the presence of other IgM antibodies, reactions must be carefully controlled and interpreted. Commercial tests are usually performed on disposable plastic, cardboard cards, or glass slides. Kits contain positive and negative controls; if the controls do not give the expected results, the test is not valid. Such tests are typically used as screening tools, which are followed by more extensive testing if the results are positive.

Reverse Passive Agglutination

In **reverse passive agglutination,** antibody rather than antigen is attached to a carrier particle. The antibody must still be reactive and is joined in such a manner that the active sites are facing outward. Adsorption may be spontaneous, or it may require some of the same manipulation as is used for antigen attachment. This type of testing is often used to detect microbial antigens. **Figure 10–12** shows the differences between passive and reverse passive agglutination.

Numerous kits are available for the rapid identification of antigens from such infectious agents as Group B *Streptococcus, Staphylococcus aureus,* streptococcal groups A and B, rotavirus, and *Cryptococcus neoformans.*[16] Rapid agglutination tests have found the widest application in detecting soluble antigens in urine, spinal fluid, and serum.[8] The principle is the same for all these tests: Latex particles coated with antibody are reacted with a patient sample containing the suspected antigen. In some cases, an extraction step is necessary to isolate antigen before the reagent latex particles are added. Organisms can be identified in a few minutes with fairly high sensitivity and specificity, although this varies for different organisms. The use of monoclonal antibodies has greatly cut

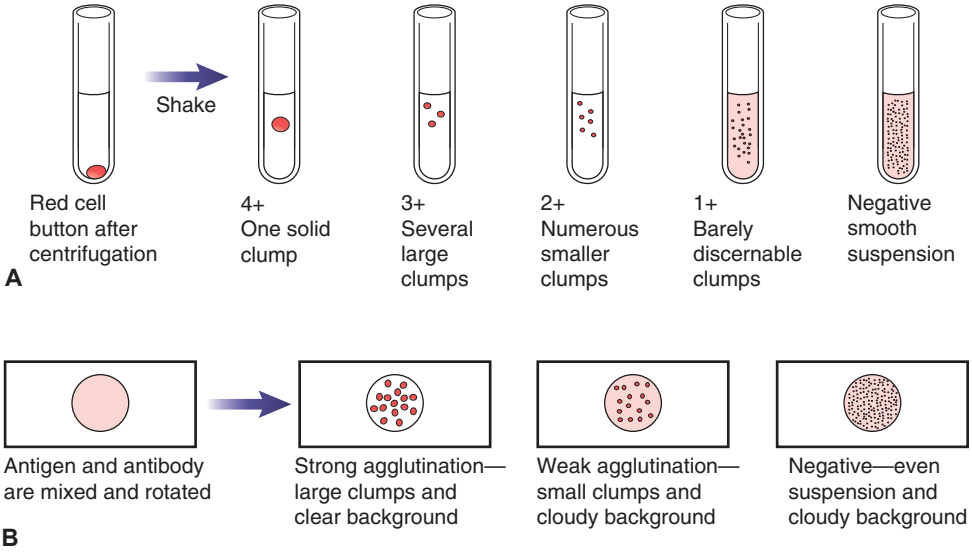


FIGURE 10–11 Grading of agglutination reactions: A. tube method. If tubes are centrifuged and shaken to resuspend the button, reactions can be graded from negative to 4+, depending on the size of clumps observed. B. Rapid slide method.

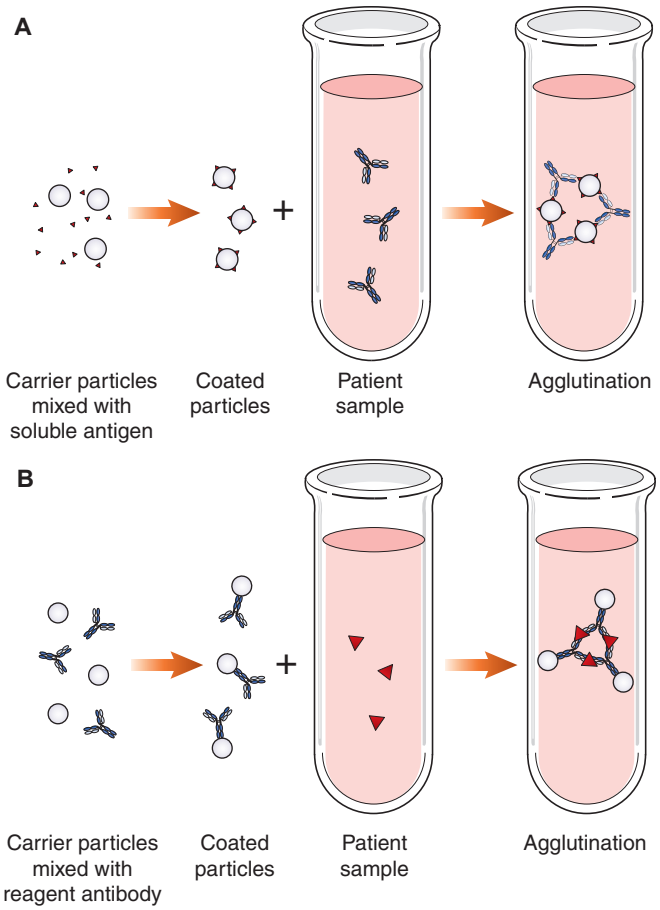


FIGURE 10–12 Passive and reverse passive agglutination. (A) Passive agglutination. Antigen is attached to the carrier particle; agglutination occurs if patient antibody is present. (B) Reverse passive agglutination. Antibody is attached to the carrier particle; agglutination occurs if patient antigen is present.

down on cross-reactivity, but there is still the possibility of interference or nonspecific agglutination.

Such tests are most often used for organisms that are difficult to grow in the laboratory or for instances when rapid identification will allow treatment to be initiated more promptly. Direct testing of specimens for the presence of viral antigens has still not reached the sensitivity of enzyme immunoassays; however, for infections in which a large amount of viral antigen is present, such as rotavirus and enteric adenovirus in infants, latex agglutination tests are very useful. Reverse passive agglutination testing has also been used to measure levels of certain therapeutic drugs, hormones, and plasma proteins such as haptoglobin and C-reactive protein.

Agglutination Inhibition

Agglutination inhibition reactions are based on competition between particulate and soluble antigens for limited antibody-combining sites. A lack of agglutination is an indicator of a positive reaction. Typically, this type of reaction involves haptens that are complexed to proteins; the hapten–protein conjugate is then attached to a carrier particle. The patient sample is first reacted with a limited amount of reagent antibody that is specific for the hapten being tested. Indicator particles that contain the same hapten one wishes to measure in the patient are then added. If the patient sample has no free hapten, the reagent antibody is able to combine with the carrier particles and produce a visible agglutination. In this case, however, agglutination is a negative reaction, indicating that the patient did not have sufficient hapten to inhibit the secondary reaction **(Fig. 10–13).** Either antigen or antibody can be attached to the particles. The sensitivity of the reaction is governed by the avidity of the antibody itself. It can be a sensitive assay capable of detecting small quantities of antigen. Tests used to detect illicit drugs such as cocaine or heroin are examples of agglutination inhibition tests.

Hemagglutination inhibition reactions use the same principle, except RBCs are the indicator particles. This type of testing has been used to detect antibodies to certain viruses, such as rubella, influenza, and respiratory syncytial virus (RSV).[5,9] RBCs have naturally occurring viral receptors. When virus is present, spontaneous agglutination occurs because the virus particles link the RBCs together. Presence of patient antibody inhibits the agglutination reaction **(Fig. 10–14).**

To perform a hemagglutination inhibition test, dilutions of patient serum are incubated with a viral preparation. Then RBCs that the virus is known to agglutinate are added to the mixture. If antibody is present, it will attach to the viral particles and prevent agglutination, so a lack of or reduction in agglutination indicates the presence of patient antibody.[5] Controls are necessary because there may be a factor in the serum that causes agglutination or the virus may have lost its ability to agglutinate.

Instrumentation

Although agglutination reactions require no complicated instrumentation to read, several chemistry analyzers have been developed using automation to increase sensitivity.[2] Nephelometry has been applied to the reading of agglutination reactions and the term *particle-enhanced immunoassay* is used to describe such reactions. When particles are used, the sensitivity can be increased to nanograms/mL.[2] For this type of reaction, small latex particles with a diameter of smaller than 1 μm are used. One such type of instrumentation system is called **particle-counting immunoassay (PACIA).**

PACIA involves measuring the number of residual nonagglutinating particles in a specimen. These particles are counted by means of a laser beam in an optical particle counter similar to the one that is designed to count blood cells. Nephelometric methods are used to measure forward light scatter. Very large and very small particles are excluded by setting certain thresholds on the instrument.

Latex particles are coated with whole antibody molecules or with $F(ab')_2$ fragments. Use of the latter reduces interference and nonspecific agglutination. If antigen is present, complexes will form and will be screened out by the counter because of their large size. An inverse relationship exists between the number of unagglutinated particles counted and the amount of unknown antigen in the patient specimen. Measurements

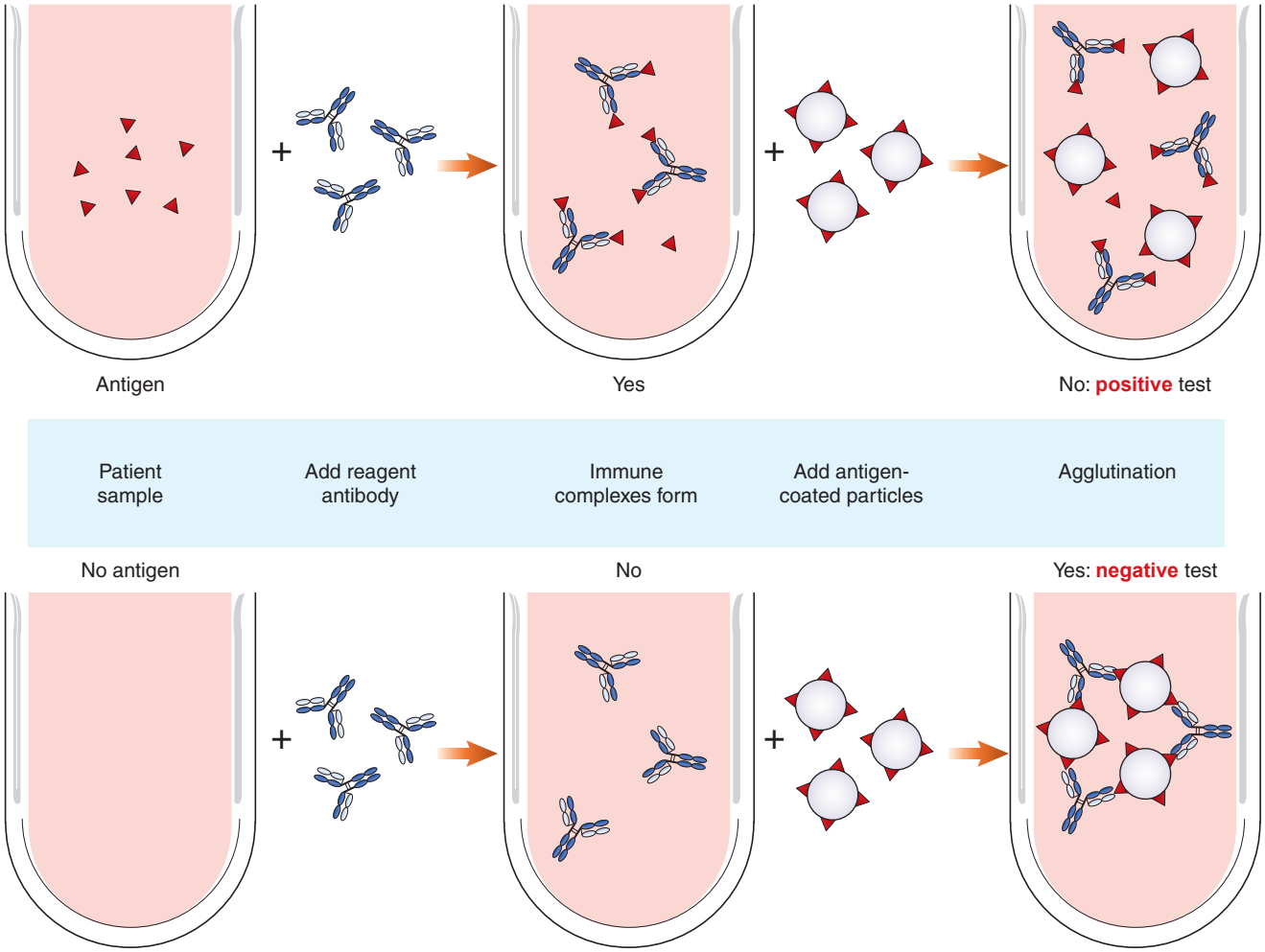


FIGURE 10–13 Agglutination inhibition. Reagent antibody is added to the patient sample. If patient antigen is present, antigen–antibody combination results. When antigen-coated latex particles are added, no agglutination occurs, which is a positive test. If no patient antigen is there, the reagent antibody combines with latex particles and agglutination results, which is a negative test.

are made by looking at the rate at which the number of unagglutinated particles decrease, called a *rate assay,* or the total number of unagglutinated particles left at the end, known as an *end-point assay.*[2,3] PACIAs have been used to measure several serum proteins, therapeutic drugs, tumor markers, and certain viral antigens.

Quality Control and Quality Assurance

Although agglutination reactions are simple to perform, interpretation must be carefully done. Techniques must be standardized regarding the concentration of antigen, incubation time, temperature, diluent, and the method of reading. The possibility of cross-reactivity and interfering antibody should always be considered. Cross-reactivity is caused by the presence of antigenic determinants that resemble one another so closely that antibody formed against one will react with the other. Most cross-reactivity can be avoided through the use of monoclonal antibody directed against an antigenic determinant that is unique to a particular antigen.

Other considerations include proper storage of reagents and close attention to expiration dates. Reagents should never be used beyond the expiration date. Each new lot should be evaluated before use and the manufacturer's instructions for each kit should always be followed. The sensitivity and specificity of different kits may vary and thus must be taken into account.

Advantages of agglutination reactions include rapidity; relative sensitivity; and the fact that if the sample contains a microorganism, the organism does not need to be viable. In addition, most tests are simple to perform and require no expensive equipment. Tests are conducted on cards, tubes, and microtiter plates, all of which are extremely portable. A wide variety of antigens and antibodies can be tested for in this manner. It must be kept in mind, however, that agglutination tests are screening tools only and that a negative result does not rule out presence of the disease or the antigen. The quantity of antigen or antibody may be below the sensitivity of the test system. Although the number of agglutination tests have decreased in recent years, they continue to play an important role in the identification of rare pathogens such as *Francisella* and *Brucella* and more common organisms such as rotavirus and *Cryptococcus,* for which other testing is complex or unavailable.

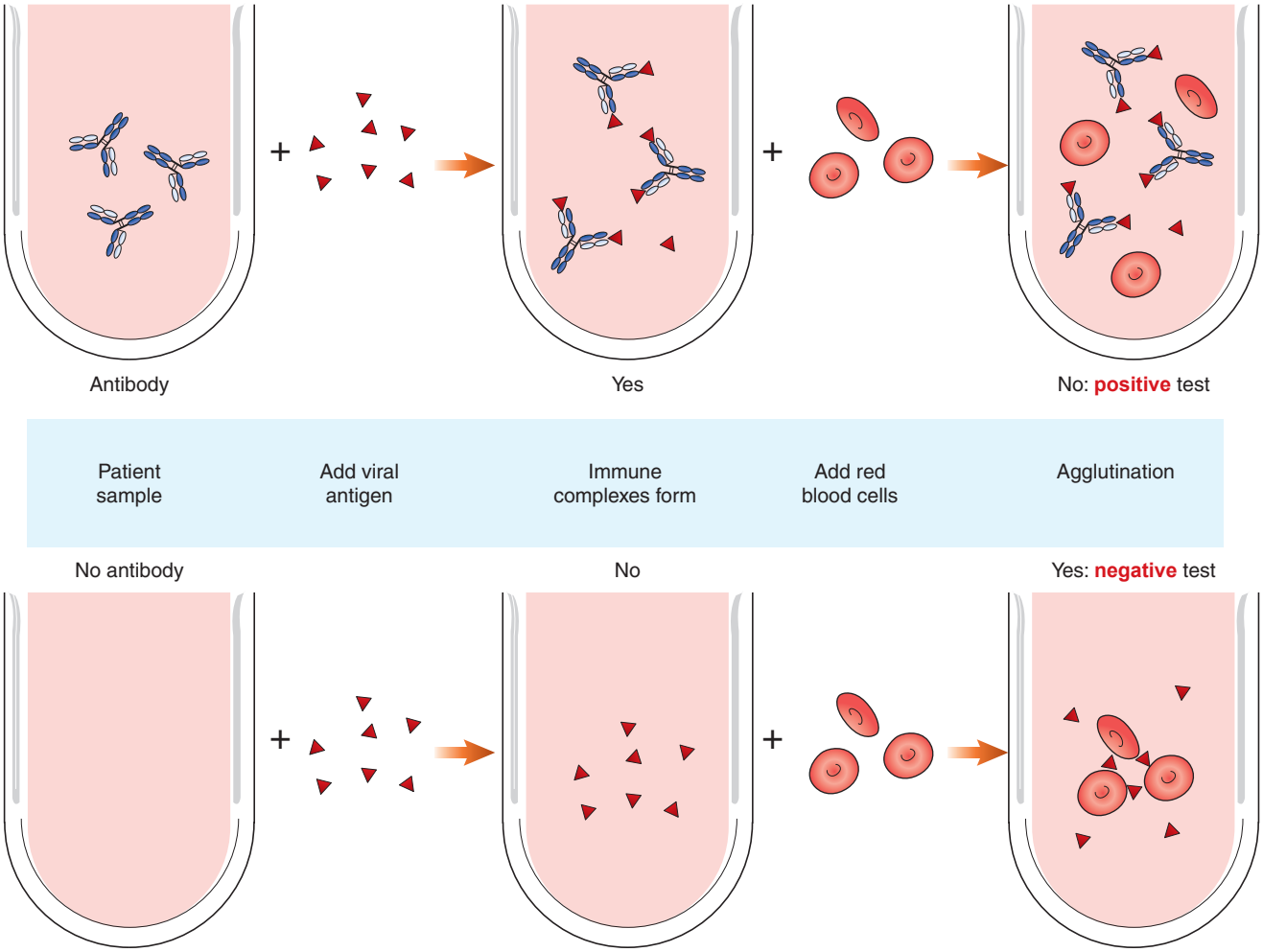


FIGURE 10–14 Hemagglutination inhibition. In the presence of certain viruses, RBCs spontaneously agglutinate. However, if patient antibody is present, then agglutination is inhibited. Thus, a lack of agglutination indicates the presence of antibody.

SUMMARY

- Precipitation involves the combination of soluble antigen with soluble antibody to produce insoluble complexes that are visible.
- Union of antigen and antibody depends on affinity, or the force of attraction that exists between one antibody-binding site and a single epitope on an antigen.
- Avidity is the sum of all attractive forces occurring between multiple binding sites on antigen and antibody.
- Maximum binding of antigen and antibody occurs when the aggregate number of multivalent sites of antigen and antibody are approximately equal.
- The concentrations of antigen and antibody that yield maximum binding represent the zone of equivalence. This is where the multivalent sites of antigen and antibody are approximately equal.
- In the prozone, when antibody is being tested for against a standard concentration of antigen, antibody is in excess and precipitation or agglutination cannot be detected.
- In the postzone, antibody has been diluted out and antigen is in excess so that manifestations of antigen–antibody combination such as precipitation and agglutination are present to a much lesser degree.
- If light scatter produced by immune complexes in solution is measured as a reduction in light intensity, this is called turbidimetry.
- Nephelometry is the technique that measures the amount of light scattered at a particular angle. Several automated instruments are based on these principles.
- When precipitation reactions take place in a gel, this is known as passive diffusion. In single diffusion, only one of the reactants travels, whereas the other is incorporated in the gel.
- In radial immunodiffusion, antibody is incorporated in a gel. Antigen is placed in wells in the gel and diffuses out. The amount of precipitate formed is directly related to the amount of antigen present.
- In Ouchterlony diffusion, both antigen and antibody diffuse from wells and travel toward each other. Precipitin lines may indicate identity, nonidentity, or partial identity, depending on the pattern formed.
- In immunofixation electrophoresis, antibody is applied directly to the gel after electrophoresis of antigens has taken

place. Compared with immunoelectrophoresis, precipitation occurs in a shorter time and bands with higher resolution are obtained.
- The process of agglutination can be divided into two steps: (1) sensitization or initial binding, which depends on the nature of the antibody and the antigen-bearing surface, and (2) lattice formation, which is governed by such factors as pH, ionic strength, and temperature.
- Because of its larger size, IgM is usually able to effect lattice formation without additional enhancement, whereas for IgG measures are necessary to see a visible reaction.
- In direct agglutination, antigens are found naturally on the indicator particle.
- In passive agglutination, antigens are artificially attached to such a particle.
- Reverse passive agglutination is so called because antibody is attached to the indicator particle.
- Agglutination inhibition is based on competition between antigen-coated particles and soluble patient antigen for a limited number of antibody sites. It is the only instance in which agglutination represents a negative test.
- PACIA looks at residual nonagglutinating particles by means of nephelometry. As agglutination occurs, clumps of antigens increase in size; these large clumps are not counted. The amount of unknown antigen in a patient specimen is therefore indirectly proportional to the number of unagglutinated particles.
- Agglutination reactions are typically used as screening tests; they are fast and sensitive and can yield valuable information when interpreted correctly.

Study Guide: Comparison of Agglutination Reactions

TYPE OF REACTION	PRINCIPLE	RESULTS
Direct agglutination	Antigen is naturally found on a particle.	Agglutination indicates the presence of patient antibody.
Indirect (passive) agglutination	Particles coated with antigens not normally found on their surfaces.	Agglutination indicates the presence of patient antibody.
Reverse passive	Particles are coated with reagent antibody.	Agglutination indicates the presence of patient antibody.
Agglutination inhibition	Haptens attached to carrier particles. Particles compete with patient antigens for a limited number of antibody sites.	Lack of agglutination is a positive test, indicating the presence of patient antigen.
Hemagglutination inhibition	Red blood cells spontaneously agglutinate if viral particles are present.	Lack of agglutination is a positive test, indicating the presence of patient antibody.

CASE STUDIES

1. A 4-year-old female was hospitalized for pneumonia. She has had a history of upper-respiratory tract infections and several bouts of diarrhea since infancy. Because of her recurring infections, the physician decided to measure her immunoglobulin levels. The following results were obtained by nephelometry:

IMMUNOGLOBULIN	NORMAL LEVEL (3–5 YRS) (MG/DL)	PATIENT LEVEL (MG/DL)
IgG	550–1,700	800
IgA	50–280	20
IgM	25–120	75

Questions

a. What do these results indicate?
b. How do they explain the symptoms? (You may want to refer back to Chapter 5 for a discussion of the function of different classes of antibody.)
c. How do nephelometry measurements compare with the use of RID?

2. A 25-year-old female who was 2 months pregnant went to her physician for a prenatal workup. She had been vaccinated against rubella, but her titer was never established. She was concerned because a friend of hers who had never been vaccinated for rubella thought she might have the disease. The patient had been on an all-day shopping trip with her friend 2 days before she saw her doctor. The physician ordered a latex agglutination test to screen for rubella as a part of the prenatal workup. The results on an undiluted serum specimen were positive, indicating that at least 10 IU/mL of antibody was present.

Questions

a. What does the positive rubella test indicate?
b. How should this be interpreted in the light of the patient's condition?

REVIEW QUESTIONS

1. In a precipitation reaction, how can the ideal antibody be characterized?
   - **a.** Low affinity and low avidity
   - **b.** High affinity and low avidity
   - **c.** High affinity and high avidity
   - **d.** Low affinity and high avidity

2. Precipitation differs from agglutination in which way?
   - **a.** Precipitation can only be measured by an automated instrument.
   - **b.** Precipitation occurs with univalent antigen, whereas agglutination requires multivalent antigen.
   - **c.** Precipitation does not readily occur because few antibodies can form aggregates with antigen.
   - **d.** Precipitation involves a soluble antigen, whereas agglutination involves a particulate antigen.

3. When soluble antigens diffuse in a gel that contains antibody, in which zone does optimum precipitation occur?
   - **a.** Prozone
   - **b.** Zone of equivalence
   - **c.** Postzone
   - **d.** Prezone

4. Which of the following statements apply to rate nephelometry?
   - **a.** Readings are taken before equivalence is reached.
   - **b.** It is more sensitive than turbidity.
   - **c.** Measurements are time dependent.
   - **d.** All of the above.

5. Which of the following is characteristic of the end-point method of RID?
   - **a.** Readings are taken before equivalence.
   - **b.** Concentration is directly in proportion to the square of the diameter.
   - **c.** The diameter is plotted against the log of the concentration.
   - **d.** It is primarily a qualitative rather than a quantitative method.

6. In which zone might an antibody-screening test be false negative?
   - **a.** Prozone
   - **b.** Zone of equivalence
   - **c.** Postzone
   - **d.** None of the above

7. How does measurement of turbidity differ from nephelometry?
   - **a.** Turbidity measures the increase in light after it passes through a solution.
   - **b.** Nephelometry measures light that is scattered at an angle.
   - **c.** Turbidity deals with univalent antigens only.
   - **d.** Nephelometry is not affected by large particles falling out of solution.

8. Which of the following refers to the force of attraction between an antibody and a single antigenic determinant?
   - **a.** Affinity
   - **b.** Avidity
   - **c.** Van der Waals attraction
   - **d.** Covalence

9. Immunofixation electrophoresis differs from immunoelectrophoresis in which way?
   - **a.** Electrophoresis takes place after diffusion has occurred in immunofixation electrophoresis.
   - **b.** Better separation of proteins with the same electrophoretic mobilities is obtained in immunoelectrophoresis.
   - **c.** In immunofixation electrophoresis, antibody is directly applied to the gel instead of being placed in a trough.
   - **d.** Immunoelectrophoresis is a much faster procedure.

10. If crossed lines result in an Ouchterlony immunodiffusion reaction with antigens 1 and 2, what does this indicate?
    - **a.** Antigens 1 and 2 are identical.
    - **b.** Antigen 2 is simpler than antigen 1.
    - **c.** Antigen 2 is more complex than antigen 1.
    - **d.** The two antigens are unrelated.

11. Which technique represents a single-diffusion reaction?
    - **a.** Radial immunodiffusion
    - **b.** Ouchterlony diffusion
    - **c.** Immunoelectrophoresis
    - **d.** Immunofixation electrophoresis

12. Which best describes the law of mass action?
    - **a.** Once antigen–antibody binding takes place, it is irreversible.
    - **b.** The equilibrium constant depends only on the forward reaction.
    - **c.** The equilibrium constant is related to strength of antigen–antibody binding.
    - **d.** If an antibody has a high avidity, it will dissociate from antigen easily.

13. Agglutination of dyed bacterial cells represents which type of reaction?

a. Direct agglutination
b. Passive agglutination
c. Reverse passive agglutination
d. Agglutination inhibition

14. If a single IgM molecule can bind many more antigens than a molecule of IgG, which of the following is higher?

a. Affinity
b. Initial force of attraction
c. Avidity
d. Initial sensitization

15. Agglutination inhibition could best be used for which of the following types of antigens?

a. Large cellular antigens such as erythrocytes
b. Soluble haptens
c. Bacterial cells
d. Coated latex particles

16. Which of the following correctly describes reverse passive agglutination?

a. It is a negative test.
b. It can be used to detect autoantibodies.
c. It is used for identification of antigens.
d. It is used to detect sensitization of red blood cells.

17. Reactions involving IgG may need to be enhanced for which reason?

a. It is only active at 25°C.
b. It may be too small to produce lattice formation.
c. It has only one antigen-binding site.
d. It is only able to produce visible precipitation reactions.

18. For which of the following tests is a lack of agglutination a positive reaction?

a. Hemagglutination
b. Passive agglutination
c. Reverse passive agglutination
d. Agglutination inhibition

19. Typing of RBCs with reagent antiserum represents which type of reaction?

a. Direct hemagglutination
b. Passive hemagglutination
c. Hemagglutination inhibition
d. Reverse passive hemagglutination

20. In a particle-counting immunoassay using reagent antibody attached to latex particles, if the particle count in solution is very low, what does this mean about the presence of patient antigen?

a. The patient has no antigen present.
b. The patient has a very small amount of antigen.
c. The patient has a large amount of antigen present.
d. The test is invalid.

11 Labeled Immunoassays

Christine Dorresteyn Stevens, EdD, MT(ASCP)

LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Describe the difference between competitive and noncompetitive immunoassays.
2. Distinguish between heterogeneous and homogeneous immunoassays.
3. Explain how the principle of competitive binding is used in radioimmunoassays.
4. Discuss criteria for selection of an enzyme for enzyme immunoassay.
5. Explain the principle of sandwich or capture immunoassays.
6. Describe applications for homogeneous enzyme immunoassay.
7. Describe uses for rapid immunoassays.
8. Compare and contrast enzyme immunoassay and radioimmunoassay regarding ease of performance, sensitivity, and clinical application.
9. Describe the difference between direct and indirect immunofluorescence techniques.
10. Relate the principle of fluorescence polarization immunoassay.
11. Explain how chemiluminescent assays are used to identify analytes.
12. Discuss advantages and disadvantages of each type of immunoassay.
13. Choose an appropriate immunoassay for a particular analyte.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Analyte
Capture assays
Chemiluminescent immunoassay
Competitive immunoassay
Direct immunofluorescent assay
Enzyme-linked immunosorbent assays (ELISA)
Fluorescence polarization immunoassay (FPIA)
Heterogeneous enzyme immunoassay
Homogeneous enzyme immunoassay
Immunochromatography
Immunofluorescent assay
Indirect immunofluorescent assays
Noncompetitive immunoassay
Radioimmunoassay (RIA)
Sandwich immunoassays

Unlabeled immunoassays, such as the precipitation and agglutination reactions that were discussed in Chapter 10, are fairly simple techniques to perform and require little in the way of sophisticated equipment. However, they are relatively insensitive because they rely on a high enough concentration of the unknown to visualize the reaction. In contrast, labeled immunoassays are designed for antigens and antibodies that may be small in size or present in very low concentrations. The presence of such antigens or antibodies is determined indirectly by using a labeled reactant to detect whether or not specific binding has taken place.

The substance to be measured, often called the **analyte,** typically is a protein. Examples include bacterial antigens, hormones, drugs, tumor markers, specific immunoglobulins, and many other substances. Analytes are bound to molecules that react specifically with them. Typically, this is antibody. One reactant, either the antigen or the antibody, is labeled with a marker so that the amount of binding can be monitored. The sensitivity and specificity of the antibody used is the key to successful results.

The development of rapid, specific, and sensitive assays to determine the presence of important biologically active molecules ushered in a new era of testing in the clinical laboratory. Labeled immunoassays have made possible rapid quantitative measurement of many important entities such as viral antigens in patients infected with HIV. This ability to detect very small quantities of antigen or antibody has revolutionized the diagnosis and monitoring of numerous diseases and has led to more prompt treatment for many such conditions.

Formats for Labeled Assays

Current techniques include the use of fluorescent, radioactive, chemiluminescent, and enzyme labels. The underlying principles of all these techniques are essentially the same. There are two major formats for all labeled assays: competitive and noncompetitive.

Competitive Immunoassays

In a **competitive immunoassay,** all the reactants are mixed together simultaneously; labeled antigen competes with unlabeled patient antigen for a limited number of antibody-binding sites. The concentration of the labeled analyte is in excess, so all binding sites on the antibody will be occupied. After separation, the amount of bound label is measured and used to determine the amount of patient antigen present. If patient antigen is present, some of the binding sites will be filled with unlabeled analyte, thus decreasing the amount of bound label **(Fig. 11–1).** Therefore, the amount of bound label is inversely proportional to the concentration of the labeled antigen, which means that the more labeled antigen that is detected, the less there is of patient antigen. This ratio can be illustrated by the following equation:

$$6Ag^* + 2Ag + 4Ab \rightarrow 3Ag^*Ab + 1AgAb + 3Ag^* + 1Ag$$

In this example, labeled and unlabeled antigens occur in a 3:1 ratio. Binding to a limited number of antibody sites will take place in the same ratio. Thus, on the right side of the equation, three of the four binding sites are occupied by labeled antigen, whereas one site is filled by unlabeled antigen. As the amount of patient antigen increases, fewer binding sites will be occupied by labeled antigen, as demonstrated by the next equation:

$$6Ag^* + 18Ag + 4Ab \rightarrow 1Ag^*Ab + 3AgAb + 5Ag^* + 15Ag$$

In this case, the ratio of labeled to unlabeled antigen is 1:3. Binding to antibody sites takes place in the same ratio and the amount of bound label is greatly decreased in comparison to the first equation. A standard curve using known amounts of unlabeled antigen can be used to extrapolate the concentration of the unknown patient antigen.[1] The detection limits of competitive assays are largely determined by the affinity of the antibody.[2] The higher the affinity of the antibody for the antigen, the more sensitive the assay will be. Refer back to Chapter 10 for a discussion of how affinity affects antigen–antibody binding.

Noncompetitive Immunoassays

In a typical **noncompetitive immunoassay,** antibody, often called capture antibody, is first passively absorbed to a solid phase such as microtiter plates, nitrocellulose membranes, or plastic beads.[2] Excess antibody is present so that any patient antigen present can be captured. Unknown patient antigen is then allowed to react with and be captured by the solid-phase antibody. After washing to remove unbound antigen, a second antibody with a label is added to the reaction **(Fig. 11–2).** In this case, the amount of label measured is directly proportional to the amount of patient antigen. This type of assay is more sensitive than competitive immunoassays.

In both types of assays, the label must not alter the reactivity of the molecule and should remain stable for the reagent's shelf

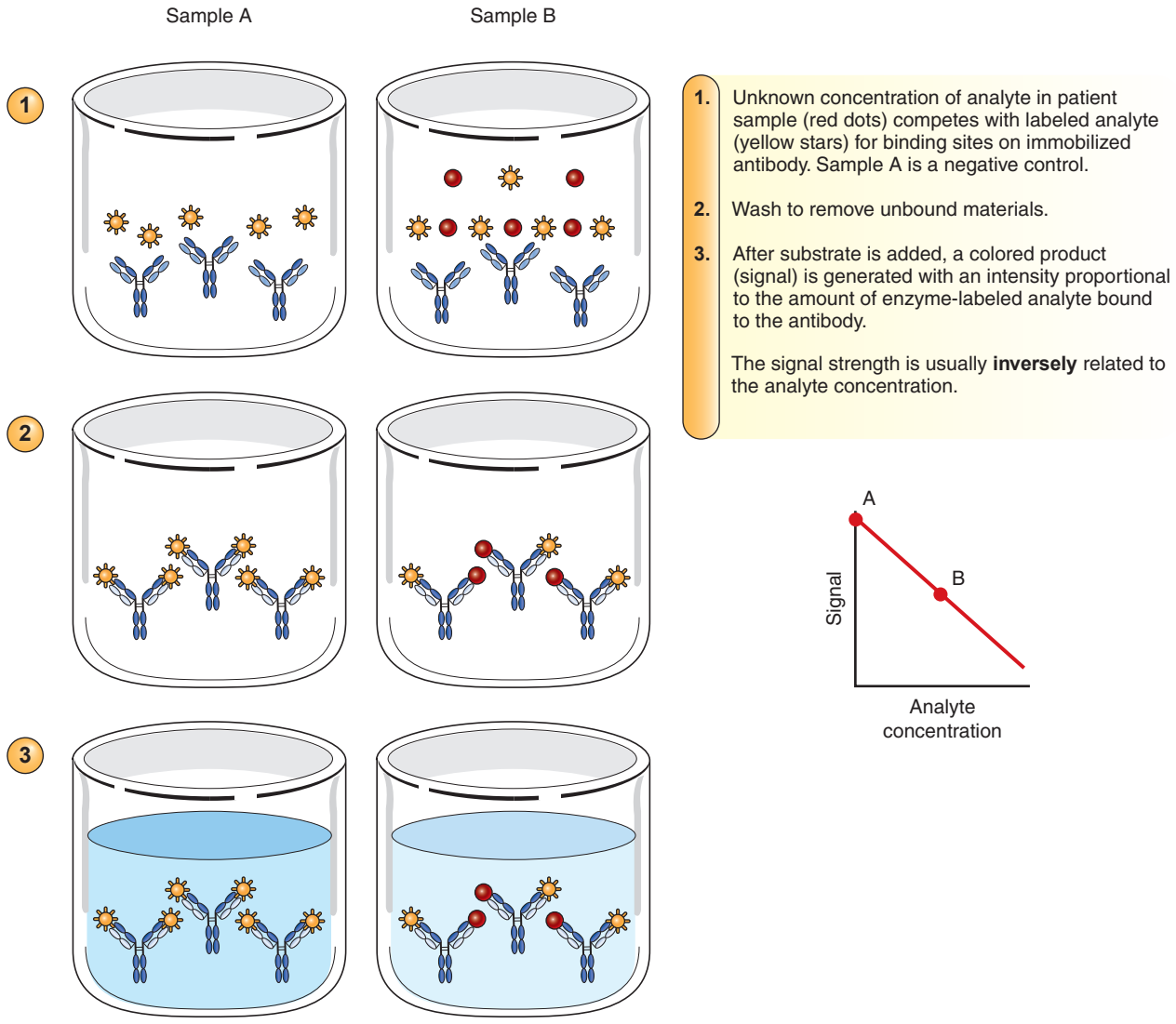


FIGURE 11–1 Principle of a competitive immunoassay.

life. Techniques using each different type of label will be discussed in a separate section.

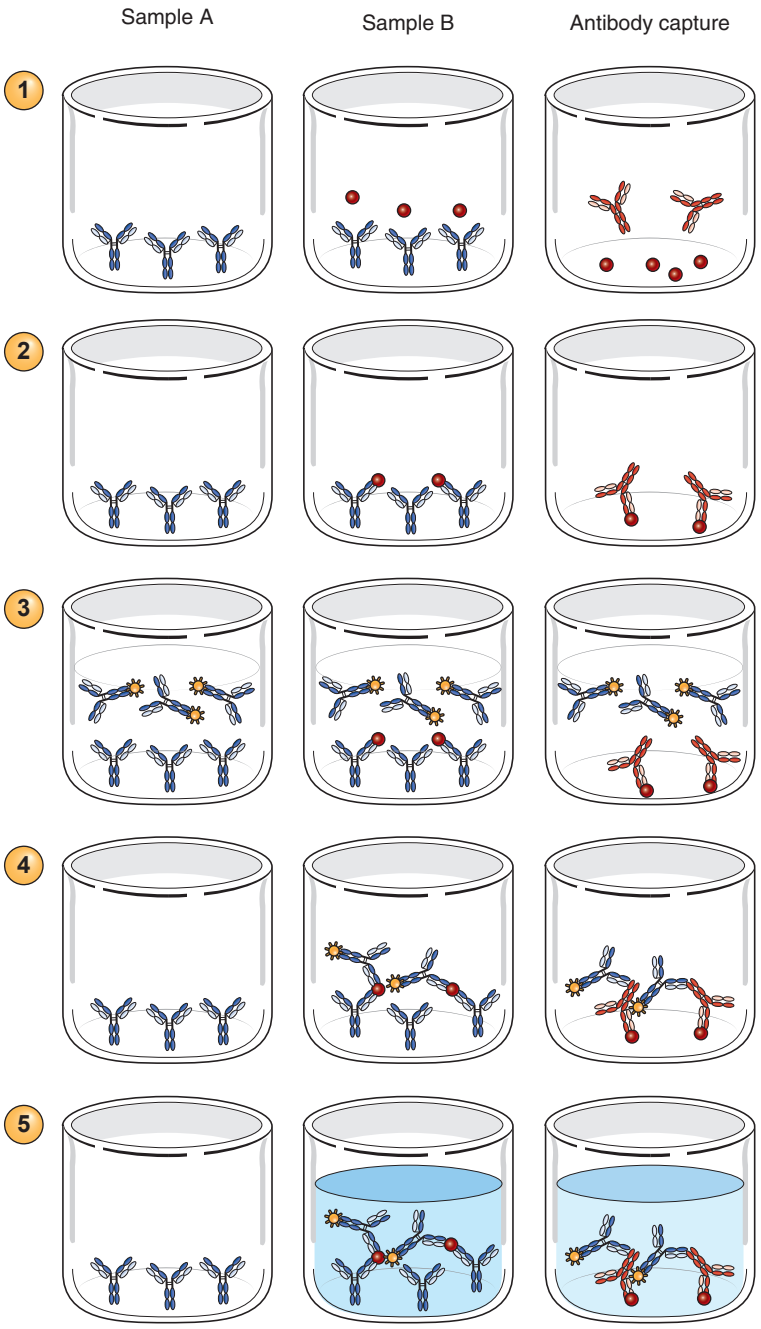
Heterogeneous Versus Homogeneous Assays

Immunoassays can also be categorized according to whether or not it is necessary to separate the bound reactants from the free ones. **Heterogeneous enzyme immunoassays** require a step to physically separate free from bound analyte. Antigen or antibody is attached by physical adsorption; when specific binding takes place, complexes remain attached to the solid phase. This step provides a simple way to separate bound and free reactants. The sample is then thoroughly washed and the remaining activity is determined.

Homogeneous enzyme immunoassays, on the other hand, do not need a separation step. The activity of the label attached to the antigen is diminished when binding of antibody and antigen occurs. Typically, homogeneous assays involve an enzyme label, chosen so that the enzyme is inactivated when bound to an antibody. This type of assay is simpler to perform because there is no washing step. The sample containing patient antigen is incubated with the labeled antigen and the antibody; the amount of activity then can be measured directly **(Fig. 11–3).**[1] Homogeneous assays are less sensitive than heterogeneous assays, however. Immunoassays using different types of labels will now be discussed according to these two categories.

Radioimmunoassay

The first type of immunoassay developed was **radioimmunoassay (RIA),** pioneered by Yalow and Berson in the late 1950s. It was used to determine the level of insulin–anti-insulin complexes in diabetic patients.[3,4] The technique proved to be valuable in measuring a number of substances such as hormones,



1. For Sample B, immobilized reagent antibodies capture analyte in patient sample (red dots) while in the Ab capture technique, immobilized reagent antigen captures patient antibodies. Sample A is a negative control.
2. Wash to remove unbound materials.
3. Add enzyme-labeled (detection) antibody that binds to a different epitope on analyte for Sample B (or binds Fc region on human Ig for Antibody capture).
4. Wash to remove unbound materials.
5. Add substrate and measure color change. Color intensity is directly related to analyte (Sample B) or human antibody (Antibody capture).

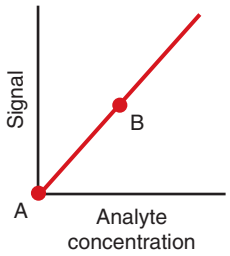


FIGURE 11–2 Noncompetitive immunoassay.

serum proteins, and vitamins that either occur at very low levels in blood plasma or are so small that they could not be detected otherwise. Yalow was honored with the Nobel Prize in 1977 for her groundbreaking work.

The assay uses a radioactive substance as a label. Radioactive elements have nuclei that decay spontaneously, emitting matter and energy. Several radioactive labels have been used, but ^{125}I has been the most popular.[1,4] It is easily incorporated into protein molecules and emits gamma radiation, which is detected by a gamma counter. Very low quantities of radioactivity can be easily measured.[2]

RIA is an extremely sensitive and precise technique for determining trace amounts of analytes that are small in size. However, the chief disadvantage is the health hazard involved in working with radioactive substances. Disposal problems, short shelf life, and the need for expensive equipment has caused laboratorians to utilize other techniques for identifying analytes in low concentration.[1,2]

Enzyme Immunoassays

Enzyme immunoassays, using enzymes as labels, were developed as alternatives to RIAs.[2,4] Enzymes are naturally occurring molecules that catalyze certain biochemical reactions. They react with suitable substrates to produce breakdown products

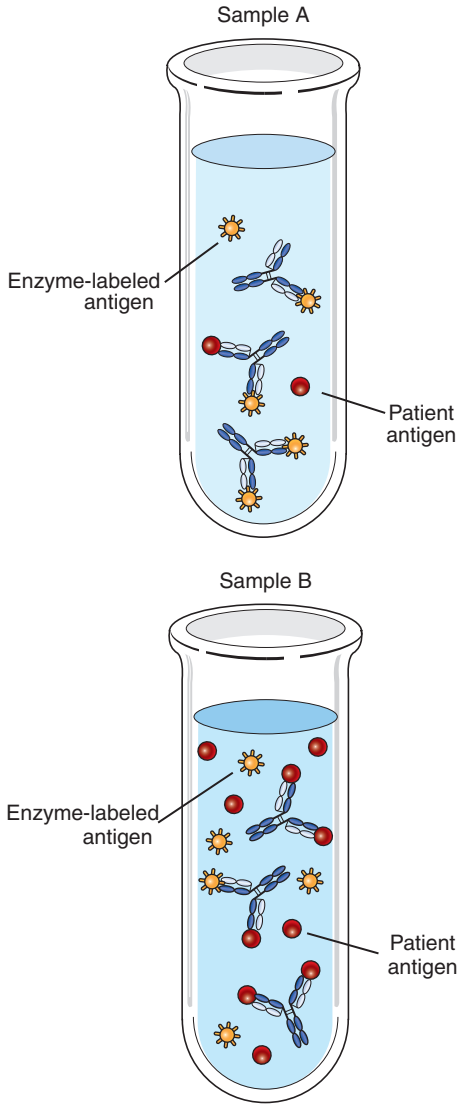


FIGURE 11–3 Homogeneous immunoassay. Reagent antibody is in solution. Patient antigen and enzyme-labeled antigen are added to the test tube. Patient antigen and enzyme-labeled antigen compete for a limited number of binding sites on the antibodies. When patient antigen is present, the enzyme label on the reagent antigen is not blocked, so color development is observed. Sample A has a low concentration of patient antigen, whereas Sample B contains more patient antigen and has stronger color development.

that may be chromogenic, fluorogenic, or luminescent. Some type of spectroscopy can then be used to measure the changes involved. As labels for immunoassay, enzymes are cheap and readily available, have a long shelf life, are easily adapted to automation, and cause changes that can be measured using inexpensive equipment.[2] Sensitivity can be achieved without disposal problems or the health hazards of radiation. Because one molecule of enzyme can generate many molecules of product, little reagent is necessary to produce high sensitivity.[1] Enzyme labels can either be used qualitatively to determine the presence of an antigen or antibody or quantitatively to determine the actual concentration of an analyte in an unknown specimen. Assays based on the use of enzymes can be found in such diverse settings as clinical laboratories, doctors' offices, and at-home testing.

Enzymes used as labels for immunoassays are typically chosen according to the number of substrate molecules converted per molecule of enzyme, ease and speed of detection, and stability.[4] In addition, availability and cost of enzyme and substrate play a role in the choice of a particular enzyme as reagent. Typical enzymes that have been used as labels in colorimetric reactions include horseradish peroxidase, alkaline phosphatase, and β-D-galactosidase.[1,4] Alkaline phosphatase and horseradish peroxidase have the highest turnover (conversion of substrate) rates, high sensitivity, and are easy to detect, so they are most often used in such assays.[4,5] Enzyme assays are classified as either heterogeneous or homogeneous on the basis of whether a separation step is necessary, as previously mentioned.

Heterogeneous Enzyme Immunoassays

Competitive Enzyme Immunoassays

The first enzyme immunoassays were competitive assays based on the principles of RIA. Enzyme-labeled antigen competes with unlabeled patient antigen for a limited number of binding sites on antibody molecules that are attached to a solid phase. After carefully washing to remove any nonspecifically bound antigen, enzyme activity is determined. Enzyme activity is inversely proportional to the concentration of the test substance. This method is typically used for measuring small antigens that are relatively pure, such as drugs and hormones.[5]

Noncompetitive Enzyme Immunoassays

Although competitive tests have a high specificity, the tendency in the laboratory today is toward the use of noncompetitive assays because they have a higher sensitivity than their competitive counterparts.[1] Most noncompetitive assays are indirect immunoassays, or so-called indirect **enzyme-linked immunosorbent assays (ELISA),** because the enzyme-labeled reagent does not participate in the initial antigen–antibody binding reaction. The enzyme-labeled reagent only binds after the initial antigen–antibody reaction has taken place. This type of assay is one of the most frequently used immunoassays in the clinical laboratory because of its sensitivity, specificity, simplicity, and low cost.[5] Antigen is typically bound to solid phase. A variety of solid-phase supports are used, including microtiter plates, nitrocellulose membranes, and magnetic latex or plastic beads.[1,2] When antigen is bound to solid phase, patient serum with unknown antibody is added and given time to react. After a wash step, an enzyme-labeled antiglobulin, or secondary antibody, is added. This second antibody reacts with any patient antibody that is bound to solid phase. If no patient antibody is bound to the solid phase, the second labeled antibody will not be bound. After a second wash step, the enzyme substrate is added. The amount of color, fluorescence, or luminescence is measured using a detection device and is compared with the amount of product according to a standard curve.[1,4] The amount of color, fluorescence, or luminescence detected is directly proportional to the amount of antibody in the specimen.

This type of assay is used to measure antibody production to infectious agents that are difficult to isolate in the laboratory and for autoantibody testing.[2] Viral infections especially are more easily diagnosed by this method than by other types of testing.[4] This technique is especially useful as a screening tool for detecting antibody to HIV, hepatitis B, and hepatitis C.[2,4] It is best employed where quantitation is not necessary and is easily applied to point-of-care and home testing.

Capture Assays

If antibody, rather than antigen, is bound to the solid phase, these assays are often called **sandwich immunoassays** or **capture assays.** Antigens captured in these assays must have multiple epitopes. Excess antibody attached to solid phase is allowed to combine with the test sample to capture any antigen present. After an appropriate incubation period, enzyme-labeled antibody is added. This second antibody recognizes a different epitope or binding site than the solid-phase antibody and completes the "sandwich." Depending upon the particular enzyme used, either a colored or chemiluminescent reaction product is detected (see Fig. 11–2). Enzymatic activity is directly proportional to the amount of antigen in the test sample.

Capture assays are best suited to antigens that have multiple determinants, such as antibodies, cytokines, proteins, tumor markers, and microorganisms, especially viruses.[2,5] When used with microorganisms, the epitope must be unique to the organism being tested and must be present in all strains of that organism. The use of monoclonal antibodies has made this a very sensitive test system. A major use of capture assays is in the measurement of immunoglobulins, especially those of certain classes. For instance, the presence of IgM can be specifically determined, thus indicating an acute infection. When capture assays are used to measure immunoglobulins, the specific immunoglobulin class being detected is actually acting as antigen and the antibody is anti-human immunoglobulin. Such indirect ELISA tests are quite sensitive because all patient antigen has a chance to participate in the reaction.

Heterogeneous enzyme assays, in general, achieve a sensitivity similar to that of RIA.[1] In sandwich assays, capture antibody on solid phase must have both a high affinity and a high specificity for this test system to be effective. However, there may be problems with nonspecific protein binding or the presence of antibodies to various components of the testing system.[1] If IgG is present, rheumatoid factor can cause false-positive results. Rheumatoid factor is an IgM antibody that reacts with IgG. See Chapter 14 for a discussion of the diseases in which rheumatoid factor may be found. If this is suspected, serum can be pretreated to avoid this problem. Sandwich assays are also subject to the hook effect, an unexpected fall in the amount of measured analyte when an extremely high concentration is present[1]. This typically occurs in antigen excess, where the majority of binding sites are filled and the remainder of patient antigen has no place to bind. If this condition is suspected, serum dilutions must be made and then retested.

Homogeneous Enzyme Immunoassays

Homogeneous enzyme immunoassays are generally less sensitive than heterogeneous assays, but they are rapid, simple to perform, and adapt easily to automation.[1,2] No washing or separation steps are necessary. Their chief use has been in the determination of low-molecular-weight analytes such as hormones, therapeutic drugs, and drugs of abuse in both serum and urine.[1,2,6] An example of a homogeneous immunoassay is the enzyme-multiplied immunoassay technique (EMIT) developed by the Syva Corporation.[6]

Homogeneous assays are based on the principle of change in enzyme activity as specific antigen–antibody combination occurs. Reagent antigen is labeled with an enzyme tag. When antibody binds to specific determinant sites on the antigen, the active site on the enzyme is blocked, resulting in a measurable loss of activity.[2] Free analyte (antigen) competes with enzyme-labeled analyte for a limited number of antibody-binding sites, so this is a competitive assay. Enzyme activity is directly in proportion to the concentration of patient antigen or hapten present in the test solution (see Fig 11-3). A physical separation of bound and free analyte is thus not necessary.

The sensitivity of homogeneous assays is determined by the following: (1) detectability of enzymatic activity; (2) change in that activity when antibody binds to antigen; (3) strength of the antibody's binding; and (4) susceptibility of the assay to interference from endogenous enzyme activity, cross-reacting antigens, or enzyme inhibitors. This technique is usually applied only to detection of small molecules that could not be easily measured by other means because it is far less sensitive than heterogeneous assays.[2]

Homogeneous assays have a number of limitations. First, only certain enzymes are inhibited in this manner. Additionally, enzymatic activity may be altered by steric exclusion of the substrate or there may also be changes in the conformation structure of the enzyme, especially in the region of the active site. Two enzymes that are frequently used in this type of assay are malate dehydrogenase and glucose-6-phosphate dehydrogenase.[2]

Enzyme immunoassays in general have achieved sensitivity similar to that of RIA without creating a health hazard or causing disposal problems. Expensive instrumentation is not needed because most assays can be read by spectrophotometry or by simply noting the presence or absence of color. Reagents are inexpensive and have a long shelf life.

Disadvantages include the fact that some specimens may contain natural inhibitors. Additionally, the size of the enzyme label may be a limiting factor in the design of some assays. Nonspecific protein binding is another difficulty encountered with the use of enzyme labels.[2] However, this technique has been successfully applied to a wide range of assays.

Rapid Immunoassays

Rapid immunoassays are membrane based, easy to perform, and give reproducible results.[2] Although designed primarily

for point-of-care or home testing, many of these have been modified for increased sensitivity and can be made semiquantitative for use in a clinical laboratory. Typically, these are designed as single-use, disposable assays in a plastic cartridge. The membrane is usually made of microporous nylon, which is able to easily immobilize proteins.[1] The rapid flow through the membrane and its large surface area enhance the speed and sensitivity of ELISA reactions.[1] Either antigen or antibody can be coupled to the membrane; the reaction is then read by looking for the presence of a colored reaction product. Some test devices require the separate addition of a patient sample, wash reagent, labeled antigen or antibody, and the substrate.

Another type of rapid assay, called **immunochromatography,** combines all the previously mentioned steps into one. The analyte is applied at one end of the strip and migrates toward the distal end where there is an absorbent pad to maintain a constant capillary flow rate.[2] The labeling and detection zones are set between the two ends. As the sample is loaded, it reconstitutes the labeled antigen or antibody and the two form a complex that migrates toward the detection zone. An antigen or antibody immobilized in the detection zone captures the immune complex and forms a colored line for a positive test, which may be in the form of a plus sign **(Fig. 11–4).** Excess labeled immunoreactant migrates to the absorbent pad. This type of test device has been used to identify microorganisms such as *Streptococcus pyogenes,* the cause of strep throat, and has been used for pregnancy testing as well as testing for cardiac troponin after a heart attack, to name just a few examples.[1,2] Test results are most often qualitative rather than quantitative.

Fluorescent Immunoassays

In 1941, Albert Coons demonstrated that antibodies could be labeled with molecules that fluoresce.[7] These fluorescent compounds, called *fluorophores* or *fluorochromes,* can absorb energy from an incident light source and convert that energy into light of a longer wavelength and lower energy as the excited electrons return to the ground state. Fluorophores are typically organic molecules with a ring structure; each has a characteristic optimum absorption range. The time interval between absorption of energy and emission of fluorescence is very short and can be measured in nanoseconds.

Ideally, a fluorescent probe should exhibit high intensity, which can be distinguished easily from background fluorescence.[1,2] It should also be stable. The two compounds most often used are fluorescein and rhodamine, usually in the form of isothiocyanates, because these can be readily coupled with

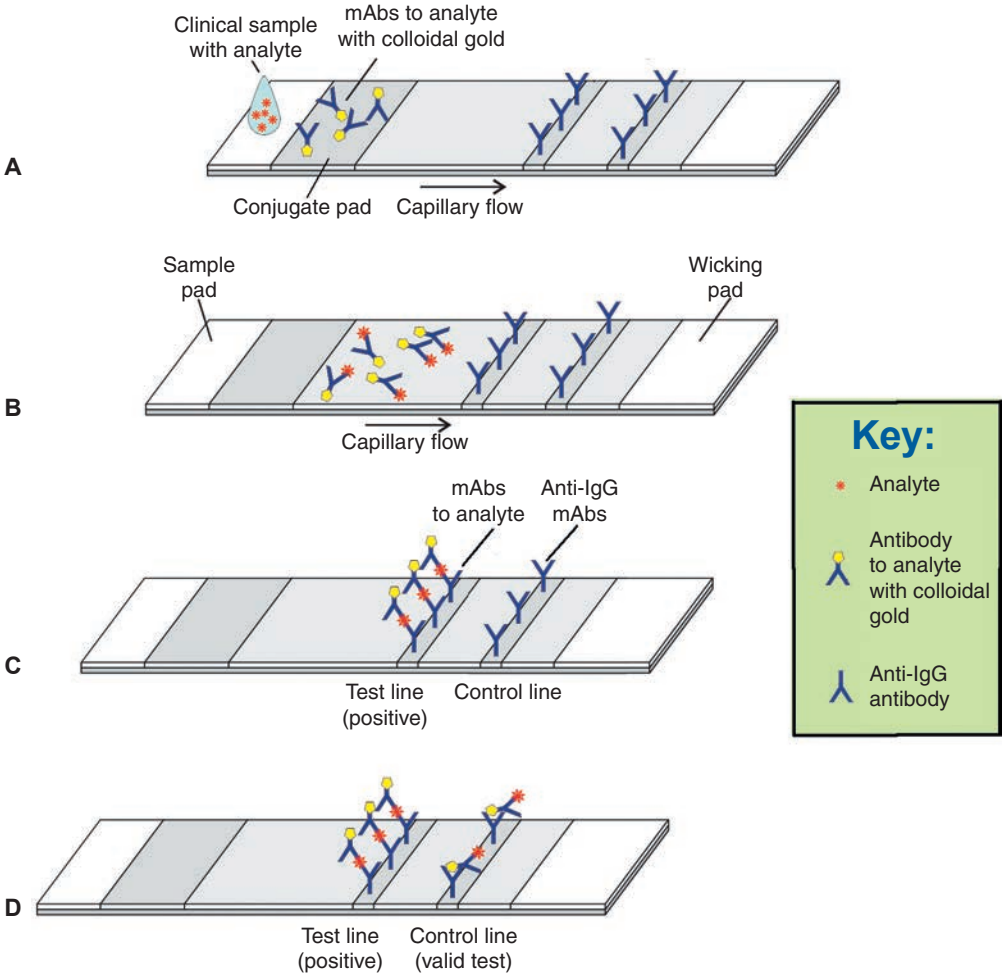


FIGURE 11–4 Immunochromatographic assay. (A) Patient sample is added to a cassette containing antibody labeled with colloidal gold. (B) Sample combines with antibody and is moved along by capillary flow. (C) Monoclonal antibody to the analyte captures the patient antigen attached to gold-labeled antibody. (D) Control line has antibody that captures colloidal gold-labeled antibody. *(Courtesy of University of Nevada School of Medicine.)*

antigen or antibody. Fluorescein absorbs maximally at 490 to 495 nm and emits a green color at 520 nm. It has a high intensity, good photostability, and a high quantum yield. Tetramethylrhodamine absorbs at 550 nm and emits red light at 585 nm. Because their absorbance and emission patterns differ, fluorescein and rhodamine can be used together. Other compounds used are phycobiliprotein, europium (β-naphthyl trifluoroacetone), and lucifer yellow VS.[1]

Fluorescent tags or labels on antibodies were first used for localization of antigen in cells or tissues.[5] Antibodies used to identify such antigens are highly specific; when bound to antigen in the tissue, the fluorescent probe attached to the antibody is detected under ultraviolet light using a fluorescent microscope.[8] This technique is called **immunofluorescent assay.** In this manner, many types of antigens can be detected either in fixed tissue sections or in live cell suspensions with a high degree of sensitivity and specificity.

The presence of a specific antigen is determined by the appearance of localized color against a dark background. This method is used for rapid identification of microorganisms in cell culture or infected tissue, tumor-specific antigens on neoplastic tissue, transplantation antigens, and CD antigens on T and B cells through the use of cell flow cytometry. (See Chapter 13 for a more complete discussion of the principles of cell flow cytometry.)

Direct Immunofluorescent Assays

Fluorescent staining can be categorized as direct or indirect, depending on whether the original antibody has a fluorescent tag attached. In a **direct immunofluorescent assay,** antibody that is conjugated with a fluorescent tag is added directly to unknown antigen that is fixed to a microscope slide. After incubation and a wash step, the slide is read using a fluorescence microscope. Antigens are typically visualized as bright apple green or orange-yellow objects against a dark background. Examples of antigens detected by the direct method include *Legionella pneumophila* and *Chlamydia trachomatis*.[9,10] Direct immunofluorescent assay is best suited to antigen detection in tissue or body fluids, whereas indirect assays, described in the text that follows, can be used for both antigen and antibody identification.[5]

Indirect Immunofluorescent Assays

Indirect immunofluorescent assays, which are more commonly used than direct assays, involve two steps. In the first step, patient serum is incubated with a known antigen attached to a solid phase. The slide is then washed and an anti-human immunoglobulin containing a fluorescent tag is added. This immunoglobulin combines with the first antibody to form a sandwich, which localizes the fluorescence. In this manner, one antibody conjugate can be used for many different types of reactions, eliminating the need for numerous purified, labeled reagent antibodies. Indirect assays result in increased staining because multiple molecules can bind to each primary molecule, thus making this a more sensitive technique.[5] Such assays are especially useful in antibody identification and have been the standard for detecting treponemal and antinuclear antibodies, as well as antibodies to a number of viruses.[11–13]

Figure 11–5 depicts the difference between the two techniques. Reading immunofluorescent assays on slides may be open to interpretation and only experienced clinical laboratorians should be responsible for reporting out slide results.

Fluorescence Polarization Immunoassays

A number of quantitative heterogeneous fluorescent immunoassays (FIAs) have been developed that correspond to similar types of enzyme immunoassays except that a fluorescent tag is used. However, many of the newer developments in FIAs are related to homogeneous immunoassays. Homogeneous FIA, just like the corresponding enzyme immunoassay, requires no separation procedure, so it is rapid and simple to perform. There is only one incubation step and no wash step; competitive binding is usually involved. The basis for this technique is the change that occurs in the fluorescent label on antigen when it binds to specific antibody. Such changes may be related to wavelength emission, rotation freedom, polarity, or dielectric strength. The amount of fluorescence measured

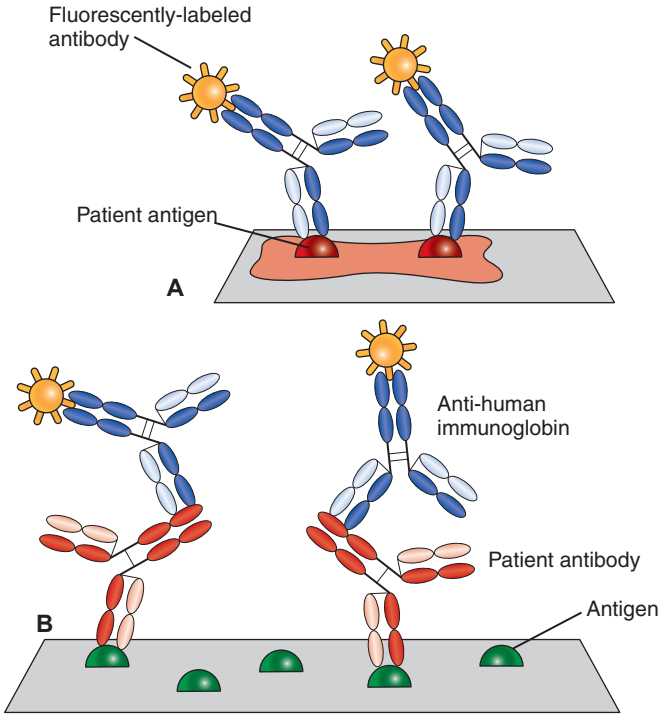


FIGURE 11–5 Direct versus indirect immunofluorescent assays. (A) In direct fluorescent assay, the patient antigen is fixed to a microscope slide and incubated directly with a fluorescent-labeled antibody. The slide is washed to remove unbound antibody. If specific antigen is present in the patient sample, fluorescence will be observed. (B) In indirect fluorescence, well-characterized tissues or cells are fixed to slides. Specific antibody in patient serum (in red) binds to the antigens on the slides. A wash step is performed and a labeled anti-human immunoglobulin is added. After a second wash step to remove any uncombined anti-immunoglobulin, fluorescence of the sample is determined. The amount of fluorescence is directly in proportion to the amount of patient antibody present.

is directly related to the amount of antigen in the patient sample. As binding of patient antigen increases, binding of the fluorescent analyte decreases; hence, more fluorescence is observed.

One of the most popular techniques developed is **fluorescence polarization immunoassay (FPIA),** which is based on the change in polarization of fluorescent light emitted from a labeled molecule when it is bound by antibody.[1,9] Incident light directed at the specimen is polarized with a lens or prism so that the waves are aligned in one plane. If a molecule is small and rotates quickly enough, the emitted light is unpolarized after it is excited by polarized light.[1,2,8] If, however, the labeled molecule is bound to antibody, the molecule is unable to tumble as rapidly and it emits an increased amount of polarized light **(Fig. 11–6).** Thus, the degree of polarized light reflects the amount of labeled analyte that is bound. A polarization analyzer is used to measure the amount of polarized light.

In FPIA, labeled antigens compete with unlabeled antigen in the patient sample for a limited number of antibody-binding sites. The more antigen that is present in the patient sample, the less the fluorescence-labeled antigen is bound and the less the polarization that will be detected. Hence, the degree of fluorescence polarization is inversely proportional to concentration of the analyte.[8]

FPIA is limited to small molecules that tumble freely in solution, usually low molecular weight analytes.[2,8] The technique has been used mainly to determine concentrations of therapeutic drugs and hormones. It requires sophisticated instrumentation and is the basis for several automated analyzers on the market today.

The use of fluorescence has the potential for high sensitivity and versatility. The main problem, however, has been separation of the signal on the label from autofluorescence produced by different organic substances normally present in serum. An additional consideration is nonspecific binding of the labeled conjugate to other proteins in serum. Binding to these molecules would increase polarization, thus falsely decreasing values. Fluorescence polarization has overcome some of these problems and has seen more widespread use. However, it requires expensive, dedicated equipment, which may limit its use in smaller laboratories.

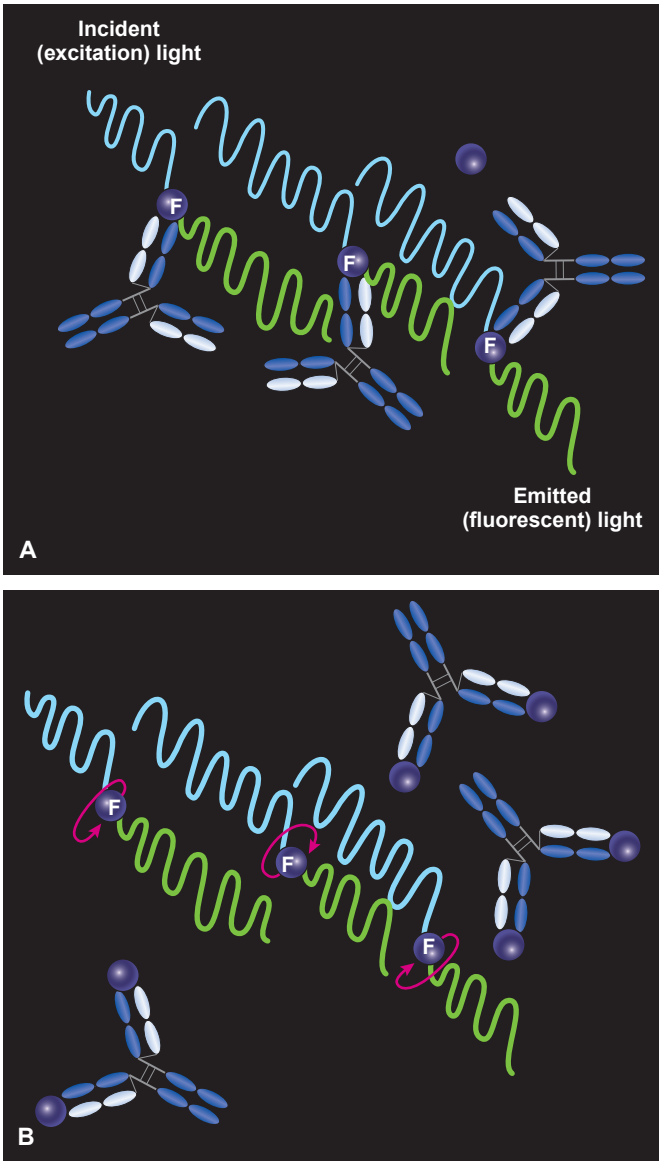


FIGURE 11–6 Fluorescence polarization immunoassay. Reagent antibody and fluorescently labeled analyte are added to the patient sample. (A) If sample analyte concentration is low, the antibody will bind most of the labeled analyte, restricting its rotation. When excited by polarized light, the emitted fluorescence will remain polarized (the light waves all oscillate with the same orientation). (B) If the patient sample has a high concentration of analyte, this unlabeled analyte will occupy most of the antibody-binding sites, leaving the labeled analyte free to rotate. The light waves emitted by the rotating labels will not be uniformly oriented (less polarization).

Chemiluminescent Immunoassays

Chemiluminescent immunoassay is another technique employed to visualize antigen–antibody combination. Chemiluminescence is the emission of light caused by a chemical reaction, typically an oxidation reaction, producing an excited molecule that decays back to its original ground state.[8] A large number of molecules are capable of chemiluminescence, but some of the most common substances used are luminol, acridinium esters, ruthenium derivatives, and nitrophenyl oxalates.[2] When these substances are oxidized, intermediates are produced that are of a higher energy state. These intermediates spontaneously return to their original state, giving off energy in the form of light. Light emissions range from a rapid flash of light to a more continuous glow that can last for hours. For example, when acridinium esters are oxidized by hydrogen peroxide under alkaline conditions, they emit a quick burst or flash of light. The light remains for a longer time with luminol.[2] Different types of instrumentation are necessary for each kind of emission.

This type of labeling can be used for heterogeneous and homogeneous assays because labels can be attached to either antigen or antibody. In heterogeneous assays, competitive and sandwich formats are the ones most often used. Smaller analytes such as therapeutic drugs and steroid hormones are measured

using competitive assays, whereas the sandwich format is used for larger analytes such as protein hormones.

Chemiluminescent assays have an excellent sensitivity; the reagents are stable and relatively nontoxic.[2,8] Because very little reagent is used, they are also quite inexpensive to perform. The relatively high speed of detection also means a faster turnaround time. Detection systems basically consist of photomultiplier tubes, which are simple and relatively inexpensive. Chemiluminescent technology is the basis for several types of automated immunoassays, which are used to detect a wide range of substances including cardiac markers, hormones, vitamin D levels, and total IgE.[14,15]

A newer technique called electrochemiluminescence immunoassay uses electrochemical compounds that generate light when an oxidation-reduction reaction occurs. Ruthenium, one of the common chemical substances used as an indicator, can be conjugated with antibody and applied to sandwich type assays. It undergoes an electrochemiluminescent reaction with another chemical substance, tripropylamine (TPA), at the surface of an electrode. When the ruthenium is oxidized and then returned to its reduced state through interaction with TPA, it gives off light that can be measured by a photomultiplier tube.[1] Magnetic beads are often used as solid phase to capture the labeled antibody.

False negative results may be obtained if some biological materials such as urine or plasma cause quenching of the light emission. Electrochemiluminescence assays have begun to be more widely applied to immunologic testing and have great potential for the future.

SUMMARY

- Labeled immunoassays were developed to measure antigens and antibodies that may be small in size or present in very low concentrations.
- The substance that is being measured is called the analyte.
- Labeling techniques include the use of radioactivity, enzymes, fluorescence, and chemiluminescence.
- There are two major types of immunoassays: competitive and noncompetitive. In competitive assays, all the reactants are added at the same time and labeled antigen competes with patient antigen for a limited number of antibody-binding sites.
- In competitive immunoassays, the concentration of patient antigen is inversely proportional to the amount of bound analyte. In other words, the greater the amount of patient antigen there is, the less the labeled antigen will be bound.
- Noncompetitive assays allow any antigen present to combine with an excess of antibody attached to a solid phase. A second antibody bearing a label is added and binds wherever there is patient antigen.
- In noncompetitive assays, the amount of the label detected is directly proportional to the amount of patient antigen present.
- Antibodies used in immunoassays must be very specific and have a high affinity for the antigen in question. Specificity helps to cut down on cross-reactivity and the affinity determines how stable the binding is between antigen and antibody. These two factors help to determine the sensitivity of such assays.
- Radioimmunoassay was the first type of immunoassay to be applied to quantitative measurements of analytes in the clinical laboratory. The original technique was based on competition between labeled and unlabeled antigen for a limited number of antibody-binding sites.
- Indirect enzyme immunoassays (ELISA) are a type of noncompetitive assay in which antigen is bound to solid phase and patient antibody is detected. After allowing sufficient time for binding to occur, a second enzyme-labeled antibody is added.
- In capture or sandwich assays, the antibody is bound to solid phase and any patient antigen is allowed to bind or be captured. A second labeled antibody is added, which also binds to patient antigen to make a "sandwich."
- Homogeneous enzyme assays require no separation step. They are based on the principle that enzyme activity changes as specific antigen–antibody binding occurs. When antibody binds to enzyme-labeled antigen, steric hindrance results in a loss in enzyme activity.
- Homogeneous assays are used for detection of low molecular weight analytes such as hormones, therapeutic drugs, and drugs of abuse.
- Simple one-step formats have been developed for heterogeneous enzyme assays. Rapid flow-through test devices are able to capture antigen or antibody in a certain spot on a membrane. The results are easy to interpret.
- Fluorochromes are fluorescent compounds that absorb energy from an incident light source and convert that energy to light of a longer wavelength.
- Direct immunofluorescent assays involve antigen detection through a specific antibody that is labeled with a fluorescent tag. The presence of fluorescence is detected with a fluorescent microscope that utilizes ultraviolet light.
- In indirect immunofluorescent assays, the original antibody is unlabeled. Incubation with antigen is followed by addition of a second fluorescent-labeled anti-immunoglobulin that detects antigen–antibody complexes.
- Fluorescence polarization immunoassay (FPIA) is a type of homogeneous fluorescent immunoassay. It is based on the principle that when an antigen is bound to antibody, polarization of light increases.
- Chemiluminescence is produced by certain compounds when they are oxidized and emit light as they return to their original ground state. Substances that do this can be used as markers in reactions that are similar to RIA and enzyme immunoassays.

Study Guide: Labeled Immunoassays

TYPE OF ASSAY	PRINCIPLE	RESULTS
Competitive	Patient antigen competes with labeled antigen for limited antibody-binding sites.	Inverse ratio: The more patient antigen is present, the less the label detected.
Noncompetitive or indirect ELISA	Excess solid-phase antigen binds patient antibody and a second labeled antibody is added.	All patient antibody is allowed to bind. Amount of label is directly proportional to the amount of patient antibody present.
Capture or sandwich	Excess solid-phase antibody binds patient antigen and a second labeled antibody is added.	All patient antigen is allowed to bind. Amount of label is directly proportional to the amount of patient antigen present.
Homogeneous	Patient antigen and enzyme-labeled antigen react with reagent antibody in solution. Enzyme label is inactivated when reagent antigen binds to antibody.	No separation step. Antibody in solution. Inverse ratio between patient antigen and amount of label detected.
Direct fluorescent	Patient antigen is attached to a slide. Specific fluorescent-labeled antibody is added.	If fluorescence is detected, patient antigen is present and the test is positive.
Indirect fluorescent	Reagent antigen is attached to a slide. Patient antibody is allowed to react. A second fluorescent-labeled antibody is added.	If fluorescence is detected, patient antibody is present and the test is positive.
Fluorescent polarization	Fluorescent-labeled antigen competes with patient antigen for a limited number of soluble antibody-binding sites.	When patient antigen binds, less reagent antigen binds and less polarization will be detected. Inverse ratio between patient antigen and amount of polarization.
Immunochromatographic	Patient sample is added to a test strip and migrates through the strip. Labeled antigen or antibody binds and is captured by a second reagent in the detection zone.	If test is positive, a line or plus sign will form on the test strip where patient antigen or antibody is captured.

CASE STUDY

A 2-year-old male child has symptoms that include fatigue, nausea, vomiting, and diarrhea. These symptoms have persisted for several days. Stool cultures for bacterial pathogens such as *Salmonella* and *Shigella* were negative. The stool was also checked for ova and parasites and the results were negative. The day-care center that the child attends has had a previous problem with contaminated water; therefore, the physician is suspicious that this infection might be caused by *Cryptosporidium,* a waterborne pathogen. However, because no parasites were found, he is not certain how to proceed.

Questions

a. Does a negative finding rule out the presence of a parasite?
b. What other type of testing could be done?
c. How does the sensitivity of testing such as enzyme immunoassay compare with visual inspection of stained slides?
d. What are other advantages of enzyme immunoassay tests?

REVIEW QUESTIONS

1. Which of the following statements accurately describes competitive binding assays?
   - **a.** Excess binding sites for the analyte are provided.
   - **b.** Labeled and unlabeled analyte are present in equal amounts.
   - **c.** The concentration of patient analyte is inversely proportional to bound label.
   - **d.** All the patient analyte is bound in the reaction.

2. How do heterogeneous assays differ from homogeneous assays?
   - **a.** Heterogeneous assays require a separation step.
   - **b.** Heterogeneous assays are easier to perform than homogeneous assays.
   - **c.** The concentration of patient analyte is indirectly proportional to bound label in heterogeneous assays.
   - **d.** Homogeneous assays are more sensitive than heterogeneous ones.

3. In the following equation, what is the ratio of bound radioactive antigen (Ag*) to bound patient antigen (Ag)?

   $$12Ag^* + 4Ag + 4Ab \rightarrow :\_\_\_Ag^*Ab + \_\_\_AgAb + Ag^* + \_\_\_Ag$$

   - **a.** 1:4
   - **b.** 1:3
   - **c.** 3:1
   - **d.** 8:4

4. Which of the following responses characterizes a capture or sandwich enzyme assay?
   - **a.** Less sensitive than competitive enzyme assays
   - **b.** Requires two wash steps
   - **c.** Best for small antigens with a single determinant
   - **d.** A limited number of antibody sites on solid phase

5. Which of the following is an advantage of enzyme immunoassay over RIA?
   - **a.** Decrease in hazardous waste
   - **b.** Shorter shelf life of kit
   - **c.** Natural inhibitors do not affect results
   - **d.** Needs to be read manually

6. Which of the following is characteristic of direct fluorescent assays?
   - **a.** The anti-immunoglobulin has the fluorescent tag.
   - **b.** Antibody is attached to a solid phase.
   - **c.** Microbial antigens can be rapidly identified by this method.
   - **d.** The amount of color is in inverse proportion to the amount of antigen present.

7. Which of the following is true of fluorescence polarization immunoassay?
   - **a.** Both antigen and antibody are labeled.
   - **b.** Large molecules polarize more light than smaller molecules.
   - **c.** When binding occurs, there is quenching of the fluorescent tag.
   - **d.** The amount of fluorescence is directly proportional to concentration of the analyte.

8. A fluorescent substance is best described as one in which
   - **a.** light energy is absorbed and converted to a longer wavelength.
   - **b.** the emitted wavelength can be seen under normal white light.
   - **c.** there is a long time between the absorption and emission of light.
   - **d.** it spontaneously decays and emits light.

9. In a noncompetitive enzyme immunoassay, if a negative control shows the presence of color, which of the following might be a possible explanation?
   - **a.** No reagent was added.
   - **b.** Washing steps were incomplete.
   - **c.** The enzyme was inactivated.
   - **d.** No substrate was present.

10. Which of the following best characterizes chemiluminescent assays?
    - **a.** Only the antigen can be labeled.
    - **b.** Tests can be read manually.
    - **c.** These are only homogeneous assays.
    - **d.** A chemical is oxidized to produce light.

11. Immunofluorescent assays may be difficult to interpret for which reason?
    - **a.** Autofluorescence of substances in serum
    - **b.** Nonspecific binding to serum proteins
    - **c.** Subjectivity in reading results
    - **d.** Any of the above

12. Which statement best describes flow-through immunoassays?
    - **a.** Results are quantitative.
    - **b.** They are designed for point-of-care testing.
    - **c.** Reagents must be added separately.
    - **d.** They are difficult to interpret.

13. Which of the following is characteristic of an indirect enzyme immunoassay?

a. The first antibody has the enzyme label.
b. All reagents are added together.
c. Color is directly proportional to the amount of patient antigen present.
d. Enzyme specificity is not essential.

14. In a homogeneous enzyme immunoassay, which best describes the enzyme?

a. Enzyme activity is altered when binding to antibody occurs.
b. The enzyme label is on the antibody.
c. Enzyme activity is directly proportional to the amount of patient antigen present.
d. Most enzymes can be used in this type of assay.

15. In an indirect immunofluorescent assay, what would be the outcome of an improper wash after the antibody-enzyme conjugate is added?

a. Results will be falsely decreased.
b. Results will be falsely increased.
c. Results will be unaffected.
d. It would be difficult to determine the effect.

16. In a heterogeneous enzyme immunoassay, if the patient sample produces more color than the highest positive control, what action should be taken?

a. Report the results out as determined.
b. Dilute the patient sample.
c. Repeat the assay using one-half the volume of the patient sample.
d. Report the results as falsely positive.

12 Molecular Diagnostic Techniques

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Describe the structure of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).
2. Identify complementary sequences.
3. Define hybridization.
4. Describe restriction enzyme mapping of DNA.
5. Discuss the principles of the polymerase chain reaction (PCR).
6. Apply dye and probe signal detection to PCR.
7. Assess template quantity by qPCR.
8. Discuss DNA and RNA amplification methods.
9. Differentiate target amplification from probe amplification and give examples of each.
10. Explain the basis of DNA chain termination sequencing.
11. List technologies for next generation sequencing (NGS).

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Amplicons
Amplification
Branched DNA (bDNA)
Chain termination sequencing
Complementary or copy DNA (cDNA)
Denatured
Deoxyribonucleic acid (DNA)
Electropherogram
Flanking
Fluorescence in situ hybridization (FISH)
Gel electrophoresis
Genetic code
Hybridization
Immunogenetics
Internal amplification control
Isothermal
Mutation
Next generation sequencing (NGS)
Nick
Nucleic acid sequences
Nucleotide
Palindromic sites
Polymerase chain reaction (PCR)
Polymorphisms
Primers
Probe
Quantitative PCR (qPCR)
Restriction endonucleases
Restriction fragment length polymorphisms (RFLPs)
Reverse transcriptase
Ribonucleic acid (RNA)
Southern blot
Strand cleavage
Strand displacement amplification (SDA)
Stringency
Threshold cycle
Transcription
Transcription-mediated amplification (TMA)
Translation
Variants

Molecular diagnostic assays are powerful tools used to gain information to aid in diagnosis and monitoring of disease and now play a central role in medicine. These techniques are based on the detection of specific **nucleic acid sequences** in microorganisms or particular cells. Such techniques may be an advantage in clinical diagnosis because detection of nucleic acids can be accomplished long before antibody detection is possible. Immunodeficiency and autoimmune diseases that have a genetic basis can also be identified more quickly. Molecular diagnostics also contribute to therapeutic decisions, transplant selection, drug efficacy, forensics, and disease prognosis.[1] Molecular techniques used in the clinical laboratory to identify unique nucleic acid sequences include enzymatic cleavage of nucleic acids, gel electrophoresis, enzymatic amplification of target sequences, and hybridization with nucleic acid probes. All of these techniques are discussed in this chapter in addition to an overview of the structure and functions of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

Characteristics of Deoxyribonucleic Acid and Ribonucleic Acid

The two main types of nucleic acids are **deoxyribonucleic acid (DNA)** and **ribonucleic acid (RNA).** DNA carries the primary genetic information within chromosomes found in each cell. RNA, in contrast, is an intermediate nucleic acid structure that helps convert the genetic information encoded within DNA into proteins that are the primary cellular component. Both DNA and RNA are macromolecules of nucleotides. A **nucleotide** is a unit composed of a phosphorylated ribose sugar and a nitrogen base. DNA and RNA have the same two purine bases, adenine and guanine, but the pyrimidine bases differ. DNA uses cytosine and thymine, whereas in RNA, cytosine is present, but uracil replaces thymine **(Fig. 12–1).**

Nucleotides

In DNA, the nitrogen base of a nucleotide—guanine, adenine, cytosine, or thymine—is attached to the 1′ carbon of the deoxyribose sugar. The ribose 5′ carbon is mono-, di-, or triphosphorylated. The ribose 3′ carbon carries a hydroxyl group (OH). Positions in the deoxyribose and nitrogen base rings are numbered as shown in **Figure 12–2.**

In contrast to the deoxyribose sugar in DNA, ribose is the primary sugar in RNA. Unlike deoxyribonucleotides, which are hydroxylated on the 3′ carbon, the phosphorylated ribose sugar in RNA carries hydroxyl groups on carbons 2′ and 3′ **(Fig. 12–3).** Furthermore, thymine is not present in RNA and is replaced by uracil.

Natural modifications of the nucleotide structure may occur through additions, substitutions, and other chemical modifications. These modifications may be enzymatically catalyzed in the cell or spontaneous. Enzymatic alteration of nucleotides in the cell is what brings about antibody diversity.

The Nucleic Acid Polymer

Nucleotides are polymerized by attachment of the sugar 3′ hydroxyl groups to the 5′ phosphate groups, forming a phosphodiester bond. A chain of nucleotides makes up one strand of RNA or DNA. Most RNA exists without a partner or complementary strand; they are single stranded. In contrast, DNA is mostly double stranded and arranged in a double helix **(Fig. 12–4).** The double helix is formed by two single DNA chains wrapped around each other. There is a complementary relationship between the two linear polymers (strands) of the double helix. All C's are across from G's and all A's are across from T's in the two corresponding strands.

The hydroxyl group on the 3′ carbon and the phosphate group on the 5′ carbon that participate in formation of the DNA polymer through phosphodiester bonds give the DNA

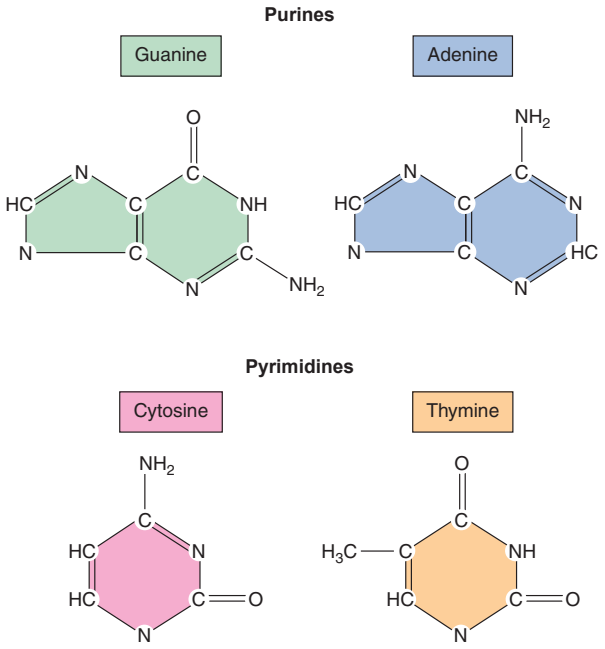

FIGURE 12–1 DNA is made up of nucleotides, each of which is composed of a phophorylated sugar and a nitrogen base. Adenine and guanine are the purine bases, and cytosine and thymine are the pyrimidine bases. *(Adapted from Buckingham L.* Molecular Diagnostics. *2nd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

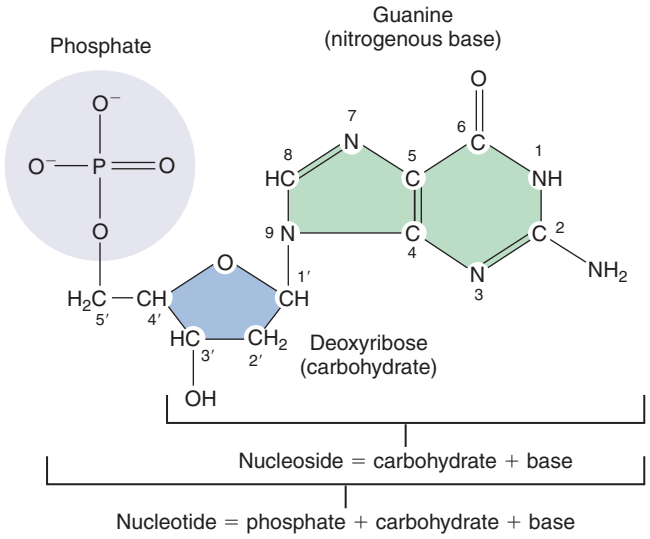

FIGURE 12–2 Nucleotide structure. Nitrogen base ring positions are numbered ordinally and the ribose ring positions are numbered with prime numbers. The 1′ ribose carbon carries the nitrogen base. The 3′ ribose carbon hydroxyl group and the 5′ ribose carbon phosphate group are important for connecting nucleotides together into a DNA chain. A nucleoside is an unphosphorylated ribose sugar carrying a nitrogen base. *(Adapted from Buckingham L.* Molecular Diagnostics. *2nd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

strands polarity, that is, a 5′ phosphate end and a 3′ hydroxyl end. Sequences are by convention ordered in the 5′ to 3′ direction. Complementary strands hydrogen bond together in an antiparallel arrangement with their 5′ phosphates on opposite ends of the double helix **(Fig. 12–5).**

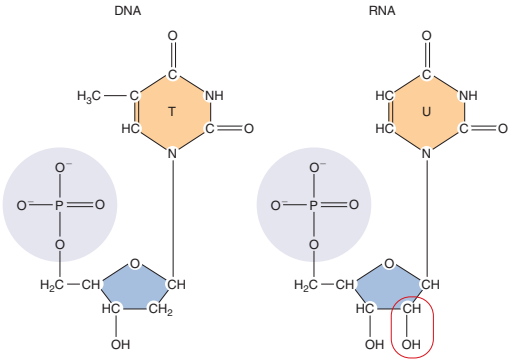

FIGURE 12–3 The ribose sugar in RNA is hydroxylated on the 2′ and 3′ carbons (right), whereas in DNA it's only hydroxylated at the 3′ carbon (left). The nitrogen base, uracil, found in RNA (right) is analogous to thymine found in DNA (left). *(Adapted from Buckingham L.* Molecular Diagnostics. *2nd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

The two chains (strands) of the DNA double helix are held together by hydrogen bonds between their nucleotide bases. Guanine (G) and cytosine (C) are complementary, that is, they will only hydrogen bond with each other. Similarly, adenine (A) and thymine (T) are complementary to each other. G pairs with C by three hydrogen bonds and A pairs with T by two hydrogen bonds. Two bases paired together in this way are called a base pair (bp). The length of a double-stranded DNA macromolecule is measured in bp. The length of a single strand of RNA (or DNA) is measured in bases (b). Metric prefixes are used to describe long strands of DNA or RNA, for example, 1,000 bp or b comprise a kilobase pair (kbp) or kilobase (kb), respectively. One million bp or b comprise a megabase pair (Mbp) or megabase (Mb), respectively.

In humans, each chromosome is a double helix of DNA. There are 46 chromosomes per nucleus—two copies of each of the 22 autosomes (nonsex chromosomes 1 to 22) and two copies of the X chromosome in females (46,XX) or one copy each of the X and Y chromosome in males (46,XY). The autosomes are numbered according to size from the largest, chromosome 1 (246 Mbp), to chromosome 22 (47 Mbp). The X chromosome, 154 Mbp, is much larger than the Y chromosome (57 Mbp). Genes are locations in chromosomes carrying information for a gene product, either protein or RNA (noncoding RNA). The 23 chromosomes carry fewer than 30,000 protein-coding genes, with two copies of each gene making up the diploid genome (two copies of each of the 23 chromosomes per cell or 46 chromosomes).

With the development of array and sequencing technology, it is possible to investigate the entirety of DNA in the cell nucleus (genome). In contrast to tests that examine one gene at a time, these genomic studies are designed to analyze all of the genes simultaneously to assess the overall genetic status of the cell.

DNA Replication

Replication of DNA is semiconservative, that is, the two strands of the DNA duplex are separated and each single strand serves

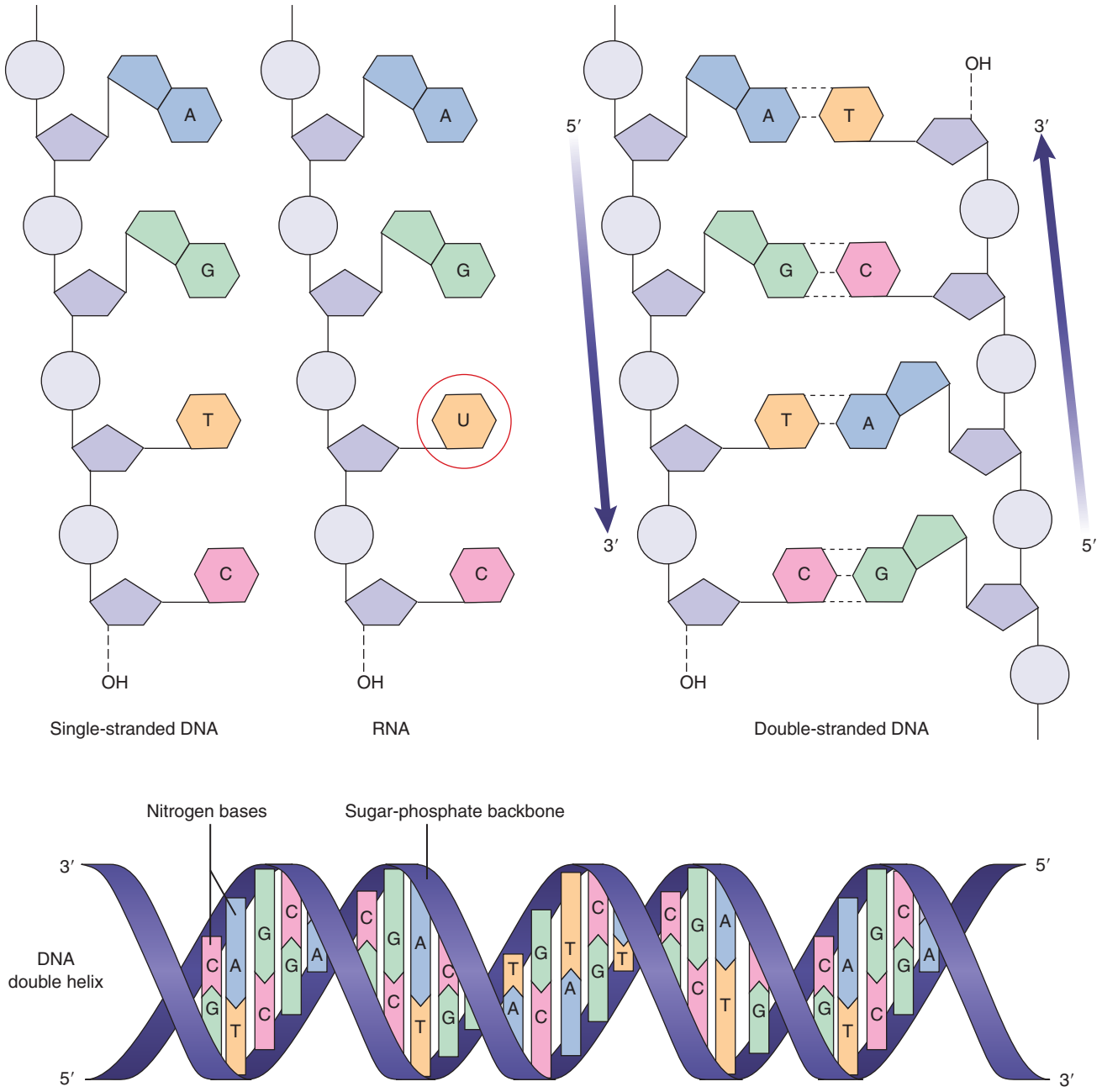

FIGURE 12–4 Nucleic acids are chains of nucleotides covalently attached, forming a sugar–phosphate backbone of phosphodiester bonds. RNA has uracil nucleotides rather than thymines and does not always have a complementary partner strand as does double-stranded DNA. In double-stranded DNA, the double helix is coiled such that areas of the double helix, the major and minor grooves, can interact with proteins and other molecules. *(Adapted from Buckingham L.* Molecular Diagnostics. *2nd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

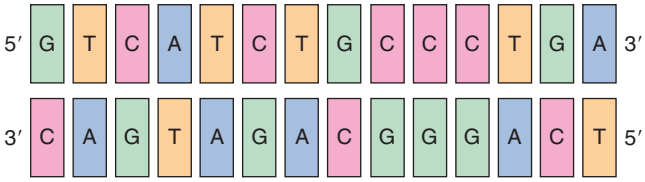

FIGURE 12–5 Complementary sequences are not identical. In each nucleotide chain, G nucleotides always hydrogen bond to C nucleotides and A nucleotides bond with T nucleotides in the complementary chain. Complementary sequences hybridize in an antiparallel arrangement.

as a template for a newly synthesized complementary strand. Two daughter strands result, each of which is an exact copy of the original molecule. DNA replication proceeds with the formation of phosphodiester bonds between the 5′ phosphate of an incoming nucleotide and the 3′ hydroxyl of the previously added nucleotide (**Fig. 12–6**). This reaction is catalyzed by DNA polymerase. The parental template strand is read from the 3′ to 5′ direction, whereas synthesis of the new strand of DNA proceeds 5′ to 3′, making the completely replicated strand and its parent strand antiparallel.

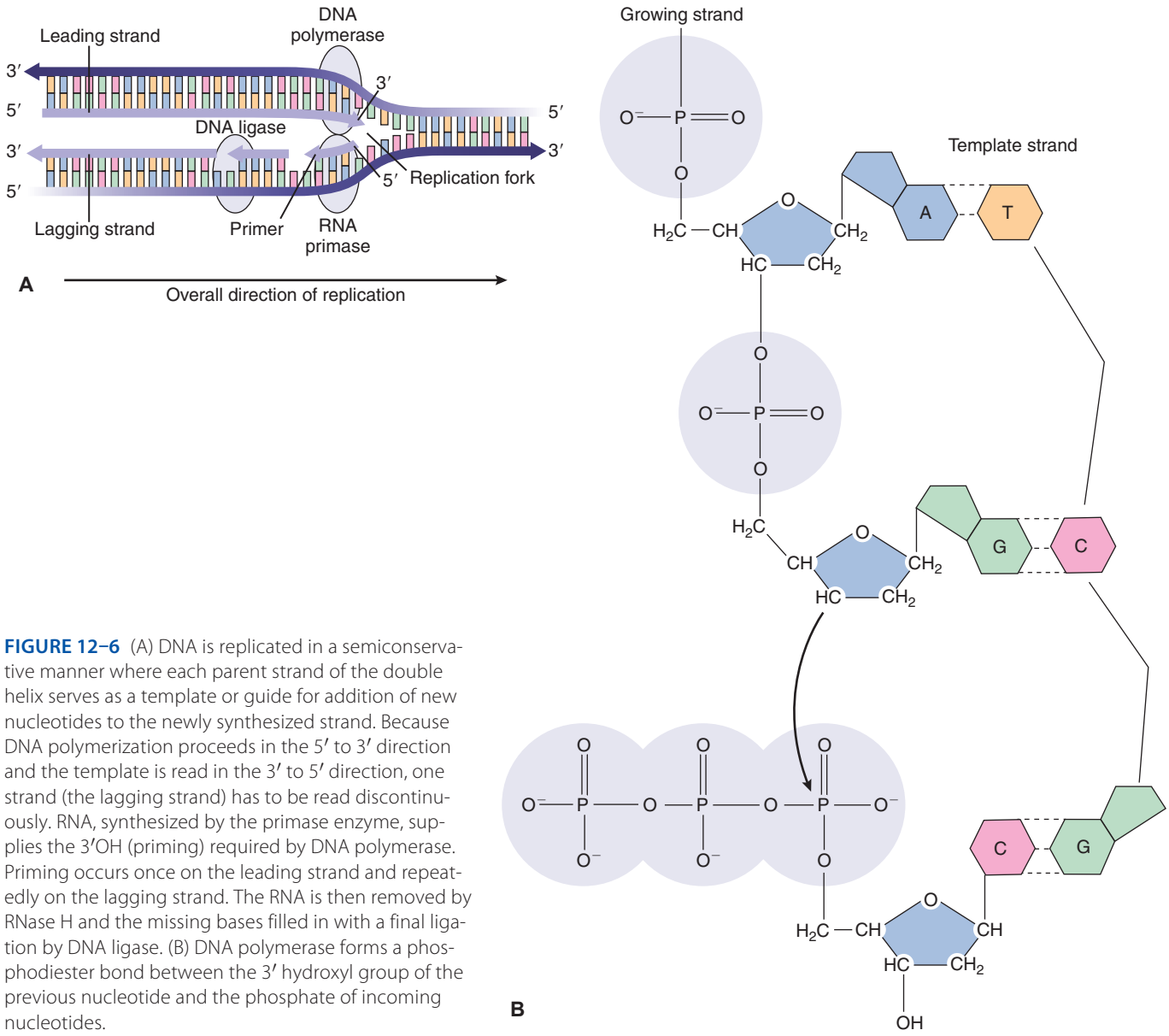

FIGURE 12–6 (A) DNA is replicated in a semiconservative manner where each parent strand of the double helix serves as a template or guide for addition of new nucleotides to the newly synthesized strand. Because DNA polymerization proceeds in the 5′ to 3′ direction and the template is read in the 3′ to 5′ direction, one strand (the lagging strand) has to be read discontinuously. RNA, synthesized by the primase enzyme, supplies the 3′OH (priming) required by DNA polymerase. Priming occurs once on the leading strand and repeatedly on the lagging strand. The RNA is then removed by RNase H and the missing bases filled in with a final ligation by DNA ligase. (B) DNA polymerase forms a phosphodiester bond between the 3′ hydroxyl group of the previous nucleotide and the phosphate of incoming nucleotides.

DNA synthesis cannot begin without a preexisting 3′ hydroxyl group. To begin synthesis in vivo, a short (~60 b) RNA molecule is synthesized by an RNA polymerase (primase) enzyme. The RNA molecule is complementary to one strand of the DNA to be copied and will hydrogen bond to the DNA template. There, it provides the preexisting 3′ hydroxyl group required by DNA polymerase to copy the DNA. This aspect of DNA synthesis is useful for directing synthesis to a specific place in DNA. In the laboratory, short synthetic DNA molecules called **primers** are used routinely to direct copying of specific regions of DNA in vitro. The **polymerase chain reaction (PCR)** is an example of such targeted DNA synthesis. The double helix is copied in a single pass such that DNA undergoing replication can be observed by electron microscopy as a replication fork. DNA synthesis on the template strand in the 3′ to 5′ direction is not simultaneously consistent with that on the 5′ to 3′ direction., Thus, one strand is copied discontinuously (lagging strand—3′ to 5′) whereas the other is copied continuously (leading strand—3′ to 5′; see Fig. 12–6).

RNA Synthesis

RNA synthesis proceeds in a manner that is chemically similar to that of DNA synthesis with some exceptions. Unlike DNA synthesis, RNA synthesis can start de novo without a primer. RNA synthesis is catalyzed by RNA polymerase, a more error prone, slower polymerase (50 to 100 bases/second) than DNA polymerase (1,000 bases/second). There are more start sites for RNA polymerization than for DNA synthesis in the cell. The bulk of DNA synthesis takes place in the S phase of the cell cycle, whereas RNA synthesis occurs throughout the cell cycle and varies depending on the cellular requirements.

RNA is copied from almost all of the genome; however, only about 2% of the RNA-coding regions are translated into protein. Some genes code for transfer RNA (tRNA) and ribosomal

RNA (rRNA), which is required for protein synthesis. Other RNA products referred to as long- and short-noncoding RNA make up the remainder of the total RNA transcribed in the cell.

Protein Synthesis

After DNA is transcribed by RNA polymerase into RNA, the RNA transcripts of protein-coding genes (messenger RNA or mRNA) are translated into protein. Each mRNA is marked by a guanidine nucleotide covalently attached to its 5′ end in an unusual 5′–5′ bond (cap) and 2 to 20 adenines at the 3′ end (polyadenylation). These structures maintain the stability of the mRNA and allow its recognition by the ribosomes. Ribosomes are composed of ribosomal proteins and ribosomal RNA. Ribosomes assemble on the mRNA for protein synthesis.

Translation means converting information from one language to another. The language held in the order or sequence of the four nucleotides in the DNA chains must be translated into the order or sequence of the 20 amino acids making up a protein chain. The nucleotide sequence of mRNA contains a three-base recognition sequence (codon) for each of the 20 amino acids, that is, the genetic code **(Table 12–1).** Each amino acid has at least one associated three-base sequence (codon) carried in mRNA. The **genetic code** (3 nucleotides/1 amino acid as shown in Table 12–1) is redundant. All amino acids except methionine and tryptophan have more than one three-nucleotide codon. There are three stop codons that terminate protein synthesis.

The codons are carried from the nucleus to the cytoplasm in mRNA to be translated into protein. The tRNA serves as an adaptor between the nucleotide sequence in the RNA and the amino acid sequence in proteins, thus completing the transfer of genetic information from DNA to RNA to protein. This flow of information from DNA to mRNA to protein is known as the central dogma of molecular biology **(Fig. 12–7).**

Mutations

A change in the nucleotide sequence is a **mutation (Table 12–2).** Depending on how frequently they occur, changes in the nucleotide sequence may also be referred to as **variants** or **polymorphisms.** These alterations may be single bps up to chromosomal structural abnormalities involving millions of bps. Molecular technology is part of **immunogenetics,** the analysis of gene mutations and polymorphisms that affect immune function. Diseases such as immunodeficiency result from mutations in genes encoding proteins that bring about the immune response.[2,3]

Table 12–1 The Genetic Code Connects the Four-Base Nucleotide Sequence to the 20 Amino Acids

AMINO ACID	ABBREVIATION	SINGLE-LETTER CODE	CODONS
Alanine	Ala	A	GCU, GCG, GCC, GCA
Arginine	Arg	R	AGA, AGG
Asparagine	Asn	N	AAC, AAU
Aspartic acid	Asp	D	GAU, GAC
Cysteine	Cys	C	UGU, UGC
Glutamine	Gln	Q	CAA, CAG
Glutamic acid	Glu	E	GAA, GAG
Glycine	Gly	G	GGU, GGA, GGC, GGG
Histidine	His	H	CAU, CAC
Isoleucine	Ile	I	AUU, AUC, AUA
Leucine	Leu	L	UUA, UUG, CUA, CUC, CUG, CUU
Lysine	Lys	K	AAA, AAG
Methionine	Met	M	AUG
Phenylalanine	Phe	F	UUU, UUC
Proline	Pro	P	CCU, CCA, CCC, CCG
Serine	Ser	S	UCU, UCC, UCA, UCG
Threonine	Thr	T	ACU, ACA, ACG, ACC
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAU, UAC
Valine	Val	V	GUU, GUA, GUC, GUG
(Stop codons)		X	UAA, UAG, UGA

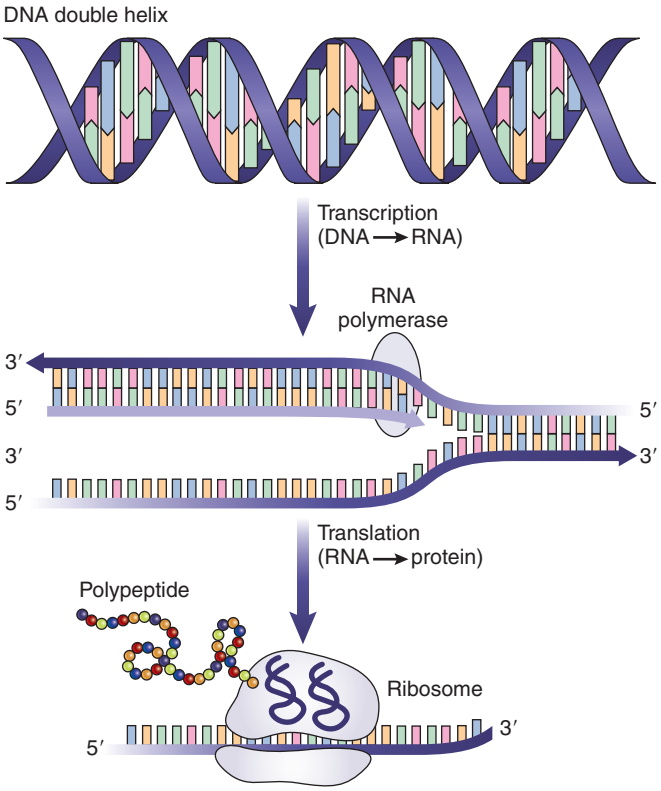

FIGURE 12–7 Messenger RNA (mRNA) is transcribed from DNA using RNA polymerase. The mRNA delivers the information to ribosomes where protein synthesis takes place. As each amino acid is added, the peptide chain continues to grow. This process, known as translation, is accomplished with the help of tRNA, which brings in individual amino acids.

Mutations can also affect the response to therapy; for example, cells expressing a mutation in the *JAK1* gene are hypersensitive to the antiproliferative and antitumorigenic effect of interferon chemotherapy, suggesting that this type of interferon should be considered as a potential therapy for acute leukemia.[4] As technology advances, immunogenetic testing will become increasingly routine in patient care.

Polymorphisms

Structurally, mutations and polymorphisms are the same thing: a change from a reference amino acid or nucleotide sequence. Polymorphisms are defined by their frequency in a population. Alterations in DNA or protein sequences shared by at least 2% of a population are considered polymorphisms. The different versions of the affected sequences are referred to as alleles. Polymorphic changes may or may not have phenotypic effects. Deleterious phenotypic changes are usually limited so that they do not reach the required frequency in a population; however, there are balanced polymorphisms that are maintained by an associated beneficial effect. A well-known example of this is the A to T base substitution in the beta-globin gene on chromosome 11 that causes sickle cell anemia. This DNA substitution results in the replacement of glutamic acid (E) with valine (V) at position 6 in the protein sequence. The mutation results in abnormal red blood cells (RBCs) that do not circulate efficiently. The deleterious effect is balanced by a beneficial phenotype of resistance to *Plasmodium* species that cause malaria.

Polymorphisms are found all over the genome. Like mutations, polymorphisms can involve a single bp (single nucleotide polymorphisms or SNPs) or millions of bps. There are about 10 million SNPs in the human genome.[5] Larger polymorphic differences are less frequent in the genome.

The most highly polymorphic region in the human genome is the major histocompatibility complex (MHC) locus on chromosome 6. The different nucleotide sequences result in multiple versions or alleles of the human leukocyte antigen (HLA) genes in the human population. These alleles differ by nucleotide sequence at the DNA level (polymorphisms) and by amino acid sequence. Each person will have a particular group of HLA alleles that are inherited from his or her parents. "Nonself" proteins are identified by the differences in the HLA alleles. The recommended nomenclature identifies HLA alleles by type, subtype, whether the allele is silent, if the allele differs in an untranslated region, and if the protein is secreted or not highly expressed.[6]

Other highly polymorphic areas of the genome include the genes coding for the antibody proteins and antigen-receptor proteins in B cells and T cells, respectively. Polymorphisms are introduced in each cell through genetic events (gene rearrangements) followed by enzymatically catalyzed sequence changes (somatic hypermutation). Thus, these sequences differ from cell to cell, allowing the generation of a large repertoire of antibodies and antigen receptors to better match any foreign antigen.

Table 12–2 DNA Changes Affect Amino Acid Sequences

MUTATION TYPE	EXAMPLE DNA CHANGE	EXAMPLE RNA CHANGE	EXAMPLE AMINO ACID CHANGE
Conservative substitution	ATC → CTC	AUC → CUC	Isoleucine → Leucine
Nonconservative substitution	CAT → CCT	CAU → CCU	Histidine → Proline
Silent	TAT → TAC	UAU → UAC	Tyrosine → Tyrosine
Nonsense	CAA → TAA	CAA → UAA	Glutamine → Stop
Frameshift	TGT AAC CAG → TGT AAA CCA G …	UGU AAC CAG → UGU AAA CCA G …	Tyr Asp Glu → Tyr Lys Pro …

Polymorphisms that create, destroy, or otherwise affect restriction enzyme sites are detected as **restriction fragment length polymorphisms (RFLPs)** or variations that differ among individuals. RFLPs are caused by variations in nucleotides within genes that change where restriction enzymes cleave the DNA. Repeat sequence polymorphisms, such as short tandem repeats (STR) and variable number tandem repeats (VNTR), are head-to-tail repeats of single bp to over 100 bp repeat units. STR has replaced RFLP for human identification (DNA fingerprinting) and HLA typing for parentage testing. STR and VNTR are also commonly used markers for engraftment monitoring after allogeneic bone marrow transplantation.

In addition to the nuclear genome, mitochondria located in the cytoplasm of eukaryotic cells carry their own genome. The mitochondrial genome is circular, containing about 16,500 bps. Polymorphisms are also found in two regions of mitochondrial DNA sequences (hypervariable regions). These polymorphisms are not transcribed into RNA and do not affect proteins. They are used for maternal inheritance testing because all maternal relatives share the same mitochondria and therefore have the same mitochondrial polymorphisms. Mitochondria provide cellular energy. Mutations in the mitochondrial genome affect high energy activities in muscle, brain and in the immune system. When an infection occurs, the immune response requires millions of cells and increased energy demands. Loss of mitochondrial function can result in increases in the frequency and length of infections. In the 1000 Genomes Project, an initiative to investigate the DNA of many diverse individuals, as many as 20% of healthy study participants had mitochondrial gene mutations.[7] Mitochondrial DNA mutations can cause other diseases, including epilepsy, as well as complex conditions such as type 2 diabetes, aging, and cancer.

Molecular Analysis

Nucleic acid tests are designed to detect changes (mutations and polymorphisms) in the DNA sequence. There are four main approaches to nucleic acid analysis: strand cleavage methods, hybridization methods, sequencing methods, and amplification methods.

Strand Cleavage Methods

One means of characterizing DNA is by cleaving it at specific locations through the use of enzymes and then separating the resulting fragments on the basis of size and charge through gel electrophoresis. This is referred to as **strand cleavage**. **Restriction endonucleases** are enzymes that recognize and bind to specific nucleotide sequences in the DNA so that they will only separate the DNA at those locations. Restriction endonuclease (restriction enzyme) cleavage methods are highly informative for investigating small genomes, such as those of microorganisms, or plasmids. They are also used for mutation and polymorphism detection on small amplified regions of human DNA. **Amplification** makes many copies of a particular DNA sequence and is used to study a small section of the genome.

There are hundreds of restriction enzymes with unique binding and cleavage sites. The restriction enzymes used in the clinical laboratory (type II restriction enzymes) recognize **palindromic sites.** These sites are nucleotide sequences that read the same 5′ to 3′ on both strands of the DNA. For example,

5′-GAATTC-3′
3′-CTTAAG-5′

is the recognition site for the restriction enzyme, *Eco*RI. Restriction enzymes are named for the organisms from which they were isolated. *Eco*RI was the first enzyme isolated from *Escherichia coli,* strain R. *Hind*III is the third enzyme isolated from *Haemophilus influenzae,* strain d. In a bacterium, restriction enzymes serve as part of a primitive immune system that allows it to recognize its own DNA and degrade any incoming foreign DNA.

DNA can be characterized by incubation with several restriction enzymes, separating the products by electrophoresis, and observing the size and pattern of the resulting fragments. DNA with a different sequence will yield different-sized bands characteristic of that DNA. These different band patterns are RFLPs. **Figure 12–8** shows the band patterns expected from digestion of two different DNA fragments, A and B. Fragment A has two sites for the endonuclease to cut, resulting in three bands on the gel. Fragment B has only one site, resulting in two gel bands. The loss of the second restriction site in fragment B is caused by a mutation in the recognition site for the endonuclease. The presence of the mutation, therefore, is inferred from the two band, rather than the three band pattern. Early work in recombinant DNA technology relied on these types of studies. RFLP analysis is applied to epidemiological studies of microorganisms and identification of resistance factors carried on extrachromosomal DNA (plasmids) in the cell.[8]

Hybridization Methods

For analysis of complex genomes, such as human DNA, restriction enzyme cleavage methods are not practical because

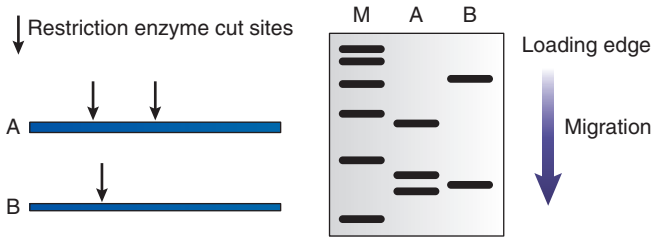

FIGURE 12–8 Restriction enzyme mapping characterizes DNA by the pattern of fragments generated when the DNA is cut with restriction enzymes. DNA double helix A has two restriction sites (arrows), whereas the DNA B has only one. When these two DNAs are digested with the restriction enzyme, the DNA A fragment will yield three fragments and DNA B will yield two. These band patterns are a characteristic of these DNAs (M = molecular weight standard, used for sizing the DNA fragments).

the DNA is too large and complex to generate readable fragment patterns. This problem was addressed by Edwin Southern in the mid-1970s.[9,10] Using the specificity of nucleic acid hybridization, he developed the **Southern blot. Hybridization** is the binding of two specific complementary nucleic acid strands together. With this method, the very informative RFLP studies could be performed directly on large and complex genomes.

Southern Blot Analysis

Southern blot analysis starts with restriction enzyme digestion of DNA isolated from the test organism or cells. The restriction fragments are then separated by agarose **gel electrophoresis.** The separated double-stranded fragments are then **denatured,** that is, separated into single strands in the gel. The treated DNA is transferred or blotted from the gel onto a nitrocellulose-based membrane **(Fig. 12–9).** At this stage, all of the millions of fragments from complex genomes will be present on the membrane, whereas only one particular gene or locus is of interest.

The key to the specificity of the Southern blot technique is the use of a probe. A **probe** is generally a nucleic acid, several hundred to a few thousand bases in length, with a known sequence that is used to identify the presence of a complementary DNA or RNA sequence in an unknown sample. Probes typically have an attached and detectable signal **(Fig. 12–10).** The signal can either be emission of radioactivity, development of color, or light. Radioactive nucleotides provide the radioactive signals, whereas the nonradioactive signals are generated by an antigen–antibody or biotin–avidin binding with the antibody or avidin conjugated to alkaline phosphatase or horseradish peroxidase enzymes that produce color or light when exposed to the color or light-emitting substrates **(Fig. 12–11).**

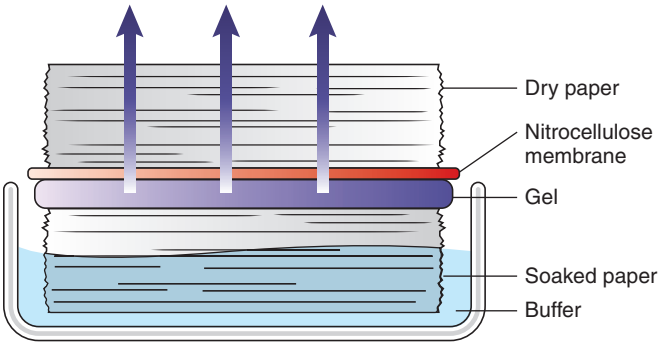

FIGURE 12–9 Capillary transfer of DNA after restriction digestion and electrophoresis from the gel to a nitrocellulose membrane was the original method of Southern blotting. Other blotting formats include vacuum transfer and electrophoretic transfer.

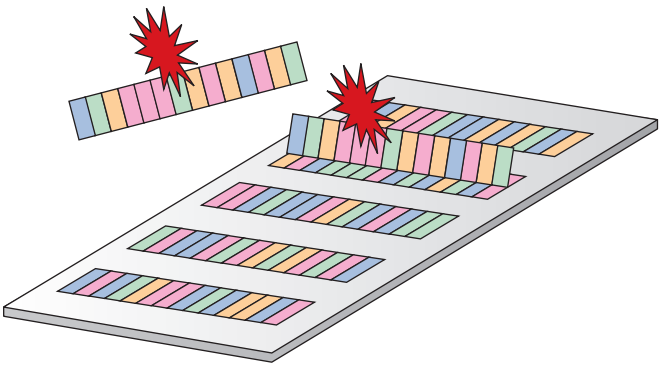

FIGURE 12–10 The labeled probe binds only to fragments on the membrane that are complementary to the nucleotide sequence of the probe.

Results of a Southern blot reveal the fragmented regions of the test DNA that are complementary to the probe. Any locus or gene can thus be analyzed for RFLP using this method. Positive human identification by DNA fingerprinting was first performed by Southern blot.[11] Southern blots can also be used for detecting deletions in mitochondrial DNA and gene rearrangements in nuclear DNA.[12,13]

A variation of the Southern blot known as northern blot is used to analyze RNA structure and expression.[14] Northern blots are mostly research tools and not used routinely for diagnostic purposes.

Array Methods

Although Southern blotting was helpful to analyze a single or a very few DNA sequences, as knowledge of genetic networks and pathways grew it became apparent that there was a need to analyze hundreds or thousands of genes at the same time. Variations of the Southern blot led to the development of array technology, where multiple targets or multiple patient samples could be investigated simultaneously. Such techniques are used for the prognosis in leukemia or diagnosis of complex diseases such as autoimmune states, where multiple nucleic acid or protein targets have to be detected.

The first array methodology used dot blot hybridization. In this method, instead of cutting and resolving DNA or RNA on a gel and blotting it to a membrane for probe hybridization, a highly specific unlabeled probe is spotted directly on a solid support. Then the test sample (nucleic acids or proteins isolated from cultures, cells, or body fluids) is labeled and hybridized to the many immobilized probes.

To assess the abnormal presence or amounts of target molecules, a normal reference is required. The use of fluorescent labels provides a number of signals that can be distinguished from one another. This allows dual detection of the test sample and a reference sample that is hybridized to the array along with it **(Fig. 12–12).** Measurement of test material can be compared with the normal reference. Arrays are used to detect amplifications or deletions in DNA, gene expression (RNA synthesis), and SNP, where there is a change in a single base.

Arrays were initially performed by manually spotting probes on nitrocellulose membranes and hybridizing labeled test DNA. This macroarray method was soon replaced by automated array printers that could deposit tens of thousands of probes on a glass slide. These microarrays, also known as gene chips, are used for a variety of applications including analysis of genetic mutations in malignancies, detection of microdeletions,[15,16] detection of viral resistance mutations, and gene expression profiling.[17,18]

Another configuration of array technology is bead arrays. These assays are based on preparations of 100 to 400 fluorescent

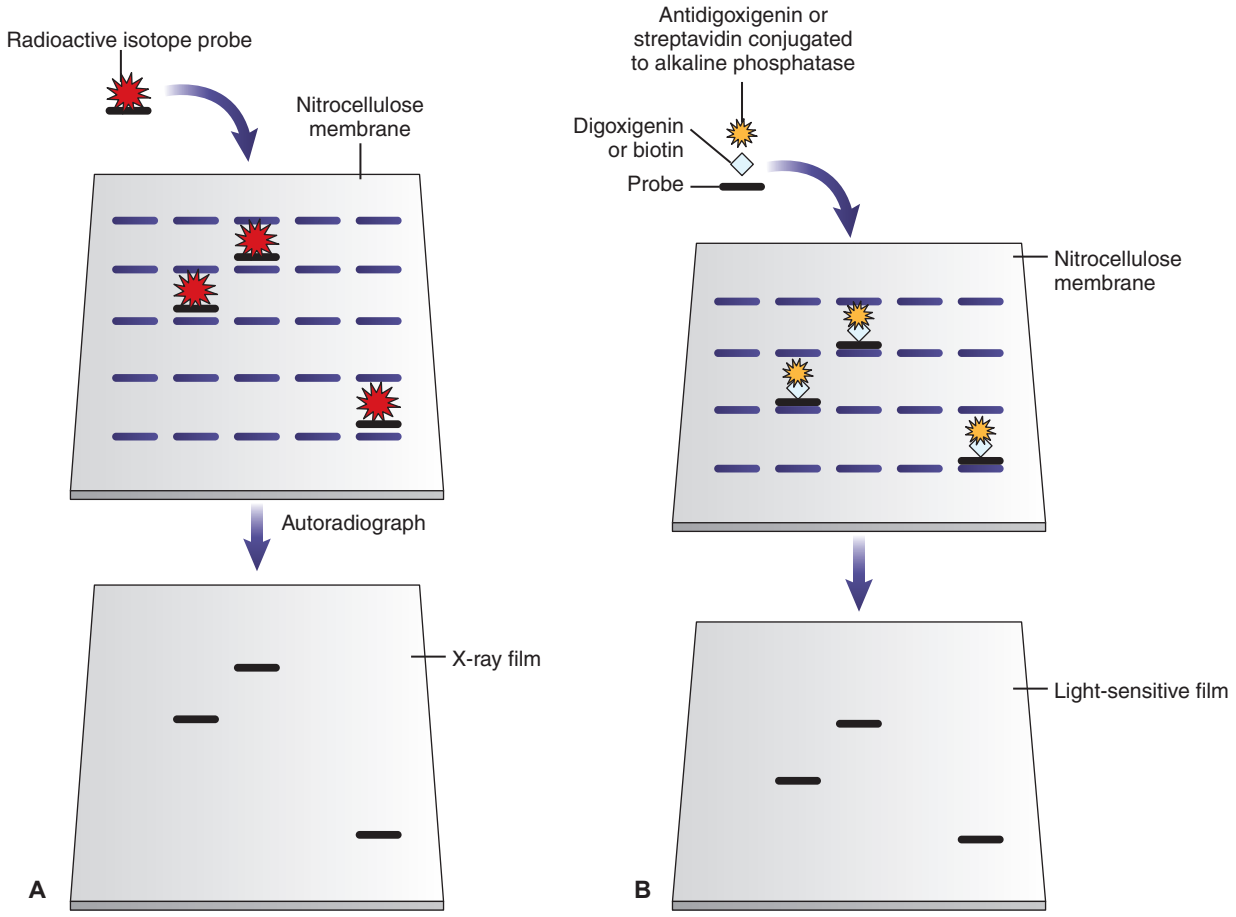

FIGURE 12–11 Probes can be either (A) radioactive or (B) nonradioactive. Nonradioactive probes produce signal through enzymatic production of color or light.

bead populations that differ by the relative amounts of two distinct dyes that are incorporated into the beads. Each bead population is attached to an antibody or other molecule that will bind specifically to the target molecules or sequences. A secondary antibody conjugated to a fluorescent signal will detect the presence of the target **(Fig. 12–13).** Bead assays are used for multiplex detection of proteins and nucleic acids **(Fig. 12–14).** Applications include tissue typing for stem cell and organ transplantation[19] and respiratory virus panels.[20]

Solution Hybridization

In solution hybridization, the probe and the target are both in solution. The target must be single stranded in order for hybridization to work. After probes and denatured targets are mixed in solution, the bound products are resolved by gel or capillary electrophoresis.

In variations of solution hybridization, DNA probe and RNA targets form hybrids, indicated by DNA probe:RNA. The target hybrids formed in solution are detected by capture on a solid support.[21] Two probes are used, both hybridizing to the target RNA. One probe, the capture probe, has an attached molecule of biotin that will bind specifically to streptavidin immobilized on a solid support **(Fig. 12–15A).** The other probe, which is the detection probe, is detected by an antibody directed against RNA:DNA hybrids or a covalently attached digoxigenin molecule used to generate chromogenic or chemiluminescent signal. This type of solution hybridization is the basis of the Hybrid Capture assays used to detect oncogenic variants of the human papilloma virus (HPV)[22,23] (see Fig. 12–15B). This method uses RNA probes complementary to the HPV DNA. The resulting RNA:DNA hybrid is captured and detected by antibodies to the hybrid. By altering the probe nucleotide sequences, HPV variants at low risk for causing cervical cancer can be distinguished from high-risk HPV types.

The ability to distinguish nucleotide sequence variants by solution hybridization has been applied to gene mutations and polymorphism analysis. Some automated systems use electrical changes in a ferrocene label to indicate the presence of a mutation or polymorphism recognized by the signal probe (see Fig. 12–15C). These systems can be used to assess respiratory viruses, cystic fibrosis, warfarin sensitivity, and thrombophilic mutations.

Fluorescence in Situ Hybridization (FISH)

In situ hybridization is an additional technique that utilizes nucleic acid probes to identify DNA. However, in this case, the target DNA is found in intact cells. In this method, probes ranging in size from a few thousand to hundreds of

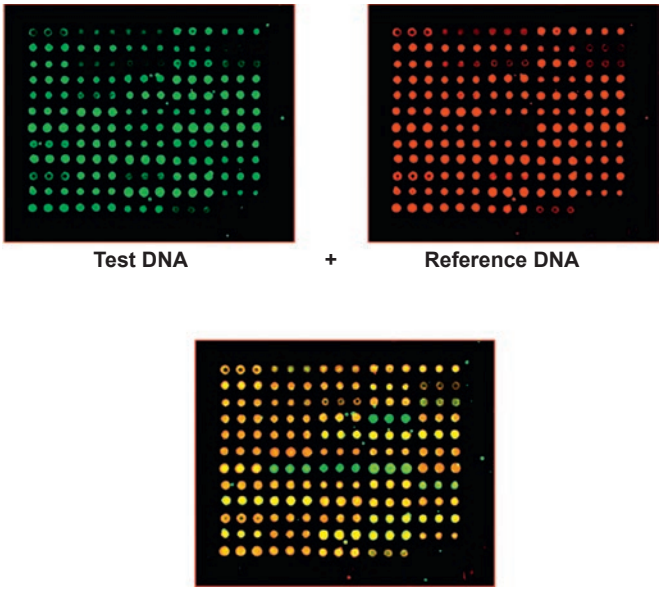

FIGURE 12–12 For array analysis, unlabeled probes are immobilized and hybridized to labeled sample material (green). A reference material (red) is hybridized to the same array. The results of the array are relative: test/reference.

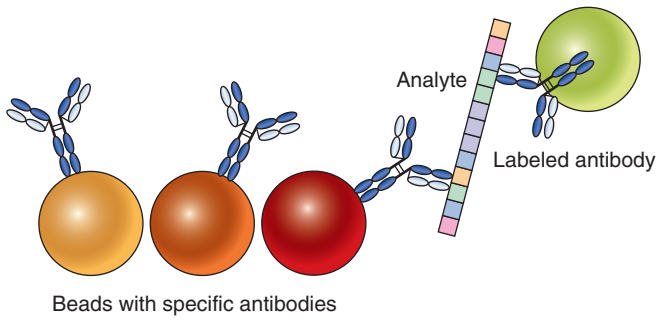

FIGURE 12–13 Three of 100 to 400 bead shades, each with antibody to a different analyte. The presence of multiple targets can be detected by which bead has associated fluorescence from the secondary antibody. Flow cytometry is used to assay each bead separately for associated fluorescence.

thousands of bases long are covalently attached to a fluorescent dye. When fluorescence is used to visualize the hybridization reaction, this is called **fluorescence in situ hybridization,** or **FISH.** For FISH, thin sections of solid tissue or deposits of cells are deposited on glass slides. The cell membranes are permeabilized to allow entry of the probe. The probes are applied to the prepared slides of cells where they hybridize to their complementary sequences. The resulting signals will show the state of the targeted gene or region. The probes are used with reference probes to centromeric genes to assess deletion or amplification **(Fig. 12–16).** A variation of FISH using chemiluminescent labeling (chemiluminescent in situ hybridization or CISH) is performed in the same way, except the slides can be read on a regular light

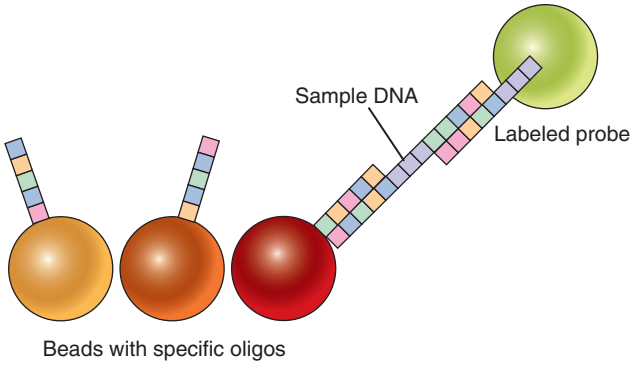

FIGURE 12–14 For nucleic acid analysis, bead array antibodies are replaced with single-stranded oligonucleotides complementary to the test nucleic acid. If present, biotinylated sample DNA will hybridize to the sequences and the biotin-specific conjugate will generate a signal.

microscope instead of expensive fluorescent microscopes and their special filters.[24,25]

In addition to identifying many different chromosomal abnormalities, FISH has been used to identify T-cell lymphomas, B-cell malignancies, and graft versus host disease after transplants. However, FISH is sensitive to the buffer and temperature conditions of hybridization (stringency). **Stringency** refers to the conditions that affect the ability of a probe to correctly bind to a specific DNA target sequence. Array methods (comparative genome hybridization) complement FISH testing in cases of multiple or complex genetic abnormalities as well as deletions and amplification of genes.[26]

Amplification Methods

The most frequently used methods in molecular diagnostics involve some aspect of amplification, that is, copying of nucleic acids. The development of the in vitro PCR reaction by Kerry Mullis[27] greatly facilitated and broadened the potential applications of gene amplification. PCR was quickly followed by other target amplification methods, such as reverse transcriptase PCR (RT-PCR), **transcription-mediated amplification (TMA),** and **strand displacement amplification (SDA).**

Polymerase Chain Reaction

PCR is an in vitro replication procedure that results in amplification of the target DNA. A PCR reaction includes all of the necessary components required for DNA replication: template (sample) DNA to be copied; deoxyribonucleotides, which will be joined together; oligonucleotide primers to prime the synthesis of the copies; DNA polymerase to covalently join the deoxyribonucleotides; and a buffer with a pH optimal for the polymerase activity. The oligonucleotide primers are key components to the specificity of the PCR reaction. Primers are synthetic single-stranded nucleic acids usually 18 to 30 bases in length. They are complementary to sequences **flanking** (on either side of and including) the region of the template DNA to be copied **(Fig. 12–17).**

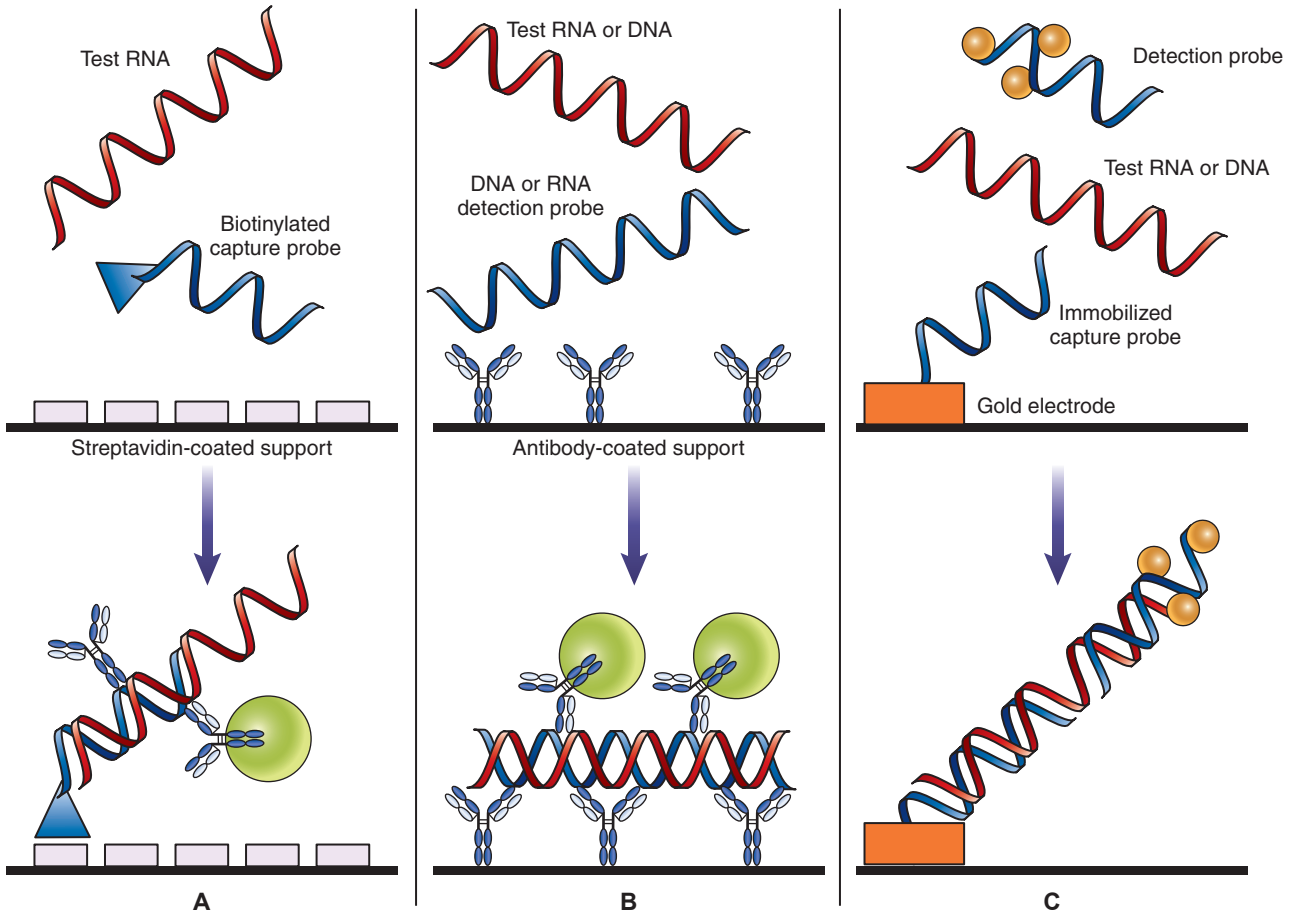

FIGURE 12–15 Variations of methods designed to detect specific DNA or RNA sequences by probe hybridization in solution. (A) Test RNA detected by a DNA capture probe with covalently attached biotin. The biotinylated probe binds to a streptavidin-coated support, whereas unbound probe and other RNAs are washed away. The bound hybrids are detected with anti DNA:RNA hybrid antibody conjugates. (B) RNA:DNA hybrids are captured by immobilized antibodies and detected with antibody conjugates or by signal protection assays. (C) Signal and capture probes hybridize to test RNA or DNA followed by electrochemical detection of ferrocene labels on the signal probe.

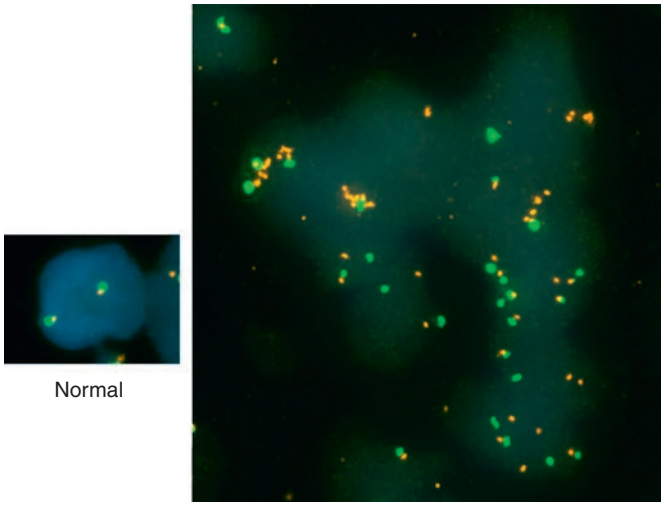

FIGURE 12–16 FISH analysis of the epidermal growth factor receptor gene. The gene probe is labeled orange, whereas a probe complementary to the centromere of chromosome 7 is labeled green. Normally there are two chromosomes, each carrying one gene in each nucleus (left). The image on the right shows gene amplification with multiple orange signals associated with single green signals.

For many procedures, premixed PCR reagents (deoxyribonucleotides, primers, and buffer) are supplied from manufacturers to which only the template DNA and, in some cases, enzyme are added. The PCR reaction mix is subjected to a computerized amplification program consisting of a designated number of cycles. A sequence of temperature changes comprises a cycle. An example of a three-step PCR cycle is (1) a denaturation step (94°C to 96°C), (2) an annealing step (50°C to 70°C), and (3) an extension step (68°C to 72°C). In one cycle, each of these temperatures will be held for 30 to 60 seconds. PCR cycles vary depending on the target DNA and the protocol. Two-step cycles are commonly used, as well as more complicated multi-step cycles. The cycle is repeated 20 to 50 times depending on the assay. The set of repeated cycles make up the PCR program. Because the amplification programs differ among assays, the program may be entered and stored in the thermal cycler for each assay once it is validated. Alternatively, prevalidated commercial assays may include preloaded thermal cyclers. For convenience, a hold at 4°C is usually added to the end of the amplification program.

The PCR amplification produces millions of copies or **amplicons** of the region of interest. At 100% PCR efficiency,

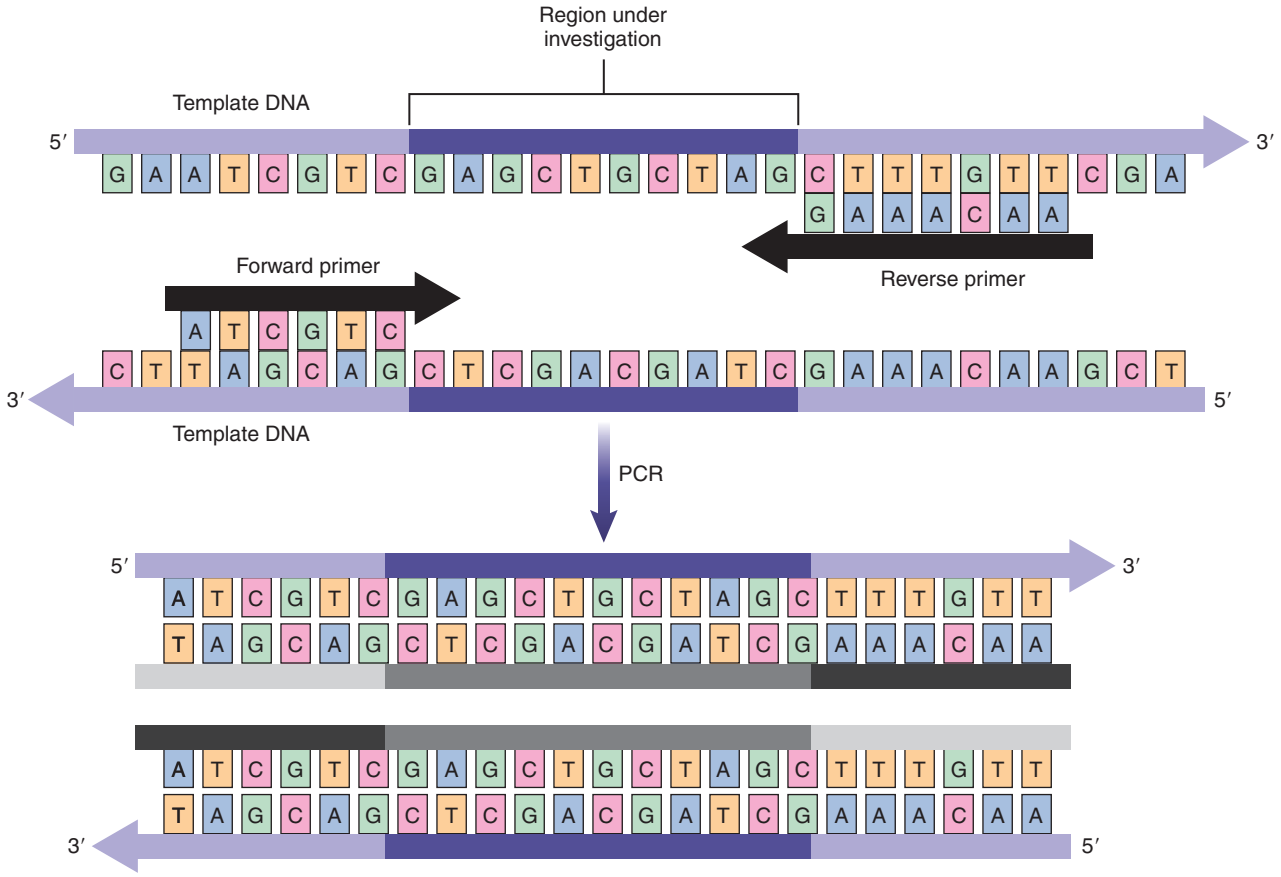

FIGURE 12–17 In the PCR reaction, short, single-stranded primers hydrogen hybridize to complementary sequences flanking the region of interest. The PCR reaction will produce millions of copies of the desired sequences. Note: The image is shortened. Primers are usually 18 to 30 bases long and the region of interest (and subsequent PCR products) range from 50 to more than 1,000 base pairs.

the number of copies will be approximately 2^n, where n is the number of cycles (20 to 50) in the amplification program. The products of the PCR reaction can be visualized by gel electrophoresis **(Fig. 12–18),** capillary electrophoresis, or other detection methods. For some applications, the presence, absence, or size of a PCR product is the test result.

A variety of modifications have been made to the PCR reaction. RT-PCR is a method of analysis for cellular RNA or qualitative detection of RNA viruses such as HIV and HCV. Infection with either of these viruses can be diagnosed by this method long before a positive antibody screen would occur. RT-PCR starts with an RNA template. **Reverse transcriptase** is a DNA polymerase that makes double-stranded DNA from RNA by reverse **transcription** (DNA-dependent RNA polymerase). The double-stranded **complementary or copy DNA (cDNA)** is thus synthesized from the RNA using reverse transcriptase in a separate step. Alternatively, enzymes that copy both RNA and DNA are used in simultaneous RT and PCR reactions that do not require a separate RT step.[28] The cDNA serves as the template for the PCR reaction.

PCR primer design introduces additional flexibility into the PCR reaction. In sequence-specific primer PCR (SSP-PCR, also called amplification refractory mutation system PCR or ARMS-PCR) primers are designed so that they will end on a potentially mutated or polymorphic bp.[29] Annealing of the last base at the 3′ end of the primer is critical for the polymerase activity. If the last base of the primer is not complementary to the template, DNA polymerase will not recognize the primer as a substrate for extension and no PCR product will be produced.

SSP-PCR is a common approach used to detect mutations and polymorphism, such as in HLA typing by SSP-PCR.[30] Unlike the 3′ end of primers, the 5′ end does not have to be complementary to the template. This allows attachment of noncomplementary sequences containing restriction enzyme recognition sites or RNA polymerase-binding sites to PCR products. The amplicons can then be conveniently inserted into plasmids for biological analyses or transcribed into RNA and translated into in vitro transcription or translation systems. Labels in the form of fluorescent molecules, biotin, or other molecules may also be covalently attached to the 5′ end of primers. This allows capture immobilization of the PCR products or detection in capillary electrophoresis systems **(Fig. 12–19).** Fluorescently labeled primers are used in PCR analysis of DNA fingerprinting,[31,32] for cancer tests

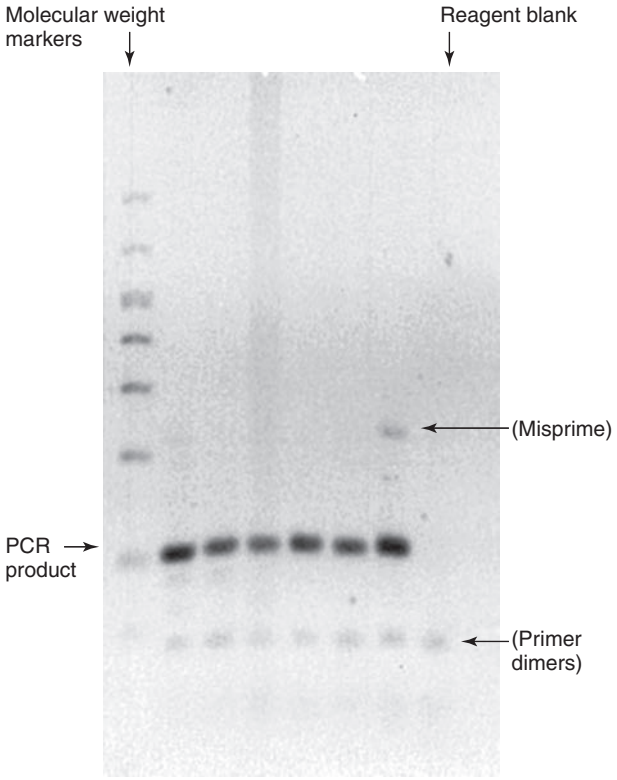

FIGURE 12–18 Detection of PCR products by gel electrophoresis. The first lane (left) contains molecular weight markers (fragments of known size). The next six lanes are PCR products, followed by a reagent blank control for contamination (lane 8). *(From Buckingham L.* Molecular Diagnostics. *2nd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

such as microsatellite instability testing in colon cancer for Lynch syndrome,[33] diagnosis of endometrial cancer,[34] and for genetic testing of Fragile X syndrome and Huntington disease.[35,36]

Quantitative PCR. Standard PCR results are interpreted as the presence, absence, or size of the PCR product at the completion of the PCR program, but quantification of starting material is not easily measured. In 1993, Higuchi et al. demonstrated that target quantification could be achieved by observing the accumulation of PCR product in real time during amplification.[37,38] Although originally termed real-time PCR or RT-PCR, the preferred term is now **quantitative PCR** or **qPCR** to avoid confusion with reverse transcriptase PCR (also RT-PCR). In qPCR, results can be seen at the end of each cycle, as opposed to PCR where measurement only occurs after all cycles are completed.

The qPCR product was followed initially by using a fluorescent dye specific for double-stranded DNA (ethidium bromide). Product labeling is currently done using a less toxic dye, SYBR Green. SYBR Green is not specific to sequence, so any product, including artifacts of the PCR reaction (misprimes and primer dimers from primer self-amplification), will produce a signal. More specific detection of product is achieved with probes. These probes generate fluorescent signals from the accumulating PCR products. Probes are sequence specific and only bind to the targeted region within the product to provide higher specificity for the intended product than SYBR Green.

Accumulation of PCR product detected using TaqMan probes through 50 PCR cycles is shown in **Figure 12–20.** The fluorescence depicted on the y axis is the strength or brightness of the fluorescence signal from the dye or probe. The fluorescence plotted versus cycle number generates a curve similar to a bacterial growth curve with a lag phase, a log phase, a linear phase, and a stationary phase (see Fig. 12–20). The length of the lag phase is assessed by the number of cycles required to reach a threshold level of fluorescence as determined by the instrument. The cycle at which the sample fluorescence reaches this value is the **threshold cycle** or Ct.

A relationship between the amount of target and the Ct values is established by generating a standard curve of dilutions of known target nucleic acid **(Fig. 12–21).** For test samples, the target is quantified relative to an internal amplification control. The **internal amplification control** is a gene target that is always present at a constant level. Internal controls also confirm negative results as true negatives and not PCR failure. Test results are quantified using the standard curve followed by normalization with the internal control.[34]

Just as PCR generated a wide variety of test methods and applications, qPCR has also been modified to address a variety of clinical questions. Both DNA and RNA targets are measured by qPCR. For RNA, cDNA made from the RNA using reverse transcriptase is the input material.

Widely used applications of qPCR and RT-qPCR include detection of microorganisms, especially viruses and other pathogens that are difficult or dangerous to culture in the laboratory.[39,40] Assessment of tumor-associated gene expression using qPCR and RT-qPCR may predict cancer recurrence.[41,42] Researchers have developed methods using qPCR for HLA typing and chimerism analysis.[43–45] Multiplex qPCR methods are performed to assess multiple targets simultaneously, such as the expression of various cytokine genes during different stages of infections.

Transcription-Based Amplification

Kwoh and colleagues developed the first transcription amplification system in 1989.[46] Commercial variations of this process include transcription-mediated amplification (TMA) (Gen-Probe), nucleic acid sequence-based amplification (NASBA) (Organon Teknika), and self-sustaining sequence replication (3SR) (Baxter Diagnostics). These three methods are similar but have variations in enzyme systems.

For TMA, RNA is the usual target instead of DNA. A cDNA copy is synthesized from the target RNA, after which transcription of the cDNA produces millions of copies of RNA products. In this technology, RNA is the primary product as well as the target. The RNA products transcribed from the cDNA can also serve as target RNA for synthesis of more cDNA **(Fig. 12–22).** The RNA products are detected by chemiluminescence with acridinium

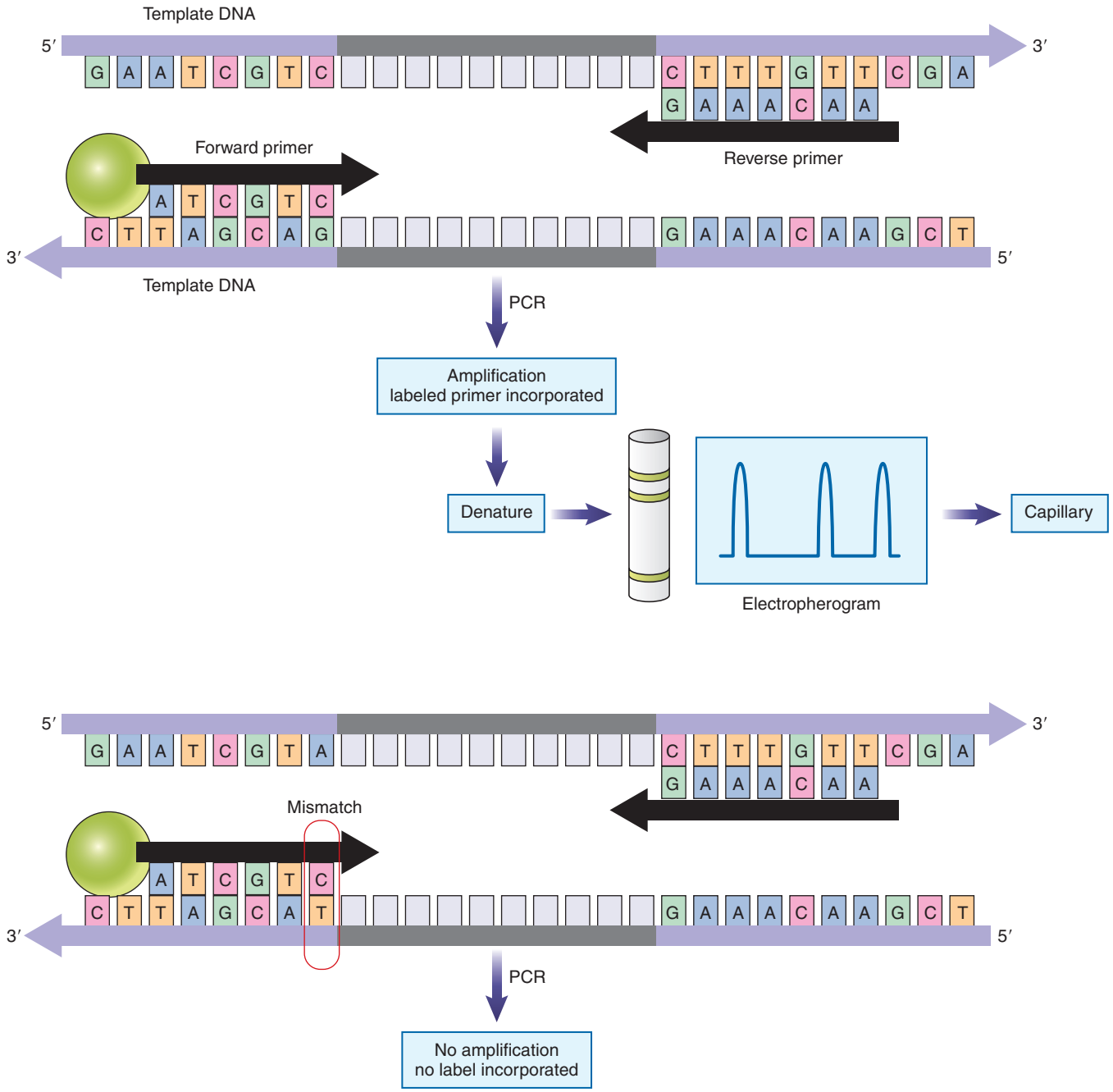

FIGURE 12–19 One PCR primer (forward or reverse) is covalently attached to a fluorescent dye, such as fluorescein, to allow detection of PCR products by capillary gel electrophoresis. The double-stranded products are denatured and diluted (left). Only the single strand of the PCR product with labeled primer will be detected (center). The output from the capillary instrument is an electropherogram (right) showing peaks of fluorescence, which are analogous to band patterns on gel electrophoresis.

ester (Gen-Probe)[47] or, in the case of NASBA, Molecular Beacon probes.[48]

The TMA process has been simplified with the addition of RNase H derived from *E coli* to degrade the RNA from the DNA and RNA hybrid, eliminating a heating step required for denaturation of the DNA and RNA hybrid to complete the cDNA synthesis. An additional modification and simplification of the procedure was the application of the reverse transcriptase derived from avian myeloblastosis virus (AMV) with inherent RNase H activity. Thus, the reaction can be run with only two enzymes, AMV reverse transcriptase and T7 RNA polymerase. These procedures have been marketed as TMA, NASBA, and 3SR.

In contrast to PCR, TMA is an **isothermal** process, meaning that the reaction proceeds at a single temperature. This process is much simpler to perform compared with PCR

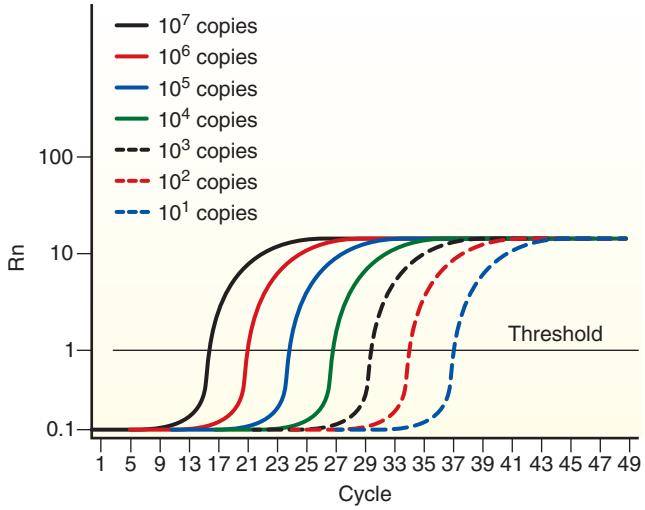

FIGURE 12–20 Real-time quantitative PCR signal generated from a TaqMan probe. The normalized fluorescence (ΔRn) is plotted against PCR cycles 1 to 50. The threshold cycle is indicated by the green line. The length of the lag phase (number of cycles required to reach a threshold level of fluorescence) is inversely correlated with the amount of starting material.

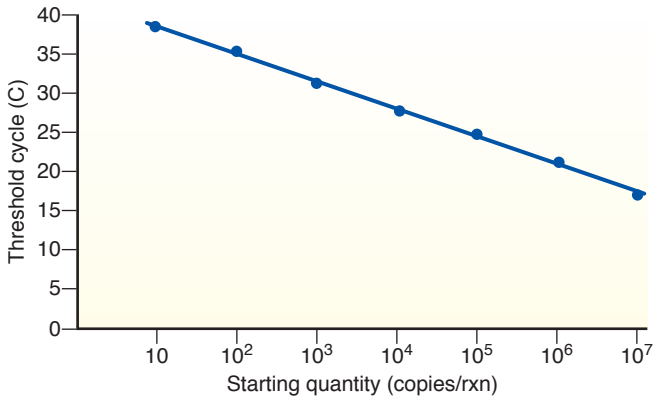

FIGURE 12–21 Ct values (y axis) were determined for serial 10-fold dilutions of a synthetic target of known concentration (x axis). The resulting standard curve is used to convert Ct values of test samples to concentration.

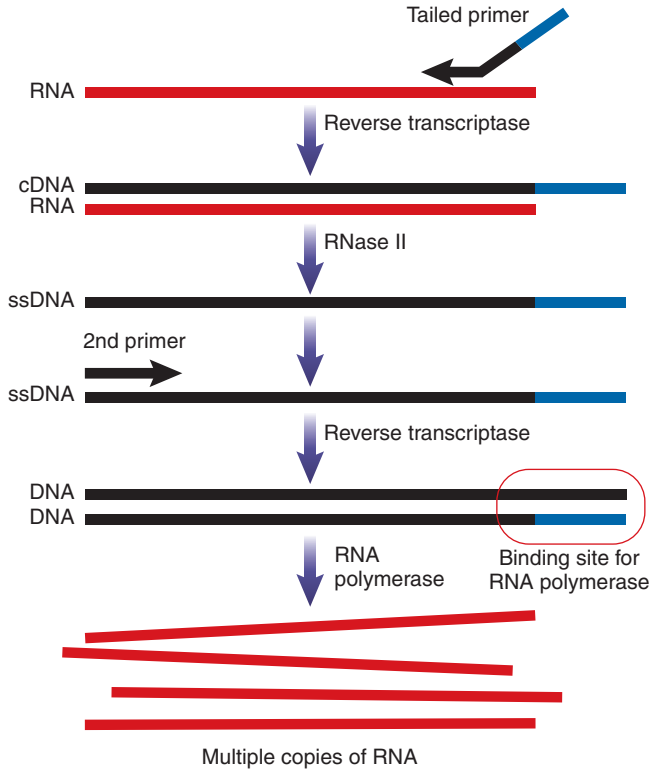

FIGURE 12–22 Transcription-mediated amplification (TMA) targets RNA. In the first step, a cDNA:RNA hybrid is synthesized by reverse transcriptase using a primer with a tail (that will ultimately form the binding site for a later enzyme). Hybridized (but not single-stranded) RNA is degraded by RNase II, leaving single-stranded DNA. This ssDNA serves as a template for reverse transcriptase to synthesize a complementary strand of DNA, including the primer tail to complete the binding site for RNA polymerase. The RNA polymerase uses dsDNA as a template to synthesize many copies of RNA. This new RNA can cycle back to step one and repeat the process, resulting in a large amplification of product.

cycling, which requires repeated heating and cooling. Targeting RNA allows for the direct detection of RNA viruses, such as hepatitis C virus, and HIV.[49,50] Targeting the RNA of organisms with DNA genomes, such as *Mycobacterium tuberculosis*, is more sensitive than targeting the DNA because each microorganism makes multiple copies of RNA, whereas it has only one copy of DNA.[51] Detection of *Chlamydia trachomatis* in genital specimens and cytomegalovirus (CMV) quantification in blood are additional applications for TMA. The high sensitivity of TMA also makes it suitable for blood screening.[52] Screening assays require high sensitivity, even at the expense of specificity to assure detection of low levels of the target analyte, such as anti-HBV antibodies. Positive screening results may have to be confirmed by a more specific method (which may be too labor intensive or expensive for screening large numbers of samples).

Probe Amplification

The number of target nucleic acid sequences in a sample is not changed in probe amplification. Rather, primers are extended or ligated into many copies of detectable probes. Examples of probe amplification are ligase chain reaction (LCR) and SDA.

Ligase Chain Reaction. The LCR amplifies synthetic probes complementary to target nucleic acid. In order for LCR to work, the entire target sequence must be known. Instead of a polymerase, LCR uses DNA ligase, an enzyme that forms one phosphodiester bond between two preexisting DNA chains—in this case, two primers. In LCR, the two primers bind immediately adjacent to each other on the target sequences and DNA ligase covalently attaches the adjacent primers together. These joined, or ligated, primers essentially

become the probe. There is no amplification of the product because the ligated primers then serve as a template for the annealing and ligation of additional primers. The probes (ligated primers) are detected by capture and signal detection **(Fig. 12–23).**

LCR requires temperature changes to drive the denaturation of the template and ligated primers. LCR was used to detect point mutations in a target sequence. The DNA mutation that occurs in the beta-globulin of patients with sickle cell disease, as compared with normal beta-globulin, was one of the first applications of LCR.[53] LCR was used at one time for detection of *Neisseria gonorrhoeae* and *C trachomatis*, but has been replaced by other amplification methods.

Strand Displacement Amplification. SDA is another isothermal amplification process. After an initial denaturation step, the reaction proceeds at one temperature. In SDA, the amplification products are the probes. After the target DNA is denatured by heating to 95°C, two primers bind close to each other, an outer and an inner primer (containing a probe) **(Fig. 12–24).** As the outer primer is extended by DNA polymerase, it displaces the product formed by the simultaneous extension of the inner primer (probe). The probe becomes the target DNA for the next stage of the process. The second stage of the reaction is the exponential probe amplification phase by extension from a **nick** (breaking one phosphodiester bond on one strand of a double-stranded DNA) formed by a restriction endonuclease enzyme. Methods using fluorescence polarization or fluorogenic probes to detect the amplified probes have been designed to test for *M tuberculosis*,[54] *C trachomatis*,[55] and *N gonorrhoeae*.[56]

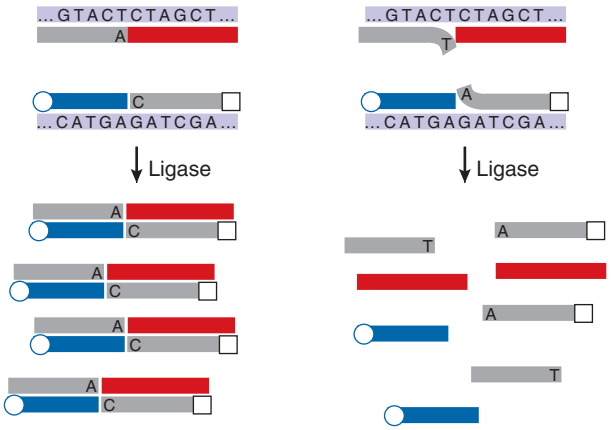

FIGURE 12–23 In the ligase chain reaction, primers hybridize on adjacent nucleotide sequences. If the primers are complementary to the template, DNA ligase will join them together to form a probe. The probe serves as a hybridization substrate for more primers to bind. Four probes are used for each reaction. One of the primers carries a capture molecule (circle) and another a signal molecule (square). When these primers are ligated, the probe will be captured, unbound primers removed, and signal detected from the joined primers. If the primers are not complementary to the template, no ligation will occur and no signal will result. *(Adapted from Buckingham L.* Molecular Diagnostics. *2nd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

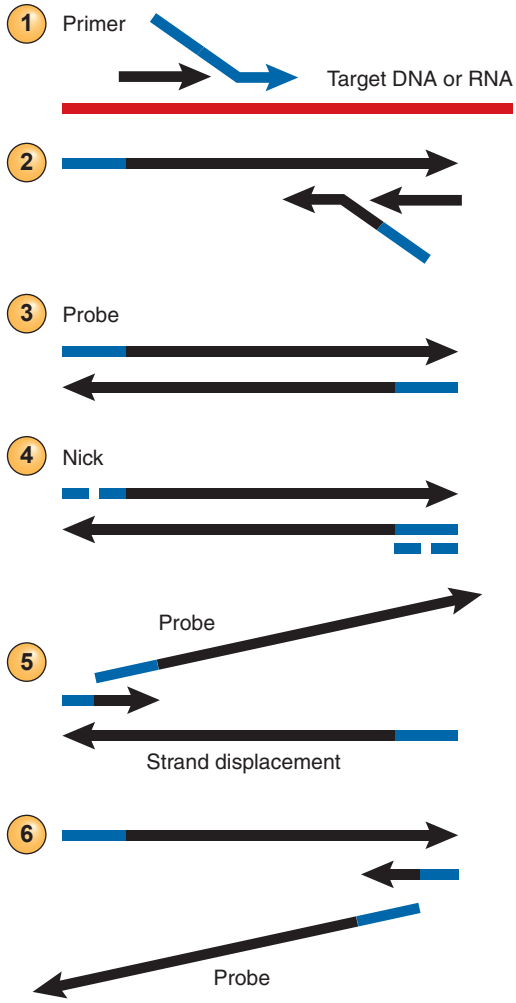

FIGURE 12–24 Strand displacement amplification showing one target strand. 1. Primers bind to single-stranded DNA at a complementary sequence. 2. A polymerase extends the primer from the 3′ end. 3. The extended primer forms a double-stranded DNA segment containing a site for a restriction enzyme at each end. 4. The enzyme binds double-stranded DNA at the restriction site and forms a nick. 5. The DNA polymerase recognizes the nick and extends the strand from that site, displacing the previously created strand. 6. Each strand can then anneal and continue the process.

Signal Amplification

In signal amplification, large amounts of signal are bound to the target sequences that are present in the sample. **Branched DNA (bDNA)** is an example of a commercially available signal amplification method. In bDNA amplification, a series of short single-stranded DNA probes are used to capture the target nucleic acid and to bind to multiple reporter molecules, loading the target nucleic acid with signal **(Fig. 12–25).** Because multiple probes hybridize to the

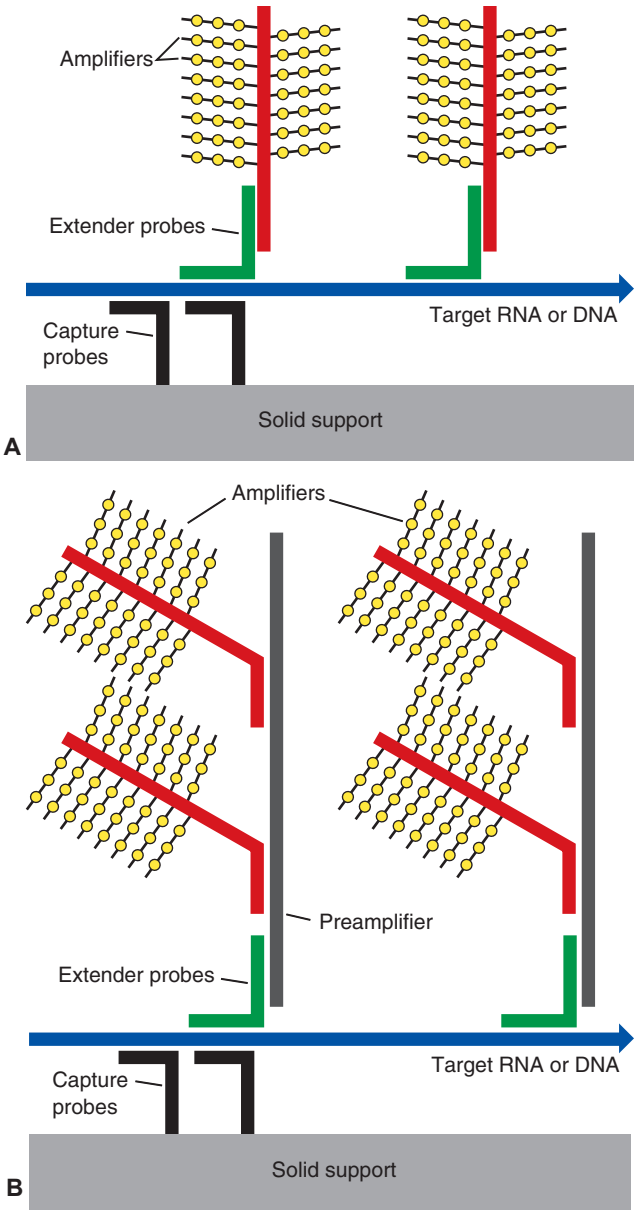

FIGURE 12–25 In branched DNA, signal is amplified through hybridization of complementary probes to the target DNA or RNA. (A) Branched DNA molecules carry multiple signals for each target molecule. (B) A second generation of the method has increased sensitivity because of the binding of additional signals. *(Adapted from Buckingham L.* Molecular Diagnostics. *2nd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

target sequences in bDNA, its sensitivity is enhanced over methods using a single probe or primer to bind to the target. This allows for multiple genotypes of the same virus to be detected by incorporating different probes that recognize slightly different sequences. Because probes are amplified and not the target, this method can be used to quantify the amount of target actually present.

The bDNA signal amplification assay has been applied to the qualitative and quantitative detection of HBV, HCV, and HIV-1. However, newer versions of qPCR methods may have better performance compared with bDNA assays. By replacing the plate support with beads, the bDNA assay has been combined with the bead array technology to provide a multiplex system that can detect 100 different targets in a single sample.[57]

Detection of HPV by hybrid capture, as described in the *Solution Hybridization* section, is also a probe amplification method. Multiple antibodies bind to the DNA:RNA hybrids, producing multiple signals from a single target molecule.

DNA Sequencing

The function of DNA is to store genetic information in the order or sequence of the four nucleotide bases in the DNA chain. Early in the history of recombinant DNA technology, researchers actively pursued the idea of sequencing or detecting the nucleotide order of the nucleic acids. Two sequencing methods emerged in the early to mid-1970s: the Maxam-Gilbert chain breakage method[58] and the Sanger **chain termination sequencing** method.[59] Sanger sequencing quickly gained popularity because it was not subject to the toxic chemicals and complex interpretation required by the Maxam-Gilbert method. Researchers have since developed alternative sequencing methods including pyrosequencing and **next generation sequencing (NGS)** methods.

Sanger (Chain Termination) Sequencing

Direct determination of the order, or sequence, of nucleotides in a DNA chain is the most explicit method for identifying genetic lesions (mutations) or polymorphisms, especially when looking for changes affecting only one or two nucleotides. Chain termination (Sanger) sequencing is a modification of the DNA replication process. It utilizes modified nucleotide bases called dideoxynucleotide triphosphates (ddNTP) **(Fig. 12–26).** The ddNTP are incorporated into the growing DNA chain, just as dNTP are. The ddNTP, however, cannot provide a 3′OH for the addition of the next nucleotide; the chain synthesis then stops (chain termination).

In a standard sequencing reaction, a short, synthetic single-stranded DNA fragment (primer) complementary to sequences

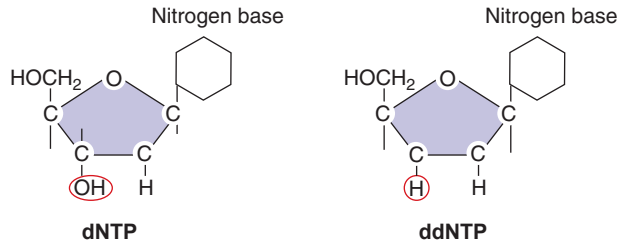

FIGURE 12–26 Dideoxyribonucleotides lack the 3′ hydroxyl group necessary for formation of the phosphodiester bond during DNA replication. *(Adapted from Buckingham L.* Molecular Diagnostics. *2nd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

flanking the region of DNA to be sequenced primes DNA synthesis **(Fig. 12–27).** Unlike PCR and other methods that require two primers, sequencing proceeds on only one DNA strand, thus using a single primer. The primer is covalently attached to a radioactive or fluorescent dye-labeled nucleotide at the 5′ end.

All other components required for DNA synthesis are added to the primer and single-stranded template, including the four dNTP bases. This DNA synthesis reaction results in polymerization of deoxyribonucleotides to make full-length copies of the DNA template. For sequencing, the reactions mixture is aliquoted to four tubes and a different ddNTP is added to each of the four reaction aliquots. DNA synthesis will stop upon incorporation of a ddNTP into the growing DNA chain (chain termination) because without the hydroxyl group at the 3′ sugar carbon, the 5′–3′ phosphodiester bond cannot be made. The newly synthesized chain therefore terminates when it encounters the ddNTP **(Fig. 12–28).** The result of the sequencing reaction is a collection of fragments termed the DNA ladder. The terminated chains can be resolved by gel electrophoresis to yield the band pattern.

In the current sequencing methods (cycle sequencing, dye-terminator sequencing), reactions with all four of the ddNTPs take place in a single tube. The region of the sample DNA to be sequenced is first amplified by PCR. The double-stranded PCR product, cleaned of residual PCR reaction components, is the sequencing template. In dye-terminator sequencing, each ddNTP is labeled with a different fluorescent dye (ddATP green, ddCTP blue, ddGTP black, ddTTP red) so that the products of the sequencing reaction are distinguished by color.

The fluorescently labeled DNA ladder is resolved by gel or capillary gel electrophoresis. Gel-based resolution will result in a series of bands of different sizes. The DNA sequence is read from the bottom to the top of the gel (smallest to largest) by which ddNTP terminated each fragment. Sequencing results from capillary gel electrophoresis are a series of fluorescent peaks, or an **electropherogram (Fig. 12–29).** The nucleotide sequences are read automatically by sequence analysis software and supplied in textual form (ACGT).

Software programs interpret and apply sequence data from automatic sequencers. These programs collect the raw data and interpret data quality. The programs report the certainty of each nucleotide base in the sequence and compare the sequence with a reference sequence or database to identify important variants.[60] HLA sequence-based typing uses this method.[61,62]

Germline or inherited variations in the DNA sequence are readily detected, usually from blood specimens. Somatic (noninherited) mutations in clinical specimens, such as cancerous tumors, are sometimes difficult to detect as they may be diluted by normal sequences that mask the somatic change.

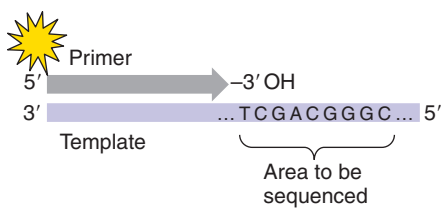

FIGURE 12–27 The labeled sequencing primer hybridizes to the template to provide a 3′OH group for formation of a phosphodiester bond by DNA polymerase. *(Adapted from Buckingham L.* Molecular Diagnostics. *2nd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

Pyrosequencing

Pyrosequencing is an alternate sequencing method developed in the 1980s.[63,64] The procedure relies on the generation of light (luminescence) when nucleotides are added to a growing strand of DNA. With this system, there are no gels, fluorescent dyes, or ddNTPs.

In the pyrosequencing reaction mix, a single-stranded DNA template is mixed with a sequencing primer, enzyme, and substrate mixes. The pyrosequencer introduces dNTPs sequentially to the reaction. If the introduced nucleotide is complementary to the base in the template strand next to the 3′ end of the primer, DNA polymerase forms a phosphodiester bond between the primer and the nucleotide, releasing pyrophosphate (PPi) (**Fig. 12–30,** left panels; also see DNA replication). The PPi is converted to ATP to energize generation of a luminescent signal. This signal indicates that the introduced nucleotide is the correct base in the sequence. The pyrosequencing reaction generates a pyrogram of luminescent peaks associated with the addition of the complementary nucleotide (see Fig. 12–30, right panel).

Because pyrosequencing produces short- to moderate-length sequence information (up to 100 bases), it is not as versatile as Sanger sequencing, which can produce reads longer than 1,000 bases, especially for de novo sequencing. Two factors have kept pyrosequencing in use. First, because pyrosequencing is less labor intensive than Sanger sequencing, it is more convenient for these types of short sequence analyses. Second, new instruments developed with the introduction of genomic sequencing or NGS use the pyrosequencing chemistry because NGS also relies on repeated sequencing of short templates. Pyrosequencing is currently used in both of these capacities.

Next Generation Sequencing (NGS)

The first human genome sequence was performed by chain termination (Sanger) sequencing. The 7-year Human Genome Project involved hundreds of sequencers and bioinformatics experts and cost billions of dollars. Using NGS, a human genome now can be sequenced by a single sequencer in a few hours for fewer than $1,000. NGS is designed to sequence large numbers of templates simultaneously, yielding hundreds of thousands of short sequences in a single run. These short sequences are then assembled into a complete genome. The development of NGS was stimulated in part by a goal to sequence the human genome for a minimal cost (less than $1,000) in order to bring what were once expensive genomic studies into the realm of

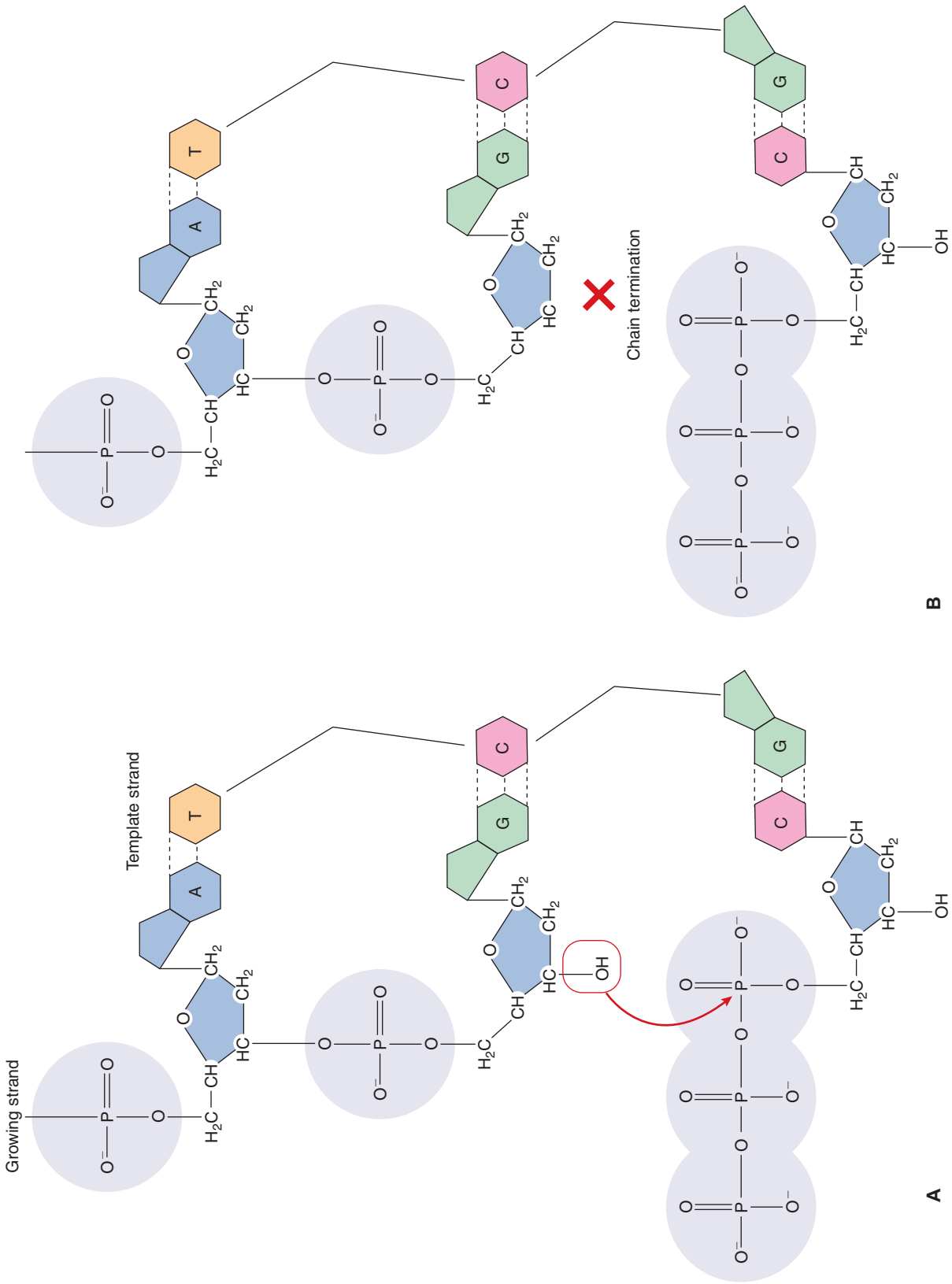

FIGURE 12–28 (A) DNA synthesis proceeds by formation of a phosphodiester bond between the ribose 3′OH of a previous nucleotide and the ribose 5′ phosphate group of the incoming nucleotide. (B) If the 3′OH is missing, as in ddNTP, synthesis terminates (right). *(Adapted from Buckingham L.* Molecular Diagnostics. *2nd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

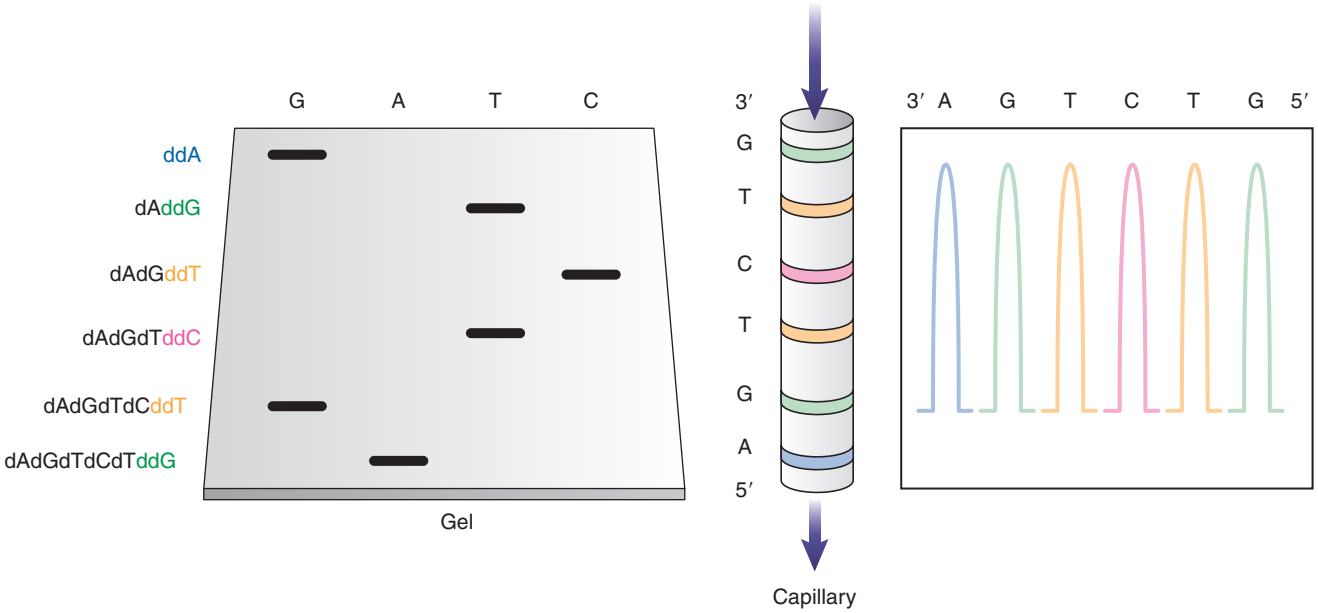

FIGURE 12–29 A sequencing ladder (left) resolved by gel electrophoresis is read from the bottom of the gel to the top of the gel (shortest fragment to the longest). The terminating base is determined by its tube (gel lane). In dye-terminator sequencing, the sequence is read automatically as fluorescently labeled fragments migrate through the gel or capillary. Each fragment passes a detector that will generate an electropherogram of fluorescent peaks (right). Sequencing software will produce a text report of the DNA sequence.

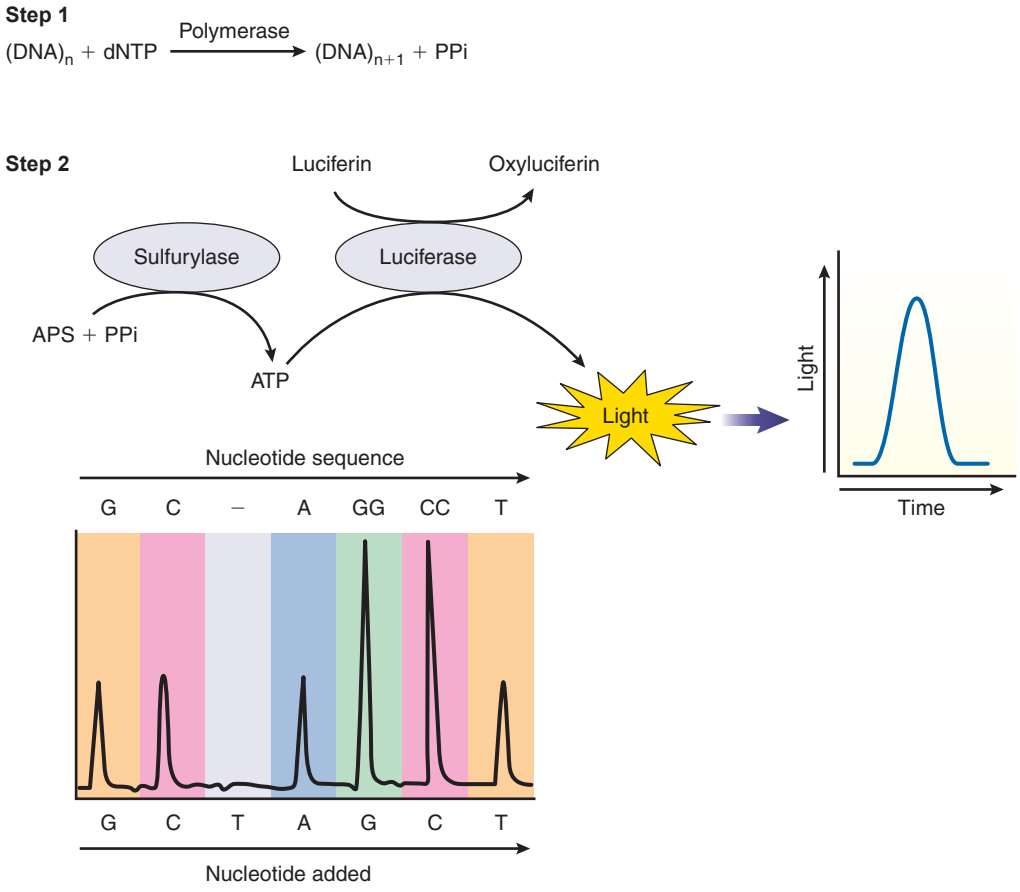

FIGURE 12–30 Pyrosequencing detects nucleotide sequence by introducing dNTPs in a predetermined order to the sequencing reaction. If the nucleotide is not complementary to the template sequence, no reaction occurs and the nucleotide is then degraded. If the nucleotide is complementary to the sequence, polymerase forms a phosphodiester bond extending the primer and releasing pyrophosphate (PPi, Step 1). The pyrophosphate then goes through a series of reactions to ultimately produce a chemiluminescent signal (Step 2). The pyrosequencing output is a pyrogram, as shown on bottom. *(Adapted from Buckingham L. Molecular Diagnostics. 2nd ed. Philadelphia, PA: F.A. Davis; 2011, with permission.)*

clinical analysis. Thousands of genomes have been sequenced by NGS as part of the 1,000 Genome Project. The goal of this project was to provide reference and variant information from ethnically diverse genomes.[65] The sequencing cost does not, however, include the expenses associated with interpretation, reporting, and data storage. These issues are currently being addressed to enable broader implementation of NGS in clinical analysis.

With NGS technology, DNA must be cut to create fragment libraries. Then these short fragments, usually 100 to 300 bp, are amplified so they can be analyzed. Adaptor sequences are added to these fragments and serve as primers for amplification. Hundreds of thousands of products from the library can be sequenced simultaneously.

For all NGS varieties, labeled nucleotides are used. Then after amplification, instrument software collects the sequence data in the form of processed images. The sequence quality is assessed and then the sequence is provided as a textual report. After a successful NGS run, areas of interest will have been sequenced from 30 to hundreds of times.

NGS has a variety of clinical applications including mitochondrial genome sequencing, sequencing for inherited disease, and gene panel sequencing for known somatic mutations. One of its most important uses is in HLA high-resolution typing.[66,67]

SUMMARY

- The two main types of nucleic acids are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). They are polymers made up of chains of nucleotides.
- Nucleotides of DNA contain a deoxyribose sugar with one of the following bases: adenine, guanine, thymine, or cytosine.
- RNA is made up of nucleotides containing a ribose sugar bonded to a similar nitrogen base as in DNA, except that instead of thymine, uracil is used.
- DNA is double stranded and arranged in a double helix, whereas RNA is typically single stranded.
- In a DNA molecule, specific base pairing occurs: adenine pairs with thymine and guanine pairs with cytosine.
- When a DNA molecule replicates, the two daughter strands separate; each is a template for a newly synthesized complementary strand.
- The high specificity of detection of nucleic acid sequences through complementary base pairing is the basis of all applications in clinical laboratory science.
- The central dogma of molecular biology refers to the fact that DNA serves as the template for messenger RNA, which in turn codes for proteins.
- Mutations and polymorphisms are changes in nucleotide sequences that may affect specific protein sequences.
- Restriction fragment length polymorphisms are changes in DNA that result in different size pieces when cleaved by restriction enzymes.
- Hybridization is the very specific binding of two complementary DNA strands or a DNA and an RNA strand. Often a probe, which has a short known nucleic acid sequence, is used to detect an unknown nucleic acid sequence.
- Hybridization techniques include Southern blot analysis, microarray technology for simultaneous assessment of multiple genes, and fluorescent in situ hybridization of specific genetic regions.
- Amplification involves copying of a specific nucleic acid sequence in order to obtain enough to identify it.
- Amplification methods include polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), and quantitative PCR (qPCR). All of these methods amplify the target DNA.
- In transcription-mediated amplification, the target is RNA instead of DNA. A cDNA copy is made of the original RNA and used to produce millions of RNA copies.
- The ligase chain reaction amplifies probes rather than the target DNA. Two primers attach to the target DNA sequence and then the primers are joined and amplified.
- Strand displacement amplification (SDA) also involves amplification of a probe rather than the original target DNA.
- Branched DNA represents a signal amplification method in which multiple probes attach to the original target sequence DNA.
- DNA sequencing allows for detection of single nucleotide polymorphisms and mutations not easily detectable with restriction enzyme mapping.
- Next generation sequencing methods allow for sequencing of a large number of small DNA sequences at one time. Whole genomes can be quickly and easily sequenced to look for inherited diseases.

CASE STUDIES

1. The FMS-like tyrosine kinase 3 (*FLT3*) gene represents one of the most frequently encountered mutations in acute myeloid leukemia. The *FLT3* mutation status aids in clinical decisions for treatment strategy. One of the *FLT3* mutations falls in the active enzyme region of the gene (kinase domain). A test for the *FLT3* kinase domain gene mutation is performed using PCR followed by digestion with the restriction enzyme, *Eco*RV. The gene mutation changes the recognition site of *Eco*RV (GATATC) such that the enzyme will not cut the DNA. For the test, DNA is isolated and the region containing the mutation is amplified by PCR using primers that specifically hybridize and allow amplification of that region. The resulting PCR product is 150 bp. The restriction enzyme digestion will separate the product into three pieces—80 bp, 59 bp, and 11 bp—in the normal DNA sequence. If the mutation is present, the restriction enzyme digestion will produce only two fragments, 139 bp and 11 bp. After digestion, the products of the reaction are separated by gel electrophoresis and the size is determined by the distance the fragments travel in the gel under the force of the electric current.

Questions

a. List the controls that would be used for the PCR reaction and restriction enzyme reactions.
b. The 11 bp fragment may not be detectable on some resolution systems. Would this preclude performance of the test by those methods?
c. Suppose the normal control yielded three products: 80 bp, 59 bp, and 11 bp. How would this affect the interpretation?
d. Suppose the internal control yielded a 150 bp product. How would this affect the interpretation?

2. A blood sample from a patient tested positive for the presence of HIV antibodies. A molecular test for the presence of HIV by qPCR was performed. The test can detect 50 to 1,000,000 viral copies per mL plasma. Previous results had shown the presence of the virus at levels of 1,500, 600, 500, 220, and 100 copies per mL over the course of treatment. The results of the qPCR test for the current specimen were negative. However, the internal amplification control for the qPCR test was also negative.

Questions

a. How would you interpret these results?
b. The test was repeated; this time, the target (HIV) amplification was negative whereas the amplification control was positive. How would you interpret these results?
c. To prepare the test report, the results are entered along with the sensitivity of the test (50 copies/mL). Should these results be reported as 0 copies/mL because nothing was detected by this qPCR test?

REVIEW QUESTIONS

1. What holds two single strands of DNA together in a double helix?
   a. 2′ carbon of deoxyribose attached to a hydroxyl group
   b. Hydrogen bonds between A and T and C and G
   c. Ribose 3′ carbon hydroxyl attached to ribose 5′ carbon phosphate
   d. Phosphorylation of nitrogen bases

2. What is the complement to the following DNA sequence?

   5′-GATCGATTCG-3′

   a. 3′-CTAGCTAAGC-5′
   b. 3′-CGAATCGATC-5′
   c. 3′-GCTTAGCTAG-5′
   d. 3′-GATCGATTCG-5′

3. How are DNA and RNA different?
   a. Only RNA contains uracil.
   b. Only DNA contains cytosine.
   c. DNA is usually single stranded.
   d. DNA is less stable than RNA.

4. What is the function of restriction endonucleases?
   a. They splice short pieces of DNA together.
   b. They cleave DNA at specific sites.
   c. They make RNA copies of DNA.
   d. They make DNA copies from RNA.

5. What is the purpose of somatic hypermutation in genes that code for antibodies?
   a. To increase diversity of the immunoglobulin repertoire
   b. To prevent further antibody formation
   c. To switch antibodies from IgM to IgG
   d. To prevent further VDJ recombination

6. What characteristic distinguishes restriction enzymes from one another?
   - **a.** Diverse binding and cutting sites
   - **b.** Ability to quickly digest DNA
   - **c.** RNA degradation capability
   - **d.** Ability of one enzyme to recognize several different binding sites

7. Which of the following is used in a Southern blot procedure?
   - **a.** A ligase joins two adjacent probes.
   - **b.** Radiolabeled nucleotides are used to synthesize DNA.
   - **c.** DNA is cleaved by enzymes and electrophoresed.
   - **d.** Many probes are placed on a small piece of glass.

8. Which best describes the PCR?
   - **a.** Two probes are joined by a ligating enzyme.
   - **b.** RNA copies of the original DNA are made.
   - **c.** Extender probes are used to create a visible product.
   - **d.** Primers are used to make multiple DNA copies.

9. What takes place during in situ hybridization?
   - **a.** RNA polymerase copies messenger RNA.
   - **b.** Hybridization takes place in solution.
   - **c.** Nucleic acid probes react with intact cells in tissues.
   - **d.** Probes are protected from degradation if hybridized.

10. What determines the specificity for PCR?
    - **a.** Nucleotide mix ratios and concentrations
    - **b.** Mono- and divalent cation concentrations
    - **c.** Primers and their annealing temperature
    - **d.** DNA polymerase

11. An antibody test for HIV within 3 months of exposure is negative. Does this guarantee a negative PCR test?
    - **a.** Yes, because if no antibodies are present, no virus is present.
    - **b.** No, PCR-detectable virus may be present before generation of detectable antibodies.
    - **c.** Yes, it has been 3 months since exposure.
    - **d.** No, but the PCR test will be less sensitive than the antibody test.

12. How do PCR and qPCR differ?
    - **a.** In qPCR, the results can be seen at the end of each cycle.
    - **b.** SYBR Green is only used in PCR.
    - **c.** PCR is an isothermal process and qPCR is not.
    - **d.** Internal amplification controls are not necessary in qPCR.

13. Which method is a signal amplification system?
    - **a.** bDNA
    - **b.** qPCR
    - **c.** PCR
    - **d.** Digital PCR

14. Which of the following amplifications is isothermal?
    - **a.** PCR
    - **b.** qPCR
    - **c.** NASBA
    - **d.** LCR

15. Consider the following results for a qPCR test for the presence of herpes virus:

Sample	Ct/Sample	Ct/Amp Control
A	22.10	21.06
B	35.02.	20.99

    Which of the following statements is true?
    - **a.** The HSV viral load in sample A is greater than in sample B.
    - **b.** The HSV viral load in sample B is greater than in sample A.
    - **c.** The HSV viral load in sample A is about the same as in sample B.
    - **d.** The absolute number of viral particles in sample B is greater than in sample A.

16. Which terminates chains when added to a DNA replication reaction?
    - **a.** dNTPs
    - **b.** ddNTPs
    - **c.** Sequencing primer
    - **d.** DNA polymerase

17. Which technique involves probe amplification rather than target amplification?
    - **a.** Southern blot
    - **b.** PCR
    - **c.** Transcription-mediated amplification
    - **d.** Ligase chain reaction

18. How does next generation sequencing (NGS) technology differ from the original Sanger chain displacement sequencing?
    - **a.** NGS is more expensive to conduct than chain displacement sequencing.
    - **b.** NGS can sequence thousands of DNA pieces faster than Sanger sequencing.
    - **c.** Sanger sequencing involves ligation and NGS does not.
    - **d.** Only Sanger sequencing has direct clinical applications.

19. Which of the following methods best describes a nucleic acid probe?

a. It attaches to double-stranded DNA.
b. It is used in transcription-mediated amplification.
c. It is used to detect specific single-stranded DNA sequences.
d. It plays a key role in DNA chain termination sequencing.

20. A hybridization reaction involves which of the following?

a. Separating DNA strands by heating
b. Binding of two complementary DNA strands
c. Increasing the number of DNA copies
d. Cleaving DNA into smaller segments

21. What is the difference between a polymorphism and a mutation?

a. Mutations only affect A and T bases.
b. Mutations are more frequently present in a population.
c. Polymorphisms are more frequently present in a population.
d. Polymorphisms are easier to detect than mutations.

13 Flow Cytometry and Laboratory Automation

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. List and describe the function of each of the major components of a flow cytometer.
2. Compare intrinsic and extrinsic parameters in flow cytometry.
3. List the advantages and disadvantages of automated testing in a clinical immunology laboratory.
4. Summarize the principle of hydrodynamic focusing within the flow cytometer.
5. Define the concept of fluorescence in flow cytometry.
6. Explain the difference between forward-angle light scatter (FSC) and side scatter (SSC).
7. Describe the difference between analyzing flow cytometry data using single-parameter histograms and dual-parameter dot plots.
8. List several clinical applications for flow cytometry.
9. Apply knowledge of various T- and B-cell surface antigens to identify various cell populations.
10. Compare advantages and disadvantages of automated immunoassay analyzers.
11. Describe the difference between a random access and a batch analyzer.
12. Define *accuracy, precision, reportable range, analytic sensitivity, analytic specificity,* and *reference interval.*

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Accuracy
Analytic sensitivity
Analytic specificity
Automatic sampling
Batch analyzers
Dual-parameter dot plot
Extrinsic parameters
Flow cytometry
Fluorochrome
Forward-angle light scatter (FSC)
Gate
Immunophenotyping
Intrinsic parameters
Precision
Random access analyzers
Reference interval
Reportable range
Side (right angle) scatter (SSC)
Single-parameter histogram

Cell Flow Cytometry

Cell **flow cytometry** is an automated system in which single cells (or beads) in a fluid suspension are analyzed in terms of their intrinsic light-scattering characteristics. The cells are simultaneously evaluated for their extrinsic properties (i.e., the presence of specific surface or cytoplasmic proteins) using fluorescent-labeled antibodies or probes. Flow cytometers, originally developed in the 1960s, did not make their way into the clinical laboratory until the early 1980s. At that point, physicians started seeing patients with a new mysterious disease characterized by a drop in circulating T helper (Th) cells. Since that time, flow cytometry has been routinely used for identifying infection with HIV, as well as **immunophenotyping** cells—identifying both their surface and cytoplasmic antigen expression. Cytometers can simultaneously measure multiple cellular or bead properties by using several different fluorochromes. A **fluorochrome,** or fluorescent molecule, is one that absorbs light across a spectrum of wavelengths and emits light of lower energy across a spectrum of longer wavelengths. Each fluorochrome has a distinctive spectral pattern of absorption (excitation) and emission. By using laser light, different populations of cells or particles can be analyzed and identified on the basis of their size, shape, and antigenic properties.

Flow cytometry is frequently used in leukemia and lymphoma diagnostics. In addition, flow cytometry has been used in functional assays for chronic granulomatous disease (CGD) and leukocyte adhesion deficiency, fetal red blood cell (RBC) and F-cell identification in maternal blood (replacing the Kleihauer-Betke assay), and identification of paroxysmal nocturnal hemoglobinuria (PNH), to give just a few examples.[1,2] Flow cytometry has now become the platform for detection of various genetic disease mutations such as cystic fibrosis, as well as tissue typing for transplantation and autoimmune and infectious disease detection. A significant advantage of flow cytometry is that because the flow rate of cells within the cytometer is so rapid, thousands of events can be analyzed in seconds, allowing for the accurate detection of cells that are present in very small numbers.

Instrumentation

The major components of a flow cytometer include the fluidics, the laser light source, and the optics and photodetectors. The data analysis and management is done by computer.[3]

Fluidics

For cellular parameters to be accurately measured in the flow cytometer, it is crucial that cells pass through the laser one cell at a time. Cells are processed into a suspension; the cytometer draws up the cell suspension and injects the sample inside a carrier stream of isotonic saline (sheath fluid) to form a laminar flow. The sample stream is constrained by the carrier stream and is thus hydrodynamically focused so that the cells pass single file through the intersection of the laser light source **(Fig. 13–1).** Each time a cell passes in front of a laser beam, light is scattered and the interruption of the laser signal is recorded.

Laser Light Source

Solid-state diode lasers are typically used as light sources. The wavelength of monochromatic light emitted by a laser in turn dictates which fluorochromes can be used in an assay. Not all fluorochromes can be used with all lasers because each fluorochrome has distinct spectral characteristics. The newer clinical instruments have three lasers—red, blue, and violet—each of which produces different colors when exciting a particular fluorochrome. This allows for as many as 10 fluorochromes, or colors, to be analyzed in a single tube at one time.

As a result of a cell passing through the laser, light is scattered in many directions. The amount and type of light scatter (LS) can provide valuable information about a cell's physical properties. Light at two specific angles is measured by the flow cytometer: **forward-angle light scatter (FSC)** and **side scatter (SSC),** also called **right angle** light scatter (SSC). FSC is considered an indicator of size, whereas the SSC signal is indicative of granularity or the intracellular complexity of the cell. Thus, these two values, which are considered **intrinsic parameters,** can be used to characterize different cell types. If one looks at a sample of whole blood on a flow cytometer where all the RBCs have been lysed, the three major populations of white blood cells (WBCs)—lymphocytes, monocytes, and neutrophils—can be roughly differentiated from each other based solely on their intrinsic parameters (FSC and SSC) **(Fig. 13–2).**

Unlike FSC and SSC, which represent light-scattering properties that are intrinsic to the cell, **extrinsic parameters** require the addition of a fluorescent probe for their detection. Fluorescent-labeled antibodies bound to the cell can be detected by the laser. By using fluorescent molecules with various emission wavelengths, the laboratorian can simultaneously evaluate an individual cell for several extrinsic properties. The

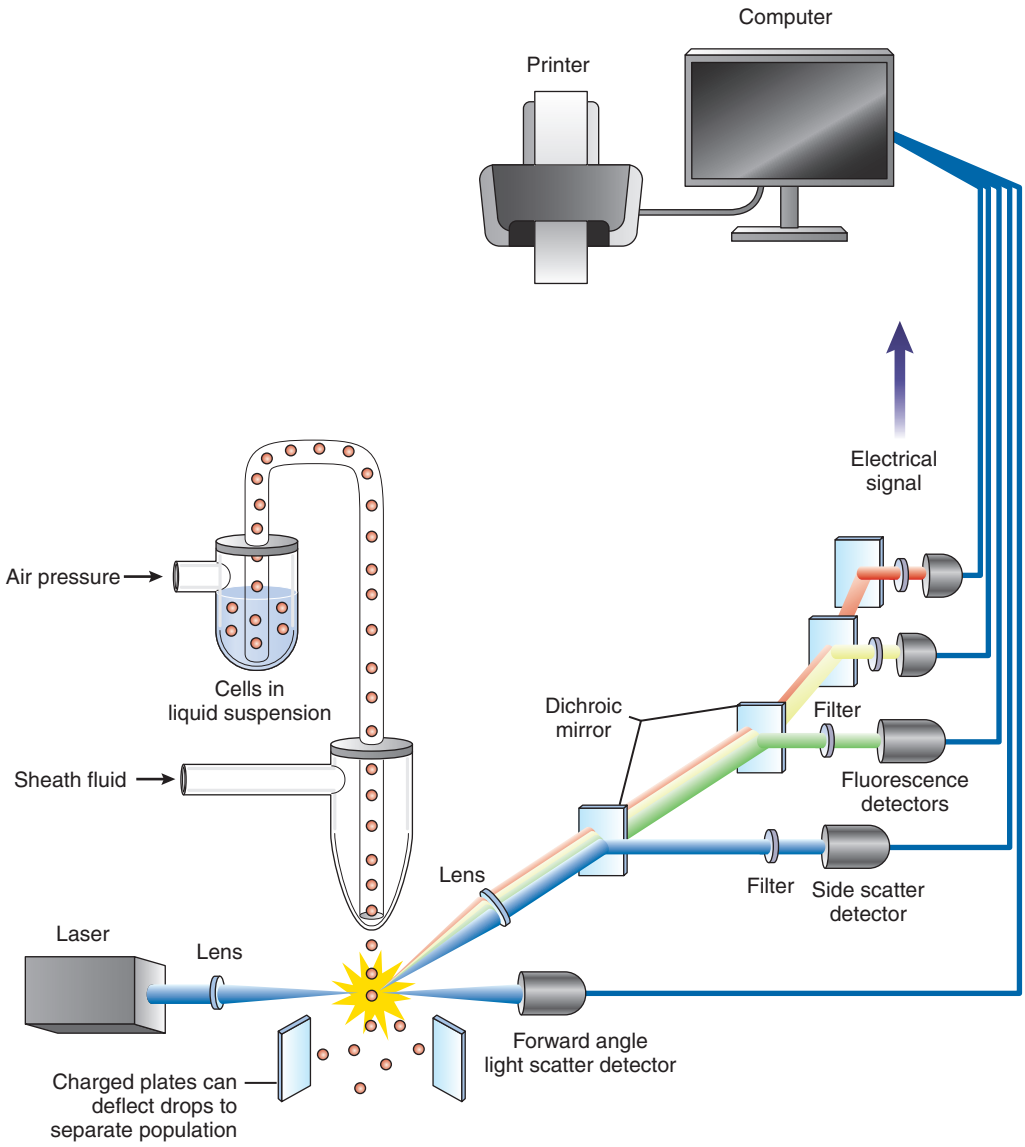

FIGURE 13–1 Flow cytometry. Components of a laser-based flow cytometer include the fluidics system for cell transportation, a laser for cell illumination, photodetectors for signal detection, and a computer-based management system. Both forward and 90-degree LS are measured, indicating cell size and type.

clinical utility of such multicolor analysis is enhanced when the fluorescent data are analyzed in conjunction with FSC and SSC.[4] The combination of data allows for characterization of cells according to size, granularity, DNA and RNA content, antigens, total protein, and cell receptors.[3]

Optics and Photodetectors

The various signals (light scatter and fluorescence) generated by the cells' interaction with the laser are detected by photodiodes for FSC and by photomultiplier tubes for fluorescence. The number of fluorochromes capable of being measured simultaneously depends upon the number of photomultiplier tubes in the flow cytometer. The specificity of each photomultiplier tube for a given band length of wavelengths is achieved through the arrangement of a series of optical filters that are designed to maximize collection of light derived from a specific fluorochrome while minimizing collection of light from other fluorochromes used to stain the cells. The newer flow cytometers actually use fiber-optic cables to direct light to the detectors.

When fluorescent light from fluorescently tagged antibodies bound to cell surfaces reaches the photomultiplier tubes, it creates an electrical current that is converted into a voltage pulse. The voltage pulse is then converted (using various methods, depending on the manufacturer) into a digital signal. The digital signals are proportional to the intensity of light detected. The intensity of these converted signals is measured on a relative scale that is generally set into 1 to 256 channels, from lowest energy level or pulse to the highest level.

Data Acquisition and Analysis

Once the intrinsic and extrinsic cellular properties of many cells (typically 10,000 to 20,000 "events" are collected for each sample) have been collected and the data digitalized, it is ready for

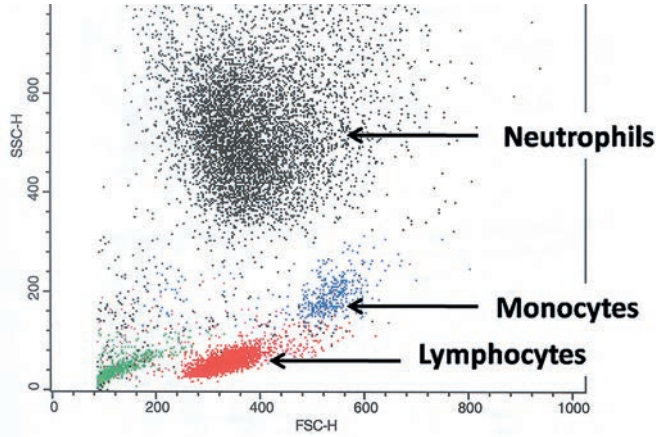

FIGURE 13–2 Peripheral blood leukocyte analysis by simultaneous evaluation of forward-angle light scatter (FSC) and 90-degree LS (SSC). Based on the intrinsic characteristics of size (FSC) and granularity (SSC) only, the three main populations of WBCs (lymphocytes, monocytes, and neutrophils) can be discriminated into individual populations.

analysis by the operator. Each parameter can be analyzed independently or in any combination. Graphics of the data can be represented in multiple ways. The first level of representation is the **single-parameter histogram,** which plots a chosen parameter (generally fluorescence) on the x axis versus the number of events on the y axis; thus, only a single parameter is analyzed using this type of graph **(Fig. 13–3).** The operator can then set a marker to differentiate between cells that have low levels of fluorescence (negative) from cells that have high levels of fluorescence (positive) for a particular fluorochrome-labeled antibody. The computer will then calculate the percentage of "negative" and "positive" events from the total number of events collected.

The next level of representation is the bivariate histogram, or **dual-parameter dot plot,** where each dot represents an individual cell or event. Two parameters, one on each axis, are plotted against each other. Each parameter to be analyzed is then determined by the operator. Using dual-parameter dot plots, the operator can draw a "**gate**" around a population of interest and analyze various parameters (extrinsic and intrinsic) of the cells contained within the gated region **(Fig. 13–4).** The gate allows the operator to screen out debris and isolate subpopulations of cells of interest. Gates can be thought of as a set of filtering rules for analyzing a very large database. The operator can basically filter the data in any way and set multiple or sequential filters (or gates).

When analyzing a population of cells using a dual-parameter dot plot, the operator chooses which parameters to analyze on both the x and y axes. He or she then divides the dot plot into four quadrants, separating the positives from the negatives in each axis **(Fig. 13–5).** Quadrant 1 consists of cells that are positive for fluorescence on the y axis and negative for fluorescence on the x axis. Quadrant 2 consists of cells that are positive for fluorescence on both the x and y axes. Quadrant 3 consists of cells that are negative for fluorescence on both the x and y axes. Quadrant 4 consists of cells that are positive for fluorescence on the x axis and negative for fluorescence on the y axis. The computer will then calculate the percentage of cells in each quadrant based on the total number of events counted (typically 10,000 to 20,000 events per sample). For example, a gate can be drawn around a population of cells based on their FSC versus SSC characteristics and the extrinsic characteristics of the gated population can be analyzed—that is, lymphocytes can be gated, after which the subpopulations of T cells (CD3+ and CD4+ or CD8+) and B cells (CD38+, CD3–) can be analyzed **(Fig. 13–6).** The absolute count of a particular cell type—for instance, CD4+ T lymphocytes—is obtained by multiplying the absolute cell count of the population of interest (e.g., lymphocytes) derived from a hematology analyzer by the percentage of the population of interest in the sample (CD3+ and CD4+ lymphocytes).[3,4] This method is considered a dual-platform analysis. The disadvantage to this type of analysis is the greater potential for added error associated with using two distinct methods to derive the absolute count. The single platform is now the method of choice to eliminate this type of error. In this method, a known quantity of beads is added to the flow cytometry tubes and a simple math calculation allows the direct calculation of absolute numbers from the individual flow cytometry tubes.

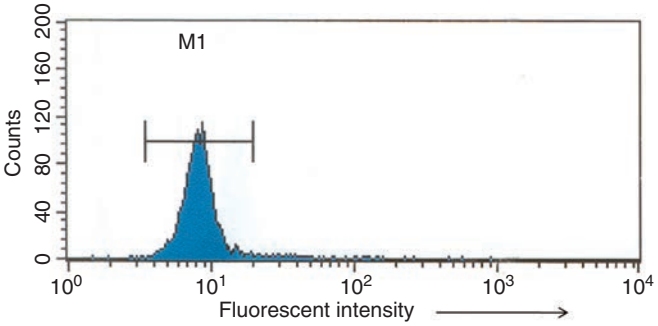

FIGURE 13–3 Example of a single-parameter flow histogram. The y axis consists of the number of events. The x axis is the parameter to be analyzed, which is chosen by the operator; it is usually an extrinsic parameter, such as a fluorescent-labeled antibody. The operator can then set a marker to isolate the positive events. The computer will then calculate the percentage of positive events within the designated markers.

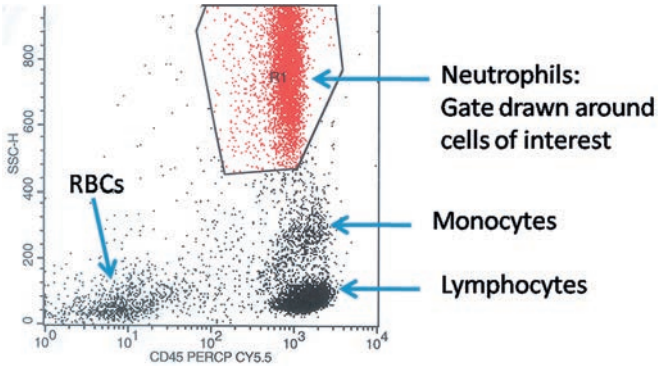

FIGURE 13–4 A dual-parameter dot plot. Both parameters on the x and y axes are chosen by the operator. In this case, lysed whole blood is analyzed on CD45 (x axis) and SSC (y axis). The operator then draws a "gate" or isolates the population of interest (e.g., granulocytes) for further analysis.

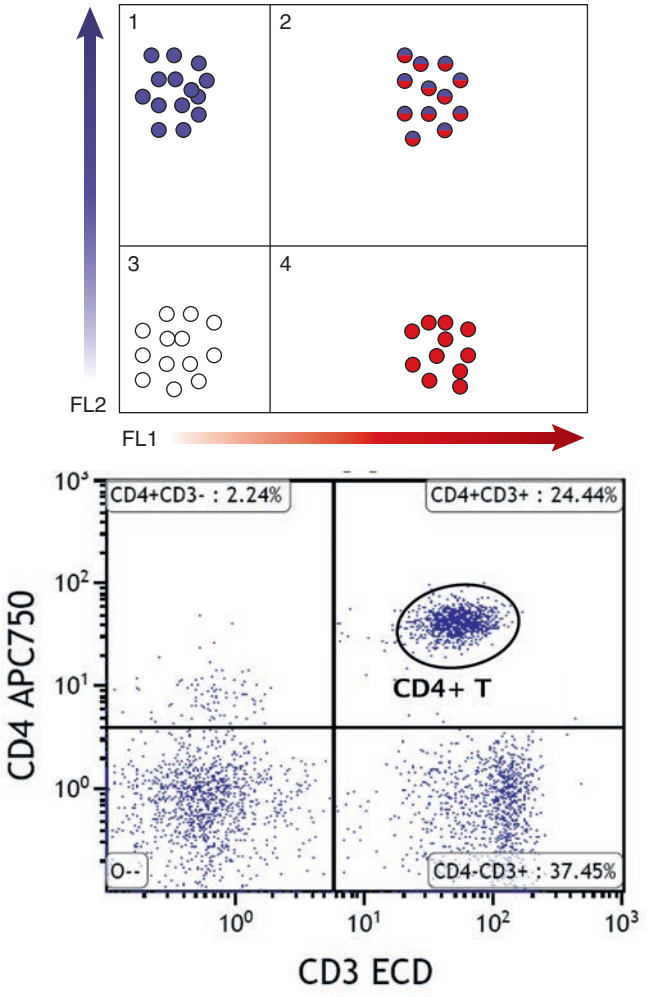

FIGURE 13–5 Quadrant analysis of a dual-parameter dot plot. The operator chooses which parameters to analyze on each axis. (A) On each axis there are positive (fluorescence positive) and negative (fluorescence negative) cells. (B) Example of a dual-parameter dot plot to identify CD4+ T cells: CD3 on the x axis and CD4 on the y axis. The cells in quadrant 2 that are positive for both CD3 and CD4 are true CD4+ T cells.

Detailed phenotypic analysis can determine the lineage and clonality, as well as the degree of differentiation and activation of a specific cell population. This information is useful for differential diagnosis or clarification of closely related lymphoproliferative disorders. Immunophenotyping requires careful selection of combinations of individual markers based on a given cell lineage and maturation.[4] Attempts to standardize individual marker panels, especially by European laboratory groups, are ongoing; however, multiple consensus panels vary from institution to institution.[5]

Sample Preparation

Samples commonly used for analysis include whole blood, bone marrow, and fluid aspirates. Whole blood should be collected into ethylenediaminetetraacetic acid (EDTA), the anticoagulant of choice for samples processed within 30 hours of collection. Heparin can also be used for whole blood and bone marrow and can provide improved stability in samples over 24 hours old. Blood should be stored at room temperature (20°C to 25°C) before processing and should be well mixed before being pipetted into staining tubes.[4,6] Hemolyzed or clotted specimens should be rejected. Peripheral blood, bone marrow, and other samples with large numbers of RBCs require erythrocyte removal to allow for efficient analysis of WBCs.

Historically, density gradient centrifugation with Ficoll-Hypaque (Sigma, St. Louis, MO) was used to generate a cell suspension enriched for lymphocytes or lymphoblasts. However, this method results in selective loss of some cell populations and is time consuming.[7] Density gradient centrifugation has mainly been replaced by erythrocyte lysis techniques, both commercial and noncommercial.[7]

Tissue specimens should be collected and transported in tissue culture medium (RPMI 1640) at either room temperature (if analysis is imminent) or 4°C (if analysis will be delayed). The specimen is then disaggregated to form a single cell suspension, either by mechanical dissociation or enzymatic digestion. Mechanical disaggregation, or "teasing," is preferred and is accomplished by the use of a scalpel and forceps, a needle and syringe, or wire mesh screen.[8] Antibodies are then added to the resulting cellular preparation and processed for analysis. The antibodies used are typically monoclonal, each with a different fluorescent tag.

Clinical Applications

Routine applications of flow cytometry in the clinical laboratory include immunophenotyping of peripheral blood lymphocytes, enumeration of CD34+ stem cells in peripheral blood and bone marrow for use in stem cell transplantation, and immunophenotypic characterization of acute leukemias, non-Hodgkin's lymphomas, and other lymphoproliferative disorders.

Immunophenotyping by flow cytometry has become an important component of initial evaluation and subsequent post-therapeutic monitoring in leukemia and lymphoma management. Flow cytometric findings have been incorporated into current leukemia and lymphoma classifications, beginning with the Revised European-American Lymphoma (REAL) classification in 1994 and, more recently, in the proposed World Health Organization (WHO) classifications.[9,10] One of the most important components of flow cytometric analysis is the stratification of hematopoietic malignancies by their lineage (i.e., B cell, T cell, or myeloid) and the degree of differentiation. Some of the more common cell-differentiation antigens are listed in **Table 13–1.**[11,12]

Knowing unique characteristics of leukemias and lymphomas and pairing those particular markers that identify these characteristics can be useful in making a more reliable diagnosis. For example, in chronic lymphocytic leukemia (CLL), typically CD5 (T-cell lymphocyte marker) is paired with CD20 (B-cell lymphocyte marker). The presence of a significant number of cells that are both CD5+ and CD20+ is an indication of CLL or mantle cell lymphoma. Common markers and fluorochrome conjugate combinations are demonstrated in **Table 13–2.**

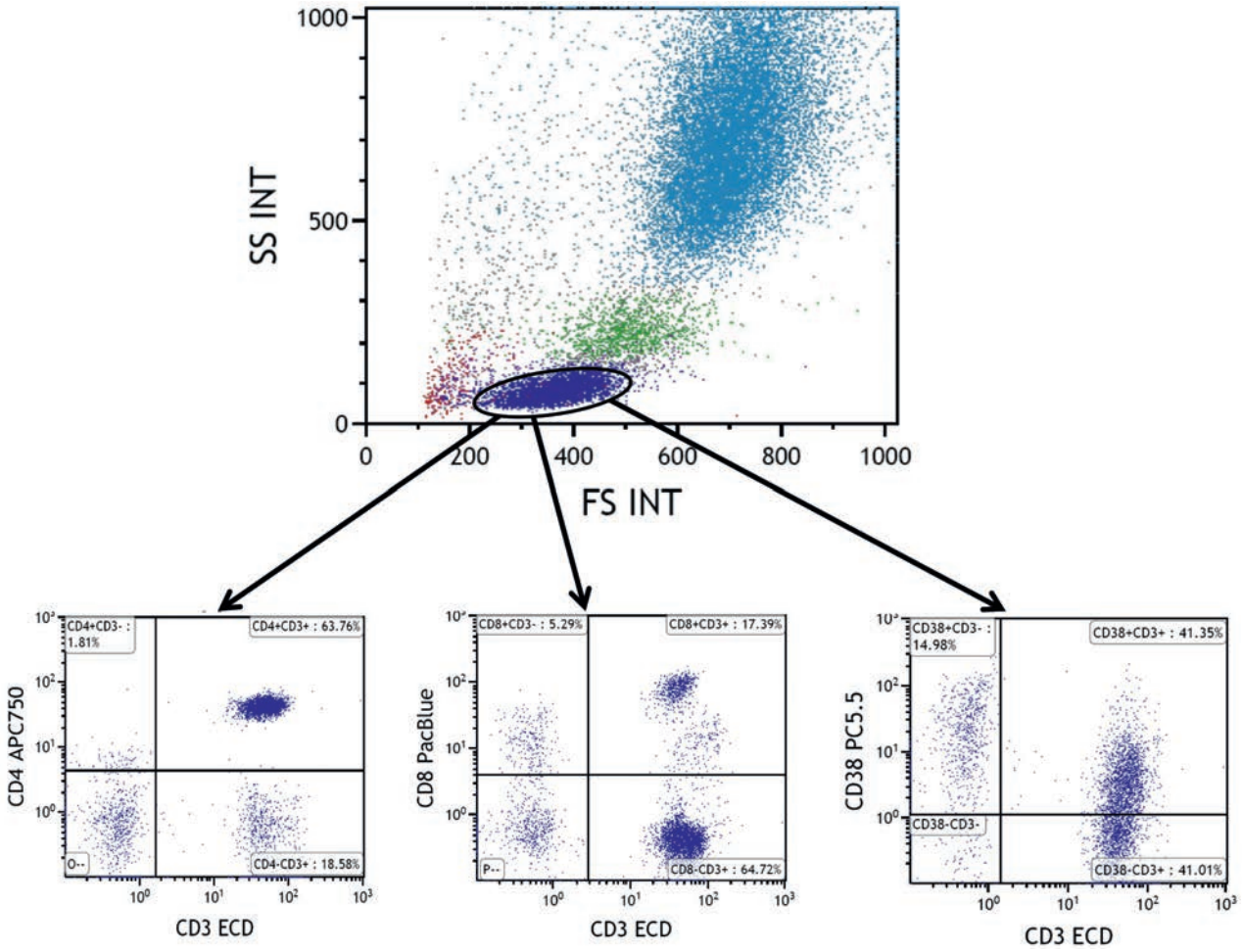

FIGURE 13–6 Gating strategy to analyze lymphocyte subsets in a sample of whole blood. Whole blood is incubated with fluorescent-labeled antibodies specific for CD3, CD4, CD8, and HLA-DR. The sample is washed, RBCs are lysed, and the sample is analyzed on the flow cytometer. To analyze using gating strategies, the sample is first plotted on FSC versus SSC. (A) A gate, or region, is drawn around the lymphocyte population. (B) On the subsequent plots of fluorescent markers, only the lymphocyte population is analyzed. The dot plot is divided into four quadrants to isolate positive from negative populations. The computer calculates the percentage of positive cells in each quadrant. The three flow contour plots are analyzing two different cell surface markers. In the first dot plot, quadrant 2 (upper right) identifies CD4+, CD3+ T helper cell lymphocytes. Quadrant 3 (lower left) identifies B lymphocyte and NK cells. Quadrant 4 (lower right) identifies CD3+ CD4– T-cell lymphocytes. In the second dot plot, quadrant 1 (upper left) identifies low intensity CD8+ CD3– NK cells. Quadrant 2 identifies CD3+ CD8+ T-cytotoxic lymphocytes. Quadrant 3 identifies any lymphocyte that is not a CD8+, CD3+, or B-cell lymphocyte. Quadrant 4 identifies CD3+ CD8– T helper cell lymphocytes. In the third contour plot, quadrant 1 identifies CD38+ CD3– B cells, quadrant 2 identifies CD38+ CD3+ activated T cells, quadrant 3 identifies CD38– CD3– cells, and quadrant 4 identifies CD3+ CD38– T cells, not activated.

Table 13–1 Surface Markers on Leukocytes

ANTIGEN	CELL TYPE	FUNCTION
CD2	Thymocytes, T cells, NK cells	Involved in T-cell activation
CD3	Thymocytes, T cells	Associated with T-cell antigen receptor; role in TCR signal transduction
CD4	T helper cells, monocytes, macrophages	Co-receptor for class II MHC; receptor for HIV
CD5	Mature T cells, thymocytes, subset of B cells (B1)	Positive or negative modulation of T- and B-cell receptor signaling
CD7	T cells, thymocytes, NK cells, pre-B cells	Regulates peripheral T-cell and NK cell cytokine production
CD8	Thymocyte subsets, cytotoxic T cells	Co-receptor for class I MHC

Table 13–1 Surface Markers on Leukocytes—cont'd

ANTIGEN	CELL TYPE	FUNCTION
CD10	B- and T-cell precursors, bone marrow stromal cells	Protease; marker for pre-B CALLA
CD11b	Myeloid and NK cells	αM subunit of integrin CR3, binds complement component iC3b
CD13	Myelomonocytic cells	Zinc metalloproteinase
CD14	Monocytic cells	Lipopolysaccharide receptor
CD15	Neutrophils, eosinophils, monocytes	Terminal trisaccharide expressed on glycolipids
CD16	Macrophages, NK cells, neutrophils	Low affinity Fc receptor, mediates phagocytosis and ADCC
CD19	B cells, follicular dendritic cells	Part of B-cell co-receptor, signal transduction molecule that regulates B-cell development and activation
CD20	B cells, T-cell subsets	Binding activates signaling pathways, regulates B-cell activation
CD21	B cells, follicular dendritic cells, subset of immature thymocytes	Receptor for complement component C3d; part of B-cell co-receptor with CD19
CD22	B cells	B, B-ALL (surface and cytoplasmic), B-CLL, HCL, PLL
CD23	B cells, monocytes, follicular dendritic cells	Regulation of IgE synthesis; triggers release of IL-1, IL-6, and GM-CSF from monocytes
CD25	Activated T cells, B cells, monocytes	Receptor for IL-2
CD33	Myeloid cell	Monocytes, macrophages, granulocytes (weak), myeloid-Pro, myeloid leukemia, AML
CD34	Hematopoietic progenitor cell	Hematopoietic-Pre, endothelial cells, immature leukemias
CD38	Plasma cell	Plasma cells, thymocytes, NK lymphocytes, early B, monocytes, multiple myelomas, ALL, acute myeloblastic leukemia
CD44	Most leukocytes	Adhesion molecule mediating homing to peripheral lymphoid organs
CD45	All hematopoietic cells	Tyrosine phosphatase, augments signaling
CD45R	Different forms on all hematopoietic cells	Essential in T- and B-cell antigen-stimulated activation
CD56	NK cells, subsets of T cells	Not known
CD57		NK cell sub, T-cell sub, small cell lung carcinoma
CD94	NK cells, subsets of T cells	Subunit of NKG2-A complex involved in inhibition of NK cell cytotoxicity
CD103	Intraepithelial lymphocytes	HCL, adult T-cell leukemia
CD138		Plasma cell, pre-B (weak)
HLA-DR	B lymphocytes, activated T lymphocytes, monocytes	Lack of expression diagnostic of M3 myeloid leukemia, T-cell activation
FCM7	B-cell subset, mantle lymphoma	Expressed in mantle cell lymphoma
Kappa chains	B cells	Light chain part of antibody molecule on B cells
Lambda chains	B cells	Light chain part of antibody molecule on B cells

ADCC = antibody-dependent cell cytotoxicity; ALL= acute lymphocytic leukemia; AML= acute myeloid leukemia; CALLA = common acute lymphoblastic leukemia antigen; CLL= chronic lymphocytic leukemia; Fc = fragment crystallizable; GM-CSF = granulocyte-macrophage colony-stimulating factor; HCL = hairy cell leukemia; HIV = human immunodeficiency virus; MHC = major histocompatibility class; NK = natural killer; PLL= prolymphocytic leukemia; TCR = CD3-αβreceptor complex.

Table 13–2 Common Markers Used for Lymphoproliferative and Myeloproliferative Studies in Clinical Flow Cytometry

DISEASE	ANTIBODIES PAIRED	INTERPRETATION
Chronic lymphocytic leukemia (CLL) and prolymphocytic leukemia	FMC7 with CD23	FMC7 negative and CD23 positive in CLL, CD23 negative in mantle cell lymphoma and FMC7 is positive
	CD5 with CD20	When a cell is both CD5 positive and CD20 positive: characteristic of CLL and well-differentiated lymphocytic lymphoma (WDLL), as well as mantle cell lymphoma
	CD19 with kappa CD19 with lambda	CD19 positive with only one light chain (kappa or lambda) is expressed in low intensity; occasionally light chains are not detected
	CD45 with CD14 (Antiglycophorin added to bone marrows)	CD45 fluorescence is brightly expressed and CD14 negative Used to determine erythroid component to the specimen
Hairy cell leukemia (HCL)	CD3 with CD23 CD11c with CD22 CD20 with CD5	CD3 negative and CD23 negative Brightly expressed CD11c and CD22, unlike CLL CD20 positive and CD5 negative (occasionally weak expression of CD5)
	CD19 with kappa CD103 with CD25	CD19 positive with one monoclonal light chain expressed CD103 is highly specific for HCL, and CD25 is usually expressed; hairy cell variants may be negative for these two markers
	CD21 with HLA-DR CD45 with CD14 (Antiglycophorin added to bone marrows)	CD21 negative and DR positive CD45 is brightly expressed and CD14 negative To determine erythroid component to the specimen, CD10 (Calla) is weakly expressed in 26% of cases
B cell Acute lymphocytic leukemia (ALL)	CD3 with HLA-DR CD5 with CD20	CD3 (T-cell receptor) negative and DR positive CD5 negative and CD20 variably positive (low intensity or negative on precursor B-cell ALL, positive on more mature B-cell ALL)
	CD19 with kappa CD19 with lambda	CD19 positive (stem cell is negative) and surface Ig negative (a mature B-cell ALL may have surface immunoglobulin)
	CD34 with CD38	CD34 positive ALL correlates with good prognosis in pediatric patients and poor prognosis in adults; CD38 is positive from stem cell to pre-B
	TdT with CD10	TdT and CD10 (Calla) positive in common ALL and pre-B ALL; CD10 positivity is associated with favorable complete treatment response and disease-free survival; CD10 is usually high intensity
	TdT with CD33 CD45 with CD14 (Antiglycophorin added to bone marrows)	CD33 negative; however, very early B-ALL may be positive CD45 dimly expressed and CD14 negative To determine erythroid component to the specimen
Myeloma plasmacytoid leukemia or lymphoma	CD3 with HLA-DR	CD3 negative; most are HLA-DR negative, although some early plasmacytoid cells may be DR positive
	CD5 with CD20 CD19 with kappa CD19 with lambda CD45 with CD38 CD40 with CD56	CD5 and CD20 negative CD19 and surface Ig negative; occasionally surface Ig is positive; cytoplasmic Ig positive CD45 negative or low intensity; CD38 is high-intensity positive Usually CD40 positive; CD56 has been reported to be positive on myeloma cells but negative on normal plasma cells
	CD10 CD138 CD45 with CD14	CD10 positivity indicates poor prognosis Syndecan-1 positive in mature plasma cells CD14 negative
T-cell acute lymphoblastic leukemia (ALL)	CD1a with CD3	CD1a positivity associated with longer disease-free survival in adult T-cell ALL; CD3 is negative in 99% (exception is mature medullary thymocyte T-cell ALL)
	CD2 with CD25 CD38 with CD7	CD2 variably expressed and CD25 negative CD38 and CD7 are positive

Table 13–2 Common Markers Used for Lymphoproliferative and Myeloproliferative Studies in Clinical Flow Cytometry—cont'd

DISEASE	ANTIBODIES PAIRED	INTERPRETATION
	CD4 with CD8	CD4 and CD8 variably expressed depending on maturity; dual expression is common
	CD5 with CD20	CD5 positive except for prothymocyte stage T-cell ALL, CD20 negative
	CD45 with CD14	CD45 positive and CD14 negative
	HLA-DR with CD34	DR positive T-cell ALL associated with a worse prognosis; CD34 in pediatric patients associated with CNS involvement and poor prognosis and predicts myeloid expression
	TdT with CD10	TdT is positive; CD10 positive T-cell ALL associated with prolonged disease-free survival
	CD19 and kappa	Negative
	CD19 with lambda	Negative
	(Antiglycophorin added to bone marrows)	To determine erythroid component to the specimen
Post-thymic T-cell leukemia or lymphoma	CD1a with CD3	CD1a negative, CD3 positive; note: peripheral T-cell lymphomas lack 1 or more pan-T cell antigen (CD3, CD2, CD5, or CD7) 75% of the time
Peripheral T-cell leukemia	CD2 with CD25	CD2 positive; CD25 positive in adult T-cell leukemias and some peripheral T-cell lymphomas
Adult T-cell leukemia	CD5 with CD7	CD5 positive; CD7 positive except in adult T-cell leukemia
	CD4 with CD8	Variable expression
	CD19 with kappa	Negative
	CD19 with lambda	Negative
	CD45 with CD14	CD45 positive and CD14 negative
	TdT with CD10	Negative
	(Antiglycophorin added to bone marrows)	To determine erythroid component to the specimen
Tγ proliferative disease (NK-like T-cell leukemia) NK-like T-cell lymphoma NK cell leukemia	CD2 with CD57	NK-like T-cell lymphoma tends to be CD56 positive, CD57 negative and is usually clinically aggressive; NK cell leukemias tend to be CD56 or CD16 positive, CD57 negative and are usually clinically aggressive; Tγ proliferative disease is CD56 negative, CD57 positive and exhibits a chronic indolent course; CD2 is usually positive for all, but there are variants
	CD3 with CD56, CD16	Surface CD3 is positive in Tγ proliferative disease and NK-like T-cell lymphoma; CD3 is negative in NK cell leukemia; for CD56 and CD57, see above
	CD11c with CD11b	Usually positive
	CD4 with CD8	Usually CD8 positive, CD4 negative; however, dual staining and CD4 positivity has been reported
	CD19	Negative
	CD45 with CD14	CD45 positive and CD14 negative
Acute myelogenous leukemia (AML)	CD11c with CD11b	CD11c positive on mature myeloid cells; CD11b positive on myelomonocytic cells, eosinophilic myelocytes, eosinophils, and neutrophils; differentiated AML usually expresses mature markers
	CD13 with CD15	Poorly differentiated AML usually lacks CD15, CD11c, CD11b, but positive for CD13
	CD33 with TdT	CD33 in the absence of CD34, HLA-DR, or CD13 suggests immature acute basophilic or mast cell leukemia; TdT is often expressed in low intensity in poorly differentiated AML
	CD14 with CD64	CD14 on early promonocytes to mature monocytes; high expression of CD14 and CD11b predicts poor outcome; CD64 on immature and mature monocytes

Continued

Table 13–2 Common Markers Used for Lymphoproliferative and Myeloproliferative Studies in Clinical Flow Cytometry—cont'd

DISEASE	ANTIBODIES PAIRED	INTERPRETATION
	CD3 with CD7	CD3 negative; some immature AML expresses CD7
	CD19 with kappa	CD19 occasionally expressed on some primitive AML
	CD19 with lambda	Surface Ig negative
	CD34 with HLA-DR	Poorly differentiated AML often expresses CD34; high-intensity CD34 has worse prognosis; CD34 coexpressed with HLA-DR has worse prognosis; CD34 coexpressed with CD has worse prognosis than CD34 alone; lack of HLA-DR indicates either APL or very immature AML
	CD10 with TdT	CD10 is present on neutrophils
	(Antiglycophorin added to bone marrows)	To determine erythroid component present in the specimen
	MPO with CD117	Myeloperoxidase (MPO) is found on fairly mature AMLs, whereas CD117 is a myeloid blast marker

ADCC = antibody-dependent cell cytotoxicity; CALLA = common acute lymphoblastic leukemia antigen; Fc = fragment crystallizable; GM-CSF = granulocyte-macrophage colony-stimulating factor; HIV = human immunodeficiency virus; IgE = immunoglobulin E; MHC = major histocompatibility class; NK = natural killer; TCR = CD3-αβ receptor complex; TdT=terminal deoxynucleotidyl transferase

CD45 is a pan-leukocyte marker present on all WBCs but with varying levels of expression based on the cell's maturity as well as lineage. This variance in expression results in varying levels of fluorescence. Blasts express lower levels of CD45 (low fluorescence) but show an increase of CD45 expression as the cell matures; therefore, mature WBCs have much brighter fluorescence compared with their earlier progenitor stages. The analysis of CD45 expression levels is useful in differentiating various populations of WBCs and in combination with SSC has replaced the FSC and SSC gating in many laboratories. However, it is always a good idea to examine the FSC and SSC plot to be sure a population is not being missed.

Immunophenotyping of WBC populations is also essential when an immunodeficiency is suspected. Enumeration of peripheral blood CD4+ T cells in HIV-infected patients remains the highest volume test performed in the flow cytometry laboratory because it is used in classifying stages of HIV disease and determining treatment options.[13] HIV type 1 (HIV-1) infections cause a rapid, profound decrease in CD4+ T-cell numbers and an expansion of CD8+ T-cell levels during the early course (12 to 18 months) of the illness.[14,15] Some individuals continue to rapidly lose CD4+ T cells and progress to AIDS, whereas others maintain relatively stable CD4+ T-cell counts and remain AIDS-free for years. During this chronic phase of HIV-1 disease, the decline in CD4+ T cells can be slow over many years because of maintenance of homeostatic mechanisms. However, as these homeostatic mechanisms start to fail, there is a further decline in CD4+ T and CD8+ T cells, which eventually leads to the development of AIDS.[16] CD4+T-cell levels are used to stage HIV disease progression, are prognostic for the development of AIDS, and are used to monitor response to antiretroviral therapy. The Centers for Disease Control and Prevention (CDC) guidelines stage HIV-1 disease into three groups by CD4+ T-cell level: greater than 500 CD4 cells/mm^3, or greater than 28% CD4 cells within lymphocytes; 200 to 500 CD4 cells/mm^3, or 14% to 28% CD4 cells; and fewer than 200 CD4 cells/mm^3, or fewer than 14% CD4 cells.[13]

Additional examples of flow cytometry use include the determination of DNA content or ploidy status of tumor cells. This analysis can provide the physician with important prognostic information.[17] Ploidy analysis is also useful in examining products of conception for molar pregnancies.

Finding a small number of abnormal cells in a particular cell population can easily be accomplished by flow cytometry. Patients who have been treated for leukemia or lymphoma can be monitored for "minimal residual disease" because statistically significant rare cell events can be easily detected. In the case of a fetal–maternal hemorrhage, using flow cytometry to detect hemoglobin F-positive cells is much more sensitive than the traditional Kleihauer-Betke method.[18,19]

Characterization of normal cell populations is another use for flow cytometry. Human leukocyte antigen typing and crossmatching for solid organ transplantation can be performed in a much faster and more accurate way than formerly utilized serological methods.[3,17]

Flow cytometry is also the method of choice for the diagnosis of several inherited chronic diseases. CGD is an X-linked and autosomal recessive disease in which neutrophils are defective in their oxidative burst. In this case, neutrophils are exposed to the dye Dihydrorhodamine 123, which is not fluorescent until oxidized. When normal neutrophils are stimulated in vitro, the dye is oxidized and becomes intensely fluorescent. Neutrophils from CGD patients are unable to oxidize the dye and do not become fluorescent after stimulation.

Paroxysmal nocturnal hemoglobinuria (PNH) is an inherited disease characterized by a hemolytic anemia. The RBCs, neutrophils, monocytes, and other cells are lacking the glycosylphosphatidylinositol (GPI) anchors by which many surface proteins are attached to the cell membrane. As a result, the RBCs are fragile. Hemolysis occurs during pH changes in the

blood, typically at night.[1] Several flow cytometry tests are available to detect this defect. One test looks for the antigens that use this anchor because these antigens will be missing or reduced in these patients. The other test detects the anchor; for example, fluorescent aerolysin (FLAER) will bind to the GPI anchor itself. Thus, normal granulocytes and monocytes will be fluorescent, whereas the fluorescence in defective ones will be reduced or absent.

Finally, another important use for flow technology in clinical diagnosis is in cytometric bead arrays. In this technology, various sizes and different fluorescent beads are used to determine multiple analytes at the same time. For example, six different sizes or colors of beads could be coated with six different autoantigens. Patient sera is then added, followed by fluorescent anti-IgG. If the patient has any autoantibodies, the respective bead will be fluorescent and can be identified and quantitated. Similarly, polymerase chain reaction (PCR) and hybridization techniques can be performed on these beads. (See Chapter 12 for a discussion of molecular techniques used to detect viral nucleic acids and various genetic mutations.) Theoretically, this technology has the potential to detect over 500 analytes from one sample of blood.

Immunophenotyping by flow cytometry relies on the use of fluorescent-labeled monoclonal and polyclonal antibodies. *Monoclonal antibodies* specific for various surface antigens are preferable to using polyclonal antibodies. The ability to produce monoclonal antibodies through hybridoma and recombinant DNA techniques has contributed greatly to the accuracy of flow cytometry and has widened its use. (See Chapter 5 for a discussion of monoclonal antibody production.)

Immunoassay Automation

In addition to flow cytometry, the use of automated technology has become more prevalent in clinical immunology laboratories with the advent of immunoassay analyzers. Reliable immunoassay instrumentation, excluding radioimmunoassay, was first available in the early 1990s. Using a solid support for separating free and bound analytes, these instruments have made it possible to automate heterogeneous immunoassays even for low-level peptides such as peptide hormones.[20] Currently, there are more than 60 different automated immunoassay analyzers that are capable of performing almost all common diagnostic immunoassays[21]; they have largely replaced manual testing, especially in larger laboratories. The driving motivation for the development of immunoassay analyzers has been the need to create an automated system capable of reducing or eliminating the many manual tasks required to perform analytical procedures and the demand to handle large volumes of samples.[22] Eliminating manual steps decreases the likelihood of error because the potential error caused by fatigue or erroneous sampling is reduced.[23] Laboratory professionals are also trying to streamline test performance to reduce turnaround time and the cost per test. Automation, in some cases, is much more accurate and precise compared with manual methods; depending on the assay platform, it may be more sensitive as well. Other potential benefits of immunoassay automation include the ability to provide more services with fewer staff; saving on controls, duplicates, dilutions, and repeats; longer shelf life of reagents and less disposal because of outdating; and the potential for automation of sample delivery with bar codes for better sample identification.[24]

Because of the wide variety of automated immunoassay analyzers available, it can be difficult to determine the best instrument for any given laboratory. **Table 13–3** offers a partial list of the many factors to consider in determining what type of analyzer will fulfill a laboratory's needs. It is important for all those involved in the instrument's selection to prioritize the properties of any analyzer to meet the demands of the laboratory.

There are currently two main types of immunoassay analyzers on the market: batch analyzers and random access analyzers **(Table 13–4). Batch analyzers** can examine multiple samples and provide access to the test samples for the formation of subsequent reaction mixtures. However, such batch analyzers permit only one type of analysis at a time. In some cases, this may be considered a drawback; stat samples cannot be loaded randomly and there cannot be multiple analyses on any one sample. Partially for those reasons, the next generation of analyzers was designed in a modular system that could be configured to measure numerous analytes from multiple samples. These types of analyzers are called **random access analyzers;** in these analyzers, many test samples can be analyzed and a number of different tests can be performed on any one sample.[25]

Automation can and does occur in all three stages of laboratory testing: the preanalytical, analytical, and postanalytical stages. For the purposes of this section, discussion is limited to automation within the analytical stage of testing.

The tasks of the analytical stage include introducing a sample, adding reagent, mixing the reagent and sample, incubating, detecting, calculating, and reporting the readout or results. All or some of these tasks may be automated on various immunoassay analyzers. **Automatic sampling** can be accomplished by several different methods: peristaltic pumps (older technology) and positive-liquid displacement pipettes (newer technology) are two examples. In most systems, samples are pipetted using thin, stainless-steel probes. Such probes have clot detectors that will automatically reject a sample if a clot is detected. They also have a liquid level sensor that can detect the lack of sample in a tube, usually because of a short draw. Samples without the proper amount of liquid in them are also rejected. Another issue associated with reusable pipette probes is carryover or contamination of one sample with material from the previous samples. Various methods have been developed to reduce carryover, including the use of disposable pipette tips to initially transfer samples and flushing the internal and external surfaces of sample probes with diluent.

Reagent use in automated immunoassay analyzers requires consideration of the following factors: handling, preparing and storing, and dispensing. Some reagents come ready for use; if they do not, the analyzer must be able to properly dilute

Table 13–3 Factors for Consideration in Selecting an Automated Immunoassay Instrument

CATEGORY	FACTOR
Analytical	Sensitivity Precision Accuracy and test standardization Linearity Interferences Carryover effects
Economic	Purchase cost Lease options Shipping and installation fees Maintenance costs Reagent costs Operator time and costs Disposable costs Training for personnel
Instrument	Maintenance requirements Automation compatibility Space requirements Utility requirements Reliability Clot error detection Hardware costs Nonwarranty service
Manufacturer	Future product plans Speed of service response Reputation Technical support Menu expansion plans Purchase of a warranty
Operational	Test menu Throughput Reagent capacity Reagent stability STAT capability Reflex testing ability Reagent kit size Training requirements Operating complexity Waste requirements Reagent storage requirements Reagent performance Downtime plans

Adapted from Remaley AT, Hortin GL. Protein analysis for diagnostic applications. In: Detrick B, Hamilton RG, Folds JD, et al., eds. Manual of Molecular and Clinical Laboratory Immunology. *7th ed. Washington, DC: American Society for Microbiology; 2006:15 and Appold K. Checklist for buying a chemistry analyzer.* Clin Lab Products. *Nov. 2013:12–15.*

reagents before they can be used. Most reagents come with bar codes that are read by the analyzer to reduce operator error; if the wrong reagent is loaded into the analyzer by mistake, the analyzer will detect the error and generate an error message. For many analyzers, reagents must be stored in laboratory refrigerators; however, in larger systems, there is a reagent storage compartment within the analyzer itself.

After reagents have been added to the samples, the next concern is proper mixing to obtain reliable results. Analyzers use different methods for mixing, including magnetic stirring, rotation paddles, forceful dispensing, and vigorous lateral shaking. Whichever method is used, it is imperative that there be no splashing between sample wells in order to prevent erroneous results.

Timed incubation is then carried out at ambient temperatures. Some analyzers have built-in incubators for temperature-controlled incubation. Heated metal blocks are widely used to incubate reagent wells or cuvettes.

Detection of the final analyte depends on the chemistry involved in the immunoassay. In the past, colorimetric absorption spectroscopy has been the principal means of measurement because of its ability to measure a wide variety of compounds. Other methods of detection include fluorescence and chemiluminescence, both of which require fluorescence detectors. With the growing trend to offer flexibility in an automated analyzer, several companies have developed analyzers that have the ability to combine chemistry and immunoassay testing on a single platform. Two examples are Beckman Coulter's UniCel DXC chemistry systems and Roche Diagnostics' COBAS analyzer series.[23]

Instrumentation can decrease turnaround time for testing, remove the possibility of manual errors, and allow for greater sensitivity in determining the presence of low-level analytes. Batch analyzers may work best if only one type of testing is performed on a large scale. Random access analyzers allow for more flexibility and include rapid processing of stat samples. In either case, any new instrument requires extensive validation before patient results can be reported with confidence.

Validation

Regardless of the instrumentation considered, proper validation of new instrumentation or methodology must always be performed. The laboratory needs to determine how it will meet Clinical Laboratory Improvement Amendment (CLIA) regulations for verifying the manufacturer's performance specifications. The regulations apply to each nonwaived test or test system brought into the laboratory for the first time. Validation of the new instrument or method must be completed before patient results using that instrument can be reported. There are multiple resources available on the topic of method validation. The Centers for Medicare and Medicaid Services has an overview of the CLIA (available at http://www.cms.gov/CLIA). The specific requirements for method validation for nonwaived and modified tests can be found at http://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/Categorization-of–Tests.html. **Table 13–5** lists other government websites with information regarding method and instrument validation.[26]

As designated by CLIA, the required verifications to be determined for a new method are accuracy, precision, analytic sensitivity, and analytic specificity to include interfering substances, reportable range, and reference intervals. **Accuracy**

Table 13–4 Automated Immunoassay Analyzers

MANUFACTURER	INSTRUMENT	OPERATIONAL TYPE	ASSAY PRINCIPLE
Abbott Diagnostics	AxSYM	Continuous random access Stat processing	FPIA, MEIA
Abbott Diagnostics	Architect Series: Ci4100, Ci8200, Ci6200, i1000SR, i2000SR, i4000SR	Batch, random access, continuous random access	Enhanced chemiluminescence
Alere	Agility DS2 DSX	Batch	EIA
Awareness Technology	ChemWell	Batch, random access	EIA
Beckman Coulter	Access UniCel DXI 800 Access UniCel DXI 600 Access	Continuous random access, STAT capability (Up to 400 tests/hr) (Up to 200 tests/hr)	Chemiluminescence, EIA
BioMérieux	VIDAS miniVIDAS (multiparametric IA)	Batch, random access STAT capability	FEIA-coated SPR Solid-phase receptacle SPR pipetting device
Bio-Rad Laboratories, Clinical Diagnosis Group	BioPlex® 2200 Multiplex Testing PhD System	Continuous random access Batch	Bead flow cytometric (multiplex) EIA
Diamedix Corporation	New Mago 4S Automated Immunoassay System Mago Plus Automated EIA Processor	Batch, random access	EIA ELISA and IFA
DiaSorin, Inc.	ETI-MAX (Germany/Italy)	Batch, random access	EIA
Dynex	DS2	Batch	EIA
Hycor Biomedical, Inc.	HYTEC 288 Plus	Random batches	EIA
Inova	BIO-FLASH Ds2	Batch, continued random access	Chemiluminescence
Inverness Medical Professional Diagnostics	AIMS (Automated IA Multiplexing System)	Batch	EIA, multiplexing or bead diagnostics
Ortho Clinical Diagnostics, a J&J Co.	VITROS 3600	Continuous random access	Chemiluminescence (enhanced)
Phadia Thermo Fisher Scientific-Phadia	ImmunoCAP Phadia Laboratory System	Continuous random access	FEIA
Siemens Medical Solutions Diagnostics	ADVIA Centaur Dimension EXL Dimension Vista 1500 IMMULITE	Continuous random access Batch, random access, continuous random access Continuous random access	Chemiluminescence Chemiluminescence, EIA Chemiluminescence
TOSOH Bioscience, Inc.	AIA	Continuous random access	Fluorescence, EIA

EIA = enzyme immunoassay; FEIA = fluoroenzyme immunoassay; FPIA = fluorescent polarization immunoassay; MEIA = microparticle enzyme immunoassay; SPR = solid-phase receptacle.

refers to the test's ability to actually measure what it claims to measure. For example, the assay may be tested using previously known positives or negatives as provided by proficiency testing or interlaboratory exchange. Also, parallel testing with an alternative method or technology is a form of accuracy testing. **Precision** refers to the ability to consistently reproduce the same result on repeated testing of the same sample. See **Figure 13–7** for a pictorial representation of the difference between accuracy and precision. CLIA '88 specifies that the standard deviation and coefficient of variation should

Table 13–5 CLIA-Related Governmental Websites

AGENCY	WEBSITE
Centers for Disease Control and Prevention	http://www.cdc.gov
Centers for Medicare and Medicaid Services	www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/Categorization-of-Tests.html
U.S. Food and Drug Administration	www.fda.gov
The College of American Pathologists Laboratory Accreditation Program Inspection Checklist for Chemistry	www.cap.org/web/home?
Clinical Laboratory Standards Institute Evaluation Standards	www.clsi.org

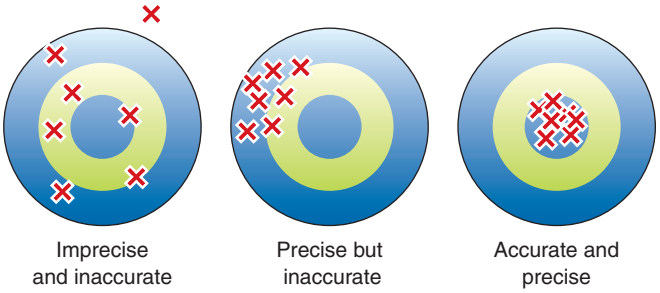

FIGURE 13–7 Pictorial representation of the difference between accuracy and precision.

be calculated from 10 to 20 day-to-day quality control results.[27] At least one normal and one abnormal control should be included in the analysis. **Analytic sensitivity** is defined as the lowest measureable amount of an analyte, whereas **analytic specificity** is the assay's ability to generate a negative result when the analyte is not present. The **reportable range** is defined as the range of values that will generate a positive result for the specimens assayed by the test procedure. Note that this may not include the entire dynamic range of the analytic instrument used to produce the result. Finally, the **reference interval** is the range of values found in healthy individuals who do not have the condition that is detected by the assay, which is used to define the expected value of a negative test.

SUMMARY

- Flow cytometry, a powerful tool to identify and enumerate various cell populations, was first used in clinical laboratories to perform CD4+ T-cell counts in HIV-infected individuals.
- A flow cytometer measures multiple properties of cells suspended in a moving fluid medium.
- As each cell or particle passes single file through a laser light source, it produces a characteristic light pattern that is measured by multiple detectors for scattered light (forward and 90 degrees) and fluorescent emissions if the cell is stained with a fluorochrome.
- Scattered light in a forward direction is a measure of cell size, whereas the side scatter determines a cell's internal complexity, or granularity.
- A single-parameter histogram shows a chosen parameter (on the x axis) versus the number of events (on the y axis).
- A dual-parameter dot plots two parameters against each other.
- A gate blocks off a particular region of cells for further analysis.
- Determining an individual's lymphocyte population is essential in diagnosing such conditions as lymphomas, immunodeficiency diseases, unexplained infections, or acquired immune diseases such as AIDS.
- Lymphoid and myeloid cells are identified using monoclonal antibodies directed against specific surface antigens. Flow cytometry is the most commonly used method for immunophenotyping of lymphoid and myeloid populations.
- Clinical immunology laboratories are now utilizing a variety of automated immunoassay analyzers to replace manual immunoassay procedures, allowing for more accurate, precise, and sensitive analysis of many analytes.
- There are many factors to consider in determining which analyzer will fill the needs of a particular laboratory, including deciding whether a batch or a random access analyzer can best serve testing needs.
- Automation is incorporated in all stages of laboratory testing: preanalytical, analytical, and postanalytical.
- Once an analyzer has been purchased, a thorough validation of all assays to be performed must be done to ensure quality; they should include at least a determination of accuracy, precision, reportable range, reference range, analytic sensitivity, and analytic specificity.
- Accuracy refers to a test's ability to measure what it actually claims to measure.
- Precision is the ability to consistently reproduce the same result on a particular sample.
- Automated analyzers are costly; however, they can reduce turnaround time and hands-on time by the technical staff and provide sensitive and precise results.

CASE STUDIES

1. A laboratory has just purchased a new immunoassay analyzer and validation is being done before patient results can be reported out. Twenty random patient samples are run by both the old and new methodology. According to the newer instrumentation, three samples that were negative by the old method are positive by the new instrument.

Questions

a. What sort of possible error—that is, sensitivity, specificity, accuracy, or precision—does this represent?
b. What steps should be taken to resolve this discrepancy?

2. A 3-year-old female is sent for immunologic testing because of recurring respiratory infections, including several bouts of pneumonia. The results show decreased immunoglobulin levels, especially of IgG. Although her WBC count was within the normal range, her lymphocyte count was low. Flow cytometry was performed to determine if a particular subset of lymphocytes was low or missing. **Figure 13–8** shows the flow cytometry results obtained.

Questions

a. What do the flow cytometry patterns indicate about the population of lymphocytes affected?
b. How can this account for the child's recurring infections?
c. What further type of testing might be indicated?

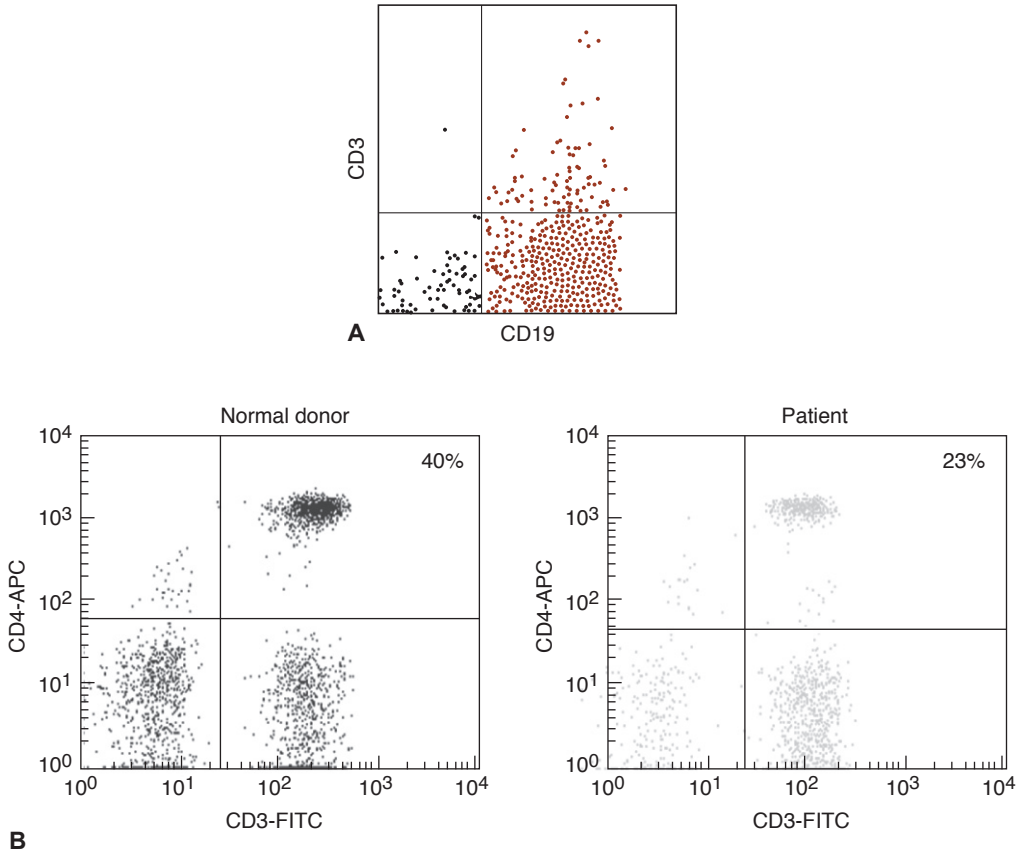

FIGURE 13–8 Flow cytometry patterns for case study. (A) Plot of CD3 versus CD19. (B) Plot of CD3 versus CD4.

REVIEW QUESTIONS

1. Flow cytometry characterizes cells on the basis of which of the following?
   - **a.** Forward and 90-degree side scatter of an interrupted beam of light
   - **b.** Front-angle scatter only of an interrupted light beam
   - **c.** Absorbance of light by different types of cells
   - **d.** Transmittance of light by cells in solution

2. Forward-angle light scatter is an indicator of cell
   - **a.** granularity.
   - **b.** density.
   - **c.** size.
   - **d.** number.

3. What is the single most important requirement for samples to be analyzed on a flow cytometer?
   - **a.** Whole blood is collected into a serum-separator tube.
   - **b.** Cells must be in a single-cell suspension.
   - **c.** Samples must be fixed in formaldehyde before processing.
   - **d.** Blood must be kept refrigerated while processing.

4. Which statement represents the best explanation for a flow cytometer's ability to detect several cell surface markers at the same time?
   - **a.** The forward scatter can separate out cells on the basis of complexity.
   - **b.** One detector can be used to detect many different wavelengths.
   - **c.** For each marker, a specific fluorochrome–antibody combination is used.
   - **d.** Intrinsic parameters are separated out on the basis of the amount of side scatter.

5. Which of the following cell surface markers would be present on a population of T helper (Th) cells?
   - **a.** CD3 and CD4
   - **b.** CD3 and CD8
   - **c.** CD3 only
   - **d.** CD4 only

6. If an analyzer consistently indicates a positive test when the analyte in question is not present, this represents a problem with
   - **a.** sensitivity.
   - **b.** specificity.
   - **c.** reportable range.
   - **d.** precision.

7. All of the following are clinical applications for flow cytometry *except*
   - **a.** fetal hemoglobin.
   - **b.** immunophenotyping of lymphocyte subpopulations.
   - **c.** HIV viral load analysis.
   - **d.** enumeration of stem cells in a peripheral blood mononuclear cell product.

8. The various signals generated by cells intersecting with a flow cytometry laser are captured by
   - **a.** bandwidth waves.
   - **b.** wave channels.
   - **c.** photomultiplier tubes.
   - **d.** flow cells.

9. Analysis of flow cytometer data of cells can be filtered in many ways by using a method of
   - **a.** "gating" in a dot plot.
   - **b.** banding of a histogram.
   - **c.** single-parameter histogram monitoring.
   - **d.** automatic sampling.

10. A newer flow cytometry technology that has the potential to detect over 500 analytes from one sample of blood is called a/an
    - **a.** RBC fragmentation assay.
    - **b.** Dihydrorhodamine 123.
    - **c.** sucrose test.
    - **d.** cytometric bead array.

11. Many flow cytometry laboratories now use the CD45 marker in combination with SSC in differentiating various populations of WBCs to replace which of the following combinations?
    - **a.** CD4 + SSC
    - **b.** CD4 + FSC
    - **c.** FSC + SSC
    - **d.** FSC + CD45

12. Which cell surface marker is present on cells seen in hairy cell leukemia?
    - **a.** CD138
    - **b.** CD33
    - **c.** CD103
    - **d.** CD34

13. CD45 is a pan-leukocyte marker expressed on WBCs in varying levels or amounts of expression, based on

a. size of a cell.
b. granularity of a cell.
c. maturity and lineage of a cell.
d. malignancy of a cell.

14. Which of the following statements best describes a single-parameter histogram?

a. Each event is represented by a dot.
b. Data is distributed in four quadrants.
c. Positive and negative events are plotted on the x and y axis.
d. A chosen parameter is plotted versus the number of events.

15. How many fluorochromes (colors) are current clinical flow cytometers capable of detecting?

a. 2
b. 6
c. 8
d. 10

16. Which type of analyzer allows one to measure multiple analytes from numerous samples, loaded at any time?

a. Batch analyzer
b. Random access analyzer
c. Front-end loaded analyzer
d. Sequential access analyzer

17. Operational considerations when selecting automated analyzers for your laboratory include all of the following *except*

a. reagent stability.
b. test menu.
c. STAT capability.
d. purchase cost.

18. Analyzers use different methods for mixing, including magnetic stirring, rotation paddles, forceful dispensing, and vigorous lateral shaking. Whichever method used, it is imperative that

a. reagents always be kept refrigerated.
b. there is no splashing or carry-over between samples.
c. samples are kept at room temperature.
d. multiple methods are not used simultaneously.

19. All of the following are benefits of automation *except*

a. greater accuracy.
b. increased turnaround time.
c. savings on controls.
d. less disposal of outdated reagents.

20. If an analyzer gets different results each time the same sample is tested, what type of problem does this represent?

a. Sensitivity
b. Specificity
c. Accuracy
d. Precision

Immune Disorders

III

14 Hypersensitivity

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Explain the concept of hypersensitivity.
2. Differentiate between the four types of hypersensitivity reactions in terms of antibody involvement, complement involvement, antigen triggers, and timing of the response.
3. Associate specific examples of clinical manifestations with each type of hypersensitivity.
4. Discuss the immunologic mechanisms involved in each of the four types of hypersensitivity reactions.
5. Provide examples of preformed and newly synthesized mediators released from IgE-sensitized mast cells and basophils and discuss their effects.
6. Discuss the influence of genetic and environmental factors on susceptibility to type I hypersensitivity responses.
7. Discuss the types of reactions that can result from latex sensitivity and their clinical manifestations.
8. Explain the underlying mechanisms of pharmacological therapy, monoclonal anti-IgE therapy, and allergy immunotherapy in the treatment of allergies.
9. Discuss the procedure, clinical applications, advantages, and limitations of skin testing for type I hypersensitivity.
10. Discuss the principles and clinical applications of allergen-specific and total-IgE testing.
11. Explain how hemolytic disease of the newborn (HDN) arises.
12. Explain the significance of a positive direct antiglobulin test.
13. Discuss the principle of cold agglutinins testing, and associate the presence of a positive result with specific disorders.
14. Discuss how skin testing for delayed hypersensitivity is performed, its clinical applications, and how to interpret the results.

CHAPTER OUTLINE

KEY TERMS

Allergen
Allergy immunotherapy (AIT)
Anaphylaxis
Anergy
Arthus reaction
Atopy
Autoimmune hemolytic anemia
Cold agglutinins
Contact dermatitis
Delayed hypersensitivity
Direct antiglobulin test (DAT)
Granulomas
Hemolytic disease of the newborn (HDN)
Histamine
Hypersensitivity
Immediate hypersensitivity
Isohemagglutinins
Leukotrienes (LT)
Paroxysmal cold hemoglobinuria
Serum sickness
Type I hypersensitivity
Type II hypersensitivity
Type III hypersensitivity
Type IV hypersensitivity

In previous chapters, the immune response has been described as a defense mechanism by which the body rids itself of potentially harmful antigens. However, in some cases, the antigen can persist, and the immune response can cause damage to the host. This type of reaction is termed **hypersensitivity,** which is defined as an exaggerated response to a typically harmless antigen that results in injury to the tissue, disease, or even death. British immunologists P. G. H. Gell and R. R. A. Coombs devised a classification system for these reactions based on four different categories. These categories, described briefly in the text that follows, are illustrated in **Figure 14–1.**

Type I hypersensitivity reactions are also known as *anaphylactic hypersensitivity.* In these reactions, exposure to an antigen induces production of specific immunoglobulin E (IgE) antibody, which binds to receptors on mast cells and basophils. Subsequent attachment of the antigen to adjacent cell-bound IgE results in degranulation with release of chemical mediators that produce characteristic allergy symptoms. In **type II hypersensitivity,** also known as *antibody-mediated cytotoxic hypersensitivity,* Immunoglobulin G (IgG) or immunoglobulin M (IgM) antibodies react with antigens on the surface of host cells. This can lead to cell damage by complement-mediated lysis or other mechanisms, dysfunction of the cell by blocking the binding of a ligand to a surface receptor, or overstimulation of a cell's function. **Type III hypersensitivity** is also referred to as *complex-mediated hypersensitivity.* In this process, IgG or IgM antibodies react with soluble antigens to form small complexes that precipitate in the tissues and activate complement. Recruitment of neutrophils to the site results in an inflammatory response that causes injury to the tissues. **Type IV hypersensitivity** differs from the other three types because sensitized T cells, rather than antibody, are responsible for the symptoms that develop. This *cell-mediated hypersensitivity* involves the release of cytokines that induce inflammation and tissue damage.

Types I through III are classified as **immediate hypersensitivity** reactions because symptoms develop within a few minutes to a few hours after exposure to the antigen. Type IV hypersensitivity is sometimes referred to as **delayed hypersensitivity** because its manifestations are not seen until 24 to 48 hours after contact with the antigen.

The four main types of hypersensitivity are discussed in more detail in the sections that follow. Although some disease manifestations may overlap among these types, knowledge of the general characteristics of each type will help you understand the immune processes that trigger such tissue damage. For each of the four types of hypersensitivity, the nature of the immune reactants is discussed, clinical examples are provided, and relevant testing is reviewed.

Type I Hypersensitivity

The type I hypersensitivity reactions are commonly thought of as *allergies.* Some examples of these reactions are hay fever, allergic asthma, hives, and systemic anaphylaxis (see the following discussion). The antigens that trigger type I hypersensitivity are called **allergens.** Examples of common allergens include peanuts, eggs, and pollen. A distinguishing feature of type I hypersensitivity is the short time lag, usually minutes, between exposure to allergen and the onset of clinical symptoms.

The first clue about the cause of type I hypersensitivity was provided by Carl Wilhelm Prausnitz and Heinz Küstner, who showed that a serum factor was responsible. In their historic experiment, serum from Küstner, who was allergic to fish, was injected into Prausnitz. A later exposure to fish antigen at the same site resulted in redness and swelling.[1] This type of reaction is known as *passive cutaneous anaphylaxis.* It occurs when serum is transferred from an allergic individual to a non-allergic individual, and the second individual is challenged at a later time with the specific allergen. Although this experiment was conducted in 1921, it was not until 1967 that the serum factor responsible for this reaction was identified as IgE.

Connections

Immunoglobulin E

Recall from Chapter 5 that IgE is the least abundant antibody class in the serum, normally accounting for less than 1% of all the immunoglobulins. This is likely because IgE is not involved in typical immune responses such as complement fixation and opsonization. IgE is unique in its ability to bind to specific receptors on mast cells and basophils. This property enables IgE to play a major role in type I hypersensitivity allergic reactions and in defense against parasites (see Chapter 22). Patients with these conditions typically have increased concentrations of IgE in their bloodstream.

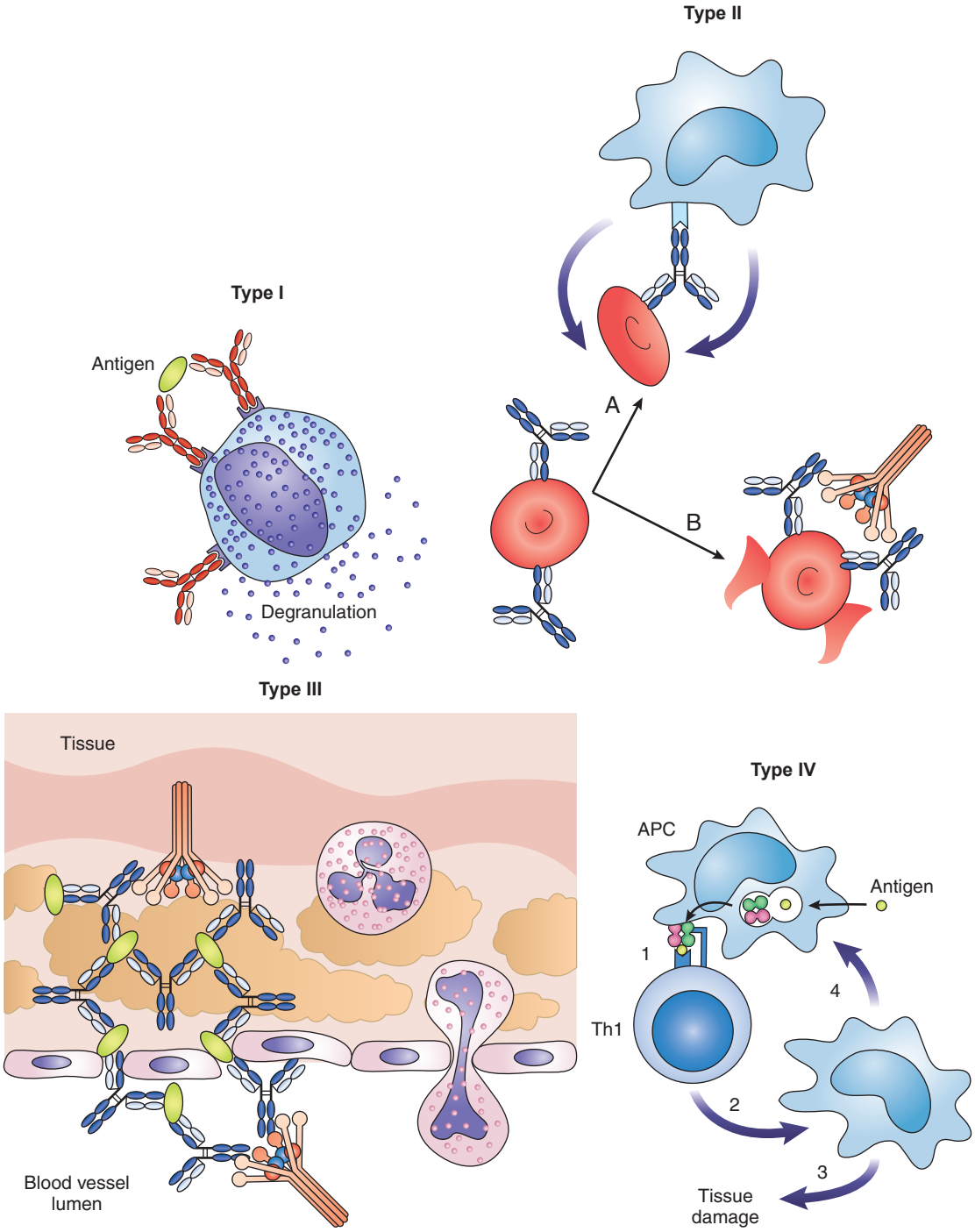


FIGURE 14–1 Immune mechanisms of hypersensitivity types I to IV.

Typically, patients who exhibit allergic hypersensitivity reactions produce a large amount of IgE antibody in response to a small concentration of allergen. IgE levels appear to depend on the interaction of both genetic and environmental factors. **Atopy**, a term derived from the Greek word *atopos* (meaning "out of place"), refers to an inherited tendency to develop classic allergic responses to naturally occurring inhaled or ingested allergens.[1] The section that follows will provide details about the immunologic mechanism which occurs in susceptible individuals to produce the symptoms of type I hypersensitivity reactions.

Immunologic Mechanism

The key immunologic components involved in type I hypersensitivity reactions are IgE antibody, mast cells, basophils, and eosinophils. The response begins when a susceptible individual is exposed to an allergen and produces specific IgE antibody. IgE is primarily synthesized by B cells and plasma cells in the lymphoid tissue of the respiratory and gastrointestinal tracts, as well as the lymph nodes. The regulation of IgE production appears to be a function of a subset of T cells called *type 2 helper cells (Th2)*.[1-3] In a normal immune response to microorganisms

and other antigens, there is an appropriate balance between the activity of the Th2 cells and the *type 1 helper cells (Th1),* which results in protective immunity that does not harm the host. However, in people with allergies, the immune response is shifted so that Th2 cells predominate. This Th2 type of response results in production of several cytokines, including IL-4 and IL-13. These cytokines are responsible for the final differentiation that occurs in B cells, initiating the transcription of the gene that codes for the epsilon-heavy chain of immunoglobulin molecules belonging to the IgE class.[1-3]

Sensitization Phase

Type I hypersensitivity occurs in two major phases: sensitization and activation **(Fig. 14–2).** A late-phase reaction may also occur in some individuals. In the sensitization phase, the IgE antibody attaches to high-affinity receptors called FcεRI, which bind the fragment crystallizable (Fc) region of the epsilon-heavy chain. Large numbers of these receptors are found on mast cells and basophils, with a single cell having as many as 200,000 such receptors.[2,4] Langerhans and dendritic cells internalize and process allergens from the environment and transport the allergen-MHC class II complex to local lymphoid tissue where synthesis of IgE occurs.[2] Binding of IgE to cell membranes increases the half-life of the antibody from 2 or 3 days to at least 10 days. Once bound, IgE serves as an antigen receptor on mast cells and basophils.

Mast cells are the principal effector cells of immediate hypersensitivity.[5] These cells are found throughout the body and in most organs tend to be concentrated around the small blood vessels, the lymphatics, the nerves, and the glandular tissue.[6] Mast cells have abundant cytoplasmic granules that store numerous preformed inflammatory mediators. They are long-lived, residing for months in the tissues. Basophils are similar, but distinct, from mast cells in terms of their appearance and function. They are present in the peripheral blood, where they represent less than 1% of the total white blood cells (WBCs). They have fewer, but larger granules than mast cells, and the concentrations of inflammatory substances in the basophil granules differ from those of the mast cell.[5] Basophils respond to chemotactic stimuli during inflammation and accumulate in the tissues, where they can persist for a few days. As you will see in the next section, subsequent binding of allergen to IgE-sensitized mast cells and basophils triggers degranulation with release of inflammatory mediators.

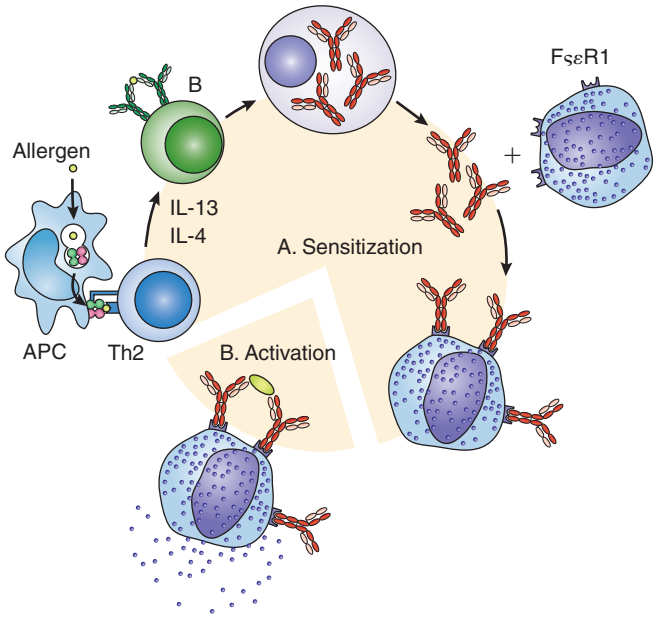


FIGURE 14–2 Type I hypersensitivity. (A) Sensitization: Formation of antigen-specific IgE that attaches to mast cells. (B) Activation: Reexposure to the same antigen, causing degranulation of mast cells and release of mediators.

Activation Phase

In the activation phase of the response, adjacent cell-bound IgE molecules are cross-linked by a bivalent or multivalent antigen, causing aggregation of the surface FcεRI receptors. This action, in turn, initiates complex intracellular signaling events involving multiple phosphorylation reactions, an influx of calcium, and secretion of cytokines.[5,7] The increase in intracellular calcium triggers rapid degranulation of the mast cells and basophils, which release chemical mediators that have been previously made and stored in the granules.[1] The most abundant preformed mediator is **histamine,** which comprises approximately 10% of the total weight of the granules in mast cells.[8] These preformed substances are referred to as *primary* mediators. Other primary mediators include heparin, eosinophil chemotactic factor of anaphylaxis (ECF-A), neutrophil chemotactic factor, and proteases.[2,9] The effects of these mediators are summarized in **Table 14–1.** Release of these substances is responsible for the early-phase symptoms seen in allergic reactions, which occur within 30 to 60 minutes after exposure to the allergen. The chemical mediators bind to receptors on target organs, most notably the skin, respiratory tract, and gastrointestinal tract, producing symptoms characteristic of an allergic response. The clinical manifestations depend on the target tissue and type of receptors activated. For example, in the skin, local swelling and redness, sometimes referred to as a *wheal-and-flare* reaction, can develop. Contraction of the smooth muscle in the bronchioles may result in airflow obstruction. Increased vascular permeability can cause hypotension or shock. Depending on the route by which an individual is exposed to the triggering allergen, one or more of these effects may be seen.

Late Phase

In addition to immediate release of preformed mediators, mast cells and basophils are triggered to synthesize other reactants from the breakdown of phospholipids in the cell membrane. These newly formed, or *secondary,* mediators include platelet-activating factor (PAF); prostaglandin (PG) D_2; **leukotrienes (LT)** B_4, C_4, D_4, and E_4; and cytokines[1,2,9] (see Table 14–1). These products are more potent than the primary mediators and are responsible for a late-phase allergic reaction that can be seen in some individuals 6 to 8 hours after exposure to the antigen. In this phase of the reaction, numerous cells, including eosinophils, neutrophils, Th2 cells, mast cells, basophils, and macrophages, exit the circulation and infiltrate the allergen-filled tissue. They release additional mediators that prolong the hyperactivity and may lead to tissue damage.[4,9,10]

Table 14–1 Mediators of Type I Hypersensitivity

	MEDIATOR	ACTIONS
Primary (Preformed)	Histamine	Smooth muscle contraction, vasodilation, increased vascular permeability
	Heparin	Smooth muscle contraction, vasodilation, increased vascular permeability
	Eosinophil chemotactic factor of anaphylaxis (ECF-A)	Chemotactic for eosinophils
	Neutrophil chemotactic factor of anaphylaxis (NCF-A)	Chemotactic for neutrophils
	Proteases (e.g., tryptase, chymase)	Convert C3 to C3b, stimulate mucus production, activate cytokines
Secondary (Newly Synthesized)	Prostaglandin PGD_2	Vasodilation, increased vascular permeability
	Leukotriene LTB_4	Chemotactic for neutrophils, eosinophils
	Leukotrienes LTC_4, LTD_4, LTE_4	Increased vascular permeability, bronchoconstriction, mucus secretion
	Platelet activating factor (PAF)	Platelet aggregation
	Cytokines IL-1, IL-3, IL-4, IL-5, IL-6, IL-9, IL-13, IL-14, IL-16, TNF-α, GM-CSF	Increase inflammatory cells in area, and increase IgE production

Eosinophils play an important role in the late-phase reaction. These cells normally compose 1% to 3% of the circulating WBCs. During allergic reactions, IL-5 and other cytokines released from the Th2 cells stimulate the bone marrow to increase production of eosinophils, and the number in the peripheral blood increases, producing eosinophilia.[4,10] The number of FcεRI receptors on eosinophils increases during the allergic response, and the eosinophil is stimulated to release a variety of toxic molecules and inflammatory mediators from its granules. These mediators are believed to contribute to the ongoing damage that occurs during chronic allergic conditions.

In individuals with persistent inflammation resulting from the late-phase reaction, such as those with chronic asthma, tissue remodeling can result. This involves structural changes, such as thickening of smooth muscle, as well as changes in connective tissue, blood and lymphatic vessels, mucus glands, and nerves.[3,4]

Genetic and Environmental Influences on Type I Hypersensitivity

The development of IgE responses and allergy appears to depend on complex interactions between genetic factors and environmental triggers. Several hundred genes associated with susceptibility to developing allergies have been identified.[11-13] These genes affect different aspects of the immune response that contribute to the pathogenesis of the type I hypersensitivity response.[4,12,13] Some of these genes affect the structure of the epithelium lining in places where allergens can enter the body, such as the skin, gastrointestinal tract, and respiratory tract. Certain polymorphisms in these genes can result in altered ability of the body's protective barriers to prevent penetration of microbes and potential allergens. Another group of genes play a role in recognition of the antigen by the innate immune system once it has entered through the epithelial barriers. Defects in these genes, which code for pattern recognition receptors such as CD14 and the Toll-like receptors (TLRs), can affect cell interactions with antigens in the initial phases of immune defense. A third group of genes can influence susceptibility to allergic disorders by affecting aspects of the adaptive immune response, such as cytokine production and the ability of T cells to differentiate into Th1 cells, Th2 cells, and T regulatory cells. Aberrations in these genes can result in dysregulation of the immune response, inducing production of cytokines that promote IgE synthesis, such as IL-4 and IL-13. In addition, allergy and asthma appear to be associated with certain HLA class II genes.[13,14] The HLA-D molecules coded for by these histocompatibility genes are known to play a role in antigen presentation and may influence the tendency to respond to specific allergens (see Chapter 3). Finally, genes that play a role in modulating the inflammatory response can influence the long-term consequences of allergies by affecting the process of tissue remodeling and repair.

Many environmental influences on the allergic response have also been identified. Exposure to infectious organisms appears to play a key role in the development of allergic disease. The increased prevalence of allergy in industrialized regions may be due, in part, to increased hygiene practices and use of antibiotics, with a consequent decrease in exposure to microbes.[4] This, in turn, could have significant effects on the immune system by altering the microbial constituent of the gut. Multiple studies in Europe, the United States, and South America have provided evidence for a protective "farm effect."[15] These studies indicate that in utero or early life exposure to the

diverse microbial populations in a farming environment provides protection against allergies by inducing development of regulatory T cells and by directing the immune system toward beneficial Th1 responses and away from Th2 atopic reactions.[15,16] In addition, exposure to stress, variations in physical factors such as temperature, and contact with environmental pollutants such as cigarette smoke and diesel exhaust fumes can intensify clinical manifestations of allergy in susceptible individuals.[4]

Clinical Manifestations of Type I Hypersensitivity

Clinical manifestations of type I hypersensitivity, or anaphylactic hypersensitivity, are common. The prevalence of allergic diseases has increased greatly in developed countries in the last 50 years, and it is estimated that 40% of the world's population has allergic sensitization to common environmental antigens such as pollen or peanuts.[17] Millions of people are affected in the United States alone, where allergies are the fifth leading cause of chronic disease in all age-groups as well as the third leading cause of chronic disease in children.[18]

The clinical manifestations caused by release of inflammatory mediators from mast cells and basophils vary from a localized skin reaction to a severe systemic response known as **anaphylaxis.** Symptoms depend on such variables as route of antigen exposure, dose of allergen, and frequency of exposure. If an allergen is inhaled, it is most likely to cause respiratory symptoms such as asthma or rhinitis. Ingestion of an allergen may result in gastrointestinal symptoms, whereas injection into the bloodstream can trigger a systemic response.

Rhinitis is the most common form of atopy, or allergy; it affects between 10% and 30% of populations worldwide.[17] Symptoms include paroxysmal sneezing; rhinorrhea, or runny nose; nasal congestion; and itching of the nose and eyes.[4,10] Although the condition itself is merely annoying, complications such as sinusitis, otitis media (ear infection), eustachian tube dysfunction, and sleep disturbances may result. Pollen, mold spores, animal dander, and particulate matter from house dust mites are examples of airborne foreign particles that act directly on the mast cells in the conjunctiva and respiratory mucous membranes to trigger rhinitis. Seasonal allergic rhinitis, triggered by tree and grass pollens in the air during the spring in temperate climates, is called "hay fever."

Asthma, derived from the Greek word for "panting" or "breathlessness," is caused by inhalation of small particles such as pollen, dust, or fumes that reach the lower respiratory tract.[1,4,10] It can be defined clinically as recurrent airflow obstruction that leads to intermittent sneezing, breathlessness, and, occasionally, a cough with sputum production. The airflow obstruction is caused by bronchial smooth muscle contraction, mucosal edema, and heavy mucus secretion. All of these changes lead to an increase in airway resistance, making it difficult for inspired air to leave the lungs. This trapped air creates the sense of breathlessness.

Food allergies are another example of type I immediate hypersensitivity reactions. Some of the most common food allergies are caused by cow's milk, eggs, nuts, soy, wheat, fish, and shellfish.[4,10] Symptoms limited to the gastrointestinal tract include cramping, vomiting, and diarrhea, whereas spread of antigen through the bloodstream may cause hives and angioedema on the skin, asthma, rhinitis, or anaphylaxis (see the text that follows).

Local inflammation of the skin, or dermatitis, can also be caused by type I immediate hypersensitivity reactions. These reactions manifest as either acute urticaria or eczema.[1,4,10] Urticaria, or hives, appear within minutes after exposure to the allergen and are characterized by severe itching, erythema (redness) caused by local vasodilation, leakage of fluid into the surrounding area, and a spreading area of redness around the center of the lesion **(Fig. 14–3).** Commonly called a wheal-and-flare reaction, this reaction is caused by release of vasoactive mediators from mast cells in the skin following contact with allergens such as pet dander or insect venom. When these reactions occur deeper in the dermal tissues, they are known as angioedema **(Fig. 14–4).** Urticaria can also appear as a result of other clinical manifestations such as anaphylaxis and food allergies. Atopic eczema can take on a variety of forms, from erythematous, oozing vesicles to thickened, scaly skin, depending on the stage of activity and age of the individual. It is a

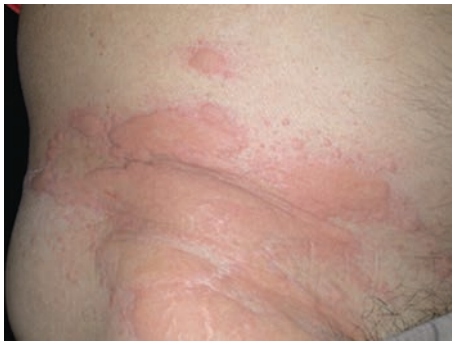


FIGURE 14–3 Urticaria (hives) caused by an immediate hypersensitivity reaction to a medication. *(From Barankin B, Freiman A.* Derm Notes. *Philadelphia, PA: F.A. Davis; 2006, with permission.)*

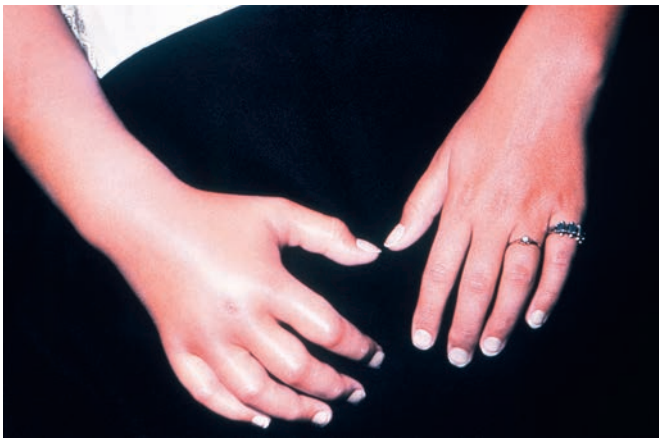


FIGURE 14–4 Angioedema caused by a yellow jacket sting on the right hand just above the middle finger. *(Courtesy of CDC/Margaret A. Parsons, Public Health Image Library.)*

chronic, itchy skin rash that usually develops during infancy, persists during childhood, and is strongly associated with allergic rhinitis and asthma.

Anaphylaxis is the most severe type of allergic response because it is an acute reaction that simultaneously involves multiple organs. It may be fatal if not treated promptly. Coined by biologists Paul Portier and Charles Richet in 1902, the term literally means "without protection." Anaphylactic reactions are typically triggered by glycoproteins or large polypeptides. Smaller molecules, such as penicillin, can trigger anaphylaxis by acting as haptens that may become immunogenic by combining with host cells or proteins. Typical agents that induce anaphylaxis include venom from bees, wasps, and hornets; drugs such as penicillin; and foods such as shellfish, peanuts, and dairy products.[1,4,10] Clinical signs of anaphylaxis begin within minutes after antigenic challenge and may include bronchospasm and laryngeal edema, vascular congestion, skin manifestations such as urticaria (hives) and angioedema, diarrhea or vomiting, and intractable shock because of the effect on blood vessels and smooth muscle of the circulatory system.[1,4,10] The severity of the reaction depends on the number of previous exposures to the antigen. This is because multiple exposures result in additional accumulation of IgE on the surface of the mast cells and basophils. Massive release of reactants, especially histamine, from the granules is responsible for the ensuing symptoms. Death may result from asphyxiation because of upper-airway edema and congestion, irreversible shock, or a combination of these symptoms.

Latex sensitivity has been a significant problem since the late 1980s after implementation of Universal Precautions by the Centers for Disease Control and Prevention and Occupational Safety and Health regulations requiring health-care workers to wear gloves when performing laboratory procedures and working with patients.[19,20] Reactions to antigens in natural rubber latex include type I hypersensitivity and contact dermatitis caused by skin irritation or type IV hypersensitivity.[19,20] Type I hypersensitivity reactions include urticaria, rhinoconjunctivitis, asthma, angioedema, and anaphylaxis. Sensitization to latex can occur as a result of direct skin contact or inhalation of airborne latex particles released when gloves are donned and removed. The risk of the latter occurring is increased when cornstarch powder is used in gloves because residual latex proteins can bind to the powder particles. Groups at particular risk for latex allergy include health-care workers; rubber industry workers; patients who have had multiple surgeries, such as children with spina bifida; and atopic individuals, particularly those who are allergic to certain foods that cross-react with latex allergens.[19,20] Prevalence of latex allergies in the general population is estimated to be 5% to 10%, whereas incidence is thought to range from 10% to 17% in health-care workers and more than 60% in patients who have undergone multiple surgeries early in life.[19] The incidence of latex sensitization has decreased in recent years to as low as 1% in countries where policies to avoid contact with latex have been implemented. These policies may include use of low-protein, powder-free gloves or gloves made from non-latex materials such as nitrile, neoprene, vinyl, or synthetic polyisoprene rubber.[19]

Treatment of Type I Hypersensitivity

Avoidance of known allergens is the first line of defense. Individuals can employ environmental interventions such as encasing mattresses and pillows in allergen-proof covers and removing a harmful food from the diet.[4] However, it is not always possible to completely eliminate contact with allergens. In these cases, pharmacological therapy is necessary to relieve acute symptoms, control chronic allergy manifestations, and, in some cases, modulate the immune response to the allergen.[4] Drugs used to treat immediate hypersensitivity vary with the severity of the reaction. Localized allergic reactions, such as hay fever, hives, or rhinitis, can be treated with antihistamines and decongestants. Asthma is often treated with a combination of therapeutic reagents, including antihistamines and bronchodilators. In cases of persistent asthma, leukotriene receptor antagonists and mast cell stabilizers are also used; in severe cases, corticosteroids can be added to block recruitment of inflammatory cells and their ability to cause tissue damage.[4] Systemic anaphylaxis is a medical emergency that requires timely injection of epinephrine, a powerful vasoconstrictor, to quickly reverse symptoms that could potentially be fatal.[4]

Another treatment approach is aimed at modulating the type I hypersensitivity response through use of an anti-IgE monoclonal antibody called omalizumab. Omalizumab is a recombinant humanized antibody that is composed of human IgG framework genes recombined with complementarity-determining region genes from mouse anti-human IgE. This antibody binds to the Cε3 domain of human IgE, which is the site that IgE normally uses to bind to FcεRI receptors.[4,21] Blocking of this site prevents circulating IgE from binding to mast cells and basophils and sensitizing them. In addition, treatment with omalizumab has been shown to downregulate cellular expression of FcεRI receptors.[21] Omalizumab has been used successfully to treat patients with moderate to severe asthma when added to conventional drug therapy. Its effectiveness for other allergic disorders is also being studied.[22]

If environmental control measures and pharmacotherapy are not successful in managing the symptoms in an individual with allergies, **allergy immunotherapy (AIT)** may be considered. The goal of AIT is to induce immune tolerance to a specific allergen by administering gradually increasing doses of the allergen over time.[4] This therapy is believed to shift the patient's immune response to the allergen to a Th1-type of response and to induce the development of T regulatory cells (Tregs) that release IL-10. This cytokine redirects the immune system to produce allergen-specific IgG4 "blocking" antibodies that combine with the antigen before it can attach to IgE-coated cells and trigger degranulation.[23,24]

Connections

Immune Tolerance

As we will discuss in Chapter 15, immune tolerance is defined as a state of immune unresponsiveness directed against a specific antigen. Development of immune tolerance to an allergen means that the type I hypersensitivity response to the allergen is inhibited. This is achieved by AIT.

The standard practice for AIT has been to administer allergens subcutaneously (i.e., under the skin) over 3 to 5 years. This practice has been shown to significantly reduce symptoms in patients with allergic rhinitis or asthma; however, it has the potential to induce anaphylaxis and must be administered in a physician's office.[25] More recently, other routes of administration that pose decreased risk of severe adverse reactions have been used, namely oral and sublingual (placement of allergen extract under the tongue). These methods of delivery have been shown to reduce symptoms associated with allergic asthma and rhinitis and to significantly decrease or eliminate allergic reactions to certain food allergens such as peanuts.[23,25] Researchers also are investigating the use of purified, recombinant allergens and allergoids, which have been chemically altered to reduce IgE epitopes.[23,24] These approaches may further increase the effectiveness of AIT while decreasing the associated risk of severe reactions.

Testing for Type I Hypersensitivity

Evaluation of patients with an allergy begins with a medical history and physical examination to assess clinical symptoms. These assessments are followed by specific in vivo skin tests and in vitro tests for IgE antibodies to confirm the presence of an allergy and to determine which allergens the patient is sensitized to.

In Vivo Skin Tests

Testing for allergies typically begins with direct skin testing because this procedure is less expensive and more sensitive than serological testing and provides immediate results.[26] Two types of skin tests are used in clinical practice: percutaneous tests (also known as prick or puncture tests) and intradermal tests. Percutaneous tests can detect hypersensitivity to a wide variety of inhaled or food allergens. In these tests, the clinician uses a needle or pricking device to introduce a small drop of allergen extract into the upper layers of the individual's skin in the inner forearm or the back. A panel of allergens is routinely used, with each applied to separate sites 2 to 2.5 cm apart. A negative control consisting of the diluent used for the allergy extract and a positive control of histamine are also included. After 15 to 20 minutes, the clinician examines the testing spots and records the reaction. In a positive test, a wheal-and-flare reaction will appear at the site where the allergen was applied **(Fig. 14–5).** Scoring of the reaction is based on the presence or absence of erythema and the diameter of the wheal, with a diameter larger than 3 to 4 mm correlating best with the presence of allergy.[26-28]

Intradermal tests use a greater amount of antigen and are more sensitive than cutaneous tests. However, they are usually performed only if prick tests are negative and allergy is still suspected because they carry a larger risk (0.05%) for anaphylactic reaction than prick tests (0.03%).[27] In intradermal testing, a 1-mL tuberculin syringe is used to administer 0.01 to 0.05 mL of test solution between layers of the skin. The test allergen is diluted 100 to 1,000 times more than the solution used for cutaneous testing. This test is performed on the inner forearm or upper arm so that if a systemic reaction occurs, a tourniquet can be applied to the arm to help stop the reaction. After 15 to 20 minutes, the site is inspected for erythema and wheal formation, and the wheal diameter is measured to determine a score.[8,26,27] Intradermal tests can be used to test for sensitivity to many allergens but have shown no benefit in the diagnosis of food allergies.[26,27]

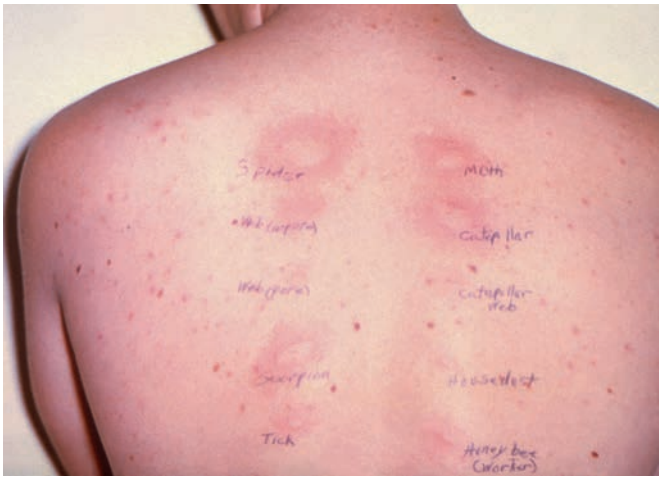


FIGURE 14–5 This individual is undergoing an allergen sensitivity test. The strongest positive reactions are to spider, moth, scorpion, caterpillar, and tick allergens as indicated by the wheal-and-flare reactions at the sites of injection. *(Courtesy of the CDC/Dr. Frank Perlman and M.A. Parsons. Public Health Image Library.)*

Although skin testing is sensitive as well as relatively simple and inexpensive to perform, it has some important limitations.[26,27] Antihistamines must be discontinued a few days before testing because they can decrease or inhibit the skin reaction. Improper technique or use of an inappropriate dilution or improperly stored allergen extract can also lead to false-negative results.[29] False-positive results can also occur; these may be caused by the patient's reaction to the diluent, preservative, or contaminants in the allergen extract or to physical trauma to the skin in patients with severe skin dermatographism or eczema.[29] In addition, there is the danger that a systemic reaction can be triggered. In cases where the risk of a harmful reaction is too large, skin disorders are present, or patients cannot discontinue medications before testing, serological testing for allergen-specific IgE antibodies is indicated.

Allergen-Specific IgE Testing

Allergen-specific IgE tests are safer to perform than skin testing; are easier on some patients, especially children or apprehensive adults; and have excellent analytical sensitivity.[30,31] These tests are useful in detecting allergies to a number of common triggers, including ragweed, trees, grasses, molds, animal dander, foods, and insect venom.

The original commercial testing method for determining specific IgE, the *radioallergosorbent test (RAST),* was introduced in 1972. In this radioimmunoassay, patient serum was incubated with a paper disk to which various allergens were covalently linked. Following a washing step to remove unbound antibody, bound IgE was detected by adding a radiolabeled

anti-IgE. After a second wash step, the amount of radioactivity detected was measured by a gamma counter and was proportional to the amount of allergen-specific IgE in the patient's sample.[30,31]

The principles of current immunoassays for serum IgE remain the same, but the newer testing methods use enzyme labels that react with substrates to produce fluorescence, chemiluminescence, or colorimetric reactions rather than radioactive labels. **Figure 14-6** illustrates the principle of these tests. All of these methods are automated immunoassays that have a high level of specificity and sensitivity. The tests can be run with a single allergen or as a multiallergen screen using a panel of allergens in a single run. A commercial noncompetitive fluoroimmunoassay is considered by most allergy specialists to be the method of choice.[32,33] In this assay, patient serum is incubated with an allergen-coated cellulose sponge that has a high binding capacity for IgE antibody. After a wash step, an enzyme-labeled anti-IgE reagent is added; following incubation, another washing step is performed to remove unbound materials. The corresponding substrate is added, and fluorescence is produced in proportion to the amount of allergen-specific IgE in the sample. The results are derived from a standard calibration curve that is linked to the World Health Organization (WHO) IgE standard. Allergen-specific IgE values are reported in kilo international units (IU) of allergen-specific antibody per liter (kUa/L), where one unit is equal to 2.42 ng/mL of IgE.[30] The method can detect IgE antibodies in the range of 0 to 100 kU/L, and 0.35 kU/L is commonly used as the cut-off for a positive test.[8,34]

Other immunoassays for allergen-specific IgE have comparable sensitivity and specificity, but the results are not interchangeable; they are likely caused by differences in the composition of the allergen reagents.[30] Because crude allergen extracts are typically used in these tests, standardization is difficult and cross-reactivity to similar but clinically insignificant antigens may be detected. This limitation has stimulated the development of recombinant allergen components produced by cloning the genes coding for these proteins and purifying the allergenic substances produced by the genetically modified cells.[30,31] Recombinant allergens are being incorporated into existing assay formats and should significantly increase the diagnostic specificity of allergy testing.

Using advanced biochemical and molecular techniques, scientists have been able to characterize more than 1,780 allergens.[35] This knowledge has led to the development of a microarray format that allows for parallel detection of IgE antibodies to more than 100 potential allergens using only 20 μL of patient serum.[35] In this system, patient serum is incubated with a biochip containing miniature spots to which the purified allergenic components have been applied. The chip contains a wide variety of antigens from foods, pollens, molds, fungi, latex, and insect venoms. An internal control is provided because all allergens are spotted in triplicate. If allergen-specific IgE is present, it will bind to the appropriate spots. The chip is scanned by a laser for fluorescence following addition of a fluorescent-labeled anti-IgE.[32] This advanced technology is highly sensitive and specific. It can theoretically use an unlimited number of natural and recombinant allergens. The technology will significantly enhance the diagnostic value of specific IgE testing.

Another development is a point-of-care lateral flow assay that can be used by primary care physicians to screen for

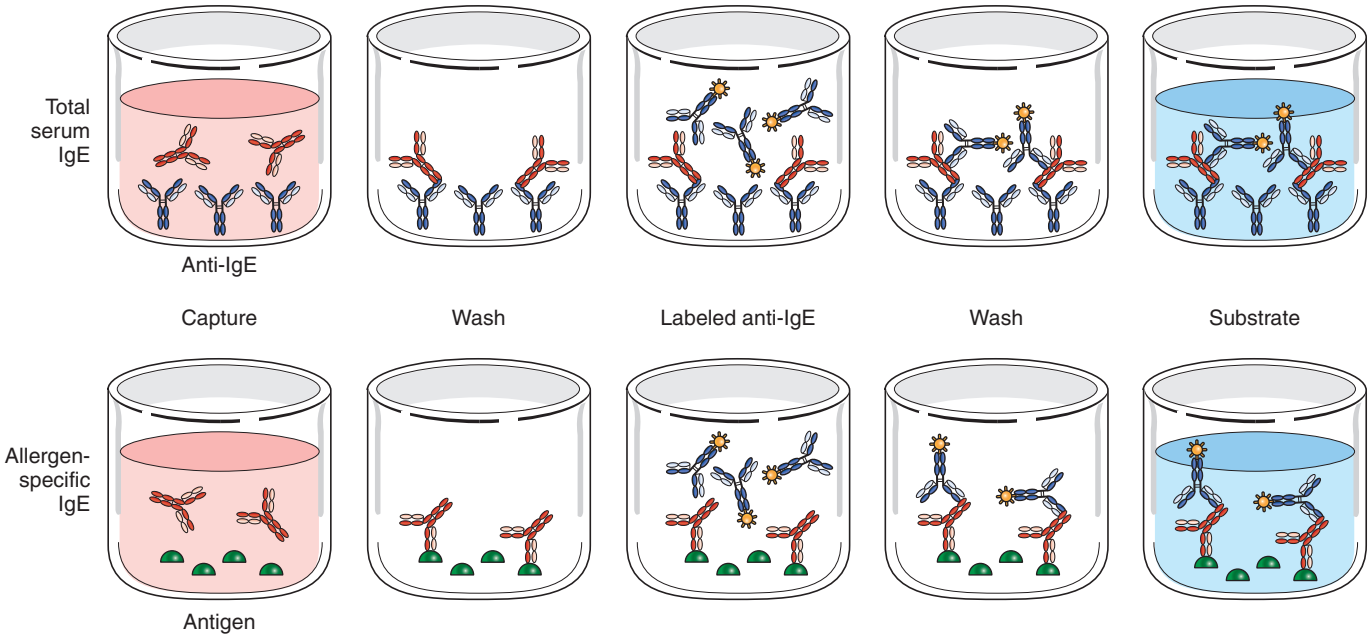


FIGURE 14–6 Comparison of noncompetitive immunoassays for total serum IgE (formerly known as RIST) and allergen-specific IgE (formerly known as RAST). IgE in the patient serum is shown in red for both tests. Total IgE is measured by capturing the antibody with solid-phase anti-IgE. A second anti-IgE immunoglobulin with an enzyme label is used to produce a visible reaction. Antigen-specific IgE is measured by using solid-phase antigen to capture patient antibody. Then a second antibody, enzyme-labeled anti-IgE immunoglobulin, is added. This combines with any bound IgE to produce a visible reaction in the presence of substrate.

reactivity to a few common allergens.[31,32] In this assay, the allergen extracts are coated onto nitrocellulose strips encased in a cassette. A drop of blood from a finger prick is added to one well in the cassette and a color-developing solution is added to a second well, driving the blood toward the antigen zone. In this semiquantitative test, the presence of allergen-specific IgE is indicated by a colored line in the corresponding allergen position. Patients with positive results can be referred to an allergist for further evaluation.

Regardless of the format of the specific IgE test used, the results should always be interpreted in light of the patient's medical history and clinical symptoms. This is because the presence of allergen-specific IgE antibody indicates sensitization to the allergen but not necessarily the presence of a clinical allergy.[30,34]

In Vitro Tests: Total IgE

Tests have also been developed to measure total serum IgE. The first test developed for the measurement of total IgE was the competitive *radioimmunosorbent test (RIST)*. The RIST used radiolabeled IgE to compete with patient IgE for binding sites on a solid phase coated with anti-IgE. Because of the expense and difficulty of working with radioactivity, RIST has largely been replaced by noncompetitive solid-phase immunoassays or nephelometry assays with enhanced sensitivity.[8,28]

In the noncompetitive solid-phase immunoassays, anti-human IgE is bound to a solid phase such as cellulose, a paper disk, or a microtiter well. Patient serum is added and allowed to react and then an enzyme-labeled anti-IgE is added to detect the bound patient IgE. The second anti-IgE antibody recognizes a different epitope than that recognized by the first antibody. The resulting "sandwich" of solid-phase anti-IgE, serum IgE, and labeled anti-IgE is washed; a colorimetric, fluorometric, or chemiluminescent substrate is then added. The amount of reactivity detected is directly proportional to the IgE content of the serum (see Fig. 14–6).[33]

Total IgE values are reported in kilo international units (IU) per liter. One IU is equal to a concentration of 2.4 ng of protein per milliliter. IgE concentration varies with the individual's age and exposure to allergens. The total IgE concentration is typically lower than 1 kU/L in cord blood, and serum IgE usually reaches adult levels at about 10 years of age. In adults, a cutoff value of 100 kU/L is considered the upper limit of normal. Levels above 100 kU/L are common in individuals with allergies.[8] However, measurement of total serum IgE is not recommended for the routine clinical evaluation of patients with suspected allergies[8,26] because values vary widely among patients and do not necessarily correlate with the presence of allergy. Patients with slightly elevated IgE levels may not have an allergy, whereas many patients with allergies have a total serum IgE concentration that falls within the reference range. Thus, allergen-specific IgE tests are considered to have more value in the diagnosis of allergies.

Total serum IgE testing is more beneficial in evaluating patients with other conditions in which IgE levels may be elevated, such as helminth infections, and certain immunodeficiencies, such as Wiskott-Aldrich syndrome, DiGeorge syndrome, and hyper-IgE syndrome.[8] Children living in areas where parasitic infections are endemic typically have serum IgE concentrations greater than 1,000 kU/L, whereas patients with hyper-IgE syndrome have extremely high IgE levels (2,000–50,000 kU/L). Measurement of total serum IgE is also helpful in monitoring patients undergoing allergen immunotherapy or treatment with the monoclonal anti-IgE antibody, omalizumab. Successful treatment results in significant reductions in total serum IgE levels and in the ratio of allergen-specific IgE to total IgE.[31]

Type II Hypersensitivity

Type II hypersensitivity is also known as antibody-mediated cytotoxic hypersensitivity. The underlying mechanism involves IgG and IgM antibodies directed against antigens found on cell surfaces. These antigens may be altered self-antigens or heteroantigens. Binding of the antibody to a cell can have one of three major effects, depending on the situation (see Fig. 14–1): (1) The cell can be destroyed; (2) the function of the cell can be inhibited; or (3) the function of the cell can be increased above normal.

Cell damage can occur by several different mechanisms, some of which involve complement as well as antibodies: (1) Activation of the classical pathway of complement can lead to the formation of the membrane attack complex and cell lysis. (2) Coating of the cell surface by antibodies can promote opsonization and subsequent phagocytosis of the cells. Opsonization can occur either through binding of IgG antibody to Fc receptors on macrophages and neutrophils or binding of cell surface C3b to complement receptors on phagocytic cells. (3) Cell damage can result from the mechanism of antibody-dependent cellular cytotoxicity (ADCC). ADCC is mediated through binding of IgG antibody to its corresponding antigen on the target cell and to Fc receptors on macrophages or natural killer cells. This binding stimulates the release of cytotoxic enzymes that destroy the cell. Clinical examples that involve destruction of cells by type II hypersensitivity include blood transfusion reactions, hemolytic disease of the newborn, and autoimmune hemolytic anemia. These conditions are discussed in the sections that follow.

A second possible effect of type II hypersensitivity is that the cell surface antibody can inhibit the function of a cell. This can occur when antibody blocks the binding of a physiological ligand to its receptor, resulting in dysfunction of the cell. An example of this effect occurs in the autoimmune disease myasthenia gravis, which affects the neuromuscular junctions. Patients with this disease produce autoantibodies to receptors on muscle cells for the neurotransmitter acetylcholine (ACH). Normally, ACH is released from the nerve endings and binds to its corresponding receptors on muscle cells, stimulating contraction in the muscle fibers and muscle movement. However, in myasthenia gravis, attachment of the autoantibody to the ACH receptor blocks the binding of ACH, leading to muscle weakness (see Chapter 15).

Sometimes, binding of an antibody to a self-antigen can have the opposite effect, stimulating the cell instead of inhibiting its function. This results in overproduction of the cell's product, such as a hormone. The classic example of this effect is an autoimmune disorder of the thyroid gland called Graves disease. Patients with Graves disease produce antibodies

against the receptor for the thyroid-stimulating hormone (TSH) on thyroid cells. TSH, a hormone produced by the pituitary gland in the brain, binds to the TSH receptors and stimulates the thyroid cells to produce hormones that increase metabolism. Normally, this process is carefully regulated by a feedback loop that signals the pituitary gland to make less TSH in the presence of high levels of thyroid hormones.[36] However, in Graves disease, the autoantibody binds to the TSH receptor, resulting in unregulated production of thyroid hormones. This leads to symptoms associated with increased metabolism, known as hyperthyroidism (see Chapter 15).

Clinical Examples of Type II Hypersensitivity

Transfusion Reactions

Transfusion reactions are examples of cell destruction that results from antibodies combining with heteroantigens. There are more than 29 different blood group systems with more than 700 different RBC antigens.[37,38] Some antigens are stronger than others and are more likely to stimulate antibody production. Major groups involved in transfusion reactions include the ABO, Rh, Kell, Duffy, and Kidd systems.[37,39] Certain antibodies are produced naturally with no prior exposure to RBCs, whereas other antibodies are produced only after contact with cells carrying that antigen.

The ABO blood group is of primary importance in considering transfusions. Anti-A and anti-B antibodies are naturally occurring antibodies, or **isohemagglutinins,** which are probably triggered by contact with identical antigenic determinants on microorganisms. Individuals do not make these antibodies to their own RBCs. Thus, a person who has type A blood has anti-B in the serum and a person with type B blood has anti-A antibodies. An individual with type O blood has both anti-A and anti-B in the serum because O cells have neither of these two antigens. The antibody formed typically belongs to the IgM class, but IgG may also be made.

If a patient is given blood for which antibodies are already present, a transfusion reaction occurs. This reaction can range from acute massive intravascular hemolysis to an undetected decrease in RBC survival. The extent of the reaction depends on the following factors:

- The temperature at which the antibody is most active
- The plasma concentration of the antibody
- The immunoglobulin class involved
- The extent of complement activation
- The density of the antigen on the RBC
- The number of RBCs transfused[40]

It is most important to detect antibodies that react at 37°C. If a reaction occurs only below 30°C, it can be disregarded because antigen–antibody complexes formed at colder temperatures tend to dissociate at 37°C.

Acute hemolytic transfusion reactions may occur within minutes or hours after receipt of incompatible blood. In this case, the individual has been exposed to the antigen before and has preformed antibodies to it. Reactions that begin immediately are most often associated with ABO blood group incompatibilities and the antibodies are of the IgM class.[1,40,41] As soon as cells bearing the antigen are introduced into the patient, intravascular hemolysis occurs because of complement activation, resulting in the release of hemoglobin and vasoactive and procoagulant substances into the plasma. This may induce disseminated intravascular coagulation (DIC), vascular collapse, and renal failure. Symptoms in the patient may include fever, chills, nausea, lower back pain, tachycardia, shock, and hemoglobin in the urine.[39,40]

Delayed hemolytic reactions occur within the first 2 weeks following a transfusion and are caused by an anamnestic response to the antigen to which the patient has previously been exposed.[41] The type of antibody responsible is IgG, which was initially present in such low titer that it was not detectable with an antibody screen. Antigens most involved in delayed reactions include those in the Rh, Kell, Duffy, and Kidd blood groups.[1,42] Rh, Kell, and Duffy antigens may also be involved in immediate transfusion reactions. In a delayed reaction, antibody-coated RBCs are removed extravascularly in the spleen or in the liver. The patient may experience a mild fever, low hemoglobin, mild jaundice, and anemia. Intravascular hemolysis does not take place to any great extent because IgG is not as efficient as IgM in activating complement. (See Chapter 5 for further details.)

Hemolytic Disease of the Newborn

Hemolytic disease of the newborn (HDN) appears in infants whose mothers have been exposed to blood-group antigens on the baby's cells that differ from their own. The mother makes IgG antibodies in response to these antigens that cross the placenta to destroy the fetal RBCs. Severe HDN is called *erythroblastosis fetalis.* The most common antigen involved in severe reactions is the D antigen, a member of the Rh blood group. HDN caused by ABO incompatibility is actually more common; however, the disease is milder, possibly because the antibodies are neutralized by A or B antigens found in fetal tissues or because the A and B antigens on the fetus' RBCs are more poorly developed or reduced in number.[43] Other antibodies associated with HDN include anti-c, anti-C, anti-E, anti-e, and less commonly those associated with the Kell, Duffy, and Kidd blood groups.[44]

Exposure usually occurs during the birth process when fetal cells leak into the mother's circulation. Typically, the first child is unaffected; however, the second and later children have an increased risk of the disease because of an anamnestic response. The extent of the first fetal–maternal bleed influences whether antibodies will be produced. If enough of the baby's RBCs enter the mother's circulation, memory B cells develop. These become activated upon re-exposure to the same RBC antigen; IgG is then produced. This antibody crosses the placenta and attaches to the fetal RBCs in a subsequent pregnancy.

Depending on the degree of antibody production in the mother, the fetus may be aborted, stillborn, or born with evidence of hemolytic disease as indicated by jaundice. As RBCs are lysed and free hemoglobin released, this is converted to bilirubin, which builds up in the plasma. There is too much of it to be conjugated in the liver, so it accumulates in the tissues. Bilirubin levels above 20 mg/dL are associated

with deposition in tissues such as the brain and result in a condition known as kernicterus. Treatment for severe HDN involves an exchange transfusion to replace antibody-coated RBCs. If serum antibody titrations during the pregnancy indicate a high level of circulating antibody, intrauterine transfusions can be performed.[1,43,44]

To prevent the consequences of HDN, all women should be screened at the onset of pregnancy. If they are Rh-negative, they should be tested for the presence of anti-D antibodies on a monthly basis. In current practice, anti-D immune globulin, called Rhogam, is administered prophylactically at 28 weeks of gestation and within 72 hours following delivery.[43] The mechanism by which Rhogam works is not completely known, but it is thought to facilitate clearance of the fetal RBCs through opsonization and therefore suppress production of maternal antibody **(Fig. 14–7).**[44] This practice has dramatically reduced the number of women who form anti-D antibodies to slightly over 1%.[45]

Autoimmune Hemolytic Anemia

Autoimmune hemolytic anemia is an example of a type II hypersensitivity reaction directed against self-antigens because individuals with this disease form antibodies to their own RBCs. Symptoms include malaise, lightheadedness, weakness, unexplained fever, pallor, and possibly mild jaundice.[46] Such antibodies can be categorized into two groups: warm reactive antibodies, which react at 37°C, and cold reactive antibodies, which only react below 30°C. Autoimmune hemolytic anemia has been estimated to occur in 1 in 50,000 to 80,000 individuals.[42]

Warm autoimmune hemolytic anemia, which accounts for more than 70% of autoimmune anemias, is characterized by formation of IgG antibody; this reacts most strongly at 37°C.[42,46] Some of these antibodies may be primary with no other disease association; others may be secondary to another disease process. Associated diseases may include viral or respiratory infections—such as infectious mononucleosis, cytomegalovirus, or chronic active hepatitis—or immunoproliferative diseases—such as chronic lymphocytic leukemia and lymphomas.[46,47] Often, the underlying cause of antibody production is unknown; this is referred to as *idiopathic autoimmune hemolytic anemia.*

In addition, certain drugs can induce production of antibodies that can cause hemolytic anemia. These drugs are capable of attaching to the RBCs directly or of forming immune complexes that attach to the RBCs. Damage to the RBCs is believed to occur through several mechanisms.[42,46] Some drugs, such as the penicillins and cephalosporins, can act as haptens after binding to proteins on the RBC membrane. These drugs stimulate the production of anti-drug antibodies that destroy the RBCs, primarily through extravascular hemolysis. The cephalosporins are also thought to modify the RBC membrane by facilitating binding of immunoglobulins and complement. Other drugs, such as quinidine and phenacetin, can stimulate the production of anti-drug antibodies that bind to the drug to form soluble immune complexes. The complexes attach loosely to the surface of the RBCs, which are cleared by intravascular hemolysis after binding of complement. Other drugs, such as methyldopa, can induce hemolytic anemia by

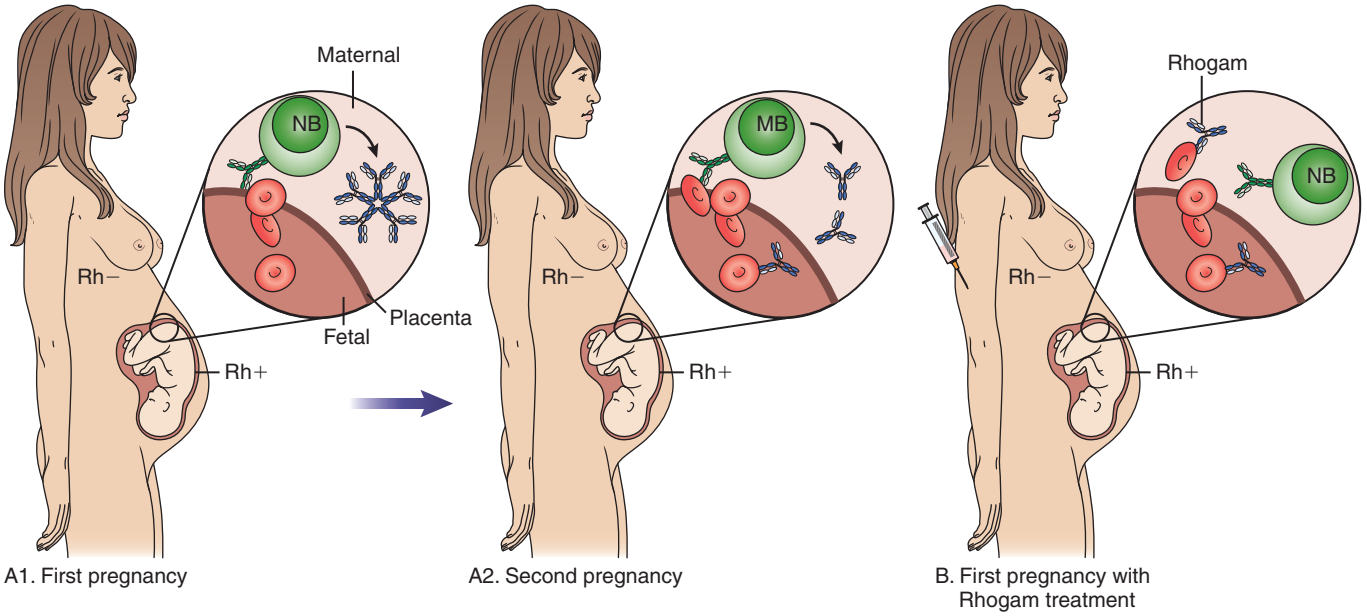


FIGURE 14–7 Hemolytic disease of the newborn (HDN) can develop in Rh-positive babies who are born to Rh-negative mothers if no treatment is administered. (A1) An Rh-negative mother may produce anti-Rh antibodies if an Rh-positive baby's RBCs enter her circulation late in gestation or during the birth process. In this primary immune response, naïve B cells (NB) differentiate and the plasma cells secrete mainly anti-Rh IgM, which does not cross the placenta; therefore, a healthy baby is delivered. However, memory B cells (MB) are also produced and (A2) if the mother becomes pregnant with another Rh-positive baby, these memory cells produce a stronger secondary immune response and secrete mainly anti-Rh IgG, which crosses the placenta and destroys the baby's RBCs, resulting in HDN. (B) Alternatively, passive vaccination of the mother with an anti-Rh IgG called Rhogam during pregnancy and upon delivery prevents the mother from mounting an active immune response, allowing her future Rh-positive children to be born normally.

stimulating production of autoantibodies against the RBC membrane.

Typically, patients exhibit symptoms of anemia because of clearance of antibody-coated RBCs by macrophages in the liver and spleen. Hemolysis is primarily extravascular because IgG is not as efficient as IgM in activating complement; however, intravascular hemolysis can also occur if complement does become activated. Severity of the hemolysis is affected by the subclass of IgG involved, with IgG3 and IgG1 being most destructive to the RBCs because they are efficient at binding complement.[46] Patients with warm autoimmune hemolytic anemia are usually treated with corticosteroids to reduce antibody synthesis or, in more serious cases, with a splenectomy to decrease RBC clearance.[46,47] Treatment with anti-CD 20 (rituximab) can be used for cases that are refractory to corticosteroids.[47,48] This antibody attaches to B cells and causes a decrease in antibody production.

Cold agglutinins are a less frequent cause of immune hemolytic anemias. By definition, cold agglutinins are autoantibodies that react with antigens on the RBC membrane at cold temperatures.[49] The reaction is reversible upon exposure to a warm temperature. These cold-reacting antibodies belong to the IgM class and most are specific for the Ii blood groups on RBCs.[42,46,47] Cold agglutinins may be transient or chronic, depending on the cause. Polyclonal cold agglutinins can be produced secondary to certain infections, most notably *Mycoplasma* pneumonia and infectious mononucleosis but also respiratory viruses and HIV.[47,48,50] In most cases, cold agglutinin production is transient and resolves in 2 to 3 weeks.[46] Persistent, high titer, monoclonal cold agglutinins have been associated with B- or plasma cell-lymphoproliferative disorders including B-cell chronic lymphocytic leukemia (CLL), B-cell lymphomas, Hodgkin disease, and Waldenstrom's macroglobulinemia as well as autoimmune diseases such as systemic lupus erythematosus (SLE). Chronic cold agglutinins can also be produced as a primary characteristic of a disease entity of unknown origin, which is known as chronic cold agglutinin disease (CCAD) or chronic hemagglutinin disease (CHD).[46,49] CHD typically occurs in persons over the age of 50 and is responsible for about 20% of autoimmune hemolytic anemia cases.[42,46]

Cold agglutinins do not cause clinical symptoms unless the individual is exposed to the cold and usually have their maximal effect when the temperature in the peripheral circulation falls below 30°C.[42,50] Under these conditions, the antibodies can bind to the RBCs to form lattices, which can obstruct the small capillaries in the skin.[49] The areas of the body most affected are those having greatest exposure to the cold, most notably the fingers, toes, earlobes, and nose. These areas develop a blue coloration known as acrocyanosis and become numb, stiff, and slightly painful.[50] The symptoms are quickly reversible when the patient returns to warm surroundings; however, in severe cases, peripheral necrosis may result.

Another consequence of cold agglutinins results from the fixation of complement. Although complement activation cannot be completed in the cold, it can proceed once the cells recirculate and reach body temperature. If the RBCs become coated with C3b, opsonization can facilitate binding to macrophages and rapid clearance of the cells in the liver. Less commonly, the entire classical pathway is activated and intravascular hemolysis occurs.[42] Both processes lead to destruction of the RBCs and corresponding symptoms of autoimmune hemolytic anemia.[46] Patients with this disease are usually treated by simply avoiding cold temperatures and keeping the extremities warm; drug therapy is only used if these measures are ineffective.[46,50]

A rare condition known as **paroxysmal cold hemoglobinuria** can also cause autoimmune hemolytic anemia. This condition occurs most often after infection with certain viral illnesses, including measles, mumps, chickenpox, and infectious mononucleosis. Patients produce a biphasic autoantibody that binds to the RBCs at cold temperatures and activates complement at 37°C to produce an intermittent hemolysis. Consequently, an acute, rapidly progressing anemia with hemoglobin in the urine is seen. The condition occurs most often as a transient disorder in children and young adults.[42,46]

Type II Reactions Involving Tissue Antigens

All the reactions that have been discussed so far deal with individual cells that are destroyed when a specific antigen–antibody combination takes place. Some type II reactions involve destruction of tissues because of their combination with antibody. Organ-specific autoimmune diseases in which antibody is directed against a particular tissue are in this category. Goodpasture's syndrome is an example of such a disease (see Chapter 15 for details). The antibody produced during the course of this disease reacts with basement membrane protein. Usually the glomeruli in the kidney and pulmonary alveolar membranes are affected.[1] Antibody binds to glomerular and alveolar capillaries; this triggers the complement cascade, which provokes inflammation. An evenly bound linear deposition of IgG in the glomerular basement membrane, which is detected with fluorescent-labeled anti-IgG, is indicative of Goodpasture's syndrome. Treatment usually involves the use of corticosteroids or other drugs to suppress the immune response.

Other examples of type II hypersensitivity reactions to tissue antigens include some of the organ-specific autoimmune diseases such as Hashimoto's disease, myasthenia gravis, and insulin-dependent diabetes mellitus. Immunologic manifestations and detection of these diseases are presented in Chapter 15.

Testing for Type II Hypersensitivity

Coombs' discovery of the antiglobulin test in 1945 made possible the detection of antibody or complement on RBCs. The **direct antiglobulin test (DAT)** is performed to detect transfusion reactions, HDN, and autoimmune hemolytic anemia. (Refer to the exercise in this text's Davis*Plus* website at davisplus.fadavis.com keyword Stevens for details.) Polyspecific anti-human globulin, which is a mixture of antibodies to IgG and complement components such as C3b and C3d, is used for initial testing. If the test is positive, it should be repeated using monospecific anti-IgG, anti-C3b, and anti-C3d to determine which of these is present.[42] If an autoimmune hemolytic anemia is caused by IgM antibody, only the tests for complement components would be positive.

The indirect Coombs' test is used in the crossmatching of blood to prevent a transfusion reaction. It can either determine the presence of a particular antibody in a patient or type patient RBCs for specific blood group antigens. The method detects in vitro binding of antibody to RBCs rather than in vivo binding. This method is a two-step process in which RBCs and antibody are allowed to combine at 37°C and then the cells are carefully washed to remove any unbound antibody. Anti-human globulin is added to cause a visible reaction if antibody has been specifically bound. Any negative tests are confirmed by quality-control cells, which are coated with antibody.

To determine the titer of a cold agglutinin antibody, the patient serum can be serially diluted and incubated overnight at 4°C with a dilute suspension of washed human type O RBCs.[49] The tubes are then gently shaken and observed for agglutination. The last tube with agglutination represents the titer. Titers of 64 or higher are considered to be clinically significant.[42,49] The agglutination should disappear after warming the tubes briefly in a 37°C water bath. Before testing, it is important to use prewarmed blood to separate the serum or plasma from the patient's RBCs.[49] Failure to do so can result in binding of the cold agglutinins to the patient's own RBCs, producing false-negative results when the patient's serum is assayed for cold agglutinin reactivity against the reagent type O cells.

Type III Hypersensitivity

Type III hypersensitivity reactions are similar to type II reactions in that IgG or IgM is involved and destruction is complement-mediated. However, in the case of type III-associated diseases, the antigen is soluble. When soluble antigen combines with antibody, complexes are formed that precipitate out of the serum. Normally such complexes are cleared by phagocytic cells; however, if the immune system is overwhelmed, these complexes deposit in the tissues. There, they bind complement, causing damage to the particular tissue. Deposition of antigen–antibody complexes is influenced by the relative concentration of both components. If a large excess of antigen is present, sites on antibody molecules become filled before cross-links can be formed. In antibody excess a lattice cannot be formed because of the relative scarcity of antigenic determinant sites (see Fig. 10–3). The small complexes that result in either of the preceding cases remain suspended or may pass directly into the urine. Precipitating complexes, on the other hand, occur in mild antigen excess and are the ones most likely to deposit in the tissues. Sites in which this typically occurs include the glomerular basement membrane, vascular endothelium, joint linings, and pulmonary alveolar membranes.[1]

Complement binds to these complexes in the tissues, causing the release of mediators that increase vasodilation and vasopermeability, attract macrophages and neutrophils, and enhance binding of phagocytic cells by C3b-mediated opsonization. If the target cells are large and cannot be engulfed for phagocytosis to take place, granule and lysosome contents are released by a process known as *exocytosis*. This process results in the damage to host tissue that is typified by type III reactions. Long-term changes include loss of tissue elements that cannot regenerate and accumulation of scar tissue. Type III hypersensitivity reactions can be local or systemic, depending on where the immune complexes deposit in the body.

Arthus Reaction

The classic example of a localized type III reaction is the **Arthus reaction**, demonstrated by Maurice Arthus in 1903. Using rabbits that had been immunized to produce an abundance of circulating antibodies, Arthus showed that when these rabbits were challenged with an intradermal injection of the antigen, a localized inflammatory reaction resulted. The reaction which is characterized by erythema and edema, peaks within 3 to 8 hours and is followed by a hemorrhagic necrotic lesion that may ulcerate.[1] The inflammatory response is caused by an antigen–antibody combination and subsequent formation of immune complexes that deposit in small dermal blood vessels[1,51] **(Fig. 14–8).** Complement is fixed, attracting neutrophils and causing aggregation of platelets. Neutrophils release toxic products such as oxygen-containing free radicals and proteolytic enzymes. Activation of complement is essential for the Arthus reaction because the C3a and C5a that are

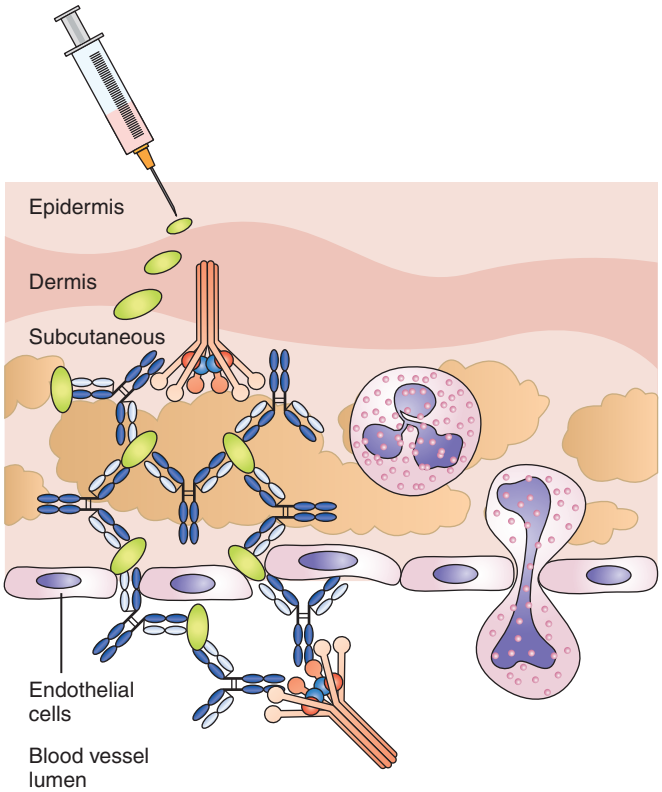


FIGURE 14–8 The Arthus phenomenon. Antigen is injected into the skin of an individual who has circulating antibody of that specificity. Immune complexes are formed and deposit on the walls of blood vessels, activating complement. Complement-generated anaphylatoxins cause vasodilation, increased vascular permeability, edema, and accumulation of neutrophils.

generated activate mast cells to release permeability factors; consequently, immune complexes localize along the endothelial cell basement membrane. The Arthus reaction can sometimes be seen in humans following booster injections with tetanus, diphtheria, or measles vaccines.[52]

Serum Sickness

Serum sickness is a generalized type III hypersensitivity reaction that results from passive immunization of humans with animal serum. Before the advent of antibiotics and vaccines, serum sickness was observed more often because horse antiserums were used to treat infections such as diphtheria, tetanus, and pneumonia.[51] These antisera were produced by immunizing horses with the corresponding antigen and provided immediate immunity to the individuals receiving them. Today, horse antivenoms are still used to treat people who have been bitten by poisonous snakes. Serum sickness can also occur after treatment of patients with mouse monoclonal antibodies for diseases such as cancer or autoimmune disorders.[1,51] Patients exposed to such animal sera can produce antibodies against the foreign animal proteins. These can combine with their corresponding antigen to form immune complexes that can deposit in the tissues and trigger the type III hypersensitivity response.

Generalized symptoms of serum sickness appear 7 to 21 days after injection of the animal serum and include headache, fever, nausea, vomiting, joint pain, rashes, and lymphadenopathy.[1] Usually this is a self-limiting disease and recovery takes a few weeks after the offending antigen is eliminated.[51] However, previous exposure to animal serum can result in a more rapid reaction with increased severity.[1]

Autoimmune Diseases and Other Causes of Type III Hypersensitivity

Type III hypersensitivity reactions can also be triggered by autologous antigens as seen in several of the autoimmune diseases. SLE and rheumatoid arthritis (RA) are two such examples. Patients with these diseases commonly produce antibodies against nuclear constituents such as DNA and histones. They may also produce an antibody against IgG called *rheumatoid factor.* The autoantibodies combine with their corresponding antigen to produce immune complexes that trigger the type III hypersensitivity response. In SLE, immune complex deposition involves multiple organs; however, the main damage occurs to the joints, skin, and glomerular basement membrane in the kidneys. In rheumatoid arthritis, immune complexes primarily cause damage to the joints. Complement enhances tissue destruction in both diseases. See Chapter 15 for a more detailed discussion of these two conditions.

Type III hypersensitivity can also be caused by a number of other factors.[1,51] These include components of vaccines, bee stings, treatment with certain drugs (for example, penicillin and sulfonamides), and infections, such as viral hepatitis and Group A Streptococcus.

Testing for Type III Hypersensitivity

In autoimmune diseases such as SLE and RA, the presence of antinuclear antibodies can be detected by a variety of methods, including indirect immunofluorescence, enzyme-linked immunosorbent assay, and fluorescent microsphere multiplex immunoassays (see Chapter 15 for details). Fluorescent staining of tissue sections has also been used to determine deposition of immune complexes in the tissues. The staining pattern seen and the particular tissue affected help to identify the disease and determine its severity. Rheumatoid factor can be detected by latex agglutination, nephelometry, or other immunoassays (see Chapter 15).

A more general method of evaluating immune complex diseases is by measuring complement levels. During periods of high disease activity, complement levels in the serum may be decreased because of binding of some of the complement to the antigen–antibody complexes. The results should be interpreted in conjunction with other clinical findings. Refer to Chapter 7 for a discussion of complement testing.

Type IV Hypersensitivity

Type IV hypersensitivity was first described in 1890 by Robert Koch. He observed that individuals infected with *Mycobacterium tuberculosis* (Mtb) developed a localized inflammatory response after receiving intradermal injections of a filtrate from the organism.[1] Type IV hypersensitivity differs from the other three types of hypersensitivity in that sensitized T cells, rather than antibodies, play the major role in its manifestations. Because symptoms peak between 48 to 72 hours after exposure to antigen, this reaction is also known as delayed hypersensitivity. Although there are different mechanisms involved in type IV hypersensitivity, the classic type IV response involves the Th1 subclass of T helper (Th) cells (see Fig. 14–1). There is an initial sensitization phase of 1 to 2 weeks that takes place after the first contact with antigen. During this phase, Langerhans cells in the skin and macrophages in the tissues capture antigen and migrate to nearby lymph nodes, where they present the antigen to naïve Th cells. The antigen-presenting cells (APCs) also release cytokines that promote differentiation of the naïve T cells into Th1 cells and other T-cell subsets and induce their proliferation. The expanded Th1 cells then migrate to the site where the antigen is located and the effector phase of the response begins. At the site, the activated Th1 cells release cytokines. IL-3 and GM-CSF induce hematopoiesis of cells of the granulocyte-macrophage lineage and chemokines such as monocyte chemotactic protein (MCP-1/CCL2) recruit macrophages to the site. In the tissues, the monocytes differentiate into macrophages and are activated by IFN-γ and TNF-β These activated macrophages release reactive oxygen species, nitric oxide, and proinflammatory mediators that recruit more macrophages to the site and stimulate them to become effective APCs, thus perpetuating the response.[1,51,53,54]

Chronic persistence of antigen leads to the development of organized clusters of cells called **granulomas,** which consist

of epithelioid-shaped and multinucleated fused macrophages with an infiltrate of lymphocytes or other WBCs. Many of these macrophages contain engulfed antigens such as intracellular bacteria. Thus, the granuloma can function to wall off the organism in a contained area, preventing its spread to other parts of the body. However, cells in the granuloma can release large amounts of lytic enzymes that can destroy surrounding tissue and promote fibrin deposition.[1] Cytotoxic T cells are also recruited and they bind with antigen-coated target cells to cause further tissue destruction.[51]

The antigens that can trigger the type IV hypersensitivity are generally one of two types: intracellular pathogens or contact antigens. The intracellular pathogens can be bacteria, fungi, parasites, or viruses. Pathogens that commonly induce delayed hypersensitivity include *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Pneumocystis carinii*, *Leishmania* species, and herpes simplex virus.[1] Contact antigens are those that come into direct contact with the skin. They include plants such as poison ivy and poison oak, metals such as nickel salts, and components of hair dyes and cosmetics.[1,55] Clinical manifestations of type IV hypersensitivity induced by these antigens are discussed in the sections that follow.

Contact Dermatitis

Contact dermatitis is among the most prevalent skin disorders, affecting an estimated 15% to 20% of the general population.[53,55] Reactions are usually caused by low-molecular-weight compounds that touch the skin. The most common causes include poison ivy, poison oak, and poison sumac, all of which release the chemical urushiol in the plant sap and on the leaves. Allergic dermatitis caused by contact with these plants affects millions of Americans every year.[56] Other common compounds that produce allergic skin manifestations include nickel; rubber; formaldehyde; hair dyes and fabric finishes; cosmetics; and medications applied to the skin, such as topical anesthetics, antiseptics, and antibiotics.[55,57,58] In addition, latex sensitization has been reported as a cause of contact dermatitis in a significant number of health-care workers (see the previous text). Most of these substances probably function as haptens that bind to glycoproteins on skin cells. Langerhans cells in the skin are thought to process the hapten–protein complexes and migrate to the regional lymph nodes where they present the antigen to Th1 cells.[53,54] Sensitization of the Th cells takes several days; however, once it occurs, its effects can last for years because of immunologic memory.[1] Cytokine production by the Th1 cells causes macrophages to accumulate and release cytokines and other substances that produce a local inflammatory response.

Contact dermatitis produces a skin eruption characterized by erythema, swelling, and the formation of papules that appears from 6 hours to several days after the exposure **(Fig. 14–9).** The papules may become vesicular with blistering, peeling, and weeping. The site usually itches. The dermatitis is first limited to skin sites exposed to the antigen, but then it spreads to adjoining areas. The duration of the reaction depends upon the degree of sensitization and the concentration of antigen absorbed. Dermatitis can last for 3 to 4 weeks after the antigen has been removed.[54] Simple redness may fade of its own accord within several days. If the area is small and localized, a topical steroid may be used for treatment. Otherwise, systemic corticosteroids may be administered. The patient also needs to avoid contact with the offending allergen.

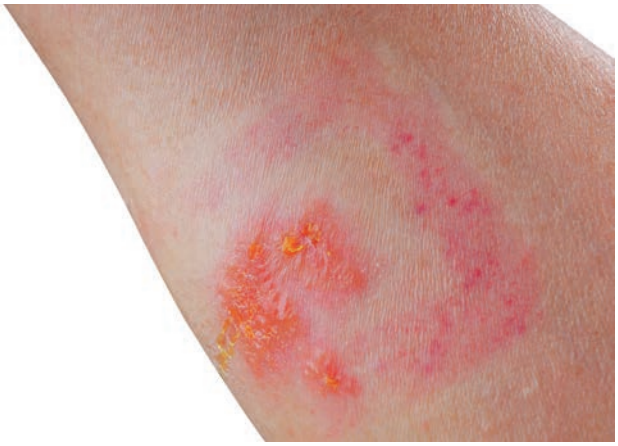


FIGURE 14–9 Contact hypersensitivity. Formation of papules occurs after exposure to poison ivy. *(Courtesy of Yong Choi/Thinkstock.)*

Hypersensitivity Pneumonitis

Evidence shows that hypersensitivity pneumonitis is mediated predominantly by sensitized T lymphocytes that respond to inhaled allergens. IgG and IgM antibodies are formed, but these are thought to play only a minor role in the pathogenesis of this disorder. Hypersensitivity pneumonitis is an allergic disease of the lung parenchyma characterized by inflammation of the alveoli and interstitial spaces. It is caused by chronic inhalation of a wide variety of antigens and is most often seen in individuals who are engaged in work or hobbies involving exposure to the implicated antigen.[59,60] Depending on the occupation and the particular antigen, the disease goes by several names: *farmer's lung, bird breeder's lung disease,* and *humidifier* or *air conditioner lung disease.* The reaction is most likely caused by microorganisms, especially bacterial and fungal spores, which individuals are exposed to from working with moldy hay, pigeon droppings, compost, moldy tobacco, infested flour, and moldy cheese, to name just a few examples. Symptoms include a dry cough, shortness of breath, fever, chills, weight loss, and general malaise, which may begin 6 to 8 hours after exposure to a high dose of the offending antigen.[59,60] Alveolar macrophages and lymphocytes trigger a chronic condition characterized by interstitial fibrosis with alveolar inflammation. Systemic corticosteroid therapy is used for treatment.

Skin Testing for Delayed Hypersensitivity

Skin testing is used clinically to detect delayed hypersensitivity responses to a variety of antigens. The tests are based on a T-cell–mediated memory response. When antigen is injected intradermally or applied to the surface of the skin, previously sensitized individuals develop a reaction at the application site.

This reaction results from infiltration of T lymphocytes and macrophages into the area. Blood vessels in the vicinity become lined with mononuclear cells and the reaction reaches a peak by 72 hours after exposure. Skin testing has been used to determine allergen sensitivity in contact dermatitis, to assess exposure to *Mycobacterium tuberculosis,* and to evaluate competency of cell-mediated immune responses in patients with immune deficiency diseases. Each of these applications is discussed in the text that follows.

The patch test is considered the gold standard in testing for contact dermatitis.[54,55] This test must be done when the patient is free of symptoms or at least has a clear test site. A nonabsorbent adhesive patch containing the suspected contact allergen is applied on the patient's back and the skin is checked for a reaction over the next 48 hours. Redness with papules or tiny blisters is considered a positive test. Final evaluation is conducted at 96 to 120 hours. All readings should be done by a skilled evaluator. False negatives can result from inadequate contact with the skin.

Skin testing for exposure to tuberculosis is a classic example of a delayed hypersensitivity reaction. The test is based on the principle that soluble antigens from *Mycobacterium tuberculosis* induce a reaction in people who currently have tuberculosis or have been exposed to *M tuberculosis* in the past. The tuberculin skin test uses an *M tuberculosis* antigen extract prepared from a purified filtrate of the organism's cell wall, called a purified protein derivative (PPD). The test is routinely performed by the Mantoux method in which 0.1 mL of 5 tuberculin units (TU) of PPD is injected intradermally into the inner surface of the forearm using a fine needle and syringe **(Fig. 14–10).**[61,62] The test site is examined between 48 and 72 hours for the presence of a hardened, raised area called induration. Interpretation of the reaction depends on the particular group in which the individual is categorized. An induration reaction of 15 mm or more is considered a positive test in individuals with no risk factors, whereas a reaction of 10 mm or greater is considered positive in recent immigrants of high prevalence countries, intravenous drug users, employees of health-care and other high-risk facilities, persons with certain clinical conditions, and children younger than 5 years of age.[62] An induration reaction of 5 mm or more is considered positive in persons who have HIV infection or other forms of immunosuppression, features on a chest x-ray consistent with tuberculosis, or recent contact with tuberculosis patients. A positive PPD test indicates that the individual has previously been exposed to *M tuberculosis* or a related organism, but it does not necessarily mean that he or she has an active tuberculosis infection. Positive test results also occur in persons who have previously received the BCG (Bacillus Calmette Guerin) vaccine for tuberculosis. The test has been an important screening tool to detect exposure in health-care workers and other individuals at risk for the infection.[63]

Skin testing can also be used to determine whether the cell-mediated arm of the immune system is functioning properly in individuals suspected of having immunodeficiency disorders. Antigens typically used for testing are from sources to which individuals have been commonly exposed such as *Candida albicans,* tetanus toxoid, Streptococcus bacteria, and fungal antigens such as trichophyton and histoplasmin.[61,64] The antigens are injected intradermally by the Mantoux method; the injection sites are then examined at 48 hours for redness and induration. Normal individuals should mount a memory response and develop a positive skin reaction to at least one of the antigens tested. Those with deficient cell-mediated immunity will display **anergy,** the absence of positive reactions for all of the common antigens used in the skin test.[64]

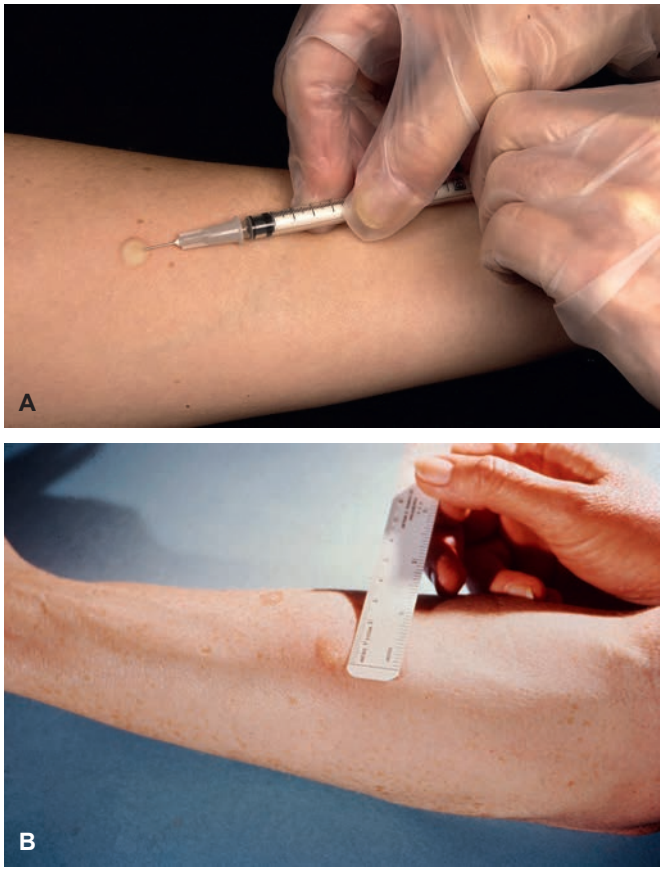


FIGURE 14–10 The Mantoux test. (A) Purified protein derivative (PPD) is injected into an individual's forearm, which causes a wheal (i.e., a raised area of the skin surface) to form at the injection site. (B) After 48 hours, the individual presented with an induration (hardened, raised area) indicating a positive reaction. *(A. Courtesy of the CDC/Gabrielle Benenson and Greg Knobloch, Public Health Image Library. B. Courtesy of the CDC/Donald Kopanoff, Public Health Image Library.)*

SUMMARY

- Hypersensitivity is an exaggerated immune response to antigens that are usually not harmful. It results in cell destruction and tissue injury.
- Gell and Coombs devised a system for classifying hypersensitivity reactions into four types based on the immunologic mechanism involved and the nature of the triggering antigen.

- Hypersensitivity types I, II, and III are antibody-mediated. Because they occur within minutes to hours after exposure to antigen, they are referred to as immediate reactions. Type IV hypersensitivity is a cell-mediated response involving T lymphocytes. Because the clinical manifestations do not appear until 24 to 72 hours after contact with the antigen, the type IV response is also referred to as delayed hypersensitivity.
- Type I hypersensitivity is a Th2 polarized immune response that involves production of IgE antibody to the inducing antigen or allergen. In the sensitization phase of this response, IgE antibody binds to high-affinity FcεRI receptors on mast cells and basophils. In the activation phase, the receptors become cross-linked when the allergen binds to adjacent IgE molecules. This cross-linking stimulates the cells to degranulate and release preformed and newly synthesized chemical mediators that cause an inflammatory response. The reaction occurs very quickly, within minutes of exposure to the inducing antigen. Cytokines produced during the response can cause a late-phase response of prolonged inflammation.
- Preformed mediators that are released from mast cells and basophils include histamine, eosinophil chemotactic factor of anaphylaxis, neutrophil chemotactic factor, and proteolytic enzymes such as tryptase. These factors cause contraction of smooth muscle in the bronchioles, blood vessels, and intestines; increased capillary permeability; chemotaxis of eosinophils and neutrophils; and decreased blood coagulability. Newly synthesized mediators such as prostaglandins, leukotrienes, and PAF potentiate the effects of histamine and other preformed mediators.
- Clinical manifestations of type I hypersensitivity include localized wheal-and-flare skin reactions (urticaria or hives); rhinitis; allergic asthma; and systemic anaphylaxis, which can be life-threatening.
- Susceptibility to allergies is based on a combination of genetic factors and environmental influences. Genes affect different aspects of the immune response that contribute to the pathogenesis of type I hypersensitivity. Some genes affect anatomical structures. Exposure to infectious organisms appears to play a key role in the development of allergic disease. Stress, variations in physical factors such as temperature, and contact with environmental pollutants can intensify clinical manifestations of allergy in susceptible individuals.
- Allergies can be treated with drugs such as antihistamines, decongestants, and corticosteroids. The monoclonal anti-IgE antibody omalizumab has been used to block the binding of IgE to mast cells and basophils in patients with moderate to severe asthma.
- Allergen immunotherapy (AIT) can be administered to patients for whom drug therapy and environmental control measures are not successful. The goal of AIT is to induce immune tolerance by administering gradually increasing doses of the allergen over time.
- The preferred method of screening for allergies is an in vivo skin prick test, in which very small amounts of potential allergens are injected under the skin. A positive test produces a wheal-and-flare reaction within 20 minutes.
- In patients unable to tolerate skin testing, in vitro testing by noncompetitive solid-phase immunoassays for allergen-specific IgE can be performed. In these assays, patient serum is incubated with a solid phase to which a specific allergen has been attached. Binding is detected with an enzyme-labeled anti-human IgE antibody and a colorimetric, fluorescent, or chemiluminescent substrate.
- Solid-phase immunoassays for total serum IgE can be used to monitor patients undergoing treatment with AIT or omalizumab or to detect patients with certain diseases characterized by elevated IgE levels. The principle of these tests is the same as that for allergen-specific IgE tests except that anti-IgE, rather than allergen, is attached to the solid phase.
- Type II hypersensitivity involves production of IgG or IgM antibodies to antigens on the surface of host cells. These antibodies can destroy the cells through complement-mediated cytolysis, opsonization and phagocytosis, or antibody-dependent cellular cytotoxicity (ADCC). In other cases, binding of the antibody to the cell surface antigen can result in dysfunction or overstimulation of the cell.
- Examples of type II reactions that involve cell damage include autoimmune hemolytic anemia, transfusion reactions, and hemolytic disease of the newborn (HDN). Myasthenia gravis is an example of a type II disorder in which the antibody blocks binding of a ligand to cell receptors, causing dysfunction of the cells. In contrast, in Graves disease the antibody produced stimulates cells after binding to cell receptors.
- The direct antiglobulin test (DAT) is used to screen for transfusion reactions, autoimmune hemolytic anemia, and HDN. In this test, washed patient RBCs are combined with anti-human globulin and observed for agglutination, indicating the presence of IgG or complement components on the cells.
- Cold agglutinin antibodies bind to RBCs at temperatures below 30°C and cause microocclusions of small blood vessels or destruction of the RBCs, mainly through opsonization and extravascular clearance by macrophages in the liver. Production of cold agglutinins may be from unknown causes or may be associated with certain infections or B cell/plasma cell lymphoproliferative disorders. Cold agglutinin titers can be determined by incubating patient serum with a dilute suspension of human type O RBCs overnight at 4°C and observing for agglutination.
- Type III hypersensitivity involves formation of IgG or IgM antibody that reacts with soluble antigen under conditions of slight antigen excess to form small complexes that precipitate in the tissues. These complexes activate complement, resulting in migration of neutrophils to the site with subsequent release of lysosomal enzymes that produce damage to the surrounding tissues.

- The Arthus reaction, characterized by deposition of antigen–antibody complexes in the blood vessels of the skin, is a classic example of a type III reaction. Other examples include serum sickness and autoimmune diseases such as SLE and RA.
- Type IV hypersensitivity is a cell-mediated mechanism that involves the activation of Th1 cells to release cytokines. As a result, macrophages and other immune cells are recruited to the area, where they induce an inflammatory reaction. Cytotoxic T cells may also cause damage to the target cells involved.
- Contact dermatitis is an example of a type IV hypersensitivity reaction. It results from exposure to chemicals released by plants such as poison ivy and poison oak, metals such as nickel, or components of hair dyes and cosmetics that act as haptens when bound to self-proteins. Hypersensitivity pneumonitis is a type IV hypersensitivity response that results mainly from occupational exposure to inhaled antigens.
- Skin testing is used to detect the type IV hypersensitivity responses in contact dermatitis and tuberculin (PPD) testing. It is also used to test for functional cell-mediated immunity to common antigens in patients suspected of having immunodeficiency diseases. Positive test results appear in 48 to 72 hours and indicate sensitization to the antigen(s) used in the test.
- All four types of hypersensitivity represent defense mechanisms that stimulate an inflammatory response to cope with and react to an antigen that is seen as foreign. In many cases, the antigen is not harmful, but the response to it results in tissue damage.

Study Guide: Comparison of Hypersensitivity Reactions

	TYPE I	TYPE II	TYPE III	TYPE IV
Immune Mediators	IgE	IgG or IgM	IgG or IgM	T cells
Synonym	Anaphylactic	Antibody-mediated cytotoxic	Complex-mediated	Cell-mediated or delayed type
Timing	Immediate	Immediate	Immediate	Delayed
Antigen	Heterologous	Cell surface: autologous or heterologous	Soluble: autologous or heterologous	Autologous or heterologous
Complement Involvement	No	Yes	Yes	No
Immune Mechanism	Release of mediators from IgE-sensitized mast cells and basophils	Cell destruction caused by antibody and complement, opsonization, or ADCC Cell function inhibited by antibody binding Cell function stimulated by antibody binding	Antigen–antibody complexes activate complement proteins. Neutrophils are recruited and release lysosomal enzymes that cause tissue damage.	Antigen-sensitized Th1 cells release cytokines that recruit macrophages and induce inflammation or activate cytotoxic T cells to cause direct cell damage.
Clinical Examples	Anaphylaxis, allergic rhinitis, allergic asthma, food allergies, urticaria	Transfusion reactions, autoimmune hemolytic anemia, hemolytic disease of the newborn, drug reactions, myasthenia gravis, Goodpasture's syndrome, Graves disease	Serum sickness, Arthus reaction, lupus erythematosus, rheumatoid arthritis, drug reactions	Contact dermatitis, tuberculin and anergy skin tests, hypersensitivity pneumonitis

CASE STUDIES

1. A 13-year-old male had numerous absences from school in the spring because of cold symptoms that included head congestion and cough. He had received antibiotic treatment twice, but he seemed to get one cold after another. A complete blood count (CBC) showed no overall increase in WBCs, but a mild eosinophilia was present. Because he had no fever or other signs of infection, his physician suggested that allergy testing be run.

Questions

a. What would account for the eosinophilia noted?
b. What tests should be run for this patient?
c. If the patient was treated with allergy immunotherapy, what test could be used to monitor his response over time?

2. A 55-year-old male went to his physician complaining of feeling tired and run down. Two months previously, he had pneumonia and was concerned that he might not have completely recovered. He indicated that his symptoms only become noticeable if he goes out in the cold. A CBC count was performed, showing that his WBC count was within normal limits; however, his RBC count was just below normal. A DAT performed on RBCs was weakly positive after incubating at room temperature for 5 minutes. When the DAT was repeated with monospecific reagents, the tube with anti-C3d was the only one positive.

Questions

a. What does a positive DAT indicate?
b. What is the most likely class of the antibody causing the reaction?
c. Why was the DAT positive only with anti-C3d when monospecific reagents were used?

REVIEW QUESTIONS

1. Which of the following is a general characteristic of hypersensitivity reactions?
   a. The immune responsiveness is depressed.
   b. Antibodies are involved in all reactions.
   c. An exaggerated immune response to an antigen occurs.
   d. The antigen triggering the reaction is a harmful one.

2. Which of the following is associated with an increase in IgE production?
   a. Transfusion reaction
   b. Activation of Th2 cells
   c. Reaction to poison ivy
   d. HDN

3. Which of the following would cause a positive DAT test?
   a. Presence of IgG on RBCs
   b. Presence of C3b or C3d on RBCs
   c. A transfusion reaction caused by preformed antibody
   d. Any of the above

4. All of the following are associated with type I hypersensitivity *except*
   a. release of preformed mediators from mast cells.
   b. activation of complement.
   c. cell-bound antibody bridged by antigen.
   d. an inherited tendency to respond to allergens.

5. Which of the following is associated with anaphylaxis?
   a. Buildup of IgE on mast cells
   b. Activation of complement
   c. Increase in cytotoxic T cells
   d. Large amount of circulating IgG

6. To determine if a patient is allergic to rye grass, the best test to perform is the
   a. total IgE test.
   b. skin prick test.
   c. DAT.
   d. complement fixation.

7. Which condition would result in HDN?
   a. Buildup of IgE on mother's cells
   b. Sensitization of cytotoxic T cells
   c. Exposure to antigen found on both mother and baby RBCs
   d. Prior exposure to foreign RBC antigen

8. What is the immune mechanism involved in type III hypersensitivity reactions?
   a. Cellular antigens are involved.
   b. Deposition of immune complexes occurs in antibody excess.
   c. Only heterologous antigens are involved.
   d. Tissue damage results from exocytosis.

9. What is the immune phenomenon associated with the Arthus reaction?

a. Tissue destruction by cytotoxic T cells
b. Removal of antibody-coated RBCs
c. Deposition of immune complexes in blood vessels
d. Release of histamine from mast cells

10. Which of the following conclusions can be drawn about a patient whose total IgE level was determined to be 150 IU/mL?

a. The patient definitely has allergic tendencies.
b. The patient may be subject to anaphylactic shock.
c. Antigen-specific testing should be done.
d. The patient will never have an allergic reaction.

11. Which of the following explains the difference between type II and type III hypersensitivity reactions?

a. Type II involves cellular antigens.
b. Type III involves IgE.
c. IgG is involved only in type III reactions.
d. Type II reactions involve no antibody.

12. Two days after administration of the PPD test, a female health-care worker developed an area of redness and induration 12 mm in size at the injection site. This result means that she has

a. an active case of tuberculosis.
b. been exposed to *M tuberculosis.*
c. developed protective immunity against tuberculosis.
d. a result in the normal range for her risk group.

13. A young woman developed red, itchy papules on her wrist 2 days after wearing a new bracelet. This reaction was caused by

a. IgE-sensitized mast cells in the skin.
b. antigen-antibody complexes in the skin.
c. damage to the skin cells by antibodies and complement.
d. an inflammatory response induced by cytokines released from Th1 cells.

14. Reactions to latex are caused by

a. type I hypersensitivity.
b. type IV hypersensitivity.
c. skin irritation.
d. all of the above.

15. To determine a cold agglutinin titer

a. patient serum should be separated from whole blood at 4°C and tested at 4°C.
b. patient serum should be separated from whole blood at 4°C and tested at 37°C.
c. patient serum should be separated from whole blood at 37°C and tested at 4°C.
d. patient serum should be separated from whole blood at 37°C and tested at 37°C.

15 Autoimmunity

Linda E. Miller, PhD, I, MB^{CM}(ASCP)SI

LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Explain the mechanisms of central and peripheral tolerance that are essential in preventing the development of autoimmunity.
2. Discuss genetic and environmental factors that are thought to contribute to the development of autoimmunity.
3. Explain the relationship between microbial infections and the development of autoimmune disease.
4. Distinguish between organ-specific and systemic autoimmune diseases and give examples of each and their associated target tissues.
5. Discuss the immunopathology and clinical manifestations of each of the following diseases: systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), granulomatosis with polyangiitis (Wegener's granulomatosis), Graves disease, Hashimoto's thyroiditis, type 1 diabetes mellitus, celiac disease, autoimmune hepatitis, primary biliary cirrhosis, multiple sclerosis (MS), myasthenia gravis (MG), and Goodpasture's syndrome.
6. Associate each of the diseases listed in Learning Outcome 5 with its corresponding autoantibodies and laboratory findings.
7. Explain the principles of laboratory methods used to screen for and confirm the presence of antinuclear antibodies (ANA).
8. Describe common immunofluorescence patterns seen in the indirect immunofluorescence (IIF) test for ANAs and their clinical significance.
9. Describe the c-ANCA and p-ANCA patterns seen in the IIF test for ANCAs and their clinical significance.
10. Discuss the clinical significance of rheumatoid factor (RF) and anti-CCP.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Anergy
Anticentromere antibodies
Anticyclic citrullinated peptide (anti-CCP or ACPA)
Antihistone antibodies
Antineutrophil cytoplasmic antibody (ANCA)
Antinuclear antibodies (ANA)
Antiphospholipid antibodies
Anti-RNP antibody
Autoantibodies
Autoimmune diseases
Autoimmune hepatitis (AIH)
Autoimmune liver disease
Autoimmune thyroid diseases (AITDs)
Celiac disease
Central tolerance
CREST syndrome
Double-stranded DNA (dsDNA) antibodies
Epigenetics
Epitope spreading
Extractable nuclear antigens (ENAs)
Fluorescent antinuclear antibody (FANA) testing
Goodpasture's syndrome
Granulomatosis with polyangiitis (PGA)
Graves disease
Hashimoto's thyroiditis
Immunologic tolerance
Molecular mimicry
Multiple sclerosis (MS)
Myasthenia gravis (MG)
Nucleolus
Nucleosome antibodies
Peripheral tolerance
Primary biliary cirrhosis (PBC)
Rheumatoid arthritis (RA)
Rheumatoid factor (RF)
Self-tolerance
Sm antigen
Superantigens
SS-A/Ro
SS-B/La
Systemic lupus erythematosus (SLE)
Thyroglobulin (Tg)
Thyroid peroxidase (TPO)
Thyroid-stimulating hormone (TSH)
Thyroid-stimulating hormone receptor antibodies (TRAbs)
Thyrotoxicosis
Thyrotropin-releasing hormone (TRH)
Tissue transglutaminase (tTG)
Type 1 diabetes mellitus (T1D)
Wegener's granulomatosis (WG)

In the early 1900s, Paul Ehrlich noted that the immune system could attack the very host it was intended to protect, a phenomenon he referred to as *"horror autotoxicus,"* or "fear of self-poisoning." Conditions in which this phenomenon occurred later became known as autoimmune diseases. **Autoimmune diseases** are disorders in which immune responses are targeted toward self-antigens and result in damage to organs and tissues in the body. These harmful effects can be caused by T-cell–mediated immune responses or **autoantibodies** that are directed against host antigens. More than 100 autoimmune diseases have been discovered, and these can involve various organ systems. Autoimmune diseases are a leading cause of chronic illness and death, affecting about 5% of the world's population, including 50 million people in the United States alone.[1-3] This chapter will begin by discussing the factors that are thought to contribute to the development of autoimmunity so that you can gain a better understanding of the underlying pathology of autoimmune disease. The discussion will then proceed to the clinical manifestations and immunopathology of specific autoimmune diseases, as well as the laboratory tests that are used in their diagnosis.

Etiology of Autoimmune Disease

Self-Tolerance

Under normal circumstances, the immune system is able to differentiate between "self" and "nonself" or "foreign," so that self-antigens are not destroyed. This fundamental concept was introduced in Chapter 2. Central to this phenomenon is **self-tolerance,** or the ability of the immune system to accept self-antigens and not initiate a response against them. Autoimmune disease is thought to result from a loss of self-tolerance.

Self-tolerance is a type of **immunologic tolerance,** or a state of immune unresponsiveness that is directed against a specific antigen, in this case, a self-antigen. In order for self-tolerance to develop, lymphocytes must be "educated" so they can distinguish between self-antigens and foreign antigens. This education takes place at two levels: central and peripheral.

Central tolerance occurs in the central or primary lymphoid organs, the thymus, and the bone marrow.[1,4] As T cells mature in the thymus, they encounter self-antigens that are normally present on the surface of the thymic epithelial cells. In a process called negative selection, T cells that express T-cell receptors (TCRs) with a strong affinity for these self-antigens are deleted by apoptosis, a physiological form of cell death (see Chapters 4 and 17 and **Figure 15–1**). Negative selection occurs with both the immature, double-positive CD4+/CD8+ cells in the cortex and with the more mature, single-positive CD4+ or CD8+ cells in the medulla. During this process, some of the self-reactive CD4+ T cells are not deleted, but instead differentiate into T regulatory (Treg) cells that can specifically inhibit immune responses to self-antigens. Similarly, as B cells mature in the bone marrow, those with receptors having a strong affinity for self-antigens are eliminated by apoptosis. Some self-reactive B cells are not deleted: rather, they are stimulated to rearrange their immunoglobulin genes so that their B-cell receptors are no longer antigen specific. This process is known as *receptor editing.* B cells that possess receptors that only weakly recognize self-antigens are induced to downregulate the expression of their receptors and develop a specific state of unresponsiveness to the antigens known as **anergy.**

Thus, there are several ways in which the central tolerance of T and B cells can be achieved. This process is not totally effective, however, and some self-reactive lymphocytes manage to escape to the secondary lymphoid organs such as the lymph nodes and spleen. Therefore, a second level of protection is

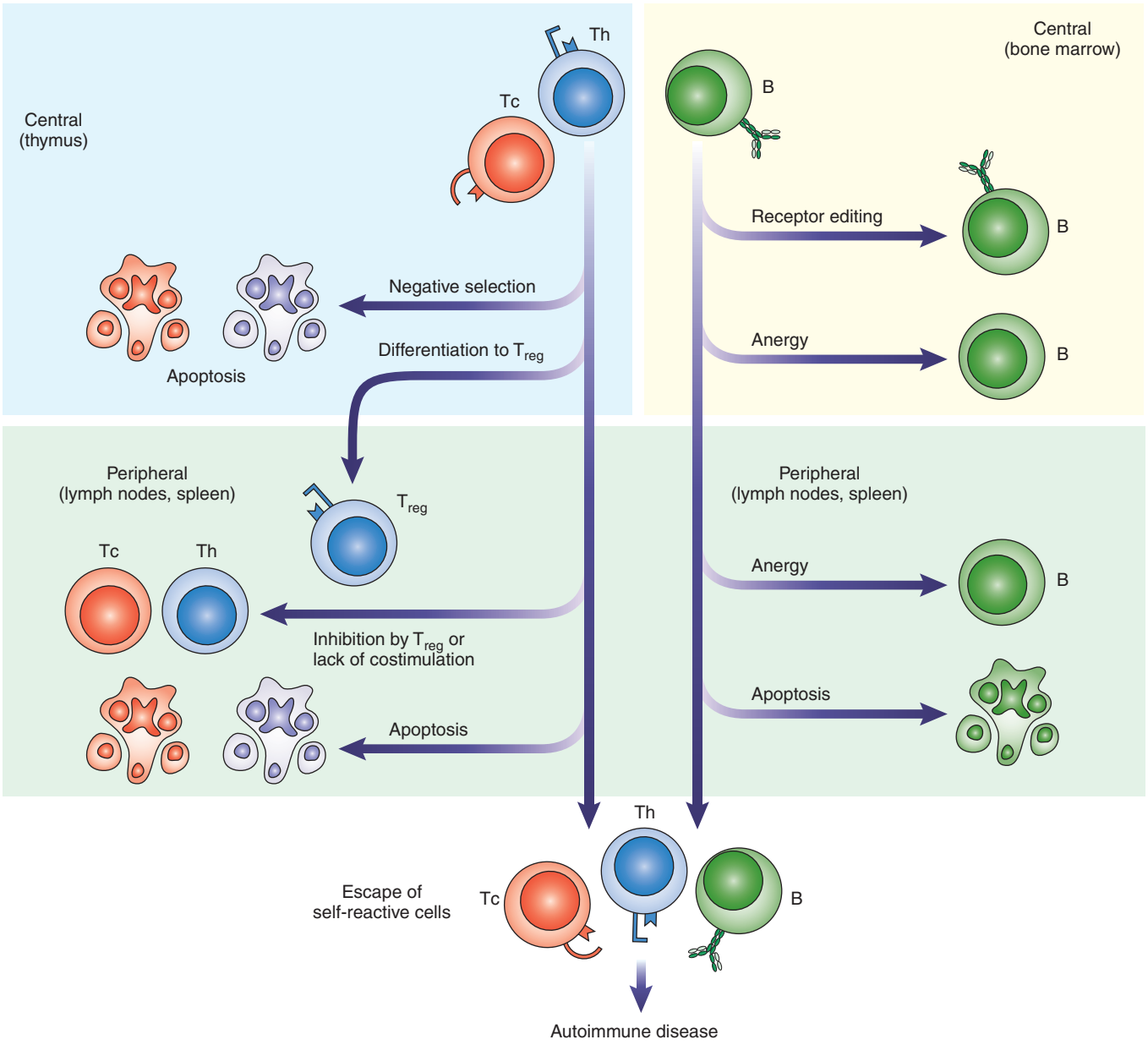


FIGURE 15–1 Mechanisms of central and peripheral tolerance.

needed. In **peripheral tolerance,** lymphocytes that recognize self-antigens in the secondary lymphoid organs are rendered incapable of reacting with those antigens.[1,4] Peripheral tolerance of T cells can result from anergy caused by the absence of a costimulatory signal from an antigen-presenting cell (APC) or binding of inhibitory receptors such as CTLA-4 (a molecule that prevents T-cell activation). Peripheral T-cell tolerance can also result from inhibition by Tregs or death by apoptosis. Self-reactive B cells in the periphery can be deleted by apoptosis, be rendered anergic after repeated stimulation with self-antigens, or receive inhibitory signals through receptors such as CD22.

In some individuals, self-tolerance can fail even after this second layer of protection; if this happens, autoimmunity can arise. The development of autoimmune disease is thought to be caused by complex interactions between genetics, exposure to environmental factors, and defects in immune regulation. Some of the major factors that are believed to contribute to autoimmunity will be discussed in the text that follows.

Genetics

There is much evidence supporting a genetic basis for autoimmune disease.[5,6] Autoimmune diseases are often more prevalent among family members than among unrelated individuals and are more prevalent among monozygotic (genetically identical) twins than dizygotic (non-identical) twins or siblings. Researchers conducting molecular studies continue to identify specific genetic polymorphisms or mutations that are associated with autoimmune diseases.

Most of the research concerning humans has focused on genes in the major histocompatibility complex (MHC) (see Chapter 3). Investigators have found that there is an association between the presence of certain human leukocyte antigen (HLA) types and the risk of developing a particular autoimmune disorder. The strongest link found is between the HLA-B27 allele and the development of ankylosing spondylitis, an autoinflammatory disease that affects the spine. Individuals who possess HLA-B27 have about a 100 times greater chance of developing the disease than individuals who do not have that allele.[4,6] Other associations with specific MHC genes are discussed in the sections on particular autoimmune diseases in this chapter. Differences in MHC genes are thought to influence the development of autoimmune disease because the specific structure of the MHC molecule can determine whether or not a self-antigen can attach to the peptide-binding cleft of the molecule and subsequently be processed and presented to T cells.[4] In addition, class II MHC molecules can sometimes be abnormally expressed on cells where they are not typically found, resulting in the presentation of self-antigens for which no tolerance has been established.

Genome-wide association studies are revealing that polymorphisms in some non-MHC genes can also be associated with development of autoimmune disease. Many of these genes influence the development and regulation of immune responses. Examples include the *PTPN22* gene, which has a role in T- and B-cell receptor signaling; the *IL2RA* gene, which is involved in T-cell activation and maintenance of Tregs; the *CTLA4* gene, which has an inhibitory effect on T-cell activation; the *BLK* gene, which is involved in B-cell activation and development; and the *AIRE* (autoimmune regulator) gene, which promotes the development of T-cell tolerance in the thymus.[4] Although most autoimmune diseases involve multiple genes (~20 to 30), single-gene mutations that can be inherited in a Mendelian fashion have been associated with rare autoimmune disorders.

Inheritance of specific genes may make an individual more susceptible to a particular autoimmune disease, but genetic makeup is not totally responsible because the majority of people with a particular gene will not develop autoimmunity. In addition, the concordance rate among monozygotic twins (i.e., the presence of autoimmune disease in both members of a pair of identical twins) is only between 20% and 30% for most autoimmune diseases.[7] Furthermore, the prevalence and severity of many autoimmune diseases vary in different geographic locations.[5] This variance suggests that environmental and other factors also play a role in the development of autoimmune disease. A discussion of some of the major factors that are believed to trigger autoimmune responses follows.

Other Endogenous and Environmental Factors

Hormonal Influence

Women are 2.7 times more likely to acquire an autoimmune disease than men; in fact, about 78% of patients with autoimmune diseases are of female gender.[8] Women also tend to develop autoimmunity at an earlier age and have a higher risk for acquiring more than one autoimmune disease as compared with men. Furthermore, females have been found to have higher absolute CD4+ T-cell counts and higher levels of circulating antibodies than men.[8] These observations suggest that there is a hormonal influence on the development of autoimmunity. Studies on the effects of hormones have shown that estrogens tend to direct the immune system in favor of a type 2 helper cell (Th2) response, resulting in more B-cell activation and antibody production, whereas androgens favor a type 1 helper cell (Th1) response with activation of CD8+ T cells. Prolactin, a hormone that stimulates production of breast milk in pregnant and nursing women, can stimulate both humoral and cell-mediated immune responses.[8] The stimulatory effects of female hormones may place women at a greater risk for developing autoimmune disease.

Tissue Trauma and Release of Cryptic Antigens

When immunologic tolerance to self-antigens occurs during the early development of lymphocytes in the thymus and bone marrow, some self-antigens may be *cryptic*, or hidden within the tissues of the host. T and B lymphocytes are shielded from these sequestered antigens and are not educated to become tolerant to them. At a later time in life, inflammation or tissue trauma could cause the cryptic antigens to be released and to suddenly be accessible to the uneducated lymphocytes, triggering an immune response.[1,4,9] Tissue damage could be caused by factors such as infections, contact with environmental toxins, or physical injury from exposure to ultraviolet (UV) radiation. This concept has also been referred to as *immunologic ignorance* and may be responsible for the production of autoantibodies to the lens of the eye following an ocular injury, autoantibodies to sperm after a vasectomy, and autoantibodies to DNA following damage to skin cells by overexposure to UV rays from the sun.

Microbial Infections

Scientists have been very interested in the association of microbial infections with the development of autoimmune disease. Bacteria, viruses, and other infectious pathogens may be able to trigger autoimmune responses in a variety of ways. A principal means by which microbes are thought to accomplish this is through molecular mimicry. **Molecular mimicry** refers to the fact that many bacterial or viral agents contain antigens that closely resemble the structure or amino acid sequence of self-antigens. Exposure to such foreign antigens may trigger immune responses that cross-react with similar self-antigens. Molecular mimicry has been postulated as a mechanism for a number of human autoimmune diseases.[9-11] The best known example involves the association between the gram-positive bacterium *Streptococcus pyogenes* and rheumatic fever, an autoimmune disorder that primarily affects the joints and the heart. Some patients who have acquired scarlet fever or pharyngitis as a result of infection with *S pyogenes* will proceed to develop rheumatic fever if they are not treated adequately with antibiotics (see Chapter 20). Symptoms develop 2 to 4 weeks after the infection and are thought to be caused by the production of antibodies to the M protein and N-acetyl

glucosamine components of the bacteria, which cross-react with cardiac myosin, causing damage to the heart.[10]

A second way that microbes might trigger autoimmunity is through a *bystander effect*.[4,9,11] In this mechanism, the microbial organism does not have to share structurally similar antigens with the host. Instead, the microorganism can induce a local inflammatory response that recruits leukocytes and stimulates APCs to release cytokines that nonspecifically activate T cells. Some of the T cells that are activated may have specificity for self-antigens. This expansion of the immune response to unrelated antigens has also been termed **"epitope spreading."**

A third way that microorganisms might induce autoimmunity is through superantigens. **Superantigens** are proteins that are produced by various microbes that have the ability to bind to both class II MHC molecules and TCRs, regardless of their antigen specificity.[12] Examples are the staphylococcal enterotoxins that cause food poisoning and toxic shock syndrome. These superantigens can act as potent T-cell mitogens by activating a large number of T cells with different antigen specificities. If some of these T cells possess specificity for a self-antigen, autoimmune responses might result.[9] Likewise, some viruses, including the Epstein-Barr virus (EBV) and cytomegalovirus (CMV), can cause polyclonal activation of B cells.[1]

Scientists have also been very interested in the complex relationship between microbiota, or normal flora, and the immune system. Research has shown that the presence of certain strains of endogenous bacteria may be associated with a greater risk for autoimmune disease.[11] These strains, as well as pathogenic microorganisms, may stimulate innate immune responses through interaction with pattern recognition receptors such as the Toll-like receptors (TLRs). This interaction triggers cell-signaling pathways that result in the production of cytokines such as IFN α, which can stimulate cells of the adaptive immune system, some of which are directed toward self-antigens. A decrease in the number and function of Tregs can perpetuate the activity of autoreactive cytotoxic T cells and hyperactive B cells that produce autoantibodies. The activated lymphocytes, in turn, produce proinflammatory cytokines that provide signals to stimulate cells of the innate system, thus producing a vicious cycle that amplifies the immune response and sustains autoimmunity.[7]

Epigenetics and Modification of Self-Antigens

Investigators have done much research in the area of epigenetics and how it may relate to the development of autoimmunity. **Epigenetics** refers to modifications in gene expression that are *not* caused by changes in the original DNA sequence.[13,14] These alterations are stable and can be inherited. They are thought to be triggered by exposure to environmental toxins, ingestion of harmful foods or drugs, or the aging process. These factors can induce epigenetic changes by increasing or decreasing methylation of cytosine bases, modifying histones, and causing abnormal regulation by microRNAs. These modifications can result in changes in the level at which genes are expressed by affecting their ability to be transcribed into mRNA, which is subsequently translated into proteins that will influence the phenotype of an individual. Over- or underexpression of certain genes in the immune system may result in homeostatic imbalances and a breakdown of self-tolerance, leading to autoimmunity.[15,16]

Sometimes, exposure to environmental factors can lead to changes at the protein level. The changes are known as *post-translational modifications* and may involve biochemical processes such as acetylation, lipidation, citrullination, and glycosylation.[6] These modifications can alter the immunogenicity of an antigen, affecting its ability to be processed by APCs and presented to T cells. Such alterations of self-antigens can make them more immunogenic, leading to autoimmune responses. For example, citrullination of collagen might play a role in the pathogenesis of **rheumatoid arthritis (RA)** and glycosylation of myelin may be involved in the pathology of multiple sclerosis (see the *Rheumatoid Arthritis* and *Multiple Sclerosis* sections in the text that follows).

Interactions Between Factors

Although the precise etiology of autoimmunity is unknown, there is much evidence that suggests that this heterogeneous disease entity is caused by complex interactions between genetic and environmental factors **(Fig. 15–2).**[1,4,6,15] Certain genes are thought to make individuals more susceptible to immune responses against self-antigens, but are not sufficient by themselves to cause autoimmune disease. Gender of the individual, tissue injury, and exposure to infectious microorganisms or other environmental agents are all believed to have significant effects on the immune system that can trigger autoimmune responses in susceptible individuals. As a result of this break in immunologic tolerance, autoreactive T cells recognize and proliferate in response to self-antigens and B cells develop into plasma cells that secrete autoantibodies. This can result in the release of proinflammatory cytokines, which, when coupled with dysfunctions in immune-regulatory cells, perpetuate the autoimmune responses. If these responses are not held in check, they can culminate in autoimmune disease.[1,7,14] Tissue injury in these disorders results from hypersensitivity reactions that involve autoantibodies to cell-surface receptors, deposition of immune complexes that contain self-antigens, and cell-mediated cytotoxicity.[1] The immunopathological mechanisms vary with specific autoimmune diseases and will be discussed in the text that follows.

Systemic Autoimmune Diseases

Autoimmune diseases can be classified as systemic or organ-specific, depending on the extent of the pathology. There is often a good bit of overlap between the two categories because some diseases may start out affecting a single organ, but progress later to affect other locations in the body. **Table 15–1** lists some of the systemic autoimmune diseases along with their corresponding target tissues and associated autoantibodies, whereas Table 15–3 later provides this information for some of the organ-specific diseases. The sections that follow will discuss three systemic autoimmune diseases and the laboratory tests that are essential to their

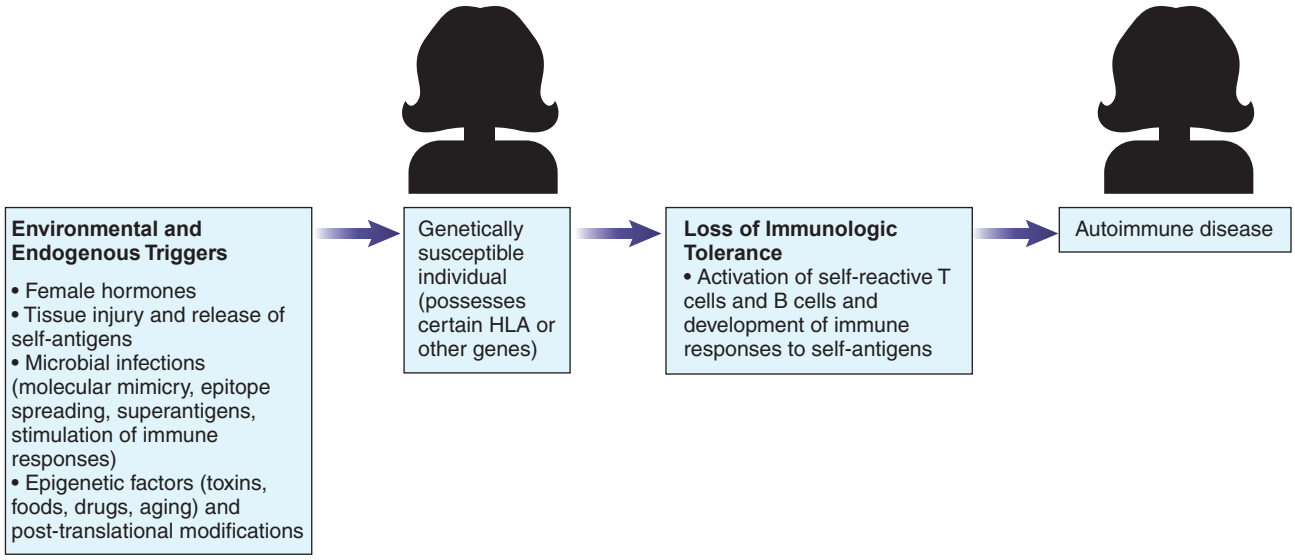


FIGURE 15–2 Interactions between genetic and environmental factors in the development of autoimmunity.

Table 15–1 Systemic Autoimmune Diseases

DISEASE	TARGET CELLS AND TISSUES	ASSOCIATED AUTOANTIBODIES
Systemic lupus erythematosus (SLE)	Multiple cells and organs throughout the body, including the skin, joints, kidneys, brain, heart, lungs	Antibodies to double-stranded DNA and other nuclear components, such as Sm (ANAs) Phospholipid antibodies Antibody to RBCs Antibody to platelets Antibody to lymphocytes Antibody to ribosomal components Antibody to endothelium Rheumatoid factor
Rheumatoid arthritis (RA)	Joints, bone; other tissues in some cases	Anti-CCP (cyclic citrullinated proteins) Rheumatoid factor Antinuclear antibodies (ANAs)
Granulomatosis with polyangiitis (Wegener's granulomatosis)	Upper respiratory system, lungs, blood vessels	Antineutrophil cytoplasmic antibodies (ANCA); c-ANCA pattern Rheumatoid factor ANAs

diagnosis: systemic lupus erythematosus, RA, and granulomatosis with polyangiitis (Wegener's granulomatosis).

Systemic Lupus Erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a chronic systemic inflammatory disease that affects between 40 and more than 200 persons per 100,000, depending on the population.[17] The peak age of onset is usually between 20 and 40 years. Women are much more likely to be affected than men, by a ratio of about 9 to 1.[18] SLE is also more common in African Americans and Hispanics than in Caucasians.[17,19] With earlier diagnosis and improved treatments, the 5-year survival rate has increased from 50% in the 1950s to greater than 90% today.[17,18,20]

Etiology

SLE appears to originate from complex interactions between environmental factors, genetic susceptibility, and abnormalities within the immune system.[21] Environmental factors thought to play a role in SLE include UV light, certain medications, and possibly infectious agents.[17,21,22] Exposure to sunlight is a well-known trigger of the photosensitive skin rashes seen in many lupus patients. Certain drugs, such as procainamide (used to treat abnormal heart rhythms), hydralazine (used for high blood pressure), and the tuberculosis drug isoniazid, can induce a transient lupus-like syndrome that resolves once the drug is stopped.[23] Hormones are also important as indicated by the significantly higher incidence of lupus in females and an increased risk of developing lupus in women that have used estrogen-containing contraceptives or hormone replacement therapy.[19] Hormones may be important because they may help regulate the transcription of genes that are central to the expression of SLE.[22]

The majority of individuals exposed to the environmental factors mentioned previously do not develop lupus, and genetic

makeup is believed to play an important role in susceptibility to SLE. More than 20 genetic loci associated with lupus in humans have been reported.[18,21] People with certain HLA types, especially HLA-A1, B8, and DR3—have an increased chance of developing lupus.[17] Another group of genes that have been associated with increased susceptibility to SLE plays a role in the clearance of immune complexes (see the text that follows).[21,24] Other lupus-associated genes include polymorphisms in genes associated with immune function, genes coding for various cytokines, and genes involved in signaling of innate immune responses.[25] These defects are thought to result in uncontrolled autoreactivity of T and B cells, which leads to the production of numerous autoantibodies.

Immunopathology

Over 100 autoantibodies associated with SLE have been discovered.[25] These include antibodies to double-stranded DNA (dsDNA), histones, and other nuclear components, as well as autoantibodies to lymphocytes, erythrocytes, platelets, phospholipids, ribosomal components, and endothelium.[25,26] The typical patient has an average of three circulating autoantibodies.[19]

B cells and the autoantibodies they produce are believed to play a central role in the pathogenic mechanisms that are responsible for this complex disease. In fact, the presence of autoantibodies can precede the onset of disease by 9 to 10 years.[26] Abnormal apoptosis of certain types of cells may occur, releasing excess amounts of cellular constituents such as DNA and ribonucleic acid (RNA). Dysfunctional removal of cellular debris by phagocytes may allow these cellular components to persist, increasing the chances for autoantibody production.[17,27]

Antibodies to dsDNA are present in 70% of patients with lupus and are highly specific for the disease.[17] Anti-dsDNA and complement proteins have been found in immune complexes that are deposited in organs such as the kidneys and skin and are thought to play a major role in the pathogenesis of SLE. It is not clear whether preformed immune complexes circulate in the bloodstream and settle in various organs in the body, whether the autoantibodies cross-react with protein components of the tissues, or whether nuclear antigens are attracted to the tissues by charge–charge interactions and then stimulate the production of autoantibodies.[17,28,29] Accumulation of IgG to dsDNA seems to be the most pathogenic because it forms complexes of an intermediate size that become deposited in the glomerular basement membrane (GBM).

Once immune complexes are formed, they cannot be cleared efficiently because of other possible deficiencies in lupus patients. These include defects in complement receptors on phagocytic cells; defects in receptors for the Fc portion of immunoglobulins; or rarely, deficiencies of early complement components such as C1q, C2, or C4 (see Chapter 7).[17,19,22] The immune complexes activate complement and initiate an inflammatory response. Leukocytes are attracted to the sites of inflammation and release cytokines that perpetuate the response, resulting in tissue damage.[17,28]

Autoantibodies to nuclear and nonnuclear antigens can also cause cellular destruction by other mechanisms. For example, antibodies to red blood cells (RBCs) can cause hemolytic anemia and antibodies to platelets can cause thrombocytopenia by antibody-mediated cytotoxic (type II) hypersensitivity.[17] Antibodies to endothelial cells can cause inflammation of the blood vessels and vascular damage in lupus, which may be responsible for the vasculitis and neuropsychiatric symptoms seen in some SLE patients.[26] Phospholipid antibodies are associated with increased miscarriage, stillbirth, and preterm delivery in pregnant women with lupus.[30,31] Neonatal lupus, which occurs in up to 8% of babies born to pregnant women with SLE, is associated with antibodies to the nuclear antigens, **SS-A/Ro** and **SS-B/La.**[30] Symptoms are transient and resolve at 6 to 8 months of age when the maternal antibodies have cleared from the infant's circulation. In utero heart block is a serious complication that occurs in 2% of fetuses whose mothers have anti–SS-A antibodies.[30]

Connections

Type III Hypersensitivity

The immune complexes generated in SLE activate complement, inducing the generation of chemotaxins such as C5a. The activation of complement results in recruitment of neutrophils, which release lysosomal enzymes that cause injury to the surrounding tissues. This is an example of a type III hypersensitivity response, which was discussed in Chapter 14.

Clinical Signs and Symptoms

The clinical signs of SLE are extremely diverse; nonspecific symptoms such as fatigue, weight loss, malaise, fever, and anorexia are often the first to appear.[31] The disease is marked by alternating relapses or flares and periods of remission.[18] Joint involvement seems to be the most frequently reported manifestation because over 90% of patients with SLE are subject to polyarthralgias or arthritis.[19,32] Typically, the arthritis is symmetric and involves the small joints of the hands, wrists, and knees.

After joint involvement, the next most common signs are skin manifestations. These can present in various forms and are experienced by about 80% of patients with lupus.[19] An erythematous rash may appear on any area of the body exposed to UV light. Less common but perhaps more dramatic is the classic butterfly rash across the nose and cheeks that appears in some SLE patients **(Fig. 15–3).** This rash is responsible for the name *lupus,* derived from the Latin term meaning "wolf-like." In discoid lupus, skin lesions have central atrophy and scarring.

Evidence of renal involvement is present in about half of all patients with lupus; nephritis is a major cause of illness and death.[28] There are several types of lesions, but the most dangerous is diffuse proliferative glomerulonephritis, characterized by cell proliferation in the glomeruli that can lead to end-stage renal disease.[19,28,33] Other conditions involving the kidneys may include deposition of immune complexes in the subendothelial tissue and thickening of the basement membrane, all of which can lead to renal failure.

Other systemic effects may include cardiac involvement with pericarditis, tachycardia, or ventricular enlargement; pleuritis with chest pain; and neuropsychiatric manifestations such as

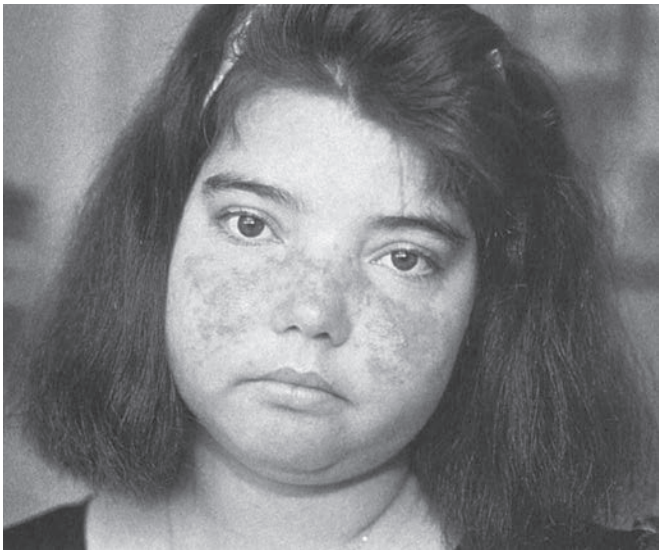


FIGURE 15–3 Butterfly rash in SLE. Characteristic rash over the cheekbones and forehead is diagnostic of SLE. The disease often begins in young adulthood and may eventually involve many organ systems. *(From Steinman L. Autoimmune disease.* Sci Am. *1993;269:107, with permission.)*

seizures, mild cognitive dysfunction, psychoses, or depression. Hematologic abnormalities such as anemia, leukopenia, thrombocytopenia, or lymphopenia can also be present.[32,33]

Drug-induced lupus differs from the more chronic form of the disease, in that symptoms usually disappear once the drug is discontinued. The most common drugs implicated are procainamide, hydralazine, chlorpromazine, isoniazid, quinidine, anticonvulsants such as methyldopa, and possibly oral contraceptives.[19,31] Typically, this is a milder form of the disease and is usually manifested as fever, arthritis, or rashes; rarely are the kidneys involved.[19,23]

In 1982, the American College of Rheumatology (ACR) established a set of clinical and immunologic criteria that could be used to define SLE for the purposes of research and surveillance; an update was published in 1997.[18] In 2012, the Systemic Lupus International Collaborating Clinics validated and further revised these criteria.[34] There are now 11 clinical criteria and 6 immunologic criteria. The clinical criteria are acute cutaneous lupus, chronic cutaneous lupus, oral ulcers, nonscarring alopecia (thinning or fragility of the hair), synovitis, serositis, renal involvement, neurological symptoms, hemolytic anemia, leukopenia, and thrombocytopenia[34] The six immunologic criteria are elevated antinuclear antibody titer, elevated anti-dsDNA titer, presence of antibody to the Sm nuclear antigen, presence of antiphospholipid antibody, low complement levels, and positive direct Coombs' test in the absence of hemolytic anemia.[34] In most cases, a patient must satisfy at least 4 of the 17 criteria, including at least one clinical criterion and one immunologic criterion, to be classified as having SLE. Although these criteria are not meant to be used for diagnosis, they reflect the major clinical and laboratory features that are associated with SLE. Some of the main laboratory tests that are helpful in diagnosis are discussed in *Laboratory Diagnosis of Systemic Lupus Erythematosus* section later in this chapter.

Treatment

The type of treatment used depends on the severity of the disease. For mild symptoms, a high dose of aspirin or other antiinflammatory drug may bring relief. For skin manifestations, antimalarials such as hydroxychloroquine or chloroquine and topical steroids are often prescribed.[19] The antimalarial drugs are thought to inhibit signaling of TLR 7, 8, and 9. Systemic corticosteroids are used for acute fulminant (severe and sudden) lupus, lupus nephritis, or central nervous system (CNS) complications because these suppress the immune response and lower antibody titers.[19,35] Other drugs have also been used; however, any immunosuppressive drug may have serious side effects and patients must be monitored closely. Monoclonal antibodies and other biological agents that target components of the immune system thought to be central to pathogenesis of lupus are being evaluated for their clinical effectiveness.[17,35]

The most common cause of death in lupus patients is infection, followed by heart disease.[31] Renal involvement is also a source of significant morbidity and mortality in this patient group. The key to successful treatment is to prevent organ damage and achieve remission. Overall, the treatments of today have come a long way in achieving this goal, as the 5-year survival rate has increased to more than 90%.[17,18,20]

Laboratory Diagnosis of Systemic Lupus Erythematosus

General laboratory tests that can be used in the initial evaluation of patients include a complete blood count (CBC), a platelet count, and urinalysis.[19] Some of the first laboratory findings in lupus patients are leukopenia and possible anemia and thrombocytopenia.[31] In addition, the erythrocyte sedimentation rate (ESR) may be elevated even though the C-reactive protein (CRP) level tends to be low or normal.

More specific laboratory tests include the quantification of complement proteins and the detection of specific autoantibodies. C3 is the most commonly measured complement protein.[19] Serum complement levels may be low during disease flares as a result of complement consumption by immune complexes. Thus, complement levels can be helpful not only in the initial diagnosis, but also for monitoring patients over time.[19,31]

When SLE is suspected, the first test typically done is a screening test for **antinuclear antibodies (ANAs)** because these are present in the majority of patients with the disease. These antibodies and the methods used to detect them are discussed in more detail in the section that follows. Phospholipid antibodies are present in some patients with lupus and are discussed in a later section.

Antinuclear Antibodies (ANAs)

Types of Antinuclear Antibodies

ANAs are autoantibodies that are directed against antigens in the nuclei of mammalian cells. ANAs are present in over 95% of patients with active lupus and are used as a major marker for the disease.[19,25,29,36] However, ANAs are not specific for SLE because they can also be detected in a significant percentage of patients with other connective tissue diseases, including mixed connective tissue disease, Sjögren's syndrome, scleroderma,

polymyositis-dermatomyositis, and RA.[37] They can also be found in some individuals with other conditions, including chronic infections, cancer, and pregnancy.[36] Furthermore, up to 5% of healthy persons and up to 30% of elderly individuals are ANA-positive.[36]

ANAs are a heterogeneous group of antibodies that have different antigen specificities. The nuclear antigens they are directed against include double-stranded (ds) and single-stranded (ss) DNA (deoxyribonucleic acid), histones, nucleosomes (DNA-histone complexes), centromere proteins, and **extractable nuclear antigens (ENAs).**[26] ENAs are a group of nuclear antigens that were so named because they were isolated in saline extracts of mammalian tissues.[38] These antigens represent a family of small nuclear proteins that are associated with uridine-rich RNA. The ENAs include ribonucleoproteins (RNP), the Sm antigen, the SS-A/Ro and SS-B/La antigens, Scl-70, Jo-1, and PM-1.[38] Some of the more common ANAs and their associated features are discussed in the text that follows and listed in **Table 15–2.**

Double-stranded DNA (dsDNA) antibodies are the most specific for SLE because they are mainly seen in patients with lupus and their levels correlate with disease activity.[25,32,36] Although they are found in only 40% to 70% of patients, the presence of these antibodies is considered diagnostic for SLE, especially when they are found in combination with low levels of the complement component C3.[39,40] Antibodies to dsDNA typically produce a peripheral or a homogeneous staining pattern on indirect immunofluorescence (IIF).[25,36,41] See the *Indirect Immunofluorescence (IIF)* section for additional information.

Antihistone antibodies can also be found in lupus patients. Histones are nucleoproteins that are essential components of chromatin. There are five major classes of histones: H1, H2A, H2B, H3, and H4. Antibodies to H2A and H2B can be detected in almost all patients with drug-induced lupus. Presence of antihistone antibody alone or combined with antibody to ssDNA supports the diagnosis of drug-induced lupus.[32,36] About 70% of other patients with SLE have elevated levels of antihistone antibodies, but the titers are usually fairly low.[19,25] High levels of antihistone antibodies tend to be associated with more active and severe SLE.[32] Antihistone antibodies are also found in RA, Felty's syndrome, Sjögren's syndrome, systemic sclerosis, and primary biliary cirrhosis, but the levels are usually lower.[25] Antihistone antibodies typically produce a homogeneous pattern in the IIF assay.[25,32,41]

Table 15–2 Common Antinuclear Antibodies

AUTOANTIBODY	CHARACTERISTICS OF ANTIGEN	IMMUNOFLUORESCENT PATTERN	DISEASE ASSOCIATION
Anti-dsDNA	dsDNA	Peripheral or homogeneous	SLE
Anti-ssDNA	Related to purines and pyrimidines	Not detected on routine screen	SLE, many other diseases
Antihistone	Different classes of histones	Homogeneous	Drug-induced SLE, other diseases
Anti-DNP	DNA-histone complex (nucleosomes)	Homogeneous	SLE, drug-induced SLE
Anti-Sm	Extractable nuclear antigen (uridine-rich RNA component)	Coarse speckled	Diagnostic for SLE
Anti-RNP	Proteins complexed with small nuclear RNA	Coarse speckled	SLE, mixed connective tissue diseases
Anti–SS-A/Ro	Proteins complexed to RNA	Finely speckled	SLE, Sjögren's syndrome, others
Anti–SS-B/La	Phosphoprotein complexed to RNA polymerase	Finely speckled	SLE, Sjögren's syndrome, others
Antinucleolar	RNA polymerase, fibrillarin, PM-1	Prominent staining of nucleoli (can be smooth, clumpy, or speckled)	SLE, systemic sclerosis
Anti–Scl-70	DNA topoisomerase I	Atypical speckled	Systemic sclerosis, scleroderma
Anti–Jo-1	Histidyl-tRNA synthetase	Fine cytoplasmic speckling	Polymyositis
Autoantibody–Anti-Centromere	Characteristics of Antigen-Antigens in the chromosome centromeres	Immunofluorescent pattern-Discrete speckled	Disease Association-CREST syndrome

Adapted from Bradwell AR, Hughes RG, Karim AR. Immunofluorescent antinuclear antibody tests. In: Detrick B, Hamilton RG, Folds, JD, eds. Manual of Molecular and Clinical Laboratory Immunology. *7th ed. Washington, DC: ASM Press; 2006:996–997.*

DNA = deoxyribonucleic acid; DNP = deoxyribonucleoprotein; RNA = ribonucleic acid; RNP = ribonucleoprotein; SLE = systemic lupus erythematosus.

Nucleosome antibodies are stimulated by DNA-histone complexes, known as nucleosomes, or deoxyribonucleoprotein (DNP). These antibodies are directed only against the complexes and not against DNA or the individual histones. Nucleosome antibodies are found in about 85% of patients with SLE and their levels correlate with disease severity.[25] They typically produce a homogeneous pattern in the IIF assay.[41]

Antibody to the **Sm antigen** is specific for lupus because it is not found in other autoimmune diseases. However, it is found in only 20% to 40% of patients with SLE, depending on the race of the population.[25] It is unclear whether titers correlate with disease activity. Antibody to a preparation of this ENA was first described in a patient named Smith, hence the name *anti-Sm antibody*. The anti-Sm antibody produces a coarse speckled pattern of nuclear fluorescence on IIF.[41]

Anti-RNP antibody is directed against RNP, which consists of several nonhistone proteins complexed to a small nuclear RNA called *U1-nRNP* (*U* for "uridine-rich"). RNP forms complexes with the Sm antigen in the nucleus, and antisera to these antigens produce a pattern of partial identity when they are reacted in the Ouchterlony double immunodiffusion test (see discussion and Figure 15–7 in the text that follows). In the IIF assay, anti-nRNP produces a coarse speckled pattern.[41] Antibodies to RNP are detected in 20% to 30% of patients with SLE, but are also found at a high titer in individuals with mixed connective tissue disease and in lower levels in patients with other autoimmune rheumatic diseases such as systemic sclerosis, Sjögren's syndrome, and RA.[25,32,36]

Lupus patients can also produce antibodies to another family of ENAs called SS-A/Ro and SS-B/La. These antigens consist of small, uridine-rich RNAs complexed to cellular proteins and were given the prefix of SS- because a large percentage of patients with Sjögren's syndrome (~70%) possess antibodies to the antigens.[36] Anti–SS-A/Ro also appears in approximately 24% to 60% of patients with SLE and has been closely associated with the presence of nephritis, vasculitis, lymphadenopathy, photosensitivity, and hematologic manifestations such as leukopenia.[25,32] Antibodies to SS-B/La are found in only 9% to 35% of patients with SLE and all of these have anti–SS-A/Ro.[40] The SS-B/La antibody is most often found in patients who have cutaneous manifestations of SLE, especially photosensitivity dermatitis.[32] Antibodies to both SS-A/Ro and SS-B/La can cross the placenta and have been associated with neonatal lupus.[25] Newborns that have anti–SS-A/Ro are more likely to develop cardiac manifestations, whereas those who have anti–SS-B/La are more likely to have other symptoms such as skin lesions. To detect the presence of these antibodies on IIF, human tissue culture cells such as HEp-2 (human epithelial) must be used because SS-A/Ro and SS-B/La antigens are not found in mouse or rat liver and kidney. A finely speckled pattern is evident.[41-43] Antibodies to the SS-A/Ro antigen are best detected on IIF if a special cell line, HEp-2000®, is used; these cells have been genetically transfected so that they hyperexpress the antigen.

The **nucleolus** is a prominent structure within the nucleus where transcription and processing of ribosomal RNA and assembly of the ribosomes takes place. Staining of the nucleolus in IIF is mainly caused by antibodies to one of three nucleolar components: fibrillarin, RNA polymerase I, and PM-1.[43] Antibody to fibrillarin is common in systemic sclerosis (also known as scleroderma) and is indicated by clumpy nucleolar fluorescence in the IIF assay.[36,40,43] Scleroderma is an autoimmune disease that primarily involves the skin and the blood vessels. Antibodies to RNA polymerase are also associated with scleroderma, but produce a speckled nucleolar pattern in IIF. Homogeneous staining of the nucleolus is associated with antibodies to the PM-1 antigen (also known as PM/Scl) and is found in polymyositis and systemic sclerosis.[36,43]

Anticentromere antibodies bind to proteins in the middle region of a chromosome where the sister chromatids are joined. These antibodies are directed against three centromere antigens of molecular weights 16kDa, 80kDa, and 120kDa.[32] They are found in 50% to 80% of patients with the **CREST syndrome**, a subset of scleroderma named after its five major features: calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia.[32] In the IIF assay, centromere antibodies produce discrete speckled staining in the nuclei of the cells.[42,43]

Methods of ANA Detection

A variety of methods have been developed to detect ANAs in patient serum. These include IIF, immunoperoxidase staining, enzyme-linked immunosorbent assay (ELISA), microsphere multiplex immunoassays (MIA), radioimmunoassay (RIA), immunodiffusion, immunoblotting (Western blot), dot blot, immunoelectrophoresis, and microarray.[32,38] Some of the more commonly used assays are discussed in the text that follows.

Indirect Immunofluorescence (IIF)

Fluorescent antinuclear antibody (FANA) testing has been the most widely used and accepted test because it is highly sensitive, detects a wide range of antibodies, and is inexpensive and easy to perform.[36,38] In addition, the antigens are in their original form and location within the cells used in the test.[25] This test has several applications. It has been used as a screening test to identify patients who have ANAs as well as patients who are negative for ANAs to provide guidance in selection of follow-up assays based on immunofluorescence patterns and to monitor ANA titers in patients during treatment.[36]

The IIF test uses a commercially prepared microscope slide onto which nucleated cells have been fixed. The human epithelial cell line, HEp-2, is the standard substrate for clinical laboratories worldwide.[25,36,38] HEp-2 cells are used because they have large nuclei with high antigen expression, allowing for high sensitivity and facilitating visualization of results.[36] Patient serum is incubated with the HEp-2 cell-coated slide, washed to remove unbound antibodies, and then allowed to react with an anti-human immunoglobulin labeled with a fluorescent tag to detect bound IgG or total immunoglobulins. Following a second incubation and wash, the slide is mounted and viewed under a fluorescent microscope using 400X magnification (40X objective and 10X eyepiece). The principle of this assay is shown in **Figure 15–4.**

The screening test is commonly performed with a 1:40 or 1:80 dilution of patient serum in order to avoid detecting low positive titers that may be seen in healthy persons, although

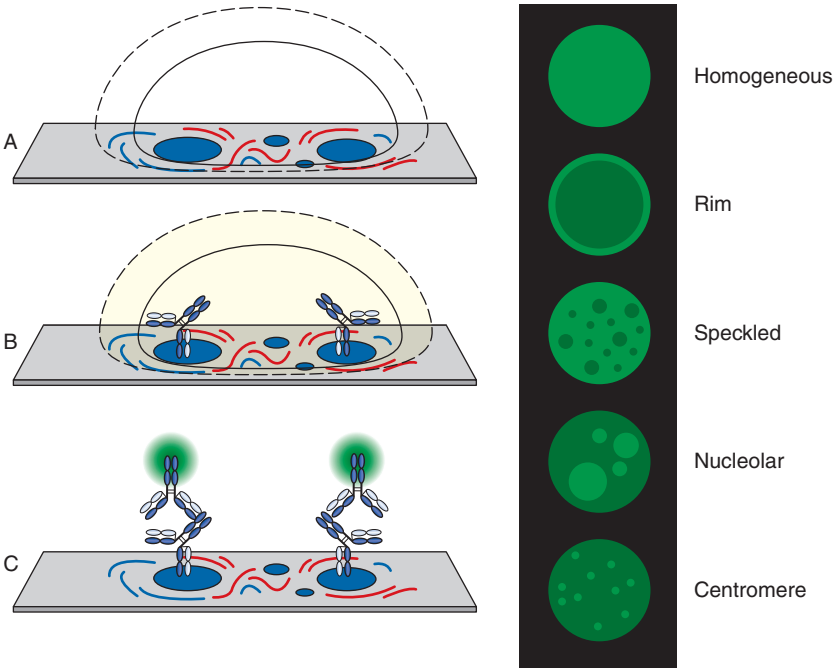


FIGURE 15–4 Fluorescent ANA test principle. (A) Microscope slide coated with HEp-2 cells (the plasma and nuclear membranes have been permeabilized). (B) Patient serum (containing antinuclear antibodies) is applied. (C) After washing, fluorescent-labeled anti-human Ig is added to detect bound autoantibodies. After a final wash, the slide is read under a fluorescent microscope. Five typical patterns in nondividing cells are shown on the right.

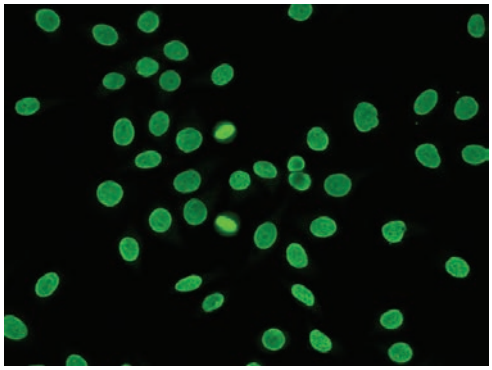
the exact dilution used for screening may vary with the laboratory and population being tested.[36] A titer of ≥160 is generally considered to be clinically significant.[38] Patient samples that are positive on the ANA screen are serially diluted and tested to determine the antibody titer, specified as the highest dilution to show nuclear fluorescence. Inclusion of a 1+ endpoint control serum can help to standardize the readings by setting the minimum level of fluorescence that is considered positive.

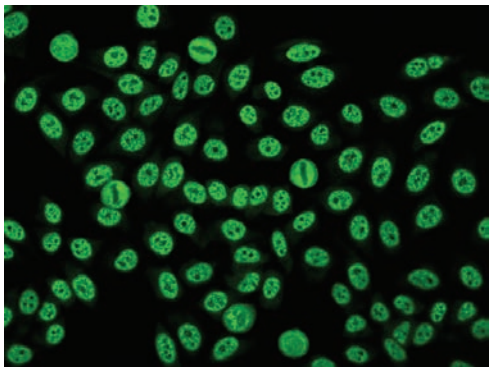
In addition to the antibody titer, the pattern of fluorescence is also reported because it can provide clues about the autoantibody present and associated diseases. Fluorescence can be within the nucleus, cytoplasm, or mitotic structures of the cell.[38] Although about 40 patterns of immunofluorescence are possible, some of the most common nuclear patterns are homogeneous, peripheral, speckled, nucleolar, and centromere **(Fig. 15–5).**[36,42-44]

- Homogeneous (also known as diffuse)—This pattern is characterized by uniform staining of the entire nucleus in interphase cells and of the condensed chromosomal region in metaphase cells. It is associated with antibodies to dsDNA (also known as native or nDNA), histones, and deoxyribonuclear protein (DNP). The homogenous pattern is found in patients with SLE, drug-induced lupus, and many other autoimmune diseases.
- Peripheral (rim or outline)—In this pattern, diffuse staining is seen throughout the nucleus, but there is a greater staining intensity around the outer circle surrounding the nucleus in interphase cells. Dividing cells show strong staining of the condensed chromatin. This pattern is primarily caused by antibodies to dsDNA and is highly specific for SLE.
- Speckled—This pattern is characterized by discrete, fluorescent specks throughout the nuclei of interphase cells. Staining is absent in the nucleolus and in the chromatin region in dividing cells. The specks can be fine or coarse, depending on the autoantibody present. The speckled pattern is associated with antibodies to ENAs and can be found in patients with SLE, Sjögren's syndrome, systemic sclerosis, and other systemic autoimmune rheumatic diseases.
- Nucleolar—Prominent staining of the nucleoli within the nuclei of interphase cells is seen in this pattern. The size, shape, and number of the nucleoli per cell are variable and staining can be smooth, clumpy, or speckled, depending on the type of antibody present. Staining may or may not be present in the dividing cells. The nucleolar pattern is primarily caused by antibodies to RNA and RNP and is seen mainly in patients with scleroderma, but can also be present in patients with other connective tissue diseases.
- Centromere—Numerous discrete speckles are seen in the nuclei of interphase cells and the chromatin of dividing cells. Most cells have 46 speckles, representing the number of chromosomes. This pattern is caused by antibodies to proteins in the centromeres of the chromosomes and is found mainly in patients with the CREST syndrome.

Mixed patterns can also be observed; in some cases, one pattern may totally or partially obscure another (for example, a homogeneous pattern might cover up a speckled pattern). In these cases, titration of the patient serum can help to distinguish between the separate patterns and an antibody titer would be reported for each one. If the FANA test is negative, no clearly discernable fluorescent pattern is observed in the nuclei of the cells. Up to 5% of SLE patients test negative, so this test cannot be used to absolutely rule out SLE.

Although the IIF method is considered the gold standard for ANA testing, it also has some important limitations. The test is time-consuming and requires a significant amount of


Homogeneous pattern


Speckled pattern

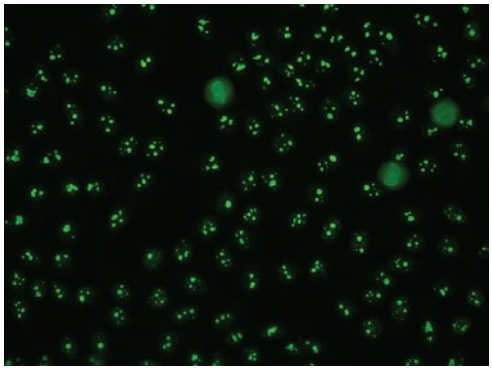

Nucleolar pattern

FIGURE 15–5 Photomicrographs showing three patterns of immunofluorescent staining for antinuclear antibodies. Examples of predominant staining patterns obtained are homogeneous—staining of the entire nucleus; speckled pattern—staining throughout the nucleus; and nucleolar pattern—staining of the nucleolus. *(Courtesy of DiaSorin, Inc., with permission.)*

technical expertise to correctly identify the fluorescent patterns. Automated IIF ANA assays have been developed that may help to reduce these problems and also allow for storage and retrieval of the fluorescent images.[41] These assays need to be validated further but hold promise for the future. However, the autoantibodies present cannot be precisely identified on the basis of the fluorescent patterns and additional tests are needed.[38,41] Some of the more common tests used to characterize ANAs are described in the section that follows.

Other Assays for ANA

ELISA and CLIA. ELISAs and chemiluminescence immunoassays for ANAs have become very popular, especially for high volume testing laboratories, because they are automated, easy to perform, and yield objective results.[38,45] These assays can test for a broad range of antibodies if multiple nuclear antigens are coated onto a single test well, or for specific ANAs if each well is coated with a single antigen. The antigens used in commercial kits are derived from tissue extracts or are produced by recombinant technology. Because of their advantages, many laboratories are using ELISA methods to screen for the presence of ANAs in addition to identifying specific ANAs. However, there is a large variation in the performance of tests produced by different manufacturers, which is influenced by the antigen preparation used. For example, one study found sensitivities of ELISA assays ranging from 69% to 98%, and specificities from 81% to 98% when they were compared with the IIF ANA method.[38] These findings suggest that immunoassays may miss a significant proportion of ANA-positive patients and also yield a significant number of false-positive results. Based on such studies, the ACR has recommended that the IIF test remain the gold standard for ANA testing and that clinical laboratories should specify the method they used when they are reporting results.[46]

Microsphere Multiplex Immunoassay (MIA). MIAs are also very popular because they are amenable to automated, high throughput testing with objective results. In these assays, the patient serum is incubated in a microtiter plate well containing a suspension of polystyrene microspheres that are coated with individual nuclear antigens or with a HEp-2 extract. Beads containing specific antigens can be differentiated by their unique shade of red created by a specific combination of infrared and red fluorescent dyes. Antibodies in the patient serum will bind only to the beads containing their specified antigens. Following a washing step to remove unbound antibodies, a phycoerythrin-labeled anti-human IgG is added. The conjugate will bind only to the beads that have bound patient antibodies and excess conjugate is removed by washing. The bead suspension is analyzed for fluorescence by a flow cytometer that has two lasers, one that identifies each bead and another that detects the amount of fluorescent conjugate attached. MIAs are more efficient than ELISAs because they allow testing for multiple antibody specificities to be performed in a single tube. Studies have shown that MIAs, like ELISAs can be variable in terms of their sensitivity and specificity when compared with IIF and that test performance varies with the specific ANA detected.[47,48] Hopefully, the future will bring improvement in assays such as the MIA and ELISA that will allow laboratories to report accurate results while reaping the full benefits of automation.

Immunofluorescence Using *Crithidia luciliae*. This IIF assay is used to detect antibodies to dsDNA. In testing for these antibodies, a purified antigen preparation that is free from single-stranded DNA (ssDNA) must be used because antibodies to ssDNA occur in many individuals with other autoimmune or inflammatory diseases. One particularly sensitive assay for dsDNA is an IIF test using a hemoflagellate organism called *Crithidia luciliae* as the substrate.[49] This trypanosome has a circular organelle called a kinetoplast that is composed mainly of dsDNA **(Fig. 15–6).** In this test, patient serum is incubated on a microscope slide coated with *C luciliae* organisms and binding

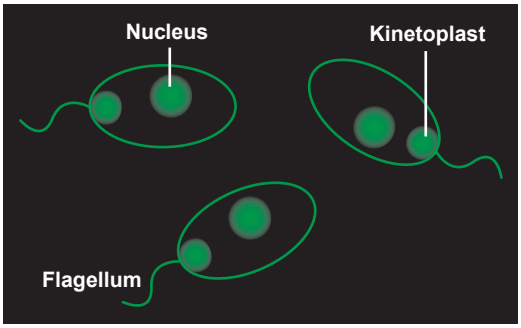


FIGURE 15–6 An illustration of *Crithidia luciliae* fixed to a microscope slide. A positive test for dsDNA will show green fluorescence in the nucleus and kinetoplast.

is detected with a fluorescent-labeled anti-Ig conjugate. Washing of the slide is performed after each step to remove unbound antibody. A positive test is indicated by a brightly stained kinetoplast. This test has a high degree of specificity, although it is less sensitive than other FANA tests.[36,45]

Ouchterlony test. ANAs can also be detected by immunodiffusion. Typically, this method is used to determine the immunologic specificity of a positive FANA test, particularly when a speckled pattern is observed. Ouchterlony double diffusion detects antibody to several of the small nuclear proteins, or ENA. These include antibodies to Sm, RNP, SS-A/Ro, SS-B/La, Scl-70 (DNA topoisomerase I), and Jo-1 (see the previous descriptions and Table 15–2). A solution containing ENA antigens is placed in a central well of an agarose plate and patient samples and controls are placed in the surrounding wells, as indicated in **Figure 15–7.** A visible precipitate is formed between the ENA well and each surrounding well that contains antibodies to any of the ENAs present (e.g., anti-Sm, anti-RNP, or antibodies to other ENAs). If an outer well does not contain antibodies to any of the ENAs, no precipitate is formed between that well and the center well. Samples in the outer wells are identified as containing antibody to a particular ENA by comparing their reactivity patterns of identity, nonidentity, or partial identity to control sera containing specific ENA antibodies. A positive reaction is indicated by immunoprecipitation lines of serological identity. (Refer to Chapter 10 for a discussion of Ouchterlony test principles.) Although this type of testing is not as sensitive as some other techniques, it is highly specific.[40]

Antiphospholipid Antibodies

Antiphospholipid antibodies are a heterogeneous group of antibodies that bind to phospholipids alone or phospholipids complexed with protein. They can affect every organ in the body, but they are especially associated with deep-vein and arterial thrombosis and with recurrent pregnancy loss.[25,50,51] Antiphospholipid antibodies have been found in up to 60% of patients with lupus, but they are also associated with several other disease states.[25,32,52] They can be identified by their ability to cause false-positive results in nontreponemal tests for syphilis, the lupus anticoagulant assay, and immunoassays for antibodies to cardiolipin or other phospholipids.[32]

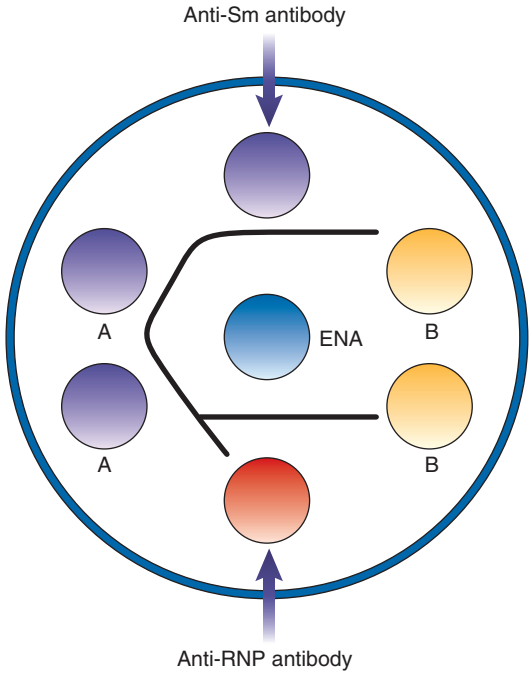


FIGURE 15–7 Extractable nuclear antibody immunodiffusion pattern. A mixture of extractable nuclear antigens (ENA), including RNP, Sm, and other soluble nuclear antigens, is placed in a central well in an agarose gel. Sm antibody and RNP antibody are run as positive controls and patient samples are placed between the controls. The pattern of precipitin lines formed indicates the antibodies present in patient serum. The arc of serological identity formed between Sm and patient A indicates that serum A contains anti-Sm antibodies. The arc of partial identity formed between serum A and RNP occurs because RNP is always found complexed to Sm antigen. RNP antibodies are not present. Serum B contains neither Sm nor RNP antibody.

The lupus anticoagulant, one of several types of antiphospholipid antibodies, was so named because it produces a prolonged activated partial thromboplastin time (APTT) and prothrombin time (PT). In patients with antiphospholipid antibodies, the APTT may be prolonged, but it is not corrected by mixing with normal plasma.[31] Ironically, patients with this antibody have an increased risk of clotting and spontaneous abortion. Platelet function may also be affected and thrombocytopenia may be present. In addition to determining the APTT and PT, there are several EIAs for antiphospholipid antibodies that are sensitive and relatively simple to perform.[25,50-52] If these antibodies are suspected, factor assays may also need to be performed to rule out any factor deficiencies or factor-specific inhibitors.

Rheumatoid Arthritis (RA)

RA is another example of a systemic autoimmune disorder. It affects about 0.5% to 1.0% of the adult population, but prevalence varies with ethnicity and geographic location.[53,54] Typically, it strikes individuals between the ages of 25 and 55.[54] Women are three times as likely to be affected as men; in addition, the prevalence of the disease is highest in women who

are more than 65 years of age.[53,55] RA can be characterized as a chronic, symmetric, and erosive arthritis of the peripheral joints that can also affect multiple organs such as the heart and the lungs.[32,54] Progress of the disease varies because there may be spontaneous remissions or an increasingly active disease in some individuals that rapidly progresses to joint deformity and disability.[56] In addition to a decline in functional ability, there is a reduced life expectancy.[54,57] In recent years, RA patients have experienced less disability and a better quality of life because of advances in therapy and earlier treatment (see the *Treatment* section in the text that follows).

Etiology

Associations of RA with more than 30 genetic regions have been discovered. The strongest associations have been between a subset of patients with RA and specific HLA-DRB1 alleles or *PTPN22* gene polymorphisms.[53,54,58] These patients are positive for **rheumatoid factor (RF)** or antibodies to CCP (see the section on *Laboratory Diagnosis*). The strongest environmental risk factor for RA is believed to be cigarette smoking, which doubles the risk of developing the disease.[53,55] Other factors have also been implicated, but their associations are weaker.[53,54] Numerous infectious agents have been proposed as possible triggering antigens for RA, but a cause-effect relationship has not been proven.[54,55]

Immunopathology

The pathology of RA is caused by an inflammatory process that results in the destruction of bone and cartilage. The lesions in rheumatoid joints show an increase in cells lining the synovial membrane and formation of a *pannus,* a sheet of inflammatory granulation tissue that grows into the joint space and invades the cartilage. Infiltration of the inflamed synovium with T and B lymphocytes, plasma cells, dendritic cells, mast cells, and granulocytes is evidence of immunologic activity within the joint.[54]

The balance between proinflammatory and anti-inflammatory cytokines in RA appears to be tipped toward continual inflammation. Proinflammatory cytokines found in synovial fluid that contribute to inflammation include IL-1, IL-6, IL-17, and TNF-α (see Chapter 6).[56,58] TNF-α plays a key role in the inflammatory process by stimulating the production of other cytokines and facilitating the transport of white blood cells (WBCs) to the affected areas.[54,56] The proinflammatory cytokines trigger the release of matrix metalloproteinases from fibroblasts and macrophages; these enzymes degrade important structural proteins in the cartilage.[58]

Local bone erosion is another feature that is characteristic of the pathology in RA. Multinucleated giant cells called osteoclasts are central to the structural damage that is seen in the bones. Osteoclasts absorb bone as part of the normal bone remodeling process that occurs in the body in response to growth and repair of damaged bone. Normally, there is a good balance between bone production and destruction. However, in RA, the osteoclasts become overly activated in the inflammatory environment of the joints. TNF-α, in conjunction with other cytokines and a molecule called RANKL (receptor activator of nuclear factor kappa-B ligand), induces the differentiation of osteoclasts and inhibits bone formation. Significant local bone destruction occurs and there is also generalized osteoporosis throughout the body.[54,58]

It is not known what role autoantibodies play in the initiation of the inflammatory response. Two key antibodies found in the disease are RF and anti-CCP. RF is an antibody that is most often of the IgM class and is directed against the Fc portion of IgG. It has been postulated that RFs may play a role in the pathogenesis of RA by increasing macrophage activity and enhancing antigen presentation to T cells by APCs.[59]

Antibodies to cyclic citrullinated proteins **(anticyclic citrullinated peptide antibody [anti-CCP or ACPA])** are a second major type of antibody associated with RA. Citrullinated proteins contain an atypical amino acid called *citrulline,* which is generated when the enzyme peptidyl arginine deiminase (PAD) modifies the amino acid arginine by replacing an NH_2 group with a neutral oxygen.[60,61] This enzyme is associated with granulocytes, monocytes, and macrophages, as well as other types of cells. Death of granulocytes and macrophages triggers production of citrullinated proteins; overexpression of these antigens may provoke an immune response in individuals with certain HLA-DRB1 alleles.[61,62] These antibodies can react with citrulline-containing components of the matrix, including filaggrin, keratin, fibrinogen, and vimentin, and are thought to correlate with the pathogenesis of RA.[54] Antinuclear antibodies are also present in some RA patients (see the previous *Antinuclear Antibodies* section).

In RA, autoantibodies such as RF and anti-CCP are thought to combine with their specified antigen, and the resulting immune complexes become deposited in the joints, resulting in a type III (or immune complex) hypersensitivity reaction. The complement protein C1 binds to the immune complexes, activating the classical complement cascade. During this process, C3a and C5a are generated, which act as chemotactic factors for neutrophils and macrophages. The continual presence of these cells and their associated cytokines leads to chronic inflammation, which damages the synovium itself.[54]

Clinical Signs and Symptoms

The initial symptoms of RA involve the joints, tendons, and bursae.[54] The RA patient commonly experiences nonspecific symptoms such as malaise, fatigue, fever, weight loss, and transient joint pain that begin in the small joints of the hands and feet. The joints are typically affected in a symmetric fashion. Joint stiffness and pain are usually present in the morning and improve during the day. The disease can progress to the larger joints, often affecting the knees, hips, elbows, shoulders, and cervical spine. Joint pain can lead to muscle spasms and limitation of motion. The ongoing inflammation, if left untreated, results in permanent joint dysfunction and deformity. Osteoporosis (bone erosion) occurs in about 20% to 30% of RA patients because of the inflammatory environment of the joints and activation of osteoclasts.[54]

Some patients with RA develop extra-articular manifestations, which occur outside of the joints.[54] These patients are most likely to have had a history of smoking, early disease

onset, and test positive for anti-CCP or RF. Extra-articular manifestations include the formation of subcutaneous nodules, pericarditis, lymphadenopathy, splenomegaly, interstitial lung disease, or vasculitis. Some patients have small masses of tissue called *nodules* over the bones. Nodules can also be found in the myocardium, pericardium, heart valves, pleura, lungs, spleen, and larynx. About 10% of patients with RA develop secondary Sjögren's syndrome, an autoimmune disorder characterized by the presence of dry eyes and dry mouth in addition to connective tissue disease. A small percent develop Felty's syndrome, a combination of chronic, nodular RA coupled with neutropenia and splenomegaly. The most common cause of death in RA is cardiovascular disease, presumably because of the acceleration of arteriosclerosis by proinflammatory cytokines released during the disease process.[55]

Diagnostic classification criteria for RA were established by the ACR in 1987 to standardize identification of RA patients for clinical trials.[63] These criteria were revised in 2010 in collaboration with the European League Against Rheumatism in order to improve the identification of patients in the early stages of disease who could benefit from new treatments.[64,65] The criteria are based on the number of joints involved, duration of symptoms, serology results for RF and anti-CCP, serum level of the acute-phase reactant, CRP, and the ESR. These important laboratory tests will be discussed in more detail.

Treatment

In the past, therapy for RA was primarily based on nonsteroidal anti-inflammatory drugs (NSAIDs) such as salicylates (aspirin) and ibuprofen. Although these agents can be used to reduce local swelling and pain, they are no longer the dominant treatment for RA. The discovery that joint destruction occurs early in the disease has prompted more aggressive treatment and the development of new drugs to prevent disease progression.[54,55,66] Disease-modifying anti-rheumatic drugs (DMARDs), most notably methotrexate, are now prescribed at the time of diagnosis. Methotrexate is thought to act by inhibiting adenosine metabolism and T-cell activation.[58] If methotrexate alone does not work, it can be combined with other DMARDs or with biological agents.

Biological agents have revolutionized the way that RA is treated because they target components of the immune system that are central to the pathogenesis of the disease.[53,54,66,67] Several key therapies for RA block the activity of the cytokine, TNF-α. Agents that act against TNF-α are classified into two categories: (1) monoclonal antibodies to TNF-α (e.g., infliximab, adalimumab, certolizumab, golimumab) and (2) TNF-α receptors fused to an IgG molecule (etanercept). All of these agents specifically target and neutralize TNF-α and have demonstrated effectiveness in halting joint damage.[67] DMARDs and biological agents have become the mainstay of RA treatment because they act on specific components of the inflammatory response and are effective in slowing the progression of joint erosion. Use of these treatments has resulted in higher rates of disease-free periods (remission) and has dramatically improved the quality of life and prognosis of RA patients.[54,66] Although these agents are effective in treating RA, patients must be monitored closely because they are at greater risk for infection.

Laboratory Diagnosis of Rheumatoid Arthritis

Diagnosis of RA is based on a combination of clinical manifestations, radiographic findings, and laboratory testing. RF is the antibody that is most often tested to aid in making the initial diagnosis. The importance of testing for the presence of RF is also reflected in the fact that it is one of the classification criteria for RA.[64,65] Recall that RF is an autoantibody, usually of the IgM class, that reacts with the Fc portion of IgG. Approximately 70% to 90% of patients with RA test positive for RF.[54,59] Thus, a negative result does not rule out the presence of RA. Conversely, a positive test result is not specific for RA because RF is also present in about 5% of healthy individuals and in 10% to 25% of those over the age of 65.[59] In addition, RF can be found in patients with other connective tissue diseases such as SLE, Sjögren's syndrome, scleroderma, and mixed connective tissue disease, as well as in people with some chronic infections.[54,59]

Manual agglutination tests using charcoal or latex particles coated with IgG have been used for many years to detect RF.[59,61] These tests, however, only detect the IgM isotype, found in about 75% of patients, and have been largely replaced by ELISA, chemiluminescence immunoassay, and nephelometric methods, which are automated, have greater precision and sensitivity, and can also detect other RF isotypes.[54,59] Testing for IgG and IgA RFs increases test specificity. The presence of both IgM and IgA, or of all three isotypes, rarely occurs in other disease states, which may help in making a differential diagnosis. In addition, testing for specific RF isotypes may have some prognostic value. Some studies have found that IgM RF levels decrease with clinical response to therapy and that elevation of IgA early in the disease appears to be associated with a poor response to TNF-α inhibitors and a worse prognosis.[59]

Laboratory testing for antibody to cyclic citrullinated peptides (anti-CCP) has been added to the classification criteria to increase the specificity for RA.[64,65] These assays are performed mainly by ELISA and use a circular synthetic form of citrullinated peptides to increase test sensitivity, which ranges from 39% to 50%.[61,62,68] About 20% to 30% of RF-negative patients are positive for anti-CCP and the presence of anti-CCP precedes the onset of RA by several years, making it a better marker for early disease.[61,62,68] The presence of anti-CCP is also associated with an increased likelihood to develop clinically significant disease activity with a worse prognosis.[62,68] Importantly, specificity of anti-CCP for RA is higher than RF, ranging from 91% to 93%.[68] Furthermore, the combination of anti-CCP and RF testing has a specificity of 98% to 100%, providing for a more accurate diagnosis of RA by allowing for better differentiation from other forms of arthritis.[68] For these reasons, most rheumatologists now recommend testing for both RF and anti-CCP.[62]

Low titers of ANAs are present in about 40% of patients. The pattern most frequently identified is the speckled pattern directed against RNP. The significance of this group of autoantibodies remains unclear because they do not appear to be directly related to pathogenesis.

Once a diagnosis of RA is made, the most helpful tests for following the progress of the disease are general indicators of inflammation, such as measurement of ESR, CRP, and complement components.[56] Typically, CRP and ESR are elevated and the levels of serum complement components are normal or increased because of increased acute-phase reactivity. CRP levels correlate well with disease activity because levels reflect the intensity of the inflammatory response.[56]

Granulomatosis With Polyangiitis (Wegener's Granulomatosis)

Granulomatosis with polyangiitis (GPA), also known as **Wegener's granulomatosis** or **WG,** is a rare autoimmune disease involving inflammation of the small- to medium-sized blood vessels, or *vasculitis.* The disease usually begins with a localized inflammation of the upper and lower respiratory tract. General symptoms include fever, malaise, arthralgias, anorexia, and weight loss. The majority of patients progress to develop a more systemic form of the disease that can affect any organ system.[69-71]

Virtually all patients have symptoms that affect the upper respiratory system and lungs.[69,71] Symptoms of the upper airway include severe or persistent rhinorrhea ("runny nose"), rhinitis, sinusitis, oral or nasal ulcers, and gingivitis. Damage to the nasal mucosa leads to drying of the nasal membranes, which become susceptible to infection and frequent bleeding. Chronic otitis media can cause perforation and scarring of the eardrums, leading to hearing loss. Tissue damage can lead to perforation of the nasal septum or collapse of the bridge of the nose. Pulmonary infiltrates are commonly observed upon x-ray of the lungs. Although patients can experience coughing, shortness of breath, chest pain, or hemoptysis (coughing up blood), they may be asymptomatic.

As the disease progresses, other organ systems become involved.[69,71] The majority of patients have renal involvement, which can range from mild glomerulonephritis with little functional impairment to severe glomerulonephritis that can rapidly lead to kidney failure. Most patients experience pain and arthritis of the large joints, which is usually symmetric but not deforming. Skin lesions may occur in patients with WG. Many patients have ocular manifestations that can potentially lead to vision loss. Other areas of the body that can be affected include the nervous system, heart, and thyroid gland. Without treatment, more than 90% of patients will die within 2 years of diagnosis.[70]

The etiology of GPA is unknown, but multiple genes are thought to be involved. The HLA-DPB1*0401 allele has been found to have a strong association with GPA in Caucasian patients, whereas the HLA-DRB1*0901 and HLA-DRB*1501 alleles are associated with increased risk in Asian and African American populations, respectively.[72-74] Although environmental factors have not been definitively identified, chronic nasal infection with *Staphylococcus aureus* bacteria has been associated with a greater rate of relapse in patients with WG.[71] *S aureus* may induce molecular mimicry because it contains peptides that bear similarity to the proteinase 3 (PR3) autoantigen.[72] Exposure to silica or to certain drugs such as hydralazine and penicillamine may also be risk factors.[72]

Most patients with GPA have antibodies to neutrophil cytoplasmic antigens; in 80% of these, the antibody is directed against an enzyme found in the azurophilic granules of neutrophils called PR3.[70,71] Antibody to the PR3 antigen is thought to play a role in the pathophysiology of the systemic vasculitis seen in GPA.[70] Events such as chronic infections can result in the release of the proinflammatory cytokine TNF-α, which stimulates neutrophils and results in migration of the PR3 antigen from the granules to the neutrophil membrane. Binding of PR3 antibodies to the PR3 antigen and Fcγ-receptors on the cell surface result in activation of the neutrophils, which adhere to the endothelial cells lining the blood vessels. There, they release reactive oxygen species and proteolytic enzymes that damage the vascular endothelium. A Th1 response follows, with release of cytokines that induce macrophage maturation and the formation of granulomatous lesions. Chronic activation of T cells within these lesions is thought to induce differentiation of autoreactive B cells into plasma cells that produce antibodies to PR3, thus perpetuating the response.

Therapy for GPA is directed toward suppression of this inflammatory response. Patients with severe forms of GPA are treated with a combination of prednisone and cyclophosphamide, which produces remission with resolution of the inflammatory lesions in most patients.[69,75] Biological agents, such as the anti-CD20 monoclonal antibody rituximab, show promise in providing an effective alternative to cyclophosphamide with reduced toxicity. Patients must then be maintained on a less potent immunosuppressive drug regimen, but infections are the main cause of death in about half of GPA patients.[75] Nonetheless, immunosuppressive therapy has greatly improved patient outcomes, with survival rates as high as 80% at 10 years after diagnosis.[71]

Clinical Criteria and Laboratory Diagnosis

According to the criteria published by the ACR, patients are classified as having WG/GPA if two of the following conditions are met: (1) nasal or oral inflammation with oral ulcers or purulent or bloody nasal discharge; (2) abnormal chest x-ray, showing presence of nodules, fixed infiltrates, or cavities; (3) urinary sediment with microhematuria or RBC casts; and (4) granulomatous inflammation on biopsy.[76] Since then, clinicians have found it useful to include a positive **antineutrophil cytoplasmic antibody (ANCA)** result (specifically, antibody to proteinase 3); see the Antineutrophil Cytoplasmic Antibodies (ANCA) section in the text that follows.[71]

General laboratory findings include a normochromic, normocytic anemia; leukocytosis; eosinophilia; and an elevated ESR. In addition, there may be a decreased concentration of albumin in the blood and mild to severe renal insufficiency. Urinalysis typically shows microhematuria, proteinuria, and cellular casts. Serological findings include an elevated CRP, elevated immunoglobulin levels, positive ANCAs (c-ANCA pattern; see the text that follows), and possibly other autoantibodies, such as RF and ANAs.[69]

Antineutrophil Cytoplasmic Antibodies (ANCAs)

ANCAs are autoantibodies that are produced against proteins that are present in the neutrophil granules. These antibodies are strongly associated with three syndromes involving vascular inflammation: GPA or WG, microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA; formerly known as Churg-Strauss syndrome); these syndromes are collectively known as ANCA-associated vasculitides (AAV).[72,77] In patients with GPA, ANCAs are mainly directed against the PR3 antigen, whereas in MPA and EGPA they are usually specific for myeloperoxidase (MPO).[72]

International consensus guidelines require that patient sera be screened for ANCAs by IIF using ethanol-fixed leukocytes as the cellular substrate.[78,79] Ethanol treatment permeabilizes the granule membranes, allowing for migration of the contents.[80] In this method, patient serum is incubated with a microscope slide containing ethanol-fixed leukocytes. Following incubation, the slide is washed to remove unbound serum and an anti-human IgG, FITC-labeled conjugate is added. Following a second incubation and wash step, the slide is viewed under a fluorescent microscope for staining of the neutrophils. Lymphocyte staining should not be present; if it is, it should be minimal.

Two patterns of fluorescence can be observed: *cytoplasmic,* also known as *c-ANCA,* and *perinuclear,* or *p-ANCA.*[80,81] The c-ANCA pattern is primarily caused by PR3-ANCA and appears as a diffuse, granular staining in the cytoplasm of the neutrophils **(Fig. 15–8A).** Staining is most intense in the center of the cell between the nuclear lobes and gradually fades at the outer edges of the cytoplasm. In the p-ANCA pattern, fluorescence surrounds the lobes of the nucleus, blending them together so that individual lobes cannot be distinguished (Fig. 15–8B). This is because the p-ANCA pattern is caused by antibodies against positively charged antigens such as MPO that migrate out of the granules after ethanol fixation and are attracted toward the negatively charged nucleus. **Table 15–3** summarizes the main features of ANCAs.

It is important to distinguish the p-ANCA pattern from ANAs, which can also stain the nuclei of the neutrophils. With ANAs, the nuclear lobes may be more clearly separated, speckled staining may be present, and the nuclei of the lymphocytes will also be stained. In addition, if a true p-ANCA pattern is present, ANA testing using HEp-2 cells as the substrate in IIF should be negative.[81] For more definitive differentiation, the test can be repeated using microscope slides with formalin-fixed leukocytes. Formalin is a cross-linking fixative that prevents the migration of antigens out of the granules. Therefore, in the presence of antibodies to MPO or other positively charged proteins, perinuclear staining will be prevented and intense, granular staining of the cytoplasm will be seen that resembles c-ANCA.

ANCA detection by IIF has a sensitivity of more than 90% for the AAV, but has a lower specificity (80% or less) because ANCAs, especially those producing the p-ANCA pattern, can be found in other conditions.[80] Therefore, samples that are positive through initial testing with IIF should be confirmed by PR3– and MPO–specific immunoassays whenever possible.[79] These assays are available in automated ELISA, chemiluminescence immunoassay, and fluorescent EIA formats.[77] By using a combination of automated immunoassays and IIF, ANCAs can be detected in nearly 100% of patients with active, systemic GPA.[73] However, failure to detect ANCAs does not rule out the presence of AAV.[80] ANCA titers are useful in monitoring disease activity, but are of limited value in predicting relapses in patients who are in remission.[77,78,80]

It is important to understand that ANCAs are not diagnostic for AAV and can be found in a variety of other disorders, including other types of vasculitis, connective tissue diseases

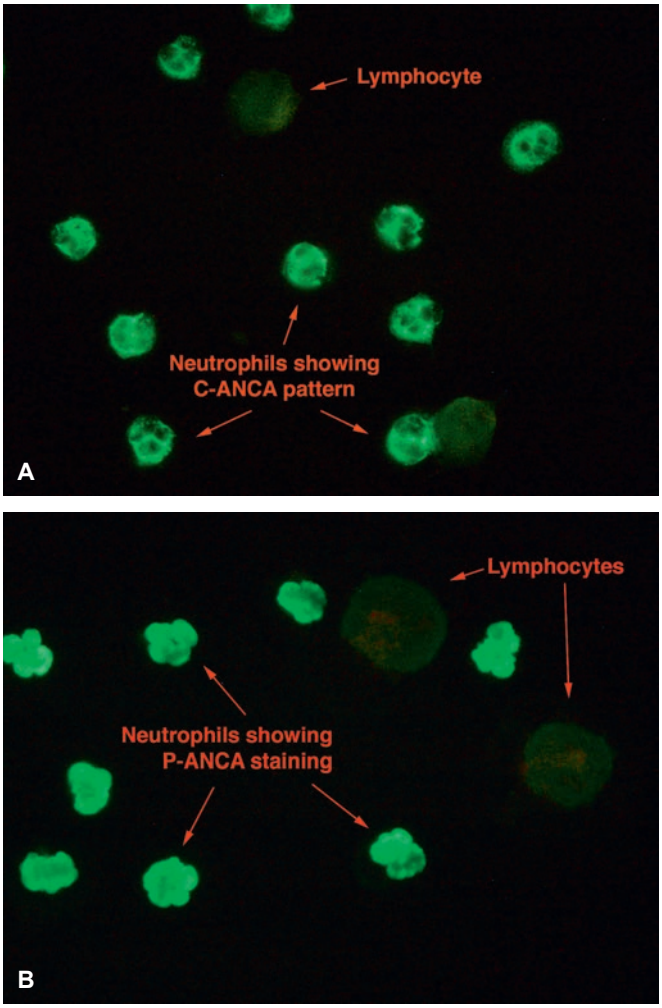


FIGURE 15–8 c-ANCA and p-ANCA fluorescent patterns. (A) c-ANCA (cytoplasmic pattern). Note the granular staining of the primary granules in the cytoplasm of the neutrophils and the more intense fluorescence between the lobes of each nucleus. The lymphocytes have negative staining. This pattern can be seen with either ethanol-fixed or formalin-fixed neutrophils. (B) p-ANCA (fluorescent pattern). This pattern is characterized by smooth or homogeneous staining of the multi-lobed nuclei in ethanol-fixed neutrophils as shown. (On formalin-fixed neutrophils, these antibodies would produce a granular cytoplasmic staining.) The lymphocytes are not stained. (Reprinted with permission from Immuno Concepts.)

Table 15–3 Antineutrophil Cytoplasmic Antibodies (ANCAs)

PATTERN ON INDIRECT IMMUNOFLUORESCENCE WITH ETHANOL-FIXED LEUKOCYTES	APPEARANCE	AUTOANTIGENS	ASSOCIATED DISEASES
c-ANCA	Diffuse, granular staining in the cytoplasm of the neutrophils, fading toward the outer edges of the cells	PR3 antigen	Granulomatosis with polyangiitis (GPA; Wegener's granulomatosis)
p-ANCA	Fluorescence surrounding the lobes of the neutrophil nuclei, blending them together	Positively charged antigens, including myeloperoxidase (MPO)	Microscopic polyangiitis (MPA) Eosinophilic granulomatosis with polyangiitis (EGPA; Churg-Strauss syndrome)

such as SLE and RA, autoimmune gastrointestinal and liver diseases, certain infections including HIV and hepatitis C, malignancy, and other diseases.[82] Patients with these conditions often have ANCAs directed toward neutrophil antigens other than PR3 or MPO, which can also be detected by IIF. A positive ANCA test must therefore be combined with clinical manifestations and histological findings of biopsy tissue to make an accurate diagnosis.

Organ-Specific Autoimmune Diseases

This chapter will refer to organ-specific autoimmune diseases as those diseases in which the immune response is directed against self-antigens that are mainly found in a single organ or gland. Although the clinical manifestations are largely related to the target area, systemic effects may sometimes also occur. Examples of organ-specific autoimmune diseases are listed in **Table 15–4;** many of these will be discussed in more detail here.

Autoimmune Thyroid Diseases (AITDs)

Autoimmune thyroid diseases (AITDs) encompass several different clinical conditions, the most notable of which are Hashimoto's thyroiditis and Graves disease. Although these conditions have distinctly different symptoms, they do share some antibodies in common; in addition, both interfere with thyroid function. The thyroid gland is located in the anterior region of the neck and is normally between 12 and 20 grams in size. It consists of units called *follicles* that are spherical in shape and lined with cuboidal epithelial cells. Follicles are filled with material called *colloid.* The primary constituent of colloid is **thyroglobulin (Tg),** a large iodinated glycoprotein from which the active thyroid hormone triiodothyronine (T3) and its precursor, thyroxine (T4), are synthesized. The enzyme **thyroid peroxidase (TPO)** plays an important role in the synthesis of these hormones by oxidizing iodine ions, allowing for their incorporation into the tyrosine residues of thyroglobulin to produce the building blocks for the hormones.[83,84]

Under normal conditions, the synthesis of T3 and T4 is tightly regulated by an endocrine feedback loop called the thyroid axis **(Fig. 15–9). Thyrotropin-releasing hormone (TRH)** is secreted by the hypothalamus to initiate the process that eventually causes release of hormones from the thyroid. TRH acts on the pituitary gland to induce release of **thyroid-stimulating hormone (TSH).** TSH, in turn, binds to receptors on the cells of the thyroid gland, causing thyroglobulin to be broken down into secretable T3 and T4. If the levels of T3 and T4 become too high, they feed back to the hypothalamus and pituitary to inhibit release of TRH and TSH, resulting in decreased production of the thyroid hormones. The presence of autoantibodies to components of the thyroid gland interferes with this process and causes under- or overactivity of the gland.[83,84]

Etiology

The genes thought to be associated with a predisposition to thyroid autoimmunity are related to immune function or are thyroid specific. A strong association between HLA-DR3 and Graves disease has been observed.[85,86] Association of Hashimoto's thyroiditis with inheritance of HLA antigens DR3, DR4, DR5, and DQ7 has been reported, but this is not consistent among different ethnic populations.[85,86] A unique feature of both Graves and Hashimoto's diseases is that HLA-DR antigens are expressed on the surface of thyroid epithelial cells, perhaps increasing the autoimmune response. Mutations in the thyroglobulin gene may allow for interaction of the protein with HLA-DR antigens, resulting in antithyroglobulin antibodies. These can be found in both Graves and Hashimoto's disease. Additionally, in Graves disease, modifications in the TSH receptor gene may allow the immune system to recognize the receptor and produce antibodies against it.[86,87]

Possible environmental triggers of AITDs include infections, certain medications, smoking, psychological stress, and pregnancy, but the strongest link is thought to be between high iodine intake and development of Hashimoto's disease. Highly iodinated thyroglobulin is thought to be more immunogenic, possibly creating or exposing more epitopes and facilitating the

Table 15–4 Organ-Specific Autoimmune Diseases

DISEASE	TARGET CELLS OR TISSUES	ASSOCIATED AUTOANTIBODIES
Addison's disease	Adrenal glands	Antibody to adrenal cells
Autoimmune hemolytic anemia	Red blood cells (RBCs)	Antibody to RBCs
Autoimmune hepatitis (AIH)	Liver	AIH-1—smooth muscle antibodies; ANAs AIH-2—anti-liver kidney microsomal antibody (anti-LKM-1); anti-liver cytosol type 1 antibody (anti-LC-1)
Autoimmune thrombocytopenic purpura	Platelets	Antiplatelet antibody
Celiac disease	Small intestine and other organs	Antitransglutaminase (tTG) Antibodies to deamidated gliadin peptides (DGPs) Endomysial antibodies
Goodpasture's syndrome	Kidneys, lungs	Antibody to an antigen in the renal and pulmonary basement membranes
Graves disease	Thyroid gland	Thyroid-stimulating hormone receptor antibodies (TRAbs) Antithyroglobulin Antithyroid peroxidase (TPO)
Hashimoto's thyroiditis	Thyroid gland	Antithyroglobulin Antithyroid peroxidase (TPO)
Multiple sclerosis	Myelin sheath of nerves	Antibodies to myelin basic protein
Myasthenia gravis	Nerve-muscle synapses	Antibodies to acetylcholine receptors (AChR) Anti-muscle-specific kinase (MuSK) Antibody to the lipoprotein LRP4
Pernicious anemia	Stomach	Parietal cell antibody, intrinsic factor antibody
Poststreptococcal glomerulonephritis	Kidneys	Streptococcal antibodies that cross-react with kidney tissue
Primary biliary cirrhosis	Intrahepatic bile ducts	Antimitochondrial antibodies (AMA)
Rheumatic fever	Heart	Streptococcal antibodies that cross-react with cardiac tissue
Scleroderma	Connective tissue	Antinuclear antibodies: anti-Scl-70, anticentromere antibody
Sjögren's syndrome	Eyes, mouth	Antinuclear antibodies, rheumatoid factor, antisalivary duct antibodies, antilacrimal gland antibodies
Type 1 diabetes mellitus	Pancreas	Anti-insulin Islet cell antibodies Anti–IA-2 and anti–IA-2βA Antibody to glutamic acid phosphatase (GAD)

antigen uptake and processing step of the adaptive immune response (see Chapter 4).[88]

Clinical Signs and Immunopathology of Hashimoto's Thyroiditis

Hashimoto's thyroiditis, also known as *chronic lymphocytic thyroiditis,* was discovered in Japan in 1912 by Dr. Hakaru Hashimoto. It is now considered to be the most common autoimmune disease, affecting about 8 out of every 1,000 individuals.[89] The disease is most often seen in middle-aged women; in addition, women are 5 to 10 times more likely to develop the disease than men.[87,88] Patients develop an enlarged thyroid called a *goiter*, which is irregular and rubbery. Patients also produce thyroid-specific autoantibodies and cytotoxic T cells. Immune destruction of the thyroid gland occurs, which results in a state of decreased thyroid function called *hypothyroidism.* Symptoms of hypothyroidism include dry skin, decreased sweating, puffy face with edematous eyelids, pallor with a yellow tinge, weight gain, fatigue, and dry and brittle hair.[84,89]

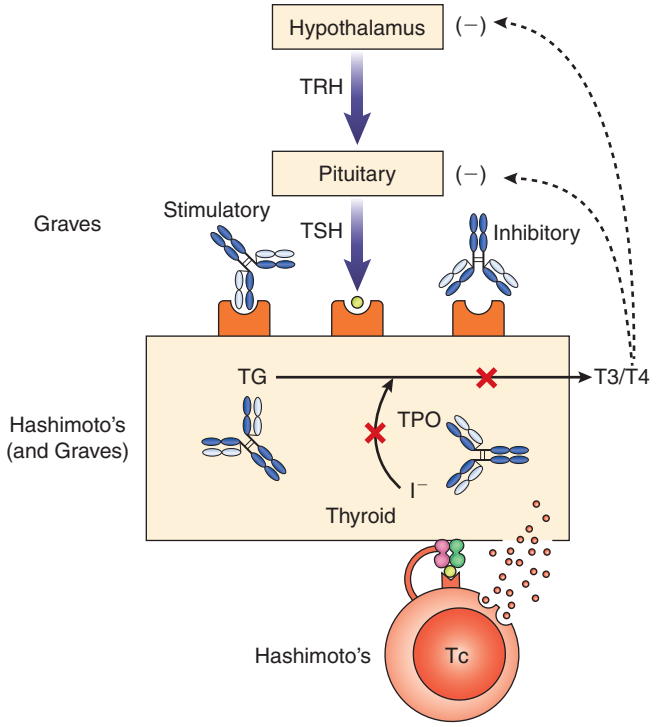


FIGURE 15–9 Autoimmune disorders in thyroid hormone synthesis and regulation. The actions of autoantibodies and cytotoxic T cells in the pathogenesis of Graves disease and Hashimoto's disease is shown. Solid arrows = stimulation; dotted arrows = feedback inhibition.

Six forms of Hashimoto's disease have been described, each with distinct pathological features.[89] In the classic form of the disease, the thyroid shows hyperplasia with an increased number of lymphocytes. Cellular types present include activated T and B cells (with T cells predominating), macrophages, and plasma cells. Pathology to the thyroid gland is mediated primarily by CD8+ cytotoxic T cells, which bind to the thyroid cells and destroy them by releasing enzymes that cause apoptosis or necrosis.[84] The immune response also results in the development of germinal centers that almost completely replace the normal glandular architecture of the thyroid and progressively destroy the thyroid gland.[89] Antibodies to Tg and TPO are produced that have the ability to fix complement; this may result in an inflammatory response that perpetuates the tissue damage.[84,86] Injury to the thyroid gland results in the symptoms associated with hypothyroidism.

Clinical Signs and Immunopathology of Graves Disease

Graves disease, in contrast to Hashimoto's thyroiditis, is characterized by *hyperthyroidism,* a state of excessive thyroid function. Graves disease is, in fact, one of the most frequently occurring autoimmune diseases and the most common cause of hyperthyroidism.[84] Women exhibit greater susceptibility to Graves disease by a margin of about 5 to 1 and most often present with the disease in the fifth and sixth decades of life.[90]

The disease is manifested as **thyrotoxicosis,** or an excess of thyroid hormones, with a diffusely enlarged goiter that is firm instead of rubbery. Clinical symptoms include nervousness, insomnia, depression, weight loss, heat intolerance, sweating, rapid heartbeat, palpitations, breathlessness, fatigue, cardiac dysrhythmias, and restlessness.[84,90] Another sign present in approximately 35% of patients is exophthalmos, in which hypertrophy of the eye muscles and increased connective tissue in the orbit cause the eyeball to bulge out so that the patient has a large-eyed staring expression **(Fig. 15–10).**[91,92] There is evidence that orbital fibroblasts express TSH receptor-like proteins that are affected by thyroid-stimulating immunoglobulin just as the thyroid is.[92,93] Localized edema in the lower legs can also occur.

The thyroid shows uniform hyperplasia with a patchy lymphocytic infiltration. The follicles have little colloid but are filled with hyperplastic epithelium. A large number of these cells express HLA-DR antigens on their surface in response to IFN-γ produced by infiltrating T cells.[94] This allows presentation of self-antigens such as the thyrotropin receptor to activated T cells. B cells, in turn, are stimulated to produce antibody.

The major antibodies involved in the pathogenesis of Graves disease are the **thyroid-stimulating hormone receptor antibodies (TRAbs)**. When TRAbs bind to the TSH receptor they mimic the action of TSH, resulting in uncontrolled receptor stimulation with excessive release of thyroid hormones (Fig. 15–9). In the end, symptoms of hyperthyroidism are produced.[83,84] Other antibodies present include anti-Tg, anti-TPO, and thyrotropin receptor-blocking antibody. The thyrotropin receptor-blocking antibody may coexist with thyroid-stimulating antibody in a number of patients.[86,93] Depending on the relative activity of blocking and stimulating autoantibodies, patient symptoms may vary from hyperthyroidism to euthyroidism (the state of normal thyroid function) to hypothyroidism, which may confound the patient's diagnosis.

Treatment of Autoimmune Thyroid Diseases

Treatment for Hashimoto's disease consists of daily oral thyroid hormone replacement therapy, with levothyroxine (T4) being the preferred drug. TSH levels should be monitored throughout treatment and are used in adjusting the dose of the drug so that normal TSH levels are maintained.[83,84]

Several different protocols are used in the treatment of Graves disease. In the United States, the first line of treatment involves radioactive iodine, which emits beta particles that are locally destructive within an area of a few millimeters. The iodine is administered for 1 to 2 years and results in a 30% to

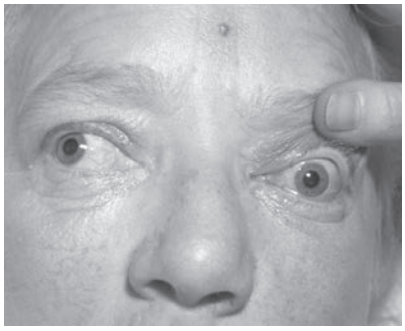


FIGURE 15–10 Exophthalmos indicative of Graves disease. *(Courtesy of CDC/Dr. Sellers/Emory University.)*

50% long-term remission rate.[84,93] Some patients, however, become hypothyroidal within 5 years; therefore, continued monitoring is essential. In Europe and Japan, the patient is first placed on antithyroid medications with beta blockers as adjuvant therapy.[84,90,93] This initial course is followed by continued drug treatment, radioactive iodine therapy, or surgery to remove part of the thyroid. Surgery is recommended for patients resistant to drug treatment, but can damage the laryngeal nerves and cause permanent hoarseness.[83,84]

Laboratory Diagnosis of Autoimmune Thyroid Diseases

Initial screening for AITDs involves measurement of circulating TSH levels. Recall that because of the thyroid axis, the TSH level is inversely related to the levels of T3 and T4. TSH is routinely measured with highly sensitive chemiluminescent immunoassays that can detect fewer than 0.1 mU/L.[83,84] A normal TSH level indicates normal thyroid function, with rare exceptions.[84] If the TSH level is abnormally high or low, laboratory tests for circulating thyroid hormone levels must be performed. Although immunoassays for total serum T3 and T4 are available, the majority of these hormones are protein bound and alterations in these hormone-binding proteins can affect test results. Therefore, it is more useful to measure unbound thyroid hormone, usually free T4 (FT4).[83,84] **Table 15–5** summarizes some of the main laboratory findings in Hashimoto's disease and Graves disease.

Patients with Hashimoto's thyroiditis will have normal or high TSH levels and low FT4 levels.[83,84] To establish an autoimmune etiology for the hypothyroidism, it is necessary to follow these findings by testing for thyroid antibodies. Today, these antibodies are most commonly detected by sensitive ELISA and chemiluminescent immunoassays.[83,95] Antibodies to TPO are the best indicator of the disease because they are found in about 95% of patients with Hashimoto's disease, but only 10% to 15% of the general population. Antibodies to Tg are less sensitive and specific because they are detected in only 60% to 80% of patients with the disease and are found more frequently than TPO antibodies in healthy persons.[83,89] Because anti-Tg antibodies are not found in all patients, a negative test result does not necessarily rule out Hashimoto's disease.

In contrast, patients with Graves disease characteristically have low or undetectable levels of TSH and increased levels of FT4.[83,84] Increased uptake of radioactive iodine also helps to confirm the diagnosis.[90] Although antibodies to TPO and Tg are found in the majority of patients, they are generally not useful in making the diagnosis. TRAbs, on the other hand, are highly indicative of Graves disease because they are present in 98% to 100% of patients; TRAbs are therefore included as one component of the diagnostic criteria for Graves disease.[90]

There are two types of tests for TRAbs: binding assays and bioassays. With binding assays, such as automated solid-phase ELISA or chemiluminescent immunoassays, a labeled TRAb reagent competes with the patient antibody for TSH receptor bound to a solid phase.[90,96] Bioassays require tissue culture and thus are difficult to perform.[95,96] However, these assays can distinguish between TRAbs with stimulatory activity versus those with inhibitory activity because they are functional assays. Current bioassays detect the ability of TRAbs to bind to TSH receptors on live cells and trigger cAMP-dependent luciferase activity. These assays show promise for use in clinical settings to evaluate patient response to therapy and individuals with alternating episodes of hyper- and hypothyroidism.[96]

Type 1 Diabetes Mellitus (T1D)

Diabetes mellitus is a group of common endocrine disorders that are characterized by *hyperglycemia* (a high level of glucose in the blood).[97] The American Diabetes Association (ADA) has classified diabetes into three main categories based on the etiology of the disease: type 1 diabetes, type 2 diabetes, and gestational diabetes. The majority of patients have type 2 diabetes, which is characterized by insulin resistance and occurs most commonly in obese individuals over the age of 40.[98] About 5% to 10% of patients with diabetes mellitus are classified as having **type 1 diabetes mellitus (T1D),** which is characterized by a complete or nearly complete deficiency in insulin. Of these patients, 90% have an immune-mediated form of the disease known as type 1A diabetes, whereas the remaining 10% are idiopathic cases with no identifiable cause (type 1B diabetes).[98-100] Gestational diabetes develops in some women during pregnancy. This section will focus on type 1A diabetes, which will be referred to as T1D. T1D was previously known as *juvenile onset diabetes* because it usually develops in children or in young adults before the age of 30.[97]

T1D is a chronic autoimmune disease that involves selective destruction of the beta cells of the pancreas. These cells are located in clusters called the *islets of Langerhans* and are responsible for the production and secretion of the hormone, insulin. Insulin plays a vital role in regulating the amount of glucose

Table 15–5 Typical Laboratory Findings in Autoimmune Thyroid Diseases

DISEASE	TSH LEVEL	FREE T4 (FT4) LEVEL	AUTOANTIBODIES
Hashimoto's thyroiditis	Normal or elevated	Decreased	Antithyroglobulin Antithyroid peroxidase (TPO)
Graves disease	Decreased or undetectable	Elevated	Thyroid-stimulating hormone receptor antibodies (TRAbs)* Antithyroglobulin Antithyroid peroxidase (TPO)

**diagnostic*

in the circulation by promoting its absorption by skeletal muscles and fat tissue so that it can be converted into energy needed by our cells. The autoimmune destruction of beta cells in T1D results in insufficient insulin production, hyperglycemia, and toxic effects on the body. Long-term effects include cardiovascular complications, renal disease, nerve damage, blindness, and infections of the lower extremities, which can lead to amputation.[97] Patients require lifelong insulin injections to control glucose levels and lower the risk of these complications.

Family studies indicate that there is an inherited genetic susceptibility to the disease, probably attributable to multiple genes. Most people with T1D carry the HLA-DR3 or DR4 gene, and there is an increased risk when both of these genes are present.[97] There is also a strong correlation between certain HLA-DQ haplotypes and type 1 diabetes mellitus.[97,101] Possible environmental influences include certain viral infections and early exposure to cow's milk.[97]

Immunopathology

Progressive inflammation of the islets of Langerhans in the pancreas leads to fibrosis and destruction of most beta cells. The subclinical period may last for years. Hyperglycemia does not become evident until 80% or more of the beta cells are destroyed. Immunohistochemical staining of inflamed islets shows a preponderance of CD8+ lymphocytes, along with plasma cells and macrophages.[97] B cells themselves may act as APCs, stimulating activation of CD4+ lymphocytes.[102] A shift to a Th1 response causes production of certain cytokines, including TNF-α, IFN-γ, and IL-1. The generalized inflammation that results is responsible for the destruction of the beta cells. Although islet autoantibodies trigger the immune response, it is not known what role they play in cell destruction. There is increasing evidence that autoimmunity to insulin itself may be central to disease pathogenesis.[102] Cell death, however, is likely caused by apoptosis and attack by cytotoxic lymphocytes.

It is apparent that autoantibody production precedes the development of T1D by months to several years.[99] Autoantibodies are present in newly diagnosed patients and in prediabetic individuals who are being monitored because they have a high risk of developing diabetes. Antibody production diminishes with time, however. Among the antibodies found are antibodies to two tyrosine phosphatase-like transmembrane proteins called *insulinoma antigen 2 (IA-2)* and *IA-2βA (phogrin); anti-insulin antibodies;* antibodies to the enzyme *glutamic acid decarboxylase (GAD-65);* antibodies to *zinc transporter 8 (ZnT8);* and antibodies to various other islet cell proteins, called *islet cell antibodies (ICAs).*[99,100,103] Many of these antigens are components of the regulated pathway that is essential for the secretion of insulin.[103]

Treatment

Daily injectable insulin has been the mainstay of therapy for T1D. Clinical trials are investigating the use of immunosuppressive drugs and biological agents to inhibit the autoimmune responses that lead to beta cell destruction and prevent disease progression in T1D patients.[104] Transplantation of pancreatic beta islet cells has been used for T1D patients who have poor glucose control, but this treatment requires continual immunosuppressive therapy in order to prevent rejection and the number of suitable donors is limited. New technologies, such as the use of stem cells to produce islet cells in vitro and methods to induce immunologic tolerance in recipients, offer hope that islet cell transplantation may encounter fewer obstacles in the future.[105]

Laboratory Diagnosis of Type 1 Diabetes Mellitus

According to the ADA, a person is considered to have diabetes if he or she meets one of four criteria: (1) a fasting glucose greater than 126 mg/dL on more than one occasion (normal value is lower than 100 mg/dL); (2) a random plasma glucose level of 200 mg/dL or more with classic symptoms of diabetes; (3) an oral glucose tolerance test of 200 mg/dL or more in a 2-hour sample with a 75 g glucose load; or (4) a hemoglobin A1c value (HbA1c) greater than 6.5%.[98] HbA1c is a glycated form of hemoglobin that is made when the RBC protein combines with glucose in the blood. The HbA1c plasma level is proportional to the life span of the circulating RBCs (up to 120 days) and reflects the average plasma glucose concentration over the previous 2 to 3 months.

Although T1D is usually diagnosed by the prime characteristic of hyperglycemia, it may be useful to perform serological tests for diabetes before beta-cell destruction occurs to the extent necessary to cause symptoms. When T1D is suspected, tests for antibodies to GAD and IA-2A can be done to confirm the diagnosis. If these results are negative, they can be followed by testing for ICA in children and for insulin antibodies in adults.[99] ICAs have been reported in the sera of more than 80% of patients newly diagnosed with T1D.[97] Antibodies to islet cells have traditionally been detected by IIF using frozen sections of human pancreas.[106] However, such assays are rather cumbersome to perform and other methods are available, including radio-immunoprecipitation assays, Western blotting, ELISA, and mass spectrometry.[103] These methods can also be used to detect antibodies to other pancreatic antigens such as insulin, GAD, and IA-2.[103,107] Combined screening for IA-2A, ICA, and GAD antibodies appears to have the most sensitivity and best positive predictive value for T1D in high-risk populations.[99,107]

Celiac Disease

Celiac disease is an autoimmune disease affecting the small intestine and other organs. It affects 0.6% to 1.0% of the world's population, but this number is thought to be an underestimate because many cases go undiagnosed.[108] Celiac disease is unique in that it is associated with a known environmental trigger—dietary gluten.[109] Gluten is a protein complex found in wheat, barley, and rye that is poorly digested by the upper gastrointestinal system. It contains an alcohol-soluble component called *gliadin* that is rich in the amino acids glutamine and proline. Gliadin is resistant to digestive enzymes in the stomach, pancreas, and small intestine and therefore remains intact in the lumen, or space within the intestines, after ingestion. If there is an increase in the permeability of the intestinal walls, possibly as a result of an infection, undigested gliadin is able to pass through the epithelial barrier of the intestine and trigger an inappropriate immune response. The immunogenicity of gliadin

is enhanced when it is acted on by **tissue transglutaminase (tTG)**, an intestinal enzyme that converts the glutamine residues in gliadin to glutamic acid.[109,110]

As a result, immunogenic peptides are generated that specifically react with HLA-DQ2 or HLA-DQ8 molecules on APCs. In fact, the presence of one of these two HLA haplotypes is a necessary condition for developing celiac disease. The majority of patients (90% to 95%) possess an HLA-DQ2 allele, whereas almost all of the remaining patients have HLA-DQ8.[108,109] The gliadin peptides that are picked up by the APC are presented to antigen-specific CD4+ T cells, which produce cytokines that activate CD8+ T cells and trigger an inflammatory response that damages the architecture of the intestinal mucosa and causes injury to the villi.[109,110] In addition, B cells are stimulated to produce antibodies to the deamidated gliadin peptides (DGPs), tTG, and endomysium (a layer of connective tissue surrounding the intestinal muscles).

Environmental factors believed to play a role in the development of celiac disease include administration of gluten in the diet of an infant younger than 4 months in the absence of breastfeeding, rotavirus infection, and overgrowth of pathogenic bacteria in the gut.[109,110] In addition, the disease is found more often in women than in men (ratio, 2-3:1) and among those who have selective IgA deficiency, Down syndrome, Turner's syndrome, or type 1 diabetes.[109,110] Several non-HLA genes involved in immune function are also thought to contribute to this autoimmune response.[111]

Clinical Symptoms and Treatment

The clinical symptoms of celiac disease vary with age.[109,110] Infants typically present with diarrhea, abdominal distention, and failure to thrive, but may also experience vomiting, irritability, anorexia, and constipation. Older children, teenagers, and adults may have the classic symptoms of diarrhea and abdominal pain or discomfort, but often have extraintestinal manifestations that make the condition difficult to diagnose. These include short stature, arthritis or arthralgia, osteoporosis, neurological symptoms, iron-deficiency anemia, and dermatitis herpetiformis (a skin disorder with itchy blistering).

Treatment of celiac disease involves placing patients on a gluten-free diet. Elimination of gluten from the diet usually results in improvement of clinical symptoms within days or weeks and healing of intestinal damage in 6 months to 2 years.[108,109] However, a significant number of patients do not adhere to a gluten-free diet because of expense, inconvenience, or social stigma; in addition, some patients have persistent symptoms despite adherence to the diet. Alternative treatments, such as the use of recombinant enzymes to digest the toxic gliadin, are being investigated.

Laboratory Diagnosis of Celiac Disease

Diagnosis of celiac disease is based on clinical symptoms, serological findings, duodenal biopsy, and presence of the HLA-DQ2 or HLA-DQ8 haplotype. Serological testing is the initial approach taken to evaluate patients suspected of having celiac disease and helps to differentiate these patients from those having conditions with similar symptoms, such as gluten sensitivity or wheat allergy.[108] It is recommended that patients follow a regular diet before serological testing because false-negative antibody results can occur in individuals on a gluten-free diet.[108,112]

The first serological test for celiac disease was produced in the 1980s and measured antibodies to gliadin; however, this test is not commonly used today because it is associated with a significant number of false-positive results.[113] Currently, detection of IgA antibodies to tTG is the serological method of choice for initial testing.[108,112,113] This is because automated, ELISA-based assays using purified human or recombinant tTG antigen have a high sensitivity (91% to 95%) and specificity (95% to 97%) for celiac disease. Rapid EIA tests are also available for detection of antibodies to tTG, but are not as sensitive or specific as the ELISA tests.[113] Serum IgA levels should be concurrently measured in these individuals because a significant number also have selective IgA deficiency and will therefore test negative in IgA-based assays.[108,112] In patients who are IgA deficient, testing for IgG anti-tTG or for IgG antibodies to DGPs can be performed. Automated ELISA tests for anti-DGPs are a more recent development and show an especially high sensitivity in children under the age of 2 to 3 years, who may test negative for other antibodies. False-positive results for anti-tTG can occur in patients with other autoimmune diseases such as type 1 diabetes. Positive anti-tTG results can be followed by testing for endomysial antibodies (EMA). EMA tests are highly specific for celiac disease, but are more costly because they are based on IIF assays using monkey esophagus or human umbilical cord tissue as the substrate.[113]

Because serology results are not absolute, biopsy of the small intestine should be performed to confirm the diagnosis. Initial biopsy results can also provide a baseline for comparing future samples for intestinal injury. Histological examination of biopsy tissue characteristically shows an increase in the number of intraepithelial lymphocytes, elongation of the intestinal crypts, and partial to total atrophy of the villi.[108] In order to minimize the occurrence of false-negative results, multiple biopsies should be obtained from different parts of the duodenum because mucosal injury may be patchy.[108,112]

HLA typing is also useful in differentiating celiac disease from other conditions, especially when serological tests and biopsy results are borderline. Absence of HLA-DQ2 or HLA-DQ8 virtually excludes a diagnosis of celiac disease because individuals who are negative for these haplotypes are highly unlikely to have the disease.[108]

Following proper diagnosis and maintenance of patients with celiac disease on a gluten-free diet, clinical symptoms usually improve, antibody titers revert to negative, and histology can return to normal. However, about 5% of patients fail to improve, usually because of nonadherence to the diet (which can sometimes be unintentional), but sometimes caused by refractory disease, incorrect diagnosis, complications of celiac disease, or simultaneous gastrointestinal disorders.[112]

Autoimmune Liver Diseases

There are three major forms of autoimmune liver disease: **autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC),** and primary sclerosing cholangitis (PSC). In AIH, the

autoimmune process targets the hepatocytes; in PBC, it affects the small, interlobular bile ducts; and in PSC, it affects the medium-sized, intra- and extrahepatic bile ducts.[114] There can also be overlap syndromes that combine features of these diseases; many patients also have other autoimmune disorders such as autoimmune thyroiditis or ulcerative colitis. This section will discuss two of these diseases, AIH and PBC, and the serological tests that are used in their diagnosis in more detail.

Autoimmune Hepatitis (AIH)

AIH, formerly known as *chronic active hepatitis,* is an immune-mediated liver disease that can lead to end-stage liver failure if left untreated. It can affect children and adults of all ages and is more common in females than in males. The clinical features of AIH can be quite variable.[115-117] About 25% of individuals are asymptomatic and are diagnosed only after abnormal liver function tests are found coincidentally when blood work is performed. Adults usually present with an unexpected onset of vague symptoms, including fatigue, nausea, weight loss, abdominal pain, itching, and maculopapular rashes. Less often, patients have symptoms of portal hypertension such as gastrointestinal bleeding or hypersplenism. Jaundice may also be present. Rarely, the initial presentation is fulminant liver failure requiring liver transplantation.

Two types of AIH can be differentiated on the basis of its autoantibody specificity (see the text that follows); these are referred to as AIH-1 and AIH-2. AIH-1 accounts for two-thirds of all AIH cases and has a female:male ratio of 4:1. AIH-2 has a female:male ratio of 10:1 and is seen mostly in children.[115,117]

Certain HLA-DRB1 and HLA-DQB1 alleles are associated with a higher risk of developing AIH; these vary in different ethnic populations.[115,118] Exposure to certain viruses or drugs has been suggested to play a role in triggering AIH, possibly through molecular mimicry and cross-reactivity between their epitopes and liver antigens, the most notable virus being hepatitis C.[115]

Common laboratory findings include elevated levels of the liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), with less prominent increases in serum bilirubin and alkaline phosphatase. Serum immunoglobulin levels, particularly IgG, are high; in adults, various autoantibodies are present, including ANAs, ANCAs, smooth muscle antibodies (SMA), anti-liver kidney microsomal antibody (anti-LKM-1), anti-liver cytosol type 1 antibody (anti-LC-1), and antimitochondrial antibodies (AMAs).[116,117]

Autoantibody profiles can distinguish between AIH-1 and AIH-2. AIH-1 patients are characteristically positive for SMA or ANA. ANAs most commonly produce a homogeneous pattern on IIF, but can sometimes produce a speckled pattern.[116,117] The SMAs are directed against actin and other components of the cytoskeleton. They can be detected by IIF on rodent kidney, stomach, or liver sections, where they produce fluorescent staining of the smooth muscle in the artery walls and other components, such as the glomeruli and tubules of the kidneys. Antibody titers are usually 1:80 or higher in adults, but can be as low as 1:20 in children.[116,117]

In contrast, patients with AIH-2 characteristically produce antibodies against LKM-1 or LC-1. On IIF using rodent tissue substrates, anti-LKM-1 stains the cytoplasm of the hepatocytes and the P3 portion of the kidney tubules. The resulting immunofluorescent pattern appears similar to that produced by mitochondrial antibodies, but can be distinguished by experts when testing is performed on multiple tissue substrates and the intensity of fluorescent staining of different components of the substrates is carefully examined. Clinically significant titers are considered to be 1:40 in adults and 1:10 in children; antibody titers correlate with disease activity.[116,117] Antibodies to LC-1 are directed against a folate-metabolizing enzyme in the liver and stain the cytoplasm of liver cells in IIF. These antibodies can be masked by anti-LKM-1 if they are also present and other methods, such as immunodiffusion, may be required to detect them.[116,117]

Liver biopsy is necessary to confirm the diagnosis of AIH and to assess the extent of liver damage. Inflammation at the portal-parenchymal boundary, known as interface hepatitis, is typical of AIH and is characterized by an infiltrate of lymphocytes, plasma cells, and histiocytes surrounding dying hepatocytes.[116,117] Histological findings, along with detection of autoantibodies, elevated IgG, and exclusion of viral hepatitis, comprise the criteria recommended by the International Autoimmune Hepatitis Group for the diagnosis of AIH.[115,117] These criteria have a sensitivity greater than 80% and a specificity greater than 95%.[115]

Laboratory findings are essential for early diagnosis, and treatment should be started promptly once the diagnosis is made. Most patients respond to the standard immunosuppressive treatment of prednisolone (+/–azathioprine) to induce remission, followed by azathioprine alone to maintain remission. However, patients must be monitored carefully because they are at increased risk of developing cirrhosis and possibly hepatocellular carcinoma. If untreated, AIH usually progresses to liver failure, at which point liver transplantation is required.[116,117]

Primary Biliary Cirrhosis (PBC)

PBC, the most common autoimmune liver disease, occurs about 10 times as often in females as in males.[118] There is a genetic link with certain HLA-DRB1, HLA-DQA1, HLA-DPB1, and HLA-DQB1 haplotypes.[118,119] PBC is an autoimmune disease that involves progressive destruction of the intrahepatic bile ducts.[118, 120-121] The destruction leads to chronic *cholestasis* (a condition in which the flow of bile is slowed or blocked), inflammation of the portal vein in the liver, and accumulation of scar tissue that can ultimately lead to cirrhosis and liver failure. Individual patients can be asymptomatic or have slowly or rapidly progressing disease. Symptoms include fatigue, pruritis (itchy skin), abdominal pain, and dry eyes and mouth; in the later stages, symptoms include jaundice, ascites, and greasy stools. The only established therapy for PBC is ursodeoxycholic acid (UDCA), a bile acid that helps move bile through the liver. Use of UDCA has helped to slow down disease progression and increase patient survival. Liver transplantation is the only effective treatment for patients who have reached end-stage liver disease.[118,120-121]

Anti-mitochondrial antibodies (AMAs) are found in the majority of patients with PBC and their presence is one of three diagnostic criteria for the disease.[118,120-121] The other two criteria

are a serum alkaline phosphatase level elevated at least 1.5 times the upper limit of normal for 6 months or more and liver biopsy showing nonsuppurative destructive cholangitis and interlobular bile duct injury. A diagnosis of PBC can be made if at least two out of three of these criteria are met. In addition, patients commonly have elevated levels of aminotransferases and serum immunoglobulins, especially IgM, because of polyclonal activation of B cells. ANAs giving perinuclear or rimlike, nuclear dot, or centromere patterns may also be present.

Traditionally, AMAs have been detected by IIF with mitochondria-rich tissue substrates such as rodent liver, kidney, or stomach sections. These antibodies produce a bright, uniform granular cytoplasmic fluorescence in the distal renal tubules, gastric parietal cells, thyroid epithelial cells, and cardiac muscle.[122] IIF uses antigens in their natural configuration and has fairly high levels of sensitivity and specificity; however, the method is manual, time-consuming, and requires a high level of expertise to correctly interpret the pattern. In addition, about 5% to 10% of PBC patients test negative for AMAs on IIF or may give atypical staining patterns that are difficult to interpret.[121]

Since the development of IIF, the target antigens of AMAs have been identified as components of the 2-oxo-acid dehydrogenase complexes that are involved in mitochondrial energy-producing pathways. Identification of these antigens allowed for the development of solid-phase ELISA assays. The mitochondrial antigens employed in these ELISAs consist of preparations of porcine or bovine heart, mixtures of recombinant subunits of pertinent antigens, designer antigens composed of particular subunits, or mixtures of native and designer antigens, depending on the commercial manufacturer. ELISAs have the advantages of automation and provision of objective results, but their performance can vary because these assays generally do not include the full spectrum of antigenic epitopes available by IIF.[122]

A cost-effective approach to testing may be to screen samples for AMAs and other autoantibodies by IIF, then follow-up with an AMA ELISA assay for confirmation. This approach would also be helpful in identifying AMAs in those patients who test negative through IIF.[122] This testing strategy would facilitate an earlier diagnosis for PBC patients, allowing the initiation of treatment that could slow down disease progression and improve patient survival. It is important to note that a diagnosis of PBC cannot be based on the presence of AMAs alone because these antibodies have also been observed in patients with other conditions such as SLE, RA, and graft-vs.-host disease, as well as a small percentage of healthy persons.[121]

Multiple Sclerosis (MS)

Multiple sclerosis (MS) is an autoimmune disorder involving inflammation and destruction of the CNS. It affects approximately 350,000 Americans and 2.5 million individuals worldwide.[123] MS is most closely associated with inheritance of a particular HLA molecule coding for the beta chain of the DR subregion, namely DRB1*1501.[123] Environmental factors that appear to be associated with MS include reduced exposure to sunlight, vitamin D deficiency, and cigarette smoking.[124]

MS is characterized by the formation of lesions called *plaques* in the white matter of the brain and spinal cord, resulting in the progressive destruction of the myelin sheath of axons. Plaques vary in size from 1 or 2 mm up to several centimeters.[123] Within the plaques, T cells and macrophages predominate; these are believed to orchestrate demyelination.[123] Antibody binds to the myelin membrane and may initiate the immune response, stimulating macrophages and specialized phagocytes called *microglial cells*.[125] The cascade of immunologic events results in acute inflammation, injury to axons and glia, structural repair with recovery of some function, and then postinflammatory neurodegeneration.[125] The Th1 cytokines IL-1, TNF-α, and IFN-γ are believed to be central to the pathogenesis of the disease, promoting changes in the endothelial cells that facilitate adherence of activated T cells and their migration across the blood–brain barrier.[124] Th17 cells are also thought to play an important role in the inflammatory response of the CNS. A Th2 response, characterized by production of IL-4, IL-5, and IL-10, may also contribute to pathogenesis.[123]

Clinical Symptoms and Treatment

Damage to the tissue of the CNS can cause visual disturbances, weakness or diminished dexterity in one or more limbs, locomotor incoordination, dizziness, facial palsy, and numerous sensory abnormalities such as tingling or "pins and needles" that run down the spine or extremities, as well as flashes of light seen on eye movement.[125] The disease most often begins in young and middle-aged adults between the ages of 20 and 50 and is twice as common in women as in men.[124] MS can be classified into four major subtypes based on the clinical course of the disease.[123,124] More than 80% of patients fall into the first subtype, relapsing-remitting MS, which is characterized by clearly defined episodes of neurological attacks with periods of full or partial recovery in between. Most patients with MS eventually develop progressive deterioration of the CNS and functional disability.

Treatment for MS is aimed at easing recovery from acute attacks and reducing the risk of future relapses.[123,126] Acute exacerbations are treated with corticosteroids to reduce inflammation. Disease-modifying agents such as IFN-β1a and IFN-β1b have been approved to treat MS for the long term.[123,126] These agents are believed to work by downregulating MHC molecules on APCs, decreasing production of proinflammatory cytokines, and upregulating anti-inflammatory cytokines.[123,126] The severity of MS can be greatly reduced by therapy with natalizumab, a humanized monoclonal antibody directed against an adhesion molecule of lymphocytes, preventing them from binding to endothelial cells and crossing the blood–brain barrier.[123,126] Agents such as these are improving the long-term prognosis of MS.[123]

Laboratory Diagnosis of Multiple Sclerosis

The diagnosis of MS is based primarily on clinical symptoms, demonstration of disseminated lesions in the white matter of the brain and spinal cord by magnetic resonance imaging (MRI), and exclusion of other possible causes. Several laboratory tests in combination are used to support the diagnosis.[123,124] Immunoglobulins are increased in the

spinal fluid in 75% to 90% of patients with MS, producing two or more distinct bands on protein electrophoresis that are not seen in the serum. These bands are referred to as *oligoclonal* and can be identified by isoelectric focusing with immunoblotting, a more sensitive technique than protein electrophoresis.[127] The IgG index, a calculated ratio of cerebral spinal fluid (CSF) IgG/albumin ÷ serum IgG/albumin, is typically elevated and may also be used in making a diagnosis, even though it is not specific for MS. Although there is not one specific antibody that is diagnostic for MS, a large percentage of patients produce antibody directed against a myelin basic protein peptide. Other antibodies are directed against components of oligodendrocytes and against myelin membranes.[125]

Myasthenia Gravis (MG)

Myasthenia gravis (MG) is an autoimmune disease that affects the neuromuscular junction. It is characterized by weakness and fatigability of skeletal muscles. It is fairly common, with a prevalence of 2 to 7 people per 10,000.[128] The disease is heterogeneous in terms of its age of onset and gender involvement. Early-onset MG (EOMG) occurs before the age of 40 and affects predominantly females, whereas the late-onset form of the disease (LOMG) occurs after the age of 40 and is seen more often in males.[129,130]

In MG, antibody-mediated damage to the acetylcholine receptors in skeletal muscle or to other proteins in the neuromuscular junction leads to progressive muscle weakness. Early signs are *ptosis* (drooping of the eyelids), *diplopia* (double vision), and the inability to retract the corners of the mouth, often resulting in a snarling appearance.[128,131] In the majority of patients, the disease progresses to a generalized form that involves more muscle groups.[131] In patients with generalized MG, muscle weakness is most noticeable in the upper limbs.[131] They can also experience difficulty in speaking, chewing, and swallowing and may be unable to maintain support of the trunk, neck, or head.[106] If respiratory muscle weakness occurs, it can be life threatening.[131] Onset of symptoms can be acute or they may develop and worsen over time.

Immunopathogenesis

Approximately 80% to 85% of patients have antibody to acetylcholine (ACH) receptors (ACHR), which appears to contribute to the pathogenesis of the disease by three mechanisms.[128] Normally, ACH is released from nerve endings to generate an action potential that causes the muscle fiber to contract. When the antibody combines with the receptor site, binding of ACH is thought to be blocked **(Fig. 15–11).** In addition, the antibody can interact with complement to damage the postsynaptic muscle membrane and can promote rapid endocytosis of the ACHRs, resulting in reduced numbers on the muscle cell membranes.[128,129]

Patients lacking anti-ACHR may produce antibodies to other proteins involved in the neuromuscular junction. About 4% of patients, mostly young females, have antibodies against muscle-specific kinase (MuSK), an enzyme that plays an important role in the development of the neuromuscular junction

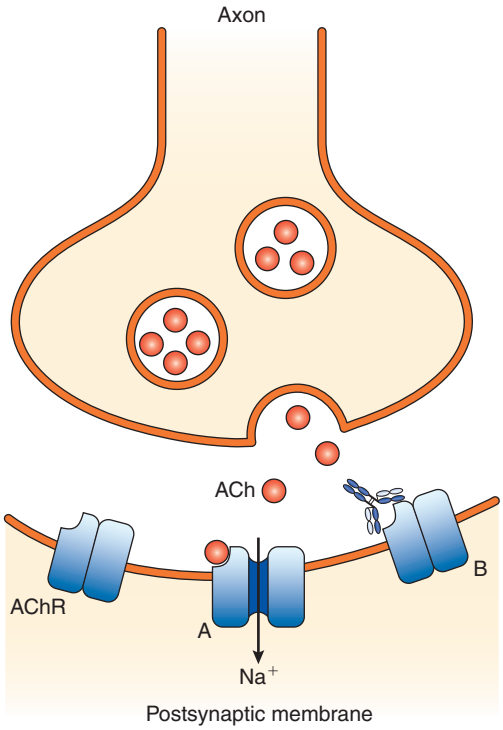


FIGURE 15–11 Mechanism of immunologic injury in MG. (A) Normal nerve impulse transmission: Acetylcholine (Ach) is released from axon vesicles and binds to receptors (AchR) on the postsynaptic membrane, opening channels, allowing sodium ions to enter. (B) In MG, antibodies to the AChRs are formed, blocking transmission of nerve impulses.

and in the clustering of ACHRs on the muscle cell membrane, which enhances the transmission of the signals from the nerve cells.[132] Consequently, there is fragmentation of the postsynaptic ACHR clusters, resulting in muscle weakness and atrophy. Symptoms are usually severe, involving the facial, bulbar, and respiratory muscles. About 2% of patients with generalized MG have antibodies against LRP4, a lipoprotein involved in the activation of MuSK. These patients are typically young females that have a mild form of the disease.[132]

The thymus also appears to play a role in the autoimmune process of MG. Thymic hyperplasia is common in EOMG patients with ACHR antibodies.[129,132] The follicles in the thymus expand and contain ectopic germinal centers with autoantibody-producing B cells. In addition, about 10% to 15% of patients with LOMG have a *thymoma,* a tumor of the thymus that may contain autoreactive T cells. It is thought that inflammation of the thymus gland may be triggered by persistent activation of TLRs by viruses such as EBV.[129] The autoreactive response is thought to be perpetuated by defective immunoregulatory mechanisms involving an imbalance between Th17 cells and Tregs, resulting in increased production of proinflammatory cytokines and B-cell growth factors.

Genetic factors have been implicated in susceptibility to MG. The HLA haplotype, A1, B8, DR3 has a strong association with EOMG, whereas the HLA antigens B7 and DR2 are more likely to appear in LOMG, and HLA-DR14-DQ5 may increase susceptibility to MuSK antibody production in MG.[129]

Treatment

Most patients with MG can have a good quality of life with appropriate treatment. Anticholinesterase agents to prevent destruction of the neurotransmitter, acetylcholine, are used as the main therapy.[128] Thymectomy should be performed on patients who have a thymoma.[131] If these treatments are not effective, immunosuppression is recommended. Treatment generally begins with high doses of corticosteroid drugs followed by other immunosuppressive drugs such as azathioprine or mycophenolate mofetil, to maintain the response.[133] Plasmapheresis or intravenous immunoglobulin can be administered to patients in crisis. Biological agents such as monoclonal antibodies or fusion proteins targeted to specific components of the immune system involved in the pathogenesis of MG offer hope to MG patients who are unresponsive to conventional therapies.[133]

Laboratory Diagnosis of Myasthenia Gravis

Laboratory tests are available to detect autoantibodies in sera from patients with MG. The most commonly used procedure is a radioimmunoprecipitation (RIPA) assay for antibody to the ACHR, which is based on precipitation of the patient's antibody with ACHRs isolated from human muscle.[134] The complex is detected with a radio-labeled snake venom called α-bungarotoxin, which binds with high affinity to a different site on the receptors. This assay is sensitive and can be used to determine antibody titers. A similar RIPA method using ^{125}I-labeled human MuSK is used to detect antibody to MuSK. Sensitivity of the method has been increased by using a two-step RIPA in which antibodies are isolated by an affinity purification procedure using Sepharose beads containing immobilized antigen before performing the steps of the RIPA.[134] This method increases the likelihood of detecting antibodies present in low concentration.

Because assays using radioactive materials are not conveniently used in the routine clinical laboratory, there has been an attempt to develop alternate methods. One approach is to use immunofluorescence cell-based assays in which patient serum is incubated with HEK293 cells expressing all four ACHR subunits. This highly sensitive assay can detect antibodies directed toward ACHR clusters in patients that were previously classified as seronegative by RIPA.[134] Other assays that have been developed include ELISA, luciferase immunoprecipitation, and fluorescence immunoprecipitation assays (FIPA). FIPA uses ACHR subunits or MuSK antigens labeled with green fluorescent protein to detect patient antibodies and has a sensitivity that is similar to RIPA.[134]

Goodpasture's Syndrome

Goodpasture's syndrome is characterized by the presence of autoantibody to an antigen in the basement membranes in the glomeruli of the kidneys and alveoli of the lungs. The basement membranes are composed of a thin, fibrous matrix that separates the epithelial cell layer within these organs from underlying connective tissue. Originally identified by Ernest Goodpasture in 1919, Goodpasture's syndrome is a rare disorder that is found mainly in Caucasians of European origin. It primarily affects two age groups—men in their 30s and men and women in their 60s and 70s.[135]

The clinical presentation of patients varies, but most patients initially experience fatigue and malaise followed by clinical signs of kidney involvement such as edema and hypertension, which can rapidly progress to acute renal failure if left untreated.[135,136] Some patients develop chronic renal failure that requires lifetime hemodialysis or kidney transplantation. About 60% to 70% of patients with Goodpasture's syndrome have pulmonary involvement and exhibit symptoms such as cough, shortness of breath, and hemoptysis (coughing up blood).[135]

Standard treatment of Goodpasture's syndrome involves administration of high dose corticosteroids to stop inflammation, followed by immunosuppressive drugs such as cyclophosphamide to inhibit further production of autoantibodies. In addition, plasmapheresis is performed to remove circulating autoantibodies. Prompt initiation of therapy is important, because symptoms can be life threatening. If started early, this therapy is successful in preventing acute renal failure in most patients.[136,137] However, the long-term prognosis is poor, with many patients developing end-stage renal disease and more than half of patients dying within 2 years of diagnosis.[136]

Etiology and Immunopathology

Although little is known about the circumstances that trigger the autoimmune response in Goodpasture's syndrome, there is a strong genetic association, as 70% to 80% of patients carry the HLA-DRB1-15 antigen.[137] Exposure to cigarette smoke and organic solvents has also been implicated in disease pathogenesis.[136,137] The autoantibodies produced in Goodpasture's syndrome are known to be specifically directed against the noncollagenous domain of the alpha-3 chain of type IV collagen.[136,137] This autoantibody reacts with collagen in the glomerular or alveolar basement membranes and causes damage by type II hypersensitivity.[136,137] Complement binding to the immune deposits attracts neutrophils, which mediate injury to the membranes by releasing chemically reactive oxygen-containing molecules and proteolytic enzymes. These immune reactants progressively destroy the renal tubular, glomerular, and pulmonary alveolar basement membranes. Loss of membrane integrity results in leakage of blood and proteins into the urine.

Laboratory Diagnosis of Goodpasture's Syndrome

Laboratory evidence of renal involvement includes gross or microscopic hematuria, proteinuria, a decreased 24-hour creatinine clearance, and elevated blood urea and serum creatinine levels.[136] Abnormally shaped RBCs and casts can be found in the urine sediment. In those patients with pulmonary involvement, decreased total lung capacity and increased uptake of carbon monoxide is evident. An iron deficiency anemia with decreased hemoglobin and hematocrit can develop if pulmonary hemorrhage is severe. The ESR and CRP level may be normal or increased.

Circulating antibodies to the GBM can be detected in about 87% of patients.[136] These antibodies can be identified by IIF,

ELISA, or Western blot. The IIF assay, long held as the standard, uses frozen kidney sections that are incubated with patient serum and then overlaid with a fluorescein-labeled anti-IgG. Results are often hard to interpret and there is a high percentage of false positives and false negatives.[138] Commercially developed ELISAs, which use the alpha-3 subunit to detect antibody, are also available and have a sensitivity between 70% and 100%, depending on the source of antigen substrate. High antibody titers are usually associated with rapidly progressing disease.[135] The Western blot technique detects antibodies to the Goodpasture basement membrane antigen as well as other human α chain proteins that have been separated by polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose paper for immunoblotting. Western blot is a highly specific test that can be used for confirmation and minimization of false-positive results.[135] Between 20% and 35% of patients are also positive for ANCAs, which are usually specific for myeloperoxidase and exhibit the perinuclear staining pattern on IIF.[135,137] ANCAs may be detectable months to years before anti-GBM and symptoms are evident.

Histological analysis is important for confirmation of the diagnosis and for assessment of tissue damage.[139] Tissue-bound anti-GBM can be detected by performing direct immunofluorescence on biopsy sections from kidney or lung specimens.[136,137,139] In patients with renal disease, these antibodies are indicated by formation of a smooth, linear, ribbonlike pattern of fluorescence along the GBM. In contrast, glomerulonephritis caused by other autoimmune diseases shows a granular pattern of immunofluorescence caused by nonspecific deposition of immune complexes in the glomeruli. Examination of renal biopsy tissue also reveals crescent formations of inflammatory macrophages in the glomeruli. In patients with pulmonary involvement, linear IgG staining of the alveolar cell walls can be seen on direct immunofluorescence of lung biopsy tissue or bronchial washings. Thus, laboratory testing plays a key role in differentiating Goodpasture's syndrome from other diseases that can cause similar symptoms and in facilitating an early diagnosis that can lead to prompt treatment and better clinical outcomes.

SUMMARY

- Autoimmune diseases result from a loss of self-tolerance, a delicate balance set up in the body to restrict the activity of T and B lymphocytes. Immunologic tolerance is achieved at two levels. Central tolerance affects potentially reactive B and T cells as they mature in the bone marrow and thymus, respectively, whereas peripheral tolerance occurs in the secondary lymphoid organs.
- Autoimmune disease is thought to result from complex interactions between the genetic makeup of an individual, exposure to environmental factors, and defects in immune regulation. Associations between certain HLA types or polymorphisms in non-MHC genes involved in the immune response have been observed for several autoimmune diseases. Other factors, including sex hormones, tissue injury, and exposure to microbial infections, are thought to trigger the development of autoimmunity in genetically susceptible individuals.
- Infectious microorganisms are believed to trigger autoimmune responses in a variety of ways, including molecular mimicry (a resemblance to self-antigens), epitope spreading (induction of a local inflammatory response that affects immune reactivity to unrelated antigens), and presence of superantigens that can bind to class II MHC molecules and several T-cell receptors, regardless of their antigen specificity.
- Autoimmune diseases can be classified as organ specific or systemic, depending on whether tissue destruction is localized or affects multiple organs. SLE, RA, and GPA are examples of systemic diseases, whereas Hashimoto's thyroiditis, Graves disease, type 1 diabetes mellitus, celiac disease, autoimmune hepatitis, primary biliary cirrhosis, multiple sclerosis, myasthenia gravis, and Goodpasture's syndrome are considered organ-specific diseases.
- Specific autoantibodies are strongly associated with the presence of certain autoimmune diseases and are useful in their diagnosis. For example, anti-dsDNA antibodies are found in SLE, anti-CCP (cyclic citrullinated proteins) antibodies are seen in RA, and antibodies against the thyroid-stimulating hormone receptor are specific for Graves disease.
- Antinuclear antibodies (ANAs) are found in the majority of patients with SLE and in a significant number of patients with other connective tissue diseases. The gold standard in ANA testing is an IIF test using the human epithelial cell line HEp-2 as the substrate. Some of the main fluorescent patterns observed in this test are homogeneous, peripheral, speckled, nucleolar, and centromere. Each pattern is correlated with the presence of certain ANAs and should be followed up by confirmatory tests to more specifically characterize the antibodies.
- Rheumatoid factor is an autoantibody directed against the Fc portion of IgG molecules. It is found in the majority of patients with rheumatoid arthritis, but is not specific for the disease because it is also present in a significant number of patients with other autoimmune diseases involving the connective tissues.
- Antineutrophil cytoplasmic antibodies (ANCAs) are strongly associated with autoimmune syndromes involving vasculitis. ANCAs are routinely detected by IIF using ethanol- or formalin-fixed leukocytes as the substrate. Two patterns of fluorescence can result: c-ANCA, a diffuse, granular staining of the cytoplasm of the neutrophils, mainly caused by antibodies against PR3 and seen in the vast majority of patients with active systemic GPA; and p-ANCA, characterized by fluorescence surrounding the nuclear lobes of ethanol-fixed neutrophils, caused by antibodies to positively charged antigens such as MPO.

CASE STUDIES

1. A 25-year-old female consulted her physician because she had been experiencing symptoms of weight loss, joint pain in the hands, and extreme fatigue. Her laboratory results were as follows: RF rapid slide test positive at 1:10; ANA rapid slide test positive at 1:40; RBC 3.5×10^{12} per L (normal is 4.1 to 5.1×10^{12} per L); WBC count 5.8×10^{9} per L (normal is 4.5 to 11×10^{9} per L).

Questions

a. What is a possible explanation for positive results on both the RF test and the ANA test?
b. What is the most likely cause of the decreased RBC count?
c. What further testing would help the physician distinguish between RA and SLE?

2. A 40-year-old female went to her doctor because she was feeling tired all the time. She had gained about 10 pounds in the last few months and exhibited some facial puffiness. Her thyroid gland was enlarged and rubbery. Laboratory results indicated a normal RBC and WBC count, but her FT4 level was decreased and an assay for antithyroglobulin antibody was positive.

Questions

a. What condition do these results likely indicate?
b. What effect do antithyroglobulin antibodies have on thyroid function?
c. How can this condition be differentiated from Graves disease?

REVIEW QUESTIONS

1. All of the following may contribute to autoimmunity *except*
   a. clonal deletion of self-reactive T cells.
   b. molecular mimicry.
   c. increased expression of class II MHC antigens.
   d. polyclonal activation of B cells.

2. Which of the following would be considered an organ-specific autoimmune disease?
   a. SLE
   b. RA
   c. GPA
   d. Hashimoto's thyroiditis

3. SLE can be distinguished from RA on the basis of which of the following?
   a. Joint pain
   b. Presence of antinuclear antibodies
   c. Immune complex formation with activation of complement
   d. Presence of anti-dsDNA antibodies

4. Which of the following would support a diagnosis of drug-induced lupus?
   a. Antihistone antibodies
   b. Antibodies to Smith antigen
   c. Presence of RF
   d. Antibodies to SS-A and SS-B antigens

5. A peripheral pattern of staining of the nucleus on IIF is caused by which of the following antibodies?
   a. Anti-Sm antibody
   b. Anti-SSA/Ro antibody
   c. Centromere antibody
   d. Anti-dsDNA

6. Which of the following would be considered a significant finding in Graves disease?
   a. Increased TSH levels
   b. Antibody to TSHR
   c. Decreased T3 and T4
   d. Antithyroglobulin antibody

7. Destruction of the myelin sheath of axons caused by the presence of antibody is characteristic of which disease?
   a. MS
   b. MG
   c. Graves disease
   d. Goodpasture's syndrome

8. Blood was drawn from a 25-year-old woman with suspected SLE. A FANA screen was performed and a speckled pattern resulted. Which of the following actions should be taken next?
   a. Report out as diagnostic for SLE
   b. Report out as drug-induced lupus
   c. Perform an assay for specific ANAs
   d. Repeat the test

9. Which of the following is a mechanism used to achieve peripheral tolerance?

a. Negative selection of autoreactive T cells in the thymus
b. Apoptosis of autoreactive B cells in the bone marrow
c. Editing of B-cell receptors that weakly recognize self-antigens in the bone marrow
d. Lack of a costimulatory signal to autoreactive T cells in the lymph nodes

10. Epitope spreading refers to

a. post-translational modifications to self-antigens.
b. modifications in gene expression that are not caused by changes in DNA sequence.
c. expansion of the immune response to unrelated antigens.
d. cross-reaction of the immune response to a pathogen with a similar self-antigen.

11. Anti-CCP (cyclic citrullinated proteins) is specifically associated with which autoimmune disease?

a. RA
b. MG
c. Autoimmune hepatitis
d. Goodpasture's syndrome

12. Which autoantibodies are strongly associated with granulomatosis with polyangiitis (Wegener's granulomatosis)?

a. ANA
b. ANCA
c. AMA
d. SMA

13. A technologist performs an IIF test for ANCAs and observes that there is an intense fluorescent staining of the nuclear lobes of the neutrophils. How can this type of staining be differentiated from a peripheral ANA pattern?

a. Perform the test on formalin-fixed leukocytes
b. Perform IIF with HEp-2 cells
c. Perform an ELISA for ANCAs
d. All of the above

14. A 20-year-old woman made an appointment to see her physician because she was experiencing intermittent diarrhea. Laboratory testing revealed that she also had an iron deficiency anemia. To determine if the patient has celiac disease, her doctor should order which of the following laboratory tests?

a. Anti-tTG
b. Antigliadin
c. Antigluten
d. All of the above

15. Antimitochondrial antibodies are strongly associated with which disease?

a. Autoimmune hepatitis
b. Celiac disease
c. Primary biliary cirrhosis
d. Goodpasture's syndrome

16 Transplantation Immunology

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. List the histocompatibility systems relevant to clinical transplantation.
2. Compare the mechanisms of direct and indirect alloantigen recognition.
3. Distinguish between an allograft, autograft, xenograft, and syngeneic graft (isograft).
4. Compare the immunologic mechanisms involved in hyperacute, acute, and chronic graft rejection.
5. Identify risk factors for graft-versus-host disease (GVHD) and the types of grafts in which this mechanism of rejection could occur.
6. List the major classes of immunosuppressive agents and their effects on the immune system.
7. Explain the principles of laboratory methods for human leukocyte antigen (HLA) typing.
8. Describe laboratory methods for detecting and identifying HLA antibodies (i.e., antibody screening, identification, and crossmatching).
9. Deduce the suitability of a possible donor for a transplant recipient, based on results of HLA typing and antibody identification.
10. Describe the nomenclature used for HLA antigens and alleles.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Accelerated rejection
Acute graft-versus-host disease (GVHD)
Acute rejection (AR)
Allograft
Autograft
Chronic rejection
Complement-dependent cytotoxicity (CDC)
Crossmatch
Direct allorecognition
Graft-versus-host disease (GVHD)
Haplotypes
HLA antibody screen
HLA genotype
HLA matching
HLA phenotype
HLA typing
Human leukocyte antigens (HLAs)
Hyperacute rejection
Immunosuppressive agents
Indirect allorecognition
Isograft
Major histocompatibility complex (MHC)
Minor histocompatibility antigens (mHAs)
Mixed lymphocyte reaction
Percent panel reactive antibody (%PRA)
Polymorphism
Syngeneic graft
Xenograft

Transplantation is a potentially lifesaving treatment for end-stage organ failure, cancers, autoimmune diseases, immune deficiencies, and a variety of other diseases. In 2015, 30,900 solid-organ (kidney, pancreas, liver, heart, lung, small intestine) transplants were performed in the United States.[1] Today, more than 50,000 hematopoietic stem cell (HSC) transplants are performed worldwide each year for a variety of indications, including cancer, autoimmune disease, immunodeficiencies, and other diseases.[2] The number of transplants performed is a testament to the numerous developments over the past few decades in patient management pre- and post-transplant and in the technologies for organ or stem cell acquisition and sharing. Of critical importance has been the growing knowledge of the immunologic mechanisms of graft rejection and **graft-versus-host disease (GVHD),** in particular the role of **human leukocyte antigens (HLAs)** and the development of pharmacological agents that promote graft survival by interfering with various components of the immune system.

The HLA system is the strongest immunologic barrier to successful allogeneic organ and HSC transplantation (see Chapter 2 for details). It consists of cell surface proteins that play a central role in thymic education of T lymphocytes, initiation of adaptive immune responses, and regulation of other immune system components. HLA proteins are found on the surface of almost all nucleated cells and are antigenically very diverse in the human population. Therefore, transplantation of a solid organ or HSCs into an allogeneic host is likely to result in graft rejection in the absence of immunosuppressive therapy.

Histocompatibility Systems

Major Histocompatibility Complex (MHC) Antigens

The classical (transplant) antigens, also known as **major histocompatibility complex (MHC)** antigens, are composed of the class I and class II proteins. Class I proteins include HLA-A, HLA-B, and HLA-C; class II proteins consist of HLA-DR, HLA-DQ, and HLA-DP. HLA proteins are encoded by a set of closely linked genes located on the short arm of chromosome 6 within the MHC region. HLA genes are inherited as **haplotypes** from parental chromosomes **(Fig. 16–1).** A haplotype is a group of closely linked alleles on a single chromosome; for example, HLA-A1, HLA-Cw7, HLA-B8, HLA-DR3, and HLA-DQ2. Offspring receive one maternal and one paternal HLA haplotype. Based on Mendelian inheritance, there is a 25% chance that any two siblings will inherit the same two haplotypes (i.e., are HLA identical), a 50% chance of them being HLA haploidentical (i.e., share one of two HLA haplotypes), and a 25% chance of them being HLA nonidentical (i.e., share neither HLA haplotype). Recombination within the HLA region can take place, resulting in the inheritance of unexpected haplotypic combinations; however, this occurs in less than 1% of families that are HLA typed.

HLA proteins are heterodimeric molecules consisting of two different polypeptide chains chemically bound to each other (see Chapter 2). They are also members of the immunoglobulin superfamily, which share structural similarities with immunoglobulin molecules.

Class I proteins are the product of the HLA-A, HLA-B, and HLA-C genes and are expressed on the cell surface

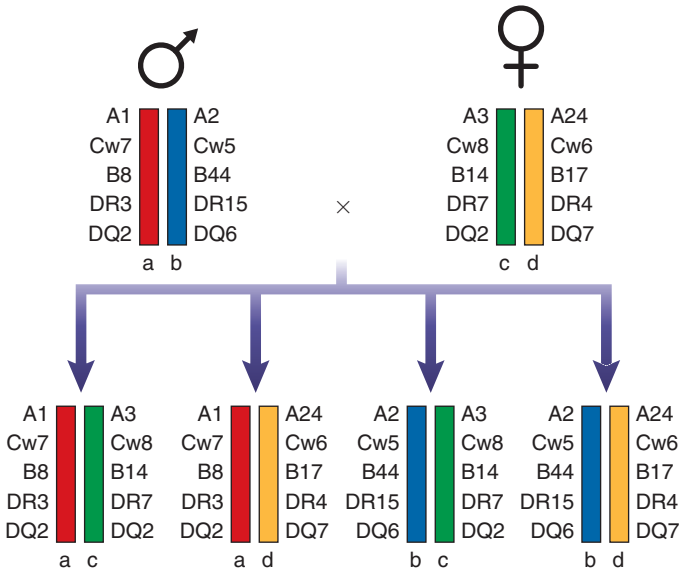

FIGURE 16–1 HLA genes are linked and inherited in Mendelian fashion as haplotypes. One paternal (a or b) and one maternal (c or d) haplotype is passed to each offspring. Four different combinations of haplotypes are possible in offspring. Elucidation of haplotype sharing between siblings is an important assessment in the search for a transplant donor. *(Figure courtesy of John Schmitz.)*

covalently bound to β2–microglobulin. Class I heterodimers are codominantly expressed on virtually all nucleated cells. Class II heterodimers are the products of the HLA-D region genes. Class II proteins are codominantly expressed primarily on antigen-presenting cells (APCs; e.g., dendritic cells, monocytes, macrophages, B lymphocytes).

As discussed in Chapter 2, HLA proteins have critical roles in the development and functioning of the innate and adaptive immune systems. They serve as recognition elements for antigen receptors on T lymphocytes, thus initiating adaptive immune responses. In addition, they serve as ligands for regulatory receptors on natural killer (NK) cells in the innate immune response. The CD8 molecule on cytotoxic T lymphocytes interacts with class I HLA proteins, whereas the CD4 molecule on the T helper (Th) cell subset interacts with class II HLA proteins.

A cardinal feature of the genes encoding HLA proteins is the extensive degree of allelic **polymorphism.** Polymorphism refers to the presence of two or more different genetic compositions among individuals in a population. The HLA system is the most polymorphic genetic system in humans; many HLA genes exist in the population and numerous combinations of these genes are possible in individuals (see **Tables 16–1 and 16–2**). This degree of polymorphism is believed to have resulted from the survival benefit of initiating an immune response to a broad array of peptides from an innumerable array of pathogenic microbes that populations encounter. Although this has successfully enabled populations to survive infectious challenges, it severely restricts the ability to transplant foreign tissues or cells between any two individuals because the HLA proteins are immunogenic and elicit robust allogeneic immune responses.

Minor Histocompatibility Antigens (mHAs)

Researchers identified a second set of transplantation antigens based on studies in mice and humans. These studies demonstrated tissue rejection in MHC-identical transplants and development of GVHD in HLA-identical sibling HSC transplants.[3] The scientists conducting these studies also observed that a "slower" rejection pace was mediated by these transplantation antigens, thus their name—**minor histocompatibility antigens (mHAs).**

mHAs are non-HLA proteins that demonstrate variation in the amino acid sequence between individuals. Both X-linked and autosomally encoded mHAs have been identified. Transplanting one individual's tissue or cells containing a polymorphic variant of one of these proteins into another individual possessing a different polymorphic variant can induce a recipient's immune response to the donor variant. CD4 or CD8 T cells recognize the variant protein in an MHC-restricted fashion and mediate the immune response. This response is analogous to the reaction to a microbial antigen. Several mHAs have been identified, including proteins encoded by the male Y chromosome, proteins for which the recipient has a homozygous gene deletion, proteins that are autosomally encoded, and proteins that are encoded by mitochondrial DNA.[3]

Table 16–1 Approximate Number of HLA Antigens and Alleles Defined at the Classical Transplant Loci*

HLA LOCUS	# ANTIGENS	# ALLELES
A	28	3,356
B	62	4,179
C	10	2,902
DRB1	24	1,860
DQB1	9	900

**Note: The number of HLA alleles identified has increased tremendously in recent years because of the availability of improved molecular techniques. The numbers in this table were current as of January 2016. To view the most up-to-date information about the HLA alleles discovered, access the website: http://hla.alleles.org/nomenclature/stats.html.*

Table 16–2 Listing of Individual HLA Antigens*

HLA-A		HLA-B			HLA-C	HLA-DR	HLA-DQ
1	66	5	44	64	1	1	1
2	68	7	45	65	2	2	2
3	69	8	46	67	3	3	3
9	74	12	47	70	4	4	4
10	80	13	48	71	5	5	5
11		14	49	72	6	6	6
19		15	50	73	7	7	7
23		16	51	75	8	8	8
24		17	52	76	9	9	9
25		18	53	77	10	10	
26		21	54	78		11	
28		22	55	81		12	
29		27	56	82		13	
30		35	57			14	
31		37	58			15	
32		38	59			16	
33		39	60			17	
34		40	61			18	
36		41	62				
43		42	63				

**Note: HLA antigen names begin with the prefix "HLA-", followed by the gene locus (A, B, C, or D) and the antigen number (e.g., HLA-A2). A "w" was originally placed after newly identified antigens at International Histocompatibility Workshops. The "w" has been retained for HLA-C antigen names to differentiate them from complement proteins (e.g., HLA-Cw1).*

MHC Class I-Related Chain A (MICA) Antigens

The MHC class I-related chain A (MICA) gene encodes a cell surface protein that is involved in gamma or delta T-cell responses. MICA is polymorphic, with over 50 allelic variants. MICA proteins are expressed on endothelial cells, keratinocytes, fibroblasts, epithelial cells, dendritic cells, and monocytes, but they are not expressed on T or B lymphocytes.[4] As such, MICA proteins could serve as targets for allograft immune responses. Antibodies to MICA antigens have been detected in as many as 11% of kidney-transplant patients and are associated with rejection episodes and decreased graft survival.[5]

ABO Blood Group Antigens

The ABO system is the only blood group system that affects clinical transplantation. ABO blood group incompatibility is a barrier to solid-organ transplantation because these antibodies can bind to the corresponding antigens that are expressed on the vascular endothelium. Binding activates the complement cascade, which can lead to very rapid rejection of the transplanted organ. This phenomenon, known as **hyperacute rejection,** occurs within minutes to hours after the vascular supply to the transplanted organ is established (see *Transplant Rejection*). Anti-A or anti-B antibodies develop in individuals lacking the corresponding blood group antigens. As such, recipient–donor pairs must be ABO identical or compatible to avoid this adverse outcome. For example, an individual of blood group A will possess anti-B antibodies and can thus receive an organ only from an ABO type A or type O donor. Likewise, a B-expressing individual has anti-A antibodies and can receive an organ only from an ABO type B or type O donor.

Transplantation approaches using plasma exchange and intravenous immunoglobulin administration have allowed successful transplantation of kidneys from ABO-incompatible donors. The procedures reduce the ABO antibody to levels that significantly lower the risk of hyperacute rejection.[6]

Killer Immunoglobulin-Like Receptors (KIRs)

Another polymorphic genetic system that affects allogeneic transplantation is the killer immunoglobulin-like receptor (KIR) system.[7] KIRs are one of several types of cell surface molecules that regulate the activity of NK lymphocytes. The KIRs contain activating and inhibitory receptors that vary in number and type on any individual NK cell. The balance of signals received by the activating and inhibitory receptors regulates the activity of the NK cell (see Fig. 3–7).

The ligands for the inhibitory KIRs have been defined as several of the class I MHC molecules, including specific HLA-A, HLA-B, and HLA-C proteins. Normally, an NK cell encounters self class I MHC proteins as it circulates in the body. This interaction between MHC protein and the inhibitory KIR maintains the NK cells in a quiescent state. If an NK cell encounters a cell with absent or decreased HLA class I expression, inhibitory receptors are not engaged and a loss of negative regulatory activity occurs, resulting in NK cell activation.

The regulatory role of KIRs has been exploited in haploidentical stem cell transplantation.[8] Stem cell donors have been selected for recipients who lack a corresponding class I MHC protein for the donor's inhibitory KIR type. This results in alloreactivity by NK cells that repopulate the recipient after transplant. These alloreactive NK cells have been shown to mediate a graft-versus-leukemia (GVL) reaction and prevent relapse after transplantation for certain types of hematologic malignancies.

Self-Antigens

In addition to these well-described alloantigen systems, humoral immune responses to self-antigens in transplant recipients have been associated with poor transplant outcomes, although a direct causal relationship has yet to be firmly established. Among the several proteins to which antibody has been described post-transplantation are angiotensin II type-1 receptor,[9] vimentin, K-alpha1 tubulin, collagen-v, and myosin.[10]

Allorecognition

Transplantation of cells or tissues is classified by the genetic relatedness of the donor and the recipient. An **autograft** is the transfer of tissue from one area of the body to another of the same individual. A **syngeneic graft** (also known as an **isograft**) is the transfer of cells or tissues between individuals of the same species who are genetically identical, for example, identical twins. An **allograft** is the transfer of cells or tissue between two genetically disparate individuals of the same species. Finally, a **xenograft** is the transfer of tissue between two individuals of different species. Most transplants fall into the category of allografts. The HLA disparity between donor and recipient that occurs with allografts and xenografts will result in a vigorous cellular and humoral immune response to the foreign MHC antigens. This response is the primary stimulus of graft rejection.

The recipient's immune system recognizes foreign HLA proteins via two distinct mechanisms—direct and indirect allorecognition **(Fig. 16–2).**[11] In **direct allorecognition,** recipient T cells bind and respond directly to foreign (allo) HLA proteins on graft cells. Although an individual T lymphocyte can recognize self-HLA + peptide, foreign HLA proteins may mimic a self-HLA + peptide complex because of similarities in the structure of the allo-HLA protein itself or to structural similarities of allo-HLA protein + peptide. Evidence suggests that virus-specific T cells may be an important source of alloreactive cells.[12] Either way, direct allorecognition is characterized by a high frequency (up to 10%) of responding T cells[11,13] compared with the responder frequency in a typical T-cell response to a foreign antigen. The **mixed lymphocyte reaction (MLR)** is an in vitro correlate of direct allorecognition. In this assay, lymphocytes from an individual needing a transplant are incubated with lymphocytes from a potential donor that have been inactivated so they cannot proliferate. A disparity in the HLA-D antigens found on the two populations of lymphocytes results in the

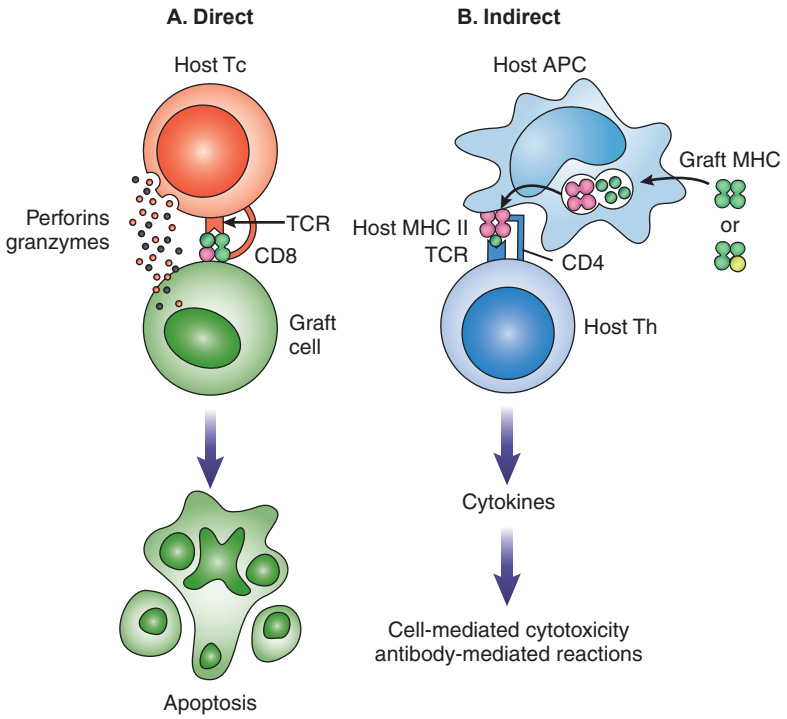

FIGURE 16–2 Direct versus indirect allorecognition. (A) In direct allorecognition, cytotoxic T cells from the host bind directly to foreign HLA (MHC I) proteins on graft cells. (B) In indirect allorecognition, APCs from the host present foreign MHC I or MHC II antigens to CD4+ Th cells, which produce cytokines that stimulate graft rejection.

proliferation of the recipient cells, which can be quantitated by incorporation of radio-labeled (^{3}H) thymidine into the DNA of the proliferating cells. The amount of radioactivity taken up by the cells increases in proportion to the amount of cell proliferation. Thus, a high level of radioactivity indicates that the recipient's T cells have divided in response to different HLA-D antigens on a potential donor's cells and that such a donor would be more likely to stimulate graft rejection.

Indirect allorecognition is the second pathway by which the immune system recognizes foreign HLA proteins.[13] Indirect allorecognition involves the uptake, processing, and presentation of foreign HLA proteins by recipient APCs to recipient T cells. It is analogous to the normal mechanism of recognition of foreign antigens. Indirect allorecognition may play a predominant role in induction of alloantibody and chronic rejection.[12]

The effector responses against transplanted allogeneic tissue include direct cytotoxicity, delayed-type hypersensitivity responses, and antibody-mediated mechanisms. Antibody can mediate antibody-dependent cellular cytotoxicity reactions and fix complement, resulting in cell death. Rejection episodes vary in the time of occurrence and the effector mechanism that is involved. The next section will cover three types of rejection: hyperacute, acute, and chronic.

Transplant Rejection

Hyperacute Rejection

As previously discussed, hyperacute rejection occurs within minutes to hours after the vascular supply to the transplanted organ is established. This type of rejection is mediated by preformed antibody that reacts with donor vascular endothelium. ABO, HLA, and certain endothelial antigens may elicit hyperacute rejection. Antibodies to these antigens may be present as a result of blood transfusion, prior transplantation, or exposure of a pregnant woman to fetal antigens of paternal origin. Binding of preformed antibodies to the alloantigens activates the complement cascade and clotting mechanisms and leads to thrombus formation. The result is ischemia and necrosis of the transplanted tissue.[14]

Hyperacute rejection is seldom encountered in clinical transplantation. Donor–recipient pairs are chosen to be ABO identical or compatible and patients awaiting transplantation are screened for the presence of preformed HLA antibodies. In addition, the absence of donor HLA-specific antibodies is confirmed before transplant by the performance of a crossmatch test (see *HLA Antibody Screening, Identification, and Crossmatching*). These approaches have virtually eliminated hyperacute rejection episodes.

Some individuals possess very low levels of donor-specific antibody in the pretransplant period. In these cases, antibody-mediated rejection may take place over several days. This has been termed **accelerated rejection.**[11,15] Similar to hyperacute rejection, accelerated rejection involves intravascular thrombosis and necrosis of donor tissue.

Acute Rejection

Days to months after transplant, individuals may develop **acute rejection (AR).** AR can be mediated by a cellular alloresponse (ACR) or by donor-specific antibody (also known as antibody-mediated response; AMR).[14,16]

ACR is characterized by parenchymal and vascular injury. Interstitial cellular infiltrates contain a predominance of CD8+ T cells as well as CD4 T cells and macrophages. CD8 cells likely mediate cytotoxic reactions to foreign MHC-expressing cells, whereas CD4 cells likely produce cytokines and induce

delayed-type hypersensitivity (DTH) reactions. Antibody may also be involved in acute graft rejection by binding to vessel walls and activating complement. The antibody induces transmural necrosis and inflammation as opposed to the thrombosis typical of hyperacute rejection. Diagnostic criteria include characteristic histological findings, deposition of the complement protein C4d in the peritubular capillaries, and detection of donor-specific HLA antibody.[16] The development and application of potent immunosuppressive drugs that target multiple pathways in the immune response to alloantigens has improved early graft survival of solid-organ transplants by reducing the incidence of AR and by providing approaches for its effective treatment.

Chronic Rejection

Chronic rejection results from a process of graft arteriosclerosis characterized by progressive fibrosis and scarring with narrowing of the vessel lumen caused by proliferation of smooth muscle cells.[17] Chronic rejection remains the most significant cause of graft loss after the first year post-transplant because it is not readily amenable to treatment. Several predisposing factors affect the development of chronic rejection, including prolonged cold ischemia, reperfusion injury, AR episodes, and toxicity from immunosuppressive drugs. Chronic rejection is also thought to have an immunologic component, presumably a delayed-type hypersensitivity reaction to foreign HLA proteins.[11] This is indicated in studies employing animal models of graft arteriosclerosis in which mice lacking IFN gamma do not develop graft arteriosclerosis. In addition, similar studies support an important role for CD4 T cells and B cells in this process. Cytokines and growth factors—secreted by endothelial cells, smooth muscle cells, and macrophages activated by IFN gamma—stimulate smooth muscle cell accumulation in the graft vasculature. Alloantibody production likely contributes to the development of chronic rejection as well.[18]

Graft-Versus-Host Disease (GVHD)

HSC transplants (and less commonly lung and liver transplants) are complicated by a unique allogeneic response—GVHD. In this condition, lymphoid cells in the graft mount an immune response against the host's histocompatibility antigens. Recipients of HSC transplants for hematologic malignancies typically have depleted bone marrow before transplantation as a result of the chemotherapy used to treat the malignancy. The individual receives an infusion of donor bone marrow or, more commonly, peripheral blood stem cells. The infused products often contain some mature T cells. These cells have several beneficial effects, including promotion of engraftment, reconstitution of immunity, and mediation of a graft-versus-leukemia effect. However, these mature T cells may also mediate GVHD.

Acute graft-versus-host disease (GVHD) occurs during the first 100 days postinfusion and manifests in the skin, gastrointestinal tract, and liver.[19] In mismatched allogeneic stem cell transplantation, the targets of GVHD are the mismatched HLA proteins, whereas in matched stem cell transplantation mHAs are targeted. The infused T cells can mediate GVHD in several ways, including a massive release of cytokines because of large-scale activation of the donor cells by MHC-mismatched proteins and by infiltration and destruction of tissue.

The incidence and severity of GVHD is related to the match status of the donor and recipient as well as other factors.[20] The recipient's medical team can take several approaches to reduce the incidence and severity of GVHD including immunosuppressive therapy in the early post-transplant period and removal of T lymphocytes from the graft. T-cell reduction, achieved via purification of donor HSCs from the peripheral blood or bone marrow collection, is very effective in lowering the incidence of GVHD, but it can also reduce the GVL effect of the infused cells and increase the incidence of graft failure. The GVL effect is similar to the GVH response but targets the recipient's malignant cells as opposed to healthy tissues of the recipients such as the skin and gastrointestinal tract.

Beyond 100 days post-transplant, patients may experience chronic GVHD. This condition resembles autoimmune disease, with fibrosis affecting the skin, eyes, mouth, and other mucosal surfaces.

Immunosuppressive Agents

The list of agents employed to suppress antigraft immune responses in solid-organ and HSC transplantation is growing. **Immunosuppressive agents** are used in several ways, including induction and maintenance of immune suppression and treatment of rejection. Combinations of different agents are frequently used to prevent graft rejection. However, the immunosuppressed state (and graft survival) induced by these agents comes at a price of increased susceptibility to infection, malignancies, and other associated toxic side effects. There are several classes of immunosuppressive agents:

Corticosteroids—Corticosteroids are potent anti-inflammatory and immunosuppressive agents used for immunosuppression maintenance. At higher doses, they are used to treat AR episodes. Steroids act by blocking production and secretion of cytokines, inflammatory mediators, chemoattractants, and adhesion molecules. These activities decrease macrophage function and alter leukocyte-trafficking patterns. However, long-term use is associated with several complications, including hypertension and diabetes mellitus.

Antimetabolites—Antimetabolites interfere with the maturation of lymphocytes and kill proliferating cells.[21] Azathioprine was the first such agent employed. It has been replaced in large part by mycophenolate mofetil, which has a more selective effect on lymphocytes compared with azathioprine and thus fewer side effects.

Calcineurin inhibitors—Cyclosporine and FK-506 (tacrolimus) are compounds that block signal transduction in T lymphocytes, resulting in impaired synthesis of cytokines such as IL-2, IL-3, IL-4, and interferon-gamma.[21] Inhibition of cytokine synthesis blocks the growth and differentiation of T cells, impairing the antigraft response. Rapamycin

(sirolimus)[22] is an agent that inhibits T-cell proliferation by binding to specific intracellular proteins, including mammalian target of rapamycin (mTOR). mTOR is an intracellular molecule involved in cellular functions such as proliferation and motility.

Monoclonal antibodies—Several monoclonal antibodies that bind to cell surface molecules on lymphocytes are used at the time of organ transplant and to treat severe rejection episodes after transplantation. Two anti-CD25 monoclonal antibodies are available for use in transplant patients.[23] Basiliximab and daclizumab both bind the CD25 (IL-2 receptor) and thus interfere with IL-2–mediated T-cell activation. They may also deplete CD25-expressing cells. An additional monoclonal antibody that targets the CD52 receptor found on T and B lymphocytes is alemtuzumab, which may be used for induction therapy at the time of transplantation.[23] A problem with some monoclonal antibody preparations is that patients can develop anti-mouse antibody that may interfere with the effectiveness of these agents. The potential for this problem to occur is being reduced with increased use of humanized and fully human monoclonal antibodies (see Chapters 5 and 25).

Polyclonal antibodies—Two polyclonal anti–T-cell antibody preparations have been used as induction agents or to treat severe rejection. Thymoglobulin is an antithymocyte antibody prepared in rabbits and ATGAM is a polyclonal antiserum prepared from the immunization of horses. Both are potent immunosuppressive agents that deplete lymphocytes from the circulation. A drawback associated with administration of polyclonal antibody preparations is the development of serum sickness because of antibody responses to the foreign immunoglobulin (see Chapter 14).

Clinical Histocompatibility Testing

Appreciation of the beneficial role of **HLA matching**[11,24] and the detrimental role of antibody to HLA proteins[15] on graft survival provided the impetus for development and application of specialized testing to aid in the selection of the most appropriate donors for patients needing transplantation. Histocompatibility laboratories provide specialized testing for both solid-organ and stem cell transplantation programs. Two main activities are carried out by these laboratories in support of transplantation: HLA typing and HLA antibody screening and identification.

HLA Typing

HLA typing is the phenotypic or genotypic identification of the HLA antigens or genes in a transplant candidate or donor. For clinical HLA testing, phenotypes or genotypes of the classical transplant antigens or genes are determined (HLA-A, HLA-B, HLA-Cw, HLA-DR, HLA-DQ). This information is used to find the most suitable donor–recipient combination from an immunologic standpoint. It must be stressed that other factors must also be considered when choosing a particular donor for any given patient, be it a solid-organ or stem cell transplant. For example, ABO compatibility and infectious disease status are important considerations in donor selection.

HLA Phenotyping

The classic procedure for determining the **HLA phenotype** is the **complement-dependent cytotoxicity (CDC)** test. Panels of antisera or monoclonal antibodies that define individual or groups of immunologically related HLA antigens are incubated with lymphocytes from the person to be HLA typed in separate wells of a microtiter plate. Each well of the plate contains a different antibody. It is important to note that multiple antisera are used for HLA typing. This requirement is based on the presence of both unique epitopes on HLA molecules (those that define the phenotypic specificity of a specific HLA antigen) and public epitopes (epitopes that are present on more than one unique HLA protein). Because responses to public epitopes are common, many sera must be used to define a pattern of reactivity that correlates with a specific HLA antigen. T and B lymphocytes are used for HLA class I typing, whereas purified B lymphocytes are used for HLA class II typing because class II antigens are not found on most T cells. After incubation with the antisera, complement is added. Binding of antibody occurs only if the lymphocytes express the HLA antigen targeted by the antisera. A complement reagent derived from rabbit serum is added to each well. If the cells possess the HLA antigen defined by the antibody in that well, complement is activated and the cells are killed. A vital dye such as eosin red or trypan blue is added to distinguish live cells from dead cells when they are viewed microscopically. The dead cells, whose membranes have been made more permeable, are able to take up the dye and appear colored, whereas the live cells, whose membranes remain intact, cannot take up the dye and remain colorless. The proportion of dead cells is estimated by microscopic examination and scored according to the following scale, established by the American Society for Histocompatibility and Genetics (ASHI):

- 1 = 0% to 10% cell death; negative
- 2 = 11% to 20% cell death; doubtful negative
- 4 = 21% to 50% cell death; weak positive
- 6 = 51% to 80% cell death; positive
- 8 = 81% to 100% cell death; strong positive
- 0 = unreadable

The principle of the CDC is illustrated in **Figure 16–3.** Using this assay, an extensive array of HLA antigens can be defined (see Table 16–2).

The CDC method has several limitations for HLA typing. Viable lymphocytes must be used, which demands timely performance of the assay. Separation of T and B lymphocytes is required for differentiation of class I versus class II antigens. In addition, the source of antisera for HLA typing is not always consistent or reliable. Thus, reagents can vary in quality or quantity over time. Finally, the level of resolution (i.e., the ability to distinguish two closely related yet distinct HLA antigens) is limited. The limits of resolution don't significantly affect the

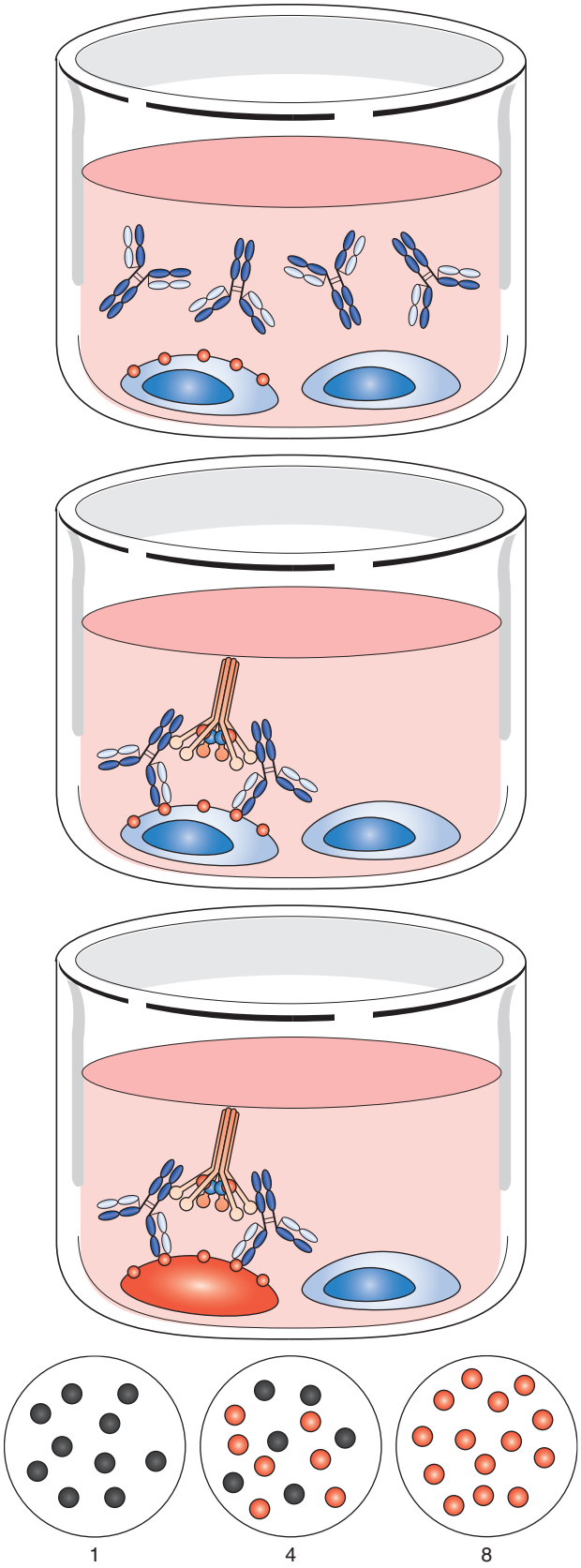

FIGURE 16–3 Principle of the complement-dependent cytotoxicity (CDC) test.

role of this technology for matching solid-organ donors and recipients. However, for allogeneic stem cell transplantation, a higher level of resolution is required. DNA-based (molecular) HLA typing methods are now commonly employed in histocompatibility laboratories because their higher resolution, reagent quality, and amenability to higher throughput formats overcome the limitations of CDC-based methods.

HLA Genotyping

Molecular-based HLA genotyping methods use polymerase chain reaction (PCR)-based amplification of HLA genes followed by analysis of the amplified DNA to identify the specific HLA allele or allele group (see Chapter 12).[25] Three DNA-based HLA typing methods are in use: polymerase chain reaction with sequence-specific PCR (PCR-SSP), PCR-sequence-specific oligonucleotide probe hybridization (PCR-SSOP), and sequence-based typing (SBT). The most common approaches for analysis involve PCR amplification of HLA genes with panels of primer pairs, each of which amplifies specific alleles or related allele groups (PCR-SSP). Only those primer pairs that bind perfectly to the target gene result in detection of an amplification product **(Fig. 16–4).** Amplification is detected by agarose gel electrophoresis. The **HLA genotype** is then identified by determining which primers resulted in amplification.

A second common approach for HLA genotyping is to perform PCR-SSOP. This involves a single PCR reaction that will amplify all HLA gene variants at a specific locus (referred to as a generic amplification). The amplified gene is then subjected to hybridization with a panel of DNA probes, each specific for a unique HLA allele or allele group. Only those probes that specifically hybridize to the amplified DNA will be detected. The HLA genotype is determined by assessing which probes hybridized **(Fig. 16–5).**

A third common method for HLA genotyping is SBT, which involves sequencing of PCR-amplified HLA genes. SBT is typically carried out using Sanger dideoxy chain terminator sequencing. A generic amplification of the HLA gene of interest is conducted, followed by a sequencing reaction using dideoxy nucleotides. The dideoxy terminators are fluorescently labeled. Incorporation into the synthesized DNA molecule is detected using automated DNA sequencers with fluorescent detectors. The sequence of the target gene is compared with an HLA sequence database to determine the specific HLA allele for the patient. SBT for HLA typing is considered the gold standard and is able to detect new allelic variants because it interrogates all nucleotides in the amplified target region as opposed to PCR-SSP and PCR-SSOP that target small stretches of previously defined nucleotides.

HLA genotyping overcomes the limitations of CDC-based HLA phenotyping. Cells do not need to be viable in order to obtain DNA for HLA genotyping. Typing reagents are chemically synthesized; thus, there is no reliance on human donors of antisera. HLA genotyping can provide varying levels of resolution that can be tailored to the specific clinical need. DNA-based typing can provide results at a level of resolution

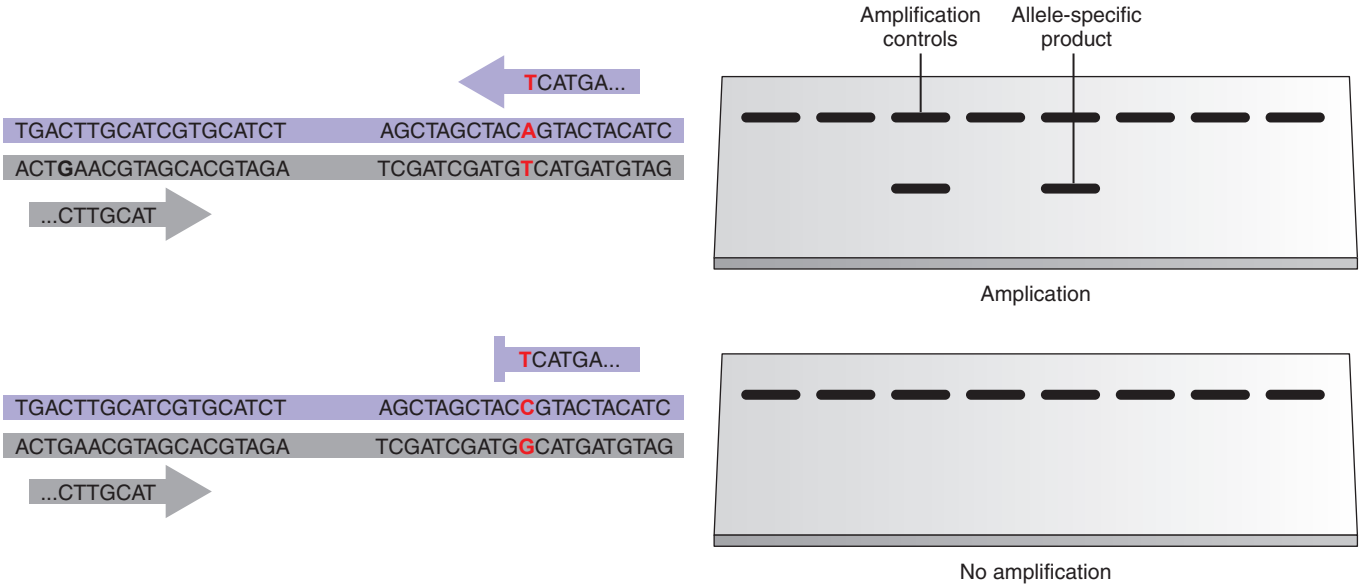

FIGURE 16–4 Principle of PCR-SSP. The sequence-specific primer ending in 3′TCATGA...5′ will be extended only from a template carrying the polymorphism shown.

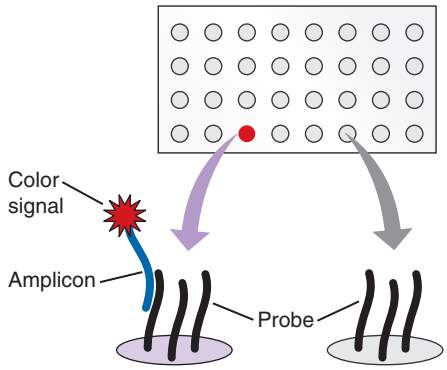

FIGURE 16–5 Principle of PCR-SSOP. Patient DNA is amplified using primers labeled with biotin or digoxigenin at the 5′ end. The amplicons are then hybridized to panels of probes immobilized on a membrane. If the sequence of the amplicon matches and hybridizes to that of the probe, a secondary reaction with enzyme-conjugated avidin or antidigoxigenin will produce a color or light signal when exposed to substrate (bottom left). If the sequence of the amplicon differs from that of the probe, no signal is generated (bottom right).

comparable to CDC-based typing (antigen equivalent) or can provide results at the allele level, which are required for matching of unrelated HSC donors and recipients. Allele-level HLA typing has demonstrated the incredible extent of polymorphism within the HLA loci (see Table 16–1). The nomenclature used for HLA typing is explained in **Figure 16–6.**

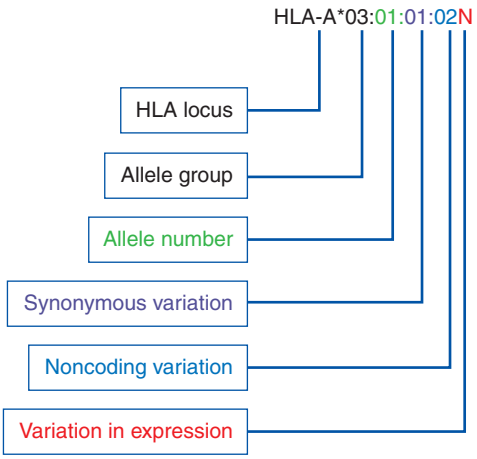

FIGURE 16–6 The nomenclature used for naming HLA alleles includes identification of the HLA locus, which is separated from the allele number by an "*". The first 2 digits of the allele number typically correspond to the phenotypic group. The next position after the ":" identifies the specific allele within the allele group. This position can accommodate up to 3 digits. The next position identifies allelic variants resulting from synonymous nucleotide substitutions (i.e., they do not result in a change in amino acid). The next position identifies allelic variants resulting from polymorphisms in noncoding regions of the gene (i.e., introns). Lastly, a letter may be added that indicates unique features of the allele such as "N" indicating the allele is null (i.e., not expressed on the cell surface). *(Figure courtesy of John Schmitz.)*

Connections

Polymerase Chain Reaction

Recall from Chapter 12 that the PCR is a molecular method that is used to produce millions of copies of a DNA segment. It is commonly used to amplify a piece of DNA to levels that can be detected in the laboratory. Amplification is achieved by a three-step cycle: (1) heat denaturation of the target DNA (in this case, DNA coding for a specific HLA allele) to form single strands; (2) cooling the reaction and binding of short, complementary nucleotide sequences called primers to the 3′ ends of the target strands to initiate replication; and (3) heating the reaction in the presence of DNA polymerase and the 4 deoxynucleotide triphosphates (i.e., the DNA building blocks) to extend the strands. These heating-cooling cycles are performed by an automated thermocycler.

HLA Antibody Screening, Identification, and Crossmatching

Antibodies to HLA antigens can be detected in candidates and recipients of solid-organ transplants by performance of a **crossmatch** test. These antibodies can develop in response to multiple blood transfusions or to prior HLA-mismatched transplants. They can also be produced by women who have had multiple pregnancies in response to paternally derived fetal antigens. Because of the potential adverse impact HLA antibodies can have on graft survival, patients awaiting solid-organ transplantation are screened periodically for their presence through an **HLA antibody screen.**[15] If detected, the HLA specificity of the antibodies is then determined so that donors possessing those HLA antigens can be eliminated from consideration for donation to that patient. Patients are tested monthly for the presence of HLA antibodies while they are waiting for an organ offer. Antibody screening and identification is also performed post-transplantation to aid in the diagnosis of antibody-mediated rejection and to assess the effectiveness of therapy for antibody-mediated rejection. Crossmatching is performed before transplant to confirm the absence of donor-specific antibody.

The methods used for antibody detection, identification, and crossmatching have changed significantly in recent years. The CDC method used for HLA typing is also used for HLA antibody detection and identification. In this case, panels of lymphocytes with defined HLA phenotypes are incubated with the patient's serum. If the serum contains HLA antibodies, they will bind to those lymphocytes in the panel that express the corresponding HLA antigen. Binding is detected by addition of a complement reagent derived from rabbit serum and a vital dye to assess cell death microscopically (see Fig. 16–3).

In some scenarios, the level of antibody in a serum sample may be below the level detectable by the CDC assay. In these cases, anti-human globulin (AHG) can be added to the CDC assay to increase the test's sensitivity. The AHG-CDC assay can detect lower levels of antibody as well as isotypes of bound antibody that don't activate complement and thus wouldn't normally be detected in the standard CDC assay. Usually 30 to 60 unique lymphocyte preparations are included in the panel; the proportion of lymphocytes in the panel that are killed by the patient's serum is referred to as the **percent panel reactive antibody (%PRA).** In addition, the specificity of the antibodies can be determined by evaluating the phenotype of the panel cells.

More recently, the determination of PRA for solid-organ allocation has been modified. Currently, a calculated PRA (cPRA) is determined for organ allocation. In this approach, the HLA antigens to which a candidate has HLA antibody are determined using solid-phase assays. These antigens are then classified as unacceptable antigens for that candidate. Accordingly, donors expressing those antigens are excluded from donation for that candidate. The proportion of potential donors in the donor pool possessing one or more of the unacceptable antigens is determined and reported as the cPRA value. For example, a recipient with an HLA-A2 antibody would have a cPRA value of 47% if 47% of potential donors are projected to be HLA-A2 expressing, based on historic HLA typing data.

Enzyme-linked immunosorbent assay (ELISA) has been developed as a substitute for CDC-based HLA antibody testing.[26] ELISA assays utilize purified HLA antigens bound to the wells of microtiter plates. Patient serum is added to the wells of the plate; if HLA-specific antibody is present, it will bind. Bound antibody is detected by the addition of an enzyme-labeled anti-immunoglobulin reagent. Addition of substrate results in a color change in the wells that have bound antibody. The wells of the ELISA plate may contain a pool of HLA antigens, thus serving as a qualitative screen for the presence of HLA antibody in a serum. Alternatively, each well may contain HLA antigens representing a single donor and thus can be used in a fashion analogous to a CDC-based analysis, allowing %PRA and specificity to be determined.

Another approach for antibody detection and identification is flow cytometry.[27] Antibody in patient serum can be incubated with beads that are coated with purified HLA antigens, either from a pool of donors, an individual donor, or a single purified or recombinant HLA protein. Beads coated with pooled HLA proteins are a sensitive qualitative screen for the presence of HLA antibody because they will detect antibodies to the majority of common HLA antigens. Beads coated with purified HLA proteins from individual donors or with a single HLA type (referred to as single-antigen beads) are used to determine the specificity of the HLA antibodies in a patient's serum; this information is used to determine the cPRA. Patient serum is incubated with the beads and bound antibody is detected by adding a FITC-labeled anti-IgG reagent **(Fig. 16–7).** A more recent version of flow cytometry-based antibody detection is the multiplex bead array system that can assess binding of patient antibodies to up to 100 different HLA antigens in a single tube using a dedicated flow-based detection system such as a Luminex bead array.[28] Flow cytometry-based methods are the most sensitive technology for detecting HLA antibodies. In addition, they can provide the most specific determination of the specificity of HLA antibodies when beads coated with a single HLA antigenic type are used.

Once a suitable donor has been identified for a particular patient, a donor–recipient crossmatch test is performed to confirm the absence of donor-specific antibody. Donor T and B lymphocytes are incubated with recipient serum in a CDC assay. Microscopic analysis is used to verify a lack of binding after the addition of complement and a vital dye to differentiate live from dead cells. Cell death is an indication of recipient antibody binding to donor HLA antigen(s). Alternatively, binding of antibody can be detected by flow cytometry using an FITC-labeled anti-IgG reagent. As for antibody screening and identification, the flow cytometric crossmatch is the most sensitive method for detecting donor-specific antibody.[15]

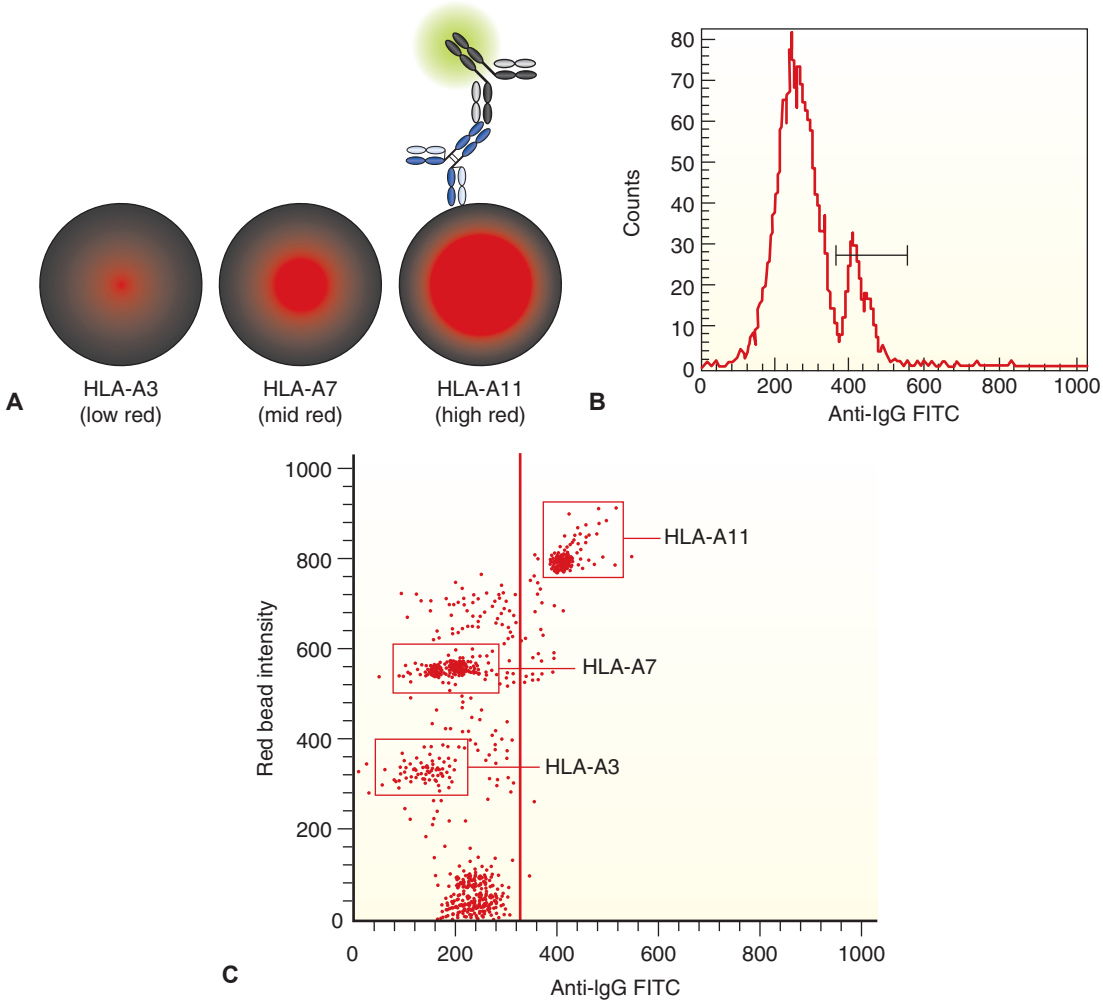

FIGURE 16–7 Flow cytometric detection and identification of antibodies. (A) Patient serum is incubated with a mixture of polystyrene beads coated with different HLA proteins (e.g., HLA-A3, HLA-A7, HLA-A11). (The beads have been labeled with various amounts of a red dye for identification.) Any HLA antibodies present in the serum will bind to the corresponding beads. After washing, patient antibodies bound to the beads are detected by the addition of a FITC-labeled anti-IgG reagent. After a second wash, the beads are analyzed for fluorescence on a flow cytometer. (B) Single-parameter histogram display of an HLA class I antibody screen using the three types of HLA-coated beads. The large peak represents beads with no bound antibody, whereas the smaller peak to the right indicates the presence of HLA antibody bound to approximately 29% of the HLA class I coated beads. This represents a positive HLA class I antibody screen. (C) The individual bead populations are identified in the dual-parameter dot plot. The beads coated with HLA-A11 have shifted to the right relative to the other beads, indicating that the HLA antibodies in this patient's serum are specific for the A11 antigen.

SUMMARY

- The immune system's ability to recognize and respond to the myriad of infectious agents that humans encounter has obvious benefits. However, the mechanisms that impart this ability make the transplantation of organs and cells between allogeneic individuals difficult.
- The targets of the response to transplanted tissues are HLA proteins and, to a lesser extent, minor histocompatibility antigens. These proteins play critical roles as antigen-presenting molecules for CD4 and CD8 T cells, resulting in the phenomenon of MHC restriction. Although an individual's immune system develops to respond to the foreign proteins presented on its own MHC antigens, it responds intensely to foreign MHC proteins. Both the humoral and cellular branches of the immune system contribute to this allogeneic immune response and mediate graft rejection.
- The likelihood of developing an immune response to a graft depends on the genetic relatedness of the transplant donor and recipient. Autografts involve transfer of tissue from one area of the body to another in the same individual. Syngeneic grafts (isografts) involve transfer of tissue between two genetically identical members of the

same species. In allografts, tissue is transplanted between genetically nonidentical members of the same species, whereas in xenografts, tissue is transplanted between members of different species.
- Because of the disparity in HLA antigens, allografts and xenografts have the potential to induce strong immune responses in the transplant recipient.
- Hyperacute rejection occurs within minutes to hours after transplantation and is mediated by preformed HLA antibodies that react with donor vascular epithelium, resulting in activation of the complement cascade and clotting mechanisms. Preformed antibodies can also cause accelerated rejection, which occurs over several days.
- Acute rejection develops days to months after transplantation and is mediated primarily by a cellular response to foreign HLA antigens involving cytokine production by CD4+ T cells and cytotoxic activity of CD8+ T cells.
- Chronic rejection can occur after the first year of transplantation through a delayed type of hypersensitivity reaction to foreign HLA proteins, resulting in graft arteriosclerosis, fibrosis, and scarring.
- Graft-versus-host disease can occur in HLA-mismatched recipients of HSC transplants or other transplants that contain lymphoid cells. In this condition, the donor's T lymphocytes destroy the recipient's cells, primarily in the skin, gastrointestinal tract, and liver.
- Even in the face of intense immune responses, transplantation has become an effective treatment for a variety of diseases because of the development of immunosuppressive agents that, in various ways, inhibit the immune system from responding to the allogeneic MHC proteins in the transplanted tissue. The classes of immunosuppressive agents include corticosteroids, antimetabolites, calcineurin inhibitors, monoclonal antibodies, and polyclonal antibodies. Unfortunately, these agents also interfere with immune responses to infectious organisms and tumors.
- Identifying the MHC antigens of donors and recipients and monitoring the allospecific immune response are critical components of clinical transplantation. Greatly improved outcomes of transplantation are seen when donor and recipient are matched for HLA types as much as possible and when the recipient does not have preformed antibodies to the donor's HLA antigens.
- The classic method of HLA phenotyping is the complement-dependent cytotoxicity test (CDC), in which HLA antigens are typed by incubating the individual's lymphocytes with a panel of antisera and reagent complement. Positive cells are identified by their ability to bind specific antisera and complement, resulting in membrane permeabilization and uptake of vital stains.
- Molecular methods such as PCR-SSP, PCR-SSOP, and SBT provide greater resolution than HLA antigen typing by determining the HLA genotype at the allele level.
- Screening and identification of preformed HLA antibodies in transplant recipients can be performed by the CDC method through incubation of patient serum with panels of lymphocytes with known HLA antigens, ELISA, traditional flow cytometry, or a flow cytometry-based multiplex bead array.
- Crossmatching is performed by incubation of recipient serum with donor lymphocytes in a CDC assay to confirm the absence of donor-specific antibody.

Study Guide: Classification of Grafts

TYPE OF GRAFT	DEFINITION	EXAMPLES
Autograft	Transfer of tissue within the same individual	Skin graft from leg to face of a burn patient Transfer of a saphenous vein from the leg of a cardiac bypass patient to his heart
Syngeneic (Iso) graft	Transfer of cells or tissues to a genetically identical individual	Transplant between identical human twins Grafts between genetically identical strains of mice
Allograft	Transfer of cells or tissues to a genetically nonidentical member of the same species	Human transplant from a cadaver donor or family member other than an identical twin Grafts between genetically nonidentical strains of mice
Xenograft	Transfer of cells or tissues to a member of a different species	Transplant of a pig valve into a human heart

Study Guide: Types of Graft Rejection

TYPE	TIMING (AFTER TRANSPLANT)	IMMUNOLOGIC MECHANISM
Hyperacute	Minutes to hours	Preformed antibodies to ABO, HLA, and certain endothelial antigens bind to donor vascular endothelium, activating complement and clotting factors. This leads to thrombus formation, ischemia, and necrosis of transplanted tissue.
Accelerated	Days	Same as for hyperacute rejection.
Acute	Days to months	Cell-mediated response to foreign MHC-expressing cells. CD4+ T cells produce cytokines and induce delayed type hypersensitivity. CD8+ T cells mediate cytotoxic reactions. Antibodies produced against HLA antigens bind to vessel walls, activate complement, and induce transmural necrosis and inflammation.
Chronic	1 year or more	Delayed type hypersensitivity response, and possibly antibodies, to foreign HLA antigens on graft. Graft arteriosclerosis and smooth muscle proliferation occur, resulting in fibrosis, scarring, and narrowing of vessel lumen.
Graft-versus-host disease (GVHD)	100 days or more	T cells in HSC, lung, or liver transplants react against foreign HLA proteins in the recipient's cells, causing massive cytokine release, inflammation, and tissue destruction in various locations throughout the body.

CASE STUDIES

1. A 40-year-old mother of three needs to have a second kidney transplant. Her first transplant was lost because of chronic rejection. The mother's HLA type, HLA antibodies, and ABO blood group status was determined. The patient was found to have antibodies to HLA-B35 by flow cytometric testing with HLA-B35 coated beads. The HLA type and blood group were also determined for two of her siblings and two close friends who are interested in donating a kidney to the patient.

Question

a. From the available donors, who would likely be the most compatible to this patient?

IDENTIFICATION	BLOOD GROUP	A	B	C	DR	DQ
Recipient	O	1,2	8,44	7,5	17,4	2,7
Sibling 1	O	1,11	8,35	7,4	17,1	2,5
Sibling 2	A	3,11	7,35	7,4	15,1	6,5
Friend 1	B	2,24	57,7	6,7	7,15	2,6
Friend 2	O	2,24	57,7	6,7	7,15	2,6

2. A 59-year-old male with leukemia needed a HSC transplant for his disease. Clinicians were hopeful that the patient's single sibling might be a suitable stem cell donor. However, the sibling was determined to be medically unsuitable for donation. As such, the transplant center conducted an unrelated donor search for this patient and a potential donor was identified. The transplant registry provided the HLA type for the donor that was determined using CDC-based testing (phenotyping). The patient's HLA type was determined at high resolution by SBT.

ID	HLA-A*	HLA-B*	HLA-C*	HLA-DRB1*	HLA-DQB1*
Patient	01:01	08:01	07:02	03:01	02:01
	02:01	44:02	02:01	15:01	06:02
Donor	1	8	7	3	2
	2	44	2	15	6

Questions

a. Is this donor–recipient pair HLA identical? Yes / No / Maybe

b. The transplant physician requested high-resolution HLA typing for the donor. Why?

REVIEW QUESTIONS

1. Which of the following responses is the type of allograft rejection associated with vascular and parenchymal injury with lymphocyte infiltrates?
   - **a.** Hyperacute rejection
   - **b.** Acute cellular rejection
   - **c.** Acute humoral rejection
   - **d.** Chronic rejection

2. Antigen receptors on T lymphocytes bind HLA class II + peptide complexes with the help of which accessory molecule?
   - **a.** CD2
   - **b.** CD3
   - **c.** CD4
   - **d.** CD8

3. Patients who have received the following types of grafts are at risk for graft-versus-host disease (GVHD) *except* for recipients of
   - **a.** bone marrow transplants.
   - **b.** lung transplants.
   - **c.** liver transplants.
   - **d.** irradiated leukocytes.

4. Which of the following properties are not exhibited by HLA molecules?
   - **a.** They belong to the immunoglobulin superfamily.
   - **b.** They are heterodimeric.
   - **c.** They are integral cell membrane glycoproteins.
   - **d.** They are monomorphic.

5. Kidney allograft loss from intravascular thrombosis without cellular infiltration 5 days post-transplant may indicate which primary rejection mechanism?
   - **a.** Hyperacute rejection
   - **b.** Accelerated humoral rejection
   - **c.** Acute humoral rejection
   - **d.** Acute cellular rejection

6. Which reagents would be used in a direct (forward) donor–recipient crossmatch test?
   - **a.** Donor serum and recipient lymphocytes + rabbit serum complement
   - **b.** Recipient serum and donor lymphocytes + rabbit serum complement
   - **c.** Donor stimulator cells + recipient responder cells + complete culture medium
   - **d.** Recipient stimulator cells + donor responder cells + complete culture medium

7. The indirect allorecognition pathway involves which one of the following mechanisms?
   - **a.** Processed peptides from polymorphic donor proteins restricted by recipient HLA class II molecules
   - **b.** Processed peptides from polymorphic recipient proteins restricted by donor HLA class I molecules
   - **c.** Intact polymorphic donor protein molecules recognized by recipient HLA class I molecules
   - **d.** Intact polymorphic donor protein molecules recognized by recipient HLA class II molecules

8. Which immunosuppressive agent selectively inhibits IL-2 receptor-mediated activation of T cells and causes clearance of activated T cells from the circulation?
   - **a.** Mycophenolate mofetil
   - **b.** Cyclosporine mofetil
   - **c.** Corticosteroids
   - **d.** Daclizumab

9. Phenotyping for HLA class II antigens requires B lymphocytes because
   - **a.** B lymphocytes express HLA class II antigens.
   - **b.** B lymphocytes do not express HLA class I antigens.
   - **c.** B lymphocytes are exquisitely sensitive to complement-mediated lysis.
   - **d.** B lymphocytes represent the majority of lymphocytes in the peripheral blood.

10. A renal transplant candidate was crossmatched with a donor that was mismatched for only the HLA-B35 antigen. The candidate was known to have an antibody specific for HLA-B35. Which of the following combinations of T- and B-cell flow cytometric crossmatch results would be expected?
    - **a.** T cell negative, B cell negative
    - **b.** T cell positive, B cell positive
    - **c.** T cell negative, B cell positive
    - **d.** T cell positive, B cell negative

11. Which of the following HLA alleles differs from A*02:01:02 by a synonymous nucleotide substitution?
    - **a.** A*01:01:01:01
    - **b.** A*02:01:03
    - **c.** A*02:02
    - **d.** A*02:03:01

12. Which one of the following donors would be expected to elicit a positive mixed lymphocyte response in lymphocytes from a patient who has the HLA-DRB1*01:01, 01:03 alleles?

a. DRB1*01:01, 01:03
b. DRB1*01:01, 01:01
c. DRB1*01:03, 01:03
d. DRB1*01:01, 01:05

13. Which of the following donors would be the most appropriate, based on ABO compatibility, for a renal transplant candidate with the ABO type = O?

a. O
b. A
c. B
d. AB

14. Which of the following HLA antigens would be expected to elicit an HLA antibody response in a kidney transplant recipient with the following HLA type: HLA-A*01,03; B*07,14; C*01,04N; DRB1*16,07?

a. HLA-A*01
b. HLA-B*14
c. HLA-C*04
d. HLA-DRB1*16

15. Suppose a 30-year-old man was found to be a suitable donor for a kidney transplant to his younger sister. This transplant would be an example of a(an)

a. autograft.
b. allograft.
c. isograft.
d. xenograft.

17 Tumor Immunology

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Describe the characteristics that differentiate cancer cells from normal cells and the process by which malignant cells are thought to develop.
2. Differentiate between tumor-specific antigens and different categories of tumor-associated antigens and recognize examples of each.
3. Summarize the uses of tumor markers in screening for cancer, diagnosing malignancy, detecting prognosis, and monitoring patient responses to treatment.
4. Identify the characteristics that should be possessed by an ideal tumor marker and explain how nonideal features can affect the clinical utility of a marker.
5. Explain the principles of immunohistochemistry as they apply to tumor marker detection.
6. Distinguish the clinical applications of each of the following tumor markers: alpha-fetoprotein (AFP), cancer antigen 125 (CA 125), carcinoembryonic antigen (CEA), human chorionic gonadotropin (hCG), and prostate-specific antigen (PSA).
7. Contrast the advantages and limitations of immunoassays for tumor markers.
8. Summarize key principles of molecular and proteomic testing for tumor markers.
9. Describe the innate and adaptive immune responses that play a role in defense against tumors and how they contribute to immunosurveillance.
10. Discuss the process of immunoediting and how this relates to mechanisms of tumor escape from the immune system.
11. Recognize the overall goal of immunotherapy and specific examples of active, passive, and adoptive immunotherapy for cancer.
12. Discuss principles and applications of molecular tests for cancer diagnosis.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Adoptive immunotherapy
Alpha-fetoprotein (AFP)
Antibody arrays
Antibody–drug conjugates
Apoptosis
Benign
Biomarker profiling
Cancer
Cancer antigen 125 (CA 125)
Cancer vaccines
Carcinoembryonic antigen (CEA)
Carcinogenesis
Carcinomas
CD45
Colony stimulating factors
Cytogenetics
Hematopoietic growth factors
Heterophile
High-dose hook effect
Human chorionic gonadotropin (hCG)
Human epithelial growth factor receptor 2 (HER2)
Immunoediting
Immunohistochemistry
Immunosurveillance
Immunotherapy
Immunotoxins
Karyotype analysis
Malignant
Metastasis
Microarray
Mutations
Neoplasm
Oncofetal antigen
Passive immunotherapy
Postzone
Prostate-specific antigen (PSA)
Proteomics
Proto-oncogenes
Sarcomas
Tumor
Tumor-associated antigens (TAAs)
Tumor-infiltrating lymphocytes (TILs)
Tumor markers
Tumor-specific antigens (TSAs)
Tumor suppressor genes

Introduction to Tumor Biology

Tumor immunology is the study of the relationship between the immune system and cancer cells. The field encompasses several areas, including the antigens associated with tumor cells, the host's immune responses to tumors, mechanisms by which tumors are thought to escape these responses, and therapeutic use of the immune system in an attempt to eradicate tumors. Tumor immunology is a major area of interest to immunologists because cancer is a leading cause of mortality worldwide, responsible for more than 8 million deaths each year.[1] The American Cancer Society states that cancer is the second most common cause of fatality in the United States, accounting for almost 1 out of every 4 deaths.[2]

Tumor immunology is best understood with a background on the origin of cancer cells and their differences from normal cells. Normally, cell growth and division are carefully regulated processes designed to rapidly produce new cells when necessary, inhibit cell division when enough cells are present, and limit cell life span through a normal physiological process of cell death called **apoptosis.** These activities make it possible for the human body to carry out functions necessary for life, such as generation of new skin cells to replace those that are dying, proliferation of lymphocytes in immune responses, and regeneration of tissue during wound healing and repair. However, if these processes are allowed to continue unchecked, they can lead to excessive cell growth and division, resulting in the development of an abnormal cell mass called a **tumor** (from the Latin, meaning "to swell") or **neoplasm** (from the Greek, meaning "new growth").

Tumors can be classified as **benign** or **malignant.** Benign tumors are composed of slowly growing cells that are well-differentiated and organized, similar to the normal tissue from which they originated.[3] These tumors are surrounded by a capsule, which secures them in place and prevents them from circulating to other parts of the body. In contrast, malignant tumors, or cancer cells, are disorganized masses that are rarely encapsulated, allowing them to invade nearby organs and destroy their normal architecture. **Cancer,** named after the Latin word for "crab," derives its name from this property of invasiveness, which can resemble the legs of a crab when viewed in microscopic tissue sections. In addition, malignant tumors commonly exhibit **metastasis,** or the ability of cells to break away from the original tumor mass and spread through the blood to nearby or distant sites in the body. Malignant tumors can vary in their degree of differentiation, from completely differentiated, or mature, to completely undifferentiated tumors that tend to grow more aggressively and have a poorer prognosis.

Malignant tumors are classified according to their tissue of origin. Approximately 80% of cancers are **carcinomas,** derived from the skin or epithelial linings of internal organs or glands; about 9% are *leukemias or lymphomas,* malignant white blood cells (WBCs) present in the circulation or lymphatic system; and about 1% are **sarcomas,** derived from bone or soft tissues such as fat, muscles, tendons, cartilage, nerves, and blood vessels.[4] To make a specific diagnosis and guide treatment decisions, physicians use staging systems based on the site and type of the primary tumor, tumor size, involvement of regional lymph nodes, presence or absence of metastasis, and degree of resemblance to normal tissue.[5] The most widely used staging scheme is the TNM system of the American Joint Committee on Cancer (AJCC).[6]

Connections

Apoptosis

Recall that apoptosis is a normal homeostatic mechanism that is necessary for maintaining normal cell numbers. This process occurs through a series of biochemical reactions within a cell that lead to chromatin condensation, DNA fragmentation, cell shrinkage, and membrane blebbing. Cell death occurs in the absence of cell lysis and does not provoke an inflammatory reaction that could damage neighboring cells. Genetic changes within a cell that cause it to become resistant to apoptosis are important in the development of cancer.

Clinical Correlations

The TNM System

In the TNM system, tumors are classified according to the size and extent of the primary tumor (T0 to T4), the degree of spread to adjacent lymph nodes (N0 to N3), and the presence or absence of distant metastases (M0 or M1). For example, breast cancer that is classified as T2N1M0 would involve a tumor between 2 to 5 cm in diameter that has spread to one to three regional lymph nodes but has not spread to distant sites.[6] For many types of cancer, the TNM criteria also correspond to one of five stages (stage 0, I, II, III, or IV). Although the specific criteria vary according to the type of cancer, in general, stage 0 represents noninvasive (in situ) carcinoma; stages I, II, and III are tumors that are larger or that have spread beyond the organ in which they first developed to nearby lymph nodes or tissues; and stage IV tumors are those that have metastasized to distant sites.[5]

The transformation of a cell into a malignant tumor is thought to be a multistep process involving a series of genetic mutations that cause the phenotype of a cell to be changed over time. This process, called **carcinogenesis,** is thought to be initiated by exposure of the host to factors in the environment that induce genetic changes in the cell. These factors include chemical carcinogens such as asbestos and cigarette smoke;[7] radiation such as ultraviolet rays from the sun and ionizing radiation from x-rays; and certain pathogenic viruses that have been linked to specific types of cancer.[3] These agents are all believed to create genetic changes, or **mutations,** in the DNA of our cells that affect the body's mechanisms that normally control cell growth.

Two major types of genes are involved in malignant transformation: **proto-oncogenes** and **tumor suppressor genes.**[3,8] Proto-oncogenes are normal genes that have a positive influence on cell proliferation and development. Sometimes, mutations in proto-oncogenes can convert them to oncogene-like genes, which have DNA sequences similar to those found in the *oncogenes* of transforming viruses. These genetic alterations—which include point mutations, chromosomal translocations, and gene amplifications—cause constitutive activation of the proto-oncogenes, resulting in changes in the cell that produce continuous cell division.

Cell division is normally inhibited by the action of tumor suppressor genes. These genes have both "gatekeeper" and "caretaker" functions.[8] The gatekeeper genes exert their effects by controlling the entry of cells into the cell cycle and preventing cells from completing the cell cycle if they contain damaged DNA. The caretaker genes are important in maintaining genetic stability by recognizing and repairing damaged DNA in a cell. If a mutation occurs in which the normal function of a tumor suppressor gene is lost, growth inhibitory signals are removed, resulting in dysregulated cell growth and genetic instability.

In most cases, the development of a cancerous tumor is believed to result from a series of mutations in proto-oncogenes and tumor suppressor genes that accumulate over a lifetime **(Fig. 17–1).** In 2000, Hanahan and Weinberg defined a cancerous cell as one that exhibits six characteristics:

- Sustained signaling of proliferation
- Resistance to cell death
- Ability to induce angiogenesis (development of new blood vessels to provide oxygen and nutrients to the tumor)
- Immortality in terms of cell division
- Invasion and metastasis
- Ability to avoid suppressors of cell growth[9]

Today, it is recognized that cancer is not a single disease, but rather a heterogeneous group of diseases that show variability in these characteristics. In fact, heterogeneity is commonly found among different cancer cells in the same patient. The extent to which a tumor exhibits these characteristics will determine its ability to survive, grow, and metastasize. This, in turn, has important implications that influence disease aggressiveness, patient prognosis, and choice of therapy.

In 2011, Hanahan and Weinberg proposed four additional hallmark characteristics of cancer cells:[10]

- Reprogramming of energy metabolism to support malignant proliferation
- Ability to evade destruction by the immune system
- Genomic instability and mutations
- Inflammatory responses that promote tumor growth

These additions to the original hallmarks recognize the important contributions of genetics and immunology to the development of cancer. In this chapter, we will discuss the interactions between tumors and the immune system in more detail.

Tumor Antigens

The concept of tumor immunology is based on the premise that tumors possess antigens that are recognized as foreign by the immune system. Tumor antigens can be broadly classified into two groups: **tumor-specific antigens (TSAs)** and **tumor-associated antigens (TAAs) (Table 17–1).** TSAs are unique to tumor cells, whereas TAAs are also found on normal cells.

Tumor-Specific Antigens (TSAs)

TSAs are unique to the tumor of an individual patient or shared by a limited number of patients with the same type of tumor.[11,12] They are coded for by viral oncogenes or by host proto-oncogenes or tumor suppressor genes that have undergone genetic mutations.[3] A well-known example of a TSA is a fusion protein that is produced in chronic myelogenous leukemia (CML) cells. This protein is a result of a reciprocal chromosome translocation commonly known as the *Philadelphia chromosome,* which involves the *BCR* (breakage cluster region) on chromosome 9 and the c-*ABL* gene on chromosome 22. C-*ABL* is a cellular proto-oncogene that codes for tyrosine kinase, a key enzyme in cell-signaling pathways that promote cell division. During the translocation, the two chromosomes break and exchange parts so that the c-*ABL* gene is combined

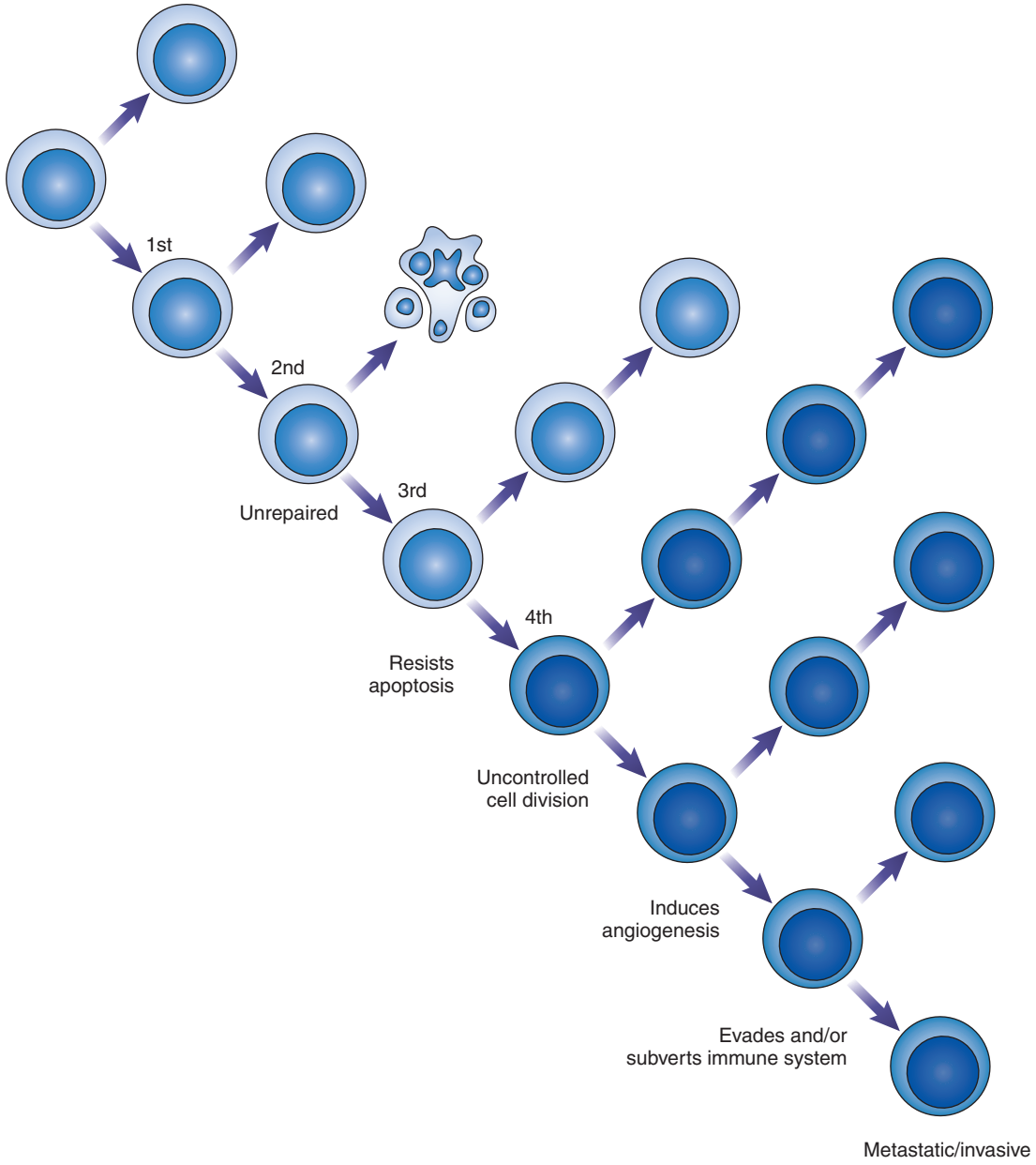

FIGURE 17–1 Genetic mutations in the evolution of cancer. As cells acquire an increasing number of mutations over time, they develop more of the characteristics that allow them to evolve into invasive cancer cells (numbers indicate mutations).

with part of the *BCR* to produce a hybrid gene that is constantly expressed **(Fig. 17–2).** The *BCR/ABL* gene rearrangements result in uncontrolled cell proliferation and are found in the majority of CML patients.

Other TSAs originate from point mutations (i.e., genetic changes involving a single base pair) in key genes involved in cell proliferation, such as the tumor suppressor gene *p53*, and the gene coding for caspase 8, an enzyme important for apoptosis (both of which are associated with head and neck tumors and squamous cell carcinoma).[13] An updated list of tumor antigens resulting from mutations can be found at http://cancerimmunity.org/peptide/mutations/.[14] TSAs also include protein antigens encoded by cancer-causing viruses **(Table 17–2).** These antigens can be found in the nucleus, cytoplasm, or plasma membrane of the associated tumor cells.

TSAs can also be produced by mutations induced by carcinogenic chemicals and radiation. Researchers are continually discovering new TSAs by using molecular techniques.[11,12] Some of these antigens can serve as targets for specifically directed therapies (see *Immunotherapy* later).

Tumor-Associated Antigens (TAAs)

In contrast to TSAs, TAAs are expressed in normal cells as well as in tumor cells. Tumor cells abnormally express these protein or carbohydrate antigens in terms of their concentration, location, or stage of differentiation.[3] Classification of these antigens may vary according to their source. This section will focus on the major categories of peptide TAAs that have been identified by the Cancer Research

Table 17–1 Tumor-Specific and Tumor-Associated Antigens*

CATEGORY	DESCRIPTION	EXAMPLES*
Tumor-specific antigens (TSAs)	Antigens that are unique to a tumor or shared by tumors of the same type	*BCR/ABL* fusion protein (CML)
Tumor-associated antigens (TAAs)	Antigens that are expressed in normal cells as well as tumor cells	
Shared TSAs (cancer/testis antigens)	Expressed in many tumors but not in most normal tissues	MAGE (melanoma)
Differentiation antigens	Expressed on immature cells of a particular lineage	CD10 (ALL) CEA (mainly in colorectal cancer) AFP (HCC) PSA (prostate cancer)
Overexpressed antigens	Found in higher levels on malignant cells than on normal cells	HER2 (mainly in some breast cancers)

**Primary cancer associations are shown in parentheses.*

AFP = alpha-fetoprotein; CD10 = cluster of differentiation 10; CEA = carcinoembryonic antigen; CML = chronic myelogenous leukemia; HER2 = human epidermal growth factor 2; MAGE = melanoma antigen gene; PSA = prostate-specific antigen.

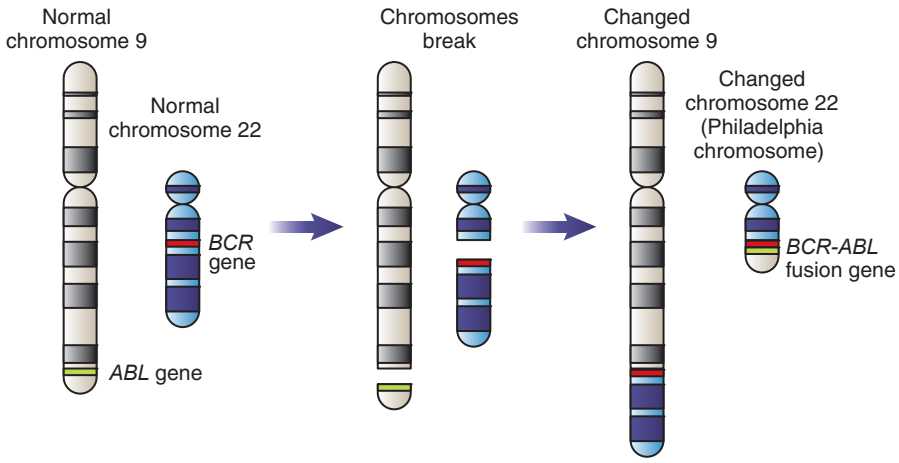

FIGURE 17–2 The *BCR/ABL* gene rearrangement characteristic of CML.

Institute: shared TSAs, differentiation antigens, and overexpressed antigens.[12]

Shared TSAs are expressed in many tumors, but not in most normal tissues.[3,12,13] The only normal cells in which they have been detected are testicular germ cells (i.e., spermatogonia and spermatocytes) and, to a lesser extent, placental trophoblasts and ovaries. Hence, these antigens have also been called *cancer/testis antigens*. They become TAAs when the transformation process causes them to be expressed on tumors originating from other cell types. These antigens have been identified on many tumors of epithelial or mesenchymal origin. The best known examples of TAAs in this category are the melanoma antigen gene (MAGE) proteins that are expressed by melanoma tumors.

A second group of TAAs contains the *differentiation antigens,* which are expressed on immature cells of a particular lineage. An example of a TAA in this group is the CD10 antigen (previously known as the CALLA, or common acute lymphoblastic leukemia antigen), which is normally found on pre-B cells but not on mature B cells. This category of antigens also includes the oncofetal or embryonic antigens that are normally expressed on developing cells of the fetus but not on cells in the adult.[3,11,12] It is thought that the genes coding for these antigens are silenced during development of the embryo, but that the process of malignant transformation allows them to be re-expressed.[11] Examples of oncofetal antigens include carcinoembryonic protein (CEA), alpha-fetoprotein (AFP), and prostate-specific antigen (PSA). Many of these antigens are tumor markers that can be detected in the clinical laboratory (see the next section).

The third category of TAAs is composed of the *overexpressed antigens,* which are found in higher levels on malignant cells than on normal cells. Genetic mutations that occur during transformation are thought to deregulate expression of these proteins, resulting in levels up to 100 times greater than normal.[3] A well-known example of a TAA in this category is the **human epithelial growth factor receptor 2 (HER2)** protein, a transmembrane receptor that binds human epidermal growth

Table 17–2 Human Viruses Associated With Cancer

VIRUS	CANCER ASSOCIATIONS
Epstein-Barr virus (EBV)	Burkitt lymphoma Hodgkin lymphoma Leiomyosarcomas Post-transplant lymphoproliferative disease Nasopharyngeal carcinoma
Hepatitis B virus (HBV)	Hepatocellular carcinoma
Hepatitis C virus (HCV)	Hepatocellular carcinoma
Human herpes virus 8 (HHV-8)	Kaposi sarcoma
Human papilloma virus (HPV)	Cervical cancer Other genital and anal cancers Head and neck cancer
Human T-lymphotropic virus I (HTLV-1)	Adult T-cell leukemia or lymphoma
Merkel cell polyomavirus	Merkel cell carcinoma (a type of skin cancer)

factor. Gene amplification in a certain type of breast cancer can result in overexpression of this protein, which serves as a marker for detection and therapy (see *Immunotherapy* later). In addition to peptide TAAs, glycolipid and glycoprotein antigens may also be overexpressed in some tumors.[11] Examples of these antigens include cancer antigen 125 (CA 125), which is associated with ovarian cancer, and cancer antigen 19-9 (CA 19-9), which is associated with pancreatic cancer. In the next section, we will discuss clinical applications of some of these markers.

Clinically Relevant Tumor Markers

Tumor markers can be defined as biological substances that are found in increased amounts in the blood, body fluids, or tissues of patients with a specific type of cancer. These substances can be produced by the tumor itself or by the patient's body in response to the tumor or related benign conditions. The concentration of a tumor marker in the serum depends on the degree of tumor proliferation, the size of the tumor mass, the proteolytic activities of the tumor, or release of the marker from dying tumor cells.[15] An elevated level of a tumor marker suggests that a significant amount of a particular type of tumor is present.

Tumor markers can be proteins, carbohydrates, oncofetal antigens, hormones, metabolites, receptors, or enzymes.[16] **Table 17–3** lists some examples of clinically relevant tumor markers in each category, along with their disease associations.

An ideal tumor marker should have seven characteristics.[15-17] A marker should:

- Be produced by the tumor itself or by the patient's body in response to the tumor
- Be secreted into a biological fluid, where it can be inexpensively and easily quantified
- Have a circulating half-life long enough to permit its concentration to rise with increasing tumor load
- Increase to clinically significant levels above the reference level while the disease is still treatable
- Have a high sensitivity; in other words, it should easily detect the majority of individuals in the population who have a particular cancer
- Have a high specificity; in other words, the marker should be absent from, or present at background levels in all individuals without the malignant disease in question to minimize false-positive test results

Table 17–3 Categories of Clinically Relevant Tumor Markers[15-17]

TUMOR MARKER CLASS	EXAMPLES	DISEASE ASSOCIATIONS
Cell surface markers	Estrogen or progesterone receptors CD markers on white blood cells (WBCs)	Prognosis for hormone therapy in breast cancer Clonality and lineage of WBC neoplasms
Proteins	Thyroglobulin (TG) Immunoglobulins (Ig) and Ig light chains (Bence Jones proteins)	Well-differentiated papillary or follicular thyroid carcinoma Multiple myeloma and lymphoid malignancies
Oncofetal antigens	Alpha-fetoprotein (AFP) Carcinoembryonic antigen (CEA)	Germ cell carcinomas, hepatocellular carcinoma Colorectal, breast, or lung cancer
Carbohydrate antigens	CA 125 CA 15-3 CA 19-9	Ovarian cancer Breast cancer Pancreatic and gastrointestinal cancers
Enzymes and isoenzymes	Prostate-specific antigen (PSA) Alkaline phosphatase (ALKP) Neuron-specific enolase	Prostate cancer Bone and liver cancer Neural tissue neoplasms
Hormones	Human chorionic gonadotropin (hCG) Calcitonin Gastrin	Germ cell carcinoma, trophoblastic tumors Medullary thyroid cancer Pancreatic gastrinoma

Unfortunately, very few of the tumor markers in clinical use are ideal because they are not tumor specific.[16] This limitation affects the performance of tumor markers in each of the four applications introduced in the next section.

Clinical Uses of Tumor Markers: Benefits and Limitations

In general, tumor markers have four major clinical applications: screening, diagnosis, prognosis, and monitoring. It is a well-established fact that patient survival rates are usually higher when cancer is diagnosed in its earliest stages. Detecting cancer early, before it begins to invade normal tissues and is difficult to target, requires good screening methods. Tumor markers can be used to screen asymptomatic individuals in a population for the presence of cancer. The individuals screened may belong to a healthy population or to a high-risk population. For example, PSA has been routinely used to screen men aged 50 or older for the presence of prostate cancer. Secondly, tumor markers can be helpful in making an initial diagnosis of cancer. For example, an elevated level of PSA might suggest the presence of prostate cancer. A third use of tumor markers is in predicting prognosis of a cancer patient. In general, an initial high concentration of a tumor marker or an increasing level of a tumor marker over time points to a poorer prognosis. A fourth use of tumor markers is to monitor known cancer patients to determine whether their treatment is working and to check for recurrence of their tumor. Decreasing levels indicate that the treatment is effective in reducing the amount of tumor harbored by the patient, whereas increasing levels indicate that the treatment is ineffective and that the number of tumor cells in the patient is increasing. Each of these applications will be discussed in more detail in the text that follows. The second half of this section will focus on traditional tumor markers that can be detected in the serum. Some of the more commonly detected markers will be discussed. This section will also introduce the role of molecular and proteomic methods in detecting newer tumor markers that are being increasingly evaluated in the clinical laboratory.

Screening for Tumor Markers

The detection of tumor markers provides an ideal way to screen for tumors because the markers can be detected by a simple blood test. However, the benefits and limitations of using a particular marker to screen a population must be considered before testing is implemented. On the positive side, screening asymptomatic individuals can lead to earlier detection of tumors with a need for less aggressive treatment. In addition, many people who have been screened can receive reassurance from true negative results. However, there are also significant disadvantages associated with screening. Besides the actual dollar costs of the screening test, harm to the individuals tested may also occur.[18,19] For example, misleading reassurance can be experienced by individuals with false-negative results. In contrast, false-positive results can lead to patient anxiety; possible harm from more invasive, unnecessary follow-up testing; and overtreatment of questionable diagnoses. For example, PSA, a marker elevated in prostate cancer, can also be increased in men with a harmless enlargement of the prostate known as benign prostatic hypertrophy (BPH) (see section on PSA). In these cases, the finding of an elevated PSA value can lead to unnecessary testing and potentially harmful treatments, such as a prostate biopsy.[17,20]

It is important to remember that the effectiveness of a tumor marker in screening for cancer depends on the sensitivity and specificity of the marker, as well as the cancer's prevalence in the population. Screening is most effective when it is conducted in populations at a high risk for developing the disease, such as certain ethnic groups or those with a family history for a particular type of cancer. For example, alpha-fetoprotein (AFP), a tumor marker for hepatocellular carcinoma, is not used for screening in the United States, but is used to screen people in China, where the incidence of liver cancer is high. In high-risk populations, the predictive value of the tumor marker will be highest. In other words, a positive test result is most likely to be found in a person who truly has the disease, whereas a negative result is most likely to occur in a person who truly does not have the disease.

Diagnosis Using Tumor Markers

A second application of tumor markers is to help physicians distinguish between different diseases with similar symptoms, a process called *differential diagnosis*. For example, if a computerized tomography (CT) scan revealed the presence of a lung nodule, histological examination of a lung biopsy could help differentiate whether the nodule was caused by cancer or another disease process, such as an infection. Follow-up staining of the biopsy for tumor markers could help determine the neoplasm's tissue origin.[21] In order to improve the sensitivity and specificity of the testing, multiple tumor markers could be examined or testing for tumor markers could be combined with other laboratory tests.[15] It is important to recognize that tumor markers can serve as a valuable aid in making a cancer diagnosis when they are used in conjunction with clinical findings and other tests, but that they are not diagnostic by themselves.

Prognosis Using Tumor Markers

A third application of tumor markers is to assess prognosis in a known cancer patient. A high concentration of a tumor marker at the time of diagnosis or increasing levels of a tumor marker

Connections

Specificity

A problem with many tumor markers is that they lack specificity for the disease they are intended to detect. Recall from Chapter 9 that specificity is the ability of a test to be negative for a person who does not have a particular disease. For example, suppose a tumor marker has a specificity of 90%. Although this may sound like a good attribute because the test will give a true negative result in every 90 out of 100 people tested, 10% of those tested will have a false-positive result. If 100,000 people in a population were tested, 10,000 would have a positive result even though they did not have the intended disease and would likely be recommended to have additional unnecessary testing or invasive procedures.

over time can indicate the presence of an aggressive tumor that has metastasized and requires rigorous treatment.[15,16] Tumor markers can also be used to determine the type of therapy that would be most effective for a patient. For example, in breast cancer, anti-HER2 agents such as trastuzumab work best in patients whose tumors overexpress the HER2 protein or gene; antiendocrine therapies such as tamoxifen are suitable for patients whose tumors overexpress the estrogen receptor.[21]

Monitoring Patient Response to Treatment Using Tumor Markers

One of the most important uses of tumor markers is to monitor known cancer patients to determine whether their treatment is effective and to check for recurrence of the tumor. This can be done because the level of a serum tumor marker often correlates with the amount of tumor in the patient. The laboratory typically determines a baseline concentration of the marker before treatment begins, followed by serial measurements over time. A significant decrease in the concentration of a tumor marker after surgery, chemotherapy, or other treatment indicates that the therapy has been effective in shrinking the tumor. In contrast, an increasing level of the marker after a return to normal indicates that the tumor has recurred and that more aggressive treatment may be needed. Elevation in a tumor marker can become evident several months before the cancer is detected by other methods.[16]

The clinical significance of monitoring a tumor marker is illustrated in **Figure 17–3.** This figure shows tumor marker levels from a hypothetical cancer patient who has been treated with surgery and two chemotherapy drugs. As expected, the level of the tumor marker in the patient's serum declined after the initial tumor mass was removed by surgery. However, after a few months, the concentration of the tumor marker began to increase, indicating that the tumor had recurred. The tumor was unresponsive to Chemotherapy #1, as reflected by the sustained elevation in the marker after treatment with that drug. This prompted a change in treatment to Chemotherapy #2, which was successful in decreasing the amount of tumor present in the patient, as indicated by the decline in the tumor marker to an undetectable level.

Serum Tumor Markers

Although there are scores of possible tumor markers in the literature, only a few have FDA approval.[17] However, many tests for non-FDA-approved markers are available to clinicians, with a notation on the laboratory report stating that results are for research use only. The National Academy of Clinical Biochemistry (NACB) has published consensus guidelines regarding the clinical use of tumor markers.[22,23] Some of the most commonly used markers are listed in **Table 17–4,** along with their clinical applications and important noncancerous conditions that can cause elevations.

Alpha-Fetoprotein (AFP)

Alpha-fetoprotein (AFP) is a 70,000 MW glycoprotein that is similar to albumin in its physical and chemical properties.[15,16] It is classified as an **oncofetal antigen** because it is synthesized by the fetal liver and yolk sac and is abundant in fetal serum, but declines to low levels (10–20 ug/L) by 12 months of age.[24] AFP is frequently elevated in patients with primary hepatocellular carcinoma (HCC) and nonseminomatous testicular cancer, but can also be elevated in other types of cancers and in nonmalignant conditions such as pregnancy and hepatitis.[15,16,23,24]

AFP is the most widely used tumor marker for HCC, serving as a tool in diagnosis, staging, prognosis, and monitoring patients undergoing therapy.[16,24] The sensitivity of AFP for HCC

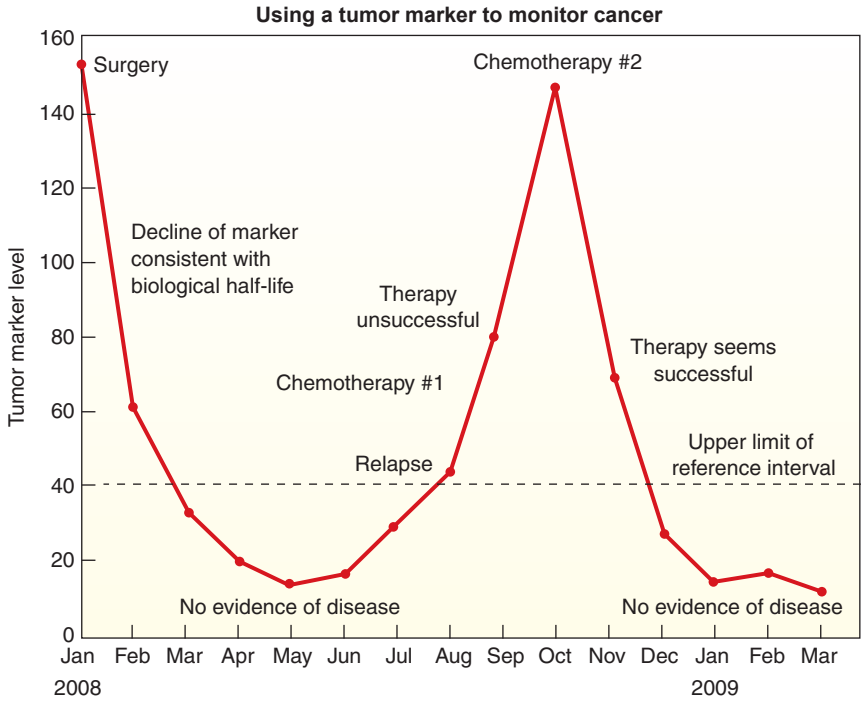

FIGURE 17–3 Tumor marker analysis. A curve showing a sample scenario monitoring a cancer patient for tumor recurrence and for therapy efficacy using levels of a tumor-associated antigen.

Table 17–4 Common Tumor Markers[15-17, 22-25]

MARKER	CANCER(S)	USES	NORMAL SOURCES	NONCANCEROUS CONDITIONS WITH ELEVATIONS	COMMENTS
AFP	Nonseminomatous testicular germ cell Liver	Screening Diagnosis and staging Prognosis Monitoring	Fetal liver and yolk sac, adult liver	Pregnancy, nonneoplastic liver disease	Screening conducted in high-risk populations for liver cancer such as those with liver cirrhosis and chronic hepatitis. In germ cell tumors, both AFP and hCG are elevated.
β2–microglobulin	B-lymphocyte malignancies	Prognosis Monitoring	Part of class I MHC molecule	Inflammatory and high cell turnover conditions	Higher levels imply poor prognosis in multiple myeloma.
Calcitonin and Ca++	Familial medullary thyroid carcinoma	Diagnosis Monitoring	Thyroid	In hypercalcemia, increased calcitonin is expected. Serum Ca++ may be low when calcitonin is elevated in medullary carcinoma.	Can be elevated in other forms of cancer.
CD markers	WBC	Diagnosis Monitoring	All WBCs	WBC increase such as infection	Different CD markers are associated with specific WBC malignancies.
CEA	Colorectal Breast Lung	Prognosis Monitoring	Tissues of endodermal origin	Renal failure, nonneoplastic liver and intestinal disease, age	Values increased with age and in smokers.
CA 125	Ovarian adenocarcinoma	Screening Diagnosis Prognosis Monitoring	Ovaries and various other tissues	Endometriosis, pelvic inflammatory disease, uterine fibroids, and pregnancy	Increases can occur during menstruation. Screening is only recommended for women with a family history of ovarian cancer.
CA 15-3	Breast cancer Can also be increased in pancreatic, lung, colorectal, ovarian, and liver cancers	Prognosis Monitoring	Mammary tissue	Benign breast disease, benign liver disease	CA 15-3 is a monoclonal antibody directed against an epitope of episialin.
CA 19-9	Pancreatic	Diagnosis Prognosis Monitoring	Sialylated Lewis^a blood group antigen	Benign hepatobiliary and pancreatic conditions	Can be elevated in some nonpancreatic malignancies. Subjects who are Lewis ^{a} and ^{b} negative persons cannot synthesize CA 19-9.
ER/PR	Breast adenocarcinoma	Prognosis	Breast	N/A	ER/PR$^+$ breast cancers benefit from estrogen or progesterone reduction therapy.

Table 17–4 Common Tumor Markers[15-17, 22-25]—cont'd

MARKER	CANCER(S)	USES	NORMAL SOURCES	NONCANCEROUS CONDITIONS WITH ELEVATIONS	COMMENTS
hCG	Nonseminomatous testicular cancer germ cell trophoblastic (hydatidiform mole, choriocarcinoma)	Diagnosis Prognosis Monitoring	Placenta	Pregnancy	hCG has a high homology with luteinizing hormone (LH). Malignancies can produce free α and β chains as well as intact α and β dimer. Immunoassays that detect only intact hCG should not be used for tumor marker detection. In germ cell tumors, both AFP and hCG are elevated.
HER2 (neu) or neu	Breast	Prognosis	Growth factor gene in all cells	N/A	Cancers associated with overexpression of HER2 (neu) or neu have a good response to monoclonal antibody therapy (trastuzumab).
Monoclonal free Ig light chains	Plasma cell, B lymphocytes	Diagnosis Monitoring Prognosis	Normal Igs are polyclonal. Few free light chains exist.	Monoclonal gammopathy of undetermined significance (MGUS), amyloidosis, nonsecretory myeloma	Bence Jones proteins are free Ig light chains in urine detected by urine immunofixation electrophoresis.
Monoclonal Igs	Plasma cell, B lymphocytes	Diagnosis Monitoring Prognosis	Normal Igs are polyclonal	Monoclonal gammopathy of undetermined significance	Monoclonal IgG/IgA—multiple myeloma. Monoclonal IgM—Waldenström's macroglobulinemia.
PSA	Prostate	Screening Diagnosis Monitoring Prognosis	No tissues other than prostate	UTI or prostatitis, benign prostatic hypertrophy	Levels directly proportional to prostate size. Many elevations are benign or not clinically significant. Screening is routinely conducted in men aged 50 and older, but is controversial. Decreased percent of free PSA and PSA velocity greater than 0.75 ng/mL/year are more strongly associated with prostate cancer. Collect specimen before ejaculation, digital rectal examination, or prostate manipulation.

Continued

Table 17–4 Common Tumor Markers[15-17, 22-25]—cont'd

MARKER	CANCER(S)	USES	NORMAL SOURCES	NONCANCEROUS CONDITIONS WITH ELEVATIONS	COMMENTS
PTH and Ca++	Parathyroid carcinoma	Diagnosis Prognosis Monitoring	Parathyroid glands	In hypocalcemia, increased PTH is expected. Serum Ca++ may be high when PTH is elevated in parathyroid carcinoma.	PTH has a short half-life, so levels are done intraoperatively to ensure complete parathyroid tumor removal.
TG	Thyroid	Monitoring	Thyroid	TG reflects thyroid mass, injury, and TSH levels. Thyroid markers (T4, TSH) are generally normal in thyroid cancer.	Assays must simultaneously test for thyroglobulin antibodies because these can cause falsely decreased results. Often tested after TSH stimulation (or less often, by withholding thyroid medication) to see if TG production by residual tumor cells occurs.

AFP = alpha-fetoprotein; CA = carbohydrate antigen; Ca++ = serum calcium; CD = clusters of differentiation in WBC; CEA = carcinoembryonic antigen; ER or PR = estrogen or progesterone receptors; hCG = human chorionic gonadotropin; Ig = immunoglobulins; PTH = parathyroid hormone; PSA = prostate-specific antigen; TG = thyroglobulin; UTI = urinary tract infection.

is 41% to 65% and its specificity ranges between 80% to 94%.[25] As a result, the utility of AFP in screening for HCC has been a matter of debate. However, screening for HCC with AFP is routinely performed in high prevalence areas of the world such as China and Southeast Asia. In the United States, AFP screening is usually conducted in patients with a high risk for HCC, along with liver ultrasound.[24] Many of these patients have liver cirrhosis because of hepatitis B or hepatitis C infection. Studies have shown that the diagnostic utility of AFP may be improved by testing specifically for the isoform AFP-L3, which has a stronger correlation with HCC, and by combining AFP with other laboratory markers, such as DCP (des-γ-carboxy-prothrombin), the liver enzyme ALT (alanine aminotransferase), and platelet count.[24,26] In addition, high levels of AFP are associated with a poor prognosis in patients with HCC, whereas decreasing levels over time indicate a good response to therapy.[16,24]

AFP is also an established tumor marker for nonseminomatous germ cell cancers of the testes (NSGCT).[16,27] This marker, along with other markers such as human chorionic gonadotropin (hCG; see section later in this chapter) and lactate dehydrogenase (LDH), plays an important role in patient diagnosis, tumor staging, therapeutic monitoring, and detection of relapse. AFP is elevated in 10% to 20% of patients with stage I NSGCT and in nearly all patients in later stages of the disease.[16] As in HCC, increased concentrations are associated with a poor prognosis, whereas declining levels reflect responsiveness to therapy.[27]

In addition to its applications as a tumor marker, AFP is widely used as a marker to detect abnormalities in the fetus. An increased level of AFP in the serum or amniotic fluid of a pregnant woman is seen with open neural tube defects such as spina bifida, whereas low levels of AFP are associated with Down syndrome.[28]

Cancer Antigen 125 (CA 125)

Cancer antigen 125 (CA 125) is a large, heavily glycosylated, mucinlike protein that is a marker for ovarian cancer. This marker is not unique to ovarian tumors because it is also found in the normal ovary as well as other tissues, including the endocervix, endometrium, fallopian tubes, pleura, pericardium, peritoneum, and epithelial tissues of the colon, pancreas, lung, kidney, prostate, breast, stomach, and gallbladder.[27,29]

CA 125 is considered the best marker for ovarian cancer.[29,30] It has multiple applications to the disease, ranging from screening and diagnosis, to prognosis and monitoring response to therapy.[23,27] Serum CA 125 levels greater than 35 kU/L are considered to be above normal.[27,29] Although 90% or more of women with ovarian cancer in stages II to IV have elevated CA 125, the marker is not recommended for screening of the general population because it lacks sensitivity and specificity. Elevated CA 125 levels are only seen in 50% to 60% of women with stage I ovarian cancer; therefore, generalized screening would miss about half of the women during the period when the disease is most treatable.[27,30] In addition, CA 125 is not specific because it can increase during pregnancy or

menstruation, or as a result of benign gynecological conditions such as endometriosis, nongynecological conditions involving inflammation, and other malignancies.[16,23,29] However, annual CA 125 testing, together with transvaginal ultrasound, is recommended for women with a family history of ovarian cancer because early detection and intervention is likely to be beneficial in this population.[27,31]

The value of CA 125 can also be seen in clinical applications other than screening. For example, an elevated CA 125 concentration combined with imaging has been shown to be highly sensitive and specific for a differential diagnosis of ovarian cancer from benign pelvic masses, especially in postmenopausal women.[27,29] Serial CA 125 testing is beneficial in monitoring a patient's response to chemotherapy and is recommended before treatment is initiated, during treatment, and during patient follow-up. Rising concentrations of CA 125 over time can predict recurrence of the disease. Persistent elevations of CA 125 or an initial CA 125 value greater than 65 kU/L point to a poor prognosis.[27]

Carcinoembryonic Antigen (CEA)

Carcinoembryonic antigen (CEA) is a glycoprotein with a molecular weight of 180,000 to 200,000, depending on the exact structure of its carbohydrate side chains.[32] CEA was the first oncofetal antigen to be discovered. In 1965, Gold and Freedman described its presence in tissues from the fetal colon and colon adenocarcinoma, as well as its absence in colon tissues from healthy adults.[32]

Increased serum concentrations of CEA are associated with colorectal cancer because CEA is the most widely used marker for that cancer.[16,32] The main application of CEA is in monitoring patients undergoing therapy for colorectal cancer.[27] It is recommended that the medical team obtain a baseline CEA value from the laboratory just before therapy, followed by CEA testing every 1 to 3 months during active treatment.[27] Increasing CEA levels are a highly sensitive indicator of liver metastasis and can detect recurrent colorectal cancer by an average of 5 months before clinical symptoms appear.[32] CEA measurement should be used in conjunction with clinical examination, radiological testing, and histological confirmation to maximize its sensitivity in detecting disease recurrence.[33] CEA levels can also be used in determining the most appropriate treatment for colorectal cancer patients because those with higher baseline levels before surgery tend to have a poorer prognosis.[19,27]

However, CEA is not recommended for colon cancer screening because of its low sensitivity and specificity in this situation.[27] CEA is not increased in all patients with colorectal cancer and elevated CEA levels can be present as a result of other conditions, including colitis, diverticulitis, irritable bowel syndrome, and nonmalignant liver disease.[27,32] Cigarette smoking can cause an increase in CEA level to nearly twice that of nonsmokers.[32] CEA levels can also be elevated in other cancers, notably those of the breast, gastrointestinal tract, pancreas, and lung.[23]

Human Chorionic Gonadotropin (hCG)

Human chorionic gonadotropin (hCG) is best known as the "pregnancy hormone" because it is synthesized by trophoblasts, cells that contribute to development of the placenta and promote implantation of the embryo. Accordingly, it rises during the first few weeks of gestation, when it can be detected in the blood and urine of pregnant women. In addition, hCG can be produced by certain malignant tumors; elevations are associated with germ cell tumors of the ovary and testes as well as choriocarcinoma, a rare type of cancer that is caused by malignant transformation of the trophoblast cells.[16,27] In these tumors, testing for hCG is recommended as an aid to diagnosis, prognosis, monitoring response to therapy, and detection of recurrence.[23]

hCG is a 45,000 MW glycoprotein that is composed of an α subunit, which is shared by luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH), and a β subunit that is unique to hCG.[15,16,27] Serological tests can measure either intact hCG or the hCG β subunit; the presence for both should be tested to monitor patients with testicular cancer. This is because some patients may only produce the β subunit and detection of only the intact form can result in false-negative results.[27] Because hCG levels can also increase in men as the result of malfunction of the testes, it is important to observe rising values in sequential tests before making a diagnosis of testicular cancer. Elevations of hCG can also occur as a result of gonadal suppression caused by chemotherapy and do not necessarily indicate tumor recurrence.[27]

Prostate-Specific Antigen (PSA)

Prostate-specific antigen (PSA) is the most widely used marker for prostate cancer. It is a 28,000 MW glycoprotein that is produced specifically by epithelial cells in the prostate gland. PSA was first discovered in semen, where its function is to regulate the viscosity of the seminal fluid to facilitate mobility of the sperm cells.[20] Its presence was subsequently noted in serum, where it is frequently elevated in patients with prostate cancer.

The specificity of PSA for the prostate gland led to its routine use as a screening test for prostate cancer. Since its approval by the FDA in 1994, PSA testing has resulted in a dramatic increase in the detection rate of early-stage prostate cancer and in the rate of 5-year patient survival.[20] Despite these successes, general screening for prostate cancer has been a controversial issue because it may potentially lead to unnecessary testing and treatment. There are several reasons for this concern. Although PSA is specific for prostate tissue, it is *not* specific for prostate cancer. PSA can also be elevated in other conditions affecting the prostate gland, such as *benign prostatic hyperplasia (BPH),* an enlargement of the prostate gland that commonly occurs as men age, or prostatitis, an inflammation of the gland occurring as a result of infection or irritation.[16,20] Transient increases in PSA levels can also occur if samples are collected shortly after ejaculation, digital rectal examination (DRE), or prostate manipulation.[16,29] As such, there is concern that general PSA screening can lead to the performance of unnecessary prostate biopsies and risk of infection and other complications.[34] In addition, many prostate cancers are slow growing and would be unlikely to cause death during an older man's remaining life span. Furthermore, the conventional treatments for prostate cancer, radical prostatectomy, and radiotherapy can be associated with

significant side effects, most notably urinary incontinence, erectile dysfunction, and problems with bowel function.[34,35] Therefore, some clinicians believe that it may be better to carefully monitor the condition over time, by active surveillance and observation ("watchful waiting"), than to initiate treatment for early-stage prostate cancer that could decrease quality of life.[35]

Large clinical trials that tested thousands of men to determine the benefits and harms of PSA screening have produced conflicting results.[34,36,37] As a result, there is disagreement among major agencies about whether PSA screening should be performed and how it should be implemented.[19,34] For example, the American Cancer Society recommends annual PSA screening in conjunction with DRE for all men over the age of 50 and the American Urological Association recommends that routine screening be conducted from ages 55 to 69 and age 70+ if life expectancy is greater than 10 years, whereas the U.S. Preventative Services Task Force does not recommend screening at all. Earlier and more frequent screening may be recommended for men at higher risk for prostate cancer, notably those with a family history of the condition, those who have a known or suspected related genetic mutation, or those of African American ancestry.[16,34] There is lack of agreement regarding what should be considered a cut-off value to distinguish between positive and negative results, but a total PSA value of 0 to 4.0 ng/mL is generally considered to be normal.[15,16,20] Prostate biopsy is recommended for men with a total PSA value greater than 4.0 ng/mL to determine whether the elevation is caused by malignancy.

Another application of PSA testing is to assist in the diagnosis of prostate cancer. Great effort has been expended to distinguish between BPH, weakly aggressive prostate cancers, and highly aggressive cancers using PSA. Modifications in PSA testing may be helpful in making this differentiation. One modification involves testing for free PSA and the naturally occurring PSA-α-1-antichymotrypsin complex, in addition to total serum PSA.[15,20,35] This combination increases the specificity of testing because the proportion of free PSA is higher in benign conditions, whereas the proportion of complexed PSA is greater in prostate cancer. Because free PSA quickly degrades at temperatures above 4°C, it is important to perform testing within 3 hours of sample collection or to store the sample at –70°C if a longer time interval is required.[15]

Another approach is to calculate the PSA velocity (PSAV), or the rate of increase in PSA values over time. PSAV is calculated as the difference in PSA concentration divided by the number of years spanning the interval between sequential tests (reported as ng/mL/year). The rationale for this approach is that PSA will increase more rapidly if a growing tumor is present. A PSAV greater than 0.75 ng/mL/year has been shown to be strongly associated with the presence of prostate cancer.[15,20,35] To rule out the possibility that an increase in PSAV is because of an infection of the prostate gland, a repeat measurement of PSAV can be conducted after a course of antibiotics is administered.[15,20]

Another proposed strategy to increase the performance of PSA testing is to calculate the PSA density (PSAD). The rationale behind this concept is that an increase in serum PSA is more likely to be caused by the occurrence of cancer in a man with a small prostate gland versus a large prostate gland.[20] PSAD is calculated as the ratio of total PSA to the prostate gland volume.[15] Although this approach appears to increase the specificity of PSA for prostate cancer, it requires performance of transrectal ultrasonography, which can be time consuming, costly, and yield a less-than-perfect measurement of the prostate gland volume.[20]

PSA testing also plays an important role in the management of patients known to have prostate cancer.[27] PSA values, in conjunction with histological observation of prostate biopsy tissue, can be used to predict the stage of prostate cancer and to guide physicians in determining optimal treatment. In addition, a rapid rise in PSAV or in the amount of time it takes for the PSA level to double are indicators of more aggressive disease.[20,27] A persistently high level of PSA after radical prostatectomy indicates that residual disease is present. When surgery is successful in removing the tumor, PSA will decrease to undetectable levels. Rising PSA levels after surgery are a sign that the malignancy is recurring and can precede other indicators of disease recurrence by many years.[27] PSA testing is also a sensitive indicator of disease recurrence in men who have undergone hormonal therapy, but is less sensitive in detecting recurrence after radiation therapy because circulating PSA levels decline more slowly after that type of treatment.[27]

Laboratory Detection of Tumors

Three types of laboratory methods are routinely used for cancer screening and diagnosis: gross and microscopic morphology of tumors, detection of tumor markers by immunohistochemistry or automated immunoassays, and molecular diagnostics to detect genetic mutations in the malignant cells. These techniques are complementary in that many of the changes in DNA and subsequent mRNA expression result in altered protein antigens or morphology. More recently, genetic markers have been introduced into the clinical laboratory. The use of protein expression patterns of cancer cells is being evaluated by scientists.

Tumor Morphology

Pathologists and histology laboratories process suspected tumor tissue with gross dissection and preparation of slides for microscopic analysis. Tumor marker antibodies, special stains, and nucleic acid probes can be applied to the slides to enhance visible features. Even so, evaluation of morphology and staining patterns can be very subjective and classification categories can be rather broad. Considerable skill is required to accurately diagnose cancer on the basis of morphology; the final diagnosis is generally made with supplemental clinical information and additional testing.

Immunohistochemistry

Immunohistochemistry detection uses labeled antibodies to detect tumor antigens in formalin-fixed or frozen tissue sections

of tumor biopsy material.[38] Before testing, formalin-fixed sections must be treated with heat to make the antigen epitopes accessible. The indirect staining method is used because larger immune complexes are formed, providing more sensitive amplification of the signal than is achieved through direct staining. An unlabeled primary antibody specific for the antigen to be detected is applied to the tissue section on a slide. Following an incubation and wash, a labeled secondary antibody directed against the Fc portion of the primary antibody is applied. The label can be an enzyme such as peroxidase, alkaline phosphatase, or glucose oxidase; or a fluorescent dye such as FITC, rhodamine, or Texas red. Immunofluorescence provides a greater dynamic range than immunochromatographic staining and is particularly useful for the identification and quantification of co-localized proteins.[39] Binding is visualized by light microscopy after the addition of the appropriate chromogen in the case of enzyme labels and by fluorescent microscopy when fluorescent labels are used.

Use of positive and negative control tissues is essential for accurate results.[38] Negative controls are necessary to ensure that the staining observed is because of antibody binding and not the background (i.e., nonspecific reactivity), whereas positive controls confirm that the antibody reagents are working properly. Normal tissue on the same slide can serve as an excellent internal control. Accuracy of the results is also increased when a broad panel of antibodies is used.

Clinically, immunohistochemistry is used as an effective technique of classifying tumors of uncertain origin because many tumors can have a similar appearance histologically.[38] To be helpful in pathological diagnosis, the marker must be differentially expressed in the tumor of origin as compared with other tumors. The first step in immunohistochemistry is to broadly classify the tumor into one of three major lineages: *epithelial, mesenchymal,* or *hematopoietic.* This can be accomplished through the use of stains for specific markers that are characteristic of each type of tissue. Routinely, *cytokeratins,* which are intermediate filaments found in all types of epithelial cells, are used as markers for tumors of epithelial lineage. There are 20 different cytokeratin subtypes, whose expression is often organ and tissue specific and dependent on the stage of differentiation of the cells. *Vimentin,* an intermediate filament that is found in most mesenchymal cells, is used as a marker to indicate the presence of a sarcoma or melanoma, but its specificity is low because it is expressed in some other tumor types as well. **CD45,** a WBC marker (see Chapter 1), can be used to identify hematopoietic malignancies.

Once these broad differentiations are made in terms of cell lineage, antibodies for more specific markers can be used for more precise classification of the neoplasm. For example, antibodies to numerous CD antigens can be used to identify the various lymphoid and myeloid cell types (see Chapter 18). In addition, antibodies to estrogen receptors, progesterone receptors, and the HER2 antigen can be used to classify breast cancer and guide decisions about appropriate therapy. Automated immunohistochemistry assays have been developed that allow for quantitation of the proteins expressed by different cell populations.[39]

In the Laboratory

Detecting Immune Complexes With Avidin

Immune complexes can be more easily detected if an additional layer is added to the reaction. A common way to add this layer is to take advantage of avidin-biotin binding. Avidin, a protein found in egg whites, has a strong affinity for the B vitamin, biotin. Therefore, in some enzyme-labeling assays, the secondary antibody is labeled with biotin and an avidin-labeled enzyme is added in the next step. This forms a larger complex that contains more enzyme molecules, increasing the signal intensity and making the assay more sensitive.

Immunoassays for Circulating Tumor Markers

Serum tumor markers are most commonly measured by immunoassays because they are highly sensitive, lend themselves to automation, and are relatively easy to use.[16] Despite their advantages, immunoassays can be affected by several factors that need to be considered when results are interpreted. These factors are related to the use of antibodies as reagents.[15,16]

First, antibody reagents from different manufacturers can vary greatly in terms of what they detect, particularly if monoclonal antibodies are used. Thus, it is important to use the same method for monitoring patients over time because results can be affected if patients change clinics or laboratories. It also means that if laboratories switch methods, they must provide a transition period during which samples are measured by both methods and specimens are archived until new data is established for each patient.[23]

Second, although antibodies are employed for their specificity, it is not absolute. Antibodies will cross-react with similar structures, which is particularly problematic when the cross-reacting substances are present in excessive amounts, as can occur with cancer. For example, the α subunit of hCG is virtually identical to the α subunit of luteinizing hormone (LH), and the β subunits of the two hormones are 80% homologous, so epitope choice is quite important. Furthermore, assay configuration influences what is measured. Tumors may produce free α and free β chains in addition to intact hCG, so an immunochemical method that detects only β chain epitopes to minimize LH interference will measure something completely different than a method that measures intact hCG.[16]

A third factor that can affect immunoassay results is antigen excess. By virtue of their unchecked growth and aggressive metabolism, some neoplasms may produce massive amounts of tumor marker molecules. The excess of tumor antigen as compared with reagent antibody can result in a **postzone** effect. Recall from Chapter 10 that postzone is a well-known limitation of immunoassays in which saturation of the antibodies with antigen inhibits the cross-linkage required to visualize the reaction. When the measurements exceed the linear range of reportable results, this phenomenon is called the **high-dose hook effect**[16] because of the shape of the curve that depicts the relationship of the concentration of the analyte (in this case,

the tumor marker) and the intensity of the reaction signal **(Fig. 17–4).** This effect can result in a falsely decreased measurement in the area of antigen excess; therefore, the sample must be diluted to determine its value within the reportable range. It is critical that criteria be developed to identify situations in which the hook effect may be present so that specimens can be systematically diluted and accurate results obtained. A related problem of antigen excess in automated systems is sample carryover, so testing of the sample adjacent to the specimen with excessive antigen may also need to be repeated.

A final problem with immunoassays is that interference can be caused by the presence of endogenous heterophile, anti-animal, or autoantibodies in the patient sample.[16,40] Autoantibodies are produced in response to self-antigens (see Chapter 15). **Heterophile** antibodies are capable of reacting with similar antigens from two or more unrelated species. These antibodies usually have low avidity, but can react with a broad range of antigens. Anti-animal antibodies are species-specific, higher avidity antibodies that are produced by patients as a result of passive immunotherapy with mouse monoclonal immunoglobulins or polyclonal antibodies of animal origin (see Chapter 25). Because the antibodies used in immunoassays are of animal origin, endogenous anti-animal antibodies in the patient sample can interfere profoundly with test results. These antibodies can affect immunoassays in more than one way, causing either falsely elevated (most commonly) or falsely decreased test results.[16,40,41] The principle of interference resulting in a false-positive result is illustrated in **Figure 17–5.** In this situation, the antibody in the patient sample binds to both the capture antibody on the solid phase and the labeled detector antibody, forming a bridge between the two antibodies in the same way the tumor marker would if it was actually present. This interference can have profound consequences on patient care. For example, a tragic case involving false-positive hCG results reported from an automated analyzer led to several women having unnecessary chemotherapy or hysterectomies for a cancer that was suspected but not actually present.[42]

Although endogenous antibodies are mostly associated with falsely increased results by mechanisms similar to those shown in Figure 17–5, false decreases are also possible. In these situations, the patient antibody binds to the marker of interest or blocks the binding of the labeled detector antibody, preventing the formation of the immune complex "sandwich" and leading to a falsely low or negative result.

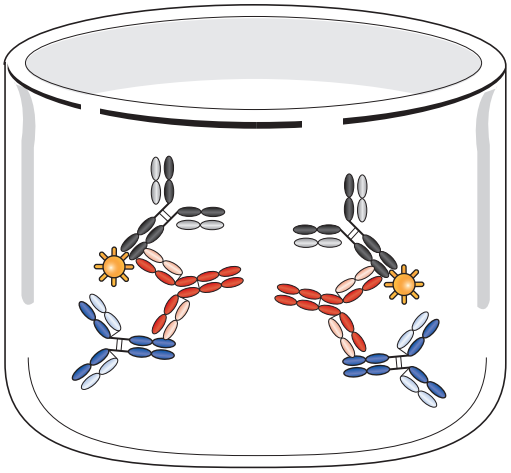

FIGURE 17–5 Interference by heterophile or anti-animal antibodies. Heterophile or anti-animal antibodies (red) can cause both false decreases and false increases, depending on their reactivity against the antibody species used in an assay. However, false increases caused by linking the capture (blue) and detection (black) antibodies together are most likely, as shown.

To confirm the presence of interfering antibodies, the sample can be diluted and the linearity of the results can be analyzed.[16] Specimens with interfering antibodies tend to exhibit nonlinear behavior. The laboratory can also test directly for the antibodies themselves. The likelihood of interference by endogenous antibodies can be reduced by pretreatment of the sample with commercial blocking reagents. These reagents are typically mouse or rabbit immunoglobulins that bind to the interfering antibodies and neutralize them.[16,40,41] Interference with tumor marker tests can also be caused by factors that can affect other immunoassays, such as icterus, lipemia, and hemolysis. In any case, patient results should not be reported until the interference issue is resolved.

Some additional recommendations should be considered in the performance of tumor marker tests.[15] Cut-off values for screening tests are typically selected under the presumption that a relatively low number of people being screened actually have cancer and that it would be worse to miss a cancer than to do further testing on a normal person to exclude cancer. Thus, a very high number of false positives is expected because of low disease prevalence. Establishment of a baseline level at initial diagnosis followed by serial testing over time can provide valuable information when a patient is being monitored for response to treatment or tumor recurrence. In this case, it is not a single absolute value of the tumor marker that is important, but rather the upward or downward trend when the marker's biological half-life is considered. When performing serial testing, each test should be performed by the same laboratory with the same test kit to minimize variations in results. Testing for multiple markers, if possible, will increase sensitivity and

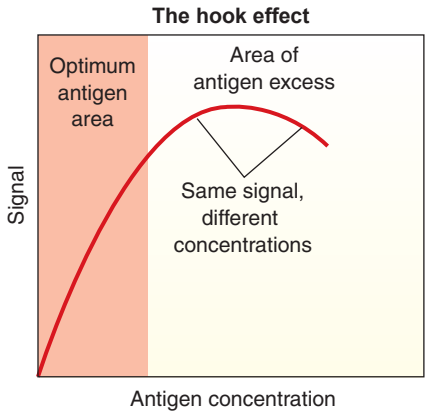

FIGURE 17–4 The high-dose hook effect. Antigen excess can saturate antibodies and the intended "sandwich" configurations cannot form, leading to a false decrease in signal.

specificity. The limitations of immunoassays have prompted a search for more specific and sensitive markers using molecular and proteomic technologies.

Molecular Methods in Cancer Diagnosis

Because cancer is a disease process that involves many genetic alterations, scientists have searched for changes in the genome that characterize the various types and subtypes of cancer. Identification of genetic mutations has become an important tool in cancer diagnosis and determination of prognosis.

Genetic Biomarkers

Molecular characterization is an essential component of testing for hematologic malignancies (see Chapter 18). For example, as previously mentioned, the chromosome 9/22 *[BCR/ABL]* gene rearrangement is a well-established biomarker for CML.[43] Rearrangements in immunoglobulin genes and T-cell receptor (TCR) genes are used to identify malignant clones of B cells or T cells, respectively, in leukemias and lymphomas. Genetic analysis is also helpful in the diagnosis of solid tumors. For example, mutations in the *MSH* genes of the DNA mismatch repair system are helpful in the diagnosis of hereditary colorectal cancer; these alterations create microsatellite instability, an alteration in the length of repetitive DNA sequences that can be visualized by molecular testing.[44] Molecular analysis of gene expression for the estrogen receptor (ER), progesterone receptor (PR), and HER2 can create a genetic profile that is used to classify breast cancer patients into subtypes that provide prognostic information and guide physicians in choosing therapeutic plans that have the best chances of success.[45,46]

Genetic biomarkers can also be used for prospective and postdiagnostic evaluation of malignancies. Prospective markers can provide valuable information regarding the risk for an asymptomatic person to develop a particular type of cancer, the growth rate of the cancer, or the development of metastatic disease. For example, women with hereditary mutations in the *BRCA1* or *BRCA2* genes carry a 40% to 80% lifetime risk for developing breast cancer, as well as an 11% to 40% lifetime risk for ovarian cancer.[47]

Postdiagnostic genetic markers are used to guide clinicians in making appropriate treatment decisions for known cancer patients. By mid-2014, the FDA had approved over 40 targeted cancer drugs for administration in cancer patients with the appropriate genetic biomarkers.[48] The list of approved therapies includes drugs that target tumors with alterations in the *BRAF* gene (metastatic melanoma), *KRAS* gene (colorectal cancer), *EGFR* gene (non-small cell lung cancer; NSCLC), *ALK* gene (NSCLC), *HER2* gene (breast cancer), and *ESR1* and *PGR* genes (breast cancer). Testing for these and other genetic markers is incorporated into the clinical practice guidelines published by the National Comprehensive Cancer Network and the College of American Pathologists. Molecular testing plays an important role in identifying responders and nonresponders to the targeted medications, so that the drugs can be given to patients who would most likely benefit from them.

Testing for biomarkers has been made possible through scientific advances in molecular techniques, including nucleic acid amplification techniques (NAAT), *fluorescent in situ hybridization (FISH),* microarray, and DNA sequencing. In NAAT, such as the *polymerase chain reaction (PCR),* millions of identical copies of a specific target sequence within a nucleic acid are synthesized in the laboratory from an original DNA template derived from the cancer cell population (see Chapter 12). These methods are used to amplify the sequence that potentially contains the genetic mutation of interest, allowing tiny changes in the sequence to be detected by the differences in fragment sizes that can be visualized by gel electrophoresis.[49]

Cytogenetics

Cytogenetics studies play a large role in the diagnosis and management of cancer. **Karyotype analysis** (arrangement of a person's chromosomes by their visual characteristics) has been used for many years to detect the chromosomal abnormalities associated with many cancers. The number of these aberrations can increase as the disease advances. One type of abnormality that can be detected by karyotyping is *aneuploidy,* a condition in which individual chromosomes are gained or lost. Another type of abnormality that can be present in cancer cells is a *deletion,* in which a portion of a chromosome has been lost. A third type of abnormality is a *rearrangement,* involving breakage of two different chromosomes and translocation of the fragments onto the opposite chromosomes (as discussed in Chapter 18).

The development of FISH has allowed chromosomal abnormalities to be characterized more precisely at a molecular level. In FISH, interphase cells from the patient's tumor are incubated with fluorescent-labeled nucleic acid probes that are complementary to the sequence of interest. Cells containing the sequence will bind the probes and can be visualized with a fluorescent microscope. In oncology, FISH is most often used to detect chromosome rearrangements and gene amplification. To detect a chromosome translocation, such as the one seen in the *BCR/ABL* rearrangement characteristic of CML, two single probes are used, each specific for one of the two chromosomes and each labeled with a different fluorochrome (for example, red and green).[49] Normally, each cell should have two red signals and two green signals. If a translocation has occurred, a fusion probe signal is generated, in which the red signal is adjacent to the green signal, producing a yellow color (see Figure 18–11B). To detect gene amplification, the probe hybridizes to the gene of interest. If that gene is overexpressed, multiple copies of the fluorescent signal representing the probe will be observed. This application of FISH is commonly used to detect the HER2 gene amplification seen in some cases of breast cancer. Although FISH is a highly specific method to detect molecular abnormalities in tumor cells, it can only detect gene sequences that are complementary to the probes used; as such, it may not detect rare, tiny deletions.

Microarrays

As more clinically significant genetic abnormalities associated with cancer have been identified, **microarray** technology has been developed to test for panels of markers, rather than individual mutations.[49] In this method, single-stranded DNA or RNA from the tumor is tagged with a fluorescent label and

incubated with known nucleic acid sequences that have been spotted onto different areas of a membrane. The sample will hybridize to any complementary sequences on the tiny spots, allowing for simultaneous testing of the specimen for multiple genes. Microarrays can also be used to compare the levels of gene expression in cancer cells with those of normal cells by using two different colors of fluorescence to tag nucleic acid from each cell type (Fig 12-12).[49] The microarray is interpreted with a fluorescent reader and analysis software. Microarrays can screen tens of thousands of gene sequences at the same time for their ability to bind to the sample nucleic acid, thus generating an enormous volume of information.

Sophisticated mathematics and computer software are necessary to analyze the vast amount of biological data. The collection and analysis of such data is referred to as *bioinformatics*. It is hoped that this approach will help uncover molecular signatures that can characterize specific tumor types and subtypes to provide better information regarding patient diagnosis and prognosis. Gene expression arrays, such as the MammaPrint and Oncotype Dx for breast cancer, are being commercially developed to aid in predicting the likelihood of cancer recurrence and guiding treatment decisions in cancer patients.[23,50]

Next Generation Sequencing

Another molecular method that provides a large amount of data is *next generation sequencing (NGS)*. With NGS, thousands of genes within the tumor can be sequenced simultaneously in just a few hours to identify genetic variations (see Chapter 12).[51] NGS can also be used to detect metastases by analyzing DNA from tumor cells circulating in the peripheral blood.[52]

NGS is playing a major role in generating an enormous volume of data for The Cancer Genome Atlas (TCGA).[52] TCGA is conducting a large-scale collaborative project funded by the National Cancer Institute and the National Human Genome Research Institute to catalogue and characterize the genomic changes that occur in cancer. The goal of the project is to identify all of the pertinent genomic alterations in several tumor types. This information is helping oncologists to better understand and interpret molecular information related to cancer diagnostics. As a result of the ongoing studies, molecular techniques have evolved from esoteric research tools into routine laboratory assays that have revolutionized cancer diagnosis and therapy.

Proteomics

Researchers are also analyzing the proteins produced by cancer cells. Analysis of the entire protein complement of a cell population is referred to as **proteomics.** This analysis is being done through the use of two-dimensional electrophoresis coupled with tandem mass spectrometry (MS/MS), surface-enhanced laser desorption/ionization mass spectrometry (SELDI-TOF), or more recently, **antibody arrays.**[39,53] The latter are typically based on a principle of a double sandwich enzyme-linked immunoassay and are available in several different multiplex formats. The most common format uses beads that are coated with specific capture antibodies to bind the target proteins and streptavidin- or fluorescent-labeled detection antibodies that can be detected by flow cytometry.[39] The advantages of antibody arrays are that they do not require fractionation or depletion of high abundance proteins to detect proteins that are present in lower concentrations. Proteomic methods may allow laboratories to identify unique patterns of proteins and their metabolites that are characteristic of particular types of cancer.[53,54] This process is called **biomarker profiling.** In the future, data generated from proteomic methods may help clinicians diagnose cancer earlier and lead to the development of personalized therapies that can effectively target the underlying biology of this highly complex disease entity.

Interactions Between the Immune System and Tumors

Paul Ehrlich proposed the idea over 100 years ago that the immune system plays an important role in eliminating tumor cells.[55] Ehrlich reasoned that, if this were not the case, we would see cancer much more frequently in animals of advanced age.[56] In the 1950s, after scientists learned more about how the immune system works, F. MacFarlane Burnet and Lewis Thomas independently proposed the theory of **immunosurveillance** to address this issue. This theory states that the immune system continually patrols the body for the presence of cancerous or precancerous cells and eliminates them before they become clinically evident.[13,57] Burnet and Thomas based their hypotheses on early studies that involved transplantation of tumor cells in genetically inbred mice.[58-60] The protective role of the immune system against tumors has also been supported by clinical evidence in humans. Notably, a significantly higher incidence of cancer has been observed in transplant patients who received immunosuppressive therapy and patients with immunodeficiency diseases than in the general population.[13,61,62] In addition, the incidence of cancer rises greatly after the age of 60, at which point there is a decline in immune function.[60] Although the validity of the immunosurveillance theory was challenged in the 1970s and 1980s, technical advances in genetics and monoclonal antibody technology have increased our knowledge in this area and there is renewed enthusiasm among scientists that the immune system indeed plays an important role in defense against tumors.[13,55,60]

Immune Defenses Against Tumor Cells

Both innate and adaptive immune responses are thought to contribute to protection of the host from cancer.[3,11,60,63] The main components of the immune system that participate in defense against tumor cells are NK cells, macrophages, cytotoxic T cells (CTLs), cytokines, and possibly T helper (Th) cells and antibodies. The mechanisms by which each of these components functions are the same as those that are involved in the elimination of pathogenic organisms **(Fig. 17–6).**

Innate Immune Responses

The key cells involved in innate immune responses to tumors are thought to be NK cells and possibly macrophages. As we

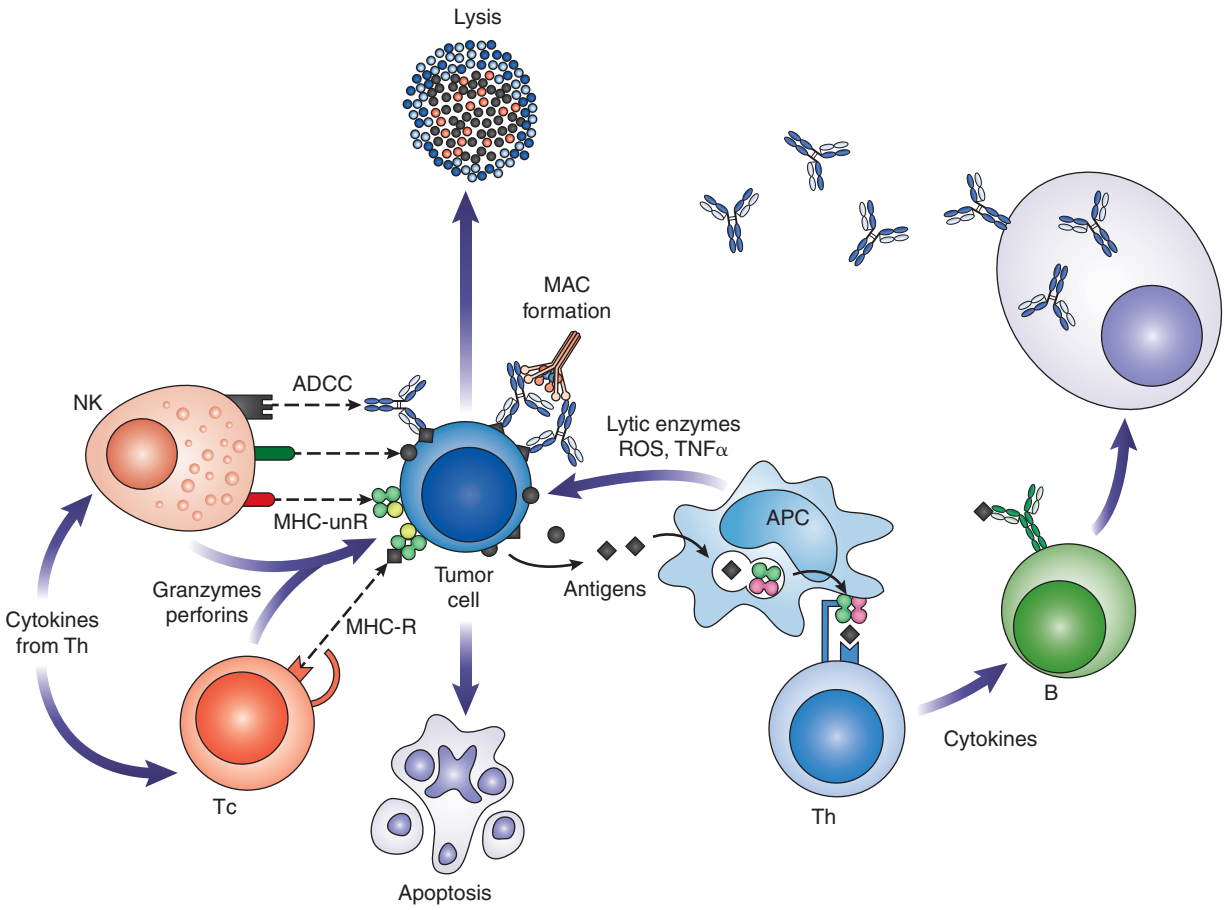

FIGURE 17–6 Immune defenses against tumor cells (MHC-R = MHC-restricted killing, MHC-unR =MHC unrestricted killing).

discussed in Chapter 3, NK cells act in a mechanism that is similar to CTLs (see the text that follows), but can kill tumor cells without prior sensitization to tumor antigens. In addition, they are activated to kill cells that lack class I MHC molecules, a property that is often seen in transformed cells (see *Immunoediting and Tumor Escape* later). Activating receptors on NK cells bind to tumor antigens or substances released from stressed tumor cells, initiating intracellular signals that promote degranulation and the release of perforin and granzymes, which ultimately kill the cells by inducing apoptosis. NK cells may also participate in antibody-dependent cellular cytotoxicity (ADCC) in the presence of tumor-specific antibodies (see the text that follows). NK cells are thought to be most effective against malignant cells circulating in the bloodstream during the early stages of tumor development. Their effectiveness in eliminating well-established solid tumors is questionable, however, and requires further study.[3,11,60]

The activity of NK cells can be increased by incubation with IL-2. In vitro culture of peripheral blood cells or tumor-infiltrating lymphocytes (TILs) with IL-2 results in the generation of *lymphokine-activated killer (LAK) cells,* which destroy tumor cells by the same mechanism as NK cells but are much more potent.[11,60] The effectiveness of cytokine-activated TILs in adoptive immunotherapy for cancer patients is an active area of investigation (see Chapter 25 and *Immunotherapy* later).

Macrophages may also play a role in innate immunity against tumors.[11,60] Macrophages activated in vitro by IFNγ have been shown to possess tumoricidal capabilities. They appear to kill tumor cells by the same mechanisms they use to kill infectious organisms, including release of lysosomal enzymes, reactive oxygen species, and nitric oxide. In addition, they produce TNF-α, a cytokine that is thought to cause necrosis of tumors by inducing local inflammation and thrombosis in the blood vessels within the cancerous mass.[11] However, the significance of macrophages in anti-tumor responses in the body is unclear.

Adaptive Immune Responses

The primary mechanism of adaptive immunity against tumors is mediated by cytotoxic T lymphocytes (CTLs).[3,11,60] CTL responses are thought to be initiated by dendritic cells, which act as antigen-presenting cells (APCs) by processing tumor antigens and displaying the peptides derived from these antigens on their surface in conjunction with class I MHC molecules. (This mechanism is known as *cross-presentation* because the exogenous tumor antigens are presented in context with class I MHC molecules rather than class II MHC molecules.) The APCs present the tumor peptide-antigen complexes to specific TCRs on the surface of the CTLs and provide costimulatory signals that promote maturation of the CTLs. The mature

CTLs use their antigen-specific TCRs to bind class I MHC-associated tumor antigens on the surface of the tumor cell. Within minutes, their granules migrate toward the plasma membrane and release cytotoxic proteins within the synapse formed between the CTL and the tumor cell. Among these proteins are perforin, which creates pores in the membrane of the tumor cell, and granzymes, which enter through the pores and cause apoptosis of the tumor target. NKT cells, which express surface antigens of both NK cells and T cells, are able to destroy tumor cells in a mechanism that is similar to the CTLs, but they have a unique type of TCR that recognizes glycolipid antigens instead of peptide antigens.[3]

Dendritic cells are also thought to activate CD4+ Th cells through presentation of tumor antigens in conjunction with class II MHC molecules.[11,60] The activated Th cells may play a role in tumor immunity by secreting cytokines such as IL-2, which can promote CTL development and enhance NK cell activity, and IFNγ, which activates macrophages and increases class I MHC expression on the tumor cell surface.

Tumor-bearing individuals can also produce antibodies against tumor antigens. In vitro studies have demonstrated that these antibodies can kill tumor cells by inducing complement-mediated lysis or ADCC.[11,60] The latter mechanism occurs when the antibodies coat the tumor cells and bind to Fc receptors on the surface of macrophages, NK cells, or neutrophils, stimulating them to release enzymes that can destroy the tumor targets. However, the relevance of these antibodies in vivo is unclear.

Immunoediting and Tumor Escape

Despite the diverse array of mechanisms employed by the immune system to attack tumors, cancer occurs at a high frequency in individuals who appear to be immunocompetent. Medical scientists have long been trying to find the answer to this intriguing puzzle. As the field of tumor immunology advances, scientists are recognizing that immunosurveillance is only part of a broader process that explains the complex relationship between the immune system and cancer. This process, termed **immunoediting,** is thought to consist of three phases: elimination, equilibrium, and escape **(Fig. 17–7).**[13,55,62]

Elimination

The elimination phase of the cancer immunoediting process is essentially the same as the immunosurveillance concept that was just discussed. If the immunologic mechanisms involved in immunosurveillance are highly effective, they will likely result in complete elimination of the tumor. If the immune responses are not completely effective, some of the tumor cells will remain in the body. The immunoediting hypothesis suggests that these cells will then enter the equilibrium phase.

Equilibrium

In this phase, tumor cells are thought to enter a state of dynamic equilibrium with the immune system, which keeps the altered cells under control so that they are not clinically evident. During this period, tumor cells may remain dormant or evolve slowly over time. The dynamic interactions between the tumor and the immune system are thought to shape the phenotype of the tumor and its ultimate outcome, hence the term *immunoediting.* As a result, the tumor may eventually be eliminated by the body, establish permanent residence in the equilibrium phase, or evolve into a phenotype that can escape the immune system and cause disease.

During the equilibrium phase, mutations can occur in the genetically unstable transformed cells. Under selective pressure from immunologic forces of attack by cells in the tumor microenvironment, some of the tumor cells may develop into genetic variants that are resistant to immune defenses.[64] These cells move past the equilibrium phase and enter the escape phase.

Escape

During this phase, the balance between immunologic control and tumor development is tipped in favor of the neoplasm and tumor growth progresses, even in the presence of anti-tumor immune responses.[13] Cancer is a heterogeneous disease and tumors have developed a variety of strategies for evading the immune system (see Fig. 17–7).[11,13,55,64] Some of the escape mechanisms employed by tumors are a result of changes in the edited tumors themselves, which lead to reduced immunogenicity. For example, some tumors downregulate the expression of tumor antigens or MHC molecules on the cell surface, making them less likely to be recognized by T cells. Other modifications may involve defects in components of the antigen-processing machinery associated with class I MHC molecules. Tumor antigens may also be masked by glycoproteins and glycolipids on the cell surface, making them inaccessible to the immune system.

Other alterations can result in tumor resistance to immune defenses. For example, impaired cell surface binding to perforin or defective apoptosis-inducing receptors such as Fas have been noted in some tumors. Another way that tumors can escape the immune system is to suppress anti-tumor immune responses. Tumors can do this directly by secreting immunosuppressive substances or indirectly by recruiting T regulatory (Treg) cells, myeloid-derived suppressor cells, or macrophages that produce cytokines such as transforming growth factor-β and IL-10, which can inhibit protective immune responses.

Another factor that may contribute to tumor progression is inflammation. Although acute inflammation may be protective to the host, chronic inflammation is believed to modify the cellular microenvironment in ways that promote the development of tumors.[56,64] Chronic inflammation and its associated proinflammatory cytokines may contribute to tumorigenesis in a number of ways, including generation of cellular stress and free radicals, production of growth factors that induce cell proliferation, enhancement of angiogenesis and tissue invasion, and suppression of adaptive immune responses.[13,56]

A clear understanding of the evasion mechanisms used by tumors and of the immune responses they suppress will be

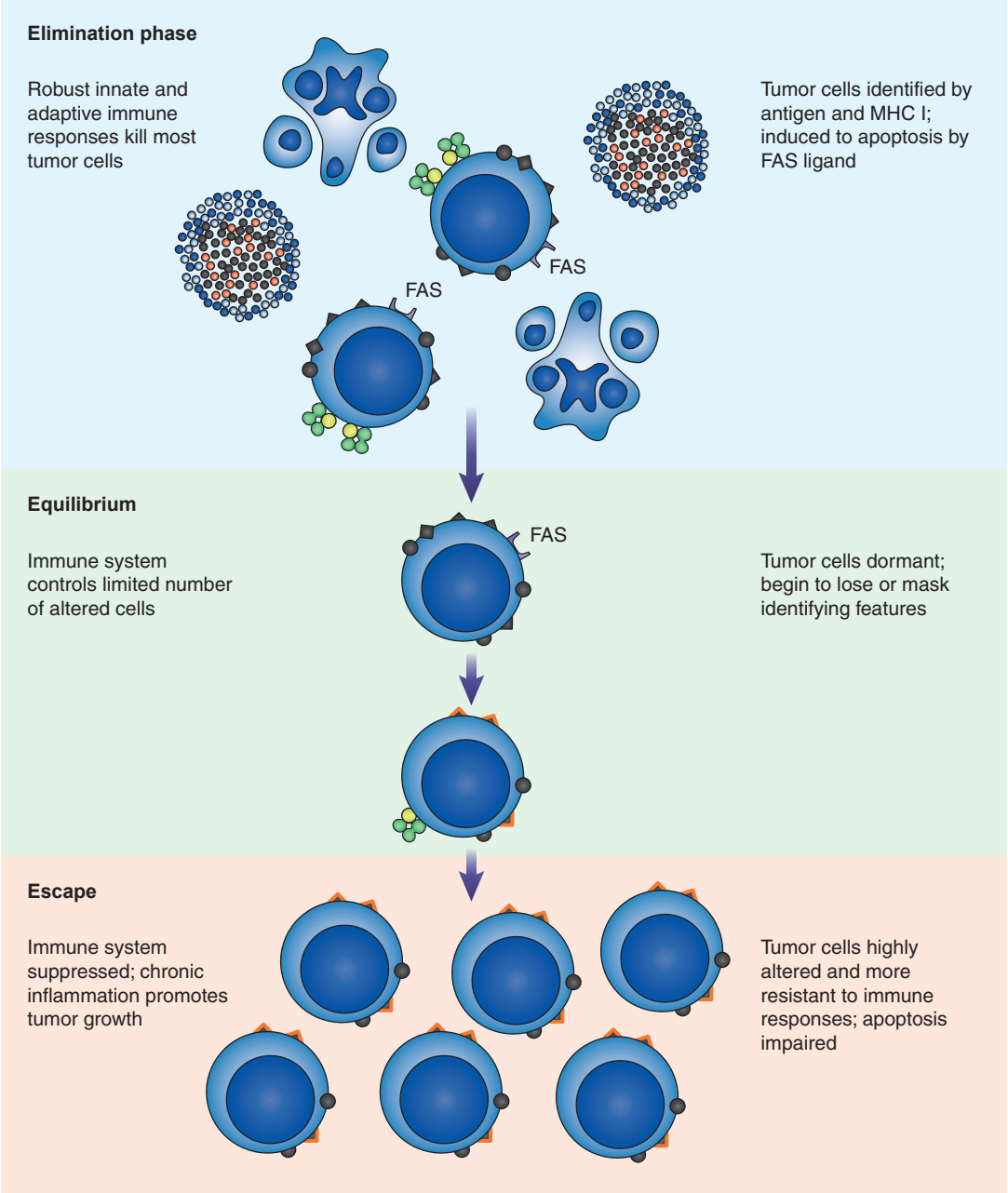

FIGURE 17–7 Relationship between the immune system and cancer: Immunoediting and mechanisms of tumor escape.

critical in developing rational approaches to targeted immunotherapy. The section that follows presents specific approaches that have been taken in an attempt to influence interactions between tumors and the immune system in order to promote tumor rejection.

Immunotherapy

Many different types of treatments can be used for cancer patients. The type of therapy used for a particular patient depends on the type of tumor present and the stage of disease. Traditional therapies include surgery to remove solid tumors, radiation therapy to reduce tumor size, and chemotherapy and hormone therapy to target residual tumor cells and metastases. Advances in medical science have allowed immunotherapy to gain a position among these conventional types of treatment in the management of cancer. The goal of **immunotherapy,** which is also known as *biological therapy* or *biological response modifier therapy,* is to harness the ability of the immune system to destroy tumor cells.

Immunotherapeutic methods can be classified into three major types: active, passive, and adoptive. With active immunotherapy, patients are treated in a manner that stimulates them to mount an immune response against their tumors. Passive immunotherapy involves administration of tumor-specific antibodies or cytokines to patients who may not be able to develop

an adequate immune response. In adoptive immunotherapy, cells from the immune system are provided to patients. Some treatments may combine different types of immunotherapy. Although it is well beyond the scope of this chapter to cover all the different protocols, the following sections present the major types of immunotherapy that have been developed and their applications to specific kinds of cancer.

Active Immunotherapy and Cancer Vaccines

The possibility of stimulating a patient's own immune system to respond to TAAs has long intrigued scientists. In 1891, the bone sarcoma surgeon, William Coley, began the first systematic study of immunotherapy.[65] In his review of the literature, he noted that cancer patients who developed an infection after surgery experienced tumor regression and had a better prognosis than patients who did not acquire an infection. Inspired by this knowledge, he decided to inject one of his cancer patients with *Streptococcus pyogenes* bacteria. To his amazement, the patient's tumor shrank and the patient became cancer free. He went on to treat additional patients and observed shrinkage of their malignant tumors, although some of the patients died from infection because the bacteria were alive and virulent. Coley then developed a less dangerous version of his treatment, which consisted of a mixture of killed *S pyogenes* and killed *Serratia marcescens* bacteria. This formulation later became known as "Coley's toxins" and was widely used by Dr. Coley and other physicians for the next 30 years to treat patients with inoperable bone and soft-tissue sarcomas.

However, Coley's treatment was controversial and many doctors who used the toxins did not get good results, particularly with other types of cancer. In 1962, the FDA refused to recognize the formulation as an effective cancer therapy and it became illegal to use Coley's toxins to treat cancer. Despite the criticism of Coley's work, the medical community later recognized that his premise of stimulating the immune system to effectively treat cancer bore some merit. Today we acknowledge Coley as the "Father of Immunotherapy."[65]

Although Coley's toxins are no longer in use today, other agents have been used to nonspecifically boost the immune system. For example, Bacillus Calmette Guerin (BCG), a live but weakened strain of *Mycobacterium bovis* bacteria, is considered to be the treatment of choice for noninvasive bladder cancer.[66] Serial treatments in which BCG is directly delivered into the urinary bladder through a catheter have been shown to be effective in reducing the progression of tumor growth and in delaying recurrence of the cancer in these patients. The exact mechanism by which BCG works is unknown, but it is believed to stimulate cytokine production by macrophages and activated lymphocytes, increase anti-tumor effects of macrophages and other cells of the innate defense system, and increase T-cell–mediated immune responses.

The development of prophylactic **cancer vaccines** offers an effective approach to active immunotherapy. These vaccines have been generated for the purpose of preventing virus-associated cancers (see Chapter 25). Notably, the human papilloma virus (HPV) vaccine is effective in preventing cervical cancer and the hepatitis B virus (HBV) vaccine has been successful in reducing the incidence of HBV infection of the liver and its associated complications, including hepatocellular carcinoma. These vaccines are routinely used and are incorporated into standard vaccination schedules.

Other cancer vaccines are being administered to patients who have already developed cancer. These vaccines are designed to induce an immune response against tumor antigens with the hope of eliminating existing tumor cells and producing long-lasting immunity. Early cancer vaccines used tumor cell lysates or whole tumor cells that were inactivated by treatments such as irradiation.[67] The advantages of this approach were that no knowledge was required about the tumor antigens themselves; the vaccine could theoretically contain all the antigenic components of the tumor. Unfortunately, clinical trials found that most killed tumor cell vaccines did not have a significant effect on patient survival; as a result, this approach is generally not used today.[68]

The focus of more recent research is on the development of antigen-specific vaccines for cancer.[11,61,67-69] In one approach to developing these vaccines, the genes that code for TSAs are identified and cloned in recombinant vectors such as viruses or bacterial plasmids. The vectors can be genetically engineered so that they produce peptides that are recognized by CTLs, which, as we discussed, are important effectors of tumor immunity. Another approach is to use synthetic peptides or full-length tumor proteins in the presence of a vaccine adjuvant. Examples of such vaccines include the HER2 antigen to treat a subset of breast cancer patients, the tumor immunoglobulin idiotype to target B-cell lymphoma, and the MAGE-3 antigen for some patients with melanoma or NSCLC.[61,69]

An interesting strategy that has gained much attention is the use of dendritic cells to immunize patients against their own tumors. In this approach, dendritic cells (DCs) are isolated from the cancer patient and incubated with the pertinent tumor antigen or transfected with the gene that codes for the antigen. The antigen-loaded DCs are then readministered to the patient, where they are believed to function as potent APCs. Sipuleucel-T (Provenge®), the only FDA-approved cancer vaccine at the time of this writing, is based on this technology. The vaccine, which is designed to treat patients with advanced prostate cancer, is produced by incubating the patient's own peripheral blood cells with a fusion protein composed of the antigen, prostatic acid phosphatase (PAP), and the cytokine GM-CSF, which is thought to promote DC activation and induce a PAP-specific T-cell response. Phase III clinical trials with Provenge have shown improvement in median overall patient survival.[70]

Although cancer vaccines show much promise for the future, they also have some important limitations. As previously mentioned, tumor cells can evade the immune response by creating an immunosuppressive microenvironment in which T cells are unable to fully exert their tumoricidal potential. It may therefore be necessary to return the tumor environment into an immunosupportive tissue before a cancer vaccine can be fully effective.[69,71] Scientists are attempting to do this by

promoting local production of certain cytokines (e.g., IL-12 and IFN-α) and using antibodies to eliminate Treg cells or block molecules that inhibit immune responses (see *Passive Immunotherapy* later).[71]

Unlike vaccines for infectious diseases, which are used to *prevent* infection, most cancer vaccines are immunotherapeutic, being administered *after* the disease has occurred. They are frequently given to patients in the advanced stages of disease when other treatment options have been exhausted. In this situation, the patient's immune system has often been compromised because of the disease process or the effects of chemotherapy; therefore, response to the vaccine may be suboptimal. In these cases, it may be more beneficial to provide the patient with components of the immune system through passive or adoptive immunotherapy to more effectively target destruction of the tumor.[67] These approaches to cancer immunotherapy will be discussed in the sections that follow.

Passive Immunotherapy

Passive immunotherapy, as previously mentioned, involves the administration of soluble components of the immune system to boost the immune response. Two approaches to passive immunotherapy in cancer patients involve the administration of cytokines to nonspecifically enhance the immune response and treatment with monoclonal antibodies to target specific tumor antigens.

Cytokines

As discussed in Chapter 6, cytokines are small proteins that play an important role in regulating immune responses by serving as chemical messengers that affect the interactions between cells of the immune system. There have been two main applications of cytokines in cancer treatment: use as hematopoietic growth factors and use as therapeutic agents.

Because chemotherapy drugs inhibit cell division, they often adversely affect the development of hematopoietic stem cells in the bone marrow, resulting in decreased production of WBCs, red blood cells (RBCs), and platelets. **Hematopoietic growth factors,** also known as **colony stimulating factors,** can be administered to patients to help them recover from or prevent these toxicities. Some of the main colony stimulating factors that have been used to treat cancer patients are granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), erythropoietin, and interleukin 11 (IL-11) (see Chapter 6).[72] G-CSF stimulates hematopoietic stem cells to develop into granulocytes, whereas GM-CSF stimulates hematopoietic stem cells to develop into granulocytes and monocytes, thus reducing the patient's risk for severe infections.[73,74] Erythropoietin stimulates production of RBCs from the bone marrow and can be used to treat patients with severe anemia.[75] IL-11 stimulates the maturation of megakaryocytes, helping patients to recover from chemotherapy-induced thrombocytopenia.[76]

The therapeutic application of cytokines is aimed at enhancing patients' immune responses to their tumors. Preclinical and clinical investigations have been conducted for the interferons (IFNs), tumor necrosis factors (TNFs), and several interleukins. Two examples of cytokines that have been widely studied are IFN-α and IL-2.

Interferons were the first cytokines that were used as biological response modifiers. IFN-α has been the most commonly used IFN in cancer therapy and has been approved by the FDA for the treatment of several types of cancer, including malignant melanoma, hairy cell leukemia, chronic myeloid leukemia, and Kaposi's sarcoma.[72] IFN-α is thought to promote anti-tumor effects by increasing tumor immunogenicity, enhancing dendritic cell responses to the tumor, enhancing Th1 responses and cell-mediated cytotoxicity, promoting tumor apoptosis, and inhibiting angiogenesis.[77] Although high doses of IFN-α are associated with better clinical responses than low doses of the cytokine, they also generate strong adverse effects, including fever, asthenia (loss of muscle strength), neutropenia, and nausea and vomiting.[11,78]

Of all the interleukins, interleukin-2 (IL-2) has been the most extensively studied. IL-2 induces T-cell proliferation and enhancement of CTL and NK cell function (see Chapter 6). However, clinical trials revealed that systemic administration of IL-2 as immunotherapy was limited because of its short half-life (fewer than 10 minutes) and serious adverse effects, including vascular leakage syndrome, marked fluid retention, and shock.[78] Although this cytokine is still used to treat metastatic melanoma and advanced renal cancer, it is rapidly cleared from the body and its most effective use may be to activate immunocompetent cells in vitro for adoptive immunotherapy (see *Adoptive Immunotherapy* later).[4,11,78]

Although cytokines continue to be incorporated in immunotherapy, their use has been limited because of the serious and sometimes life-threatening side effects associated with high-dose systemic treatment as previously discussed. The cytokine network is very complicated and administration of a cytokine can have multiple, and sometimes unwanted, effects.[4] For example, in addition to its immunostimulatory effects, IL-2 is also thought to be necessary for the generation and maintenance of Treg cells, which can be involved in enhancing tumor growth. Studies are now underway to see if some of these obstacles can be overcome through more localized administration of cytokines, use of cells that are genetically engineered to express specific cytokine genes, or therapies using small doses of cytokines combined with each other or with chemotherapy drugs.[72,78,79]

Monoclonal Antibodies

Monoclonal antibodies take a more specific approach to immunotherapy. As we discussed in Chapter 5, these antibodies are derived from a single clone of cells, providing for an abundant source of highly specific antibodies directed toward one particular epitope of an antigen. Monoclonal antibodies in cancer immunotherapy have been directed against seven major categories of antigens: CD antigens, glycoproteins, glycolipids, carbohydrates, vascular targets, stromal and extracellular antigens, and growth factors.[80] These antibodies have different mechanisms of action, depending on their target.[81] The major approaches to monoclonal antibody therapy are discussed in the text that follows and summarized in **Table 17–5.**

Table 17–5 Approaches to Cancer Immunotherapy Using Monoclonal Antibodies

TARGET OF THERAPY	MECHANISM OF ACTION	EXAMPLES
Surface antigens on tumor cells	Opsonization Complement-mediated cytotoxicity ADCC	*Rituximab,* a MAb* directed against the CD20 antigen on B cells; used to treat B-cell neoplasms *Alemtuzumab,* a MAb directed against mature lymphocyte antigen, CD52; used to treat chronic lymphocytic leukemia and T-cell lymphomas
Cell surface receptors	Block signaling pathways involved in cell proliferation and survival	*Panitumumab,* a MAb directed against epidermal growth factor receptor (EGFR), used to treat colorectal cancer *Trastuzumab,* a MAb directed against HER2, used to treat breast and gastroesophageal tumors with overexpressed HER2
Antigens involved in angiogenesis	Inhibit formation of blood vessels necessary for delivery of oxygen and nutrients to the tumor	*Bevacizumab,* a MAb directed against vascular endothelial growth factor (VEGF); for treatment of glioblastoma, colon, lung, and renal cancers
Molecules that block T-cell activation and proliferation by binding to molecules on antigen-presenting cells	Enhance anti-tumor-specific T-cell responses by preventing T-cell inhibition	*Ipilimumab,* a MAb directed against CTLA-4 (cytotoxic T-lymphocyte antigen 4); for treatment of metastatic melanoma *Nivolumab* and *Lambrolizumab,* MAbs directed against PD-1 (programmed death-1); used to treat melanoma, colon cancer. and other tumors
Antibody–drug conjugates (immunotoxins) directed against TSAs	Deliver potent toxic molecules directly to tumor cells	*Brentuximab vedotin,* an immunotoxin directed against the CD30 antigen; used to treat Hodgkin lymphoma and systemic anaplastic large cell lymphoma *Trastuzumab-DM1,* an immunotoxin directed against the HER2 antigen; for treatment of HER2-positive metastatic breast cancer

**MAb = monoclonal antibody.*

Some monoclonal antibodies are directed against antigens found on the surface of the tumor cells. These antibodies are believed to destroy the tumor through the same mechanisms that are used to attack infectious organisms, namely opsonization, complement-mediated cytotoxicity, and ADCC.[80,81] A second group of immunotherapeutic monoclonal antibodies target surface receptors involved in intracellular pathways that lead to the growth and immortality of cancer cells. These receptors are expressed at higher-than-normal levels on epithelial cancers of the colon, breast, lung, head, and neck. Therapeutic antibodies bind to these receptors and block cell signals that are necessary for activation of molecular pathways involved in cell growth and survival.[80,81]

A third group of monoclonal antibodies target antigens involved in angiogenesis or the formation of blood vessels that are necessary to provide the oxygen and nutrients needed for tumor growth.[80,81] Many of the antibodies in this category are directed against the vascular endothelial growth factor (VEGF) family of proteins or their receptors. A fourth group of monoclonal antibodies boost the immune response to the tumor by blocking inhibitory pathways that inactivate T cells.[80] This approach uses monoclonal antibodies to inhibitory receptors such as cytotoxic T-lymphocyte antigen 4 (CTLA-4), which prevents T-cell activation when bound to the CD80 (B7-1) or CD86 (B7-2) proteins on APCs, and PD-1, a receptor on T cells that inhibits T-cell proliferation when it is bound to programmed death ligand 1 (PD-L1).[81,82]

One strategy to increase the effectiveness of monoclonal antibodies involves linking them to potent cytotoxic drugs that can be taken up by the tumor cells. These products are known as **antibody–drug conjugates** or **immunotoxins.** They reduce the systemic side effects of the toxins by localizing a small number of toxic molecules directly to the tumor cells using a tumor-specific antibody.[81] After binding to an antigen on the tumor surface, the conjugate is quickly internalized by the cancer cell through receptor-mediated endocytosis and transported to the lysosomes. The cytotoxic drug is released from its antibody into the cytoplasm of the tumor cell, where it exerts potent toxic effects.[83,84]

The first immunotoxins, derived from the plant toxin ricin, or toxins from diphtheria-causing or *Pseudomonas* bacteria, had some effectiveness against tumors, but produced toxic side effects.[85] Improvements in linker technology and conjugate design have led to the development of products

that are more effective and have fewer side effects.[84,85] Newer generation antibody–drug conjugates are made from modified toxins that can be genetically engineered by cloning genes for antibody fragments with genes for the adapted toxin.[83] Two FDA-approved preparations that have shown promise are brentuximab vedotin, an immunotoxin directed against the CD30 antigen that is used to treat Hodgkin lymphoma (HL) and systemic anaplastic large cell lymphoma (sALCL); and trastuzumab-DM1, which has specificity for the HER2 antigen and is used to treat patients with HER2-positive metastatic breast cancer.[81,83,84]

Monoclonal antibodies have been used to treat almost all major subtypes of cancer[81]; as a result, this treatment modality has been established as one of the most successful therapeutic strategies for cancer in the last 20 years.[80] A listing of FDA-approved monoclonal antibodies and other drugs in oncology can be found at http://www.centerwatch.com/drug-information by searching in the FDA-approved-drugs category for the term *oncology*.

Although monoclonal antibodies are making their way into the clinic, they have limitations.[81] Some patients develop hypersensitivity reactions to the antibody proteins. This problem has been substantially reduced as monoclonal antibody technology has evolved from making purely mouse antibody products to manufacturing fully human products (see Chapters 5 and 25).[82] Monoclonal antibody treatment can also cause toxicity if the target antigen is expressed on normal cells. Therapy with monoclonal antibodies may be ineffective if the antibodies are unable to permeate through tumor tissues or bind their target antigen molecules with high affinity. Finally, cancer cells can develop resistance to monoclonal antibodies, analogous to the way that bacteria can develop resistance to antibiotics. Cancer scientists are researching several new approaches to monoclonal antibody therapy that may overcome these limitations in the future.[86]

Adoptive Immunotherapy

Scientists have reasoned that because cell-mediated immunity is so important in defense against tumors, transfer of cells of the immune system to cancer patients may effectively assist them in eliminating tumor cells. This approach, known as **adoptive immunotherapy,** is discussed in detail in Chapter 25.

Early experiments conducted in mice in the 1960s showed that lymphoid cells from mice immunized with certain tumors were able to protect against tumor growth when they were transplanted into genetically identical mice; this response was enhanced in the presence of IL-2.[58,59,87] In pioneering studies conducted in the late 1980s by Dr. Steven Rosenberg and his colleagues, it was discovered that adoptive immunotherapy could be applied to the treatment of human cancer.[88-90] These scientists isolated lymphocytes from surgically removed tumors of patients with metastatic melanoma and grew them in the laboratory in the presence of IL-2. They found that these cells, referred to as **tumor-infiltrating lymphocytes (TILs),** demonstrated potent cytolytic activity against autologous melanoma cells. Taking these findings a step further, they prepared expanded populations of TILs from melanoma patients in the laboratory and infused them back into the same patients in the presence or absence of IL-2. These early studies found only a slight improvement in patient response, but demonstrated the potential value of adoptive immunotherapy for human cancer.

Subsequent modifications of technique resulted in significantly improved patient outcomes. Instead of administering the entire population of TILs, cells are subcultured and individually tested for their reactivity to the tumor **(Fig. 17–8).**

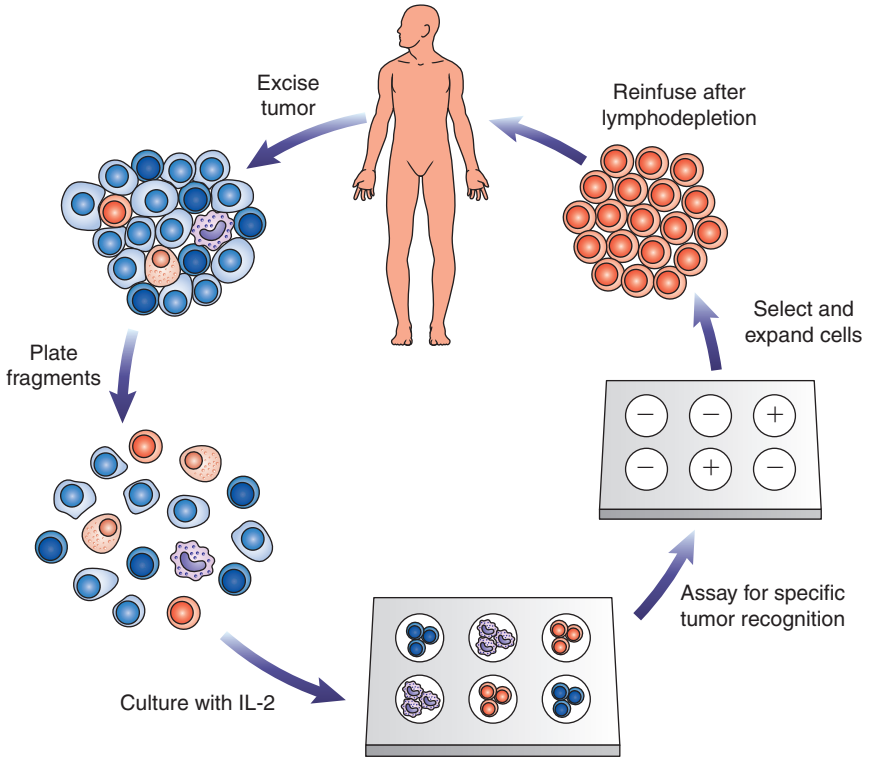

FIGURE 17–8 Adoptive immunotherapy with tumor-infiltrating lymphocytes (TILs). The patient's tumor is surgically removed and cut into fragments, which are cultured in vitro with IL-2. The cultures are screened for lymphocytes with potent anti-tumor activity. Positive cultures are expanded further in the presence of IL-2 and are infused into the cancer patient. Before infusion, the patient has been treated with high-dose chemotherapy or radiation to deplete immunosuppressive cells.

Only the cultures that show potent anti-tumor activity are selected for further expansion and infusion into patients. In addition, the effectiveness of the therapy has been improved by pretreating patients with total body irradiation or high-dose chemotherapy before conducting the adoptive cell transfer. This pretreatment is thought to eliminate cells that could exert immunosuppressive effects before the treatment begins.[91,92] Adoptive therapy with autologous TILs has shown good clinical response rates in patients with malignant melanoma but has not yet been successful with other types of cancer, which are probably less immunogenic.[93]

Alternative treatments being investigated involve the use of genetically engineered T cells. These approaches may be advantageous because they allow for the design of T cells that are targeted toward specific tumor antigens. One method to construct these genetically engineered cells involves isolating T cells from patients with good anti-tumor responses and cloning the genes for their TCRs into viral vectors that can be used to infect T cells from the patient to be treated.[94]

A second approach involves isolating TCR genes from humanized mice that have been immunized with the tumor antigen of interest and cloning these into recombinant vectors to deliver the sequences to T cells from the cancer patient. Humanized mice are mice into which human cells or tissues have been transplanted. Immunodeficient strains of mice are used so that they do not reject the human transplant. These mice are widely used models for studying the effects of therapeutic agents on human cells before they are allowed to be administered to humans.

A third method is to generate *chimeric antigen receptors (CAR)*. CARs are most often constructed by combining the antigen-binding variable fragment of a monoclonal antibody to a tumor antigen with intracellular domains of the TCR that provide activating signals to the T cells. The CAR approach has become very popular because CARs can target tumor antigens in an MHC-independent manner, so that the product can be universally used instead of being restricted for patients with a particular HLA type.[67,91,94]

Although adoptive immunotherapy looks promising, some toxicities have been reported.[95] In addition, adoptive-based therapies are expensive, personalized treatments that are available in only a limited number of sites worldwide. It is hoped that advances in technology will make it possible to mass-produce tumor-specific T cells for use in adoptive immunotherapy in the future.[91]

SUMMARY

- Cancer arises from exposure of the host to environmental factors that induce mutations in proto-oncogenes and tumor suppressor genes. The former influence cell proliferation and the latter regulate entry of cells into the cell cycle, maintain genetic stability, and repair damaged DNA.
- Malignant tumors consist of immortal cells that resist apoptosis and proliferate in an unregulated manner. They can also induce angiogenesis, invade nearby tissues, and spread to distant sites in the body. These characteristics are influenced by mutations, genomic instability, and inflammatory responses of the immune system.
- The concept of tumor immunology is based on the premise that tumor cells possess antigens that can be recognized as foreign by the immune system. Some antigens are *tumor specific,* or unique to a particular type of tumor cell. However, most are *TAAs* that are expressed by normal cells as well as tumor cells. The latter can be classified as shared TSAs, which are expressed in many tumors, but not in most normal tissues; differentiation antigens, which are expressed on immature cells of a particular lineage; or overexpressed antigens, which are found in higher levels on malignant cells than on normal cells.
- Tumor markers are biological substances that are increased in the blood, body fluids, or tissues of patients with a particular type of cancer. They can be detected by immunohistochemistry, automated immunoassays, or molecular methods, and are used in cancer screening and diagnosis, determining patient prognosis, and monitoring patient response to therapy.
- The ideal tumor marker should be produced by the tumor or by the patient's body and be secreted into a biological fluid, where it can be easily and inexpensively quantified. It should rise with increasing tumor load and have a high sensitivity and specificity. Most tumor markers do not possess these characteristics and are therefore not suitable for screening the general population.
- Tumor markers are best used to monitor patient response to therapy by performing serial measurements over time. If therapy is effective, the amount of tumor marker will decrease. Ineffective therapy and recurrence of cancer is indicated by an increase in the tumor marker level. Ideally, these increases would precede other signs of disease recurrence by several months.
- Some commonly used serum tumor markers and their primary cancer associations are presented in Table 17–4.
- Tests for circulating tumor markers are most commonly performed by highly sensitive, automated immunoassays. Their results can be affected by reagent variability among manufacturers, cross-reactivity with similar antigens, tumor antigen excess (producing a postzone, or high-dose hook effect), or presence of heterophile, anti-animal, or autoantibodies in the patient sample. Therefore, results should always be considered in conjunction with clinical factors and other laboratory tests.
- Molecular techniques such as PCR, FISH, and microarray are commonly used in clinical testing for tumor markers.

DNA sequencing is being used increasingly to identify mutations associated with cancer.

- The hypothesis of immunosurveillance states that the immune system continually patrols the body for cancer cells and eliminates them before they become clinically evident.
- There are innate and adaptive immune responses against tumor cells. Innate responses are mediated by NK cells and macrophages. Adaptive immune responses are mediated by CTLs, antibodies, activated Th cells and cytokines.
- Tumor cells are thought to escape these responses through the process of immunoediting, which allows some of the tumor cells to develop into genetic variants that are resistant to immune defenses. This process can involve avoiding immune recognition by downregulation of surface class I MHC molecules, resistance to apoptosis, suppression of anti-tumor immune responses, and chronic inflammation.
- An understanding of the interactions between the immune system and the tumor can help in the development of rational immunotherapies, whose purpose is to harness the ability of the immune system to destroy tumor cells. Immunotherapeutic approaches include stimulation of the immune system with bacterial products or cancer vaccines, passive therapy with cytokines, monoclonal antibodies, or antibody–drug conjugates, and adoptive immunotherapy with tumor-infiltrating lymphocytes or genetically engineered T cells.

Study Guide: Clinical Applications of Tumor Markers

CLINICAL APPLICATION	PURPOSE AND COMMENTS	BENEFITS	LIMITATIONS	EXAMPLE
Screening	To identify cancer in asymptomatic individuals in a population.	Detection of cancer at an early stage.	False-positive results can lead to patient anxiety, unnecessary testing, and overtreatment. False-negative results can give misleading reassurance.	PSA
Diagnosis	To identify cancer in a particular patient. Presence of the marker or an elevation of the marker above normal levels suggests the presence of the cancer.	Helps distinguish between diseases with similar clinical manifestations.	Tests by themselves are not diagnostic and should be used in conjunction with other tests and clinical findings.	PSA
Prognosis	To predict the clinical outcome of a cancer patient and aid in therapeutic decision making. An initial high concentration or an increasing level of the tumor marker over time indicates a worse prognosis.	Used to identify the level (mild to aggressive) and type of therapy that is best for a particular patient.		HER2
Monitoring	To observe the response of a cancer patient to treatment. Effective treatment is indicated by decreasing levels of the marker over time. Increasing levels of the tumor marker indicate that the therapy is not effective.	Elevations can indicate tumor recurrence before other signs become evident.	The cancer must be positive for the marker before treatment begins.	CA 125

CASE STUDIES

1. A 45-year-old woman went to her physician's office after noticing a lump during her breast self-examination. She had a strong family history of breast cancer. The lump was detected on mammography and was found to be a 0.5-cm mass that was adherent to her skin. Analysis found her CA 15-3 levels to be 60 IU/mL, which is double the upper limit of the reference interval. After surgery, the levels of CA 15-3 dropped, but at a rate that was slower than the biological half-life. They remained above 30 IU/mL. The tumor morphology indicated malignancy, so it was tested for HER2 expression, which was elevated, and estrogen and progesterone receptors, which were negative.

Questions

a. Do you think that there is a residual tumor? If so, why?
b. In addition to chemotherapy, what other therapy would you recommend? Why?
c. What type of therapy is unlikely to be successful? Why?

2. A 66-year-old male went to his urologist complaining of frequent urination with only small volumes of urine, creating great urgency. During the DRE, the urologist felt an enlarged prostate with no distinct nodules or abnormal areas. The patient's serum level of prostate-specific antigen (PSA) was determined and compared with the level from the previous year. The physician also asked that the bound-to-total PSA ratio be determined. The test results are shown in the data that follows.

Laboratory Results

TEST	PATIENT RESULTS	REFERENCE INTERVAL
PSA October 2015	3.8 ng/mL	0–3.5 ng/mL
PSA October 2014	3.5 ng/mL	0–3.5 ng/mL
Bound/free PSA	25.8%	≥23.4%

Questions

a. Do any tissues other than the prostate produce PSA? Could there be another source of the PSA in this case?
b. What is PSA velocity?
c. Should this man have a biopsy? Do you think this man has cancer? Why?
d. At what point would PSA testing no longer be recommended for this patient?

REVIEW QUESTIONS

1. How can normal cells become malignant?
   a. Overexpression of oncogenes
   b. Underexpression of tumor suppressor genes
   c. Viral infection
   d. All of the above

2. Which of the following best summarizes the concept of tumor development via immunoediting?
   a. Tumor cells produce cytokines that are toxic to T cells.
   b. Tumor cells that can escape the immune system have a growth advantage over tumor cells that are destroyed during immunosurveillance.
   c. T-cell activity causes an increase in MHC expression on tumor cells that allows them to escape the immune system.
   d. Secreted tumor-associated antigen saturates T-cell receptors and makes T cells incapable of binding to tumor cells.

3. Which of the following is an example of a tumor-specific antigen?
   a. *BCR/ABL* fusion protein
   b. CEA
   c. CA 125
   d. PSA

4. Most tumor markers are not used to screen the general population because they
   a. cannot be inexpensively quantified.
   b. do not rise to high enough levels in the presence of cancer.
   c. can also be elevated in conditions other than the cancer.
   d. vary too much between patients belonging to different ethnic populations.

5. Both AFP and hCG exhibit serum elevations in
   a. pregnancy.
   b. ovarian germ cell carcinoma.
   c. nonseminomatous testicular cancer.
   d. all of the above.

6. Suppose a patient with ovarian cancer had a serum CA 125 level of 50 kU/L at initial diagnosis. After her tumor was surgically removed, her CA 125 level declined to 25 kU/L. She received chemotherapy drug #1; after 1 year, her CA 125 level was 40 kU/L. She was then given chemotherapy drug #2 and her CA 125 level rose to 60 kU/L. These results indicate that
   a. surgery was effective in removing the patient's tumor.
   b. chemotherapy drug #1 was more effective than chemotherapy drug #2.
   c. both chemotherapy drug #1 and chemotherapy drug #2 were effective.
   d. neither chemotherapy drug #1 nor chemotherapy drug #2 were effective.

7. All of the following are recommended for cancer screening in the groups indicated *except*
   a. CA 125/women of reproductive age.
   b. AFP/subjects at high risk for liver cancer.
   c. PSA/men over 50 with at least 10 years of life expectancy.
   d. none of the above.

8. The best use of serum tumor markers is considered to be in
   a. screening for cancer.
   b. initial diagnosis of cancer.
   c. monitoring patients undergoing cancer treatment.
   d. determining patient prognosis.

9. In order to use a tumor marker to monitor the course of the disease, which of the following must be true?
   a. The laboratory measures the marker with the same method over the entire course of the patient's treatment.
   b. The marker must be released from the tumor or, because of the tumor, into a body fluid that can be obtained and tested.
   c. The marker's half-life is such that the marker persists long enough to reflect tumor burden but clears fast enough to identify successful therapy.
   d. All of the above.

10. Which of the following markers could be elevated in nonmalignant liver disease?
    a. AFP
    b. CEA
    c. CA 15-3
    d. All of the above

11. Each of the following markers is correctly paired with a disease in which it can be used for patient monitoring *except*
    a. CEA/choriocarcinoma.
    b. CA 15-3/breast adenocarcinoma.
    c. CA 125/ovarian adenocarcinoma.
    d. CA 19-9/pancreatic adenocarcinoma.

12. Which of the following is a marker used in immunohistochemical staining to identify tumors of epithelial origin?
    a. Cytokeratins
    b. Vimentin
    c. CD45
    d. CD10

13. Which of the following assays would you recommend to test for chromosomal rearrangements such as the *BCR/ABL* translocation seen in CML?
    a. PCR
    b. FISH
    c. Microarray
    d. Next generation sequencing

14. Innate immune responses thought to be involved in defense against tumors include
    a. NK cell-mediated apoptosis.
    b. MHC I-restricted T-cell–mediated destruction.
    c. ADCC.
    d. all of the above.

15. A woman with breast cancer is treated with a monoclonal antibody to HER2. This is an example of
    a. a cancer vaccine.
    b. an immunotoxin.
    c. passive immunotherapy.
    d. active immunotherapy.

18 Immunoproliferative Diseases

Donald C. Lehman, EdD

LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Compare and contrast leukemias and lymphomas.
2. Describe some of the cellular properties and genetic changes that occur during malignant transformation of hematologic cells.
3. Cite the cellular characteristics used in the classification scheme recommended by the World Health Organization for identification of the hematopoietic neoplasms.
4. Differentiate between Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL).
5. Differentiate between acute leukemias and chronic leukemias and discuss an example of each type.
6. Associate specific CD markers with selected hematologic malignancies.
7. Differentiate between monoclonal gammopathy of undetermined significance (MGUS) and other plasma cell dyscrasias.
8. Correlate specified clinical manifestations and laboratory results with multiple myeloma or Waldenström macroglobulinemia.
9. Indicate the ways in which laboratory tests can be used to diagnose and follow the progression of immunoproliferative disorders.
10. Explain the underlying principles of serum and urine protein electrophoresis (UPE), immunofixation electrophoresis (IFE), immunosubtraction, and serum free light chain (sFLC) analysis.
11. Contrast serum protein electrophoresis (SPE) and IFE results seen in monoclonal gammopathies with those observed in polyclonal increases in immunoglobulins.
12. Discuss the types of genetic abnormalities that are frequently seen in hematologic malignancies and the laboratory methods used to detect them.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Bence Jones proteins
Cryoglobulins
Fluorescence in situ hybridization (FISH)
Hairy cell leukemia
Heavy-chain diseases
Hodgkin lymphoma (HL)
Immunophenotyping
Immunosubtraction (Immunotyping)
Leukemias
Lymphomas
Monoclonal gammopathies
Monoclonal gammopathy of undetermined significance (MGUS)
Multiple myeloma
Non-Hodgkin or lymphocytic lymphomas (NHLs)
Oncogene
Paraprotein (M protein)
Plasma cell dyscrasias
Polyclonal
Proto-oncogene
Waldenström macroglobulinemia

This chapter focuses on malignancies of the immune system that involve cells of lymphoid lineage. The lymphoid malignancies can be broadly classified as leukemias, lymphomas, and plasma cell dyscrasias. In **leukemias,** the malignant cells are primarily present in the bone marrow and peripheral blood. In **lymphomas,** the malignant cells arise in lymphoid tissues, such as the lymph nodes, tonsils, or spleen. There can be an overlap between the sites affected by leukemias and lymphomas, especially when the malignancy is far advanced. However, it is generally most useful to classify the malignancy according to the site where it first arose, rather than the sites it can ultimately involve.

The **plasma cell dyscrasias** (disorders) primarily include multiple myeloma and Waldenström macroglobulinemia. These commonly involve the bone marrow, lymphoid organs, and other nonlymphoid sites. They are considered biologically distinct and are not classified as either leukemias or lymphomas. However, plasma cells may be found in the blood late in the course of myeloma. This phenomenon is sometimes referred to as *plasma cell leukemia.* **Monoclonal gammopathy of undetermined significance (MGUS)** is a premalignant condition that can develop into multiple myeloma, Waldenström macroglobulinemia, or other lymphoproliferative disorders over time.

This chapter presents an introduction to some of the lymphoid malignancies that are commonly evaluated by the clinical laboratory. It is not intended to provide a comprehensive discussion of hematopoietic malignancies. The chapter will also cover key principles and applications of some of the laboratory tests that are essential to the diagnosis and monitoring of lymphoid malignancies.

Malignant Transformation of Hematologic Cells

Cell Properties

Hematologic malignancies are characterized by excessive accumulation of cells in the blood, bone marrow, or other lymphoid organs. This accumulation may occur because of rapid proliferation of the cells (i.e., excess production) or failure of the cells to undergo *apoptosis* (a normal physiological process of cell death). Malignancy may reflect the result of an initially normal process in which regulatory steps to control the level of cell proliferation have failed. In addition to a failure of growth regulation, mutations can result in arrested maturation of a cell. Thus, some malignant hematopoietic cells may not develop into properly functioning mature cells but may remain at an earlier stage of differentiation and continue to replicate. Malignant and premalignant proliferation of cells can occur at any stage in the differentiation of the lymphoid lineages.

Cells of the immune system are at great risk for malignant transformation because the features that characterize the development of malignancy are also a normal part of the immune response. For example, as we discussed in Chapter 4, proliferation of T and B lymphocytes is an integral part of the immune response to an antigenic stimulus. In addition, gene rearrangements are a normal part of lymphocyte maturation and somatic hypermutations occur in the immunoglobulin genes of the B cells during immunoglobulin class-switching and the generation of high-affinity antigen receptors. Despite being affected by abnormal regulation, malignant lymphoid cells generally retain some or all of the morphological and functional characteristics of their normal counterpart—for example, their characteristic cell surface antigens or secretion of immunoglobulin. These characteristics are often used to classify lymphoid malignancies.

The immune system is naturally diverse and heterogeneous in its response against a wide range of potential pathogens. Normal immune responses are **polyclonal** (i.e., cells with different features such as antigen specificity all proliferate in response to an immune stimulus). In contrast, malignancies are thought to arise from excessive proliferation of a single mutant parent cell to form a clone of genetically identical cells that are similar in their appearance and surface markers. Suspicion of a hematologic malignancy is raised when there are elevated numbers of a specific population of lymphocytic cells in the bloodstream, bone marrow, or lymphoid tissues.

Genetic Changes

Malignancies are generally multifactorial in origin. As we discussed in Chapter 17, malignant transformation is thought to be a multistep process involving exposures to environmental agents such as chemical carcinogens and radiation, which induce a series of genetic mutations. The key genes involved are **proto-oncogenes,** which are involved in normal cell growth and division, and *tumor suppressor genes,* which control cell division by regulating the progression of cells through the cell cycle and maintaining genetic stability of the cells by repairing damaged DNA. Changes in these genes can result

in uncontrolled cell proliferation. Alterations in proto-oncogenes can convert them into **oncogenes,** which are involved in malignant transformation. *Aneuploidy* (an abnormal number of chromosomes) and deletions of specific chromosome regions are common secondary events that are rarely specific to a particular type of lymphoma but provide valuable prognostic information.

The genetic alterations in malignant cells of hematopoietic origin include point mutations involving a change in a single nucleotide base, duplications or deletions of specific genes, and chromosome translocations in which two different chromosomes break apart and exchange genetic material. The detection of translocations is of particular value in the diagnosis of disease.

Many of the translocations involve an exchange between the immunoglobulin or T-cell receptor (TCR) loci with various partner chromosomes, leading to abnormal proto-oncogene expression. For example, some of the hematologic malignancies are characterized by translocations involving the proto-oncogene *c-MYC,* which stimulates the transcription of several other genes involved in cell proliferation.[1] Overexpression of the *c-MYC* gene can occur as a result of a rearrangement in which c-*MYC* is placed under the control of a different gene promoter sequence. For example, a translocation involving the *c-MYC* gene on chromosome 8 and the immunoglobulin μ gene on chromosome 14 [t(8;14)] is believed to be involved in the pathogenesis of some cases of Burkitt lymphoma, a B-cell malignancy. As a result of persistent *c-MYC* expression, several genes that are involved in cell proliferation are activated beyond normal levels. The resulting high levels of c-MYC protein drive the affected cells to continually proliferate. Many small noncleaved cell lymphomas are also associated with the t(8;14) translocation.

Other hematologic malignancies are associated with genes that affect apoptosis. For example, most cases of follicular lymphoma have a t(14;18) gene translocation, in which portions of chromosome 14 (which contains the Ig heavy-chain genes) and chromosome 18 (which contains an anti-apoptotic gene called *BCL-2*) are exchanged.[2] This exchange results in the rearrangement and constitutive overexpression of *BCL-2.* The *BCL-2* gene induces production of an inner mitochondrial membrane protein that blocks apoptosis. Therefore, the cells affected by this translocation do not die normally. Even though the altered cells do not proliferate at an increased rate, an excessive number of cells accumulate because their survival is enhanced compared with normal cells.

Other characteristic translocations result in the production of a novel fusion protein. For example, chronic myelogenous leukemia is characterized by a translocation between the *BCR* (breakage cluster region) on chromosome 9 and the c-*ABL* proto-oncogene on chromosome 22 (see Fig. 17–2). This results in a BCR/ABL fusion protein, which codes for a continuously activated tyrosine kinase enzyme, causing unregulated cell division. Scientists have developed the anticancer drug Gleevec to specifically target the abnormal protein produced by this gene translocation.[3,4] Gleevec slows cell growth by inhibiting the activity of the altered kinase.

Classification of Hematologic Malignancies

The classification of hematologic malignancies has undergone many changes over the years, as new laboratory techniques have been developed and more knowledge has been gained about these diseases. In the 1950s and 1960s, classification and diagnosis of hematologic malignancies was based primarily on abnormalities in the morphological features of the malignant cells, which were viewed on peripheral blood or bone marrow samples fixed onto microscope slides and treated with stains such as Wright-Giemsa. Advances in understanding basic lymphocyte biology led to major rethinking of the use of classification schemes based solely on cell morphology. Discovery of surface markers on T and B lymphocytes in the 1970s and 1980s allowed this information to be incorporated into the diagnosis of hematologic malignancies. The 1990s and 2000s witnessed a tremendous expansion of knowledge about the molecular changes of the tumors. Thus, today, investigators and clinicians are using a combination of morphological, immunologic, cytogenetic, and molecular techniques to assist in the classification of hematologic malignancies.

A number of schemes have been used over the years to classify the hematologic malignancies, including the French-American-British (FAB) Cooperative Group consensus criteria for leukemias and myelodysplastic syndromes[5,6] and the Revised European American Lymphoma (REAL) classification for leukemias and lymphomas.[7]

The REAL scheme became the basis for the classification scheme for all types of hematologic malignancies which was adopted in 2001 and updated in 2008 by the World Health Organization (WHO)[8,9] This widely accepted system is considered the "gold standard" in the classification of tumors for diagnosis and determination of appropriate therapy. The 2008 WHO update classifies hematologic malignancies into 12 major groups and numerous subgroups. These groupings are based on cell lineage; specific cancers are further defined by their immunologic markers and genetic features, as well as their morphological and cytochemical staining properties. Some of the recognized types of lymphomas, leukemias, and plasma cell dyscrasias are discussed in more detail in this chapter. The 12-group classification scheme will continue to evolve as new knowledge is gained about the characteristics that typify the various disease entities.

Leukemias

Leukemias can be broadly divided into two groups based on the cell type from which they originated: myelogenous and lymphocytic. The myelogenous leukemias are derived from the common myeloid precursor and encompass the granulocytic, monocytic, megakaryocytic, and erythrocytic leukemias. This section will not cover this group in detail, but will briefly present a classic genetic change associated with chronic myelogenous leukemia and how it is identified in the clinical laboratory. The focus of this section will be the lymphocytic leukemias, which originate from mature lymphocytes or their precursors.

Each of the two groups of leukemias can be further divided into acute or chronic types. Chronic leukemias are usually slowly progressive and compatible with extended survival. However, they are generally not curable with chemotherapy. By contrast, acute leukemias are generally much more rapidly progressive but have a higher response rate to therapy. Acute lymphoblastic leukemias are characterized by the presence of lymphoblasts in the peripheral blood. Lymphoblasts are immature lymphocyte precursors. They are small to medium-sized cells that contain little cytoplasm, dense nuclear chromatin, and indistinct nucleoli **(Fig. 18–1).** This section will discuss two major types of lymphocytic leukemias: acute lymphocytic leukemia and chronic lymphocytic leukemia.

Acute Lymphocytic Leukemia (ALL)

Acute lymphocytic leukemia (ALL) (also known as acute lymphoblastic leukemia) is characterized by the presence of very poorly differentiated precursor cells (blast cells) in the bone marrow and peripheral blood. These cells can also infiltrate soft tissues, leading to organ dysfunction. ALL is usually seen in children between 2 and 5 years of age and is the most common form of leukemia in this age group. ALL is a treatable disease with a remission rate of 90% and a cure rate of 80% in children.[10] Remission and cure rates are lower in adults with ALL.

Immunologically, there are four types of ALL: CALLA (CD10)-expressing precursor B-cell ALL, pre–B-cell ALL without CALLA (CD10), T-cell ALL, and mature B-cell ALL. CALLA (CD10)-expressing precursor B-cell ALL is the most common ALL, whereas pre-B cell ALL is the second most common. Mature B-cell ALL is rare.

Cytogenetics studies provide information that aids in the diagnosis and prognosis of patients with ALL. In patients with precursor B-cell ALL, *hyperdiploidy,* in which the malignant cells contain more than 46 chromosomes, is associated with a good prognosis, whereas *hypodiploidy* is associated with a poorer prognosis. The most common translocation in ALL of B-cell origin, t(12:21)(p13;q22), also known as *TEL-AML-1,* is associated with an excellent prognosis in children.[11,12]

Connections

Molecular and Cytogenetic Analysis

Compare the lymphoblasts in Figure 18–1 to the normal lymphocyte shown in Figure 1–8. Although distinct differences can be seen in the morphology of the two cell types, not much else can be determined from simply observing the cells. Flow cytometry studies have added much detail to the traditional microscope-based laboratory evaluation of hematologic malignancies. Detection of cell surface markers provides insight into the lineage and maturation stage of the malignant cell type, which can be used to make a more accurate diagnosis. Molecular and cytogenetic analysis detect genetic mutations and chromosomal abnormalities in the cells, providing doctors with a more precise diagnosis and information about the patient's prognosis, which they can use to select the most effective therapy for the patient.

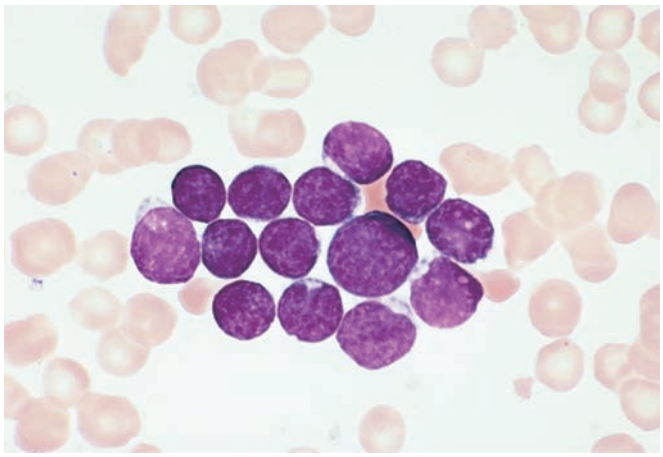

FIGURE 18–1 Lymphoblasts in the peripheral blood. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009.)*

Chronic Lymphocytic Leukemia or Lymphoma

The chronic lymphocytic leukemias or lymphomas are a group of diseases almost exclusively of B-cell origin.[13] They include chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL). The WHO considers CLL and SLL a single disease with different clinical presentations. Both reveal the B-cell marker CD19 but weakly express CD20.

CLL is a common hematopoietic malignancy that involves the expansion of a clone of B cells that have the appearance of small mature lymphocytes. In about 5% of cases, the malignant clone is T-cell derived. The cytologically normal, but dysfunctional, lymphocytes accumulate in the bone marrow and blood as well as in the spleen, lymph nodes, and other organs. CLL primarily occurs in patients over 50 years of age with a two-to-one male-to-female predominance. It is the most common leukemia in adults. Patients usually present with an increase in the peripheral blood lymphocyte count, which may be an incidental finding on a routine physical examination. Anemia and thrombocytopenia are usually absent at the time of diagnosis. However, as the malignant lymphocytes continue to increase in number, replacement of normal elements in the bone marrow leads to anemia and thrombocytopenia. Lymph node enlargement is prominent early in the disease. CLL is compatible with a long survival. Various treatments can help control or reduce symptoms but are not curative.

Hairy Cell Leukemia

Hairy cell leukemia is a rare, slowly progressive disease characterized by infiltration of the bone marrow and spleen by leukemic cells without the involvement of lymph nodes. It has a four-to-one male predominance and is seen in individuals over 20 years of age. Patients usually present with bone marrow disease and pancytopenia because of bone

marrow infiltration; however, the blood lymphocyte count is usually not very high. Splenomegaly is striking, whereas lymphadenopathy is generally absent. The clinical presentation can resemble several other B-cell neoplasms including CLL, SLL, and splenic marginal zone lymphoma.

The malignant lymphocytes are round and often have irregular "hairy" cytoplasmic projections from their surfaces **(Fig. 18–2).** They typically are not evident on bone marrow or spleen preparations. The nuclei are often oval and occupy a large percentage of the cell volume. The malignant cells express mature B-cell markers—CD19, CD20, and CD22—and the IL-2 receptor, CD25, which are not specific for hairy cell leukemia. In contrast, CD103 is highly specific and sensitive for hairy cell leukemia.[14] Although dim staining of CD123 is found in other disorders, bright staining is seen in hairy cell leukemia. These cells characteristically contain acid phosphatase isoenzyme 5, which can be detected histochemically and provides resistance to tartrate treatment. An accurate diagnosis is made in most cases because of the cytomorphological and immunophenotypic characteristics. Polymerase chain reaction (PCR) for the detection of mutated gene *BRAF-V600E* has been shown to be a sensitive and specific test for the diagnosis of hairy cell leukemia.[15]

Lymphomas

The lymphomas have commonly been referred to as **Hodgkin lymphoma (HL)** and the **non-Hodgkin, or lymphocytic lymphomas (NHLs).** Each of these entities will be discussed in more detail in the text that follows.

Hodgkin Lymphoma (HL)

HL is one of the most common lymphomas, with a reported incidence of about 3 cases per 100,000 people in the Western world.[16] This often-curable disease occurs both in young adults and the elderly. Peripheral lymph nodes are primarily involved, although numerous organs such as the liver, lung, and bone marrow can be affected. The disease is divided into nodular lymphocytic-predominant HL (NLPHL) and classic HL, which includes nodular sclerosis, mixed cellularity, lymphocyte-rich, and lymphocyte-depleted HL. Classic HL is characterized by the presence of Hodgkin and Reed-Sternberg (RS) cells in affected lymph nodes and lymphoid organs **(Fig. 18–3).** RS cells are typically large with a bilobed nucleus and two prominent nucleoli. This gives the cell an "owl's-eyes" appearance. Hodgkin cells resemble RS cells except that the nuclei are not bilobed and have a single nucleolus. NLPHL is characterized by large lymphocyte-predominant cells. The malignant Hodgkin and RS cells are generally of B-cell lineage.

The REAL/WHO classification recognizes a basic distinction between NLPHL and classic HL, reflecting the differences in clinical presentation, morphology, phenotype, and molecular features. RS cells in all subtypes of classic HL have a similar antigenic profile. They are all CD30+ and about 80% of the cases are CD15+. Expression of the B-cell marker CD20 is weak or absent. Some cells also express T-cell markers (e.g., CD3). Hodgkin RS cells were confirmed as originating from B cells with the discovery of the presence of clonal and somatically mutated immunoglobulin heavy and light chain gene rearrangements. Despite their origin as B cells, Hodgkin RS cells exhibit a decrease in typical B-cell gene products. The extent of this gene downregulation is unique among B-cell lymphomas.[17] RS cells of NLPHL have a different morphology compared with those found in classic HL; they are referred to as *lymphocytic* and *histiocytic cells.* These cells rarely express CD30 or CD15 but instead express the B-cell antigens CD19 and CD20, which are typically absent on RS cells of classic HL.

The lymphocyte-rich form of HL represents about 5% of the cases of HL and tends to occur in slightly older individuals. Nodular sclerosis HL is the most common subtype, representing about 70% of cases and having the best prognosis. It is characterized by infiltration of a mixture of normal macrophages, lymphocytes, and granulocytes in affected tissues along with small numbers of RS cells. There is also marked fibrosis, dividing affected lymph nodes into nodules. Mixed cellularity HL also has

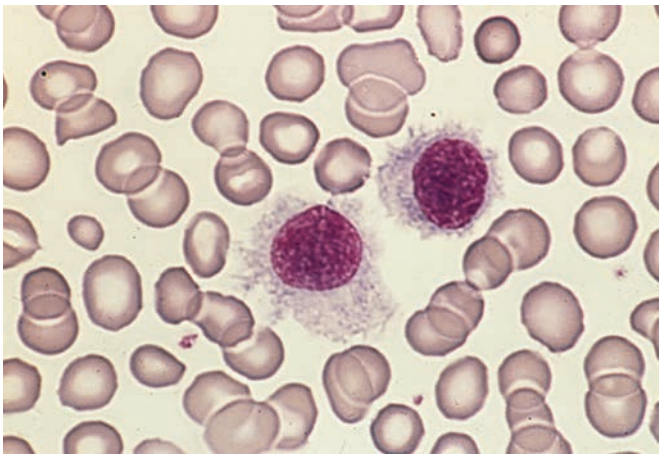

FIGURE 18–2 Hairy cell leukemia. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009.)*

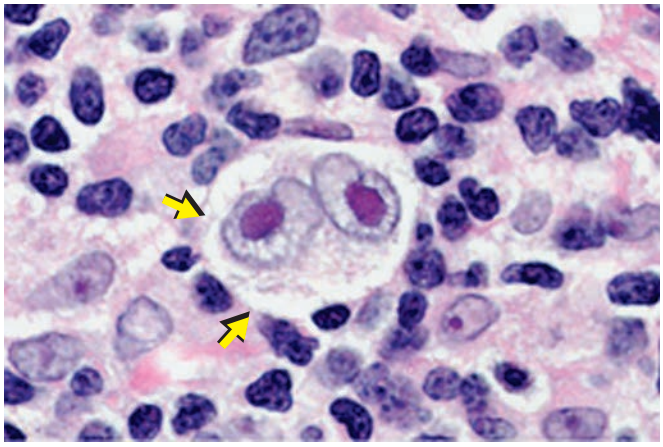

FIGURE 18–3 Reed-Sternberg cell (arrows). *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009.)*

a mixed infiltrate of normal cells but with less fibrosis and greater numbers of RS cells. It accounts for about 25% of cases. Lymphocyte-depleted HL has diffuse fibrosis, few infiltrating normal cells, the greatest number of RS cells, and the worst prognosis compared with other HL subtypes.[18]

Hodgkin RS cells interact with numerous cells including CD4+ and CD8+ T cells, B cells, plasma cells, macrophages, and others. RS cells secrete cytokines and chemokines, some of which attract these cells to the tumor. The presence of this mixed population of cells is unique among lymphomas. The nontumor cells often account for 99% of the cells in the tumor. Hodgkin RS cells are not usually found in the peripheral bloodstream.

Epidemiological studies suggest that HL has an infectious etiology. Patients with HL often have elevated levels of antibody to Epstein-Barr virus (EBV), the causative agent of infectious mononucleosis; EBV nucleic acid has been demonstrated in Hodgkin RS cells.[19,20] A history of infectious mononucleosis has been associated with an increased risk of HL, particularly in EBV-positive HL in younger adults, whereas no increase of risk between infectious mononucleosis and EBV-negative HL has been found.[21] Although the specific mechanism of tumorigenesis is unknown, EBV is known to preferentially infect B cells and immortalize them in vitro. In addition, viral proteins can induce activation of key signaling pathways, producing phenotypic changes seen in EBV-infected B cells.

Non-Hodgkin Lymphoma (NHL)

NHL includes a wide range of neoplasms. Over two-thirds of the patients are greater than 60 years of age and the incidence is greater in men than women.[22,23] Immunosuppression seems to be the greatest risk factor for NHL; in fact, an increase in cases corresponded to the emergence of human immunodeficiency virus (HIV). Other conditions associated with increased risk for NHL include certain autoimmune diseases, congenital immunodeficiency disorders, organ transplantation, and exposure to certain infectious agents.

Overall, B-cell lymphomas represent about 85% to 90% of all NHL cases; the remainder involves T cells and NK cells. B-cell lymphomas generally begin in the germinal centers of lymph nodes. Although the lymphoma can begin in any tissue, the gastrointestinal tract is the most common extranodal site for NHL. The most common NHL is diffuse large B-cell lymphoma (DLBCL), which accounts for 30% to 40% of NHL. DLBCL is a heterogeneous group of diseases characterized by diffuse growth of large atypical cells without a hallmark pattern of surface markers.

The next most common type of B-cell lymphoma, accounting for about 20%, is follicular lymphoma, which originates in the follicles of the lymphoid organs and is characterized by a much more aggressive course than DLBCL. Follicular lymphoma is often disseminated at the time of diagnosis; the spleen, liver, and bone marrow are frequently involved. Marginal-zone B cell, peripheral T cell, small B lymphocytic, and mantle cell lymphoma each constitute between 5% and 10% of all lymphoma cases. Some of these lymphomas tend to be slowly progressive and compatible with long-term survival, whereas others are typically highly aggressive and rapidly fatal if not treated. The various B-cell lymphoma types can be divided into three broad groups for prognostic purposes. The low-risk first group includes CLL, follicular lymphomas, and mucosa-associated lymphoid tissue (MALT) lymphomas. The intermediate-risk second group includes DLBCL and Burkitt lymphoma. The high-risk third group includes mantle cell lymphoma and lymphoblastic lymphoma. MALT lymphoma is often associated with autoimmune conditions such as Sjögren's syndrome and Hashimoto's thyroiditis.

Some lymphomas, such as SLL, originate from small lymphocytes that are awaiting their first encounter with an immunogen. Small B-cell lymphoma is primarily a disease of the elderly; the median age is 72 years.[24] These lymphomas are indolent but inexorable diseases that are compatible with survival for up to a decade. They progress to prolymphocytic leukemia in 10% to 30% of cases and to large-cell lymphoma or other aggressive lymphoid malignancies in 10% to 15% of cases.

Other B-cell lymphomas, such as diffuse large-cell lymphoma or lymphoblastic lymphoma, derive from rapidly dividing cells. Lymphoid cells undergo proliferation at two stages in their development: an early cycle as they first emerge from the bone marrow and a later cycle in response to immunogen exposure. Thus, rapidly proliferative lymphomas can correspond to either early or late stages of normal development. These lymphomas behave aggressively; if untreated, they can cause death in less than a year.

Three characteristics usually identify lymphomas as having a B-cell origin: (1) surface immunoglobulin, which is found on no other cell type; (2) other cell surface proteins such as CD19 and CD20 that are both sensitive and specific for B cells; and (3) rearranged immunoglobulin genes. In almost all cases, both the surface immunoglobulin and the rearranged immunoglobulin genes have features of clonality.

The T-cell and NK-cell lymphomas are more difficult to characterize than B-cell lymphomas because in cases that are morphologically not clearly malignant, no easy way exists to assay their clonality. Also, a number of T-cell syndromes progress stealthily from atypical but nonclonal proliferations into clonal malignancies. In cases that are not clearly malignant based on their morphology, two ancillary methods of establishing clonality are available. The first is to use molecular techniques to detect a clonal rearrangement of the TCR gene. In benign populations, each cell exhibits a slightly different rearrangement, but in malignant proliferations, the population of cells uniformly expresses the same rearrangement. A second method is to demonstrate by flow cytometry that the suspicious population of T cells uniformly fails to express an antigen that is normally expressed on all T cells.

The clinical presentation of NHL varies and depends on the patient's age, lymphoma subtype, and site of involvement. Most individuals present with painless lymphadenopathy. More aggressive forms cause fulminant symptoms such as weight loss, fever, and night chills. NHLs are staged, I through IV, using the Ann Arbor classification system. Staging is based on the number of lymph nodes affected, their location, and if

extranodal organs are involved. Computed tomography (CT) scan, magnetic resonance imaging (MRI), and positron emission tomography (PET) are commonly used for disease assessment. A number of new treatments have improved the survival rate; however, outcomes are variable. Elderly patients in general have a poor outcome response to therapy. In addition, current smokers with NHL have a greater mortality rate compared with those who never smoked.[25]

Plasma Cell Dyscrasias

The plasma cell dyscrasias include several related syndromes: multiple myeloma, Waldenström macroglobulinemia, and the premalignant conditions MGUS and smoldering multiple myeloma (SMM). These conditions are characterized by the overproduction of a single immunoglobulin component called a myeloma protein **(M protein),** or **paraprotein,** by a clone of identical plasma cells. M protein may also be rarely associated with other lymphoproliferative disorders, such as NHL or primary amyloidosis. Laboratory evaluation is important in the diagnosis and differentiation of these conditions. Diagnosis and monitoring of the plasma cell dyscrasias depend heavily on detecting and quantitating the M protein.

Monoclonal Gammopathy of Undetermined Significance (MGUS)

MGUS is a common premalignant condition that is present in about 3.5% of individuals aged 50 or older.[26] People with MGUS produce a monoclonal immunoglobulin but do not have symptoms of organ damage or other laboratory findings that are associated with multiple myeloma or the other plasma cell dyscrasias. MGUS is usually diagnosed incidentally when patients with various nonspecific symptoms have laboratory testing such as serum protein electrophoresis (SPE).[27,28] The International Myeloma Working Group (IMWG) has identified three criteria that define the presence of MGUS: (1) a serum monoclonal protein concentration of less than 3 g/dL; (2) a plasma cell count of lower than 10% of the total cells in the bone marrow; and (3) the absence of signs or symptoms associated with multiple myeloma, known as the CRAB features (increased serum calcium, renal failure, anemia, lytic bone lesions).[29]

Research studies have found that patients with MGUS have an average lifetime risk of developing multiple myeloma or other related disorders of about 1% per year.[26] MGUS patients who produce an IgG or IgA monoclonal Ig typically progress to multiple myeloma, patients who produce an IgM monoclonal Ig can develop Waldenström macroglobulinemia or other lymphoproliferative disorders, and patients with monoclonal light chains can develop light chain multiple myeloma, amyloidosis, or light chain deposition diseases.[27] Greater risk of disease progression has been associated with production of an M protein that is not IgG, a monoclonal Ig concentration of 1.5 g/dL or greater, and an abnormal free light chain (κ:λ) ratio (see the text that follows).[28,30] Currently, no treatments have been discovered to prevent or delay the progression of MGUS.[26] Lifelong follow-up of MGUS patients with medical examinations and pertinent laboratory testing (e.g., SPE, complete blood count (CBC), kidney function tests, serum calcium levels) is recommended to identify the development of malignancy before serious complications occur.[26,28] Treatment is not recommended unless symptomatic disease develops.

Multiple Myeloma

Multiple myeloma, sometimes called *plasma cell myeloma,* is a malignancy of mature plasma cells that accounts for about 10% of all hematologic cancers.[31] It is the most serious and common of the plasma cell dyscrasias. It is usually diagnosed in persons between 40 and 70 years of age with a peak age of 65 years. Men are slightly more likely (56%) than women to develop multiple myeloma. The American Cancer Society estimates there are 30,330 new cases of multiple myeloma diagnosed each year in the United States and 12,650 myeloma-related deaths each year.[32] Patients progress from asymptomatic MGUS to SMM to the symptomatic disease, multiple myeloma. In fact, all cases of multiple myeloma are thought to be preceded by MGUS or smoldering multiple myeloma (SMM).[33] Patients with multiple myeloma typically have excess plasma cells in the bone marrow, a monoclonal immunoglobulin component in the plasma or urine, and lytic bone lesions. The plasma cells infiltrating the bone marrow may be morphologically normal or may show atypical or bizarre cytological features. Malignant plasma cells phenotypically express CD38, CD56, and CD138. Approximately 20% of myeloma cells express CD20. Unlike normal plasma cells, multiple myeloma cells have the ability to divide at a slow rate.

The immunoglobulin produced by the malignant clone can be of any type, with IgG being the most common (50%), followed by IgA and light chains only. Very often, the production of heavy and light chains by the malignant plasma cells is not well synchronized and an excess of kappa or lambda light chains may be produced. In about 10% of cases, the myeloma cells exclusively produce light chains. These monoclonal light chains can be found in the blood, but are rapidly excreted in the urine, where they are known as **Bence Jones proteins.** Rarely do myelomas produce IgM, IgD, IgE, or heavy chains only. Very rarely, two or more distinct M proteins are produced or a myeloma might not produce a detectable secretory product. The level of normal immunoglobulin is often decreased in proportion to the amount of abnormal immunoglobulin (M protein) present in the serum because of the large number of myeloma cells.

The clinical manifestations of multiple myeloma are primarily skeletal, hematologic, and immunologic. Hematologic problems are often related to the failure of the bone marrow to produce a normal number of hematopoietic cells because myeloma cells progressively replace them **(Fig. 18–4).** The low number of hematopoietic precursors in the bone marrow leads to anemia, thrombocytopenia, and neutropenia. High levels of M protein can interfere with coagulation factors, leading to abnormal platelet aggregation and abnormal platelet function. These abnormalities, coupled with thrombocytopenia, make

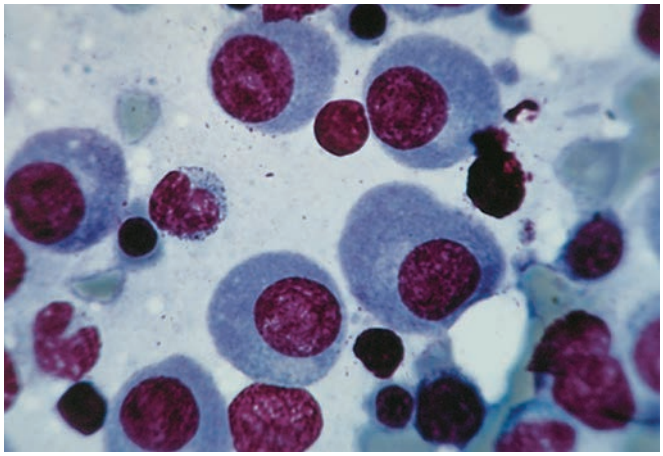

FIGURE 18–4 Bone marrow biopsy sample, showing replacement of marrow by plasma cells. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009.)*

hemorrhaging, bruising, and purpura, common complications of multiple myeloma.

Multiple myeloma preferentially involves bone, producing multiple lytic lesions, that often lead to bone pain and pathological fractures **(Fig. 18–5).** Bone loss is caused by a complex interaction between the myeloma cells and normal cells of the bone. The myeloma cells trigger increased osteoclast activity and decreased osteoblast activity. Morbidity is generally caused by bone disease. The multiple myeloma cells are dependent on the bone marrow microenvironment and interaction with several cells including osteoclasts, osteoblasts, and dendritic cells. Bone pain, usually involving the spine or chest, is the most common presenting symptom of multiple myeloma. Hypercalcemia is very common because the myeloma promotes bone reabsorption. In advanced disease, the hypercalcemia itself can reach life-threatening levels. Despite the dependence of multiple myeloma cells on the bone marrow, tumors occasionally are found in the spleen and liver. These tumors are typically more aggressive.[34]

When immunoglobulin levels in the blood are sufficiently high, they may cause the formation of rouleaux, stacklike formations of red blood cells (RBCs) that can be seen on examination of a peripheral blood smear. The excess production of the abnormal immunoglobulin is accompanied by a progressive decrease in the normal immunoglobulins. This leads to a deficiency of normal antibody responses and a higher incidence of infectious diseases. Hyperviscosity can develop when the level of M protein in the plasma is high. Because viscosity depends on the concentration and size of the molecule in solution and IgM is the largest of the immunoglobulins, hyperviscosity is most often seen with IgM-producing tumors. Hyperviscosity syndrome is also sometimes seen with an IgG3-producing myeloma because IgG3 is the largest of the IgG subclasses.

The type and severity of clinical manifestations depend on the type of immunoglobulin component produced. Up to 15% of patients with multiple myeloma develop light-chain deposition disease or amyloidosis. These are two related disorders

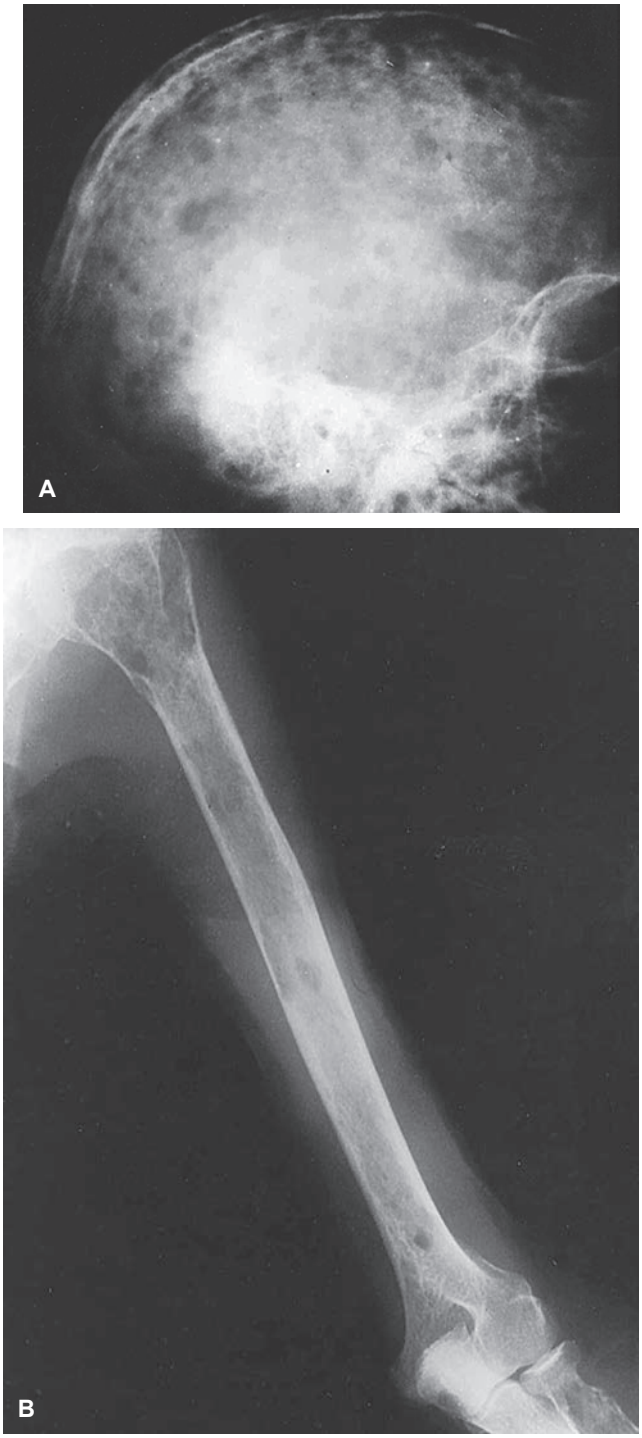

FIGURE 18–5 Bone marrow lesions seen in a patient with multiple myeloma. (A) Lytic skull lesions. (B) Left humerus. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009.)*

in which free light chains or fragments of immunoglobulin are deposited in the tissues. Amyloid fibers stain with the dye Congo red and show apple-green birefringence when viewed with a polarizing microscope. Light chains can be identified in tissue sections by immunofluorescence or immunohistochemical staining with specific antibodies. The deposition of antibody-derived material results in organ dysfunction. The

kidneys are most often affected, but every tissue in the body can develop amyloid deposits. Cardiomyopathy, peripheral neuropathy, hepatosplenomegaly, and ecchymoses (areas of skin discoloration caused by blood in the tissues) are the most common manifestations.

Patients with multiple myeloma can develop either acute or chronic renal failure. As many as two-thirds of patients with multiple myeloma exhibit some degree of renal insufficiency. Patients with myelomas that produce light chains or IgD are much more likely to develop renal failure than those with other types. Renal insufficiency caused by Bence Jones proteins is seen in about 50% of patients. After infection, this is the second leading cause of death. Bence Jones proteins are thought to be directly toxic to tubular epithelial cells and can damage the kidneys by precipitating in the tubules, causing intrarenal obstruction. The median survival for patients with multiple myeloma is approximately 6 years. Prognosis is generally best with IgM disease and worst with IgG disease. Evidence of a deletion in chromosome 13 has a significant negative impact on outcome.[35,36] Although increased treatment options have improved the median mortality rate, multiple myeloma is still an incurable disease. Almost all patients will relapse.

Criteria for the diagnosis of multiple myeloma include plasma cells comprising greater than 10% of bone marrow cells and the evidence of end-organ damage such as bone marrow lesions (detectable by radiographs), hypercalcemia, renal insufficiency, and anemia. Serum M protein of 3 g/dL or more and urinary M protein of 200 mg/day or more are indicative of multiple myeloma.[37,38] Serum M proteins can be detected by protein electrophoresis, immunofixation, and free light chain assays. It is important to differentiate among monoclonal free light chains, heavy chains, and gamma globulins. Patients with monoclonal gammopathy can have MGUS, SMM, multiple myeloma, or one of several other clonal expansions of plasma cells or B cells. **Fluorescence in situ hybridization (FISH)** of bone marrow for the detection of translocation events associated with multiple myeloma can be performed to assess the risk-stratification of the disease.[38]

An important feature supporting the diagnosis of multiple myeloma is the presence of Bence Jones protein in the urine. About 60% to 70% of the patients with myeloma excrete Bence Jones protein in the urine, which can be detected by specific techniques, such as immunofixation electrophoresis (IFE), or nonspecific techniques, such as heat precipitation.

Waldenström Macroglobulinemia

Waldenström macroglobulinemia is a malignant proliferation of IgM-producing lymphocytes which is also known as a lymphoplasmacytic lymphoma. It is a rare condition; only about 1,500 cases are reported annually in the United States.[8] More cases occur in males than females and the median age at onset is about 70 years. Waldenström macroglobulinemia is only about 10% to 20% as common as multiple myeloma and the etiology of this disease is unknown. However, because approximately 20% of the cases exhibit familial clustering, genetic factors are thought to be involved in at least some cases.[39] The presence of MGUS increases the risk of Waldenström macroglobulinemia 200-fold over the general population.[39]

The malignant cells in Waldenström macroglobulinemia are B cells or plasma cells. The B cells produce the B-cell markers CD19, CD20, CD22, and CD79a. The markers CD3, CD5, CD10, and CD103 are not expressed.[39] The tumor cells always infiltrate the bone marrow and are sometimes found in the spleen and lymph nodes. In bone marrow aspirates, the lymphocyte count can be within the reference range or be severely elevated. The tumor cells can have a variety of presentations, described as small lymphocytes, plasmacytoid lymphocytes, and cells resembling mature plasma cells. Typically one morphotype will predominate.

The clinical signs and symptoms of Waldenström macroglobulinemia are variable and are caused by infiltration of the malignant cells into the bone marrow, spleen, and lymph nodes with the overproduction of monoclonal IgM. Signs and symptoms often include weakness, fatigue, anemia, bleeding, and occasionally plasma hyperviscosity. Anemia is attributed to overgrowth of tumor cells in the bone marrow, displacing erythropoiesis. Thrombocytopenia and leukopenia are less commonly seen. At diagnosis, the median hemoglobin concentration is 100 g/L.[40] Bleeding can be caused by a combination of thrombocytopenia and interference of platelet function by monoclonal IgM. The monoclonal IgM can accumulate in any tissue, forming deposits that lead to inflammation and tissue damage. Because early diagnosis is possible, hyperviscosity is uncommon. Lytic bone lesions, hypercalcemia, and renal tubular abnormalities are rare, differentiating this disease from multiple myeloma.

All individuals with Waldenström macroglobulinemia have elevated serum monoclonal protein, referred to as *macroprotein* or *IgM paraprotein,* that migrates in the gamma region during SPE. However, the concentration varies widely and it is not possible to define a concentration that differentiates this disease from other B-cell lymphoproliferative disorders. IgM levels do not affect survival rate or correlate with symptoms. Patients with serum IgM concentrations over 5,000 mg/dL can be asymptomatic, whereas patients with levels of 500 mg/dL can have significant bone marrow infiltration and pancytopenia.[41] The presence of IgM paraprotein is not specific for Waldenström macroglobulinemia. SPE should be used to evaluate the amount of the monoclonal protein; the presence of IgM should be confirmed by IFE (see the section in the text that follows). In 70% to 80% of the patients, the light chain is κ. Bence Jones proteinuria is present in about 10% of the cases.[25] Serum β_2–microglobulin levels are generally above the reference range's upper limit of 3.0 mg/dL. Because of the variability in IgM concentration, the WHO describes Waldenström macroglobulinemia as a lymphoproliferative lymphoma involving the bone marrow with IgM paraprotein at any concentration.[42]

In 10% to 20% of patients, the IgM paraproteins behave as **cryoglobulins.** Cryoglobulins precipitate at cold temperatures and can occlude small vessels in the extremities in cold weather. Occlusion of small vessels can lead to hypoxia and the development of skin sores or even necrosis of portions of the fingers or toes. Cryoglobulins can also contribute to

plasma hyperviscosity. Cryoglobulins can be detected when a blood or plasma sample is refrigerated in the clinical laboratory. The precipitate forms at low temperatures and dissolves upon warming.

Some of the clinical symptoms are caused by autoantibody activity of the monoclonal IgM antibody. Some IgM paraproteins have specificity for the *i* or *I* antigens and will agglutinate RBCs in the cold *(cold agglutinins)*. Antibodies can bind to RBCs producing an autoimmune hemolytic anemia. Coating of the RBCs can produce rouleaux, which can be demonstrated on peripheral blood smears. A thrombocytopenic purpura-like syndrome can develop from paraprotein binding to thrombocytes. In addition, IgM can be demonstrated against polyclonal IgG. This results in immune complex disease characterized by vasculitis, affecting small vessels of the skin, kidneys, liver, and peripheral nerves.

Approximately 20% of the patients with Waldenström macroglobulinemia will present with peripheral neuropathy.[43] It appears that the monoclonal IgM in these cases is directed against glycoproteins or glycolipids of the peripheral nerves, causing symptoms of neuropathology.

Asymptomatic patients do not require treatment, but they should be monitored. Treatment includes anti-tumor chemotherapy and plasmapheresis to reduce blood viscosity. Survival time depends on the disease stage. Patients with low-stage disease have a median survival time of about 12 years compared with 3.5 years for those with high-stage disease.[42]

Heavy-Chain Diseases

The **heavy-chain diseases** are rare B-cell lymphomas that are characterized by the production of a monoclonal immunoglobulin (Ig) heavy chain.[44,45] Genetic mutations in the affected B cells result in the production of abnormal heavy chains that have lost part of their CH1 or variable domain so they are incapable of binding to Ig light chains.[46] These diseases are classified according to the type of heavy chain produced, which can be alpha (α), gamma (γ), or mu (μ).

Alpha heavy-chain disease is the most common of the three types and is seen most often in young adults in their 20s or 30s who live in the Mediterranean region, including northern Africa and the Middle East.[44,45] It has been associated with poor hygiene, poor nutrition, and chronic bacterial and parasitic infections. The disease is a lymphoma that involves the MALT and can occur as one of three forms: gastrointestinal, respiratory, or lymphomatous. Most patients have the gastrointestinal form, which is characterized by intestinal malabsorption with diarrhea, abdominal pain, and weight loss. The monoclonal α chains can be identified in patient serum through reaction with anti-IgA in IFE (see the text that follows). Because of their abnormal structure and tendency to polymerize, they may not be evident by SPE, which can appear normal or demonstrate a broad band that migrates to the α-2 or β region.[46] Histological testing of biopsy tissue obtained from the small intestine or other affected areas demonstrates an infiltration of plasma cells and mature B cells. Early diagnosis is important because treatment with antibiotics in the early stage of the disease can improve prognosis.[45] Patients unresponsive to antibiotics or those who have progressed to later stages of the disease are treated with chemotherapy.

Gamma chain disease is a very rare disorder that has been found in people around the world, usually appearing between the ages of 60 and 70.[44] One-fourth of the patients also have an autoimmune disease such as rheumatoid arthritis (RA).[46] The disease is heterogeneous and can present in one of three forms: disseminated lymphoma with lymphadenopathy and generalized symptoms such as fever and weight loss; localized disease with lymphoma limited to the bone marrow; or localized disease involving areas outside of the lymph nodes, such as the skin.[46] The abnormal gamma chains tend to migrate to the β region on SPE, where they may be masked by other proteins. Serum IgG is elevated and the abnormal protein can be seen by IFE, which demonstrates a monoclonal γ band in the absence of monoclonal light chains. Immunohistochemistry studies reveal the presence of malignant B cells and plasma cells in the bone marrow, spleen, lymph nodes, or other involved areas such as the skin. Patients are treated with chemotherapy and the prognosis is highly variable, ranging from 1 month to more than 20 years.[46]

Mu heavy-chain disease is an extremely rare disorder that has only been diagnosed in about 40 people throughout the world, mainly Caucasian males aged 50 to 60 years.[44,46] The majority of patients also have a lymphoid malignancy that resembles CLL or SLL.[46] Thus, patients have symptoms that are related to the associated lymphoma, such as splenomegaly (enlargement of the spleen), hepatomegaly (enlargement of the liver), and anemia. More than half of patients have a normal SPE pattern, but IFE typically reveals μ polymers of various sizes that are not associated with κ or λ light chains.[46] The urine does not usually contain μ heavy chains, but demonstrates the presence of free monoclonal light chains (Bence Jones proteins) in more than half of patients.[45] Bone marrow aspirates reveal a mixture of plasma cells containing prominent cytoplasmic vacuoles and small round lymphocytes resembling those of CLL.[46] Patients are treated with chemotherapy and overall survival is variable, ranging from under 1 month to more than 10 years.[46]

Role of the Laboratory in Evaluating Immunoproliferative Diseases

Diagnosis of a hematologic malignancy is usually suggested by a patient's medical history and clinical symptoms and confirmed by laboratory testing. Laboratory evaluation of a patient suspected of having an immunoproliferative disorder begins with performance of a CBC and differential and examination of the cell populations on a peripheral blood smear. Blood samples from some patients with hematologic malignancies may show a decrease in RBCs (anemia) or platelets (thrombocytopenia) because of crowding out of normal hematopoietic precursors by the malignant cell population. A decrease in normal white blood cells (WBCs) may also be evident and can result in increased susceptibility to infections. Microscopic examination of cell morphology in the peripheral blood smear can provide important clues about the lineage of the malignant cell population. Differentiation between cells of monocytic or granulocytic origin and

those of lymphoid origin can also be accomplished by the use of various cytochemical stains such as peroxidase and Sudan Black B. Once abnormalities are detected in the CBC and differential, a bone marrow aspirate and biopsy are obtained to confirm the diagnosis. Although microscopic examination of the bone marrow cells can confirm the presence of a malignant population, specialized tests are required to more precisely identify the cells of interest.

This section will discuss three types of specialized tests that are used in the diagnosis and monitoring of patients with lymphoproliferative disorders: immunophenotyping by flow cytometry, evaluation of immunoglobulins, and genetic testing. The laboratory can assess the immunophenotype of hematopoietic cells in the blood, bone marrow, or lymphoid tissues by flow cytometry. This is done by detecting cell surface antigens that are characteristic of a specific lineage and stage of differentiation. This technology serves as an excellent complement to microscope-based traditional diagnostic methods and adds distinctive, discriminatory capabilities that are unmatched by any other diagnostic technique. By performing immunophenotyping, the laboratory can determine whether the malignant cell population consists of B cells, T cells, NK cells, plasma cells, or cells of myeloid origin.

A second role of the laboratory is in evaluating the amount and characteristics of the immunoglobulins produced by malignant B cells or plasma cells. Because the B-cell lineage develops into plasma cells that produce antibody, malignancies of B cells are sometimes associated with excessive or abnormal antibody production. The concentrations and characteristics of the immunoglobulins in the patient's serum or urine can be used to diagnose and evaluate the plasma cell dyscrasias.

Third, the laboratory is involved in the assessment of genetic and chromosomal abnormalities in hematopoietic malignancies. Genetic techniques play an important role in routine clinical practice. Cytogenetic analyses are used to detect chromosomal abnormalities such as translocations. Molecular techniques such as microarray and the PCR can be used to detect mutant sequences within genes that have been linked to particular diseases.

Immunophenotyping by Flow Cytometry

Immunophenotyping, or the analysis of cell surface marker expression, is commonly used in the diagnosis and classification of leukemias and lymphomas.[8,47] Because the malignant cells express markers that often correspond to those of their normal precursors, insight into their lineage of origin and stage of maturation can often be determined by this technique.

The presence of cluster of differentiation (CD) antigens on the surface of hematopoietic cells is routinely detected by flow cytometry. **Table 18–1** lists some of the clinically relevant markers. In immunophenotyping, clinical samples containing cells that are potentially neoplastic are incubated with panels of antibodies that are specific for the relevant antigens. The clinical laboratory determines the specific antibodies used for testing on the basis of the suspected disease, the type of sample, and the amount of sample available. Each antibody in a single reaction tube is labeled with a different fluorescent dye.

Table 18–1 Markers Commonly Detected by Flow Cytometry in the Analysis of Hematologic Malignancies*

CELL TYPE	ASSOCIATED MARKERS
T cells	CD1, CD2, CD3, CD4, CD5, CD7, CD8, TCR alpha-beta, TCR gamma-delta
B cells	CD10, CD19, CD20, CD22, CD23, CD79a, CD103, kappa (surface and cytoplasmic), lambda (surface and cytoplasmic)
Myeloid cells and monocytes	CD11b, CD13, CD14, CD15, CD33, CD64, CD117, myeloperoxidase
Miscellaneous	CD11c, CD16, CD25, CD26, CD30, CD34, CD38, CD41, CD42b, CD45, CD56, CD57, CD61, HLA-DR, glycophorin, TdT, CD123, CD138, CD200

**Modified from ARUP Laboratories. Laboratory Test Directory. Leukemia/Lymphoma Phenotyping by Flow Cytometry. http://ltd.aruplab.com/Tests/Pub/2008003. Accessed July 2, 2015.*

Thus, cells that express a specific antigen are bound by the corresponding antibody and emit fluorescence of a particular color. The fluorescence, along with cell size and other cell characteristics, is analyzed by flow cytometry (see Chapter 13). This allows the antigenic profile, or immunophenotype, of the cell population to be determined. **Table 18–2** lists the CD markers typically found on selected hematologic malignancies of lymphoid origin.

Flow cytometry is ideal for fluids such as blood, in which cells are naturally suspended, but it is also useful for lymphoid tissues, from which single-cell suspensions can be easily made. The advantages of flow cytometry are largely based on its ability to very rapidly and simultaneously analyze multiple-cell properties, including size, granularity, and surface and intracellular antigens, even in small samples. The quantitative nature of the data produced, both with regard to cell population distributions and to expression of individual cell antigens, offers objective criteria for the interpretation of results.

However, laboratorians and clinicians must recognize that malignant cells can differ from their normal counterparts (e.g., B-cell ALL versus normal B cells) in terms of the antigens that they characteristically express. This difference can occur in any of the following ways:

1. There may be a gain of antigens not usually expressed by the normal cell type or lineage.
2. There may be abnormally increased or decreased levels of the antigens expressed by the malignant cells, or in some cases a complete loss of normal antigens.
3. The malignant cells may express antigens at inappropriate times during the maturation process.
4. There may be a homogeneous expression of antigens that are typically heterogeneously expressed by the normal counterpart.[47,48]

Table 18–2 Surface Markers Characteristic of Selected Leukemias and Lymphomas

HEMATOPOIETIC MALIGNANCY	CHARACTERISTIC SURFACE MARKERS*
Classic Hodgkin lymphoma	CD15+ (most), CD30+, CD3 +/–, CD20 – or weak, CD45–
Nodular lymphocytic predominant Hodgkin lymphoma (NLPHL)	CD19+, CD20+, CD45+, CD15–, CD30–
B-cell acute lymphocytic leukemia (B-ALL)	CD10+, CD19+, CD22+, CD34+, TdT+
T-cell acute lymphocytic leukemia (T-ALL)	CD1a+, CD2+, CD5+, CD7+, TdT+
Chronic lymphocytic leukemia (CLL)	CD5+, CD19+, CD20 (weak+), CD23+
Hairy cell leukemia	CD19+, CD20+, CD22+, CD25+, CD103+, CD123+
Multiple myeloma	CD38+, CD56+, CD138+, ~20% are CD20+
Waldenström macroglobulinemia	CD19+, CD20+, CD22+, CD79a+, CD3–, CD5–, CD10–, CD103–

** CD = cluster of differentiation, + = positive, – = negative, TdT = Terminal deoxynucleotidyl transferase.*

Because there is no single surface marker that is specific for a particular hematologic malignancy, the laboratory must use a panel of carefully selected antibodies to identify the markers necessary for making an accurate diagnosis.[8]

Evaluation of Immunoglobulins

As we discussed in Chapter 5, the basic immunoglobulin unit consists of two identical heavy chains and two identical light chains, covalently linked by disulfide bonds. The structure of the heavy chain defines the class, or isotype, of the antibody (e.g., γ heavy chain in IgG, μ heavy chain in IgM, etc.). The two types of light chains (κ and λ) can each occur in combination with any of the heavy-chain types. The heavy and light chains each contain constant and variable regions. The constant region contains the sites of immunoglobulin that bind to cell receptors and sites involved in complement fixation. The variable regions contain the *idiotypes*, which are responsible for the antigen specificity of the antibody. Normally, immunoglobulins in plasma are heterogeneous, because they have a variety of idiotypes that recognize a variety of different antigens. The variability in the isotype means that they also vary in their physical characteristics, such as molecular weight and charge.

B cells differentiate into antibody-producing plasma cells by maturation through several stages. Each B cell recognizes only a single antigenic site or epitope. An early B-cell precursor is stimulated to proliferate and mature when it encounters an immunogen that it recognizes. When a foreign molecule enters the body, the many different epitopes on it each stimulate a B-cell response, leading to the production of an array of different antibodies. However, in disorders such as multiple myeloma, proliferation of one clone of transformed plasma cells leads to overproduction of an immunoglobulin of a single class and antigen specificity. These disorders are called **monoclonal gammopathies,** because the diseases involve proteins that are produced by a single clone of plasma cells; these proteins are found mainly in the gamma region of a SPE analysis. The antibody produced by the malignant plasma cells is referred to as an M (monoclonal) protein or *paraprotein* (i.e., an abnormal protein). All of the antibody proteins produced by the clone of plasma cells are identical in terms of their heavy chains, light chains, and idiotypes.

The initial tests used to screen for the presence of a monoclonal gammopathy are serum immunoglobulin levels and SPE (see the text that follows). Quantitative measurement of immunoglobulin levels in the serum is routinely performed by nephelometric methods, or in smaller laboratories, by radial immunodiffusion (RID) (see Chapter 10). Because each plasma cell produces only one type of immunoglobulin, the persistent presence of an elevated amount of a single immunoglobulin class suggests malignancy. In contrast, an increase in the amount of total immunoglobulin, without an increase in any one specific class, is characteristic of nonmalignant conditions such as infections or autoimmune diseases.

Serum Protein Electrophoresis (SPE)

SPE is a technique in which serum proteins are separated on the basis of their size and electric charge, as discussed in Chapter 5. SPE results in five regions: albumin, as well as the alpha 1, alpha 2, beta, and gamma globulins. IgG, IgM, IgD, and IgE migrate in the gamma globulin region, whereas IgA migrates as a broad band in the beta and gamma regions. **Figure 18–6,** panel A, shows a stylized drawing of the protein distribution in normal serum. As can be seen, immunoglobulins normally show a range of mobilities because they are derived from many clones of plasma cells and have different variable region sequences. The SPE pattern for a polyclonal increase in serum immunoglobulins is shown in panel B. Note the broad mobility, but increased height of the gamma globulin peak. Polyclonal increases in serum immunoglobulins are seen in a variety of disorders, including infections, autoimmune diseases, liver diseases, and some immunodeficiency states (e.g., hyper-IgM syndrome). The SPE result in panel C depicts a monoclonal immunoglobulin, which is increased in concentration and has limited mobility because it is produced by an identical clone of plasma cells. This is illustrated by the tall, narrow peak in the gamma region.

Additional evaluation of serum immunoglobulins by IFE is performed if the SPE shows a monoclonal component, if there

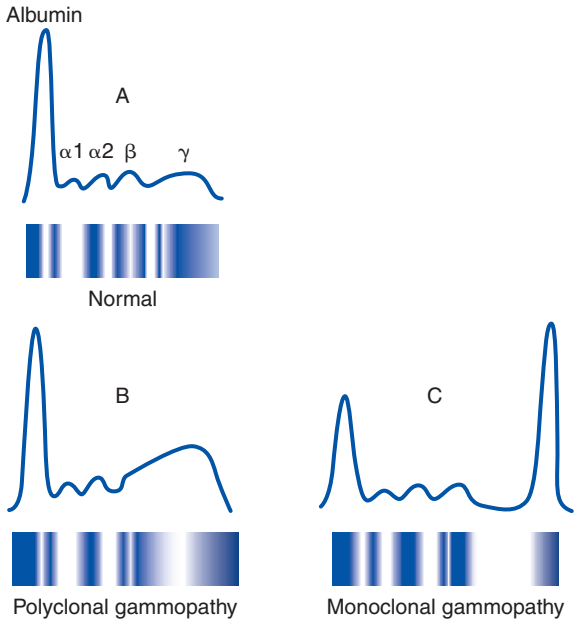

FIGURE 18–6 Serum protein electrophoresis of normal and abnormal samples. The lower portion of each panel is a representation of a stained agarose electrophoresis gel. The intensity of staining corresponds to the amount of protein in each region of the gel. In the upper portion of each panel is a densitometer tracing of a gel similar to the one beneath it. In the upper panel showing a normal serum sample, the largest peak is albumin. The globulin regions are as indicated.

is a significant quantitative abnormality of serum immunoglobulins, or if the clinical picture strongly suggests a plasma cell dyscrasia. Myeloma in which only light chains are produced may not be detected on SPE because the light chains are rapidly cleared in the urine. Therefore, additional studies on a random or 24-hour urine sample may be indicated even in the presence of a normal SPE.

Immunofixation Electrophoresis (IFE)

Serum Immunofixation Electrophoresis

The performance of IFE is typically the next step in evaluating a monoclonal gammopathy. IFE is a highly sensitive and specific assay that is used to identify the type of monoclonal protein present in a sample. It can be performed by manual or automated capillary electrophoresis systems. In IFE, serum samples are electrophoresed in six separate lanes on an agarose gel and specific antisera are applied directly to the lanes. The antisera used are selected to detect the most common M proteins and are directed against:

- Whole human serum (lane 1)
- Anti-γ (to detect IgG) (lane 2)
- Anti-α (to detect IgA) (lane 3)
- Anti-μ (to detect IgM) (lane 4)
- Anti-κ (to detect kappa light chains) (lane 5)
- Anti-λ (to detect lambda light chains) (lane 6)

The antibodies combine with the immunoglobulin proteins in the sample to form complexes that are visualized by staining. **Figure 18–7** illustrates the principle of IFE. Areas of diffuse staining indicate polyclonal immunoglobulins, whereas monoclonal bands produce narrow, intensely stained bands **(Fig. 18–8).**

Interpretation of IFE results requires a high level of expertise. When the monoclonal protein is in high concentration and the amount of polyclonal immunoglobulin of the same class is low, the bands produced in IFE gels are usually clear and easy to identify, as shown in Figure 18–8. However, the presence of higher levels of normal immunoglobulins can make it difficult to distinguish a minor monoclonal band against a background of normal proteins.[49] These M proteins can sometimes be identified by a technique called *immunosubtraction* (see section that follows). Poorly resolved bands can also be caused by poor technique during the electrophoresis

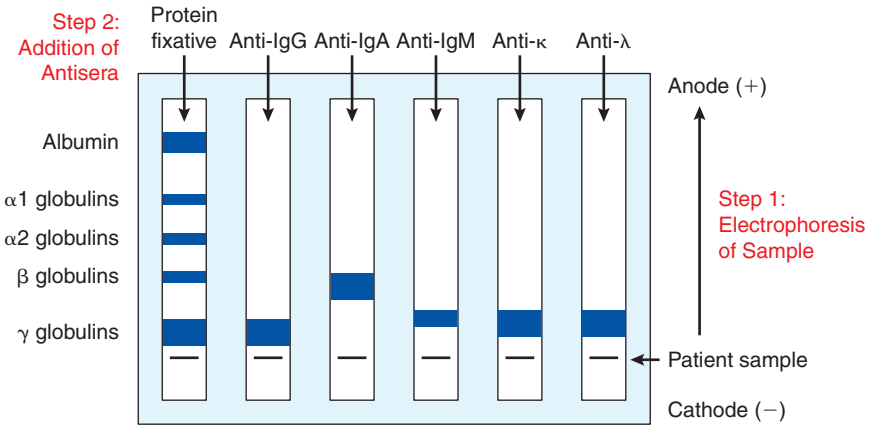

FIGURE 18–7 Principle of immunofixation electrophoresis (IFE). IFE involves two major steps. In step 1, proteins in the clinical sample (serum, urine, or CSF) are applied to an agarose gel and separated according to their surface charge under the influence of an externally applied electrical field. At a pH of 8.6, the proteins acquire a negative charge and move toward the positively charged anode. Five major protein fractions result: γ globulins, β globulins, α1 globulins, α2 globulins, and albumin. Immunoglobulins are located primarily in the γ globulin fraction, but can also migrate into the β globulin fraction. In step 2, specific antisera are added to each one of the lanes and react with their corresponding protein to produce a precipitin band. A protein fixative is added to lane 1, which binds to all of the major protein fractions. The precipitin bands can be visualized after staining the gel with a protein stain and destaining with acetic acid to remove background color.

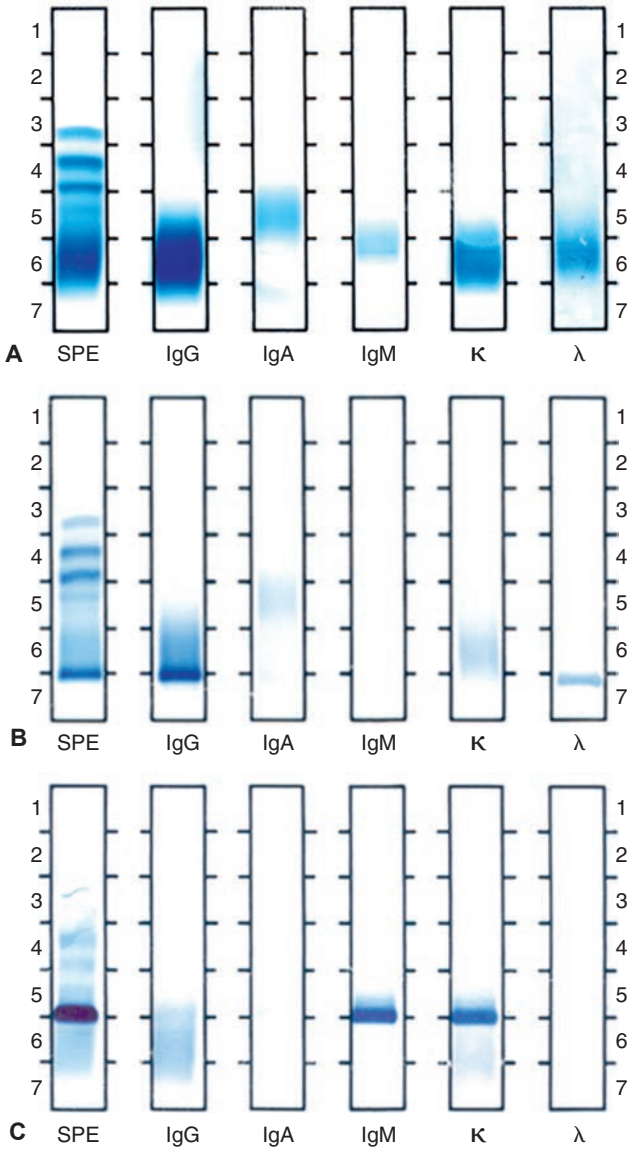

FIGURE 18–8 Sample IFEs from normal serum and sera from patients with monoclonal gammopathies. (A) Normal serum. Note the broad, diffuse bands in all the immunoglobulin lanes. These represent polyclonal antibodies that are heterogeneous in terms of their antigen specificity and structure and therefore migrate to slightly different positions on the gel. (B) Serum from a patient with a monoclonal IgG, λ antibody. Note the discrete, narrow bands in the IgG and lambda lanes. The monoclonal antibody molecules produced by the patient are all identical and migrate to the same position on the gel. (C) Serum from a patient with monoclonal IgM, κ antibody. Note the discrete, narrow bands in the IgM and kappa lanes. *(Linda Miller.)*

or antisera application steps. Other situations can also affect the quality of the results.[49,50] For example, plasma is not recommended because fibrinogen can adhere to the β region of the gel and cause monoclonal proteins to stick nonspecifically, resulting in distinct bands in all of the antisera tracks. Samples with large amounts of rheumatoid factor or immune complexes can also produce unusual results, such as precipitin bands at the place of application. An extreme excess of monoclonal immunoglobulin can cause a *postzone* effect that produces a clear zone in the center of the band **(Fig. 18–9)**.

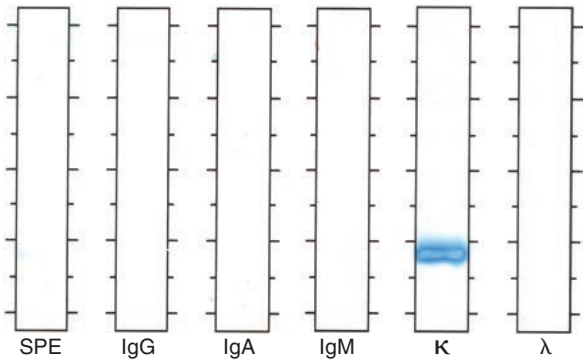

FIGURE 18–9 Postzone effect in immunofixation electrophoresis (IFE). IFE was performed on a 24-hour urine sample that was concentrated 50x by manual ultrafiltration (original protein concentration = 22 mg/dL). Note the clear area in the center of the κ band on the gel, reflecting decreased immunoprecipitation caused by an excess of κ chains relative to the amount of anti-κ reagent used in the κ lane. *(Linda Miller.)*

Connections

Postzone Effect in IFE

Recall from Chapter 10 that equivalent amounts of antigen and antibody are required for optimal formation of immune complexes and precipitation. Extreme antigen excess results in the formation of small immune complexes that cannot be visualized. This is called a *postzone* effect. When a sample with a very large concentration of an M protein is reacted with antisera on an IFE gel, precipitation will be visible on the outer edges of the band, where the reaction is in equivalence, but will not appear in the center of the band, where the reaction is in the postzone area. The problem can be corrected by diluting the sample and repeating the IFE procedure.

Immunosubtraction

A variation of immunofixation, called **immunosubtraction** or **immunotyping**, is a sensitive procedure that uses capillary electrophoresis to identify monoclonal immunoglobulin components. In this technique, antibodies to each heavy or light chain isotype are added to separate capillary runs of the patient's specimen. The binding of the antibody to its heavy or light chain antigenic target changes the electrophoretic mobility of the patient's immunoglobulin molecule. Monoclonal peaks thus "disappear" in the presence of antibodies to their components, allowing typing to occur by subtraction of peaks. **Figure 18–10** shows an example of a monoclonal IgG kappa protein detected by immunotyping.

Urine Immunofixation Electrophoresis (IFE)

Urine protein electrophoresis (UPE) and urine IFE also play an important role in the diagnosis of multiple myeloma and other plasma cell dyscrasias. As previously discussed, some patients with these disorders produce an excessive amount of free monoclonal Ig light chains (i.e., Bence Jones proteins). Because these proteins are rapidly cleared from the circulation, they may not be detectable on serum IFE. However, the excess light

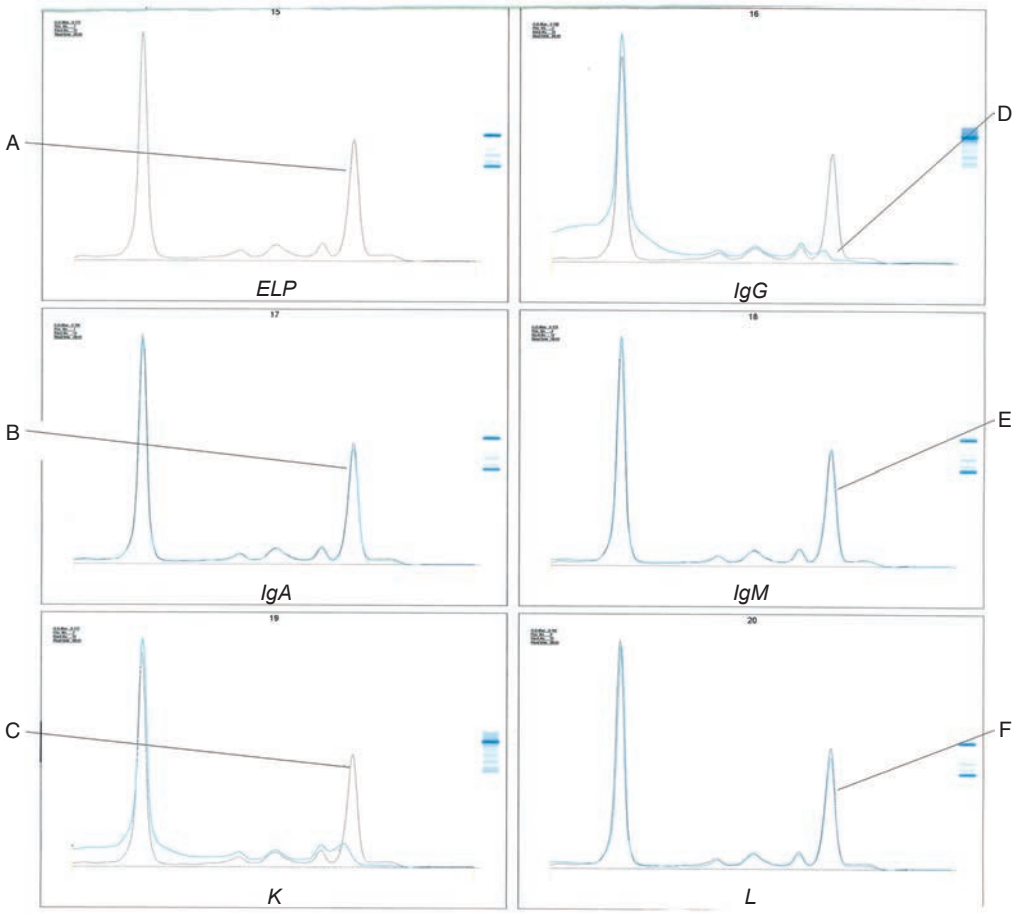

FIGURE 18–10 Immunotyping of a monoclonal IgG, kappa protein. Panel A: Serum protein electrophoresis. The arrow points to the M-spike in the gamma region. Panel B: Electrophoresis with anti-alpha heavy chain. This pattern is overlaid with the original serum protein electrophoresis. The patterns are identical. Panel C: Electrophoresis with anti-kappa light chain. The M-spike has disappeared, indicating the peak contains a kappa light chain (see arrow). Panel D: Electrophoresis with anti-gamma heavy chain. The M-spike has disappeared, indicating the peak contains IgG (see arrow). Panel E: Electrophoresis with anti-mu heavy chain. This pattern is overlaid with the original serum protein electrophoresis. The patterns are identical. Panel F: Electrophoresis with anti-lambda light chain. This pattern is overlaid with the original serum protein electrophoresis. The patterns are identical. *(Courtesy of Dr. Thomas Alexander.)*

chains are excreted into the urine and can be identified using UPE and urine IFE. The free light chains are believed to contribute to renal disease by depositing in the glomeruli and tubules of the kidneys and by their involvement in the development of renal casts.[51] Therefore, the IMWG has recommended that patients with plasma cell dyscrasias be routinely monitored by UPE and urine IFE.[52] These tests can also be used to assess the effect of therapy on the production of free monoclonal light chains and can indicate more extensive renal damage when large proteins such as intact immunoglobulins, which are normally retained in the blood, are demonstrated in the urine.

Urine samples for UPE and IFE are typically collected over a 24-hour period.[53] The total protein concentration is determined and the sample is treated by filtration or centrifugation to remove any sediment that could interfere with performance of the tests. In addition, the samples must be concentrated so that enough protein is present to produce visible bands on the gel. Concentration can be accomplished by one of two ways. The first way involves the use of urine concentrators with ultrafiltration membranes that can retain large proteins (usually 10,000 daltons or more), but allow water, salts, and other small molecules to pass through, thus reducing the sample volume. In the process, the sample flows through a chamber containing the absorbent membranes and is collected when it reaches the desired volume and concentration. The second way involves the use of centrifugal concentrators that concentrate the sample by high-speed filtration. Centrifugal concentrators are faster than ultrafiltration membranes and can produce higher concentration factors. They are recommended for the preparation of urine samples in capillary electrophoresis systems.

In both systems, the desired concentration factor is based on the amount of protein in the urine sample and is determined by dividing the starting sample volume by the final volume.[53] The concentrated sample is then applied to the electrophoresis

gel and the tests are completed as previously described for SPE and serum IFE.

Serum Free Light Chain Analysis (sFLC)

Although UPE and urine IFE are sensitive methods for the detection of free monoclonal light chains, they have some limitations.[54] For example, the requirement for a 24-hour urine collection can delay testing. In addition, interpretation of the results is subjective and can be difficult when a low level of free light chains is present with a high level of proteinuria, which can create a high degree of background staining. Automated tests for serum free light chains (sFLC) became commercially available in 2001 and offer advantages over the traditional methods for urine testing described previously.

The sFLC assays are latex-enhanced immunoassays that measure free kappa and lambda light chains in the serum. The assays employ polyclonal antibody reagents that recognize a diverse range of FLC epitopes that are normally hidden when the light chains are bound to heavy chains in intact immunoglobulins. This allows for quantitative measurement of free κ and free λ chain concentrations, as well as calculation of a κ/λ ratio (normally, 0.26–1.65).[54] An abnormal κ/λ ratio outside of the reference range, along with an increase of either κ and λ, is a sensitive indicator for the presence of a malignant plasma cell clone, which is characteristic of a monoclonal gammopathy.

The sFLC assays are highly sensitive, being capable of detecting concentrations less than 1 mg/L.[53] This sensitivity allows them to detect monoclonal FLCs in patients previously thought to be negative for monoclonal immunoglobulin production by serum or urine IFE.[54] Based on the quantitative nature and high level of sensitivity of sFLC testing and clinical studies, the IMWG developed consensus guidelines that recommend the use of the sFLC assay along with SPE and serum IFE to screen for multiple myeloma and related disorders.[49,53,54,55] In addition, the group stated that the sFLC can replace the 24 h urine IFE to screen for most plasma dyscrasias (except for light chain amyloidosis). The experts also concluded that sFLC measurements are valuable in monitoring patients with plasma cell disorders and in helping to determine patient prognosis.

Evaluation of Genetic and Chromosomal Abnormalities

As previously discussed, researchers have identified the specific mutations associated with malignant transformation for many of the hematologic malignancies. These genetic alterations are an integral part of the classification system outlined by the WHO.[8] The detection of chromosome translocations is of particular value in diagnosis of hematologic malignancies. **Table 18–3** lists chromosome translocations that are characteristic of specific leukemias and lymphomas.

Often, these translocations can be detected by cytogenetic techniques. Malignant lymphoid cells can be made to proliferate in vitro and their metaphase chromosomes can be examined for grossly visible abnormalities that correspond to characteristic translocations. Traditional cytogenetic evaluation by karyotyping has been supplemented by a technique known as FISH. This technique is used to directly identify a specific region of DNA in a cell. It involves the preparation of short sequences of single-stranded DNA, called *probes,* which are complementary to the DNA sequences of interest. These probes bind to the complementary chromosomal DNA and, because they contain a fluorescent label, allow the location of those DNA sequences in the chromosomes to be visualized. **Figure 18–11** shows an example of a FISH result obtained from a patient with a hematologic malignancy. Probes can be used on chromosomes, interphase nuclei, or tissue biopsies. FISH is rapid and quite sensitive and does not require cell culture because interphase chromosomes are used. However, it only provides information about the specific DNA sequence being detected by the probe used in the test.

Lymphoid malignancies can also be evaluated by molecular techniques to identify abnormalities that are too subtle or diverse to be detected by karyotyping or FISH. Laboratorians can use molecular methods to find microdeletions or clonal rearrangements of the immunoglobulin genes in B-cell malignancies or of the TCR genes in T-cell malignancies. The PCR is the most widely used technique to detect these gene rearrangements.[56] To detect B-cell clonality, for example, the PCR amplifies the immunoglobulin heavy-chain gene sequence containing the variable and joining genes (see Chapter 5). The primers used in the assay are designed to detect conserved sequences so that most of the immunoglobulin genes in the sample will be amplified to detectable amounts, which can be seen on gel electrophoresis. If a malignant B-cell clone is present, a sharp band will migrate to a specific position on the gel, representing the unique V-D-J IgH gene rearrangement contained in the cells of that clone (**Figure 18–12,** lanes 3, 6, 9, and 10). In contrast, normal polyclonal B cells produce a diverse array

Table 18–3 Cytogenetics Characteristic of Selected Leukemias and Lymphomas

HEMATOPOIETIC MALIGNANCY	CHARACTERISTIC CYTOGENETICS*
Burkitt lymphoma	Most commonly t(8;14) [IgH/myc]; also t(2;8) and t(8;22)
Follicular lymphoma	t(14;18) [IgH/Bcl2]
Mantle cell lymphoma	t(11;14) [IgH/cyclin D1]
B-cell acute lymphoblastic leukemia (B-ALL)	t(12;21) [TEL-AML-1]
Chronic myelogenous leukemia (CML)	t(9;22) [bcr/abl]

**t = translocation; IgH = immunoglobulin heavy-chain gene.*

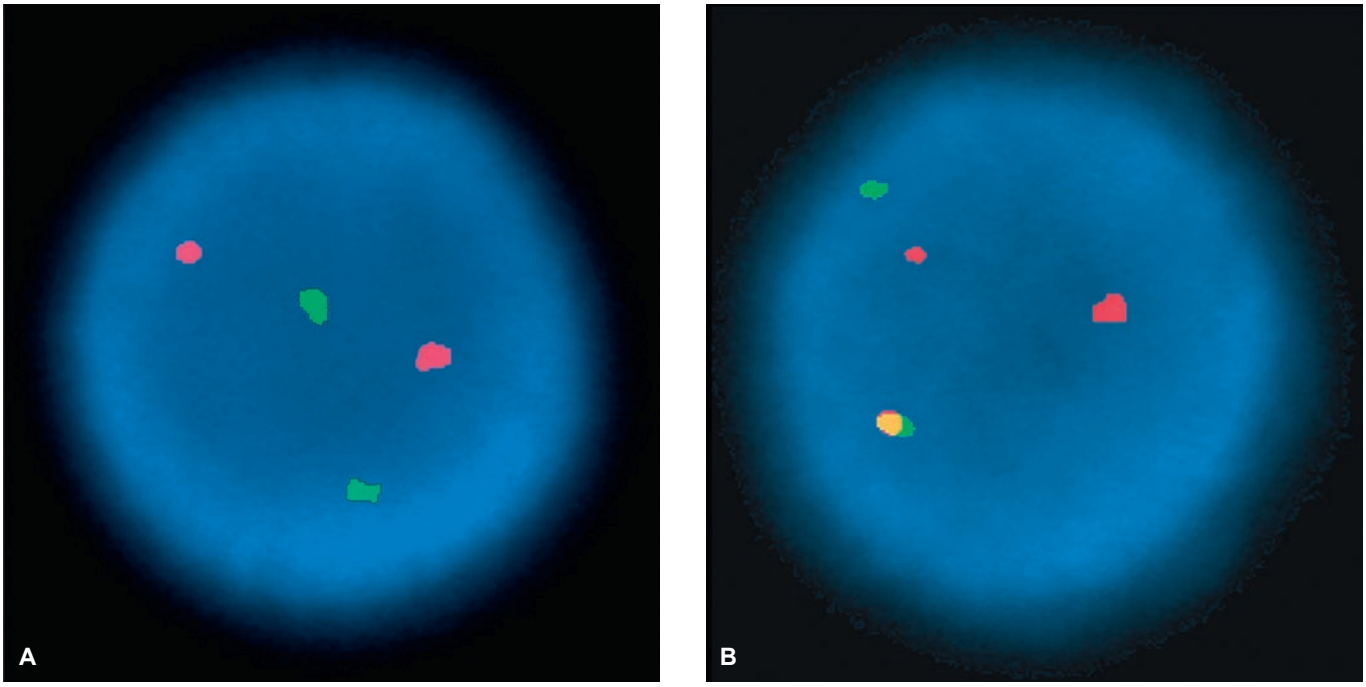

FIGURE 18–11 FISH (fluorescence in situ hybridization) demonstrating a chromosomal translocation. In this assay, interphase cells were hybridized with molecular probes complementary to specific regions on chromosomes 9 and 22. The green signal represents the *BCR* (breakpoint cluster region) on chromosome 22 and the red signal represents the *ABL1* proto-oncogene on chromosome 9. A yellow signal represents the *BCR/ABL1* fusion caused by a 9;22 chromosome rearrangement. (A) A normal test result, showing two green signals representing two copies of chromosome 22 and two red signals representing two copies of chromosome 9. (B) Result from a patient with a translocation between chromosomes 9 and 22 [t(9;22)(q34.1;q11.2)]. The yellow signal indicates the fusion between the *BCR* and *ABL1* loci on the rearranged chromosome 22, which is also known as the Philadelphia chromosome. The smaller red signal detects the residual *ABL1* sequences present on the rearranged chromosome 9. This translocation is found in patients with CML (chronic myelogenous leukemia) as well as some individuals with AML (acute myeloid leukemia) and ALL (acute lymphoblastic leukemia). *(Courtesy of the Cytogenetics Laboratory, SUNY Upstate Medical University.)*

of V-D-J IgH gene rearrangements, which migrate to different positions on the gel, producing a smear (see **Fig. 18–12,** lanes 4, 8, and 11). Similarly, unique rearrangements in the T-cell receptor genes can be amplified by PCR to detect a malignant T-cell clone.

Although PCR and FISH are used to detect abnormalities in targeted genes, other molecular techniques can assay larger areas of the genome. DNA microarray technology enables efficient analysis of thousands of genes in the human genome in a single hybridization experiment by using a panel of molecular probes (see Chapter 12). The probes are complementary to portions of specific genes or chromosome regions and are spotted onto separate locations on a small glass slide or nylon membrane. Genomic DNA is isolated from a clinical sample, labeled with a fluorescent dye, and incubated with the microarray. Fluorescent spots will be visible in the locations where the sample DNA has bound. This technique is being used clinically to detect small genetic changes called single nucleotide polymorphisms (SNPs), as well as larger chromosome deletions or additions that result in copy number variations (CNVs) that may occur in hematologic malignancies.[57] Next generation sequencing (NGS) of tumor cells is also making its way into routine clinical practice. This technique is being used to analyze the nucleotide sequence of all of the genes (i.e., whole genome sequencing), the coding regions of the genome (referred to as exome sequencing), or the transcriptome (i.e., messenger RNA) in samples from cancer patients, including those with hematologic malignancies.[58,59] The tremendous amount of data resulting from these analyses is allowing clinicians to identify genetic profiles associated with specific hematologic malignancies and is revealing new genetic alterations that are associated with the pathobiology of lymphoid neoplasms. Microarrays and NGS are enabling a more comprehensive analysis of the neoplastic genome which is being translated into better patient diagnosis and more precise, targeted therapies for the hematologic malignancies.

Connections

B-Cell Maturation and Rearrangements

Recall from Chapter 5 that rearrangements in the immunoglobulin heavy and light chain genes occur during B-cell maturation. The rearrangements occur randomly so that each B cell possesses a unique sequence of variable-heavy (V), diversity (D), and joining (J) gene segments. This unique sequence is retained as the B cell proliferates. Thus, the sequence can be used as a marker for the B-cell clonality that is characteristic of B-cell malignancies. It is visualized as a band of a distinct size when it is amplified by PCR and run on gel electrophoresis.

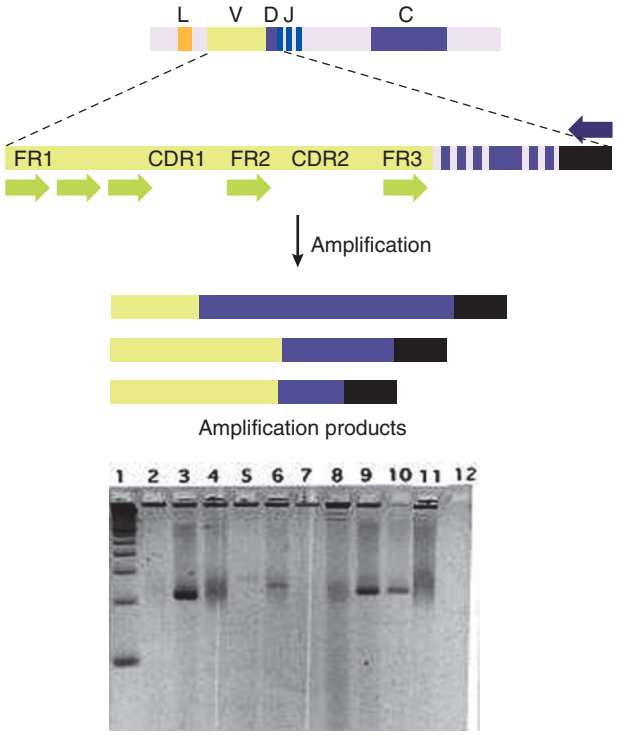

FIGURE 18–12 Immunoglobulin heavy-chain gene rearrangement by PCR with amplification from the variable region. Forward primers complementary to the variable region and reverse primers complementary to the joining region are used to amplify the diversity region (top). In a polyclonal specimen, amplification products in a range of sizes will result. These products produce a dispersed pattern on an ethidium bromide-stained agarose gel (lanes 4, 8, 11). If at least 1% of the sample is representative of a monoclonal gene rearrangement, that product will be amplified preferentially and revealed as a sharp band by gel electrophoresis (lanes 3, 6, 9, and 10). *(From Buckingham L.* Molecular Diagnostics. *2nd ed. Philadelphia, PA: F.A. Davis; 2012.)*

SUMMARY

- Cells of the immune system can undergo malignant transformation because of exposure to environmental factors or genetic mutations that result in excessive cell proliferation, failure to undergo apoptosis, or arrested maturation.
- Proto-oncogenes are normal genes that are involved in cell growth and division. Alterations in these genes can convert them into oncogenes, which are involved in malignant transformation. Several of the hematologic malignancies involve chromosome translocations in which two chromosomes break apart and exchange portions. These gene rearrangements result in the overexpression of proto-oncogenes, causing excessive cell proliferation or inhibition of normal cell death.
- Malignancies of lymphocytes, both lymphomas and leukemias, are commonly encountered in clinical practice. The WHO classification of these disorders depends on their morphological features, cytochemical staining, immunophenotype as determined by flow cytometry, and cytogenetics.
- Examples of hematologic malignancies include Hodgkin lymphoma, non-Hodgkin lymphomas, acute lymphocytic leukemias (ALL), chronic lymphocytic leukemias or lymphomas (CLL), and hairy cell leukemia.
- Plasma cell dyscrasias result in abnormal immunoglobulin secretion. These disorders are malignancies of the plasma cells that are characterized by production of a monoclonal immunoglobulin (M protein or paraprotein).
- In multiple myeloma, the M protein is usually IgG or IgA, but can be of any immunoglobulin class. Waldenström macroglobulinemia is a malignancy of plasmacytoid lymphocytes that produces an IgM paraprotein. Some patients produce free monoclonal light chains and excrete the excess in the urine, where they are known as Bence Jones proteins.
- Identification and quantification of the paraprotein are central to the diagnosis and monitoring of these conditions. SPE is used to detect the presence of an M protein, which is then characterized by immunofixation electrophoresis (IFE). Presence of a monoclonal immunoglobulin is indicated by a discrete, narrow band that migrates to a restricted position on the gel, whereas polyclonal immunoglobulins are diverse and produce broad, diffuse bands.
- Analysis of urine by UPE and IFE is important in the detection of Bence Jones proteins, which can contribute to renal damage. Before testing, the urine must be concentrated by manual filtration or high-speed centrifugal filtration to yield enough protein to produce visible banding on the gel.
- Serum free light chain assays are latex-enhanced immunoassays that measure free κ and λ light chains in patient serum. These are sensitive assays that can rapidly detect low levels of free light chains. An increase in κ or λ, along with an abnormal κ:λ ratio, indicates the presence of a malignant plasma cell clone.
- The cellular origin of a lymphoid malignancy is determined in the laboratory by flow cytometry. Fluorescent-labeled antibodies are used to identify CD markers on the surface of the malignant cells to determine their lineage and stage of maturation. This procedure is called "immunophenotyping."
- Evaluation of genetic and chromosomal abnormalities is a rapidly evolving area of laboratory practice. These alterations can be detected by a variety of cytogenetic and molecular techniques, including FISH, PCR, microarray, and NGS. These methods allow for the detection of abnormalities such as chromosome translocations, nucleotide deletions, and unique V-D-J gene rearrangements that are characteristic of specific hematologic malignancies.

CASE STUDIES

1. A 63-year-old male visits his primary care physician complaining of fatigue and shortness of breath, upper back pain, and a cough that has become productive the last 2 days. The patient was febrile and appeared acutely ill. A chest x-ray revealed pneumonia and the following significant laboratory results were found: RBC count of 4.1×10^{12}/L (reference range 4.6 to 6.0×10^{12}/L), hemoglobin 13 g/dL (reference range 14.0 to 18.0 g/dL), WBC count 4.8×10^{9}/L (reference range 4.5 to 11.0×10^{9}/L), and an erythrocyte sedimentation rate of 12 mm/hr (reference range 0 to 9 mm/hr). Based on these results, the physician ordered serum immunoglobulin levels. The following results were reported: IgG 3,250 mg/dL (reference range 600 to 1,500 mg/dL), IgM 48 mg/dL (reference range 75 to 150 mg/dL), and IgA 102 mg/dL (reference range 150 to 250 mg/dL).

Questions

a. What disease(s) should you suspect? Why?
b. What additional tests could help confirm the diagnosis and what results would you expect to find?

2. A 47-year-old man presented with fever, pneumonia, and splenomegaly. His hemoglobin was 11.5 g/dL, the WBC count was 2,700/mm^3, and the platelet count was 70,000/mm^3. A bone marrow biopsy revealed, among the normal bone marrow cells, numerous diffuse cells 10 to 14 μm in diameter with abundant, clear to lightly basophilic or eosinophilic cytoplasm. The surface of the cells exhibited delicate broad projections. The nuclei were oval and indented with variable chromatin and no prominent nucleoli. Immunohistochemical analysis revealed that the leukemic cells were positive for CD20, DBA44 (a B cell marker), CD68, and annexin A1. Expression of CD20, CD11c, CD25, and CD103 was demonstrated by flow cytometry.

Questions

a. What disease(s) should be considered in the differential diagnosis?
b. What is the significance of the immunophenotyping results?

REVIEW QUESTIONS

1. Bence Jones proteins consist of
   a. monoclonal IgG.
   b. IgG–IgM complexes.
   c. free κ or λ light chains.
   d. free μ heavy chains.

2. Which of the following would be the best indicator of a malignant clone of cells?
   a. Overall increase in antibody production
   b. Increase in IgG and IgM only
   c. Increase in antibody directed against a specific epitope
   d. Decrease in overall antibody production

3. All of the following are features of malignancy *except*
   a. excess apoptosis.
   b. rapid proliferation.
   c. clonal proliferation.
   d. chromosomal mutations.

4. All of the following features are commonly used to classify lymphoid neoplasms *except*
   a. cell of origin.
   b. presence of gene translocations.
   c. exposure of the patient to carcinogens.
   d. morphology or cytology of the malignant cells.

5. Hodgkin lymphoma is characterized by
   a. proliferation of T cells.
   b. excess immunoglobulin production.
   c. an incurable, rapidly progressive course.
   d. the presence of Reed-Sternberg cells in lymph nodes.

6. Chronic leukemias are characterized as
   a. usually being of B-cell origin.
   b. being curable with chemotherapy.
   c. usually occurring in children.
   d. following a rapidly progressive course.

7. Which of the following is characteristic of heavy-chain diseases?
   a. Usually of B-cell origin
   b. Rare lymphomas
   c. Production of abnormal Ig heavy chains
   d. All of the above

8. Flow cytometry results on a patient reveal a decrease of cells with CD2 and CD3. What does this indicate?
   a. Lack of B cells
   b. Lack of T cells
   c. Lack of monocytes
   d. Lack of natural killer cells

9. Which of the following is true of Waldenström macroglobulinemia but not multiple myeloma?

a. Hyperviscosity syndrome is often present.
b. A single protein-producing clone is elevated.
c. The cancerous cell is a preplasma cell.
d. Bence Jones proteins are present in the urine.

10. The presence of anemia, bone pain, thrombocytopenia, and lytic bone lesions is suggestive of

a. Hodgkin lymphoma.
b. hairy cell leukemia.
c. chronic lymphocytic leukemia.
d. multiple myeloma.

11. The presence of an M protein on immunofixation electrophoresis (IFE) is indicated by

a. broad, diffuse banding.
b. a narrow, discrete band.
c. a few well-defined bands in the IgG lane.
d. a single band at the point of application in all of the lanes.

12. Surface immunoglobulin on a leukemic cell indicates a(n)

a. B cell.
b. T cell.
c. macrophage.
d. autoimmune disease.

13. Which of the following is *not* a requirement for urine testing by IFE?

a. Collection of a 24-hour sample
b. Concentration of the sample
c. Dilution of the sample
d. Removal of sediment

14. Multiple myeloma is characteristically preceded by

a. chronic hypogammaglobulinemia.
b. *Helicobacter pylori* infection.
c. non-Hodgkin lymphoma.
d. monoclonal gammopathy of undetermined significance.

15. Which serum free light chain (sFLC) assay result indicates presence of a malignant plasma cell clone?

a. An abnormal κ:λ ratio
b. A decrease in κ and λ concentrations
c. A decrease in IgG, IgA, and IgM concentrations
d. An increase in immunoglobulin concentrations over a 24-hour period

19 Immunodeficiency Diseases

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Differentiate between primary immunodeficiency diseases and secondary immunodeficiency diseases.
2. Indicate the general immunologic defects associated with each of the nine categories of primary immunodeficiency diseases.
3. Associate examples of specific immunodeficiencies with each category.
4. Describe the types of infections typically associated with defects in the B-cell, T-cell, myeloid, or complement systems.
5. Recognize the association between immunodeficiency states and the risk of developing malignancy.
6. Explain the immunologic defects and clinical manifestations associated with selected primary immunodeficiency diseases.
7. Select appropriate laboratory tests to screen for and confirm the presence of specific congenital immunodeficiencies.
8. Correlate laboratory results with the presence of different types of primary immunodeficiencies.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Agammaglobulinemias
Ataxia-telangiectasia (AT)
Bruton's tyrosine kinase (Btk) deficiency
Chronic granulomatous disease (CGD)
Common variable immunodeficiency (CVI)
DiGeorge anomaly
Immunodeficiencies
Immunofixation electrophoresis (IFE)
Inflammasome
Mitogen
Oxidative burst
Primary immunodeficiencies (PIDs)
Purine-nucleoside phosphorylase (PNP) deficiency
Secondary immunodeficiency
Severe combined immunodeficiency (SCID)
Transient hypogammaglobulinemia
Wiskott-Aldrich syndrome (WAS)

Immunodeficiencies are disorders in which a part of the body's immune system is missing or dysfunctional. People with these conditions have a decreased ability to defend themselves against infectious organisms and are more susceptible to developing certain types of cancer. The clinical symptoms associated with immunodeficiencies range from very mild or subclinical to severe, recurrent infections or failure to thrive. Immunodeficiencies can be inherited or acquired secondary to other conditions such as certain infections, malignancies, autoimmune disorders, and immunosuppressive therapies. An example of a **secondary immunodeficiency** is the acquired immunodeficiency syndrome (AIDS), which is caused by the human immunodeficiency virus (HIV). AIDS is discussed in detail in Chapter 24. This chapter focuses on the **primary immunodeficiencies (PIDs),** which are *inherited* dysfunctions of the immune system. Several of the most important immunodeficiency syndromes show X-linked inheritance and, therefore, affect primarily males. Others show autosomal recessive or autosomal dominant inheritance.[1]

More than 200 different congenital forms of immunodeficiency have been reported, including defects in lymphoid cells, phagocytic cells, regulatory molecules, and complement proteins.[1] With the exception of immunoglobulin A (IgA) deficiency, the PIDs are rare disorders with a combined incidence of about 1 in 1,200 live births.[2] In spite of their rarity, it is important for physicians to consider the possibility of PID in children with recurrent infections because early detection and treatment can help prevent the development of serious, long-term tissue damage or overwhelming sepsis. Early diagnosis can also provide the opportunity for appropriate genetic counseling, carrier detection, and prenatal diagnosis for other family members.[3] The clinical laboratory plays an essential role in identifying these important diseases. This chapter serves as an introduction to the PIDs and the laboratory methods that are used to detect the presence of these disorders.

Clinical Effects of Primary Immunodeficiencies

The PIDs can affect one or more parts of the immune system, depending on the specific disease. Some PIDs have their primary effect on B cells and humoral immunity, whereas others mainly affect the cell-mediated branch of the adaptive immune system. Other conditions involve components of the innate defense system such as the phagocytic cells, complement, or NK cells. **Figure 19–1** illustrates points in the development of the immune system at which some PIDs exert their main effects.

The types of infection or symptoms displayed by a patient can give important clues regarding the specific immunodeficiency present. In general, defects in humoral immunity (antibody production) result in pyogenic (i.e., pus-forming) bacterial infections, particularly of the upper and lower respiratory tract. Recurrent sinusitis and otitis media (i.e., ear infections) are common. The clinical course of viral infections in patients with predominantly antibody deficiencies is not significantly different from that in normal hosts, with the exception of hepatitis B, which may have a fulminant course in patients with **agammaglobulinemias,** conditions in which antibody levels in the blood are significantly decreased.

Defects in T-cell–mediated immunity result in recurrent infections with intracellular pathogens such as viruses, fungi, and intracellular bacteria. Differentiating a primary cellular deficiency from HIV-induced immunodeficiencies is essential for proper treatment. Patients with congenital T-cell deficiencies almost always develop mucocutaneous candidiasis, a yeast infection that involves the skin, nails, and mucous membranes. They are also prone to disseminated viral infections, especially with latent viruses such as herpes simplex, varicella zoster, and cytomegalovirus. Because T cells also play an important role in tumor immunity, patients with these conditions are more susceptible to developing certain types of cancer. Age-adjusted rates of malignancy in patients with immunodeficiency disease are 10 to 200 times greater than those observed in immunocompetent individuals.[2] Most of the malignancies are lymphoid and may be related to persistent stimulation of the remaining immune cells, coupled with defective immune regulation.

Defects in other components of the immune system also have significant consequences. For example, neutrophils are the first line of defense against invading organisms; defects in neutrophil function are usually reflected in recurrent pyogenic bacterial infections or impaired wound healing. Abnormalities in macrophage function will have effects on both the innate and the adaptive defenses because macrophages are involved in the nonspecific phagocytosis of microorganisms during inflammation as well as in the processing of antigens and their presentation to T cells in humoral and cell-mediated immune responses. Reduction in the macrophage population by splenectomy is associated with an increased risk of overwhelming bacterial infection accompanied by septicemia. The complement system,

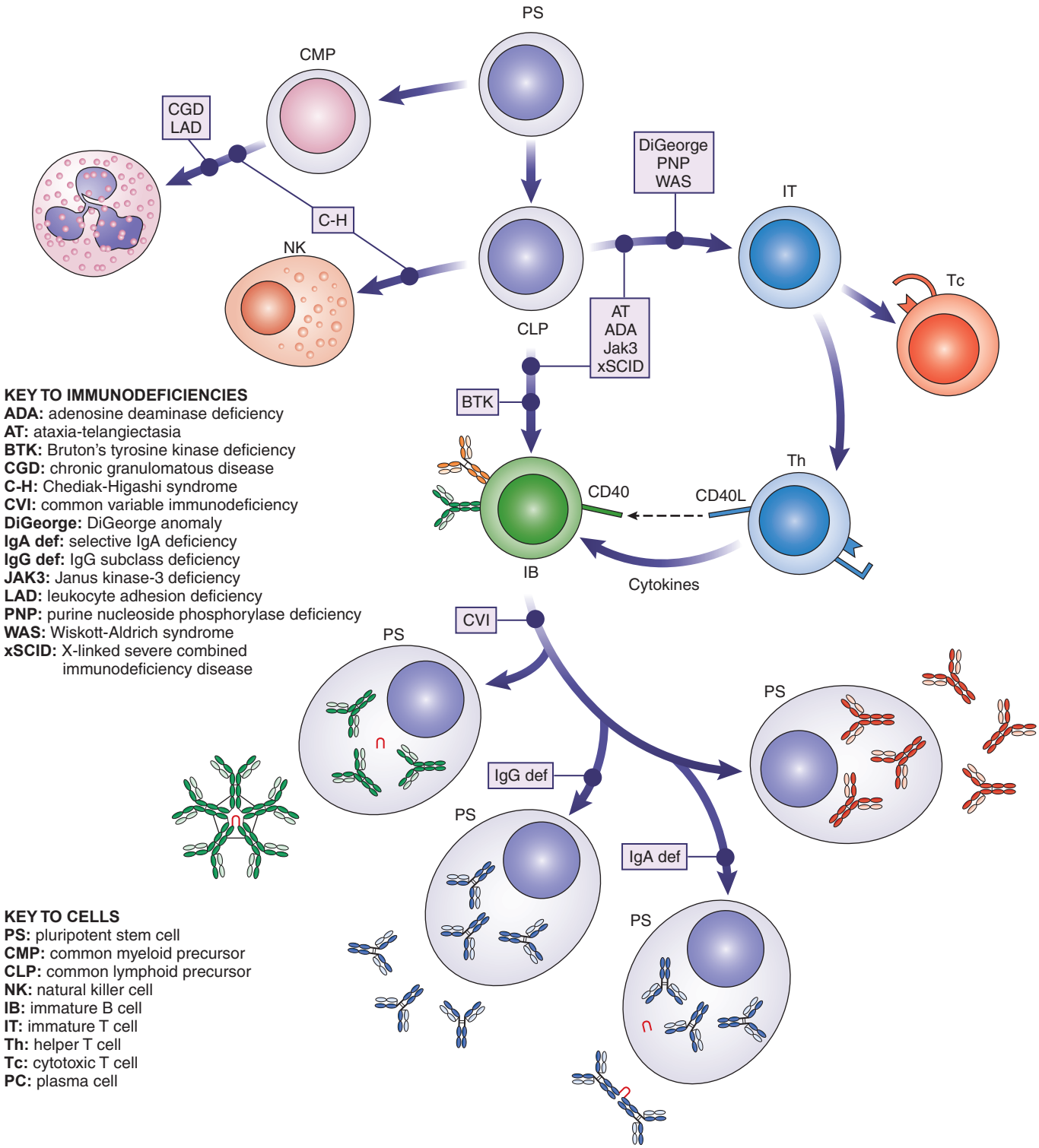

FIGURE 19–1 Examples of primary immunodeficiency diseases and their effects on the immune system.

as discussed in Chapter 7, is activated directly by antigens or by antigen–antibody complexes to produce biologically active molecules that enhance inflammation and promote lysis of microorganisms. Deficiencies of complement components result in recurrent bacterial infections and autoimmune-type manifestations. The severity of the conditions varies with the particular complement component that is deficient.

The components of the immune system play unique, but overlapping, roles in the host defense process. Therefore, defects in any one of the cellular or humoral components result in distinct clinical manifestations, as previously discussed. However, because the components of the immune system interact extensively through many regulatory and effector networks, a defect in one branch of the system may affect other aspects of immune

function as well. In many cases, it appears that deficiency of one component of the immune system is accompanied by hyperactivity of other components. This may occur because persistent infections continuously stimulate the available immune cells or because a compensatory mechanism has been activated to correct for the deficient immune function.

In addition, the deficiency may involve a component that normally exerts regulatory control over other components of the immune system—control that is lacking in the deficiency state. For instance, T helper (Th2) cells secrete cytokines that regulate the development of B cells into plasma cells. A defect in Th2 cell function, such as a deficiency in CD40L (a molecule involved in binding to cell receptors during T-dependent immune responses), removes or creates an imbalance in the regulation of those immune responses. Whatever the mechanism, many partial immunodeficiency states are associated with allergic or autoimmune manifestations, currently referred to as *autoinflammatory disorders* (see discussion in the text that follows).

The Nine Categories of Primary Immunodeficiencies

In the past, the immunodeficiencies have been broadly classified as defects in T cells, B cells, phagocytes, complement proteins, and other components of the innate immune system. As scientific knowledge has been gained about the complexity of these disorders, experts have recognized that such a broad classification is overly simplistic.[4] In 2014, the International Union of Immunologic Societies (IUIS) updated their classification of PIDs by grouping them into nine different categories based on their characteristic clinical features, immunologic defects, and genetic abnormalities.[1] The IUIS has also published diagnostic flow charts to aid in classifying patients into a disease entity based on clinical symptoms and laboratory results.[5] The nine categories are:

- Category 1: Combined Immunodeficiencies
- Category 2: Combined Immunodeficiencies With Associated or Syndromic Features
- Category 3: Predominantly Antibody Deficiencies
- Category 4: Diseases of Immune Dysregulation
- Category 5: Congenital Defects of Phagocyte Number, Function, or Both
- Category 6: Defects in Innate Immunity
- Category 7: Autoinflammatory Disorders
- Category 8: Complement Deficiencies
- Category 9: Phenocopies of Primary Immunodeficiencies

Although the PID diseases are separated into these categories, some diseases are listed in more than one category because they possess overlapping features. The following sections describe the main characteristics of each category and examples of specific PIDs that have been included in each category. Category 3, Predominantly Antibody Deficiencies, is discussed first because the conditions in this category are the most common immunodeficiencies, representing about 50% of the PIDs.[2]

Category 3: Predominantly Antibody Deficiencies

This category encompasses conditions in which the main characteristic is low levels of serum immunoglobulins. Immunoglobulins migrate in the "gamma region" of the serum protein electrophoretic profile (discussed in Chapter 5). Therefore, deficiencies of immunoglobulins have been termed agammaglobulinemias. The mechanisms of the agammaglobulinemias include genetic defects in B-cell maturation or mutations leading to defective interactions between B and T cells.[1] A wide range of immunoglobulin deficiency states have been reported and involve virtually all combinations of immunoglobulins and all degrees of severity. In some cases, only a single isotype of one immunoglobulin class is deficient, whereas all of the other isotypes are normal. Only the more common and well-characterized syndromes are described here. These are summarized in **Table 19–1.**

In evaluating immunoglobulin deficiency states, it is important to remember that blood levels of immunoglobulins change with age. The blood level of IgG at birth is about the same as

Table 19–1 Characteristics of Selected Predominantly Antibody Deficiencies (Category 3)

CONDITION	DEFICIENCY	LEVEL OF DEFECT	PRESENTATION
Transient hypogammaglobulinemia of infancy	All antibodies; especially IgG	Slow development of helper function in some patients	2–6 months; resolves by 2 years
Selective IgA deficiency	IgA; some also with reduced IgG2	IgA-B cell differentiation	Often asymptomatic
Btk deficiency	All antibody isotypes reduced	Pre–B-cell differentiation	Infancy
Common variable immunodeficiency	Reduced antibody; many different combinations	B cell; excess T suppression	Usually 20–30 years of age
Isolated IgG subclass deficiency	Reduced IgG1, IgG2, IgG3, or IgG4	Defect of isotype differentiation	Variable with the class and degree of deficiency
CD154 deficiency	Reduced IgG, IgA, IgE, with elevated IgM	B-cell switching	Variable

the adult level, reflecting transfer of maternal IgG across the placenta. The IgG level declines over the first 6 months of life as maternal antibody is catabolized. Levels of IgA and IgM are very low at birth. The concentrations of all immunoglobulins gradually rise when the infant begins to produce antibodies at a few months of age in response to environmental stimuli. IgM reaches normal adult levels first, around 1 year of age, followed by IgG at about 5 to 6 years of age. In some normal children, IgA levels do not reach normal adult values until adolescence. Therefore, it is important to compare a child's immunoglobulin levels to age-matched reference ranges.

Transient Hypogammaglobulinemia of Infancy With Normal Numbers of B Cells

All infants experience low levels of immunoglobulins at approximately 5 to 6 months of age; however, in some babies the low levels persist for a longer time. Because these children do not begin synthesizing immunoglobulins promptly, they can experience severe pyogenic sinopulmonary and skin infections as protective maternal IgG is cleared. Cell-mediated immunity is normal and there may be normal levels of IgA and IgM.[1,6] IgG appears to be the most affected, dropping to at least 2 standard deviations (SDs) below the age-adjusted mean with or without a depression of IgM and IgA.[6] Immunoglobulin levels in infants with this condition usually normalize spontaneously, often by 9 to 15 months of age. The mechanism of this **transient hypogammaglobulinemia** is not known. These patients have normal numbers of circulating CD19+ B cells. This condition does not appear to be X-linked, although it is more common in males. The cause may be related to a delayed maturation of one or more components of the immune system, possibly Th cells.[6]

X-Linked Bruton's Tyrosine Kinase (Btk) Deficiency

Bruton's tyrosine kinase (Btk) deficiency, first described in 1952, is X chromosome linked, so this syndrome affects males almost exclusively. Patients with X-linked agammaglobulinemia lack circulating mature CD19+ B cells and exhibit a deficiency or lack of immunoglobulins of all classes.[1,7,8] Furthermore, they have no plasma cells in their lymphoid tissues. The patients do, however, have pre-B cells in their bone marrow.[8] Because of the lack of B cells, the tonsils and adenoids are small or entirely absent and lymph nodes lack normal germinal centers. T cells are normal in number and function. About half of the patients have a family history of the syndrome. They develop recurrent bacterial infections beginning in infancy as maternal antibody is cleared. The patients most commonly develop sinopulmonary infections caused by encapsulated organisms such as streptococci, meningococci, and *Haemophilus influenzae*. Other infections seen include bacterial otitis media, bronchitis, pneumonia, meningitis, and dermatitis.[6] Some patients also have a susceptibility to certain types of viral infections, including vaccine-associated poliomyelitis. In general, live virus vaccines should not be administered to immunodeficient patients.

X-linked hypogammaglobulinemia results from arrested differentiation at the pre–B-cell stage, leading to a complete absence of B cells and plasma cells. The underlying genetic mechanism is a deficiency of an enzyme called the Btk in B-cell progenitor cells.[9,10] Lack of the enzyme apparently causes a failure of immunoglobulin VH gene rearrangement (see Chapter 5). The syndrome can be effectively treated by administration of intramuscular or intravenous immunoglobulin preparations and vigorous antimicrobial treatment of infections. The syndrome can be differentiated from transient hypogammaglobulinemia of infancy by the absence of CD19+ B cells in the peripheral blood, the abnormal histology of lymphoid tissues, and its persistence beyond 2 years of age. Immunologists have also described patients with a similar clinical presentation to Btk who have a genetic defect that is inherited in an autosomal recessive manner.[1,6,8]

Selective IgA Deficiency

Selective IgA deficiency is the most common congenital immunodeficiency, occurring in about 1 in 500 persons of American or European descent.[6] Most patients with a deficiency of IgA are asymptomatic. Those with symptoms usually have infections of the respiratory and gastrointestinal tract and an increased tendency to develop autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), celiac disease, and thyroiditis. Allergic disorders and malignancy are also more common.[7] About 20% of the IgA-deficient patients who develop infections also have an IgG2 subclass deficiency. If the serum IgA is lower than 5 mg/mL, the deficiency is considered severe. If the IgA level is two SDs below the age-adjusted mean but greater than 50 mg/dL, the deficiency is partial. Although the genetic defect has not been established, it is hypothesized that lack of IgA is caused by impaired differentiation of lymphocytes to become IgA-producing plasma cells.[6]

IgE antibodies specifically directed against IgA are produced by 30% to 40% of patients with severe IgA deficiency. These antibodies can cause anaphylactic reactions when blood products containing IgA are transfused.[7] Because many patients with severe IgA deficiency have no other symptoms, the IgA deficiency may not be detected until the patient experiences a transfusion reaction, resulting in the production of anti-IgA antibodies. Therefore, products for transfusion to known IgA-deficient patients should be collected from IgA-deficient donors or cellular products should be washed to remove as much donor plasma as possible.

Most gamma globulin preparations contain significant amounts of IgA. However, replacement IgA therapy is not useful because the half-life of IgA is short (around 7 days) and intravenously or intramuscularly administered IgA is not transported to its normal site of secretion at mucosal surfaces. Furthermore, administration of IgA-containing products can induce the development of anti-IgA antibodies or provoke anaphylaxis in patients who already have antibodies.

Common Variable Immunodeficiency (CVI)

Common variable immunodeficiency (CVI) is a heterogeneous group of disorders with a prevalence of about 1 in 25,000.[11] Although this is a low incidence, it does make CVI the most common PID with a severe clinical syndrome.[12] Patients usually begin to have symptoms in their 20s and 30s,

but age at onset ranges from 7 to 71 years of age. The disorder can be congenital or acquired, or familial or sporadic, and it occurs with equal frequency in men and women. CVI is characterized by hypogammaglobulinemia that leads to recurrent bacterial infections, particularly sinusitis and pneumonia. In addition, up to 20% of CVI patients develop herpes zoster (shingles), a much higher incidence than in immunologically normal young adults. There is usually a deficiency of both IgA and IgG, but selective IgG deficiency may occur. CVI is often associated with a spruelike syndrome characterized by malabsorption and diarrhea. CVI is also associated with an increased risk of lymphoproliferative disorders, gastric carcinomas, and autoimmune disorders.[7] The most common autoimmune manifestations of CVI are immune thrombocytopenia and autoimmune hemolytic anemia. Other symptoms may include lymphadenopathy, splenomegaly, and intestinal hyperplasia.[7]

CVI is diagnosed by demonstrating a low serum IgG level in patients with recurrent bacterial infections. Additionally, blood group isohemagglutinins, or the so-called naturally occurring antibodies, are typically absent or low. In contrast to X-linked agammaglobulinemia, most patients with CVI have normal numbers of mature B cells. However, these B cells do not differentiate normally into immunoglobulin-producing plasma cells. Three major types of cellular defects have been found in CVI patients. In some cases, T cells or their products appear to suppress differentiation of B cells into plasma cells. Secondly, T cells may fail to provide adequate help to support terminal differentiation of B cells. Finally, there appears to be a primary defect in the B-cell line in some patients. CVI is often a diagnosis of exclusion, where an immunodeficiency is present with no specific genetic defect defined.[8]

CVI can usually be effectively treated with intramuscular or intravenous immunoglobulin preparations.[13] However, because of their low levels of secretory IgA, patients are still susceptible to respiratory and gastrointestinal infections; the clinician should be vigilant for these infections and treat them vigorously with antibiotics.

Isolated IgG Subclass Deficiency

IgG subclass deficiencies are conditions where the level(s) of one or more of the four IgG subclasses is (are) more than two SDs below the mean age-appropriate level.[6] Normally, about 70% of the total IgG is IgG1, 20% IgG2, 6% IgG3, and 4% IgG4. Therefore, a deficiency of a single subclass may not result in a total IgG level below the normal range. In patients with recurrent infections, levels of the different subclasses should be measured if the total IgG level is normal but the clinical picture suggests immunoglobulin deficiency.[6]

Most IgG antibodies directed against protein antigens are of the IgG1 and IgG3 sub-classes, whereas most IgG antibodies against carbohydrate antigens are IgG2 or IgG4. Thus, deficiencies involving IgG1 or IgG3 lead to a reduced capability of responding to protein antigens such as toxins, whereas selective deficiencies of IgG2 can result in impaired responses to polysaccharide antigens, which cause recurrent infections with polysaccharide-encapsulated bacteria such as *Streptococcus pneumoniae* and *H influenzae*.[6] A variety of genetic defects have been associated with IgG subclass deficiency. These include heavy-chain gene deletions and transcriptional defects. The most common subclass deficiency is IgG4, with IgG1 deficiency being the least common, although IgG4 subclass deficiency may have the least clinical significance.[6]

Category 1: Combined Immunodeficiencies

This category contains diseases in which there are defects in both humoral (B cell) and cell-mediated (T cell) immunity. These deficiencies result from mutations that affect development of both types of lymphocytes or cause defective interaction between the two antigen-specific limbs of the adaptive immune system. Because helper T-cell functions are necessary for normal differentiation and antibody secretion by B cells, a severe defect of T-cell function will have effects on immunoglobulin levels as well. Combined deficiencies are referred to using a shorthand notation of $T^{+/-}B^{+/-}NK^{+/-}$ with the $^+$ or $^-$ superscript denoting whether or not each cell type is present in the deficiency.[7]

Severe Combined Immunodeficiency (SCID)

The most serious of the congenital immunodeficiencies is **severe combined immunodeficiency (SCID).** SCID is actually a group of related diseases that all affect T- and B-cell function but with differing causes. A mutation in the interleukin-2 receptor gamma (IL2RG) gene located on the X-chromosome is the most common form of the disease, accounting for approximately 46% of the cases in the United States today.[2,14] The mutation occurs with a frequency of about 1 in 50,000 births.[15] The IL2RG gene codes for a protein chain called the common gamma chain that is common to receptors for interleukins-2, 4, 7, 9, 15, and 21.[14] Normal signaling cannot occur in cells with defective receptors, thus halting natural maturation.[2,15] This may result in either a $T^-B^+NK^+$ or a $T^-B^+NK^-$ phenotype, depending on whether or not there is an additional defect in the *JAK3* gene.[7] *JAK3* is required for processing an interleukin-binding signal from the cell membrane to the nucleus. No antibody production or lymphocyte proliferative response follows an antigen or mitogen challenge in such cases.

Several autosomal recessive forms of SCID, which affect both males and females, have also been discovered.[7] For example, a *JAK3* deficiency may be found without the common gamma chain deletion. The lack of the intracellular kinase *JAK3* means that that lymphocytes are unable to transmit signals from IL-2 and IL-4.[2,11] These patients have a $T^-B^+NK^-$ phenotype and symptoms are similar to the X-linked form of the disease.[7] The *JAK3* gene is located on chromosome 19, region p12. Other autosomal recessive forms of SCID are discussed in the paragraphs that follow.

About 15% to 20% of the patients with SCID have an adenosine deaminase (ADA) deficiency, leading to a $T^-B^-NK^-$ phenotype.[7] The ADA gene is located on chromosome 1, region q21. ADA deficiency affects an enzyme involved in the metabolism of purines, similar to another form of PID, the PNP deficiency. In ADA deficiency, toxic metabolites of purines accumulate in lymphoid cells and impair proliferation of both B and T cells. In both ADA and PNP deficiencies, there is a progressive

decrease in lymphocyte numbers. A number of different mutations have been found to lead to ADA deficiency and the degree of immunodeficiency correlates with the degree of ADA deficiency. Patients with only mildly reduced ADA activity may have only a slight impairment of immune function.[7]

Other molecular defects have also been identified as causes of SCID. Infants with a lack of both T and B cells but with functioning NK cells were found to have a mutation in a recombinase activating gene (RAG-1 or RAG-2).[14] These mutations cause a profound lymphocytopenia because of the inability of T and B cells to rearrange deoxyribonucleic acid (DNA), a process that is necessary to produce functional immunoglobulins or T-cell receptors (TCRs).[11] Patients with RAG-1 or RAG-2 deficiencies have decreased class II MHC molecule expression. HLA class II molecules are intimately involved in antigen presentation; thus, this defect profoundly impairs the immune response.[7,14] Another molecular defect that has been identified is a mutation in the gene encoding a common leukocyte protein called CD45. It is a transmembrane phosphatase that regulates signal transduction of T- and B-cell receptors.[11,14]

Patients with SCID generally present early in infancy with infection by nearly any type of organism. Oral candidal yeast infections, pneumonia, and diarrhea are the most common manifestations. The administration of live vaccines can cause severe illness. Unless immune reconstitution can be achieved by bone marrow transplantation or by specifically replacing a deficient enzyme, patients with SCID die before they are 2 years old.[14]

ADA deficiency is a special case because it presents a good opportunity for enzyme replacement therapy or somatic cell gene therapy. Although ADA is normally located within cells, its deficiency can be treated by maintaining high plasma levels of ADA. For some patients, red blood cell (RBC) transfusion can raise ADA to near normal levels. However, bovine ADA conjugated with polyethylene glycol (PEG) has a longer half-life than native ADA and can raise the ADA level up to three times higher than normal. This treatment increases T-cell production and specific antibody responses. Side effects of ADA–PEG appear to be minimal. However, this therapy is very expensive and is primarily used in patients for whom a suitable bone marrow donor cannot be found or for those patients who are too sick to undergo marrow transplantation.[16] Human cord blood stem cell transplantation has been used with moderate success.[17] Studies are currently underway to attempt to treat ADA deficiency by transfecting a normal ADA gene into patients' T cells or stem cells. These cells could then be reinfused into the patient. The gene therapy has actually been curative in some cases, but many obstacles remain to be overcome.[13]

Purine-Nucleoside Phosphorylase (PNP) Deficiency

Another immunodeficiency state for which a specific enzymatic basis has been defined is **purine-nucleoside phosphorylase (PNP) deficiency.** PNP deficiency is a rare autosomal recessive trait.[11] The condition presents in infancy with recurrent or chronic pulmonary infections, oral or cutaneous candidiasis, diarrhea, skin infections, urinary tract infections, and failure to thrive. PNP deficiency affects an enzyme involved in the metabolism of purines. It produces a moderate to severe defect in cell-mediated immunity, with normal or only mildly impaired humoral immunity.[11] The number of T cells progressively decreases because of the accumulation of deoxyguanosine triphosphate, a toxic purine metabolite. The levels of immunoglobulins are generally normal or increased. About two thirds of PNP-deficient patients also have neurological disorders, but no characteristic physical abnormalities have been described. Because of the relatively selective defect in cell-mediated immunity, PNP deficiency can be confused with neonatal HIV infection. The two conditions can usually be distinguished by specific tests for HIV (see Chapter 24) and by assays for PNP activity.

Category 2: Combined Immunodeficiencies With Associated or Syndromic Features

This category differs from Category 1 in that the diseases in Category 2 are characterized by nonimmunologic features in addition to the combined immunodeficiency. Diseases in this category are typically caused by defects in cell-mediated immunity, which indirectly lead to problems with the other branches of the immune response. Often, these diseases can result from abnormalities at different stages of T-cell development.[1] Many different molecular defects can result in a similar clinical picture (as in SCID). This is because T cells provide helper functions that are necessary for normal B-cell development and differentiation. Some of the more common defects of cellular and combined cellular and humoral immunity are summarized in **Table 19–2.**

In general, defects in cellular immunity are more difficult to manage than defects in humoral immunity. When immunoglobulin production is deficient, replacement therapy is often very effective. However, there is usually no soluble product that can be administered to treat a deficiency of cell-mediated immunity. Transplantation of immunologically intact cells, usually in the form of allogenic bone marrow, is often required to reconstitute immune function.

Patients with severe defects in cell-mediated immunity may develop graft-versus-host disease (GVHD; see Chapter 16). Transfused lymphocytes are normally destroyed by the recipient's T-cell system. However, a severe defect in the T-cell system allows the donor lymphocytes to survive, proliferate, and attack the tissues of the recipient as foreign. GVHD can occur in any patient with a severe defect in cell-mediated immunity (e.g., in bone marrow transplant recipients) and can be fatal. Irradiation of cell-containing blood products (platelet concentrates, packed RBCs, and whole blood) before transfusion destroys the ability of the donor lymphocytes to proliferate and prevents development of GVHD in immunodeficient recipients. It should be noted that a defect in humoral immunity does not predispose an individual to GVHD.

GVHD also occurs in patients who have received a bone marrow transplant as therapy for a congenital immunodeficiency. The closer the match between the genetic constitution of the patient and the graft donor, the less severe the GVHD

Table 19–2 Characteristics of Selected Combined Immunodeficiencies (Category 1) and Combined Immunodeficiencies With Associated or Syndromic Features (Category 2)

CONDITION	DEFICIENCY	LEVEL OF DEFECT	PRESENTATION
Category 1			
CD40 ligand deficiency	T cells with effects on antibody production	Defective isotype switching with increased or normal IgM but decreased concentrations of other isotypes	1–2 years of age
SCID	Both T and B cells	ADA, purine metabolism; RAG-1/RAG-2; *JAK3;* common gamma chain receptor; others	Infancy
PNP deficiency	T cells; some secondary effects on antibody production	PNP, purine metabolism	Infancy
Category 2			
DiGeorge anomaly	T cells; some secondary effects on antibody production	Embryologic development of the thymus	Neonatal, with hypocalcemia or cardiac defects if severe; abnormal mental delay; development; incomplete forms may present later with infection
WAS	Reduced IgM and T-cell defect	CD43 expression	Usually infancy; with thrombocytopenia, small platelets, and eczema
AT	Reduced IgG2, IgA, IgE, and T lymphocytes	DNA instability	Infancy, with involuntary muscle movements and capillary swelling

ADA = adenosine deaminase; AT = ataxia-telangiectasia; PNP = purine-nucleoside phosphorylase; SCID = severe combined immunodeficiency; WAS = Wiskott-Aldrich syndrome.

is likely to be. Thus, although bone marrow transplantation can potentially cure the immune defect, it can also have serious, lifelong complications of its own. Examples of specific immunodeficiencies in this category are discussed in the text that follows.

Wiskott-Aldrich Syndrome (WAS)

Wiskott-Aldrich syndrome (WAS) is a rare X-linked recessive syndrome that is defined by the triad of immunodeficiency, eczema, and thrombocytopenia.[18] WAS is usually lethal in childhood because of infection, hemorrhage, or malignancy. Milder variants have also been described such as an X-linked form of thrombocytopenia.

The laboratory features of WAS include a decrease in platelet number and size with a prolonged bleeding time.[7] The bone marrow contains a normal or somewhat increased number of megakaryocytes. There are abnormalities in both the cellular and humoral branches of the immune system related to a general defect in antigen processing. As a result, patients display a severe deficiency of the naturally occurring IgM antibodies to ABO blood group antigens (isohemagglutinins). Absence of isohemagglutinins is the most consistent laboratory finding in WAS and is often used diagnostically. Patients with WAS can have a variety of different patterns of immunoglobulin levels, but they usually have low levels of IgM, normal levels of IgA and IgG, and increased levels of IgE.[7] These patients also have persistently increased levels of serum alpha-fetoprotein, which can also be a useful diagnostic feature.

The primary molecular defect in the syndrome appears to be an abnormality of the integral membrane protein CD43, which is involved in the regulation of protein glycosylation.[8] The gene responsible for the defect, called the *WAS* gene, is located on the X chromosome, region p11. Abnormalities cause defective actin polymerization and affect its signal transduction in lymphocytes and platelets.[8]

Platelets have a shortened half-life and T lymphocytes are also affected, although B lymphocytes appear to function normally. Splenectomy can be very valuable in controlling the thrombocytopenia. Current treatment for this immunodeficiency is transplantation of bone marrow or cord blood stem cells from an HLA identical sibling.[13]

DiGeorge Anomaly

DiGeorge anomaly is a developmental abnormality of the third and fourth pharyngeal pouches that affects thymus development in the embryo. All organs derived from these embryonic structures can be affected. Associated abnormalities include mental retardation, absence of ossification of the hyoid bone, cardiac anomalies, abnormal facial development, and thymic hypoplasia.[7] The severity and extent of the developmental defect can

be quite variable. Many patients with a partial DiGeorge anomaly have only a minimal thymic defect and, thus, near normal immune function.[7] However, about 20% of children with a defect of the third and fourth pharyngeal pouches have a severe and persistent decrease in T-cell numbers.[11] These children tend to have severe, recurrent viral and fungal infections. Severely affected children usually present in the neonatal period with tetany (caused by hypocalcemia resulting from hypoparathyroidism) or manifestations of cardiac defects.

The possibility of immunodeficiency can be overlooked if the association between the presenting abnormality and a possible thymic defect is not recognized. The immunodeficiency associated with the DiGeorge anomaly is a quantitative defect in thymocytes. Not enough mature T cells are made, but those that are present are functionally normal. The immunodeficiency of DiGeorge syndrome can be treated with fetal thymus transplantation. Bone marrow transplantation has also been successful in some patients, as has administration of thymic hormones.

Most patients with DiGeorge syndrome show a deletion in chromosome 22, region q11,[1,19] although this anomaly is not required for diagnosis.[7] The q11 region of chromosome 22 deletion is also associated with velocardiofacial syndrome (VFS) and other syndromes.[20]

Ataxia-Telangiectasia (AT)

Ataxia-telangiectasia (AT) is a rare autosomal recessive syndrome characterized by cerebellar ataxia (involuntary muscle movements) and telangiectasias (capillary swelling resulting in red blotches on the skin), especially on the earlobes and conjunctiva. Blood vessels in the sclera of the eyes may be dilated and there may also be a reddish butterfly area on the face and ears. Ninety-five percent of patients exhibit increased levels of serum alpha-fetoprotein.[11] The incidence of this disease is between 1:10,000 to 1:100,000, although as much as 1% of the population is heterozygous for the gene.[21] Abnormal genes produce a combined defect of both humoral and cellular immunity.[7,21] Antibody response to antigens, especially polysaccharides, is blunted. The levels of IgG2, IgA, and IgE are often low or absent, although the pattern can be quite variable. In addition, the number of circulating T cells is often decreased. Death usually occurs in early adult life from either pulmonary disease or malignancy.[22]

Patients with AT have a defect in a gene that is apparently essential to the recombination process for genes in the immunoglobulin superfamily. The *AT* gene is located on chromosome 11, region q22. This abnormality results in a defective kinase involved in DNA repair and in cell cycle control.[15] Rearrangement of TCR and immunoglobulin genes does not occur normally.[11] Patients' lymphocytes often exhibit chromosomal breaks and other abnormalities involving the TCR genes in T cells and immunoglobulin genes in B cells. These are sites of high levels of chromosomal recombination and errors that occur during gene rearrangements may not be repaired properly. The syndrome is associated with an even greater risk of lymphoid malignancy than other immunodeficiency syndromes, presumably because the failure to properly repair DNA damage leads to the accumulation of mutations. The only effective therapy for AT is allogeneic bone marrow transplantation.

Category 4: Diseases of Immune Dysregulation

Category 4 includes many diseases with normal numbers of T or B cells but with reduced control over their functions. Many of the diseases in the category also have features of autoimmunity.[1] The autoimmune lymphoproliferative syndrome (ALPS), for example, may involve mutations in genes coding for caspase enzymes involved in apoptosis. Defective apoptosis in the thymus may lead to autoreactive cells in the circulation. The CD25 deficiency is manifested by a lack of T regulatory (Treg) cells, which leads to lymphoproliferation and autoimmunity. Mutations in the *FoxP3* gene, which is required for Treg differentiation, may show a similar clinical presentation. Chediak-Higashi syndrome, an immunodeficiency with hypopigmentation (loss of skin color) caused by a mutation in the *LYST* gene, is characterized by a reduced number of natural killer (NK) cells and neutrophils, as well as an increased production of inflammatory proteins. Peripheral blood smears from patients with Chediak-Higashi syndrome show granulocytic inclusions attributed to enlarged lysosomes.[1]

Category 5: Congenital Defects of Phagocyte Number, Function, or Both

Category 5 classifies the PIDs which are characterized by abnormalities in phagocytic cells. Recall from Chapter 1 that the majority of cells that perform phagocytosis are neutrophils. Neutrophils play a crucial role in the immediate and nonspecific response to invading organisms by responding before specific antibody and cell-mediated immune responses can be mounted. In addition, neutrophils are even more effective at ingesting and killing organisms coated with specific antibody and thus continue to play an important role in host defense even after an adaptive immune response is established. To destroy invading organisms, neutrophils must adhere to vascular endothelial lining cells, migrate through the capillary wall to a site of infection, and ingest and kill the microbes (see Chapter 3). Defects affecting each of these steps can lead to an increased susceptibility to pyogenic infections.

Chronic Granulomatous Disease (CGD)

Chronic granulomatous disease (CGD) is a group of disorders involving inheritance of either an X-linked or autosomal recessive gene that affects neutrophil microbiocidal function. The X-linked disease accounts for 70% of the cases and tends to be more severe.[7] Symptoms of CGD include recurrent suppurative infections, pneumonia, osteomyelitis, draining adenopathy, liver abscesses, dermatitis, and hypergammaglobulinemia. Typically, catalase-positive organisms such as *Staphylococcus aureus, Burkholderia cepacia,* and *Chromobacterium violaceum* are involved in addition to fungi such as *Aspergillus* and *Nocardia.*[7,21] Infections usually begin before 1 year of age and the syndrome is often fatal in childhood.

CGD is the most common and best characterized of the neutrophil abnormalities. Several specific molecular defects have been described in this syndrome, all of which result in the inability of the patient's neutrophils to produce the reactive forms of oxygen necessary for normal bacterial killing. Three different autosomal recessive genes are involved and all affect subunits of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.[23] Normally, neutrophil stimulation leads to the production of reactive oxygen molecules, such as hydrogen peroxide (H_2O_2), by NADPH oxidase reactivity on the plasma membrane. The plasma membrane enfolds an organism as it is phagocytized and hydrogen peroxide is generated in close proximity to the target microbe. Neutrophil granules fuse with and release the enzyme myeloperoxidase into the forming phagosome. The myeloperoxidase uses the hydrogen peroxide to generate the potent microbicidal agent, hypochlorous acid (see Chapter 3).[23]

The process of generating partially reduced forms of oxygen by stimulated neutrophils was first detected as an increase in oxygen consumption. Therefore, this response was originally termed the neutrophil "respiratory burst." A more correct term is **oxidative burst.** A genetic defect in any of the several components of the NADPH oxidase system can result in the CGD phenotype by making the neutrophil incapable of generating an oxidative burst.

CGD was historically diagnosed by measuring the ability of a patient's neutrophils to reduce the dye nitroblue tetrazolium (NBT). NBT reduction is caused by the production of hydrogen peroxide and other reactive forms of oxygen. Reduction converts the nearly colorless NBT into a blue precipitate that can be assessed visually on a microscope slide.[23] More recently, a flow cytometric assay has been used. In this assay, neutrophils are labeled with dihydrorhodamine (DHR). DHR will fluoresce when it is reduced. The neutrophils are then activated using phorbol myristate acetate (PMA), which is mitogenic for neutrophils. The resultant oxidative burst will reduce the DHR, resulting in fluorescence that may be quantitated on a flow cytometer. Neutrophils from CGD patients will be unable to undergo the oxidative burst and show less fluorescence than normal neutrophils.[23] This technique is more objective and quantitative than the traditional NBT technique.

Although therapy with granulocyte transfusions may allow resolution of an acute infectious episode, it is impossible to provide enough granulocytes to treat the chronic condition. Administration of cytokines, such as interferon, may increase the oxidative burst activity in some patients. Continuous use of antibiotics can greatly reduce the occurrence of severe infections.[15] Bone marrow transplantation or use of peripheral blood stem cells may result in a permanent cure.[23]

Other Microbiocidal Defects

Several other recognized defects can result in impaired neutrophil microbiocidal activity. Neutrophil glucose-6-phosphate dehydrogenase deficiency leads to an inability to generate enough NADPH to supply reducing equivalents to the NADPH oxidase system. This shortfall leads to a defect in hydrogen peroxide production and a clinical picture similar to that of CGD. Myeloperoxidase deficiency is relatively common, occurring in about 1 in 3,000 persons in the United States. Deficient patients may have recurrent candidal infections. Defects of neutrophil secondary granules have been described also. However, the molecular nature of the defects is unknown.[13]

Leukocyte Adhesion Deficiency (LAD)

Even if microbiocidal activity is normal, neutrophils cannot perform their functions properly if they fail to leave the vasculature and migrate to a site of incipient infection. Adhesion receptors on leukocytes and their counterreceptors on endothelial cells and the extracellular matrix play important roles in these activities. In leukocyte adhesion deficiency (LAD), there is a deficiency in a protein called CD18, which is a component of adhesion receptors on neutrophils and monocytes (CD11b or CD11c) and on T cells (CD11a).[7,23] The CD18 deficiency is transmitted through autosomal recessive inheritance and has variable expression. This defect leads to abnormal adhesion, motility, aggregation, chemotaxis, and endocytosis by the affected leukocytes. The defects are clinically manifest as delayed wound healing, chronic skin infections, intestinal and respiratory tract infections, and periodontitis. A defect in CD18 can be diagnosed by detecting a decreased amount of the CD11/18 antigen on patient leukocytes by flow cytometry.[23]

Another type of adhesion molecule deficiency (LAD II) has also been characterized. In this disorder, a carbohydrate molecule involved in adhesive interactions, CD15s, or sialyl-Lewis X, is deficient.[23]

Category 6: Defects in Innate Immunity

Category 6 represents a new part of the PID classification, mirroring the explosive increase in knowledge and understanding of the innate immune system. At least one disease, chronic mucocutaneous candidiasis, which was previously classified as a T-cell defect, is now included in this classification.[1] Researchers identified two forms of this entity involving mutations in genes coding for IL-17. Other rare entities classified under this heading include mutations in Toll-like receptors (TLRs). For example, TLR3 deficiency results in herpes simplex encephalitis. Defects in TLR signaling pathways, such as IRAK4 deficiency, can also occur. Both types of defects can lead to bacterial infections. Few clinical laboratory assays are currently available for assessing innate immune system functional capabilities. Diagnosing these entities is based upon clinical presentation, which leads to molecular analyses to identify specific genetic mutations.

Category 7: Autoinflammatory Disorders

Autoinflammatory disorders are subdivided into two classifications: those involving the inflammasome and noninflammasome conditions. The **inflammasome** is a protein oligomer that contains caspase enzymes and other proteins associated with apoptosis. The inflammasome is located primarily in myeloid cells and may be activated by various microbial substances. Once activated, the inflammasome stimulates the production of the proinflammatory cytokines IL-1 and IL-18.[24]

Genetic defects involving the inflammasome include the Hyper IgD syndrome, also referred to as periodic fever syndrome, and Muckle-Wells syndrome. Hyper IgD is caused by

a deficiency of mevalonate kinase, an enzyme involved in a sterol synthesis pathway. The syndrome has been seen primarily in northern European populations. Diagnosis includes clinical presentation of recurrent fevers, followed by IgD testing. Muckle-Wells syndrome is caused by a mutation in the *CIAS1* gene coding for cryopyrin, a component of the inflammasome. Patients may present with urticaria and amyloidosis. Molecular assays are necessary to confirm the diagnosis.[1]

Defects not involving the inflammasome include tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) and early-onset inflammatory bowel disease (IBD). TRAPS is caused by a mutation in the *TNFRSF1A* gene, which codes for a TNF receptor and may result in recurrent fevers, as well as ocular and joint inflammation. Early-onset IBD is caused by mutations in genes coding for IL-10 or its receptor.[1]

Family history may be helpful in diagnosing diseases of Category 7. Muckle-Wells syndrome and TRAPS show autosomal dominant inheritance, whereas Hyper IgD and early-onset IBD are autosomal recessive.

Category 8: Complement Deficiencies

Complement consists of a series of proteins that work in a cascade to assist in antibody destruction of cells, as described in Chapter 7. The complement system is also part of the innate immune system and can work as part of the inflammatory system to directly eliminate a potential pathogen. Deficiencies in each of the major complement components have been described, leading to various clinical sequalae.[25]

Deficiencies in the early complement components, C1q, C4, and C2, are usually associated with a lupuslike syndrome. Deficiency of C2 is believed to be the most common complement component deficiency. A C3 deficiency may also have a lupus-like clinical presentation, but is more likely to involve recurrent infections with encapsulated organisms. Deficiencies of the later components of complement (C5–C9) are often associated with recurrent *Neisseria meningitidis* infections. A deficiency of C1 esterase inhibitor has been found in patients with hereditary neuroangioedema. Most complement deficiencies appear to be inherited in an autosomal recessive manner and are likely caused by random changes in DNA nucleotide sequence as opposed to genetic deletions.[6]

Category 9: Phenocopies of Primary Immunodeficiencies

This category comprises a new classification of PIDs. Disorders that fall into this category have an inherited genetic component but also include an acquired component, such as somatic mutations or autoantibody production.[1] New knowledge of these factors has led to reclassification of some PIDs. For example, chronic mucocutaneous candidiasis, a disease that was once classified as a cell-mediated deficiency, is now included in Category 9. This disease is induced by a genetic mutation in the *AIRE* gene, but also involves an antibody to either (or both) IL-17 and IL-22.[1] Mutations in the *nRAS* or *kRAS* genes are also associated with diseases that fall into this category.

Laboratory Evaluation of Immune Dysfunction

When performing diagnostic testing for immunodeficiency, it is important for laboratorians to compare the results for a patient with appropriate age-matched controls. In tests of cellular function, the patient's cells need to be tested in parallel with cells from a normal control. If an abnormal test result is obtained, it should be confirmed by repeat testing.

Screening Tests

Screening tests are used for the initial evaluation of a suspected immunodeficiency state. Most of these tests can be performed routinely in any hospital laboratory. The evaluation of possible immunodeficiency starts with a patient history, followed by a complete blood count (CBC) and white blood cell (WBC) differential, which may reveal a reduced lymphocyte count. Thrombocytopenia with small platelets can be detected in WAS.

Measurement of the levels of serum IgG, IgM, and IgA and levels of the subclasses of IgG are used to screen for defects in antibody production. Assay for isohemagglutinins is easily performed by the transfusion service. By the age of 2, a child should have naturally occurring IgM antibodies against ABO blood group antigens. The absence of these antibodies suggests an abnormal IgM response.

An overall assessment of antibody-mediated immunity can be made by measuring antibody responses to antigens to which the population is exposed normally or following vaccination. This can be easily done by measuring the titer of the specific antibody produced in response to immunization with a commercial vaccine such as diphtheria/tetanus. In an unimmunized child, the development of tetanus or diphtheria antibodies is determined 2 weeks after immunization. In a previously immunized patient, the response to a booster injection can be evaluated, normally 4 to 6 weeks post vaccination. A wide range of other protein and polysaccharide antigens can also be used in these tests. This technique is often used to evaluate a possible IgG subclass deficiency. IgG1 and IgG3 isotypes normally respond to protein antigens, such as tetanus and diphtheria. IgG2 normally responds to polysaccharide antigens, such as those in the *H influenzae* and *S pneumoniae* vaccines.[6]

Delayed hypersensitivity-type skin reactions can be used to screen for defects in cell-mediated immunity (see Chapter 14). These tests are generally performed by the clinician and not by laboratory personnel. Delayed cutaneous hypersensitivity is a localized cell-mediated reaction to a specific antigen. The prototype is the tuberculin skin test. An antigen to which most of the population has been exposed, such as candida, mumps, or tetanus toxoid, is injected intradermally. The presence of induration 48 to 72 hours later indicates a cell-mediated immune response. A negative test is not always informative because the patient may not have been previously exposed to the test antigen. Researchers have recently developed in vitro assays to measure cell-mediated immunity, which are detailed in the text that follows.

Screening for complement deficiencies usually begins with a CH50 assay.[25] This procedure determines the level of functional complement in an individual. Undetectable CH50 levels may indicate a deficiency of a specific component. Based upon the clinical history, individual component assays would be indicated. The laboratorian should be aware, however, that low CH50 levels may be caused by complement consumption and do not, by themselves, indicate a complement deficiency.

Defects in neutrophil oxidative burst activity may be detected by a flow cytometric assay as previously mentioned. Neutrophils labeled with DHR are stimulated to undergo an oxidative burst by exposure to a mitogen. The oxidative burst will reduce the DHR, producing fluorescence, which can be measured objectively by flow cytometry. Flow cytometry can also be used to confirm a diagnosis of LAD type 1 by looking for the expression of the CD18 antigen.

Confirmatory Tests

If the screening tests detect an abnormality or the clinical suspicion is high, more specialized testing will probably be necessary to precisely identify an immune abnormality. Some of the tests used for confirming an immunodeficiency state are listed in **Table 19–3.**

Enumeration of classes of lymphocytes in the peripheral blood is performed by flow cytometry. Even though types of lymphocytes cannot be distinguished morphologically, they exhibit different patterns of antigen or surface immunoglobulin expression that correlate with functional characteristics. Before flow cytometric analysis, antibodies to antigens specific for different types of lymphocytes are labeled with a fluorescent probe. These antigens are generally referred to by a cluster of differentiation (CD) number (see Chapters 1 and 13). The antibodies are allowed to react with the patient's peripheral blood mononuclear cells in a direct immunofluorescence assay and RBCs in the sample are lysed. The flow cytometer is used to count the WBCs that are labeled with each fluorescent antibody. Lymphocytes can then be assigned to specific types based on antigen expression: B cells (CD19), T cells (CD3), T helper (Th) cells (CD3/CD4), cytotoxic T cells (CD3/CD8), and NK cells (CD16 or CD56). Flow cytometry is objective and quite reliable in detecting those defects that result in a decrease in one or more types of lymphocytes. For example, an absence or profound decrease in the number of CD3 cells would be consistent with DiGeorge syndrome. An absence of CD19+ B cells suggests Btk deficiency. One should remember, however, that in the actual clinical arena, secondary immunodeficiencies are more common than PIDs. For example, in the laboratory of the author (TA), the most common cause of absent B cells is not Btk deficiency, but patients treated with rituximab, a monoclonal anti-CD20 antibody. This antibody, used to treat leukemic, transplant, and autoimmune patients, destroys B cells, resulting in no detectable CD19+ or CD20+ cells. **Figure 19–2** is a flow cytometry histogram from a patient treated with rituximab. Note that no CD19+ B cells are detectable.

Table 19–3 Specialized Confirmatory Tests for Immunodeficiencies

SUSPECTED DISORDER	SPECIALIZED TESTS
Humoral immunity	B-cell counts by flow cytometry B-cell proliferation in vitro (e.g., mitogen assays) Histology of lymphoid tissues
Cell-mediated immunity	T-cell counts by flow cytometry (total and T-cell subsets) T-cell function in vitro (e.g., mitogen assays) Quantiferon TB assay Cylex ImmuKnow assay Enzyme assays (ADA, PNP)
Phagocyte defects	Leukocyte adhesion molecule analysis (CD11a, CD11b, CD11c, CD18) Phagocytosis and bacterial killing assays Chemotaxis assay Enzyme assays (myeloperoxidase, glucose-6-phosphate dehydrogenase, components of NADPH oxidase)
Complement	Specific component assays

NADPH = nicotinamide adenine dinucleotide phosphate.

Most of the genes associated with PIDs have been identified and localized. Thus, genetic testing is available for many conditions, including the DiGeorge deletion, the Wiskott-Aldrich gene, and the IL2RG mutations. Although genetic testing is useful to understand the pathology of the disease, it is often not required for making a diagnosis. Genetic testing of family members of affected patients may be helpful in determining who may be at risk of developing the disease or passing it on to offspring.

T-cell function can be measured by assessing the ability of isolated T cells to proliferate in response to an antigenic stimulus or to T-cell mitogens in culture, such as phytohemagglutinin (PHA) or Concanavalin A (Con A). A **mitogen** is a substance that stimulates mitosis in all T cells or all B cells, regardless of antigen specificity. Classically, the T-cell response may be measured by quantitating the uptake of radioactive thymidine, a precursor of DNA. Increased thymidine uptake suggests cell division and activation. This assay requires experienced technologists, a radioactive materials license, and laboratory-determined reference ranges.

More recently, antigen- or mitogen-stimulated T-cell activation has been measured without the use of radioactive materials. The FDA has cleared three such assays for diagnostic use. The Quantiferon TB assay and the T-Spot assay measure an individual's response to *Mycobacterium tuberculosis* antigens. Following overnight whole blood activation with TB antigens, gamma interferon secreted by activated Th1 cells, is quantitated by either an enzyme-linked immunosorbent assay (ELISA) or ELISPOT procedure. Either of these assays may be used as an in vitro assessment of exposure to *M tuberculosis*.

The third assay, the Cylex ImmuKnow assay, measures total T-cell activity. This test uses the mitogen PHA to activate T cells.

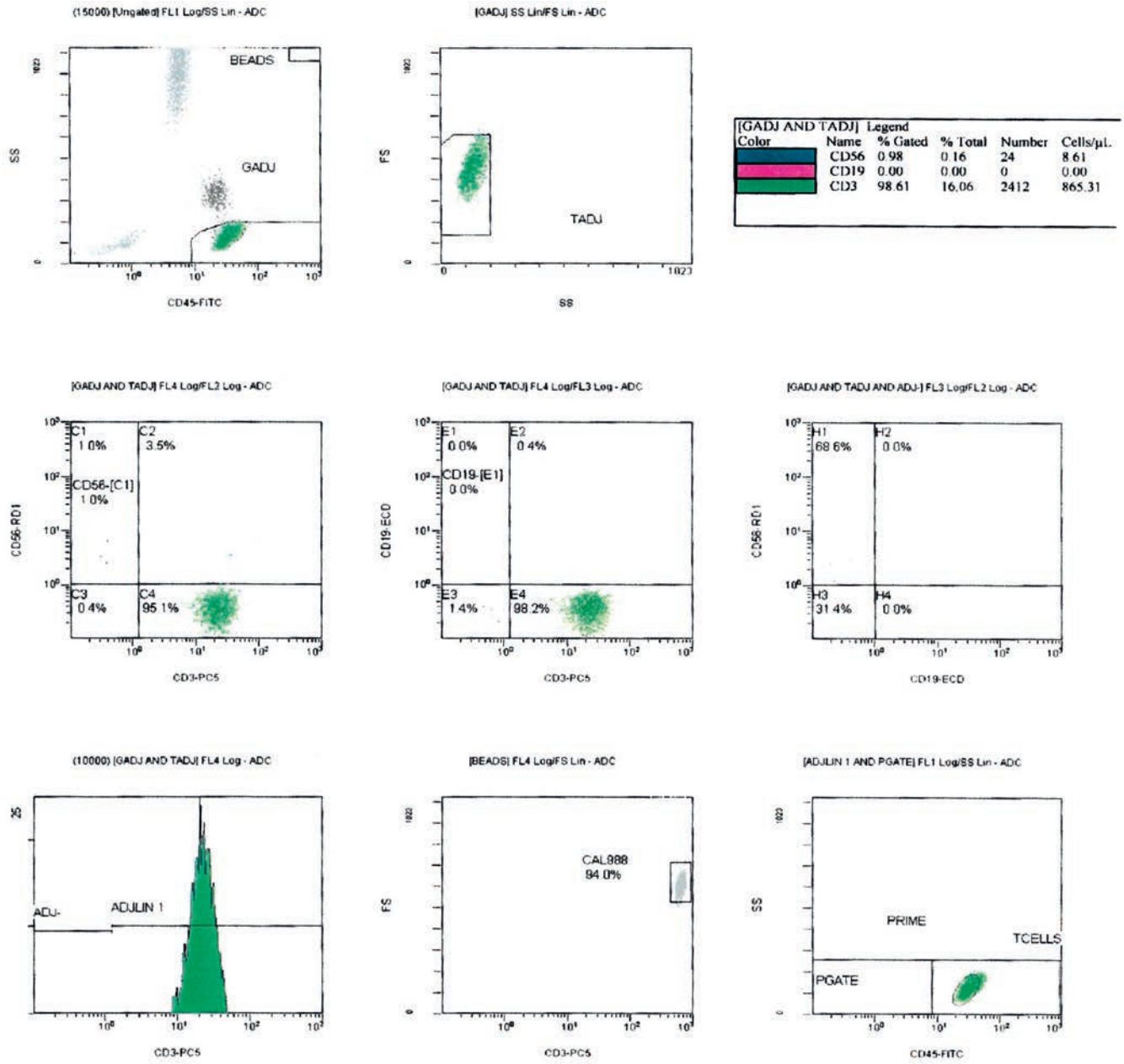

FIGURE 19–2 Flow cytometry histogram of peripheral blood stained with antibodies to CD3, CD19, and CD56. Note that no CD19+ cells are detected in this patient sample. *(Courtesy of Dr. Thomas Alexander.)*

Following incubation, ATP production is measured by a fluorescent immunoassay technique. This test is a general measurement of T-cell function and is often used to monitor individuals receiving immunosuppressive therapy. The assay may be used to determine overall T-cell functional capabilities in an individual suspected of a PID. PHA is also used as a positive control in the Quantiferon and T-Spot procedures.

Newborn Screening for Immunodeficiencies

Several states have begun to include PID testing as part of their newborn screening programs.[26] This testing is typically performed to look for the presence of TCR excision circles (TRECs).[27] TRECs, identified by quantitative PCR (see Chapter 12), are present in T cells that have undergone alpha-beta receptor gene rearrangements. They are the genetic material that has been removed from the germline DNA during alpha VJ and beta VDJ recombination **(Fig. 19–3).** Their absence indicates a lack of functional T cells, allowing early identification of T-cell related defects leading to SCID. DiGeorge and other non-SCID diseases, such as Omenn syndrome, have also been detected using this method. Another technique not normally applied to newborn screening, whole exome sequencing, has been used to identify AT in two infants and will likely see increased use in the future.[28]

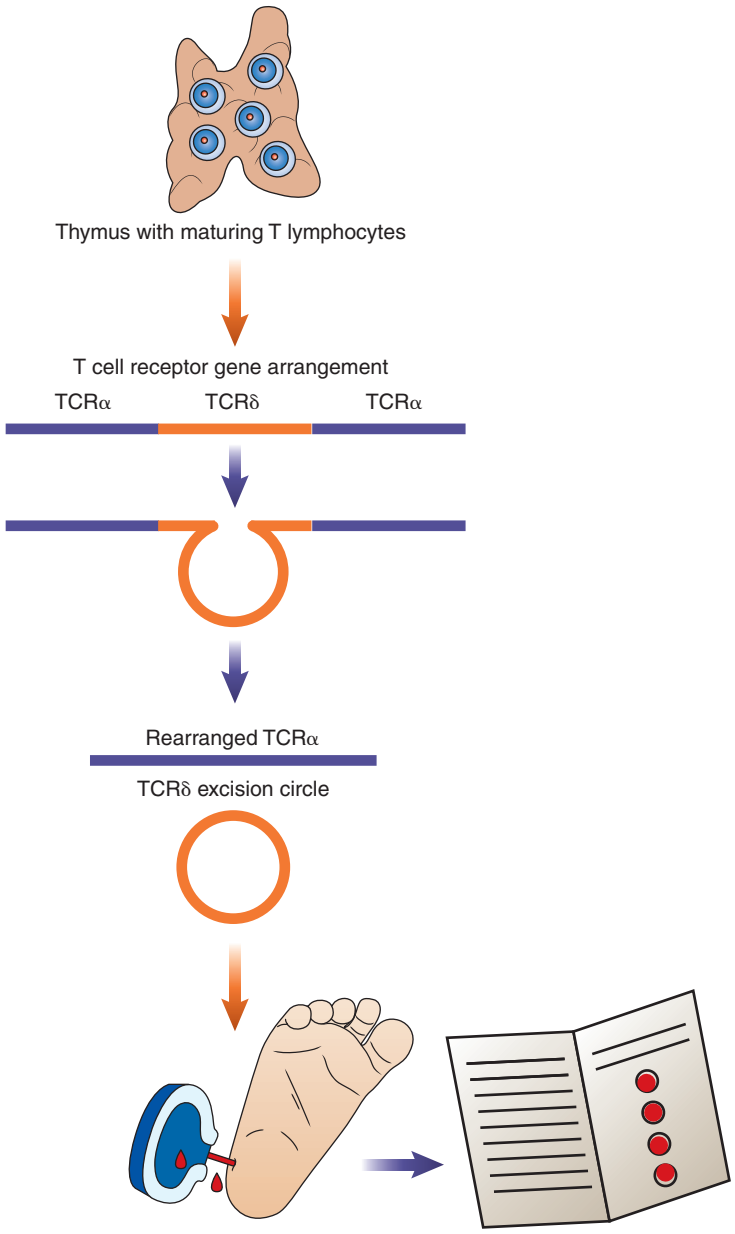

FIGURE 19–3 Newborn testing for T-cell receptor excision circles (TRECs) formed by normal T-cell receptor gene rearrangement.

Connections

T-Cell Receptors (TCRs)

Recall from Chapter 12 that the TCR for antigen is composed of two different chains: an α chain and a β chain. During T-cell maturation, rearrangement of specific V and J genes that code for the variable region of the α chain and specific V, D, and J genes that code for the variable region of the β chain occur to produce a TCR that is specific for a particular antigen (Fig. 19–3). In the process, the intervening sequences are cut out to form circles called TRECs.

Evaluation of Immunoglobulins

Quantitative measurement of serum immunoglobulins is used in the workup of both immunodeficiency states and some lymphoproliferative disorders. This is usually performed by assays involving nephelometry, but may also be performed by radial immunodiffusion (RID). In addition, serum protein electrophoresis (SPE) can be quantitative if the total serum protein is determined and the results are read using a densitometer.

Serum Protein Electrophoresis (SPE)

SPE is a technique in which molecules are separated on the basis of their size and electrical charge. SPE allows reproducible separation of the major plasma proteins. See Chapter 5 for details. The serum protein electrophoretic profile is traditionally divided into five regions: albumin, alpha$_1$ globulins, alpha$_2$ globulins, beta globulins, and gamma globulins. Some laboratories now use six regions, dividing the beta region into the beta$_1$ (transferrin) and beta$_2$ (complement component C3) regions. IgG, IgD, and IgE migrate in the gamma globulin region, whereas IgM and IgA overlap the beta and gamma regions. Immunoglobulins normally show a range of mobilities.

Additional evaluation of serum immunoglobulin is performed if the SPE shows a monoclonal component or if there is a significant quantitative abnormality of serum immunoglobulins. Classically, SPE has been performed using agarose gels. More recently, SPE may be performed using capillary electrophoresis (described in Chapter 12), which eliminates the need for gels and the drying and staining processes associated with the agarose-based technique.

Immunofixation Electrophoresis (IFE)

Another method of characterizing immunodeficiencies is **immunofixation electrophoresis (IFE)** (see Chapter 18). In IFE, serum samples are electrophoresed, just as for SPE, and then specific antibody is applied directly to the separating gel. The antibody–antigen complexes form and are visualized by staining. Polyclonal immunoglobulins are indicated by areas of diffuse staining, whereas monoclonal immunoglobulins produce narrow, intensely stained bands (see Fig. 19–4 later in the Case Studies). Lack of bands indicates immunodeficiency of one or more immunoglobulin classes. For accurate interpretations, specific immunoglobulin isotype levels, determined by nephelometry, are necessary.

Bone Marrow Biopsy

A bone marrow aspirate and biopsy is indicated in any evaluation of a monoclonal gammopathy or immunodeficiency state. It is important in establishing the diagnosis of such disorders and for excluding other diseases. The bone marrow specimen will be analyzed microscopically and by flow cytometry. Cytogenetic analysis may also be performed to detect the specific genetic anomalies associated with the PID diseases.[9,14,29]

Family History

The importance of obtaining a full family history as part of the PID diagnosis process cannot be overemphasized. The mode of inheritance, if known, can rule in or rule out many of the PIDs. The disease may be X-linked, such as in the common gamma chain mutation, WAS, or the CD154 deficiency; autosomal dominant, such as in Hyper IgE or Muckle-Wells syndrome; or autosomal recessive, such as in AT or LAD. CGD may be either X-linked or autosomal recessive. Although obtaining a family history is not the responsibility of the clinical immunology laboratory, these diseases are not identified solely by laboratory testing. A team approach among the laboratory, clinicians, medical geneticists, and the family is required for a final diagnosis. Laboratory results are most useful when integrated into the entire clinical picture. Laboratories should not test in a vacuum!

SUMMARY

- Immunodeficiencies are disorders in which part of the body's immune system is missing or dysfunctional. There are two types of immunodeficiencies—primary (i.e., inherited), and secondary (acquired because of other factors such as infections, malignancies, or immunosuppressive drugs).
- Defects in the development and regulation of individual parts of the immune system can affect the humoral or cell-mediated branches of the adaptive immune system or various components of the innate defense system, such as the phagocytic cells or the complement system.
- The primary immunodeficiencies (PIDs) have been classified into nine categories based on their clinical features, immunologic defects, and genetic abnormalities.
- Defects in antibody-mediated immunity typically lead to recurrent infections with pyogenic bacteria, particularly of the respiratory and intestinal tracts.
- Defects in T-cell–mediated immunity generally lead to recurrent infections with intracellular pathogens, such as viruses, fungi, and intracellular bacteria.
- Defects in phagocyte function generally lead to pyogenic bacterial infections, often of the skin. Immunodeficiency states range from quite mild to lethal.
- Therapy to reconstitute the deficient immune component must begin as soon as possible in an attempt to prevent permanent organ damage or death caused by infection.
- PIDs must be suspected clinically and are diagnosed with the help of screening tests to measure leukocyte counts and immunoglobulin levels, followed by specialized laboratory testing, such as flow cytometry, to determine numbers of specific lymphocyte subsets.
- Laboratory results correlate with the presence of a specific PID. For example, patients with Bruton's tyrosine kinase (Btk) deficiency typically have decreased serum concentrations of all the immunoglobulins and decreased numbers of CD19+ B cells, whereas patients with chronic granulomatous disease (CGD) would be expected to have decreased fluorescence in the DHR assay, which measures oxidative burst in neutrophils.
- Newborn screening is available to detect SCID, a combined immunodeficiency disease involving defective T-cell function. The screening assay looks for the absence of TRECs, T-cell receptor excision circles that have been removed during normal gene rearrangements that occur during T-cell development.

Study Guide: Classification of Primary Immunodeficiencies

CATEGORY	DESCRIPTION	PROTOTYPIC DISEASES
1. Combined immunodeficiencies	Low to absent humoral and cellular capabilities	SCID (common gamma chain deficiency, *JAK3* deficiency, ADA deficiency, MHC deficiencies) PNP deficiency
2. Combined immunodeficiencies with associated or syndromic features	Low to absent humoral and cellular capabilities with additional, nonimmunologic anomalies	Wiskott-Aldrich syndrome, ataxia-telangiectasia, DiGeorge syndrome
3. Predominantly antibody deficiencies	One or more immunoglobulin isotypes are decreased	Bruton's thymidine kinase (Btk) deficiency, transient hypogammaglobulinemia of infancy, common variable immunodeficiency, selective IgA deficiency, IgG subclass deficiencies
4. Diseases of immune dysregulation	Loss of T regulatory or other controlling mechanisms	Chediak-Higashi syndrome, autoimmune lymphoproliferative syndrome, CD25 deficiency
5. Congenital defects of phagocyte number, function, or both	Reduction in phagocytic function, number,or both	Chronic granulomatous disease, cyclic neutropenia, leukocyte adhesion deficiency
6. Defects in innate immunity	Interruption in signaling pathways of innate immune cells	Chronic mucocutaneous candidiasis, TLR3 deficiency, IRAK4 deficiency
7. Autoinflammatory disorders	Diseases usually associated with recurrent fevers with or without infections	Hyper IgD syndrome, familial Mediterranean fever, TNF receptor associated periodic syndrome
8. Complement deficiencies	Loss of individual components	C2 deficiency, C3 deficiency, C6 deficiency, hereditary angioedema
9. Phenocopies of PID	PID associated with an acquired mutation or autoantibody	Autoimmune lymphoproliferative syndrome, chronic mucocutaneous candidiasis

Adapted from Al-Herz W, Bousfiha A, Casanova J-L, et al. Primary immunodeficiency diseases: an update on classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency. Front. Immunol. *2014;5(162):1–33.*

Study Guide: Screening Tests for Immunodeficiencies

SUSPECTED DISORDER	SCREENING TESTS
All immunodeficiencies	Complete blood cell count, white blood cell differential count
Humoral immunity	Serum IgG, IgA, IgM levels, IgG subclass levels, isohemagglutinin titers (IgM), IgG antibody response to protein and polysaccharide antigens
Cell-mediated immunity	Delayed hypersensitivity skin tests (i.e., Candida, diphtheria, tetanus, PPD) Chest x-ray (thymus shadow) TREC screening
Phagocyte defect	DHR reduction test NBT dye test
Complement	CH50 (classical pathway) Serum complement levels (e.g., C3, C4)

NBT = nitroblue tetrazolium; PPD = purified protein derivative.

CASE STUDIES

1. A 7-month-old male child was diagnosed with bacterial meningitis. Previously he had been hospitalized with bacterial pneumonia. Laboratory testing results were as follows: RBC count: normal; WBC count: 22×10^9/L (normal is $5–24 \times 10^9$/L); differential: 70% neutrophils, 15% monocytes, 5% eosinophils, and 10% lymphocytes; and SPE: no gamma band present.

Questions

a. What possible conditions do these results indicate?
b. How are these conditions inherited?
c. What type of further testing do you recommend?

2. A 37-year-old female presents with a history of recurrent upper respiratory infections. She states that she was always a sick child, usually with respiratory infections, but occasional diarrhea would also occur. She has received countless antibiotic regimens over the years. The physician orders a SPE and immunoglobulin levels. The SPE is read as a low gamma level with no monoclonal proteins detected. Levels of IgG, IgM, and IgA are below the reference ranges. The physician then orders an immunofixation assay.

Question

a. **Figure 19–4** contains four patient immunofixations. Which pattern would be most representative of the expected pattern for this patient?
b. Explain why you chose this answer.

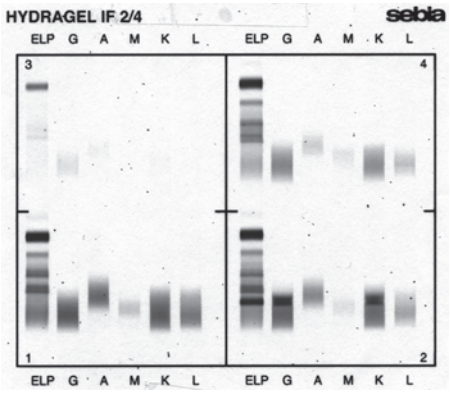

FIGURE 19–4 Four different immunofixation patterns.

REVIEW QUESTIONS

1. Patients with which immunodeficiency syndrome should receive irradiated blood products to protect against the development of GVHD?
   a. Bruton's thymidine kinase (Btk) deficiency
   b. Selective IgA deficiency
   c. SCID
   d. CGD

2. T-cell subset enumeration by flow cytometry would be most useful in making the diagnosis of which disorder?
   a. Bruton's thymidine kinase (Btk) deficiency
   b. Selective IgA deficiency
   c. SCID
   d. Multiple myeloma

3. What clinical manifestation would be seen in a patient with myeloperoxidase deficiency?
   a. Defective T-cell function
   b. Inability to produce IgG
   c. Defective NK cell function
   d. Defective neutrophil function

4. Defects in which branch of the immune system are most commonly associated with severe illness after administration of live virus vaccines?
   a. Cell-mediated immunity
   b. Humoral immunity
   c. Complement
   d. Phagocytic cells

5. Which of the following statements applies to Bruton's thymidine kinase (Btk) deficiency?
   a. It typically appears in females.
   b. There is a lack of circulating CD19+ B cells.
   c. T cells are abnormal.
   d. There is a lack of pre-B cells in the bone marrow.

6. DiGeorge anomaly may be characterized by all of the following *except*
   a. autosomal recessive inheritance.
   b. cardiac abnormalities.
   c. parathyroid hypoplasia.
   d. decreased number of mature T cells.

7. A 3-year-old boy is hospitalized because of recurrent bouts of pneumonia. Laboratory tests are run and the following findings are noted: prolonged bleeding time, decreased platelet count, increased level of serum alpha-fetoprotein, and a deficiency of naturally occurring isohemagglutinins. Based on these results, which is the most likely diagnosis?
   a. PNP deficiency
   b. Selective IgA deficiency
   c. SCID
   d. Wiskott-Aldrich syndrome

8. Which of the following is (are) associated with ataxia-telangiectasia?
   a. Inherited as an autosomal recessive
   b. Defect in both cellular and humoral immunity
   c. Chromosomal breaks in lymphocytes
   d. All of the above

9. A 4-year-old boy presents with recurrent wound and soft-tissue infections. Which of the following assays should be considered for diagnosing his presumed PID?
   a. DHR reduction
   b. CD4 quantitation
   c. CD19 quantitation
   d. CD56 quantitation

10. A patient with a deficiency in complement component C7 would likely present with
    a. recurrent *Staphylococcal* infections.
    b. recurrent *Neisserial* infections.
    c. recurrent *E coli* infections.
    d. recurrent *Nocardia* infections.

11. A *FoxP3* gene mutation may lead to a deficiency of what cell type?
    a. T helper cells
    b. T cytotoxic cells
    c. B cells
    d. T regulatory cells

12. The Cylex ImmunoKnow assay is useful in determining functional capabilities of which cell type?
    a. T cells
    b. B cells
    c. NK cells
    d. Neutrophils

13. Recurrent, periodic fevers may be associated with increased production of which immunoglobulin?
    a. IgG
    b. IgM
    c. IgD
    d. IgE

14. Chronic mucocutaneous candidiasis, a PID that was previously thought to be a cell-mediated deficiency, is now classified as which type of PID?
    a. Autoinflammatory disorder
    b. Complement deficiency
    c. Predominantly antibody deficiency
    d. Innate immunity deficiency

15. Prenatal screening for SCID involves detecting
    a. Tregs.
    b. TRECS.
    c. THELPS.
    d. TCYTOS.

IV

Serological and Molecular Diagnosis of Infectious Disease

20 Serological and Molecular Detection of Bacterial Infections

James L. Vossler, MS, MLS(ASCP)SM

LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Differentiate between commensalistic, mutualistic, and parasitic relationships.
2. Differentiate between pathogenicity, virulence, and infectivity.
3. Cite structural features of bacteria that contribute to increased virulence.
4. Describe host defenses against bacteria and the means by which bacteria can evade the immune system.
5. Compare and contrast endotoxins and exotoxins with respect to biological activity, immunogenicity, and the genetic encoding for the production of the two toxin categories.
6. Cite the five general laboratory means of detecting the causative agent of a bacterial infection.
7. Explain the principle of lateral flow immunochromatographic assays.
8. List the exotoxins produced by Group A streptococci and the roles they play in contributing to the virulence of *Streptococcus pyogenes.*
9. Describe the symptoms and pathogenesis of acute rheumatic fever and poststreptococcal glomerulonephritis.
10. Explain the principle, interpretation, and clinical significance of the antistreptolysin O (ASO), antideoxyribonuclease B (anti-DNase B), and streptozyme tests.
11. Recognize the role *Helicobacter pylori* plays in gastrointestinal ulcers and the virulence factors that contribute to infection by this organism.
12. Discuss the various types of tests that may be performed to detect *H pylori* infection.
13. Describe the respiratory and dermatological manifestations of *Mycoplasma pneumoniae* infections.
14. Discuss the use of serology for the diagnosis of *M pneumoniae* infections, including the clinical value of detecting cold agglutinins.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

15. Describe the epidemiology of Rocky Mountain spotted fever with respect to etiologic agent, transmission, and pathogenesis.
16. Select the appropriate serological and molecular techniques to diagnose Rocky Mountain spotted fever.

KEY TERMS

Acute rheumatic fever
Anti-DNase B
ASO titer
Commensalistic
Endotoxin
Exotoxin
Group A streptococci (GAS)
Helicobacter pylori
Impetigo
Indigenous microbiota
Infectivity
Lancefield groups
Lateral flow immunochromatographic assay (LFA)
Microbiome
Mutualistic
Mycoplasma pneumoniae
Parasitic
Pathogenicity
Plasmids
Poststreptococcal glomerulonephritis
Rickettsiae
Rocky Mountain spotted fever (RMSF)
Scarlet fever
Streptococcus pyogenes
Streptolysin O
Streptozyme
Symbiotic
Typhus
Urease
Virulence
Virulence factors

The collection of microorganisms that exists on the body—bacteria, viruses, and single-celled prokaryotic organisms (e.g., yeast and fungi)—is referred to as the human **microbiome.** The bacteria comprising the human microbiome keep us healthy in many ways. They protect us against disease-causing bacteria, aid in digesting our food, produce certain vitamins, and stimulate both the innate and adaptive immune systems. The establishment of an organism that leads to host injury is referred to as an "infection." When a microbe causes damage to host cells or altered physiology that results in clinical signs and symptoms of disease, the phrase "infectious disease" applies. Traditional means of determining the cause of a bacterial infection have relied largely on growing the organism in culture and using stains to view the organism under the microscope. These infections can also be identified by immunoassays that detect bacterial antigens or antibodies and by sophisticated molecular techniques that detect nucleic acid from the organisms. This chapter will begin with an introduction to the human–microbe relationship and factors that influence the interactions between bacteria and the immune system. The chapter will then discuss laboratory methods that are commonly used to detect bacterial infections in the context of selected pathogenic bacteria.

Human–Microbe Relationships

When an individual is born, a dynamic relationship begins between the human host and the bacteria in the environment. Very quickly, various bacteria establish themselves on the surfaces of an individual, including the gastrointestinal tract, creating a **symbiotic** relationship. The bacteria and the host "live together," often maintaining a long-term interaction. Symbiotic bacteria that reside on and colonize these surfaces are referred to as the **indigenous microbiota** (previously known as "normal flora"). The bacterial populations that exist on the body are not homogenous; they vary in composition and numbers, depending on the area of the body.

The microbial populations outnumber human cells by ten to one.[1] Collectively, they may account for 2 to 6 pounds of an individual's body weight.[2] Although it was previously thought that a relatively limited number of bacteria make up the human microbiome, we now know that the microbial community is actually very diverse. Furthermore, over 90% of the bacteria that comprise the human microbiome cannot be cultured in vitro, most likely because their growth depends on specific conditions or substances that have not been duplicated in the laboratory.[3]

Our relationship with our indigenous microbiota exists through co-evolution, co-adaptation, and codependency between the bacteria and the host. For a microorganism to survive, the organism needs to colonize the host and acquire nutrients. Importantly, it must not stimulate the host's immune response (in the case of the indigenous microbiota) or it must avoid or circumvent the immune responses. Once established, it needs to be able to replicate and disseminate to a preferred site in the body for survival and eventually be transmitted to a new susceptible host.

Three types of symbiotic relationships can exist between humans and bacteria. In **commensalistic** relationships, there is no apparent benefit or harm to either organism. The indigenous microbiota is often referred to as "commensals" or the "commensalistic bacteria," describing bacteria that are recovered in culture that do not represent a pathogen. An example of a commensalistic organism is *Staphylococcus epidermidis,* which colonizes and inhabits the human skin. In a **mutualistic** relationship, both humans and the bacteria benefit. One example is the *Lactobacillus* species that colonizes the epithelial surfaces of the vaginal canal. The human host provides conditions (temperature, atmosphere, nutrients) that allow the bacteria to grow and multiply; in exchange, the bacteria produce lactic acid, which prevents colonization of bacteria and yeast that may cause disease. Although the vast majority of the interactions between the host and members of the microbiome are harmless, the encounter with specific organisms or viruses occasionally results in harm to the host. In this case, a **parasitic** relationship exists between the other organisms and the host.

As previously mentioned, the establishment of an organism that leads to host injury is referred to as an *infection.* Although there is harm to the host, not all infections are symptomatic. In many instances, the infection may be *subclinical;* in other words, there are no signs or symptoms. An example is infection with *Chlamydia trachomatis,* a sexually transmitted organism. Only about 10% of infected males and 5% to 30% of infected females will show symptoms when infected with *C trachomatis.*[4,5] The organism may then be transmitted to other individuals or may result in complications such as pelvic inflammatory disease in women.

Several terms are used to describe the interaction between the infecting organisms and the host. The terms *infectivity, pathogenicity,* and *virulence* are often used interchangeably; however, each term has different meanings when discussing an organism's ability to cause an infection. **Infectivity** refers to an organism's ability to establish an infection. More specifically, infectivity describes the proportion of individuals exposed to a pathogen through horizontal transmission (i.e., person-to-person spread) who will become infected. Another term that is used with similar meaning is *contagious.* For example, the measles virus is extremely contagious and has a high degree of infectivity.

Pathogenicity refers to the inherent capacity of an organism to cause disease. This is a qualitative trait of the organism determined by its genetic makeup. Some organisms, such as the human immunodeficiency virus (HIV), are considered to be primary or true pathogens that are capable of causing harm to a majority of individuals that have intact immune systems. Although an organism may be pathogenic in nature, it may not always cause disease. The outcome of the host–pathogen interaction is determined by several factors, including the host's immunologic status. Some microorganisms may only cause disease or infection in individuals who have compromised immune systems because of factors such as chemotherapy, radiation therapy, or various chronic diseases. These organisms are referred to as "opportunistic pathogens."

Virulence is a quantitative trait that refers to the extent of damage, or pathology, caused by the organism. For example, *Yersinia pestis,* the causative agent of bubonic and pneumonic plague, is considered to be extremely virulent and is likely to cause severe illness and death upon infection unless antibiotics are administered. If the bacterial strain is not capable of causing disease, the organism is said to be "avirulent." Not all members of a bacterial species are necessarily capable of causing disease. For example, *Escherichia coli* resides as commensal bacteria in the gastrointestinal tract but only some strains of *E coli* are capable of causing diarrheal disease.

The degree of damage is mediated by specific virulence factors. **Virulence factors** may increase an organism's pathogenicity by contributing to the organism's ability to establish itself on or in the host, invade or damage host tissue, or evade the host immune response.

Bacterial Virulence Factors

Bacterial properties or features that determine whether an organism is pathogenic and able to cause disease are referred to as "virulence factors." Factors that increase a bacterium's virulence may be classified as either structural components (e.g., endotoxin is a component of the cell walls of certain bacteria) or as extracellular substances produced by the bacteria, such as exotoxins. Various types of bacterial virulence factors are discussed in the sections that follow.

For an organism to be pathogenic, it needs to possess genetic determinants that allow for production of either the structural components or the extracellular products that contribute to its virulence. Genetic determinants located on the bacterial chromosome are generally responsible for the production of structural or surface molecules, which help the organism to attach to and colonize the host. The genetic information needed to produce the extracellular substances that are virulence factors is most frequently located on independent genetic elements called plasmids. **Plasmids** are self-replicating genetic elements that are located in the bacteria's cytoplasm and contain a limited number of genes. In addition, plasmids are mobile genetic elements that can be transferred between bacteria through various mechanisms. Acquisition of exogenous DNA that codes for the production of virulence factors can convert an avirulent strain into a virulent strain.

Structural Virulence Features

Bacterial cells are classified as prokaryotic cells, whereas human cells are classified as eukaryotic cells. Although prokaryotic

bacterial cells are relatively simple, they have a well-developed cell structure and contain a number of structural and genetic features that are not found in eukaryotic cells. One significant difference is that the bacterial chromosome is not enclosed inside of a membrane-bound nucleus, but instead resides inside the bacterial cytoplasm. Also housed in the cytoplasm are internal cellular structures, such as ribosomes, mesosomes, and potentially plasmids, as well as other cytoplasmic inclusion bodies. Not present in bacterial cells are membrane-bound organelles such as the mitochondria found in eukaryotic cells **(Fig. 20–1).**

Other significant differences between eukaryotic and prokaryotic cells are features of the cell membrane and the presence of a cell wall in bacteria. Similar to the plasma membrane in eukaryotic cells, the bacterial plasma membrane plays a regulatory role in the transport of molecules in and out of the cell. The cell membrane in bacteria also contains various enzyme systems responsible for energy generation. In eukaryotic cells (except for plants and fungi) the plasma membrane is the outer surface of the cell. In contrast, bacteria have a cell wall as their outermost feature. The bacterial cell wall prevents osmotic lysis and confers shape and rigidity to the bacteria. Peptidoglycan is the primary component providing the shape and rigidity. The majority of antibiotics used to treat bacterial infections target the production of peptidoglycan in the cell wall.

The bacterial cell wall structure has two different variations, which are classified as either gram-positive or gram-negative depending on their staining characteristics. Both gram-positive and gram-negative cell walls have features that increase an organism's virulence, as we will discuss later. One of the features found in the cell wall of gram-negative bacteria is the lipopolysaccharide (LPS) layer. There are three components that make up LPS—an outer core polysaccharide, an inner core polysaccharide, and lipid A. When released from a dead or dying cell, a portion of LPS called lipid A is exposed. Lipid A, also referred to as **endotoxin,** is a powerful stimulator of the cytokine system that leads to a variety of systemic manifestations and potentially fatal endotoxic (gram-negative) shock (discussed later in this chapter).

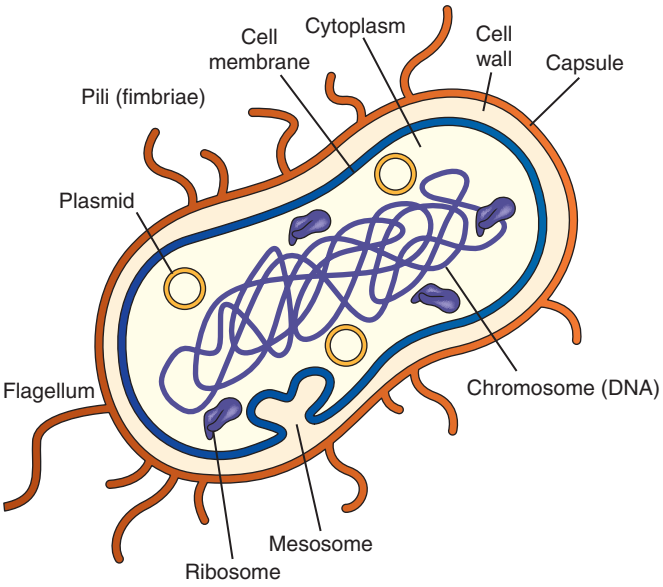

FIGURE 20–1 Cross section of a bacterial cell.

To be successful in establishing an infection, bacteria must first adhere to and colonize the host tissue. Furthermore, to persist in the host, they must be capable of evading the immune system. Structural features are generally involved in the adherence of bacteria or the evasion of the immune system. Whiplike structures called flagella can facilitate adherence as well as movement of the bacteria toward a host cell.

Bacteria have surface structures or molecules that can bind specifically to complementary receptors on specific cells of host tissues. The most common surface structures involved in attachment are fimbriae, which are composed of protein. The fimbriae involved in specific attachment to prokaryotic surface ligands are referred to as the common pili. Other pili used to exchange genetic information, called the F or sex pili, are fewer in number than common pili. The sex pili may also function in the attachment to host cells.

In addition to allowing the organism to attach to and colonize tissues, pili play a role in resisting phagocytosis by white blood cells (WBCs) and may undergo antigenic variation to evade the adaptive immune response. For example, the ability of *Neisseria gonorrhoeae* to rearrange portions of its genome can change the antigenic makeup of its pili, allowing them to adhere specifically to human mucosal epithelial cells and resist phagocytosis. Another example is the ability of ***Streptococcus pyogenes***-associated pili to attach to host cells because of its production of F protein, which attaches to fibronectin. *S pyogenes* also can resist phagocytosis because of the production of M protein. Most strains of *E coli* that colonize the gastrointestinal tract do not adhere to epithelial cells of the small intestine. The ability of certain strains of *E coli* to cause gastrointestinal disease is associated with the expression of specific pili. Enterotoxigenic *E coli* (ETEC) strains have factor antigen I (CFA) pili that adhere to the cells in the small intestine; the organism then produces the toxins that cause diarrhea.

Attachment may also occur via adhesions or surface molecules on the bacterial cell. Attachment caused by the presence of surface molecules is referred to as afimbrial (non-pili dependent) attachment. Host cell receptors recognized by bacterial surface molecules include proteoglycans, laminins, collagens, elastin, and hyaluronan. Glycoproteins such as fibrinogen, fibronectin, and vitronectin are also potential receptors for bacteria.

A major structural feature that plays an important role in increasing an organism's virulence is the presence of a capsule, which is usually polysaccharide in nature. Capsules contribute to the organism's ability to resist innate and adaptive immune responses through a variety of means. They can block the attachment of antibodies, inhibit activation of complement, or act as a decoy when capsular material is released into the surrounding host environment. One of the most important features of a capsule is its role in blocking phagocytosis by WBCs. For example, *Streptococcus pneumoniae's* primary

determinant of virulence is a polysaccharide capsule that prevents the ingestion of pneumococci by alveolar macrophages and elaborates capsular antigens to which immunoglobulins bind. Strains of bacteria that do not possess a capsule are, in most cases, avirulent and not able to produce disease.

Extracellular Virulence Factors

Extracellular substances produced by bacteria also contribute to an organism's virulence by breaking down primary or secondary defenses of the body, damaging the host tissue and cells, or facilitating the growth and spread of the organism. Substances that perform the latter function are called *invasins.* Several of the invasins include hyaluronidase, collagenase, phospholipases, lecithinases, coagulase, and various kinases.

Endotoxin and Exotoxins

Bacteria may also produce two types of toxins—endotoxin and exotoxins. Endotoxin (lipid A) is found in the LPS layer of the cell walls of all gram-negative bacteria. When the cell dies, the LPS layer is removed from the cell, releasing endotoxin (lipid A) in the host. The effects of endotoxin are complex. Endotoxin induces a variety of host responses, including the production of cytokines such as IL-1, IL-6, IL-8, tumor necrosis factor (TNF), and platelet-activating factor, which stimulate production of prostaglandins and leukotrienes. This results in inflammation, increased heart rate, increased body temperature (fever), and a decrease in blood pressure. In addition, endotoxin activates the complement cascade, resulting in the formation of the anaphylatoxins C3a and C5a, which cause vasodilation and increase vascular permeability. The alternative coagulation cascade is also activated, producing coagulation, thrombosis, and acute disseminated intravascular coagulation (DIC), leading to hemorrhage and shock. A potential consequence of endotoxin release is septic shock, a life-threatening illness that is usually the result of a gram-negative *bacteremia,* or presence of bacteria in the blood. With septic shock, there is a large scale release of inflammatory mediators that results in massive vasodilation and hypotension. Some refer to this as a "cytokine storm." If the condition worsens, there is widespread organ damage including renal failure, liver dysfunction, heart damage, and eventual death. Although immunogenic, endotoxin does not elicit a protective immune response, so no vaccine is available against this bacterial component.

Unlike endotoxin, which has multiple effects on the body, **exotoxins** have a very specific and targeted activity. They are protein molecules that are released from living bacteria (mostly gram-positive bacteria) and are considered to be some of the most potent molecules known to harm living organisms. Exotoxins may be classified as neurotoxins, cytotoxins, or enterotoxins, according to their effect on cells. Exotoxins bind to specific receptors on host cells. Most exotoxins have several subunits that bind to the receptor (B subunits) and a subunit that is actually responsible for the specific activity of the toxin (A subunit). An example of a cytotoxin is the diphtheria toxin, which interferes with protein synthesis in epithelial cells. Neurotoxins include the tetanus toxin, which prevents the release of inhibitory transmitters from the presynaptic membrane of neuromuscular cells, leading to continuous excitement of muscle cells and spasms. Botulism toxin works in a reverse manner—the toxin prevents the release of acetylcholine (ACh), resulting in paralysis of the motor system. Examples of enterotoxins are toxins A and B produced by *Clostridium difficile,* which cause fluid secretion (diarrhea), mucosal injury, and inflammation **(Fig. 20–2).** Exotoxins are extremely immunogenic and induce production of protective antibodies. Inactivated exotoxins called "toxoids" are used for some vaccines. For example, the toxin of *Corynebacterium diphtheria* is used in the vaccine to prevent diphtheria.

Some exotoxins may act as "superantigens." Unlike other antigens, these "superantigens" are not processed by antigen-presenting cells (APCs). Instead, they bind directly to class II

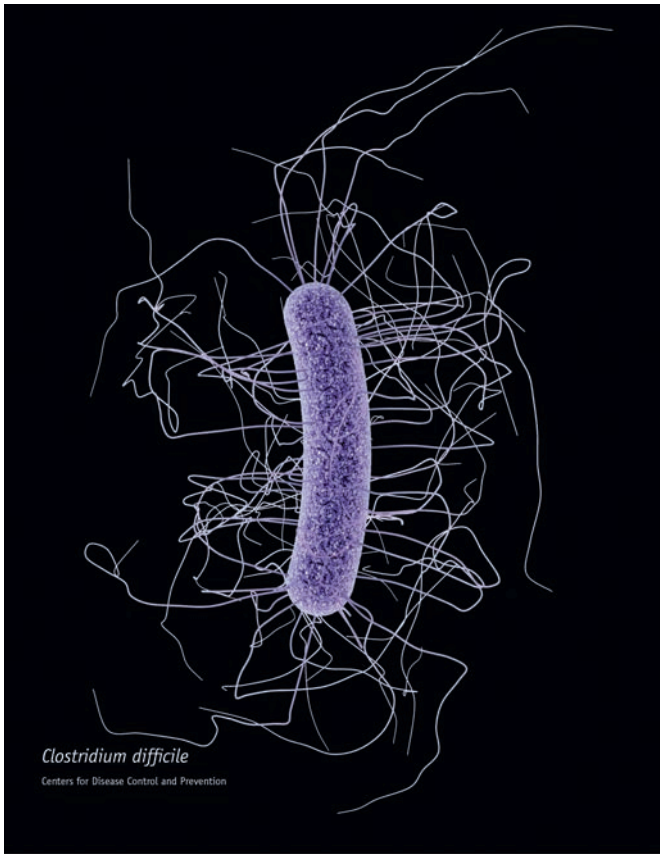

FIGURE 20–2 A three-dimensional (3D) computer-generated image of a single gram-positive *Clostridium difficile* bacillus. *(Courtesy of the CDC/James Archer. Public Health Image Library.)*

Clinical Correlations

Capsular Antigens and Vaccine Development

Most capsules evoke a strong humoral immune response and are used for the development of vaccines. For example, the vaccines for *Haemophilus influenzae* type b, *S pneumonia,* and certain types of *Neisseria meningitidis* consist of antigens derived from bacterial capsules (see Chapter 25).

major histocompatibility complexes (MHC II) on APCs outside of the normal antigen-binding groove. The MHC II receptor binds to the T-cell receptor (TCR) with the whole antigen (toxin) attached. Normally, only 0.0001% of T cells are activated in an immune response. However, a "superantigen" induces activation of up to 20% of T cells, resulting in a massive release of cytokines.[6] The systemic events brought on by the release of large amounts of cytokines leads to what is referred to as "toxic shock syndrome." Examples include the TSST-1 toxin produced by *Staphylococcus aureus* and the superantigens produced by *S pyogenes,* the cause of streptococcal toxic shock syndrome (STSS).

Immune Defenses Against Bacterial Infections and Mechanisms of Evasion

Immune Defense Mechanisms

Although bacteria may possess various features that increase their virulence, they must be able to circumvent or overcome the host's defense mechanisms. As discussed in previous chapters, both innate and adaptive responses may occur after an encounter with foreign antigens.

The first line of defense against potential pathogens is intact skin and mucosal surfaces that serve as structural barriers. In addition, the epithelial surface may have enzymes and nonspecific antimicrobial defense peptides (ADPs) and proteins that have antimicrobial activity. One example of an enzyme with specific antimicrobial activity is lysozyme, which is found in many secretions, including tears and saliva. Lysozyme destroys the peptidoglycan found in the cell wall of bacteria, especially gram-positive bacteria. Other enzymes include ribonucleases, which destroy RNA and have antimicrobial and antiviral activities. The body excretes a wide variety of ADPs and proteins that play a role in the innate defenses of the body. Some of the ADPs and proteins are only secreted by specific tissues or cells. One group of soluble peptides is the defensin peptides. *Defensins* are produced constitutively by the cells in the body.

The three main classes of defensins are alpha, beta, and theta. Alpha defensins are produced by neutrophils, certain macrophage populations, and Paneth cells of the small intestine. This class of defensins is believed to disrupt the microbial membrane. Beta defensins are produced by neutrophils as well as epithelial cells lining the various organs, including the bronchial tree and genitourinary system. They are believed to increase resistance of epithelial cells to colonization. Theta defensins are not found in humans.

Many antimicrobial proteins contribute to the innate immune response. For example, complement proteins can promote chemotaxis. Interleukins are involved in the regulation of immune responses and inflammatory reactions. Prostaglandins are involved in the dilation and constriction of blood vessels and modulation of inflammation. Leukotrienes are involved in inflammation and fever.

Acute phase reactants also play important roles. For example, C-reactive protein (CRP) activates the complement system and promotes phagocytosis by macrophages. Haptoglobin binds free plasma hemoglobin, which deprives the bacteria of iron. Ceruloplasmin is a glycoprotein with bactericidal activities.

Bacteria, fungi, and viruses possess pathogen-associated molecular patterns (PAMPs), which are structural patterns consisting of carbohydrates, nucleic acids, or bacterial peptides. PAMPs are recognized by pattern recognition receptors (PRRs) expressed on the cells of the innate immune system. The engagement of the PRR with the appropriate PAMP triggers the release of immune mediators such as cytokines and chemokines, boosts production of various defensins and proteins, and initiates phagocytosis. The phagocytic process is enhanced by the activation of the alternative complement cascade, which is triggered by microbial cell walls or other products of microbial metabolism.

Adaptive immune responses include the production of antibodies directed against bacterial antigens or extracellular products produced by bacteria such as exotoxin. Antibody formation is the main defense against extracellular bacteria. The binding of antibodies to invading bacteria is referred to as opsonization (see Chapter 4).

Cell-mediated immunity (CMI), the other branch of the adaptive immune response, is helpful in attacking intracellular bacteria, such as *Mycobacterium tuberculosis, Legionella pneumophila, Listeria monocytogenes,* and *Rickettsia* species. Through the mechanism of delayed type hypersensitivity, CD4 T cells produce cytokines, which activate macrophages to release enzymes that destroy the bacteria (see Chapter 14). The recruitment of inflammatory cells results in the formation of granulomas that surround the bacteria-infected cells to help prevent the spread of infection. Cytotoxic T cells are also recruited to the site of infection and mount an antigen-specific attack on the infected cells.

Bacterial Evasion Mechanisms

Bacteria have developed several ways to inhibit the immune system or make it more difficult for immune responses to occur. Three main mechanisms used by bacteria involve avoiding antibody, blocking phagocytosis, and inactivating the complement cascade **(Fig. 20–3).** Bacteria can evade antibodies by altering their bacterial antigens, a process called antigenic

Connections

Toll-Like Receptors (TLRs)

Recall from Chapter 3 that one of the main groups of PRRs are the Toll-like receptors (TLRs). TLRs are expressed on key cells of the innate immune system such as macrophages and dendritic cells. They recognize molecules that are commonly found in microbial pathogens but not on host cells. Once TLRs have bound to their ligands, cell-signaling pathways are triggered that result in the production of cytokines that enhance the inflammatory response, resulting in more efficient pathogen destruction.

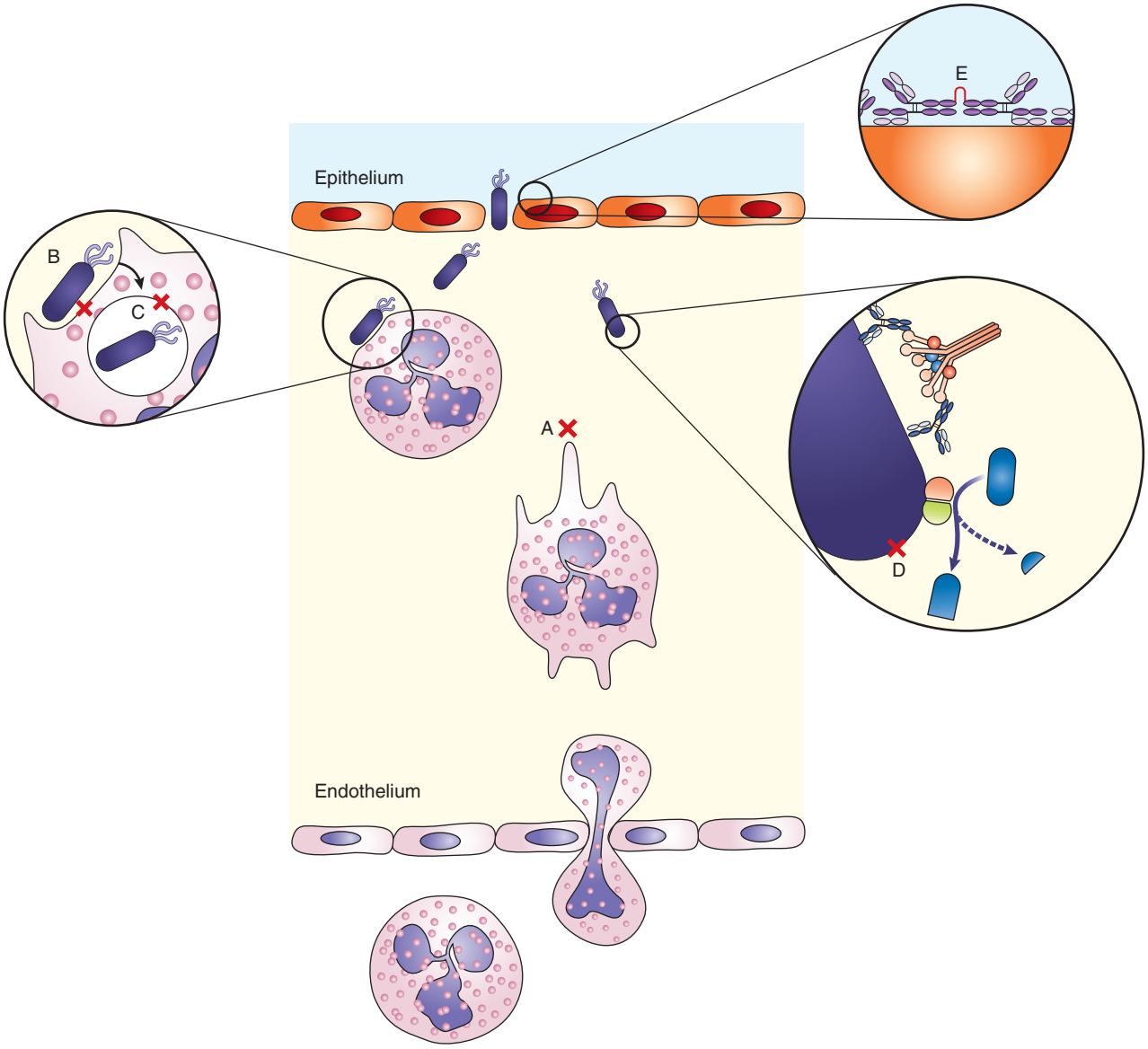

FIGURE 20–3 The strategies bacteria use to evade host defenses. (A) Inhibiting chemotaxis. (B) Blocking adherence of phagocytes to the bacterial cells. (C) Blocking digestion. (D) Inhibiting complement C3b binding. (E) Cleaving IgA.

variation. They can also coat themselves with host proteins such as fibrin or immunoglobulin molecules (*S aureus*) or fibronectin (*Treponema pallidum*), or hide their surface molecules through antigenic disguise (the hyaluronic acid capsule of *S pyogenes*). Bacteria can also evade the specific immune response through downregulation of MHC molecules and production of proteases that degrade IgA.[7] *N gonorrhoeae*, *H influenzae*, and *Streptococcus sanguinis* are all examples of bacteria that can cleave IgA.[8,9]

Most of the evasion mechanisms target the process of phagocytosis. Bacteria can mount a defense at several stages in the phagocytic process, including chemotaxis, adhesion, and digestion.[7,8] Some pathogens such as *N gonorrhoeae*, for example, inhibit the release of chemotactic factors that would bring phagocytic cells to the area. The cell walls of *S pyogenes* produce an M protein that interferes with adhesion to the phagocytic cell. Additionally, the presence of a polysaccharide capsule found in such organisms as *N meningitidis*, *S pneumoniae*, *Y pestis*, and *H influenzae* inhibits the binding of neutrophils and macrophages needed to initiate phagocytosis.[9]

Microorganisms use several different mechanisms to resist digestion. Some bacteria can block fusion of lysosomal granules with phagosomes after being engulfed by the phagocyte. *Salmonella* species are able to do this, as can *M tuberculosis* and *Mycobacterium leprae*. In *M tuberculosis* and *M leprae* infection, each bacillus is contained in a membrane-enclosed fluid compartment called a pristiophorus vacuole (PV), which does not fuse with the lysosomes because of the complexity of the acid-fast cell walls.

An additional mechanism of resisting digestion involves the production of extracellular products after the bacteria are phagocytized. The primary effect is the release of lysosomal contents into the cytoplasm of the phagocytic cells, subsequently killing the WBC. Examples include leukocidin, produced by *S aureus;*

listeriolysin O, produced by *L monocytogenes;* and streptolysin, produced by *S pyogenes.*[8]

The last major defense some bacteria use is to block the action of complement. Organisms mentioned previously that produce a capsule do not bind the complement component C3b, which is important in enhancing phagocytosis.[9] Such organisms cannot easily be phagocytized unless coated by opsonins. Additionally, some organisms express molecules that disrupt one or more of the complement pathways. Protein H, produced by *S pyogenes,* binds to C1 but does not allow the complement cascade to proceed further.[9] Another example is *Streptococcus agalactiae,* also known as Group B streptococcus or GBS. GBS has a capsule that is rich in sialic acid (a common component of host cell glycoproteins), causing degradation of C3b and making the organism resistant to complement-mediated phagocytosis.

Laboratory Detection and Diagnosis of Bacterial Infections

Five general ways can be used to detect the causative agent of a bacterial infection: (1) culture or growth of the causative agent, (2) microscopy, (3) detection of bacterial antigens in the clinical sample, (4) molecular detection of bacterial DNA or RNA, and (5) serology, or detection of antibodies produced in response to the infection.

Bacterial Culture

Traditional means of determining the cause of a bacterial infection rely largely on growing the organism in culture. Various broth and solid media may be used to recover the organism. Some media may contain substances that enhance the growth of certain organisms and are referred to as enriched media. Selective media contain substances or antibiotics that suppress the growth of commensalistic bacteria and support the growth of other bacteria. Differential media contain substrates that allow for the differentiation of bacteria based on their ability to use the substrate. For example, MacConkey agar selects for gram-negative bacteria and differentiates between lactose and nonlactose fermenting bacteria. Some organisms, such as *Bordetella pertussis,* the causative agent of whooping cough, have very specific growth requirements for which specialized media must be used.

Although culture is the primary laboratory means of diagnosing bacterial infections, the culturing of bacterial pathogens has limitations. There are a number of bacterial pathogens for which clinically useful culture systems are not available. For other organisms, recovery in culture may take too long to be clinically useful. For example, although ***Mycoplasma pneumoniae*,** a leading cause of community acquired pneumonia, can be cultured, culturing is a challenge. The organism is extremely fastidious (difficult to grow) and may take weeks to recover in the laboratory. Other organisms present a danger to the laboratory technologist if they are not grown and handled using the most rigorous safety precautions (e.g., *Y pestis*).

Microscopic Visualization

Visualization of the causative agents using microscopic techniques is most often done using differential or fluorescent stains. Examples include the Gram stain for differentiating gram-positive and gram-negative bacteria **(Fig. 20–4),** the acid-fast stain for the detection of *Mycobacteria tuberculosis* **(Fig. 20–5),** and the Giemsa stain for the detection of the causative agents of malaria. Direct fluorescent antibody assay, or DFA, involves the use of antibody conjugated with a fluorescent label to detect specific bacteria in a sample. Currently, many DFAs are being replaced by molecular tests because they lack sensitivity or because the reagents are not widely available. Although the various staining methods are not difficult to perform, a trained microscopist is necessary for proper interpretation. Another limitation of microscopy is that not all organisms may be visualized through microscopic means.

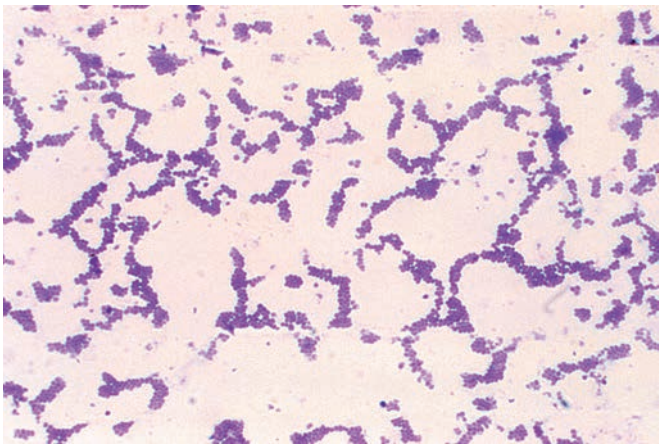

FIGURE 20–4 Photomicrograph of spherical (cocci) gram-positive *S aureus* bacteria magnified 320X. *(Courtesy of the CDC/Dr. Richard Facklam, Public Health Image Library.)*

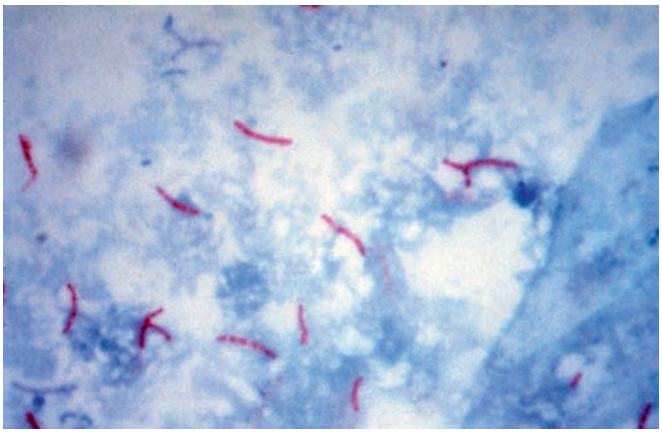

FIGURE 20–5 Photomicrograph of *Mycobacterium tuberculosis* bacteria using acid-fast Ziehl-Neelsen stain; magnified 1000X. The acid-fast stains depend on the ability of mycobacteria to retain dye when treated with mineral acid or an acid–alcohol solution. *(Courtesy of the CDC/Dr. George P. Kubica, Public Health Image Library.)*

Antigen Detection

Antigen detection assays are available for a wide variety of bacteria, viruses, parasites, and fungi in clinical samples. Testing methodologies include latex agglutination (LA), enzyme-linked immunosorbent assay (ELISA), and lateral flow immunochromatographic (LFA) assays (discussed later in this chapter). The LA and LFA assays are advantageous because of the relative ease by which the tests can be performed, their low cost, and the rapid turnaround time. Many of the LA and LFA assays are classified as "CLIA waived" tests. Under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), simple, low-risk tests can be "waived" (i.e., laboratories performing the testing are not subject to routine inspection) and may be performed in physicians' offices and various other locations. Bacteria and viruses for which antigen detection is widely used include *S pyogenes* (strep throat), *L pneumophila* (Legionnaire's disease), rotavirus (pediatric diarrheal disease), respiratory syncytial virus, and influenza A and B. Antigen detection assays are highly specific, and because of advances in technology, sensitivities of the assays have improved dramatically. In many cases, antigen detection assays, particularly LFA, have replaced other methods used to detect infections with bacteria, viruses, and fungi.

Molecular Detection

The rapid developments in the field of molecular diagnostics have allowed for the increased availability and use of nucleic acid-based assays in the clinical laboratory to detect pathogenic microorganisms. Compact hybridization and gene amplification assays that are easy to perform have made their way into physician office laboratories. The most widely known molecular technology is polymerase chain reaction (PCR) in which specific genetic sequences are amplified and detected. The development of real-time PCR or quantitative PCR (qPCR) has allowed for results to be obtained in a few hours, as compared with several days for traditional PCR. For some infectious agents, such as *N gonorrhoeae* and *C trachomatis,* nucleic-acid–based assays are widely available and clearly the choice for detection.

Although these assays are more sensitive than other methods, there are limitations associated with nucleic acid-based testing. At the time of this writing, there are relatively few FDA-approved assays on the market and the cost of the instrumentation and the disposables are prohibitive to many organizations. As more assays receive FDA approval and additional technological advances occur, the use of molecular-based assays for the detection of agents responsible for various infectious diseases will become even more widespread.

Serological Diagnosis

Serology has historically been used to detect and confirm infections from organisms that are difficult to grow or for which other laboratory methods of diagnosing the infection are not available. Serology may also be useful in determining the causative agent when the clinical symptoms are not specific enough to identify the cause of the infection. For certain organisms (e.g., *Anaplasma, Ehrlichia, Chlamydophila pneumoniae, Chlamydophila psittaci, Coxiella burnetii, Leptospira, Rickettia* spp., and *T pallidum*), serological testing remains useful and, in some cases, is the best means of detecting exposure to or infection with an organism. Detection of either IgM or IgG antibodies may indicate recent or previous exposure to an organism and antibody titers may be used to assess reactivation or reexposure to an infectious agent.

The primary disadvantage of using serology in diagnosis is that there is usually a delay between the start of the infection and the production of antibodies to the infecting microorganism. Although IgM antibodies may appear relatively early (7 to 10 days after exposure), some infections have a rapid course and the need to initiate therapy limits the detection of IgM antibodies as a diagnostic tool in those instances. In some cases, demonstration of a high IgG antibody titer in the initial stage of infection is diagnostic; however, the high titer may be caused by a past infection and the patient's symptoms may have an entirely different cause. When testing for the presence of IgG antibodies, it is ideal to collect serum samples during both the acute and convalescent phases of the illness so that a rising titer to the suspected pathogen can be observed. Another limitation of serology is that immunosuppressed patients may be unable to mount an antibody response.

The rest of this chapter addresses the immunologic response to several important bacteria that cause invasive disease. Serological and molecular testing play a major role in detecting and diagnosing the cause of these common pathogens.

Group A Streptococci (*Streptococcus Pyogenes*)

Classification and Structure

Streptococci are gram-positive cocci that are spherical, ovoid, or lancet-shaped organisms often arranged in pairs or chains when observed on Gram stain **(Fig. 20–6).**[10] The streptococci are initially identified by their effect on sheep red blood cells (RBCs) when grown in culture. Those that completely lyse or hemolyze the blood cells are classified as being β-hemolytic. If the organisms only partially hemolyze the cells, causing them to appear green, they are classified as being α-hemolytic. If the organisms exhibit no effect on the cells, they are referred to as being γ-hemolytic. The β-hemolytic streptococci are further classified according to a group-specific carbohydrate composition that

Connections

Antibody Response Curve

A serological pattern of IgM+, IgG– generally indicates an early stage, acute infection, whereas an IgM–, IgG+ pattern usually signifies a past exposure. The best indication of a current infection is a four-fold rise in antibody titer when comparing two serum samples collected from a patient during the beginning and later stages of the infection. This is a good time to review the typical antibody response curve shown in Chapter 5.

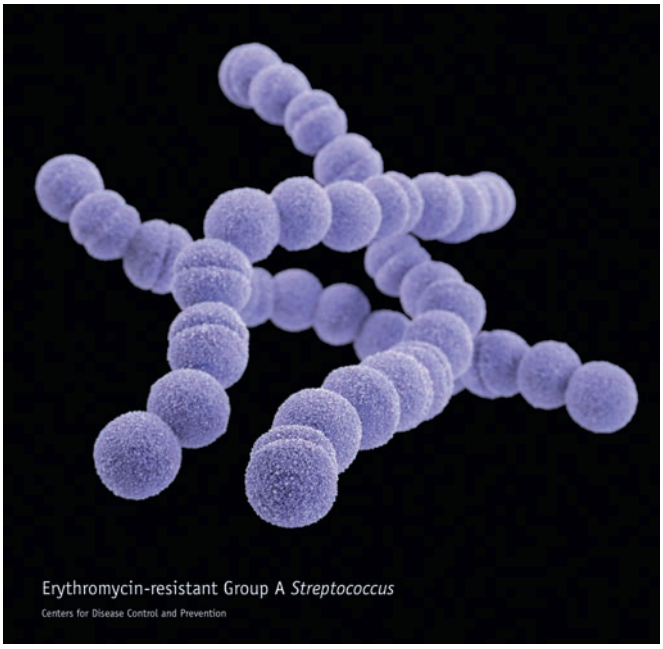

FIGURE 20–6 A three-dimensional (3D) computer-generated image of a group of erythromycin-resistant Group A streptococcus (GAS), also known as *S pyogenes* bacteria. *(Courtesy of the CDC/James Archer, Public Health Image Library.)*

divides these bacteria into 20 groups designated A through H and K through V. These are known as the **Lancefield groups,** based on the pioneering work of Dr. Rebecca Lancefield. A member of the β-hemolytic streptococci is *S pyogenes,* which includes the Group A carbohydrate. **Group A streptococci (GAS)** are a major cause of bacterial pharyngitis (a throat infection) and childhood impetigo (a skin infection). Additional cell wall components, the M and T proteins, allow for further classification and differentiation **(Fig. 20–7).** The M protein is a filamentous

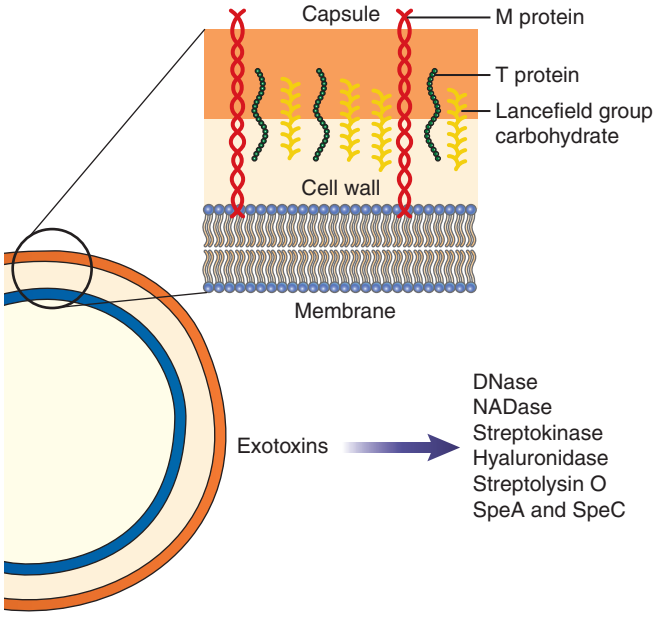

FIGURE 20–7 Diagram of antigenic components of *Streptococcus pyogenes.*

molecule consisting of two alpha-helical chains twisted into a ropelike structure that extends out from the cell surface. Some strains possess a hyaluronic acid capsule outside the cell wall that contributes to the bacterium's antiphagocytic properties.

Serotyping and molecular techniques can be used to identify a particular strain of GAS. Serotyping involves identification of the M protein antigens by precipitation with type-specific antisera. More than 80 different serotypes have been identified by this method. However, serotyping has limitations, including limited availability of typing sera, new M protein types that do not react with the antisera, and difficulty in interpreting the results.[11] Genotyping techniques involving PCR amplification of a portion of the *emm* gene, which codes for the M protein, followed by sequence analysis circumvents these problems.[12] Pulsed field gel electrophoresis (PFGE) has also been used for epidemiological studies. In PFGE, DNA from Group A streptococcus is separated by using an alternating current to obtain a unique pattern or "fingerprint." The patterns from multiple sources may be compared when there is a Group A streptococcal outbreak.[13]

Virulence Factors

GAS is one of the most common and ubiquitous pathogenic bacteria and causes a variety of infections. The M protein is the major virulence factor of GAS and has a net-negative charge at the amino-terminal end that helps to inhibit phagocytosis. In addition, the presence of the M protein limits deposition of C3 on the bacterial surface, thereby diminishing complement activation.[14,15] The M protein, along with lipoteichoic acid and protein F, help GAS attach to host cells. Immunity to GAS appears to be associated with antibodies to the M protein. There are more than 100 serotypes of this protein and immunity is serotype-specific.[15,16] Therefore, infections with one strain will not provide protection against another strain.

Additional virulence factors include various exotoxins that may be produced during the course of an infection. Pyrogenic exotoxins A, B, and C are responsible for the rash seen in scarlet fever and also appear to contribute to pathogenicity.[16,17] Additional extracellular substances include the enzymes **streptolysin O,** deoxyribonuclease B (DNase B), hyaluronidase, nicotinamide adenine dinucleotide (NAD), and streptokinase. Antibodies produced against these substances are useful in the diagnosis of infection and for the potential development of complications or sequelae associated with GAS infection (discussed later in the chapter).

Clinical Manifestations of Group A Streptococcal Infection

S pyogenes can be responsible for infections ranging from pharyngitis (a throat infection) to life-threatening illnesses such as necrotizing fasciitis and streptococcal toxic shock syndrome. The two major sites of infections in humans are the upper respiratory tract and the skin, with pharyngitis ("strep throat") and streptococcal pyoderma (a skin infection) being the most common clinical manifestations.[18] Symptoms of pharyngitis include fever, chills, severe sore throat, headache,

tonsillar exudates, petechial rash on the soft palate, and anterior cervical lymphadenopathy **(Fig. 20–8).**[18] The most common skin infection is Streptococcal pyoderma (also known as **impetigo**), characterized by vesicular lesions on the extremities that become pustular and crusted **(Fig. 20–9).** Such infections tend to occur in young children.[16] Other complications include otitis media, erysipelas, cellulitis, puerperal sepsis, and sinusitis. Septic arthritis, acute bacterial endocarditis, and meningitis also can result from a pharyngeal infection.[14,16] Humans are the primary reservoir for GAS and transmission of GAS is from person to person.

A small percentage of individuals develop **scarlet fever.** Although usually associated with pharyngeal infections, scarlet fever may occur with streptococcal infections at other sites. Symptoms include a fever of over 101°F, nausea, vomiting, headache, and abdominal pain. A distinct scarlet rash initially appears on the neck and chest and then spreads all over the body. Scarlet fever results from infection with a GAS that elaborates streptococcal pyrogenic exotoxins (erythrogenic toxins). Streptococcal pyrogenic exotoxin A (SpeA) and Streptococcal pyrogenic exotoxin C (SpeC) can act as superantigens that may induce toxic shock syndrome. Toxic shock syndrome is a life-threatening multisystem disease that often initiates as a skin or soft-tissue infection and may proceed to shock and renal failure because of overproduction of cytokines.[10,18]

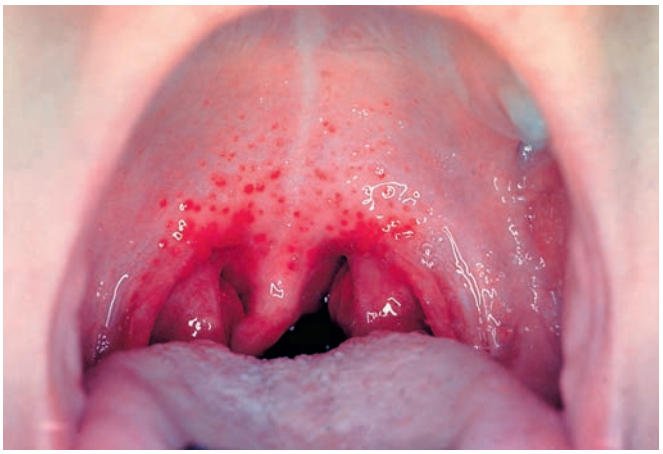

FIGURE 20–8 Pharyngitis, or sore throat, is characterized by swelling and reddening of the pharynx. Note the inflammation of the oropharynx and petechiae, or small red spots on the soft palate caused by *Streptococcal pharyngitis. (Courtesy of the CDC/Henry F. Eichenwald, Public Health Image Library.)*

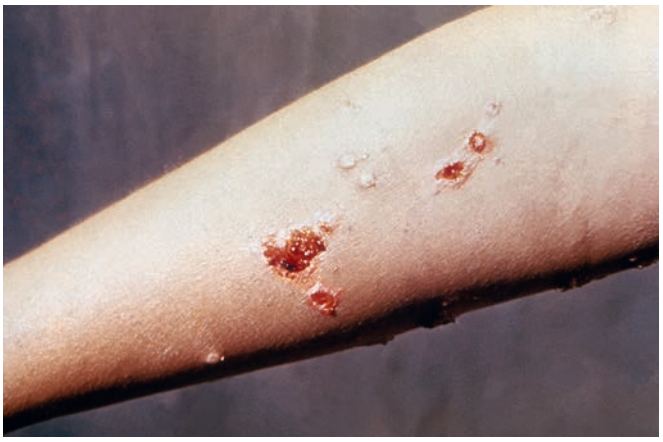

FIGURE 20–9 Impetigo is a dermatological streptococcal infection that is characterized by thick, golden-yellow discharge that dries, crusts, and sticks to the skin. It is also caused by the *S aureus* bacteria. *(Courtesy of the CDC/Dr. Herman Miranda, Public Health Image Library.)*

Necrotizing fasciitis may occur when a GAS skin infection invades the muscles in the extremities or the trunk.[19] The onset is quite acute and is a medical emergency. Exotoxins produced by *S pyogenes* cause a rapidly spreading infection deep in the fascia, resulting in ischemia, tissue necrosis, and septicemia if not treated promptly. The disease may be associated with predisposing conditions such as chronic illness in the elderly or varicella in children, but healthy persons can be affected as well.[19,20] Reporting of necrotizing fasciitis and toxic shock syndrome is part of a surveillance program conducted by the Centers for Disease Control and Prevention. Although the incidence of this syndrome has declined in the United States, a significant number of cases are still reported each year.[11]

Group A Streptococcal Sequelae

The reason GAS receives so much attention is the potential for the development of two serious sequelae, **acute rheumatic fever** and **poststreptococcal glomerulonephritis.**[10] The sequelae result from the host response to infection. Serological testing plays a major role in the diagnosis of these two diseases, because the organism itself may no longer be present by the time symptoms appear.

Acute rheumatic fever develops as a sequela to pharyngitis or tonsillitis in 2% to 3% of infected individuals. It does not occur as a result of skin infection. The latency period is typically 1 to 3 weeks after onset of the sore throat. Characteristic features of acute rheumatic fever include fever, pain caused by inflammation in the joints, and inflammation of the heart. The disease is most likely caused by antibodies or CMI originally produced against streptococcal antigens that cross-react with antigens present in human heart tissue.[10,21]

Chief among the antibodies thought to be involved are those directed toward the M proteins, which have at least three epitopes that resemble antigens in heart tissue, permitting cross-reactivity to occur. Titers of some antibodies may remain high for several years following infection.[11]

The second main complication following a streptococcal infection is acute glomerulonephritis, a condition characterized by damage to the glomeruli in the kidneys. This condition may follow infection of either the skin or the pharynx, whereas rheumatic fever follows only upper respiratory tract infections.[18] Glomerulonephritis caused by a streptococcal infection is most common in children between the ages of 2 and 12 and is especially prevalent in the winter.[22]

Symptoms of glomerulonephritis may include hematuria, proteinuria, edema, and hypertension. Patients may also experience malaise, backache, and abdominal discomfort.[23] Renal function is usually impaired because the glomerular filtration

rate is reduced, but renal failure is not typical. The most widely accepted theory for the pathogenesis of poststreptococcal glomerulonephritis is that it results from deposition of immune complexes containing streptococcal antigens in the glomeruli. These immune complexes stimulate an inflammatory response that damages the kidneys and impairs function because of release of the lysosomal contents of leukocytes and activation of complement.[10,22,23]

Laboratory Diagnosis

Culture

Diagnosis of acute streptococcal infections typically is made by culture of the organism from the infected site. The specimen is plated on sheep blood agar and incubated. If Group A streptococcus is present, small translucent colonies surrounded by a clear zone of β hemolysis will be visible **(Fig. 20–10).** Identification is made on the basis of susceptibility to bacitracin, testing for L-pyrrolidonyl-β-naphthylamide (PYR) activity, or through Lancefield typing.[18]

Detection of Group A Streptococcal Antigens

As an alternative to culture, rapid assays have been commercially developed to detect Group A streptococcal antigens directly from throat swabs. The Group A antigens are extracted by either enzymatic or chemical means and the process takes anywhere from 2 to 30 minutes, depending on the particular technique.

Lateral flow immunochromatographic assays (LFA) are increasingly being used for the detection of bacterial, viral, fungal, and parasitic antigens in clinical samples. LFA have largely replaced enzyme immunoassay (EIA) and LA assays to detect the antigens. FAs are widely used in outpatient clinics, physician offices, and urgent care facilities for the rapid diagnosis of streptococcal pharyngitis.

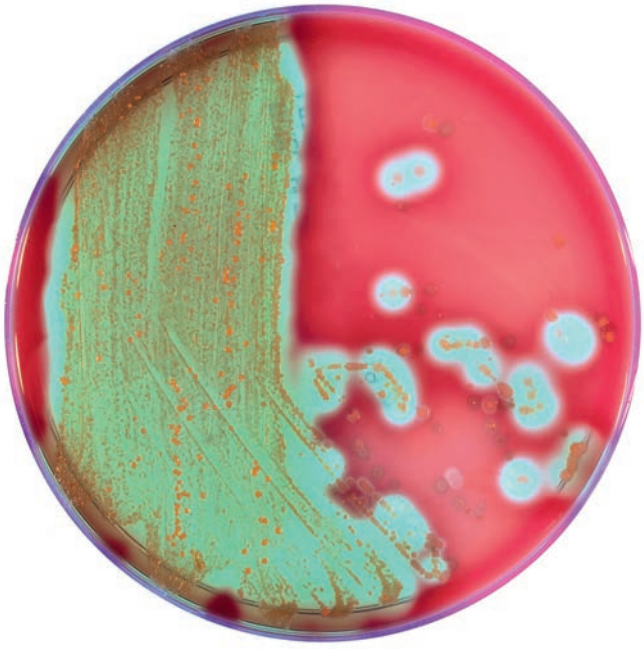

FIGURE 20–10 Throat culture plate showing a positive result for beta hemolytic Group A streptococci *(S pyogenes).* Bacterial colonies not producing beta hemolysis represent indigenous microbiota of the oropharynx. *(James Vossler.)*

The assays are technically easy to perform, allow for single sample testing because of the incorporation of an internal control, and in many cases are more sensitive than traditional laboratory methods and other antigen detection methods (see Fig. 11–4).[24] In the LFA, strep A antigen extracted from a throat swab reacts with an enzyme-labeled antibody on a test membrane. The antigen–antibody complex is captured by another antibody at a specific location on the membrane, where a colored line is produced if the sample is positive for the antigen. An example of an LFA used for the detection of GAS from a throat swab is shown in **Figure 20–11.**

Many of the assays require no more than 2 to 5 minutes of hands-on time. The specificity and sensitivity of the assays in many instances are higher than other methodologies.[25] Although the assays have high sensitivities, cultures should be performed when rapid test results are negative.[14,17] Molecular methods, including hybridization of specific rRNA sequences and real-time PCR, have also been developed as a means to rapidly detect Group A streptococcal infections.[12]

Detection of Streptococcal Antibodies

Culture or rapid screening methods are extremely useful for diagnosis of acute pharyngitis. However, serological diagnosis must be used to identify acute rheumatic fever and poststreptococcal glomerulonephritis because the organism is unlikely to be present in the pharynx or on the skin at the time symptoms appear.[18] Group A streptococci elaborate more than 20 exotoxins and it is the antibody response to one or more of these that is used as documentation of nonsuppurative disease. Some of the exotoxin products include streptolysins O and S; deoxyribonucleases A, B, C, and D; streptokinase; NADase; hyaluronidase; diphosphopyridine nucleotidase; and pyrogenic exotoxins.[25]

The antibody response to these streptococcal products is variable because of several factors, such as age of onset, site of infection, and timeliness of antibiotic treatment. The most diagnostically important antibodies are the following: anti-streptolysin O (ASO), anti-DNase B, anti-NADase, and anti-hyaluronidase (AHase). Assays for detection of these antibodies can be performed individually or through use of the **streptozyme** test which detects antibodies to all these products (see *Streptozyme Testing* later). During Group A streptococcal infections, other antibodies are made to cellular antigens, such as the Group A carbohydrate and the M protein.[13] Generally, detection of these antibodies is done in research or reference laboratories because commercial reagents are not available.

Serological evidence of disease is based on an elevated or rising titer of streptococcal antibodies. The onset of clinical symptoms of rheumatic fever or glomerulonephritis typically coincides with the peak of antibody response. If acute and convalescent phase sera are tested in parallel, a four-fold rise in titer is considered significant. The use of at least two tests for antibodies to different exotoxins is recommended because production of detectable ASO does not occur in all

FIGURE 20–11 BinaxNOW® lateral flow assay. The BinaxNOW® Strep A Test immunochromatographic assay for the qualitative detection of *Streptococcus pyogenes* Group A antigen from throat swab specimens. To perform the test, a throat swab is inserted into the test card. Extraction reagents are added from dropper bottles. The test card is closed to bring the extracted sample in contact with the test strip. Strep A antigen captured by immobilized anti-strep A reacts to bind conjugated antibody. Immobilized species antibody captures the second visualizing conjugate. The test is interpreted by the presence or absence of visually detectable pink-to-purple colored lines. A positive result is indicated by production of both a Sample Line and a Control Line (shown on left), whereas a negative assay will produce only the Control Line (shown on right). *(BinaxNOW® is a trademark of the Alere Scarborough, Inc. Used with permission.)*

patients. The most commonly used tests are those for ASO and anti-DNase B.[18]

Antistreptolysin O (ASO) Testing

ASO tests detect antibodies to the streptolysin O enzyme produced by Group A streptococcus. This enzyme is able to lyse RBCs. Presence of antibodies to streptolysin O indicates recent streptococcal infection in patients suspected of having acute rheumatic fever or poststreptococcal glomerulonephritis following a throat infection.

The classic hemolytic method for determining the **ASO titer** was the first test developed to measure streptococcal antibodies. This test was based on the ability of antibodies in the patient's serum to neutralize the hemolytic activity of streptolysin O. The traditional ASO titer involved preparing dilutions of patient serum to which a measured amount of streptolysin O reagent was added. These were allowed to combine during an incubation period after which reagent RBCs were added as an indicator. If enough antibodies were present, the streptolysin O was neutralized and no hemolysis occurred. The titer was reported as the reciprocal of the highest dilution demonstrating no hemolysis. This titer could be expressed in either Todd units (when the streptolysin reagent standard is used) or in international units (when the World Health Organization international standard is used).[13]

The range of expected normal values varies, depending on the patient's age, geographic location, and season of the year. ASO titers tend to be highest in school-age children and young adults. Thus, the upper limits of normal must be established for specific populations.[13] Typically, however, a single ASO titer is considered to be moderately elevated if the titer is at least 240 Todd units in an adult and 320 Todd units in a child.[13]

Because of the labor-intensive nature of the traditional ASO titer test and because the streptolysin O reagent and the RBCs used are not stable, ASO testing is currently performed by nephelometric methods. Nephelometry has the advantage of being an automated procedure that provides rapid, quantitative measurement of ASO titers.[13] The antigen used in this technique is purified recombinant streptolysin. When antibody-positive patient serum combines with the antigen reagent, immune complexes are formed, resulting in an increased light scatter that the instrument converts to a peak rate signal. All results are reported in international units, which are extrapolated from the classic hemolytic method described previously. When using the nephelometric method, individual laboratories must establish their own upper limits of normal for populations of different ages.

ASO titers typically increase within 1 to 2 weeks after infection and peak between 3 to 6 weeks following the initial symptoms (e.g., sore throat).[21] However, an antibody response occurs in only about 85% of acute rheumatic fever patients within this period. Additionally, ASO titers usually do not increase in individuals with skin infections.[26]

Anti-DNase B Testing

Testing for the presence of **anti-DNase B** is clinically useful in patients suspected of having glomerulonephritis preceded by streptococcal skin infections because ASO antibodies often are

not stimulated by this type of disease.[17] In addition, antibodies to DNase B may be detected in patients with acute rheumatic fever who have a negative ASO test result.

DNase B is mainly produced by Group A streptococci, so testing for anti-DNase B is highly specific for Group A streptococcal sequelae.[14] Macrotiter, microtiter, EIA, and nephelometric methods have been developed for anti-DNase testing. The classic test for the measurement of anti-DNase B activity is based on a neutralization methodology. If anti-DNase B antibodies are present, they will neutralize reagent DNase B, preventing it from depolymerizing DNA. Presence of DNase is measured by its effect on a DNA-methyl-green conjugate. This complex is green in its intact form; however, when hydrolyzed by DNase, the methyl green is reduced and becomes colorless. An overnight incubation at 37°C is required in some testing methodologies to permit antibodies to inactivate the enzyme. Tubes are graded for color, with a 4+ indicating that the intensity of color is unchanged and a 0 indicating a total loss of color. The result is reported as the reciprocal of the highest dilution demonstrating a color intensity of between 2+ and 4+. Normal titers for children between the ages of 2 and 12 years range from 240 to 640 units.[21]

Nephelometry provides an automated means of testing that can be used for rapid quantitation of anti-DNase B. In this method, immune complexes formed between antibodies in patient serum and DNase B reagent generate an increase in light scatter. Results are extrapolated from values from the classic method and are reported in international units per mL.[13]

Streptozyme Testing

The streptozyme test is a slide agglutination screening test for the detection of antibodies against streptococcal antigens. The streptozyme test measures antibodies against five extracellular streptococcal antigens: anti-streptolysin (ASO), anti-hyaluronidase (AHase), anti-streptokinase (ASKase), anti-nicotinamide-adenine dinucleotide (anti-NAD), and anti-DNAse B antibodies. The streptozyme test is positive in 95% of patients with acute poststreptococcal glomerulonephritis because of GAS pharyngitis.[18]

In this test, sheep RBCs are coated with streptolysin, streptokinase, hyaluronidase, DNase, and NADase so that antibodies to any of the streptococcal antigens can be detected. Reagent RBCs are mixed with a 1:100 dilution of patient serum. Hemagglutination represents a positive test, indicating that antibodies to one or more of these antigens are present. The test is rapid and simple to perform, but it appears to be less reproducible than other antibody tests. In addition, more false positives and false negatives have been reported for this test than for the ASO and anti-DNase B assays.[18] Because a larger variety of antibodies are included in this test, the potential is higher for detection of streptococcal antibodies. However, single-titer determinations are not as significant as several titrations performed at weekly or biweekly intervals following the onset of symptoms.[12] The streptozyme test should be used in conjunction with the ASO or anti-DNase B tests when sequelae of Group A streptococcal infection are suspected and is especially useful when negative or borderline ASO results are obtained. (See the streptozyme test laboratory exercise online at Davis*Plus*.)

Helicobacter *Pylori*

First isolated from humans in 1982, ***Helicobacter pylori*** is now recognized as a major cause of both gastric and duodenal ulcers.[27,28] The organism resides in the mucus layer, the gastric epithelium, and occasionally the duodenal epithelium.[29] This gram-negative, microaerophilic spiral bacterium is observed in 30% of the population in developed countries and more than 90% of the population in developing countries. In developing countries, more than 70% carry *H pylori* by age 10, with carriage being nearly universal by the age of 20. Conversely in the United States, there is little colonization during childhood. The rates gradually increase during adulthood, reaching a prevalence of 50% among persons older than 60 years.[30] The high incidence of colonization in developing countries where living conditions are more crowded and sanitation conditions are suboptimal suggests that fecal–oral transmission is the most likely route.[30] Since 1994, the National Institutes of Health has recommended that individuals with gastric or duodenal ulcers caused by *H pylori* be given antibiotic treatment along with anti-ulcer medications. If untreated, *H pylori* infection will last for the patient's life and may lead to gastric carcinoma.[31]

H Pylori Virulence Factors

A major virulence factor of *H pylori* is the production of the protein CagA, which is highly immunogenic. The organism has the ability to inject the CagA protein into the gastric epithelial cells. Once the CagA protein is in the epithelial cells, changes occur in the function of the cell's signal transduction pathways and in the structure of the cytoskeleton.[32,33]

A second virulence factor is vacuolating cytotoxin, or VacA. The *VacA* gene codes for a toxin precursor. Epidemiological studies have shown that if the *CagA* and *VacA* genes are present in the strain of bacteria infecting the individual, there is a higher risk of developing gastric or peptic ulcers or gastric carcinoma.[33,34]

Pathology and Pathogenesis

Unlike many other bacteria, *H pylori* is able to survive and multiply in the gastric environment. This occurs because of several characteristics of the bacteria. Its spiral shape and flagella help the organism to be highly motile and to penetrate the viscous mucus layer in the stomach. The organism produces large amounts of **urease**, providing a buffering zone around the bacteria that protects it from the effects of the stomach acid. In addition, the acid-labile flagella are coated with a flagellar sheath, protecting them from the acidic environment of the stomach.

The pathology and mechanism of action leading to tissue damage is not clearly understood. Neutrophil-induced mucosal damage may be the result of the ammonia produced by urease.

The ammonia has been shown to induce the release of chlorinated toxic oxidants from the neutrophils, which causes inflammation and damage to the mucosal cells. Once established below the mucosal layer, the organism does not invade the tissue. More likely, the pathology represents the host's response to extracellular products produced by the bacteria. Antibodies are formed against the signaling molecules, CagA and VacA. Strains from individuals with stomach ulcers produce higher levels of VacA than strains from individuals without stomach ulcers.[35]

The outcomes associated with *H pylori* exposure vary.[36] Not all individuals harboring the organism go on to develop disease, suggesting that the interaction between the host (perhaps because of genetic predisposition) and the bacteria play a role.[37-41] In some hosts, the organism is not able to establish itself. In others, asymptomatic colonization may persist or hyperacidity may result in duodenal ulceration. Treatment of *H pylori* consists of triple therapy with bismuth salts, metronidazole, and amoxicillin. Although highly effective, 10% to 40% of individuals will have treatment failure because of poor patient compliance or resistance to metronidazole.[42] An increased risk of developing gastric carcinoma and mucosa-associated lymphoid tumors (MALTomas) has also been shown.[43,44]

Diagnosis of *H Pylori* Infection

Detection of *H pylori* may be achieved by the invasive techniques of endoscopy or biopsy, or through noninvasive techniques including serological analysis, fecal antigen detection, and demonstration of urease production with urea breath tests. The most specific test to detect *H pylori* infection is culture, but the sensitivity is usually lower than other methods because the organism is not evenly distributed throughout the gastric tissue.[45]

Endoscopy and Biopsy

Endoscopy and biopsy are the most expensive and invasive methods for diagnosing an infection with *H pylori*. However, histological examination of the tissue may reveal a great deal of information regarding the lesion. One method of testing for *H pylori* involves the detection of urease from a biopsy taken from the antrum of the stomach. The antrum is the portion of the stomach before the pyloric sphincter or valve responsible for releasing stomach contents into the intestines. An example of a test that detects urease in a tissue biopsy is the CLOtest. The CLOtest (for *Campylobacter*-like organisms) detects urease activity in gastric mucosal biopsies. During the endoscopy, a small biopsy is taken (1–3 mm). The specimen is placed in the test cassette, resealed following the manufacturer's instructions, and sent to the laboratory. If urease is present, the yellow gel will turn a hot pink color because of an increase in pH in the presence of urease. If urease is not present, the gel will remain yellow **(Fig. 20–12).** A majority of the tests will turn positive within 20 minutes; however, the test should be held and reexamined after 24 hours to allow time for detection of a low-level infection. The test is easy to use and results can be detected in a short period of time, making it ideal for rapid diagnosis of *H pylori* infections.[46]

FIGURE 20–12 CLOtest. The CLOtest rapid urease method uses a tissue biopsy to detect *Helicobacter pylori*. The test consists of a well of indicator gel sealed inside a plastic cassette. The gel contains urea, phenol red, buffers, and a bacterial static agent to prevent the growth of contaminating urease-positive organisms. If the urease from *H pylori* is present in the tissue sample, it changes the gel from yellow (bottom cassette) to bright magenta (top cassette). A majority of positive tests change color within 20 minutes. The test is reviewed after 24 hours, because a low-level positive infection may not become detectable until then. *(Courtesy of Halyard Health, Inc., Irvine, CA. Used with permission.)*

Noninvasive Detection Methods

Procedures for detecting *H pylori* that do not require the use of endoscopy include urea breath testing, enzyme or lateral flow immunoassays for the detection of bacterial antigens in the feces, molecular tests for *H pylori* DNA, and serological testing. In the urea breath test, the patient ingests urea labeled with radioactive carbon (^{14}C) or a nonradioactive ^{13}C. Urea is metabolized to ammonia and bicarbonate. The bicarbonate is excreted in the breath and the labeled carbon dioxide is measured by detection of radioactivity for ^{14}C or mass spectrometry analysis for ^{13}C. This breath technique has excellent sensitivity and specificity and is helpful in determining if the bacteria have been eradicated by antimicrobial therapy; however, in most cases it involves the use of radioactivity.[10,45]

Because of the potential for treatment failure, analysis of stool samples before and after antimicrobial therapy for *H pylori* antigens is done to determine if the bacteria have been eliminated following treatment.[45] ELISA tests as well as LFA methods are available. Because of the potential for asymptomatic carriage of the organism, stool antigen testing for initial diagnosis of *H pylori* infection is not recommended.

Researchers also have developed molecular testing to detect *H pylori* DNA.[45,46] However, PCR-based methods, which detect the presence of the organism in fecal samples, cannot distinguish between living and dead *H pylori*. Real-time PCR technology has

been developed to determine the patient's bacterial load and has shown good correlation with the urea breath test. At the time of this writing, FDA-approved molecular assays for *H pylori* are not available for clinical use.

Detection of *Helicobacter Pylori* Antibodies

Serological testing is a primary screening method of determining infection with *H pylori.* Infections from this organism result in production of IgG, IgA, and IgM antibodies. Most serological tests in clinical use detect *H pylori*-specific antibodies of the IgG class. Although IgM antibody is produced in *H pylori* infections, testing for its presence lacks clinical value because most infections have become chronic before diagnosis. Thus, IgG is the primary antibody found. IgA testing has a lower sensitivity and specificity than IgG testing, but it may increase sensitivity of detection when used in conjunction with IgG testing.[45]

The presence of antibodies in the blood of an untreated patient indicates an active infection because the bacterium does not spontaneously clear. Antibody levels in untreated individuals remain elevated for years. In treated individuals, the antibody concentrations decrease after about 6 months to about 50% of the level the patient had during the active infection. Therefore, convalescent testing should be performed 6 months to a year after treatment, which requires that the acute serum sample be stored for up to a year.[47] A decrease in antibody titer of more than 25% must occur for treatment to be considered successful.[47]

Measurement of the antibodies may be done with several techniques, including ELISA, immunoblot, and rapid tests using LA or LFA. Several LFAs are approved for CLIA-waived testing by physician office laboratories. The method of choice for the detection of *H pylori* antibodies is the ELISA technique, which is reliable and accurate.[47] Tests employing antigens from a pooled extract from multiple and genetically diverse strains yield the best sensitivity because *H pylori* is so heterogeneous. Very few, if any, patients produce antibodies to all of the *H pylori* antigens; most patients produce antibodies against the CagA and VacA proteins. Antibodies to these two proteins indicate a more severe case of gastritis or an increased risk of developing gastric carcinoma.[47]

When compared with other techniques for antibody detection of *H pylori,* ELISA tests are sensitive, specific, and cost effective for determining the organism's presence in untreated individuals.[45] However, because antibodies are not rapidly cleared after treatment, antibody testing is not as well suited for determining eradication of infection as are other methods. Additionally, individuals who are immunocompromised (the elderly or immunosuppressed individuals) may have a false-negative result with antibody testing.

Rapid assays for the detection of *H pylori* antibodies are also available. It is recommended that samples with positive rapid test results be tested by an ELISA method for correlation.[45]

Mycoplasma Pneumoniae

Mycoplasma is a member of a unique group of organisms that belong to the class *Mollicutes.* Mycoplasmas represent the smallest known free-living life forms (150–250 nm) and have a small genome. Various members colonize plants, animals, and insects in addition to being human pathogens.[48] These extracellular parasites attach to and exist on the surface of host cells using attachment organelles and adhesion molecules specific for their host cells. They absorb their nutrients from the host cells to which they are attached.[49] The organisms lack cell walls (thus lacking peptidoglycan), have sterols in their cell membrane, and have complex growth requirements, making culture difficult and time consuming.

Mycoplasma Pneumoniae Pathogenesis

The best-known *Mycoplasma* is *M pneumoniae,* which is a leading cause of respiratory infections worldwide.[50] *M pneumoniae* infections are found in all age groups, with a majority of the infections involving the upper respiratory tract.[51] *M pneumoniae* is spread from one person to another by respiratory droplets. Relatively close association with an infected individual appears to be necessary for transmission of the organism.[51,52] Unlike most respiratory infections, the incubation period is 1 to 3 weeks. The infection has an insidious onset which differs from the acute onset observed with respiratory viruses such as influenza and adenovirus. Typically, there is development of a fever, along with headache, malaise, and a cough—the clinical hallmark of a *M pneumoniae* infection. Depending on the age, approximately 5% to 10% of individuals progress to tracheobronchitis or pneumonia.[51]

Originally, pneumonia caused by *M pneumoniae* was referred to as "atypical pneumonia" because the infection could not be treated with penicillin. This is because the organism lacks the cell wall to which penicillin is directed against. In many cases, the pneumonia is mild, oftentimes appearing as a cold, and symptoms are generally mild enough that bed rest or hospitalization is not required. The infection is often referred to as "walking pneumonia" because individuals often do not stay home from work or school and still participate in their daily activities. Although many infections are mild, *M pneumoniae* accounts for 20% of all hospitalizations for pneumonia in the United States.[52,53] *M pneumoniae* may remain in the respiratory tract for several months after resolution of the infection, causing chronic inflammation and a lingering cough.[50] Based on nucleic acid detection of the organism, there is increasing evidence that *M pneumoniae* may initiate or exacerbate asthma.[54]

Dermatological Manifestations

Up to 7% of individuals with *M pneumoniae* develop Stevens–Johnson syndrome, or erythema multiforme major, a condition in which the top layer of the skin dies and sheds. The syndrome is considered a medical emergency that usually requires hospitalization.[55] The conjunctivae as well as the joints and various organs in the genitourinary and gastrointestinal tract may also be involved. The cause of Stevens–Johnson syndrome is not clearly known, but it may be caused by the immune response of the host or to augmented sensitivity to antibiotics while being treated for *M pneumoniae*.[54-56]

Another manifestation of *M pneumoniae* infection is Raynaud syndrome, which is a transient vasospasm of the digits in which the fingers turn white when exposed to the cold. Although the exact cause is unclear, it may be related to the development and action of cold agglutinins in the body (see Chapter 14).[57] Other extrapulmonary manifestations of Raynaud syndrome include arthritis, meningoencephalitis, pericarditis, and peripheral neuropathy.[58]

Immunology of *Mycoplasma Pneumoniae* Infection

In addition to stimulating the production of many proinflammatory and anti-inflammatory cytokines and chemokines, several classes of antibodies are produced in the course of a *M pneumoniae* infection. As with any infection in which there is a humoral response, the body produces antibodies that neutralize the microorganism. *M pneumoniae* also induces the production of autoantibodies, including agglutinins directed against the lungs, brain, cardiolipins, and smooth muscle.[58,59]

The cold isoagglutinins observed with *M pneumoniae* infection are among the most studied agglutinins by researchers. They are oligoclonal IgM antibodies directed against the altered I antigens found on the surface of human RBCs.[60] These antibodies can agglutinate the RBCs at temperatures below 37°C. Development of the antibodies is thought to result from cross-reaction of antibodies formed against *M pneumoniae* and the I antigen on human RBCs or from alteration of the RBC antigen by the organism.[60]

Laboratory Diagnosis of *Mycoplasma Pneumoniae* Infection

Laboratory means of detecting *Mycoplasma* infection may involve culturing of the organism, detection of *M pneumoniae*-specific antibodies in serum, and detection of *M pneumoniae*-specific antigens or nucleotide sequences directly in patient specimens.

Detection of *Mycoplasma Pneumoniae* by Culture

Although culturing has been considered the gold standard for diagnosis, culturing for the organism is rarely carried out in the clinical laboratory because of the fastidious nature of the organism. Collection and transport of the specimen differs from traditional methods used for culturing other microorganisms.[53] The transport media may be trypticase soy broth with 0.5% albumin, SP4 medium, or a viral transport medium. If the sample cannot be plated immediately, it should be frozen at –70°C. Culturing requires the use of specialized media designed for the recovery of *Mycoplasma.* Growth of the organism takes several weeks in most cases. If the culture is successfully performed, the growth produces a "mulberry" colony with a typical "fried egg" appearance.

Detection of Antibodies to *Mycoplasma Pneumoniae*

Detection of *M pneumoniae*-specific IgM immunoglobulin is the most useful diagnostic test because it likely indicates a recent infection. Enzyme-linked immunoassays have been the most widely used methods for antibodies and can detect IgM or IgG directed against *M pneumoniae*.[61] Although IgM is the primary immunoglobulin response to infection, testing for the presence of IgG antibodies is necessary; the reason is that adults may only elicit an IgG response because of reinfection with the organism. ELISA methods have a specificity of more than 99% and a sensitivity of 98%.[61]

Detection of Cold Agglutinins

For many years, before the development of antigen-specific serological tests, laboratory diagnosis of *M pneumoniae* involved testing for cold agglutinins. The agglutinins are capable of clumping RBCs at 4°C. The reaction is reversible when the samples are warmed to 37°C. Cold agglutinins develop in about 50% of patients with *M pneumoniae* infection.[60] These antibodies are produced early in the disease (7–10 days) and can typically be detected at the time the patient seeks medical attention. The titer peaks at 2 to 3 weeks and antibodies are present for 2 to 3 months after infection.[60]

Although once considered the primary means of diagnosing *Mycoplasma* infection, the assay is not very specific (50% to 70%) nor is it very sensitive.[62] Testing for cold agglutinins is no longer recommended because the development of cold agglutinins occurs in other circumstances, including some viral infections and collagen vascular diseases.[63] However, a titer of 1:64 or greater, along with the clinical presentation of the patient, is suggestive of infection with *M pneumoniae.* It should be noted that cold agglutinins may be found in patients with infections whose clinical presentations resemble *M pneumoniae* infection such as mononucleosis caused by Epstein-Barr virus (anti-i) and cytomegalovirus (anti-I).[64,65]

Molecular Diagnosis of Mycoplasma Infections

Molecular methods will, in all likelihood, become the gold standard for the diagnosis of *Mycoplasma* infections. Although some laboratories offer "home brew" assays, very few laboratories offer a molecular assay for the detection of *Mycoplasma* in clinical samples. Before 2012, there were no FDA-approved assays available for the detection of *M pneumoniae.* BioFire Diagnostics, Inc. (Salt Lake City, UT), now part of the BioMerieux corporation, received FDA approval in 2012 for its FilmArray® Respiratory Panel. Using nested multiplex PCR, the assay is able to detect 20 respiratory viruses and bacteria including *B pertussis, C pneumoniae,* and *M pneumoniae.*[66] As additional assays are developed and receive FDA approval, the molecular diagnosis of *Mycoplasma* infection will become more widespread and will likely replace serology as the primary means for the diagnosis of *M pneumoniae* infections.

Rickettsial Infections

Members of the *Rickettsiaceae* family cause a variety of infections in man and animals. Because of recent advances in molecular technology, various members originally belonging to the genus ***Rickettsiae*** have now been reclassified. These obligate intracellular, gram-negative bacteria now include the genera *Rickettsia*

(Rickettsiosis) and *Orientia* (*Orientia tsutsugamushi* causing scrub typhus). Additional members are the *Ehrlichia* group including *Ehrlichia* (Ehrlichiosis), *Anaplasma* (Anaplasmosis), *Neorickettsia* (associated with helminths), and *Neoehrlichia*.[67]

Agents of *Rickettsia*-Related Disease

The genus *Rickettsia* is made up of two distinct groups: the spotted fever group (SFG) and the **typhus** group (TG). Each is responsible for a different set of diseases **(Table 20–1).** In the United States, the main *Rickettsial* disease is **Rocky Mountain spotted fever (RMSF),** caused by *R rickettsia* (SFG), with approximately 2,500 cases reported each year.[68]

Epidemic typhus, caused by *Rickettsia prowazekii* (TG), is the most prevalent member of the TG globally. Typhus fever (also known as epidemic typhus) occurs in conditions of poor hygiene and overcrowding such as in prisons and refugee camps. Epidemic typhus was responsible for over 3 million deaths in World War I.[69] Once prevalent throughout the globe in the first half of the 20th century, only a few foci of epidemic typhus still exist in the world today (Ethiopia, Burundi, Rwanda, part of Mexico).[70] Individuals traveling to areas with large homeless populations and regions that have recently experienced war or natural disasters leading to poor hygiene and crowded conditions (such as refugee camps) are at risk of acquiring typhus fever.

Table 20–1 Classification of Select *Rickettsiae* Known to Cause Disease in Humans

ANTIGENIC GROUP	DISEASE	SPECIES	VECTOR	ANIMAL RESERVOIR(S)	GEOGRAPHIC DISTRIBUTION
Anaplasma	Human granulocytic anaplasmosis	*Anaplasma phagocytophilum*	Tick	Small mammals, rodents, and deer	Primarily United States, worldwide
Ehrlichia	Human monocytic ehrlichiosis	*Ehrlichia chaffeensis*	Tick	Deer, wild and domestic dogs, domestic ruminants, and rodents	Common in United States, probably worldwide
	Ehrlichiosis	*E muris*	Tick	Deer and rodents	North America, Europe, Asia
	Ehrlichiosis	*E ewingii*	Tick	Deer, wild and domestic dogs, and rodents	North America, Cameroon, Korea
Neoehrlichia	Human neoehrlichiosis	*Neoehrlichia mikurensis*	Tick	Rodents	Europe, Asia
Neorickettsia	Sennetsu fever	*Neorickettsia sennetsu*	Trematode	Fish	Japan, Malaysia, possibly other parts of Asia
Scrub typhus	Scrub typhus	*Orientia tsutsugamushi*	Larval mite (chigger)	Rodents	Asia-Pacific region from maritime Russia and China to Indonesia and North Australia to Afghanistan
Spotted fever	Rocky Mountain spotted fever	*Rickettsia rickettsii*	Tick	Rodents	North, Central, and South America
	Rickettsiosis	*Rickettsia aeschlimannii*	Tick	Unknown	South Africa, Morocco, Mediterranean littoral
	Queensland tick typhus	*Rickettsia australis*	Tick	Rodents	Australia, Tasmania
	Boutonneuse fever or Mediterranean spotted fever	*Rickettsia conorii*	Tick	Dogs, rodents	Southern Europe, southern and western Asia, Africa, India

Continued

Table 20–1 Classification of Select *Rickettsiae* Known to Cause Disease in Humans—cont'd

ANTIGENIC GROUP	DISEASE	SPECIES	VECTOR	ANIMAL RESERVOIR(S)	GEOGRAPHIC DISTRIBUTION
Typhus fever	Epidemic typhus, sylvatic typhus	*Rickettsia prowazekii*	Human body louse, flying squirrel ectoparasites, *Amblyomma* ticks	Humans, flying squirrels	Central Africa, Asia, Central America, North America, and South America
	Murine typhus	*Rickettsia typhi*	Flea	Rodents	Tropical and subtropical areas worldwide

Adapted from Centers for Disease Control and Prevention. CDC Health Information for International Travel, 2016. *New York, NY: Oxford University Press; 2016. Online version accessed November 17, 2015.*

Each of the species responsible for the various *Rickettsial* diseases has a variety of animal reservoirs. The vectors responsible for the transmission are arthropods (ticks, mites, lice, or fleas) which transmit the organism through its bite after feeding on an infected animal **(Fig. 20–13).**[71] The one exception is typhus fever (epidemic louse-borne typhus) which is transmitted when an infected human body louse excretes *R prowazekii* onto the skin while feeding and the individual becomes infected by rubbing louse fecal matter or crushed lice into the bite wound. Except for *R prowazekii* (epidemic typhus), humans are accidental hosts for *Rickettsia* and *Rickettsia*-related organisms.[71] *Rickettsia* and *Rickettsia*-related organisms have worldwide distribution; however, certain members have a specific geographic distribution. For example, *Rickettsia japonica* is found only in Japan, whereas *R rickettsii* is found in the Western hemisphere. Some species, such as *Rickettsia typhi,* are found everywhere in the world.[72]

The members of the *Rickettsia* genus and *Anaplasma* and *Ehrlichia* cause a number of clinical diseases. Because of the prevalence of RMSF in North and South America, this chapter will focus on RMSF.

FIGURE 20–13 The Rocky Mountain wood tick, *Dermacentor andersoni,* is a known North American vector of *Rickettsia rickettsii. (Courtesy of the CDC/Dr. Christopher Paddock and James Gathany, Public Health Image Library.)*

Rocky Mountain Spotted Fever

Epidemiology

RMSF is caused by *R rickettsii.* The organism is transmitted to the human host by the bite of a tick. In the United States, the organism is transmitted by the American dog tick *(Dermacentor variabilis),* the Rocky Mountain wood tick *(Dermacentor andersoni),* and the brown dog tick *(Rhipicephalus sanguineus).* Although called *Rocky Mountain* spotted fever, five states—North Carolina, Oklahoma, Arkansas, Tennessee, and Missouri—account for over 60% of RMSF cases in the continental United States.[73] The occurrence of the disease has seasonal variation corresponding to tick activity, being most prevalent between May and September.[70] The organism is transmitted transstadially in the tick (i.e., it remains present in the tick as the tick progresses from the nymph state to the adult) and is transmitted transovarially (from generation to generation through the eggs of the tick), allowing for maintenance of the organism in the tick population. Transmission occurs when the tick bites the host for a blood meal. When the tick has fed after 6 to 10 hours, the organism is injected into the host from the salivary glands.

Pathogenesis

Once introduced into the skin, the organisms spread via the lymphatic and circulatory system, where they attach to and invade their target cells, the vascular endothelium, by means of the OmpA and OmpB ligands.[74-77] The organisms multiply by binary fission inside the endothelial cells, are released, and infect adjacent cells. This leads to hundreds of contiguous infected cells, producing the lesions and skin rash associated with the infection. The main pathophysiological event caused by the infection is endothelial cell damage, which leads to increased vascular permeability, resulting in edema, hypovolemia, hypotension, and hypoalbuminemia.[78,79]

Clinical Manifestations

The symptoms observed with RMSF occur approximately 2 to 14 days (median 7 days) after a tick bite. Before the development of the hallmark rash, a large percentage of patients will experience a quite severe headache, nausea, vomiting, abdominal pain,

diarrhea, and abdominal tenderness. The fever usually begins within the first 3 to 5 days after the onset of symptoms, and the rash usually appears 3 to 5 days after the onset of the fever. The rash typically starts on the hands and soles of the feet and proceeds to the trunk, although it may start on the trunk in some individuals **(Fig. 20–14).** With the classic form of RMSF, death occurs 7 to 15 days after the onset of symptoms if appropriate therapy is not provided. With the fulminant (i.e., severe and sudden onset) form of RMSF, death occurs within the first 5 days. The resolution or fatal outcome of the disease is largely related to the timeliness of initiating appropriate therapy. Immediate treatment with doxycycline reduces the severity of the infection.[80,81]

Diagnosis of RMSF

Initial diagnosis is often made clinically after ruling out a large variety of other conditions, including typhoid fever, measles, rubella, enteroviral infection, and respiratory tract infection. The overlapping symptoms, or clinical presentation, during the initial stages of the disease can make the diagnosis of RMSF extremely difficult. The diagnosis of fulminant RMSF is even more difficult because of its rapid course. The rash develops shortly before death, if at all; therefore, antibodies to *R rickettsii* do not have time to develop.

Serological and Molecular Diagnosis

The organism infects the endothelial cells and does not circulate until the disease has severely progressed. Therefore, culturing for the organism and molecular methods is not always useful. If the patient has a rash, molecular diagnosis using DNA from the skin lesions is of value. (Note: At the time of this writing there are no FDA-approved assays.) Serology is the usual method for confirming the diagnosis of RMSF, but this is a retrospective diagnosis. Antibodies to *R rickettsia* develop 7 to 10 days after the onset of symptoms and a majority of patients do not show antibodies during the first week of illness. For a successful outcome, therapy needs to be initiated before that time.

The gold standard for the serological diagnosis of RMSF is the indirect immunofluorescence assay (IFA) with *R rickettsii* antigen, performed on two paired serum samples to demonstrate a significant (four-fold) rise in antibody titers.[81] For many years, antibodies produced in patients with *Rickettsial* infections were detected by an agglutination test known as the Weil-Felix test, which was based on cross-reactivity of the patient's antibodies with polysaccharide antigens present on the OX-19 and OX-2 strains of *Proteus vulgaris* and the OX-K strain of *Proteus mirabilis*. This method lacks sensitivity and specificity and should not be relied on.

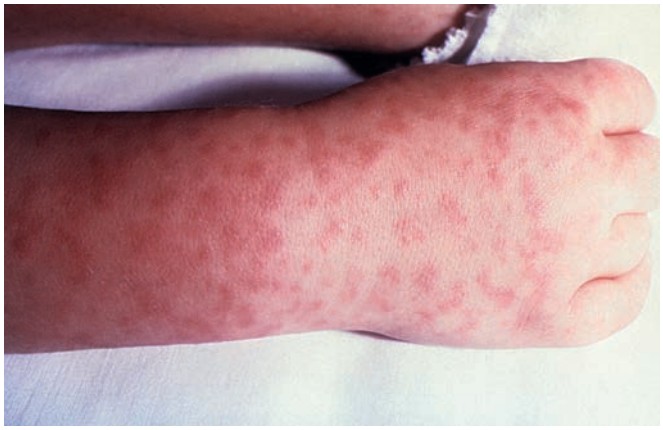

FIGURE 20–14 The characteristic spotted rash of Rocky Mountain spotted fever, the most severe and most frequently reported rickettsial illness in the United States. The disease is caused by *Rickettsia rickettsii*. (Courtesy of the CDC, Public Health Image Library.)

SUMMARY

- The indigenous microbiota varies at different sites of the body.
- The symbiotic relationship that exists between bacteria and humans is beneficial in protecting against infection, stimulating the immune system, aiding in digestion of food, and producing various vitamins.
- Humans exist in a commensalistic relationship with the bacteria that comprise the human microbiome. The encounter with some microbial organisms results in a parasitic relationship in which there is harm to the host that may result in an infection.
- Pathogenicity refers to the ability of an organism to cause disease, virulence refers to the extent that a pathogen causes damage to the host, and infectivity refers to the ability of an organism to spread from one host to another.
- To be virulent, an organism must possess structural features or produce extracellular substances that allow it to invade or cause damage to the host. These are referred to as virulence factors.
- Live bacteria may produce exotoxins that are generally specific to a particular bacterial organism and have specific modes of action on the host. Exotoxins are highly immunogenic and antibodies formed against them are protective.
- Endotoxin, or lipid A, is part of the gram-negative bacterial cell wall that is released from dead bacteria. Endotoxin has a broad range of systemic effects on the body because it induces the release of cytokines that can lead to septic shock. Endotoxin, although immunogenic, does not result in the production of protective antibodies.
- Nonspecific immune defenses (phagocytosis, production of antimicrobial defense peptides, various proteins) contribute heavily to the body's ability to overcome a bacterial infection.
- Laboratory detection of the causative agent of a bacterial infection includes culturing of the organism, visualization of the bacteria in clinical specimens, detection of bacterial antigens in the clinical specimen, detection of the pathogen's DNA or RNA, and demonstration of antibodies formed against the agent through serological means.
- Lateral flow immunochromatographic assays (LFAs) are increasingly being used for the detection of bacterial, viral, fungal, and parasitic antigens. Many LFAs have sensitivities that exceed other testing methods. The principle behind

LFAs is the movement of a liquid sample containing the analyte of interest along a strip that passes through various zones containing labeled antibodies specific to the analyte. If the antigen–antibody complex is present, it is captured by another antibody at the end of the strip, resulting in the development of a visible line.

- *Streptococcus pyogenes* (Group A streptococci or GAS) is the primary cause of bacterial pharyngitis. It is also a primary cause of a skin infection called impetigo. Untreated GAS infections may result in sequelae: acute glomerulonephritis or rheumatic heart disease.
- The production of exotoxins contributes to the infections caused by GAS. Streptolysin O, hyaluronidase, deoxyribonuclease B (DNase B), and streptokinase all play a role in the infections associated with GAS.
- Scarlet fever occurs in a small percentage of individuals infected with GAS and is caused by the production of erythrogenic exotoxins that may also result in toxic shock syndrome.
- The laboratory diagnosis of GAS infection includes culture and antigen detection for acute infection, and anti-streptolysin O and anti-DNase B antibody detection for GAS sequelae.
- The streptozyme test measures antibodies against five extracellular streptococcal antigens–anti-streptolysin (ASO), anti-hyaluronidase (AHase), anti-streptokinase (ASKase), anti-nicotinamide-adenine dinucleotide (anti-NAD), and anti-DNAse B antibodies. The streptozyme test is positive in 95% of patients with acute poststreptococcal glomerulonephritis caused by GAS pharyngitis.
- *Helicobacter pylori* is the leading cause of gastric and duodenal ulcers and is associated with gastric carcinoma (stomach cancer). *H pylori* produces a large amount of urease that protects the organism from the acidic environment in the stomach.
- Serological testing is the primary screening method of detecting *H pylori* infection. Testing for urease is also used to detect and diagnose an infection with *H pylori*. Detection of *H pylori* antigen in stool samples can be used to monitor the effectiveness of treatment for *H pylori* infections.
- *Mycoplasma pneumoniae* is a leading cause of community acquired pneumonia. Infection with *M pneumoniae* may not require bed rest or hospitalization and oftentimes is referred to as "walking pneumonia."
- Infection with *M pneumoniae* may result in dermatological manifestations causing Stevens–Johnson syndrome. Raynaud syndrome, another manifestation that may be observed with *M pneumoniae*, is a reversible variable vasospasm of the digits in which the fingers turn white when exposed to the cold.
- Production of cold agglutinins is observed in 50% of individuals with *M pneumoniae*. Demonstration of cold agglutinins is neither specific nor sensitive when detecting infection by the organism. Detection of *M pneumoniae*-specific IgM immunoglobulin is the most useful diagnostic assay because it likely indicates a recent infection.
- Rocky Mountain spotted fever (RMSF) is caused by *Rickettsia rickettsii* and is transmitted to the human host by the bite of a tick.
- The main pathophysiological event caused by RMSF is the endothelial cell damage leading to increased vascular permeability, which then results in edema, hypovolemia, and hypotension. Various cytokines are released and damage to the host may have a fatal outcome if therapy is not initiated in a timely fashion.
- The gold standard for the serological diagnosis of RMSF is the indirect immunofluorescence assay (IFA) with *R rickettsii* antigen performed on two paired serum samples to demonstrate a significant (four-fold) rise in antibody titers.

Study Guide: Immune Defenses Against Bacterial Infection

INNATE DEFENSES	ADAPTIVE IMMUNE RESPONSES
Skin and mucosal surfaces	Antibodies produced against bacterial antigens promote opsonization and complement binding
Antimicrobial defense peptides and proteins on epithelial surfaces (e.g., lysozyme, defensins)	Antibodies produced against bacterial exotoxins have neutralizing activity
Other proteins (e.g., complement proteins, interleukins, prostaglandins, leukotrienes)	T-cell–mediated immune responses attack intracellular bacteria
Acute phase reactants (e.g., CRP, haptoglobin)	
Pattern recognition receptors (e.g., TLRs on macrophages and dendritic cells)	
Phagocytosis by neutrophils and macrophages	

Study Guide: Bacterial Virulence Factors

VIRULENCE FACTOR	DESCRIPTION	MECHANISM OF PATHOGENESIS	EXAMPLE(S)
Pili	Hairlike structures on the surface of bacteria	Adherence to and colonization of host tissue Resistance to phagocytosis Transfer of genetic material	Enterotoxigenic *E coli* pili adhere to cells in small intestine
Adhesion molecules	Molecules on surface of bacteria	Attach to a variety of host cell receptors such as proteoglycans, collagen, fibrinogen	Fibronectin binding proteins of *S pyogenes* facilitate attachment to host cells
Capsule	A polysaccharide layer surrounding the cell wall of some bacteria	Blocks phagocytosis Blocks attachment of antibodies for opsonization Inhibits complement activation Acts as a decoy when released	Capsule of *S pneumoniae* bacteria prevents phagocytosis by alveolar macrophages
Endotoxin	Lipid A component of lipopolysaccharide on cell walls of gram-negative bacteria; released when bacteria die	Powerful stimulator of cytokine production	Gram-negative bacterial infection involving bacteremia can cause septic shock
Exotoxins	Neurotoxins, cytotoxins, and enterotoxins released from live bacteria	Bind to specific receptors on host cells Some can act as superantigens that activate numerous T cells	Tetanus neurotoxin prevents transmitter release from neuromuscular cells, resulting in continuous muscle spasms Exotoxins from *S pyogenes* can cause toxic shock syndrome

CASE STUDIES

1. A 6-year-old boy was brought to the pediatric clinic. His mother indicated he had been ill for several days with fever and general lethargy. The morning of the visit, the boy told his mother that his back hurt and she had observed what appeared to be blood in his urine. History and physical examination indicated a well-nourished child with an unremarkable health history other than a severe sore throat with fever 3 weeks prior that was medicated with aspirin and throat lozenges. This child's temperature was 101.5°F and the physician noted edema in the child's hands and feet. Blood and urine specimens were collected for a rapid GAS antigen test, streptozyme test, complete blood cell count, and urinalysis. Laboratory test results were as follows:

Complete Blood Count
RBC count: normal
Platelet count: normal
WBC count: 12.7 × 10^9/L (normal = 4.8–10.8 × 10^9/L)

Urinalysis
Color: red (normal = straw)
Clarity: cloudy (normal = clear)
Protein: 2+ (normal = negative/trace)
Blood: large (normal = none)

Rapid GAS Antigen Test
Negative

Streptozyme
Positive 1:600

Questions

a. What disorder is indicated by the child's history, physical examination, and laboratory test results?
b. What was the most likely causative agent of the sore throat preceding the current symptoms?
c. Discuss the most widely accepted theory explaining the physiological basis for this disease. Why didn't the physician order a throat culture?
d. What is the significance of the urinalysis results?
e. What is the significance of the streptozyme test results?

2. A 36-year-old female was seen by her physician because she had been experiencing flu-like symptoms along with a sore throat and chills for the past 3 days. She

was also having difficulty breathing. The patient had a temperature of 100.2°F and was producing a moderate amount of sputum. Her physician decided that the probable diagnosis was some type of pneumonia and ordered the following laboratory tests to be performed: complete blood count, sputum culture, tests for influenza virus, and *M pneumoniae* and cold agglutinin titers. The results were as follows:

Complete Blood Count
RBC: normal
WBC: 11.7 × 10^9/L (normal = 4.8–10.8 × 10^9/L) [somewhat elevated]

Sputum Culture
Negative

Mycoplasma Titers
IgM: none detected
IgG: 1:16

Cold Agglutinin Titer
Positive 1:128

Questions

a. What is the most probable cause of the pneumonia?
b. What is the significance of the *Mycoplasma* titer results?
c. Should additional *Mycoplasma* titers be ordered as a follow up?
d. What is the significance of the cold agglutinin titer?

REVIEW QUESTIONS

1. All of the following are protective mechanisms against bacteria *except*
a. production of antimicrobial defense peptides.
b. phagocytosis.
c. activation of complement.
d. release of lipid A from the bacterial cell.

2. All of the following are characteristics of streptococcal M proteins *except*
a. it is the chief virulence factor of Group A streptococci.
b. it provokes an immune response.
c. antibodies to one serotype protect against other serotypes.
d. it limits phagocytosis of the organism.

3. An ASO titer and a streptozyme test are performed on a patient's serum. The ASO titer is negative, the streptozyme test is positive, and both the positive and negative controls react appropriately. What can you conclude from these test results?
a. The ASO is falsely negative.
b. The patient has an antibody to a streptococcal exoenzyme other than streptolysin O.
c. The patient has not had a previous streptococcal infection.
d. The patient has scarlet fever.

4. Which of the following applies to acute rheumatic fever?
a. Symptoms begin after *S. pyogenes* infection of the throat or the skin.
b. Antibodies to Group A streptococci are believed to cross-react with heart tissue.
c. Diagnosis is usually made by culture of the organism.
d. All patients suffer permanent disability.

5. Which of the following indicates the presence of anti-DNase B activity in serum?
a. Reduction of methyl green from green to colorless
b. Clot formation when acetic acid is added
c. Inhibition of red blood cell hemolysis
d. Lack of change in the color indicator

6. Which of the following is considered to be a nonsuppurative complication of streptococcal infection?
a. Acute rheumatic fever
b. Scarlet fever
c. Impetigo
d. Pharyngitis

7. All of the following are ways that bacteria can evade host defenses *except*
a. presence of a capsule.
b. stimulation of chemotaxis.
c. production of toxins.
d. lack of adhesion to phagocytic cells.

8. Antibody testing for Rocky Mountain spotted fever may not be helpful for which reason?
a. It is not specific.
b. It is too complicated to perform.
c. It is difficult to obtain a blood specimen.
d. Antibody production takes at least a week before detection.

9. Which of the following enzymes is used to detect the presence of *H pylori* infections?
a. DNase
b. Hyaluronidase
c. Urease
d. Peptidase

10. Which of the following reasons make serological identification of a current infection with *Helicobacter pylori* difficult?

a. No antibodies appear in the blood.
b. Only IgM is produced.
c. Antibodies remain after initial treatment.
d. No ELISA tests have been developed.

11. *M pneumoniae* infections are associated with the production of which antibodies?

a. Cold agglutinins
b. Antibodies to ATPase
c. Antibodies to DNase
d. Antibodies to *Proteus* bacteria

12. Which of the following best describes the principle of the IFA test for detection of antibodies produced in Rocky Mountain spotted fever?

a. Patient serum is applied to a microtiter plate coated with a monoclonal antibody directed against the target antigen. A detection antibody labeled with biotin and directed against the target antigen is added. After addition of a substrate, a color reaction develops indicating presence of the antigen.
b. Specific antibodies in the serum sample attach to the antigens fixed to a microscope slide. In a second step, the attached antibodies are stained with fluorescein-labeled anti-human immunoglobulin and visualized with the fluorescence microscope.
c. The serum sample is treated chemically to link the target antibodies to a fluorophore. The labeled sample is applied to a microscope slide to which the antigen has been attached. Following a wash step, the slide is examined for fluorescence.
d. Patient serum is applied to a slide to which a specific antigen is bound. Following a wash step, a chromogenic dye is applied that binds to the Fc region of IgG and IgM antibodies. After a second wash step, the slide is examined for fluorescence.

13. Which of the following is true regarding exotoxins and endotoxins?

a. Both endotoxin and exotoxins are highly immunogenic allowing for the development of protective antibodies and vaccines.
b. Endotoxin has targeted activity whereas exotoxins have systemic effects when released.
c. Endotoxin is released from the cell wall of dead bacteria, whereas exotoxin is released from live bacteria.
d. Both endotoxin and exotoxins bind to specific receptors on a bacterial cell leading to cell lysis.

14. Characteristics of a bacterial capsule include which of the following?

a. It cannot be used for vaccine development.
b. It is composed of peptidoglycan.
c. It is an important mechanism for protecting a bacterium against ingestion by PMNs.
d. It is what causes bacteria to stain as gram-negative.

15. Which of the following statements regarding *Helicobacter pylori* is *not* true?

a. It is associated with an increased risk of gastric carcinoma.
b. It is the cause of most cases of acute food poisoning in the United States.
c. It is a major cause of peptic ulcers in the United States.
d. It is positive for urease.

21 Spirochete Diseases

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Describe identifying characteristics of *Treponema pallidum* and *Borrelia burgdorferi*.
2. Explain how syphilis and Lyme disease are transmitted.
3. Discuss the different stages of syphilis.
4. Discuss the advantages of direct fluorescent staining for *T pallidum* over dark-field examination without staining.
5. Define *reagin*.
6. Distinguish treponemal from nontreponemal tests for syphilis.
7. Describe the principle of the following tests for syphilis: Venereal Disease Research Laboratory (VDRL), rapid plasma reagin (RPR), fluorescent treponemal antibody absorption (FTA-ABS), and *T pallidum* particle agglutination (TP-PA).
8. Provide reasons for false-positive nontreponemal test results.
9. Compare and contrast typical results of treponemal and nontreponemal testing during the various stages of syphilis.
10. Discuss the advantages and limitations of polymerase chain reaction (PCR) and enzyme immunoassay (EIA) testing for syphilis.
11. Explain the traditional and reverse algorithms for syphilis and discuss their advantages and limitations.
12. Select appropriate laboratory methods for testing of cerebrospinal fluid (CSF) for neurosyphilis and testing for congenital syphilis.
13. Describe early and late manifestations of Lyme disease.
14. Relate various aspects of the immune response to the stages of Lyme disease.
15. Compare immunofluorescence assay (IFA), EIA, and immunoblot testing for Lyme disease as to sensitivity and ease of performance.
16. Discuss causes of false positives and false negatives in serological testing for Lyme disease.
17. Discuss the clinical manifestations and laboratory testing associated with *Borrelia miyamotoi* infections.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Borrelia burgdorferi
Borrelia miyamotoi
Chancre
Congenital syphilis
Flocculation
Fluorescent treponemal antibody absorption (FTA-ABS) test
Gummas
Immunoblotting
Lyme disease
Nontreponemal tests
Particle agglutination (PA) tests
Prozone
Rapid plasma reagin (RPR) test
Reagin
Syphilis
T pallidum particle agglutination (TP-PA) test
Treponema pallidum
Treponemal test
Venereal Disease Research Laboratory (VDRL) test

Spirochetes are long, slender, helically coiled bacteria containing *axial filaments,* or periplasmic flagella, which wind around the bacterial cell wall and are enclosed by an outer sheath.[1] These gram-negative, microaerophilic bacteria exhibit a characteristic corkscrew flexion or motility. Diseases caused by these organisms have many similarities, including a localized skin infection that disseminates to numerous organs as the disease progresses, a latent stage, and cardiac and neurological involvement if the disease remains untreated. This chapter discusses clinical manifestations and laboratory testing for the two major spirochete diseases, syphilis and Lyme disease, and provides an introduction to relapsing fever, a more recently recognized spirochete infection. Serological testing plays a key role in diagnosis of these diseases because isolation of the organisms themselves is difficult to accomplish in the laboratory and clinical symptoms are not always apparent.

Syphilis

Syphilis is the most commonly acquired spirochete disease in the United States.[2,3] It is typically spread through sexual transmission. Although the incidence of syphilis in the United States reached an all-time low of 2.2 cases per 100,000 individuals in 2000,[4] it slowly increased to 6.3 cases/100,000 people in 2014.[3] Homosexual transmission between men was responsible for much of this increase.[3,5,6] In fact, in 2000 this group accounted for 6% of the syphilis cases in this country; in 2005, over 60% of syphilis cases occurred in this group. By 2012, more than 80% of cases were within this group. Despite the current emphasis on safe sexual practices, syphilis remains a major health problem in many areas of the world; more than 10 million cases have been reported globally.[7] Early detection of syphilis is of major importance because treatment with antibiotics in the early stages of the disease can usually provide a cure. This section discusses characteristics of the organism that causes syphilis, its clinical manifestations, and laboratory methods essential to its diagnosis.

Characteristics of the Organism

The causative agent of syphilis is ***Treponema pallidum,*** subspecies *pallidum,* a member of the family *Spirochaetaceae.* Organisms in this family have no natural reservoir in the environment and must multiply within a living host. Three other pathogens in this group are so morphologically and antigenically similar to *T pallidum* that all but one are classified as subspecies.[8] These other organisms are *T pallidum* subspecies *pertenue,* the agent of yaws; *T pallidum* subspecies *endemicum,* the cause of nonvenereal endemic syphilis; and *Treponema carateum,* the agent of pinta. Yaws is found in the tropics, pinta is found in Central and South America, and endemic syphilis is found in desert regions.

T pallidum (which will hereafter be used to refer to the subspecies *pallidum*) varies in length from 6 to 20 mm and in width from 0.1 to 0.2 mm, with 6 to 14 coils **(Fig. 21–1).**[1,9] The outer membrane of *T pallidum* is a phospholipid bilayer with very few exposed proteins. Several identified membrane proteins, called *treponemal rare outer membrane proteins (TROMPs),* have been characterized.[8] It appears that the scarcity of such proteins delays the host immune response.

Mode of Transmission

Pathogenic treponemes are rapidly destroyed by heat, cold, and drying, so they are almost always spread by direct contact. Sexual transmission is the primary mode of dissemination; this occurs through contact of abraded skin or mucous membranes with an open lesion. Approximately 30% to 50% of the individuals who are exposed to a sexual partner with active lesions will acquire the disease.[9] Congenital infections can also occur during pregnancy. Transmission to the fetus is possible in mothers with clinically latent disease.

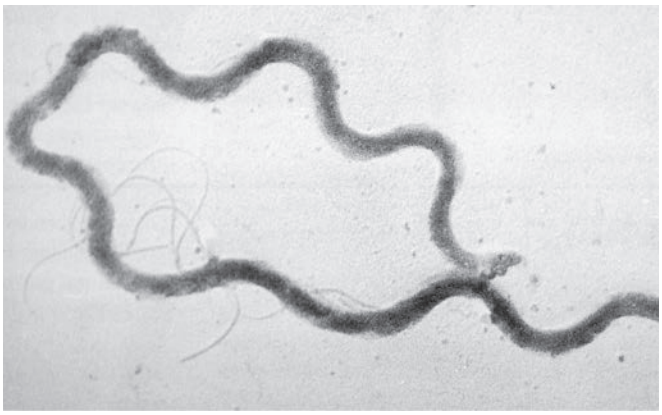


FIGURE 21–1 *Treponema pallidum.* Electron micrograph showing the coils and periplasmic flagella. *(Courtesy of CDC.)*

Other potential means of transmission include parenteral exposure through contaminated needles or blood, but this is extremely rare. For the past 30 years, the lack of transfusion-transmitted syphilis in the United States has actually called into question the necessity of testing potential donors for presence of the disease.[10] However, current guidelines by the American Red Cross require that people wait 12 months after treatment for syphilis before donating blood.[11] Because syphilis can only be transmitted by means of fresh blood products, the use of stored blood components has virtually eliminated the possibility of transfusion-associated syphilis.[9]

Stages of the Disease

Untreated syphilis can progress through four stages: primary, secondary, latent, and tertiary. Once contact has been made with a susceptible skin site, endothelial cell thickening occurs with aggregation of lymphocytes, plasma cells, and macrophages.[12] The initial lesion, called a **chancre,** develops between 10 and 90 days after infection, with an average of 21 days.[13] A chancre is a painless, solitary lesion characterized by raised and well-defined borders **(Fig. 21–2).** In men these usually occur on the outside of the penis, but in women they may appear in the vagina or on the cervix and thus may go undetected. This *primary stage* lasts from 1 to 6 weeks, during which time the lesion heals spontaneously.

About 25% of patients who are untreated in the primary stage progress to the *secondary stage,* in which systemic dissemination of the organism occurs. This stage is usually observed about 1 to 2 months after the primary chancre disappears; however, in up to 15% of reported cases, the primary lesion may still be present.[14] Symptoms of the secondary stage include generalized lymphadenopathy, or enlargement of the lymph nodes; malaise; fever; pharyngitis; and a rash on the skin and mucous membranes.[9,14] The rash may appear on the palms of the hands and the soles of the feet.[9] Involvement of the central nervous system (CNS) may occur earlier than previously suspected because viable organisms have been found in the cerebrospinal fluid (CSF) of several patients with primary or secondary syphilis.[2] Approximately 40% of patients with secondary syphilis may exhibit neurological signs such as visual disturbances, hearing loss, tinnitus, and facial weakness.[9] Lesions persist from a few days up to 8 weeks and spontaneous healing occurs, as in the primary stage.

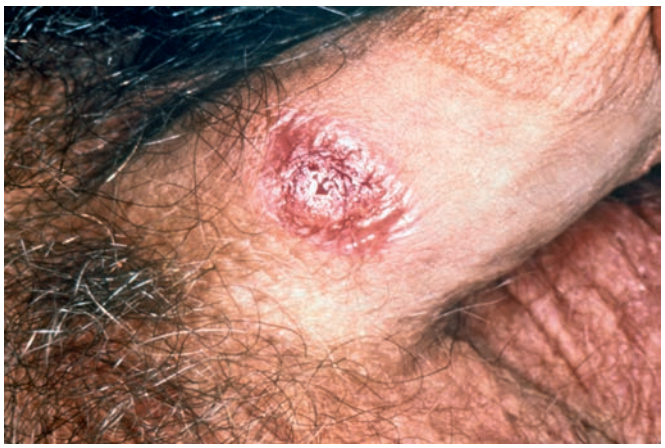


FIGURE 21–2 Primary chancre in the early stage of syphilis. *(Courtesy of the CDC/Dr. N. J. Fiumara, Public Health Image Library.)*

Clinical Correlations

The Great Imitator

Patients with syphilis can be difficult to diagnose because their clinical presentations can vary widely. Because the symptoms of syphilis can mimic those of many other diseases or conditions, the disease has often been referred to as "The Great Imitator."

The *latent stage* follows the disappearance of secondary syphilis. This stage is characterized by a lack of clinical symptoms. It is arbitrarily divided into early latent (fewer than 1 year's duration) and late latent, in which the primary infection has occurred more than 1 year previously. Patients are noninfectious at this time, with the exception of pregnant women, who can pass the disease on to the fetus even if they exhibit no symptoms.

About one-third of the individuals who remain untreated develop *tertiary syphilis.*[9,13] This stage occurs most often between 10 and 30 years following the secondary stage.[9,13] Tertiary syphilis has three major manifestations: gummatous syphilis, cardiovascular disease, and neurosyphilis.

Gummas are localized areas of granulomatous inflammation that are most often found on bones, skin, or subcutaneous tissue. Such lesions contain lymphocytes, epithelioid cells, and fibroblastic cells.[14] They may heal spontaneously with scarring or they may remain destructive areas of chronic inflammation. It is likely that they represent the host response to infection.

Cardiovascular complications usually involve the ascending aorta and symptoms are caused by destruction of elastic tissue in the aortic arch.[14] The destruction may result in aortic aneurysm, thickening of the valve leaflets causing aortic regurgitation, or narrowing of the ostia, producing angina pectoris.[9]

Neurosyphilis is the complication most often associated with the tertiary stage, but it actually can occur any time after the primary stage and can span all stages of the disease. Immunodeficient individuals such as HIV patients are susceptible to early neurosyphilis.[15] During the first 2 years following infection, CNS

Connections

Gummas

A gumma is a form of granuloma characteristic of tertiary syphilis. As discussed in Chapter 14, granulomas are organized clusters of white blood cells (WBCs) and epithelial cells that are formed as a result of a type IV hypersensitivity reaction. This cell-mediated mechanism develops in response to chronic persistence of the antigen. Granulomas can form in patients with various infectious diseases, including leprosy, tuberculosis, cutaneous leishmaniasis, yaws, and syphilis.

involvement often takes the form of acute meningitis. Late manifestations of neurosyphilis include degeneration of the lower spinal cord with partial paralysis and chronic progressive dementia. It usually takes more than 10 years for these to occur; both are the result of structural CNS damage that cannot be reversed. Fortunately, symptoms of tertiary syphilis are now very rare because of early detection and effective treatment with antibiotics such as penicillin.[9,13]

Congenital Syphilis

Congenital syphilis occurs when a woman who has early syphilis or early latent syphilis transmits treponemes to the fetus. Due in large measure to a national plan launched by the Centers for Disease Control and Prevention (CDC), the occurrence of congenital syphilis dropped from 529 cases in the year 2000 to 322 cases in 2012,[16,17] despite increases in primary and secondary syphilis. Although the disease can be transmitted at any stage of pregnancy, typically the fetus is most affected during the second or third trimester. Fetal or perinatal death occurs in approximately 10% of the cases.[14]

Infants who are liveborn often have no clinical signs of disease during the first few weeks of life. Some may remain asymptomatic, but between 60% and 90% of these infants develop later symptoms if not treated at birth.[18] Such infants may exhibit clear or hemorrhagic rhinitis, or runny nose. Skin eruptions, in the form of a maculopapular rash that is especially prominent around the mouth, the palms of the hands, and the soles of the feet, are also common.[19] Other symptoms include generalized lymphadenopathy, hepatosplenomegaly, jaundice, anemia, painful limbs, and bone abnormalities.[2,9,16] Neurosyphilis may occur in up to 60% of infants with congenital disease.[20]

Nature of the Immune Response

The primary body defenses against treponemal invasion are intact skin and mucous membranes. Once the skin is penetrated, T cells and macrophages play a key role in the immune response. Primary lesions show the presence of both CD4+ and CD8+ T cells. Cytokines produced by these cells activate macrophages; it is ultimately macrophage phagocytosis that heals the primary chancre.[14] The protective role of antibodies is uncertain, however, as coating the treponemes with antibodies does not necessarily bring about their destruction.[8] *T pallidum* is also capable of coating itself with host proteins, which delays the immune system's recognition of the pathogen.[9] The rare treponemal proteins, or TROMPS, are important in triggering the activation of complement, which ultimately kills the organism.[8] However, the chronic nature of the disease is an indicator that the organisms are able to evade the immune response. Treponemes may persist in the host for years if antibiotic therapy is not obtained.

Laboratory Diagnosis

Traditional laboratory tests for syphilis can be classified into three main types: direct detection of spirochetes, nontreponemal serological tests, and treponemal serological tests. These vary in their ability to detect syphilis at different stages of the disease. Principles and procedures of each type of testing are discussed in the text that follows. Special considerations in laboratory testing for neurosyphilis and congenital syphilis are also introduced.

Direct Detection

Direct detection of spirochetes can be accomplished by dark-field microscopy or fluorescent antibody testing. The performance of either test requires that the patient have active lesions.

Dark-Field Microscopy. Primary and secondary syphilis can be diagnosed by demonstrating the presence of *T pallidum* in exudates from skin lesions.[2] In dark-field microscopy, a dark-field condenser is used to keep all incidental light out of the field except for that captured by the organisms themselves. It is essential to have a good specimen in the form of serous fluid from a lesion. The serous fluid is usually obtained by cleaning the lesion with sterile saline and rubbing it with clean gauze. Pathogenic treponemes are identified on the basis of characteristic corkscrew morphology and flexing motility.[9]

Because observation of motility is the key to identification, specimens must be examined as quickly as possible before they dry out. False-negative results can occur when there is a delay in evaluating the slides, an insufficient specimen, or pretreatment of the patient with antibiotics.[9] Thus, a negative test does not exclude a diagnosis of syphilis. In addition, an experienced microscopist should perform testing. If a specimen is obtained from the mouth or the rectal area, morphologically identical nonpathogenic microbes can be found that must be differentiated from the true pathogens.

Fluorescent Antibody Testing. The use of a fluorescent-labeled antibody is a sensitive and highly specific alternative to dark-field microscopy. Testing can be performed by either a direct method, which uses a fluorescent-labeled antibody conjugate to *T pallidum,* or an indirect method using antibody specific for *T pallidum* and a second labeled anti-immunoglobulin antibody.[2] An advantage of these methods is that live specimens are not required. A specimen can be brought to the laboratory in a capillary tube and fixed slides can be prepared for later viewing. Treponemes can be washed off the slide even after fixing; therefore, each slide must be handled individually and rinsing must be carefully done.[2] The use of monoclonal antibodies has made fluorescent antibody testing very sensitive and specific.[2] However, monoclonal antibodies can still cross-react with other subspecies of *T pallidum,* which must be taken into account when making a diagnosis.

Serological Tests

If a patient does not have active lesions, as may be the case in secondary or tertiary syphilis, then serological testing for antibodies is the key to diagnosis. Serological tests can be classified as nontreponemal or treponemal, depending on the reactivity of the antibody that is detected. Nontreponemal tests have traditionally been used to screen for syphilis because of their high sensitivity and ease of performance. However, false-positive results are common because of the nonspecific nature of the antigen. Therefore, any positive results must be confirmed by

Connections

Complement Fixation

The first nontreponemal serological test for syphilis was developed in 1906 by the bacteriologist August Paul von Wassermann. This test used a crude liver extract from a fetus that was infected with syphilis as the source of the lipid antigen. The Wasserman test was based on the principle of complement fixation. Patient serum was incubated with cardiolipin antigen in the presence of rabbit serum as the source of complement; this was followed by a detection system consisting of antibody-coated sheep red blood cells (RBCs). If the patient serum contained cardiolipin antibody, complexes were formed that bound the reagent complement and the indicator RBCs were not lysed. In contrast, if cardiolipin antibody was not present in the patient serum, the reagent complement was free to react with the antibody-sensitized sheep RBCs to cause hemolysis.

a more specific **treponemal test,** which detects antibodies to *T pallidum.*

Nontreponemal Tests. **Nontreponemal tests** determine the presence of an antibody that forms against cardiolipin, a lipid material released from damaged cells. This antibody has sometimes been referred to as **reagin.** It is found in the sera of patients with syphilis and several other disease states. An antigen complex consisting of cardiolipin, lecithin, and cholesterol is used in the reaction to detect the nontreponemal reagin antibodies, which are either of the IgG or IgM class.

The term *reagin* as it applies to syphilis should not be confused with the same word that was originally used to describe IgE. They are not the same. Fortunately, the term *reagin* in reference to IgE is rarely used today.

The most widely used nontreponemal tests are the **Venereal Disease Research Laboratory (VDRL) test** and the **rapid plasma reagin (RPR) test.** These tests are based on flocculation reactions in which patient antibody complexes with the cardiolipin antigen. **Flocculation** is a specific type of precipitation that occurs over a narrow range of antigen concentrations. The antigen consists of very fine particles that clump together in a positive reaction.

Typical serological results for nontreponemal tests during the course of untreated and treated syphilis are shown in **Figure 21–3.** In general, nontreponemal tests are positive within 1 to 4 weeks after the appearance of the primary chancre.[2] Titers usually peak during the secondary or early latent stages. In primary disease, between 13% and 41% of individuals appear nonreactive; however, by the secondary stage almost all patients have reactive test results.[2] However, testing of sera from patients in the secondary stage is subject to false negatives because of the **prozone** phenomenon (antibody excess). In this case, a nonreactive pattern that is typically granular or rough in appearance is seen.[2] If a prozone is suspected, serial two-fold dilutions of the patient's sera should be made to obtain a titer.

Cardiolipin antibody titers tend to decline in the later stages of the disease, even if the patient remains untreated. After several years, about 25% of untreated syphilis cases become nonreactive for reagin.[9] This decline occurs more rapidly in individuals who have received treatment. A first-time infection, if in the primary or secondary stage, should show a four-fold decrease in titer by the third month following treatment and an eight-fold decrease by 6 to 8 months.[14] Following successful treatment, tests typically become completely nonreactive within 1 to 2 years.

The VDRL test, which was designed by the Venereal Disease Research Laboratories, is both a qualitative and quantitative slide flocculation test for serum that includes a modification for use on spinal fluid.[21] Antigen for all tests must be prepared fresh daily and in a highly regulated fashion. The antigen is an alcoholic solution of 0.03% cardiolipin, 0.9% cholesterol, and

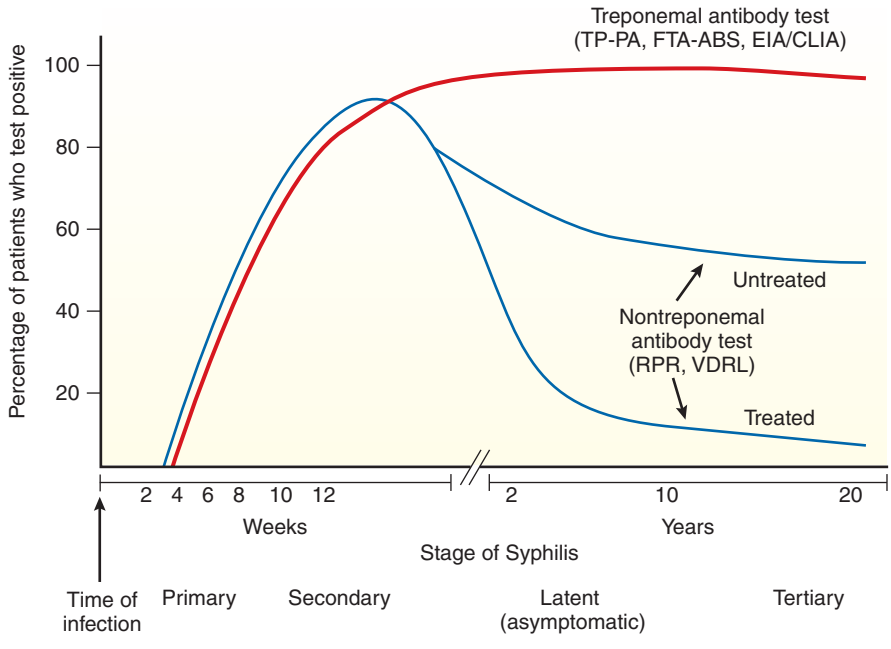


FIGURE 21–3 Typical nontreponemal and treponemal antibody patterns in syphilis. Adapted from Peeling RW, Ye H. Bulletin of the World Health Organization 2004; 82(6): 439-446.

0.21% lecithin. The antigen suspension is prepared by adding the VDRL antigen with a dropper to a buffered saline solution while continuously rotating the mixture on a flat surface; attention must be paid to required rotation speed and timing. A daily calibrated Hamilton syringe is used to deliver one drop of antigen for the slide test. If the delivery is off by more than 2 drops out of 60, the syringe must be cleaned with alcohol and recalibrated.

The serum specimens to be tested are heated at 56°C for 30 minutes to inactivate complement, after which 0.05 mL is pipetted into a ceramic ring of a glass slide. Three control sera—nonreactive, minimally reactive, and reactive—are pipetted into separate rings on the glass slide in the same manner. Sera and patient samples are spread out to fill the entire ring. One drop (1/60 mL) of the VDRL antigen is then added to each ring. The slide is rotated for 4 minutes on a rotator at 180 rpm. It is read microscopically to determine the presence of flocculation, or small clumps. The results are recorded as reactive (medium to large clumps), weakly reactive (small clumps), or nonreactive (no clumps or slight roughness).[21] Tests must be performed at room temperature within the range of 23°C to 29°C (73°F to 85°F) because results may be affected by temperature changes. All sera with reactive or weakly reactive results must be tested using the quantitative slide test, in which two-fold dilutions of serum ranging from 1:2 to 1:32 are initially used. Sera yielding positive results at the 1:32 dilution are titered further.

The RPR test is a modified VDRL test involving macroscopic agglutination. The cardiolipin-containing antigen suspension is bound to charcoal particles; this makes the test easier to read. The suspension is contained in small glass vials, which are stable for up to 3 months after opening. The antigen is similar to the VDRL antigen with the addition of ethylenediaminetetraacetic acid (EDTA), thimerosal, and choline chloride, which stabilize the antigen and inactivate complement so that serum does not have to be heat-inactivated before use. Patient serum (approximately 0.05 mL) is placed in an 18-mm circle on a plastic-coated disposable card using a capillary tube or Dispenstir device. Antigen is dispensed from a small plastic dispensing bottle with a calibrated 20-gauge needle. One free-falling drop is placed onto each test area and the card is mechanically rotated under humid conditions.[2] Cards are read under a high-intensity light source; if flocculation is evident, the test is positive **(Fig. 21–4).** All reactive tests should be confirmed by retesting using doubling dilutions in a quantitative procedure. The RPR test appears to be more sensitive than the VDRL in primary syphilis.[9]

Treponemal Tests. Treponemal tests detect antibody directed against the *T pallidum* organism or against specific treponemal antigens. (See Figure 21–3 for typical treponemal antibody results during various stages of syphilis.) Treponemal tests usually become positive before nontreponemal tests, although patients with early primary syphilis may be nonreactive.[14] In secondary and latent syphilis, tests are usually 100% reactive. Once a patient is reactive, that individual remains so for life. Although there are fewer false positives compared with reagin tests, reactivity is seen with other treponemal diseases, notably yaws and pinta.[9]

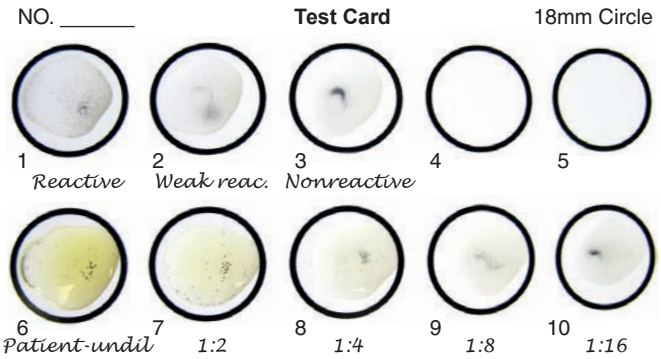


FIGURE 21–4 RPR test results. Well 1: reactive control, showing large clumps. Well 2: weakly reactive control, showing small clumps. Well 3: nonreactive control, showing slight roughness and a "tail" upon swirling. Wells 6 to 10 show results of a serially diluted sample of patient serum with a titer of 8. *(Linda Miller.)*

Two main types of manual treponemal tests are the indirect **fluorescent treponemal antibody absorption (FTA-ABS) test** and agglutination tests. Because these tests are highly specific for syphilis, they have been used to confirm positive nontreponemal test results. More recently, automated immunoassays for treponemal antibodies have been developed. Their applications will be discussed later.

The FTA-ABS test is one of the earliest confirmatory tests. In this test, a dilution of heat-inactivated patient serum is incubated with a sorbent consisting of an extract of nonpathogenic treponemes (Reiter strain), which removes antibodies that cross-react with treponemes other than *T pallidum.* Diluted patient samples and controls are applied to individual wells on a test slide fixed with the Nichols strain of *T pallidum*. Following a 30-minute incubation at 37°C, the slides are washed and air-dried and antibody conjugate (anti-human immunoglobulin conjugated with fluorescein) is added to each well. Slides are re-incubated as before and washed to remove excess conjugate. Mounting medium is applied and coverslips are placed on the slides. They are then examined under a fluorescence microscope.

If specific patient antibody is present, it will bind to the *T pallidum* antigens. The antibody conjugate will, in turn, only bind where patient immunoglobulin is present and bound to the spirochetes. When slides are read under a fluorescence microscope, the intensity of the green color is reported on a scale of 0 to 4+. No fluorescence indicates a negative test, whereas a result of 2+ or above is considered reactive.[2] A result of 1+ means that the specimen was minimally reactive and the test must be repeated with a second specimen drawn in 1 to 2 weeks.[2] Experienced personnel are needed to read and interpret fluorescent test results. The FTA-ABS is highly sensitive and specific, but it is time consuming to perform and has been replaced in many laboratories with particle agglutination methods.

The **particle agglutination (PA) tests** originally used sheep RBCs coated with *T pallidum* antigen and were referred to as MHA-TP (microhemagglutination assay for *T pallidum* antibody). Current PA tests for *T pallidum,* such as the Serodia ***T pallidum* particle agglutination (TP-PA) test,** use colored gelatin particles coated with treponemal antigens and are more sensitive in detecting primary syphilis.[14,22] In the Serodia TP-PA

test, patient serum or plasma is diluted in microtiter plates and incubated with either *T pallidum*-sensitized gel particles or unsensitized gel particles as a control. Presence of *T pallidum* antibodies is indicated by agglutination of the sensitized gel particles, which form a latticelike structure that spreads to produce a smooth mat covering the surface of the well. If a sample is negative for the antibody, the gel particles settle to the bottom of the well and form a compact button **(Fig. 21–5).**

A variety of automated immunoassays have been developed for the detection of antibodies to *T pallidum*. These include enzyme immunoassays (EIAs), chemiluminescent immunoassays (CLIA), and multiplex flow immunoassays (MFI). EIAs have been manufactured in a variety of formats, including one- or two-step sandwich assays, one-step competitive assays, and immune capture assays.[23,24] In the sandwich assays, antibodies in the patient sample bind to recombinant *T pallidum* antigens coated onto microtiter plate wells. An enzyme-labeled antibody or antigen conjugate and substrate are added to detect binding. In the immune capture format, microtiter wells are coated with antibody to IgM or IgG and are reacted with patient serum. Antigens that are labeled with an enzyme are then added **(Fig. 21–6).** The capture EIA tests are especially useful in diagnosing congenital syphilis in infants because they look for the presence of IgM, which cannot cross the placenta. They can also be used in monitoring response to therapy in the early stages of syphilis because many patients are negative for IgM treponemal antibodies 6 to 12 months after treatment.[23] In competitive EIAs, treponemal antibody in the patient sample competes with an enzyme-labeled treponemal antibody conjugate for *T pallidum* antigens bound to microtiter plate wells.[24] In a comparative study of several EIAs, test sensitivities ranged from 94.7% to 99.1% and test specificities were determined to be 100% for all of the assays evaluated.[23,24]

CLIAs are available as a one-step sandwich technique in which the patient sample is incubated with paramagnetic microparticles that have been coated with *T pallidum* antigens

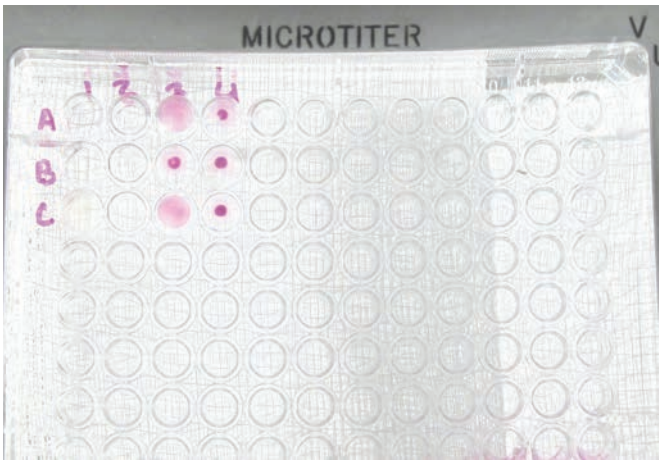


FIGURE 21–5 TP-PA test results. Row A: positive control. Row B: negative control. Row C: serum from a positive patient. *T pallidum*-sensitized gel particles were placed in column 3 of each row and unsensitized gel particles were pipetted into column 4 of each row. Positive wells (A3 and C3) are indicated by a diffuse mat of particles that spread over the surface of the well, whereas negative results are indicated by a compact button. *(Linda Miller.)*

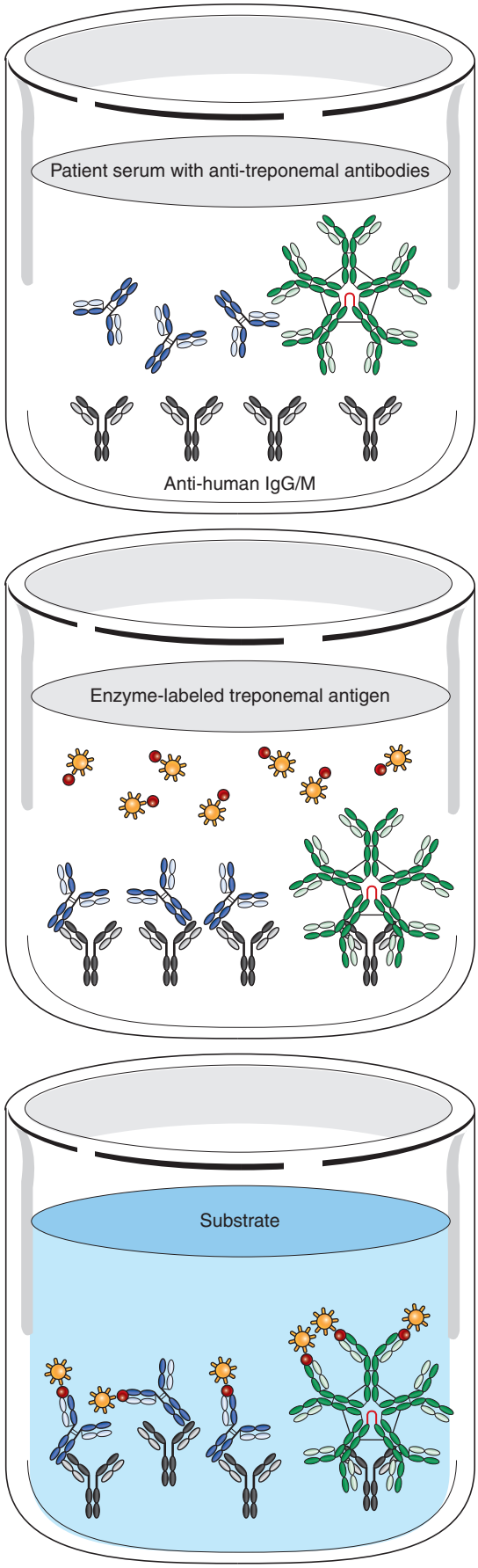


FIGURE 21–6 Antibody capture enzyme-linked immunosorbent assay (ELISA) test. Only specific anti-treponemal antibody will react with enzyme-labeled antigen.

linked to a chemiluminescent derivative.[23,24] After a wash step to remove unbound material, a catalyst is added and a chemical reaction occurs, producing emissions of light if the test sample is positive. The number of relative light units (RLUs) is proportional to the amount of treponemal antibody in the sample. CLIAs have many advantages as compared with EIAs, including a higher sensitivity in the early stages of syphilis, faster performance, and more stable reagents.[23]

MFI involves incubation of the patient sample with microspheres coated with recombinant *T pallidum* antigens. Microspheres that have bound immune complexes are detected after addition of a phycoerythrin-labeled reporter antibody and are analyzed by flow cytometry. This method can simultaneously detect antibodies to multiple *T pallidum* antigens in a small volume of sample and has a rapid turn-around time.[25] A study comparing MFI and several treponemal EIAs to the FTA-ABS found a 95.4% to 98.4% agreement in test performance.[26]

Clinical Applications. Nontreponemal tests are sensitive, inexpensive, and simple to perform. Thus, they have been very useful as a screening tool for syphilis. In addition, because antibody titers can be determined by testing serial dilutions of the patient sample, these methods have also been useful in monitoring the progress of the disease and in determining the outcome of treatment. Their main disadvantage is that they are subject to false positives. Transient false positives occur in diseases such as hepatitis, infectious mononucleosis, varicella, herpes, measles, malaria, and tuberculosis, as well as during pregnancy.[2] Chronic conditions causing sustained false-positive results include systemic lupus erythematosus (SLE), leprosy, intravenous drug use, autoimmune arthritis, advanced age, and advanced malignancy.[2,14]

A reactive nontreponemal test should be confirmed by a more specific treponemal test. In pregnancy, this is especially important because nontreponemal titers from a previous syphilis infection may increase nonspecifically.[2,9] Titers can be considered to be nonspecifically increased if lesions are absent, the increase in titer is less than four-fold, and documentation of previous treatment is available.[2]

Although treponemal tests are usually reactive before *nontreponemal* tests in primary syphilis, they suffer from a lack of sensitivity in congenital syphilis and neurosyphilis. Nontreponemal tests should be used for these purposes.[2,9] Treponemal tests are more difficult to perform, and have been traditionally used as confirmatory tests to distinguish false-positive from true-positive *nontreponemal* results. They also help establish a diagnosis in late latent syphilis or late syphilis because they are more sensitive than nontreponemal tests in these stages.[2]

Testing Algorithms. The traditional testing algorithm for syphilis involves screening the sample with a nontreponemal test and confirming any positive results with a more specific treponemal test **(Fig. 21–7).** This testing strategy is recommended by the CDC.[27]

Because of the development of sensitive, automated methods for treponemal antibodies that can be easily performed in the clinical laboratory, a change in the testing strategy for syphilis has been proposed. The newer reverse sequence algorithm method is becoming increasingly popular, especially among large reference laboratories.[28] Under this scheme, the testing order is reversed from the traditional algorithm in that patient samples are screened by an automated treponemal immunoassay and positive results are confirmed by a nontreponemal test. This algorithm has several advantages over the traditional algorithm. The first advantage is cost. Automated testing can be performed on LIS-interfaced high-throughput analyzers as opposed to labor-intensive manual methods, saving time and reducing errors.[29] Secondly, this algorithm can potentially detect more early, late, and treated syphilis cases because of the higher sensitivity of the specific treponemal tests. In the traditional algorithm, these may be missed because testing stops with a negative nontreponemal test result and the treponemal specific test is not run.

In the reverse algorithm, if the initial assay is negative, no further testing is done unless early syphilis is suspected (i.e., before seroconversion). If the automated assay result is positive and the subsequent RPR is positive, then the results are considered positive for syphilis. However, discrepant results can be obtained in some cases, with the initial automated test result being positive and the RPR that follows giving a negative result. This combination of results can be problematic because it could be caused by a false-positive treponemal antibody test result, a past syphilis infection, or early primary syphilis, in which patients have not yet produced nontreponemal antibodies. To help distinguish between these possibilities, the CDC recommends that if laboratories choose to use the reverse algorithm, all discrepant results should be tested reflexively using the TP-PA test as a secondary confirmatory treponemal

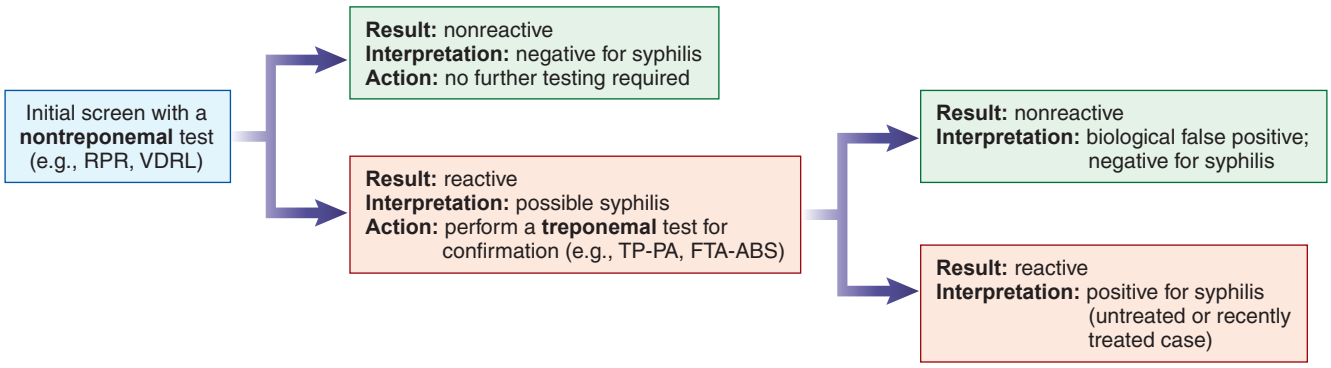


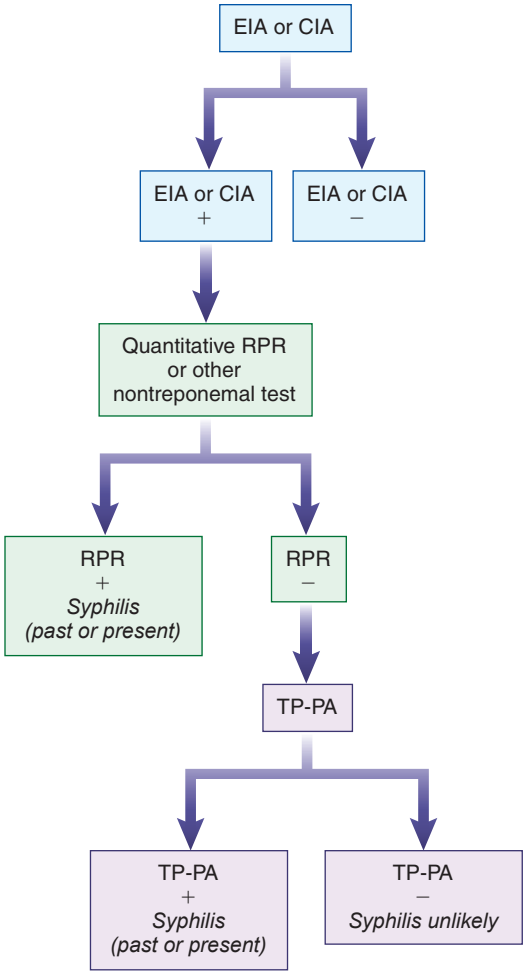
FIGURE 21–7 Traditional testing algorithm for syphilis.

test.[29,30] If the TP-PA is positive, then late or latent syphilis or previous history of syphilis would be considered. If negative, then it would be considered negative for syphilis at the time of testing. Careful evaluation of the patient's history should be considered regarding possible reevaluation at a later date **(Fig. 21–8)**.

Molecular Testing by Polymerase Chain Reaction (PCR)

PCR technology, which involves isolating and amplifying a specific sequence of DNA, has been used to test for the presence of treponemes in whole blood, spinal fluid, amniotic fluid, various tissues, and swab samples from syphilis lesions. Although there are many variations of this procedure, DNA is basically extracted from the sample and then replicated using a DNA polymerase enzyme and a primer pair to start the reaction (see Chapter 12). One variation is real-time PCR, which is automated, faster, and more sensitive than traditional PCR.

PCR is an extremely sensitive technique capable of detecting as little as one treponeme in some clinical samples.[31] Sensitivity is highest in patients with primary syphilis, but is greatly reduced in detecting disease in secondary syphilis. [29,32,33] Clinical availability of PCR is currently limited; however, in the future PCR could be a useful tool for diagnosis when serological testing is inconclusive and may provide a viable alternative to dark-field microscopy in directly detecting the organism in ulcers from patients with primary disease.[29,32,33] PCR may also be helpful in detecting treponemes in the blood of neonates with symptoms of congenital syphilis and in the CSF of patients suspected of having neurosyphilis.[33] Better standardization of PCR may help the method to gain more widespread use in testing for syphilis in the future.



EIA/CIA = enzyme immunoassay/chemiluminescence immunoassay; TP-PA = treponema pallidum particle agglutination.

FIGURE 21–8 CDC-recommended algorithm for reverse sequence syphilis screening (treponemal test screening followed by nontreponemal test confirmation). Despite these recommendations for reverse sequence screening, CDC continues to recommend the traditional algorithm with reactive nontreponemal tests confirmed by treponemal testing. *(Adapted from Centers for Disease Control and Prevention, reference [33].)*

Special Diagnostic Areas

Congenital Syphilis. Nontreponemal tests for congenital syphilis performed on cord blood or neonatal serum detect the IgG class of antibody in addition to IgM.[34] It is difficult to differentiate passively transferred IgG maternal antibodies from those produced by the neonate, so there are problems in establishing a definitive diagnosis. Late maternal infection may result in a nonreactive test because of low levels of fetal antibody. Additionally, testing the infant's spinal fluid for the presence of treponemes often lacks sensitivity.[35] Nontreponemal titers in the infant that are higher than those in the mother may be a good indicator of congenital disease, but this does not always occur.[2]

Several approaches have focused on detecting IgM antibodies in the infant. An FTA-ABS test for IgM alone lacks sensitivity and the test is subject to interference because of the presence of rheumatoid factor.[35] However, an IgM capture assay is more sensitive and a Western blot assay (see Chapter 24 for details) using four major treponemal antigens has demonstrated a high sensitivity and specificity.[2]

Currently, it is recommended that in high-risk populations, nontreponemal tests be performed on both the mother and infant at birth, regardless of previously negative maternal tests. Because symptoms are not always present at birth, if congenital syphilis is suspected because of maternal history, tests should be repeated on infant serum within a few weeks.[18] If infection is present in the infant, the titer will remain the same or will increase. The Western blot test is recommended to confirm congenital syphilis.[9]

Cerebrospinal Fluid. CSF is typically tested to determine whether treponemes have invaded the CNS. Such testing is usually more reliable if CNS symptoms are present. The VDRL test and some of the newer ELISA tests are the only ones routinely used for the testing of spinal fluid.[2,9] For VDRL spinal fluid testing, the antigen volume used is less than the serum test and is at a different concentration. In addition, different slides are used (Boerner agglutination slides). The test is read microscopically as in the VDRL serum test. If a test is reactive, two-fold dilutions are made and retested following the same protocol.

A positive VDRL test on spinal fluid is diagnostic of neurosyphilis because false positives are extremely rare.[9] However, sensitivity is lacking because samples from fewer than 70% of patients with active neurosyphilis give positive results.[9,14] If a negative test is obtained, other indicators such as increased lymphocyte count and elevated total protein (45 mg/dL) are used as signs of active disease.[12] PCR has been advocated in diagnosing neurosyphilis and may play an important role in CSF testing in the future.[9,36]

Lyme Disease

Lyme disease was first described in the United States in 1975 when an unusually large number of cases of juvenile arthritis appeared in a geographically clustered rural area around Old Lyme, Connecticut (hence the disease name), in the summer and fall. Two mothers recognized this and brought it to the attention of health officials. Because of the epidemiological features of this newly described "Lyme arthritis," transmission by an arthropod vector was suggested.[37] In 1982, the agent was isolated and identified as a new spirochete. It was given the name ***Borrelia burgdorferi*** after Willy Burgdorfer (first author in the original description).[38] The clinical features of Lyme disease were soon recognized to extend beyond arthritis; it is now known to be a multisystem illness involving the skin, nervous system, heart, and joints. Lyme disease is the most common vector-borne disease in the United States; over 36,307 confirmed or probable cases were reported in 2013. The number of cases being reported per year has doubled since 1991. According to the CDC, this reflects both a true increase in the frequency of the disease as well as better recognition and reporting.[39]

Characteristics of the Organism

Several species of *Borrelia* are known to be the causative agents of Lyme disease. In North America, it is exclusively *B burgdorferi sensu stricto,* whereas in Europe several species are known to cause Lyme disease (*Borrelia afzelii, Borrelia garinii, Borrelia sensu stricto,* and occasionally other *Borrelia* species).[40,41] All share similar characteristics; for simplicity, they will be referred to as *B burgdorferi.* The organism is a loosely coiled spirochete, 5 to 25 mm long and 0.2 to 0.5 mm in diameter.[1,40] The outer membrane, which consists of glycolipid and protein, is extremely fluid and only loosely associated with the organism. Several important lipoprotein antigens, labeled OSP-A through OSP-F, are located within this structure and are actually encoded by plasmids.[41] Surface proteins allow the spirochetes to attach to mammalian cells.

Just underneath the outer envelope are 7 to 11 *endoflagella* or *periplasmic flagella.* These run parallel to the long axis of the organism and are made up of 41 kDa subunits that elicit a strong antibody response. This immunodominant characteristic is of diagnostic importance because the response is not only strong but is also very early. Unfortunately, the flagellin subunit has homology to that of other nonpathogenic and pathogenic spirochetes, notably *B recurrentis* and *T pallidum,* causing cross-reactivity in serological testing.[1,42] Because of this, a large number of uninfected people have low levels of antibodies to the 41 kDa protein.[43] Although this is usually not a problem in the current diagnostic scheme of testing, it can become an issue when these individuals become ill with certain viruses that are known polyclonal B-cell activators (such as Epstein-Barr virus). In these cases, this normally low-level antibody becomes high enough to cause biological false positivity.

The organism divides by binary fission approximately every 12 hours. It can be cultured in the laboratory in a complex liquid medium (Barbour-Stoenner-Kelly) at 33°C, but it is difficult to isolate from patients. The spirochetemia is short-lived and generally found only early on in illness.[40] Cultures often must be incubated for 6 weeks or longer to detect growth and are therefore of little diagnostic utility.[1]

The main reservoir host is the white-footed mouse *(Peromyscus leucopus),* although in California and Oregon the spirochete is also harbored by the dusky-footed woodrat.[41] Vectors are several types of *Ixodes* ticks: *Ixodes scapularis* in the Northeast and Midwest United States **(Fig. 21–9),** *Ixodes pacificus* in the West, *Ixodes ricinus* in Europe, and *Ixodes persulcatus* in Asia. White-tailed deer are the main host for the tick's adult stage. Nymphs and adult stages of the tick can transmit the disease. The peak feeding is in the late spring, early summer, and the fall, which corresponds to the peak biphasic occurrence of Lyme disease.[1] The tick must feed for a period of time before the spirochete can be transmitted. Most agree that the risk for transmission is very low when ticks have fed for fewer than 36 hours; one study found that transmission is still low at 72 hours.[42]

Stages of the Disease

Lyme disease resembles syphilis in that manifestations occur in several stages. These have been characterized as (1) localized rash, (2) early dissemination to multiple organ systems, and (3) a late disseminated stage often including arthritic symptoms.[44,45] These stages are not always sharply delineated; therefore, it may be easier to view Lyme disease as a progressive infectious disease that involves diverse organ systems.

The clinical hallmark of early infection is the rash known as erythema migrans (EM), which appears between 2 days and

FIGURE 21–9 Adult tick *I scapularis,* which transmits Lyme disease. *(Courtesy of the CDC/Michael L. Levin, PhD, and Jim Gathany, Public Health Image Library.)*

2 weeks after a tick bite.[46] EM begins as a small red papule where the bite occurred, then rapidly expands to form a large ringlike erythema and often a central area that exhibits partial clearing **(Fig. 21–10).** The clinical diagnosis of early Lyme disease relies on the recognition of this characteristic rash, which should be at least 5 cm in diameter. At this stage, the patient may be asymptomatic or have nonspecific flu-like symptoms.[47-49] The EM usually continues to expand for over a week; even if untreated, it gradually fades within 3 to 4 weeks. Unfortunately, approximately 20% of patients do not develop the rash.[45,47,48] At this early stage, the antibody response is minimal and most serologies are negative.

Early dissemination occurs via the bloodstream in the days to weeks following the EM rash. The skin, nervous system, heart, or joints may be affected. Approximately 10% to 15% of patients will display multiple skin lesions.[41] Migratory pain often occurs in the joints, tendons, muscles, and bones. If treatment is not obtained, neurological or cardiac involvement develops in about 15% of patients within 4 to 6 weeks after the onset of infection.[41,50] The most prevalent neurological sign is facial palsy, a peripheral neuritis that usually involves one side of the face.[41,50] Pain and weakness can occur in the limb that was bitten. Some patients develop sleep disturbances, mild chronic confusional states, or difficulty with memory and intellectual functioning.[45] An aseptic meningitis can also be seen.[49]

Late Lyme disease may develop in some untreated patients months to years after acquiring the infection.[51] The major manifestations of late Lyme disease are arthritis, peripheral neuropathy, and encephalomyelitis. These symptoms usually respond well to conventional antibiotic treatment, but treatment-resistant arthritis has been associated with particular HLA–DRB alleles.[41,44] Despite resolution of objective manifestations of *Borrelia* infection after antibiotic treatment, a small percentage of patients develop chronic fatigue, concentration and short-term memory problems, and musculoskeletal pain.[52] These symptoms can last longer than 6 months in some cases.

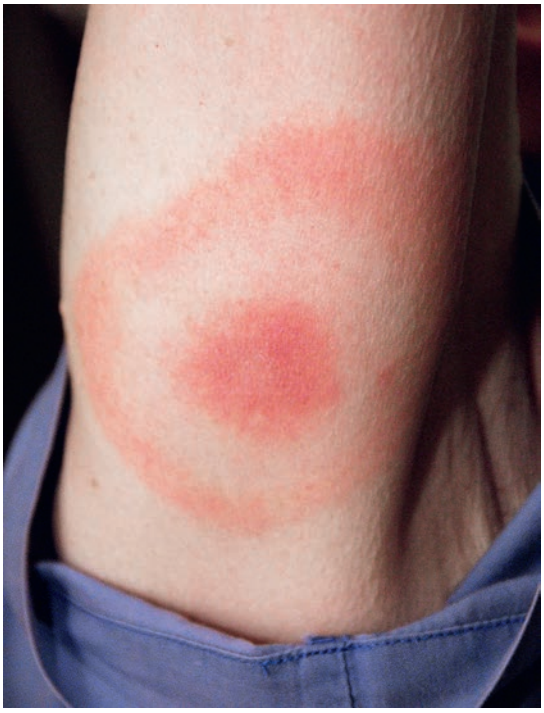


FIGURE 21–10 Erythema chronicum migrans rash, which appears after a tick bite in Lyme disease. *(Courtesy of the CDC/Jim Gathany, Public Health Image Library.)*

Nature of the Immune Response

The immune response in Lyme disease is highly variable and complex. A well-documented humoral and cellular response is known to exist. Spirochete lipoproteins also trigger production of macrophage-derived cytokines, which further enhance the immune response.[41] However, the clinical effectiveness of these responses is certainly questionable and not necessarily protective, because late Lyme disease occurs despite high levels of circulating antibody and cellular responses.

Laboratory Diagnosis

Diagnosis of Lyme disease is a clinical one, with laboratory testing used as supporting evidence. Unfortunately, the clinical diagnosis is often difficult for the reasons previously discussed. If the characteristic rash is present, this can be used as a presumptive finding, but as many as 20% of patients do not get or do not recognize the rash.[45,46,48] Direct isolation of the organism from skin scrapings, spinal fluid, or blood is possible, but the yield of positive cultures is extremely low. Therefore, culture is not used as a routine diagnostic tool.

The antibody response is variable and may not be detectable until 3 to 6 weeks after the tick bite. The IgM response occurs first followed by the IgG response. The IgG response does not peak until the third and fourth weeks of infection.[46,47] These antibody responses are also not mutually exclusive and can be variable (e.g., an IgM response can occur in late Lyme disease). In most cases of acute early Lyme disease (first 2 weeks), serological testing is too insensitive to be diagnostically helpful.[51] If patients with symptoms are tested in fewer than 7 days after infection, seropositivity is only about 30%.[47,53] Therefore, the decision to start treatment for early Lyme disease must be made before seroconversion, similar to many acute infectious diseases. However, untreated seronegative patients having symptoms for 6 to 8 weeks are unlikely to have Lyme disease and other possible diagnoses should be pursued.[44] Antibiotic therapy begun shortly after the appearance of EM may delay or abrogate the antibody response. In chronic Lyme disease, negative serologies have been attributed to previous antibiotic therapy; however, the scientific support of this theory is not strong.[52]

The CDC recommends a two-tiered approach to providing laboratory support for the diagnosis of Lyme disease.[54,55] It is recommended that patients with clinical evidence of Lyme disease be screened with an IFA or EIA test. If this serology is positive or borderline, a Western blot should be performed on that specimen as supplemental testing. Lyme testing should not be performed in the absence of supporting clinical evidence. A positive test performed under these circumstances has only a 6% positive predictive value (even when done in an endemic area), whereas it rises to greater than 97% when clinical symptoms and history

are present and consistent with Lyme disease.[55] Some of the current testing procedures are discussed next.

Immunofluorescence Assay (IFA)

The IFA was the first test used to evaluate the antibody response in Lyme disease, followed by various forms of EIAs shortly thereafter. The IFA assay is fairly easy to develop, which is why it is usually the first assay on the market in many infectious disease arenas. Basically, doubling dilutions of patient serum are incubated with commercially prepared microscope slides coated with antigen from whole or processed spirochetes. Following a wash step to remove unbound material, an anti-human globulin with a fluorescent tag attached is added and reacts with any specific antibody bound to the spirochetes on the slide. After a second wash step, the slide is viewed under a fluorescent microscope. Typically, a test result is only considered positive if a titer of 1:256 or higher is obtained,[53] although this varies between manufacturers. As previously mentioned, specimens obtained in the first few weeks are usually negative because the level of antibody present is below the detection limit of this (and other) assays.[53] As might be expected, other closely related organisms such as *B recurrentis* (relapsing fever), *T denticola* and others (associated with periodontal disease), and *T pallidum* (syphilis) may cross-react and cause biological false-positive results.[42] Autoimmune connective tissue diseases such as rheumatoid arthritis (RA) and SLE can also produce false positives in the IFA assay for Lyme disease and the FTA assay for syphilis.[56] An astute technologist can recognize a false positive by the beaded fluorescent pattern it produces. Reading of fluorescent patterns tends to be very subjective and requires highly trained individuals. However, if performed correctly by experienced personnel, the test can provide sensitive and accurate results. This test is best suited for low-volume testing.

Enzyme Immunoassay (EIA)

EIA testing is quick, reproducible (not subjective), relatively inexpensive, and lends itself well to automation and high-volume testing.[53] Antigen preparations used in the assay include crude sonicates of the organism, purified proteins, synthetic proteins, and recombinant proteins. The manufacturer's selected antigen is then coated onto 96-well microtiter plates or strips by various proprietary methods. Patient sera is added and allowed to incubate with the antigen. After a washing step, anti-human globulin conjugated with an enzyme tag such as alkaline phosphatase is added to each well. Adding specific substrate produces a color change. Plates are read in a spectrophotometer and the antibody is quantitated based on color intensity. EIAs provide objective results and the titer is based on a continuum range rather than serial dilutions of patient sera. Thus, a more accurate measurement of the specific antibody is possible.[53]

Similar to the IFA, drawbacks of EIA include a lack of sensitivity during the early stages of Lyme disease and specificity problems. The sensitivity for early serum specimens has been reported to be anywhere from 58% to 92%.[57] The differences in using various antigens is usually manifested in trade-offs between increasing sensitivity at the expense of specificity versus increasing specificity at the expense of sensitivity. Unfortunately, the technical adaptations of the EIA have not resulted in high enough sensitivity and specificity to replace the two-tier method of testing.[51] The specificity issues are generally similar to the IFA assay; however, there are no clues (beaded pattern) that a particular sample may be a false positive. As with IFA, false positives occur with syphilis and other treponemal diseases such as yaws and periodontal disease, as well as relapsing fever and leptospirosis.[1,57] If serum is absorbed to decrease cross-reactivity, this also decreases specific Lyme antibody titers. Additionally, patients with infectious mononucleosis, Rocky Mountain spotted fever, and other autoimmune diseases also have been known to be positive with EIA.[53] Lyme disease patients do not test positive with RPR, so this may be helpful if syphilis is in the differential diagnosis.[53]

Western Blot

Immunoblotting, or Western blotting, is used as a confirmatory test for samples that initially test positive or equivocal by EIA or IFA. It is the second test in the CDC-recommended two-tier testing scheme for Lyme disease.[46,57] Current CDC recommendations do not advise testing seropositive or borderline patients for IgM antibodies if they have had symptoms for more than 4 weeks for the reasons previously outlined. Serological evidence of Lyme disease in these patients is indicated by a positive result in the IgG immunoblot.[46,53]

The Lyme disease immunoblot is very complex **(Fig. 21–11)** and does not provide the same level of confidence as simpler systems such as HIV.[58,59] In Lyme disease, the immunoblot is generally referred to as supplemental testing. The technique consists of electrophoresis of *Borrelia* antigens in an acrylamide gel

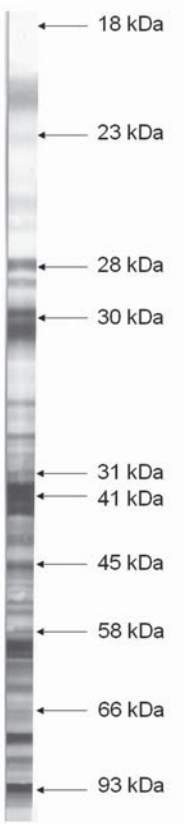


FIGURE 21–11 Immunoblot for Lyme disease.

and then transfer of the resulting pattern to nitrocellulose paper. This step is performed by the manufacturer and nitrocellulose antigen strips are provided in the test kit. These strips are reacted with patient serum and developed with an anti-human globulin (either anti-IgG or anti-IgM) to which an enzyme label is attached. The further incubation with the enzyme's substrate allows for visualization of any antibody that has bound to a particular antigen. The reactivity is then scored and interpreted.

Ten proteins are used in the CDC-recommended interpretation of this test. They are designated by their molecular weights: 18, 23, 28, 30, 39, 41, 45, 58, 66, and 93 kDa.[46] For a result to be considered positive for the presence of specific IgM antibody, two of the following bands must be present: 23(Osp C), 39, and 41 (flagellin) kDa.[60,61] An IgG immunoblot is considered positive if any 5 of the 10 bands previously listed are positive.[60,61] Because of the complexity of the Lyme immunoblots, testing and interpretation of blots should be done only in qualified laboratories that follow CDC-recommended evidence-based guidelines on immunoblot interpretations.[62]

Polymerase Chain Reaction (PCR)

In testing for Lyme disease, the PCR has found a niche in certain scenarios. Although only a few organisms need to be present for detection under optimal conditions, the number of spirochetes in infected tissues and body fluids is low, making specimen collection, transport, and preparation of DNA critical to the accuracy of the test results.[51]

Several probes for target DNA that is present only in strains of *B burgdorferi* have been made and used in PCR testing.[51,63] The procedure involves extracting DNA from the patient sample followed by amplification using specific primers, DNA polymerase, and nucleotides. Once a sufficient amount of the unknown DNA is made, this is combined with a known DNA probe to see if hybridization takes place. The single-stranded *Borrelia* DNA probe will bind only to an exact complementary strand, thus positively identifying the presence of the organism's DNA in the patient sample. This is much more specific than testing for antibody because there is little cross-reactivity. Specificity of recent PCR studies has ranged from 93% to 100%. However, sensitivity remains problematic. In a series of studies, the median sensitivity of PCR on skin biopsies was 69%; of blood components, 14%; of CSF, 38%; and of synovial fluid, 78%. However, the range of sensitivities in any one type of specimen is quite large, suggesting that testing remains to be standardized.[9,51] Furthermore, it would be hard to clinically justify a skin biopsy for PCR as a diagnostic method for an EM rash in most cases. However, in difficult diagnostic neurological and arthritic cases, PCR on CSF and synovial fluid is often employed. PCR for *Borrelia* still has limited availability.

Treatment

Borrelia is sensitive to several orally administered antibiotics, including penicillins, tetracyclines, and macrolides.[44,45] Prophylaxis, full-course treatment, or serological testing of all patients with tick bites is not recommended. A single dose of doxycycline may be offered to adults and children over 8 years of age when the tick can be reliably identified and treatment can begin within 72 hours of tick removal. Macrolides are not recommended as a first-line therapy for early Lyme disease because they are less effective. Under rare circumstances, an individual with a tick bite may be fully treated: (1) if the tick was identified as a nymph or adult *I scapularis;* (2) if the tick was attached for more than 36 hours; (3) if treatment can be started within 72 hours of tick removal; (4) if the local infection rate of ticks is over 20%; or (5) if doxycycline is not contraindicated.[44] Neuroborreliosis requires the use of intravenous antibiotic therapy.

Currently there are no effective vaccines for humans. A human vaccine made with the OSP-A surface antigen has had limited usefulness, has been associated with side effects, and has been recalled from the market.[64,65] There are renewed efforts to create a new vaccine, but as of this writing no vaccines have been approved for clinical use.

Relapsing Fever Group—*Borrelia Miyamotoi*

Relapsing fever is a relatively new disease, which was identified by a reversal of the typical disease discovery pathway. Typically, discoveries of new infectious diseases start with the association of a group of patients with a similar illness for which there is no known etiology. These findings, in turn, lead to efforts to isolate and define the causative organism. In the case of ***Borrelia miyamotoi***, the exact opposite occurred. In 1995, a novel *Borrelia* species was isolated from an *Ixodes* tick in Japan. This new species subsequently was found in North America in 2001 and in Eurasia in 2002. The discoveries confirmed the wide geographical distribution and the ecological niche of this new *Borrelia* species within the vectors of Lyme disease. These findings then led to efforts using specific assays for *B miyamotoi* to identify patients hospitalized with flu-like illness and a history of a recent tick bite. Using these methods, a Russian group identified a group of 46 patients with *B miyamotoi* infection in 2011.[66,67] This finding was confirmed in the United States by multiple studies in 2013.[68,69]

Characteristics of the Organism

B miyamotoi is closer phylogenetically to the relapsing fever *Borrelia* group than it is to *B burgdorferi.* However, similar to *B burgdorferi,* it has at least one mammalian reservoir host; like *B burgdorferi,* the white-footed mouse is the preferred host in the Eastern United States. The infection prevalence of questing nymphs in endemic areas is quite different from *B burgdorferi.* It is 20% to 50% in *B burgdorferi* and only 1% to 5% for *B miyamotoi.* Unlike *B burgdorferi,* but similar to several other relapsing fever *Borrelia* species, vertical transmission from the female adult tick to its offspring occurs in addition to the horizontal transmission from the reservoir host to the tick.[70] This transmission to offspring has possible implications for transmission to humans because the extremely small tick larvae are potentially infectious.

Stages of the Disease

Because this is a new disease, the disease characteristics are likely to change as more experience with the disease is gained. Initial studies suggest this is a flu-like illness. Unlike Lyme disease, an EM rash, arthritis, and facial palsies are uncommon.[67] The lack of a rash may, in fact, explain some of the documented "Lyme cases" where no rash was observed. Upon infection of the host, both *B burgdorferi* and *B miyamotoi* exhibit a short-lived bacteremia; however, *B burgdorferi* is at low density, whereas *B miyamotoi* is a very high density bacteremia. The opposite occurs in the skin infection: *B burgdorferi* is at high density and *B miyamotoi* is at low density.[70] The conclusions drawn from this are that the infectious period for *B miyamotoi* appears to be restricted to the bacteremia stage, whereas this period for *B burgdorferi* extends to the much longer skin involvement period. This may explain the lower infectivity rate of *B miyamotoi* in ticks.

Laboratory Diagnosis

The laboratory diagnosis of *B miyamotoi* infection is still in its infancy. At the time of this writing, all diagnostics are basically research and investigational only and there are no commercial FDA-approved kits. What follows is a brief description of some of the assays used to date; if history repeats, the commercial counterparts will follow shortly.

Direct detection by culture or PCR—Although *B burgdorferi* can be cultured, it is not useful as a diagnostic tool because it is a low yield procedure. Attempts to culture *B miyamotoi* in media have not been successful to date. However, if improved culture techniques are developed, it may be a viable diagnostic option because of the high density bacteremia. It is also possible that PCR testing of blood may become a feasible diagnostic method in the future.[66,67]

Antibody detection—The Russian study, which first found the disease associated with *B miyamotoi* infection, demonstrated, not surprisingly, that these patients were positive in the Lyme antibody assays.[67] Thus, it is possible that some of the diagnosed Lyme patients could actually be *B miyamotoi* patients instead. The 2013 U.S. study that confirmed *B miyamotoi* infection used an ELISA assay, which detected antibodies against GlpQ proteins (made from *B hermsii*). These antibodies are specific for the relapsing fever *Borrelia* and not *B burgdorferi*.[69]

SUMMARY

- Syphilis and Lyme disease are the two major diseases caused by spirochetes. Spirochetes are distinguished by the presence of axial filaments that wrap around the cell wall inside a sheath and give the organisms their characteristic motility.
- Syphilis is caused by the organism *Treponema pallidum,* subspecies *pallidum*. The disease is acquired by direct contact, usually through sexual transmission.
- Syphilis can be separated into four main clinical stages:
  1. The primary stage is characterized by the presence of a painless ulcer called a chancre at the site of initial contact.
  2. An untreated patient may progress from the primary stage to the secondary stage, in which systemic dissemination of the organism occurs and symptoms such as generalized lymphadenopathy, malaise, sore throat, and skin rash appear.
  3. Disappearance of the secondary stage is followed by a lengthy latent stage in which patients are usually free of clinical symptoms.
  4. About one-third of the individuals who remain untreated develop tertiary syphilis. This late stage disease is characterized by three major clinical manifestations: granulomatous inflammation (gummas), cardiovascular disease, and neurosyphilis. Early diagnosis and treatment help to prevent later complications.
- Direct laboratory diagnosis involves detecting the organism from a lesion and using dark-field microscopy, fluorescence microscopy, or PCR. If an active lesion is not present, diagnosis must be made on the basis of serological tests.
- Nontreponemal serological tests determine the presence of antibody to cardiolipin, also known as reagin (e.g., VDRL and RPR tests). These tests are fairly sensitive and simple to perform; however, they lack specificity, so specimens with positive results must be confirmed with a more specific treponemal antibody test.
- Traditional treponemal antibody tests include the FTA-ABS and particle agglutination (TP-PA). These tests detect antibody formed against the organism itself. Treponemal tests are more specific and sensitive in early stages of the disease.
- Titers of treponemal antibodies remain detectable for life, whereas nontreponemal titers decline after successful treatment.
- New developments in testing include EIA and CLIA technology and PCR. EIA and CLIA tests for antibody to specific treponemal antigens and separation of antibodies by class is possible. For large-volume testing, the EIA or CLIA is commonly used as a screening test, followed by confirmation with the RPR or VDRL (reverse screening algorithm).
- Lyme disease is the most common vector-borne infection in the United States. The organism responsible is the spirochete *Borrelia burgdorferi,* which is transmitted by the deer tick.
- Although an expanding red rash is often the first symptom noted in Lyme disease, the disease can be characterized as a progressive infectious syndrome involving diverse organ systems. Despite antibiotic treatment, a small percentage of patients continue to have fatigue; concentration and short-term memory problems; and musculoskeletal pain, which may last 6 months or longer. These symptoms may be caused by persistence of infection.

- The presence of IgM and IgG to *B burgdorferi* cannot usually be detected until 3 to 6 weeks after symptoms initially appear. Current testing protocol involves screening with IFA or EIA and follow-up of equivocal or positive tests with immunoblotting. All serological findings must be interpreted carefully and in conjunction with clinical diagnosis.
- *Borrelia miyamotoi* relapsing fever group is the newest tick-borne disease. Clinical features are similar to Lyme disease and may explain some of the variations seen in "Lyme" disease. Antibodies present in this disease cross-react with some of the Lyme diagnostic tests. Much is still to be elucidated regarding this disease.

Study Guide: Comparison of Tests Used for the Diagnosis of Syphilis

TEST	ANTIGEN	ANTIBODY	COMMENTS
Direct Microscopic			
Dark-field	*T pallidum* from patient	None	Requires active lesion; must have good specimen, experienced technologist; inexpensive
Fluorescent antibody	*T pallidum* from patient	Anti-treponemal antibody with fluorescent tag	Requires active lesion; more specific than dark-field; specimen does not have to be live
Nontreponemal			
VDRL	Cardiolipin	Anti-cardiolipin (Reagin)	Flocculation; good for screening tests, treatment monitoring, spinal fluid testing; false positives are common
RPR	Cardiolipin	Anti-cardiolipin (Reagin)	Modified VDRL with charcoal particles; more sensitive than VDRL in primary syphilis
Treponemal			
FTA-ABS	Nichols strain of *T pallidum*	Anti-treponemal	Confirmatory; specific, sensitive; may be negative in primary stage
Serodia TP-PA (formerly, MHA-TP)	Gel particles sensitized with *T pallidum* sonicate (formerly sensitized sheep RBCs)	Anti-treponemal	Not as sensitive as FTA-ABS
EIA, CLIA, MFI	Treponemal antigen	Anti-treponemal	Sensitive, automated testing provides objective results; used to screen for syphilis in some large laboratories; EIAs have been developed as competitive, sandwich, or capture immunoassays that can detect IgM or IgG antibodies
PCR	Nontreponemal DNA in patient sample is amplified	None	Highest sensitivity is in primary stage syphilis; availability is limited

CLIA = chemiluminescent immunoassay; DNA = deoxyribonucleic acid; EIA = enzyme immunoassay; FTA-ABS = fluorescent treponemal antibody absorption; MFI = multiplex flow immunoassay; MHA-TP = microhemagglutination assay for antibodies to Treponema pallidum; *PCR = polymerase chain reaction; RPR = rapid plasma reagin; TP-PA =* T pallidum *particle agglutination; VDRL = Venereal Disease Research Laboratory.*

Study Guide: Comparison of Tests for the Diagnosis of Lyme Disease

TEST	ANTIGEN	ANTIBODY	COMMENTS
IFA	Whole or processed *B burgdorferi*	Anti-*Borrelia* antibody from patient, anti-human globulin with fluorescent tag	Initial test for Lyme disease; labor intensive to perform; false positives; subjective;
EIA	Sonicated *B burgdorferi*	Anti-*Borrelia* antibody from patient, anti-human globulin with enzyme tag	Initial test for Lyme disease; easy to perform; false positives; more sensitive than IFA
	Purified flagellin protein	Anti-flagellin antibody from patient, anti-human globulin with enzyme tag	Initial test for Lyme disease; easy to perform; highly specific; sensitive in early Lyme disease
	C6 peptide	Conserved region of surface lipoprotein (VlsE)	Easy to perform; highly specific; sensitive in early and late Lyme disease
Western blot or immunoblot	Antigens of *B burgdorferi* separated by molecular weight	Detects antibodies (IgG or IgM) to individual *B burgdorferi* antigens	Technically difficult to perform; scoring the blot can be challenging
PCR	None. *B burgdorferi* DNA in patient sample is amplified	None	Availability is limited

DNA = deoxyribonucleic acid; EIA = enzyme immunoassay; IFA = immunofluorescence assay; PCR = polymerase chain reaction.

CASE STUDIES

1. A 30-year-old woman saw her physician to complain about repeated episodes of arthritislike pain in the knees and hip joints. She recalled having seen a very small tick on her arm about 6 months before the development of symptoms. No rash was ever seen, however. Laboratory tests for RA and SLE were negative. An EIA test conducted on the patient's serum for Lyme disease was indeterminate.

Questions

a. Does the absence of a rash rule out the possibility of Lyme disease?
b. What might cause an indeterminate EIA test?
c. What confirmatory testing would help determine the cause of the patient's condition?

2. A mother who had no prenatal care appeared at the emergency department in labor. The physician safely delivered a baby boy who appeared to be normal. The physician obtained a blood sample from the mother for routine screening. An RPR test performed on the mother's serum was positive. The mother had no obvious signs of syphilis and denied any past history of the disease. She indicated that she had never received any treatment for a possible syphilis infection. Cord blood from the baby also exhibited a positive RPR result.

Questions

a. Is the baby at risk for congenital syphilis?
b. What is the significance of a positive RPR on a cord blood test?
c. How should these results be handled?

REVIEW QUESTIONS

1. False-positive nontreponemal tests for syphilis may occur because of which of the following?
   - **a.** Infectious mononucleosis
   - **b.** Systemic lupus
   - **c.** Pregnancy
   - **d.** All of the above

2. In the fluorescent treponemal antibody absorption (FTA-ABS) test, what is the purpose of absorption with Reiter treponemes?
   - **a.** It removes reactivity with lupus antibody.
   - **b.** It prevents cross-reactivity with antibody to other *T pallidum* subspecies.
   - **c.** It prevents cross-reactivity with antibody to nonpathogenic treponemes.
   - **d.** All of the above.

3. Which test is recommended for testing cerebrospinal fluid for detection of neurosyphilis?
   - **a.** RPR
   - **b.** VDRL
   - **c.** FTA-ABS
   - **d.** Enzyme immunoassay

4. Advantages of direct fluorescent antibody testing to *T pallidum* include all of the following *except*
   - **a.** reading is less subjective than with dark-field testing.
   - **b.** monoclonal antibody makes the reaction very specific.
   - **c.** slides can be prepared for later reading.
   - **d.** careful specimen collection is less important than in dark-field testing.

5. Which of the following is true of nontreponemal antibodies?
   - **a.** They can be detected in all patients with primary syphilis.
   - **b.** These antibodies are directed against cardiolipin.
   - **c.** Nontreponemal tests remain positive after successful treatment.
   - **d.** The antibodies are only found in patients with syphilis.

6. Which syphilis test detects specific treponemal antibodies?
   - **a.** RPR
   - **b.** VDRL
   - **c.** FTA-ABS
   - **d.** Agglutination

7. Which of the following is true of treponemal tests for syphilis?
   - **a.** They are usually negative in the primary stage.
   - **b.** Titers decrease with successful treatment.
   - **c.** In large-volume testing, they are often used as screening tests.
   - **d.** They are subject to a greater number of false positives than nontreponemal tests.

8. An RPR test done on a 19-year-old woman as part of a prenatal workup was negative but exhibited a rough appearance. What should the technologist do next?
   - **a.** Report the result out as negative.
   - **b.** Do a VDRL test.
   - **c.** Send the sample for confirmatory testing.
   - **d.** Make serial dilutions and do a titer.

9. Treponemal EIA tests for syphilis are characterized by all of the following *except*
   - **a.** they are adaptable to automation.
   - **b.** they are useful in monitoring antibody titers in syphilis patients undergoing therapy.
   - **c.** subjectivity in reading is eliminated.
   - **d.** they can be used to distinguish between IgG and IgM antibodies.

10. Which of the following tests is the most specific during the early phase of Lyme disease?
    - **a.** IFA
    - **b.** EIA
    - **c.** Immunoblotting
    - **d.** detection of *B burgdorferi* DNA by PCR

11. False-positive serological tests for Lyme disease may be caused by all of the following *except*
    - **a.** shared antigens between *Borrelia* groups.
    - **b.** cross-reactivity of antibodies.
    - **c.** resemblance of flagellar antigen to that of *Treponema* organisms.
    - **d.** a patient in the early stage of the disease.

12. A 24-year-old man who had just recovered from infectious mononucleosis had evidence of a genital lesion. His RPR test was positive. What should the technologist do next?
    - **a.** Report out as false positive.
    - **b.** Do a confirmatory treponemal test.
    - **c.** Do a VDRL.
    - **d.** Have the patient return in 2 weeks for a repeat test.

13. A 15-year-old girl returned from a camping trip. Approximately a week after her return, she discovered a small red area on her leg that had a larger red ring around it. Her physician had her tested for Lyme disease, but the serological test was negative. What is the best explanation for these results?

- **a.** She definitely does not have Lyme disease.
- **b.** The test was not performed correctly.
- **c.** Antibody response is often below the level of detection in early stages.
- **d.** Too much antibody was present, causing a false negative.

14. The reverse screening algorithm for syphilis testing

- **a.** is the CDC preferred algorithm.
- **b.** is more labor intensive than the "traditional" method.
- **c.** has a high number of false positives that must be resolved by doing a TP-PA test.
- **d.** is more prone to transcription errors in reporting.

15. *Borrelia miyamotoi* infection

- **a.** may explain some cases of supposed Lyme disease where no rash was found.
- **b.** is a new lethal tick-borne disease.
- **c.** is carried by the common dog tick.
- **d.** is another name for Southern Tick Associated Illness (STARI).

22 Serological and Molecular Diagnosis of Parasitic and Fungal Infections

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Explain why a host has more difficulty overcoming parasitic diseases than those caused by bacteria or viruses.
2. Discuss potential outcomes of host and parasite interactions.
3. Cite strategies used by parasites to evade host defenses.
4. Discuss the role of immunoglobulin E (IgE) antibody and eosinophils in parasitic infections.
5. Discuss the roles of serological and molecular assays in the diagnosis of parasitic infections.
6. Discuss the role serology plays in the diagnosis of *Toxoplasma gondii* and cite the limitations of serological testing for toxoplasmosis in the newborn.
7. List possible limitations associated with parasitic serology.
8. Cite the role that rapid antigen detection systems (RDTS) play in the detection of parasitic diseases.
9. Briefly describe the principle of lateral flow assays.
10. List factors that have led to a notable increase in fungal infections in the past 25 years.
11. Describe the etiological and physiological factors to be examined when a mycosis is suspected.
12. Cite the four types of clinical manifestations that fungi can produce.
13. Describe the types of immune defenses mounted by the host in response to fungal infections and identify the immune response that plays the most important role in responding to a fungal infection.
14. Discuss the role of serological and molecular testing in the diagnosis of fungal infections.
15. Recognize the clinical diseases and epidemiology of aspergillosis, candidiasis, cryptococcosis, histoplasmosis, and coccidioidomycosis.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Antigenic concealment
Antigenic mimicry
Antigenic shedding
Antigen switching
Antigenic variation
Aspergillosis
C-type lectin receptors (CLRs)
Candidiasis
Coccidioidomycosis
Conidia
Cryptococcosis
Ectoparasites
Fungi
Helminths
Histoplasmosis
Hyphae
Immunologic diversion
Immunologic subversion
Lateral flow assays
Mycelial fungi
Mycosis (mycoses)
Parasites
Protozoa
Rapid antigen detection systems (RDTS)
Toll-like receptors (TLRs)
Yeast
Toxoplasma gondii

The detection and diagnosis of fungal and parasitic infections relies on laboratory methods as well as the patient's clinical symptoms, medical history, geographic location, and travel history. Traditional laboratory diagnosis is based on microscopic observation of the causative agent in clinical material and, in the case of fungi, recovery of the agent in culture. Technological advancements have provided newer methodologies that are an improvement over conventional methods for the detection of fungal and parasitic infections. Direct antigen detection, molecular assays, and newer serological assays have emerged. Although the sensitivity and specificity of serological assays for fungal and parasitic infections have improved over the years, the antigens used in parasitic and fungal assays are often cruder than those used in tests for viral and bacterial diagnosis, thus decreasing their specificity and sensitivity. As of 2014, there have been relatively few FDA-approved serological-based assays, limiting their use.

Despite the development of newer assays, the diagnosis of parasitic and fungal infections still remains a challenge. In many cases, the clinical presentations overlap or are nonspecific. Because of the shared antigenicity of several genera and species, the ability to distinguish a specific causative agent based on serology is sometimes not possible. Many fungal infections are opportunistic infections that occur in individuals who are immunocompromised. As such, those individuals may not mount a humoral antibody response, limiting the utility of serologically based assays. In addition, many of the parasitic diseases occur in impoverished countries. Because of the lack of resources and expertise in these countries, the development and use of serological assays has been hindered. Finally, for many of the agents, diagnostic assays (serological or molecular) are not available. This chapter provides information on the immunologic aspects of parasitic and fungal infections along with a discussion of available serological and molecular assays for the detection of the organisms that cause these infections.

Parasitic Immunology

Parasites are microorganisms that survive by living off of other organisms, referred to as *hosts*. Three types of organisms may cause parasitic infections: protozoa, helminths, and ectoparasites. **Protozoa** are a diverse group of single-celled organisms that can live and multiply inside of human hosts. Giardiasis is an example of a protozoal infection that can occur from drinking water infected with the parasites. **Helminths,** or parasitic worms, are multicelled organisms that can live either alone or in humans. They include flatworms, tapeworms, and roundworms. **Ectoparasites** are multicelled organisms that live on the skin. Common ectoparasites infecting humans include *Pediculus humanus capitis* (head lice), *Pthirus pubis* (pubic lice or crabs), and *Sarcoptes scabiei* (scabies). Other ectoparasites include fleas, ticks, and mites.

Mortality from infectious diseases declined substantially in the United States during the first eight decades of the 20th century because of improvements in living conditions, medical care, and sanitation. Beginning in 1981 that trend reversed, largely because of the AIDS epidemic. With the decline of AIDS in the United States, that trend is again heading downward. In 1996, a 7% drop in infectious disease deaths was recorded.[1,2] However, in many areas of the globe, parasitic diseases are on the rise, particularly in the tropic and subtropic regions, because of rapid and unplanned growth in the cities. The World Health Organization (WHO) reported that globally, one-third of all deaths are caused by infectious and parasitic diseases. In 2010, malaria alone was the underlying cause of death for 1.2 million people, including more than 700,000 children younger than 5 years and over 520,000 people aged 5 years or older.[3] Whereas malaria, tuberculosis, and HIV are well known for their impact in the developing world, there are a number of neglected tropical diseases (NTDs) that cause significant morbidity and mortality around the globe. A vast majority of the NTDs are caused by parasites. These diseases include leishmaniasis, schistosomiasis (snail fever), African trypanosomiasis (sleeping sickness), onchocerciasis (river blindness), and lymphatic filariasis (elephantiasis).[4,5] In the United States, trichomoniasis, giardiasis, cryptosporidiosis, and toxoplamosis are the most common parasitic infections.

Although effective vaccines have been developed for bacterial and viral agents, the development of vaccines for parasitic diseases has remained elusive. A variety of roadblocks have hindered the development of vaccines against parasitic agents. Parasites are complex organisms, many with elaborate life cycles; over time, they have developed well-honed mechanisms for immune evasion. In addition, the immune responses to parasitic organisms are not fully understood, which also contributes to the lack of vaccine development in this area. This chapter covers the various strategies used by parasites to evade the immune response and survive in a host, as well as the use

of serological and molecular techniques in the diagnosis of parasitic diseases.

The immune responses to bacterial infections are more clearly understood than the immune responses to parasitic infections. Bacteria are unicellular organisms with relatively simple life cycles. In addition, there is limited epitope variation in bacteria, allowing for the immune system to mount a response more easily. The immune response to parasitic infections differs from that associated with bacterial infections, mostly because of the multicellular nature of parasites. Chandra[6] described general concepts that need to be considered in relation to host immune responses to parasites: (1) Heterogeneity with respect to life cycles and antigenic expression is a key feature of parasitic agents. (2) Many parasitic infections are chronic in nature. (3) The mechanisms of immune evasion are significantly different from those of bacterial infections. (4) Many parasites develop significant genetic and antigenic variation in a relatively short period. (5) The innate immunity in the natural hosts may be genetically determined. (6) Humans, as well as animals, differ widely in their ability to handle the complex antigens found in parasites.

Immune Responses to Parasites

When an organism encounters a host, the eventual outcome depends on a variety of factors. These include the number of organisms or size of the inoculum, the multiplication rate of the organism, and the virulence factors possessed by the organism. The degree to which the organism establishes an infection or is eradicated by the host depends on the organism's ability to mobilize sufficient host defenses for removal or the organism's ability to overcome those defense mechanisms.

If the parasite is able to establish itself, one of several possible outcomes may occur: death of the host, eradication of the parasite, or, as is the case of the majority of parasitic infections, establishment of a persistent infection. The interaction and outcomes that parasites have within their hosts have been categorized into six different levels[7] **(Table 22–1).**

The most severe outcome is that the host is killed. Death of the host may be caused by a variety of reasons, ranging from the parasite overwhelming the host defenses to specific features of the parasite that contribute to the death of the host. One such example is *Plasmodium falciparum*. *P falciparum* rapidly multiplies in the host and produces an erythrocyte membrane protein-1 (PfEMP1) that binds to the endothelium in the blood vessels, resulting in the small blood vessels becoming clogged. When this occurs in the brain, the result is cerebral malaria, which can be fatal.

Death of the host is not the best strategy for a parasite. If the parasite were to totally elude the host immune system and was sufficiently virulent, the parasite would kill the host on which its survival depends. If the host dies, then so does the parasite. Any parasite's survival depends on its ability to live in a peaceful manner with its host while living and feeding off the host.

Defenses to parasitic infection involve both innate and acquired (adaptive) immune mechanisms. The innate or nonspecific immune response may result in the destruction and removal of the parasite, thus preventing establishment of an infection. The nonspecific immune defenses can include activation of cells that may destroy the parasite by phagocytosis, release of cytokines (e.g., TNF-α, IL-1, IL-10, IL-12, type I interferons, and chemokines) that enhance the immune response, or activation of the complement system, resulting in enhanced recognition by the immune system (see Chapters 3, 4, and 7). Similar to other organisms that may cause infection or disease, parasites have evolved strategies to evade natural nonadaptive host defenses. These include killing or avoiding being killed by phagocytes, interfering with complement's alternate pathway, production of iron-binding molecules, and blocking interferons.

If the innate immunity is unsuccessful in eliminating the parasite, the parasite may be eliminated through activation of the adaptive immune responses. This results in either a humoral or a cell-mediated response to the parasite (see Chapter 4). In some parasites, complete immunity can be achieved through the humoral immune response. Specific antibody can damage

Table 22–1 Potential Outcomes of Host and Parasite Interactions

LEVEL	HOST AND PARASITE INTERACTION	DESCRIPTION
1	Natural resistance	No invasion of host by parasite
2	Symbiosis	Colonization of host with parasite with benefit to both
3	Commensalism	Colonization of host with parasite with no benefit or harm
4	Sterilizing immunity	Parasite invades host and causes disease; host develops immunity and is cured
5	Concomitant immunity	Parasite invades host and causes disease; host develops an immune response and has some resistance to the parasite but is not cured
6	Ineffective immunity	Parasite invades host and causes disease; host does not develop resistance to the parasite and is not cured

Adapted from Playfair JHL. Effective and ineffective immune responses to parasites: evidence from experimental models. Curr Top Microbiol Immunol. *1978;80:37–64.*

protozoa, neutralize parasites by blocking attachment to the host cell, prevent the spread of the parasite, promote complement lysis, enhance phagocytosis, and destroy the parasite through antibody-dependent cellular cytotoxicity (ADCC). Many parasitic infections are characterized by eosinophilia and high levels of immunoglobulin E (IgE). The IgE antibody binds to mast cells and basophils in the host (see Chapter 14). When specific antigen–antibody combinations occur, mast cells degranulate and release chemotactic factors, histamine, prostaglandins, and other mediators. One of the most important mediators released is eosinophil chemotactic factor, which attracts eosinophils to the infected area. Eosinophils can destroy some parasites by degranulation or through ADCC. Scientists believe that the ability to produce IgE evolved mainly to protect the host from parasitic infections.[8] Studies indicate that high levels of anti-parasitic IgE correlate with resistance to reinfection by the parasite.[9,10]

Parasite Survival Strategies

In many host and parasite relationships, the host's adaptive defenses reduce the parasite load to low levels; however, they fail to eliminate the parasite completely and transmission continues. For example, schistosomes (a type of helminth) have mechanisms that can downregulate the host's immune system. This immune modulation promotes the parasite's survival but also limits severe damage to the host. As a result, adult schistosomes can live in the human host for up to 40 years before finally being eliminated.[11,12]

Parasites have developed a variety of strategies to evade adaptive defenses that are more complicated than those for evading innate defenses. Survival strategies include antigenic concealment, antigenic variation, antigenic shedding, antigenic mimicry, immunologic diversion, and immunologic subversion.

In **antigenic concealment**, parasites hide their antigens from the host. One way in which parasites can conceal their antigens is by remaining inside of the host's cells without their antigens being displayed. If a parasite becomes sequestered within host cells, the parasite is hidden from the immune system and protected. Most parasites infect only a few cell types in their host. To infect a host, the parasite must reach its specific target cell. Once the parasite reaches its target cell, some parasites have developed strategies for entering and surviving within the host cell. The host cannot recognize the parasites while they reside inside cells.[13] Examples of parasites that have an intracellular phase in their life cycle include *Plasmodium* species, *Trypanosoma cruzi, Leishmania,* and *Cryptosporidium parvum.*

Another process of evasion some parasites employ is **antigenic variation.** Evasion from the immune response depends on variation occurring in the parasite antigens that are recognized by the host's immune system. There are three main mechanisms for antigenic variation. The first mechanism involves the parasite's ability to generate novel antigens by random mutation. Some parasites have evolved mechanisms by which random mutations occur with a frequency sufficient to evade the immune system on an ongoing basis. An example of this is the antigenic variation of the malaria parasite through single nucleotide point replacement. The second mechanism of antigenic variation may occur through genetic recombination. Rearrangement of genes within an organism allows for the development and expression of new epitopes on the surface of the parasite for which a previous immune response has not taken place. The ability to rearrange genes and rapidly change surface molecules contributes to the virulence of *P falciparum* and *T cruzi.*[14] The third mechanism is gene switching. Gene switching is the most dramatic form of antigenic variation observed in parasites. Organisms may carry upwards of one thousand different genes, allowing for the expression of distinct surface molecules. An organism employing this mechanism can switch from the use of one gene to another, thus persisting while the immune system is trying to catch up with it. Examples of parasites that use this mechanism are the trypanosomes, *Trypanosoma gambiense,* and *Trypanosoma rhodesiense.* These organisms are able to alter their surface glycoproteins to produce an unlimited group of variable antigen types.[15,16] The process begins when the host produces antibody, mainly IgM, to the one antigen, thereby reducing the infection. The parasite responds by swapping genes, thus changing its antigen and making the current antibody ineffective. Gene switching may occur very rapidly, within 5 to 6 days. The host must then produce a new antibody. This process of **antigen switching** can continue for long periods of time.

A factor that must be considered with respect to antigenic variation is the parasite's life cycle. Parasites are large organisms compared with bacteria and viruses. Furthermore, they have complex life cycles and are antigenically diverse. The parasite's development and progression through its life cycle are adapted to the physiology and behavior of the host. Not all of the phases of a parasite's life cycle necessarily occur in one host. A particular host may be the definitive host that harbors the adult or sexual stage of the parasite (e.g., *Taenia saginata* and *Taenia solium*—the beef and pork tapeworms, respectively—where humans are the only definitive host), an intermediate host in which the parasite lives during the larval or asexual stage (e.g., the malarial parasites where humans are the intermediate host), or an accidental or dead-end host in which a relationship is not required for propagation or continuation of the parasite

Connections

IgE Antibodies and Parasites

IgE antibodies are best known for their role in allergic reactions. However, they also play an important role in the defense against parasites such as helminths, which are too large to be phagocytized. Killing of the parasites is accomplished by ADCC. In this mechanism, the Fc portions of the parasite-specific IgE antibodies bind to specific receptors on the surface of eosinophils, which are then stimulated to release enzymes from their granules that destroy the parasite. The concentration of IgE and the number of eosinophils in the peripheral blood are increased, indicating their importance in defense against parasitic infections.

(e.g., *Echinococcus granulosus,* the causative agent of hydatid cysts, where humans are the dead-end host).

Many parasites have complex life cycles that involve several hosts. Different antigens may be expressed, depending on the life cycle stage in the different hosts. The parasite often undergoes complex growth cycles and differentiation in preparation for transmission to its next host. In doing so, an organism's surface antigens vary considerably while in a single host. An example is *Leishmania.* The parasite enters the host through the bite of an insect vector as a trypomastigote in the bloodstream, which is then phagocytized by macrophages, where it transforms into an amastigote. The amastigotes differentiate into nonreplicating trypomastigotes and the cells rupture, releasing them into the bloodstream. Additional host cells of various types are then infected and the trypomastigotes once again form amastigotes inside these cells. Trypomastigotes circulating in the blood are acquired by an insect vector, where they differentiate to form epimastigotes and, finally, trypomastigotes. The two forms of trypomastigotes, as well as the amastigotes that occur in the human host, express very different antigens, thus making an immune response difficult.[17]

In addition to variation of antigen expression, parasites may evade the immune system through **antigen shedding.** Similar to bacteria that shed capsular material into the host environment to evade the immune system, some parasites also exhibit antigenic shedding. *Entamoeba histolytica* is one example of a parasite that can shed antigens from its cell surface.[13] Although antibody is formed against the parasite, the antibody attaches to the shed antigen rather than the parasite, allowing the parasite to escape the immune response.

Antigenic mimicry may occur when the parasite expresses epitopes that are similar, if not identical, to host molecules. The similarity between host and parasitic antigens may suppress the immune response and protect the invading parasite from being recognized and eliminated by the immune system.[18] An example is the antigenic similarity between human host antigens and those of the *Schistosoma* species.[19] The immune response may result in host and parasite cross-reactivity, which may lead to autoimmunity, manifested by the presence of autoantibodies or T cells with autoreactivity.[20,21] An autoimmune response has been linked to the cardiac and intestinal symptoms that occur in the late stages of Chagas disease that may occur because of an infection with *T cruzi*, a bloodborne parasite.[13]

Some organisms enhance their survival by modulating the immune system. The ability to modulate the immune response is an important strategy employed by some parasites to enable their survival in the host. Some parasites can subvert the immune system. **Immunologic subversion** is achieved by avoiding the effector mechanisms of the immune response. Effector molecules include complement and cytokines. Effector cells include plasma cells, T helper (Th) cells, and cytotoxic T cells. For example, some parasites can subvert cytotoxic T cells by producing decoy HLA molecules. Parasites may also subvert the Fc function of antibodies by making Fc receptor homologues or they can subvert complement by making homologues of complement control proteins (CCPs). **Immunologic diversion** occurs when the parasite causes the immune system to produce proteins that divert the attention of the immune system. An example is the ability of some parasites to cause an increase in production of beta interferon (IFN-β), which allows for increased parasite survival. For example, IFN-β has been shown to decrease the ability of macrophages to kill *Leishmania* by significantly reducing the release of superoxide from these cells.[22] Another example by which parasites can divert the immune system is seen with *P falciparum*-infected erythrocytes (IE), which have the potential to interact with B cells in different parts of the body, inducing them to divide and differentiate into antibody-secreting cells. The malaria parasite, as well as some protozoa, viruses, and bacteria, produce immunoglobulin-binding proteins (IBPs) that act as polyclonal B-cell activators. This results in the expansion of numerous B-cell clones and in antibody production not specific for the parasite. Thus, the IBPs may act as an evasion mechanism to divert specific antibody responses.[23]

Serodiagnosis of Parasitic Diseases

In instances where demonstration of the parasite in biological or tissue samples is not possible, serological testing is the gold standard for diagnosis. Serological-based assays can be divided into those that detect parasitic antigens and those that detect antibodies against the parasite. These tests include enzyme-linked immunosorbent assays (ELISA), indirect immunofluorescence, indirect hemagglutination, whole protozoan or antigen-coated particle agglutination, radioimmunoassays (now rarely used), and newer rapid diagnostic tests (RDTs). Previously, many of these assays used relatively crude antigens, making their sensitivity and specificity unpredictable. Advances in immunochemistry and molecular biology have allowed for the development of assays that have markedly improved sensitivity and specificity. However, assays utilizing these newer technologies are costly and not widely available in those countries that have the highest occurrence of parasitic diseases.[24]

ELISA-based assays have been used to detect antigens of parasites that cause human and animal infections, such as amebiasis, babesiosis, fascioliasis, cutaneous and visceral leishmaniasis, cysticercosis, echinococcosis, malaria, schistosomiasis, toxocariasis, toxoplasmosis, trichinosis, and trypanosomiasis.[25,26]

Whereas some parasitic diseases are readily diagnosed by demonstrating the causative agent in clinical material, such as infection with intestinal helminths, in which the worm's eggs are easily detected in fecal specimens, demonstration of other parasitic agents is difficult or not possible (e.g., toxoplasmosis). In those cases, serological assays can be very useful. **Table 22–2** indicates the usefulness of serological testing for the diagnosis of various parasitic diseases.[25] Although serology may not be helpful in the diagnosis of some parasitic diseases, serology can play a role in epidemiological investigations.

Toxoplasmosis

Although advances have been made in the serological assays for some parasites, commercially available ELISA assays still vary considerably in their sensitivity and specificity. One such example is the detection of antibodies against the protozoan

Table 22–2 Usefulness of Antibody Detection for Parasitic Diseases

SEROLOGY INDICATED	SEROLOGY MAY BE USEFUL	SEROLOGY NOT INDICATED
Amebiasis (extraintestinal)	Amebiasis	Anisakiasis
Chagas disease	Amebic meningoencephalitis (caused by free-living amebae)	Ascariasis
Clonorchiasis	Anaplasmosis/Ehrlichiosis	Capillariasis
Cysticercosis	Babesiosis	Cryptosporidiosis
Hydatidosis	Lyme disease	Hookworm
Filariasis (lymphatic; suspect cases when microfilariae cannot be identified in blood)	Paragonimiasis (eggs not detectable in sputum or feces)	Malaria
Leishmaniasis (cutaneous and visceral)		Trichuriasis
Schistosomiasis (ectopic cases; chronic cases when eggs cannot be demonstrated in feces or urine)		
Toxocariasis (visceral and ocular)		
Toxoplasmosis		
Trichinellosis		

Adapted from Maddison SE. Serodiagnosis of parasitic diseases. Clin Microbiol Rev. *1991;4(4):457–469.*

Toxoplasma gondii. *T gondii* has a high prevalence around the world and can infect all species of animals and birds. Nearly 24% of the U.S. population over age 12 is infected with this parasite, which usually remains dormant.[27] Reactivation may occur if the individual becomes immunosuppressed. Members of the cat family *(Felidae)* serve as the definitive hosts for *T gondii*.

The life cycle of *T gondii* has three stages: the *tachyzoite,* which rapidly multiplies in the intermediate host (e.g., humans) and in nonintestinal epithelial cells of the definitive host (e.g., the cat); the *bradyzoite,* which forms the tissue cysts; and the *sporozoite,* which is found in the oocyst[28] **(Fig. 22–1).** There are three routes by which *T gondii* is potentially transmitted to humans. Humans can become infected with *T gondii* by eating raw or insufficiently cooked infected meat (e.g., pork, mutton, or wild game) that contains the cysts or uncooked foods that have come into contact with the infected meat. Toxoplasmosis may also occur when humans ingest oocysts from cat feces present in a litter box or in the soil. Third, the tachyzoites, which are observed during the primary infection, can be transmitted transplacentally to the unborn fetus **(Fig. 22–2).** The incubation period for *T gondii* in adults ranges from 10 to 23 days following ingestion of undercooked meat and from 5 to 20 days after ingestion of oocysts from cat feces. Following infection, the organism reproduces asexually and forms tissue cysts that remain for the life of the host. In the immunocompetent individual, a majority of initial *Toxoplasma* infections are asymptomatic or may only present with a mild lymphadenopathy. Immunity is usually sufficient to contain the latent infection. Toxoplasmosis can cause serious symptoms in the brain and other organs in immunocompromised patients, as well as in the developing fetus following congenital infection.

If the individual becomes immunosuppressed because of HIV infection, malignancy, or immunosuppressive drugs, reactivated toxoplasmosis can occur. The tissue cysts rupture and release the active bradyzoite, which results in clinical disease.[29] *T gondii* infection in the immunocompromised individual can be severe or even fatal.[30] In immunosuppressed individuals, the organism can invade the central nervous system (CNS), leading to toxoplasma encephalitis. Over 95% of the cases are caused by reactivation of a latent infection.[31]

Another concern is the organism's ability to be passed to the fetus during pregnancy. If a mother has been infected before pregnancy occurs, then the fetus is protected because of maternal antibodies. However, if the woman becomes infected just before or during pregnancy, congenital transmission of the organism can occur. Congenital toxoplasmosis may result in a miscarriage, a stillborn child, or mental deficits later on in life. Up to one-half of pregnant women who become infected can transmit *T gondii* across the placenta. The trimester in which the infection was acquired influences the incidence and severity of congenital toxoplasmosis. The transmission risk during the first trimester of pregnancy is 10% to 25%, whereas the transmission risk is 60% to 90% during the third trimester.[32,33] *Toxoplasma* infection can result from congenital infection or infection after birth.

Although the diagnosis of toxoplasmosis can be made by demonstrating the parasite in stained tissue samples or CSF, diagnosis is usually made through serological means. A combination of tests needs to be performed to determine whether an individual has been recently infected or had a previous infection with *T gondii*. The sensitivity and specificity of different commercial kits vary widely and misinterpretation of the results, particularly in determining the presence and significance

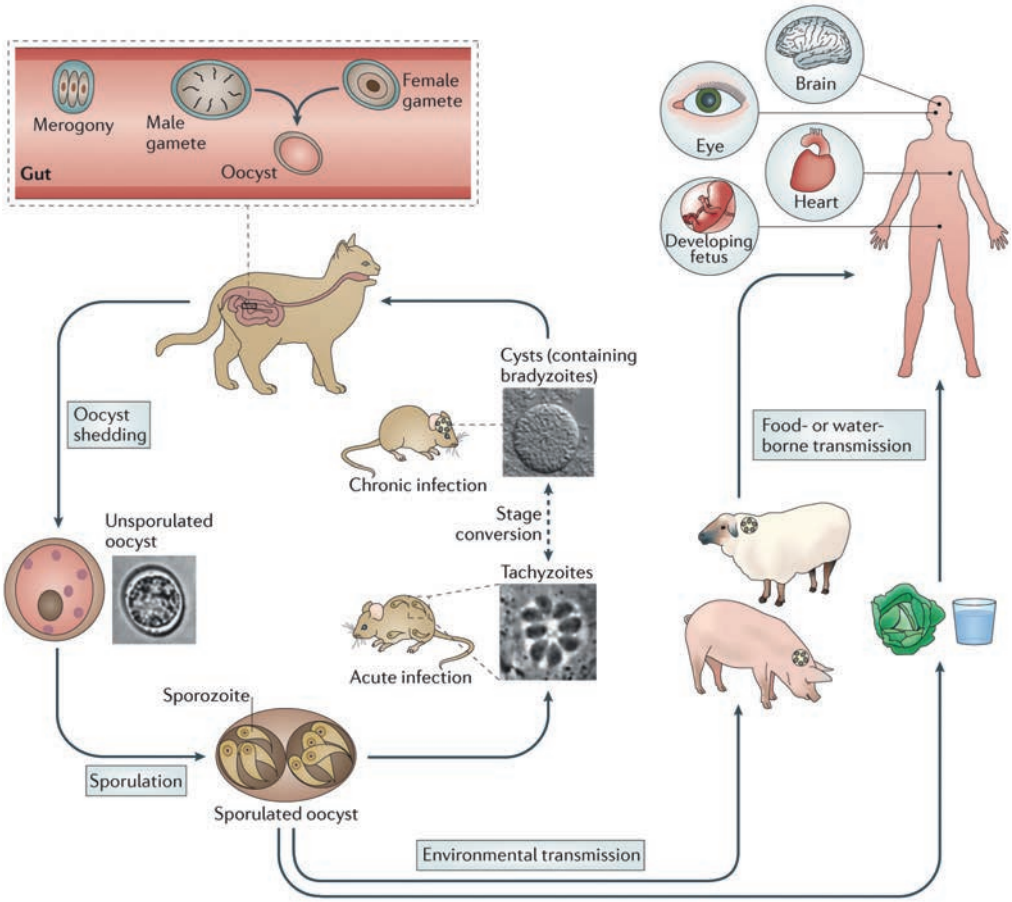

FIGURE 22–1 *Toxoplasma gondii* life cycle. Sexual replication of *T gondii* occurs in cats, the definitive host for the parasite. *T gondii* gametes are formed after merozoites replicate within enterocytes of the cat gut, a process known as merogony. The gametes fuse to form diploid oocysts, which are shed in cat feces and undergo meiosis in the environment to produce eight haploid progeny sporozoites. Asexual replication occurs in intermediate hosts such as rodents. Rapidly replicating forms called tachyzoites disseminate within the host during acute infection and can differentiate into slow-growing forms called bradyzoites inside tissue cysts. Other intermediate hosts or cats can acquire the infection by ingesting the tissue cysts, re-initiating the sexual phase of the life cycle. Oocysts can survive in the environment for a long time and develop into sporulated oocysts, which can be transmitted to intermediate hosts such as farm animals through contaminated food and water. Humans become infected by ingesting tissue cysts in undercooked meat or sporulated oocysts in contaminated water. *(Reprinted by permission from Macmillan Publishers Ltd:* Nature Reviews Microbiology*, 10:766–778, copyright 2012.)*

of IgM antibodies, may occur. This is concerning because serology results can influence decisions regarding continuation or termination of pregnancies.[34] IgM antibodies may persist for up to 18 months after infection with *T gondii*. Therefore, the FDA has recommended that anti-*Toxoplasma* IgM tests should be interpreted with caution and that the sole results of a single assay should not be used in determining a recent infection.[35] The FDA has published guidelines for the interpretation of *T gondii* serology results **(Table 22–3).**

The greatest value of testing for IgM antibodies is in determining whether a woman has had a recent infection. If no IgM antibodies are detected and only IgG is detected, this excludes a recent infection before pregnancy. One of the problems when testing for *Toxoplasma*-specific IgM antibodies is that the current assays lack specificity. If only IgM antibodies are detected, additional testing should be performed. A second blood sample should be obtained from the patient 2 weeks after the first sample is collected and tested together with the first specimen. The second sample should demonstrate high levels of IgM and IgG antibodies if the first sample was collected early in the infection. If both specimens show the presence of IgM but absence of IgG, the IgM result should be considered to be a false positive.

When both IgM and IgG antibodies are detected and the patient is pregnant, an IgG avidity test should be performed. Determination of the avidity of the antibody has proven useful in determining whether the IgG antibodies are from a recent or a previous infection. The presence of high avidity IgG indicates an infection in the past, whereas IgG antibodies with a low avidity suggest a more recent infection. In that low avidity antibodies may persist for a prolonged period of time, their presence does not necessarily mean that the infection was recently acquired. However, the presence of high avidity antibodies indicates an infection for at least 3 to 5 months and does not pose a risk to the fetus.[36-39] The assay for IgG avidity utilizes a wash buffer containing urea to differentiate low

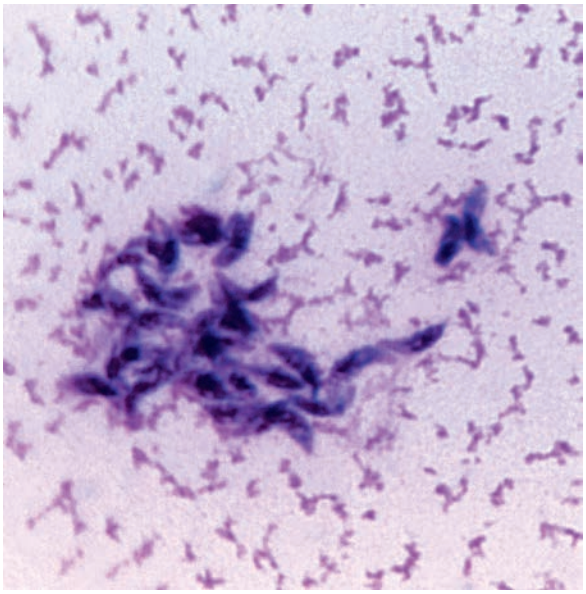

FIGURE 22–2 *Toxoplasma gondii* tachyzoites in mouse ascitic fluid stained with Giemsa stain. *(Courtesy of the CDC/Dr. L.L. Moore, Jr., Public Health Image Library.)*

Connections

High Avidity Antibodies

The avidity of an antibody molecule represents the strength by which its antigen-binding sites can bind to an antigen. During the course of an immune response, somatic mutations occur in the immunoglobulin genes in the B cells, causing them to produce antibodies of higher avidity. This results in tight binding of the antibodies to the antigen, such as a lock and key. The presence of high avidity antibodies is a sign that the immune response has been going on for quite some time (see Chapter 5).

avidity from high avidity antibodies. Antibodies with low avidity dissociate from the antigen in the presence of urea. The avidity is determined by running the assay in duplicate using buffer with and without urea. The avidity index (AI) is determined by dividing the *T gondii*-specific IgG signal (optical density; O.D.) of the set washed with urea-containing buffer by the *T gondii*-specific IgG O.D. of the set washed with non-urea buffer. AI values lower than 0.20 indicate the presence of low avidity antibodies, values between 0.20 and 0.25 suggest intermediate avidity, and values greater than 0.25 are obtained with antibodies of high avidity.[40]

When using serology to detect congenital toxoplasmosis, several differences exist. Because of the passive transfer of IgG

Table 22–3 General Guidelines for Interpretation of *Toxoplasma Gondii* Serology Results

IgG RESULT	IgM RESULT	REPORT AND INTERPRETATION (EXCEPT INFANTS)
Negative	Negative	No serological evidence of infection with *Toxoplasma.*
Negative	Equivocal	Possible early acute infection or false-positive IgM result. Obtain new specimen for IgM and IgG testing. If result for the second specimen remains the same, the patient is probably not infected with *Toxoplasma.*
Negative	Positive	Possible acute infection or possible false-positive IgM result. Obtain new specimen for IgM and IgG testing. If result for the second specimen remains the same, the IgM reaction is probably a false positive.
Equivocal	Negative	Indeterminate: obtain a new specimen for testing or retest this specimen for IgG using a different assay.
Equivocal	Equivocal	Indeterminate: obtain a new specimen for IgM and IgG testing.
Equivocal	Positive	Possible acute infection with *Toxoplasma.* Obtain new specimen for IgM and IgG testing. If the result for the second specimen remains the same or if the IgG becomes positive, both specimens should be sent to a reference laboratory with experience with diagnosing *Toxoplasma* infection.
Positive	Negative	Infected with *Toxoplasma* for 6 months or longer.
Positive	Equivocal	Infected with *Toxoplasma* for probably more than 1 year or false-positive IgM reaction. Obtain a new specimen for IgM testing. If results with the second specimen remain the same, both specimens should be sent to a reference laboratory with experience with diagnosing *Toxoplasma* infection.
Positive	Positive	Possible recent infection within the past 12 months or false-positive IgM result. Send the specimen to a reference laboratory with experience with diagnosing *Toxoplasma* infection.

Adapted from Centers for Disease Control and Prevention. DPDx—Laboratory identification of parasitic diseases of public health concern: toxoplasmosis. www.cdc.gov/dpdx/toxoplasmosis/dx.html. Accessed April 20, 2016.

across the placenta, infants whose mothers are chronically infected will be born with toxoplasma IgG antibodies of maternal origin. Serological diagnosis can be made 5 or 10 days after birth by demonstration of positive *Toxoplasma* IgM or IgA antibody titers, respectively, in newborn sera. Detection of anti-*Toxoplasma* IgA antibodies is more sensitive than detection of IgM antibodies. It should be noted that commercial assays currently used in the United States have not been cleared by the FDA for diagnostic testing of infants. Therefore, samples from neonates suspected of having congenital toxoplasmosis should be sent to the Toxoplasma Serology Laboratory, Palo Alto, California, the site that has the most experience with infant testing.

Rapid Antigen Detection Systems (RDTS)

Rapid antigen detection systems (RDTS) (also called **lateral flow assays**), are based on immunochromatographic antigen detection and are widely used in many diagnostic laboratories. RDTS detect soluble proteins through their ability to bind to capture antibodies contained in a nitrocellulose strip. The clinical sample is placed on the strip and eluted by adding a few drops of buffer that contains a labeled antibody. A colored band representing the antigen–antibody complex can then be seen on the membrane. Many of the assays are stable at room temperature, easy to perform and interpret, and cost effective. Many clinical laboratories have discontinued the use of ELISA assays in favor of immunochromatographic assays. For example, several years ago, ELISA-based assays were the predominant platform for the detection of *Giardia* and *Cryptosporidium.* Today, many clinical laboratories are using lateral flow assays. One example is the Xpect Giardia/Cryptosporidium assay from Remel (Lexena, Kansas) **(Fig. 22–3).**

Because of their advantages, many RDTS can be used in rural regions. Assays have also been developed for the rapid diagnosis of malaria using this platform.[41] The assays can be used in the field and allow for differentiation between the more deadly *P falciparum* and *Plasmodium vivax.*[42] At the time of this writing, the BinaxNOW® Malaria Test (Alere Inc., Waltham, MA) is the only available RDTS for malaria in the United States. The test detects the histidine-rich protein II (HRPII) specific to *P falciparum* (P.f.) and a pan-malarial antigen common to all four malaria species that can infect humans—*P falciparum, P vivax* (P.v.), *Plasmodium ovale* (P.o.), and *Plasmodium malariae* (P.m.) **(Fig. 22–4).**

Limitations of Parasitic Serology

As with all laboratory testing, it is important to use methodologies that are the most straightforward and cost effective for each test. However, it is also important to consider the specificity and sensitivity of the method when choosing a procedure. Currently, there is no external proficiency testing program offered in the area of parasitic serology except for diagnosis of toxoplasmosis.[43] Therefore, it is very difficult to evaluate the quality of commercial assays that are currently available. In the United States, commercial kit manufacturers must obtain FDA approval before selling their products. The FDA requires only that a new method be equivalent to a method that has already been approved. Researchers at the CDC have expressed concern that, over time, the quality of new test kits may drift in a negative direction because a new kit may not be quite as good as the one used for comparison but may still receive approval.[44]

In addition to the inability to evaluate an assay's performance using proficiency testing, a specific test may not detect a parasitic disease in an individual because of the inability of the assay to detect all species of the parasite causing the infection. For example, some serological tests, such as those for schistosomiasis, only detect antibodies that are species-specific. Therefore, the

FIGURE 22–3 Remel Xpect® Giardia/Cryptosporidium immunochromatographic immunoassay. The test uses sample wicking to capture *Giardia* and *Cryptosporidium* antigens on discrete test lines containing antigen-specific antibodies for each organism. *(Reprinted by permission Thermo Fisher Scientific, Lenexa, Kansas.)*

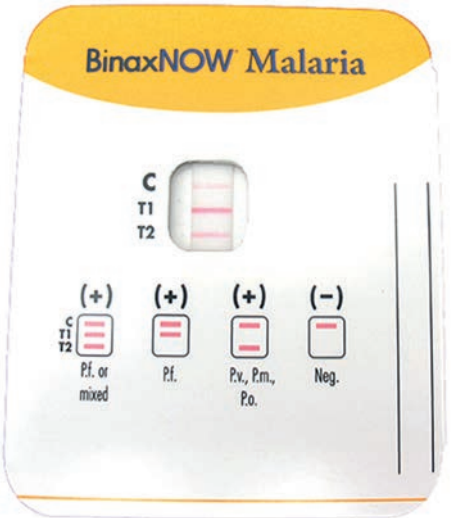

FIGURE 22–4 BinaxNOW® Malaria test. The immunochromatographic assay targets the histidine-rich protein II (HRPII) antigen specific to *Plasmodium falciparum* (P.f.) and a pan-malarial antigen common to all four malaria species capable of infecting humans—*P falciparum, P vivax* (P.v.), *P ovale* (P.o.), and *P malariae* (P.m.)—circulating in human venous and capillary EDTA whole blood. *(Reprinted by permission BioMed Central Ltd,* Malaria Journal *14;10:300, copyright 2011.)*

same sample can give a negative result in one test and a positive result in another. Also, the type of specimen submitted from a patient may give conflicting results. In one reported example, samples of serum, stool, and urine from a patient suspected of having schistosomiasis were submitted to a commercial laboratory for testing. Although the serum result was antibody negative and no parasites were found in the stool, the urine examination demonstrated eggs of *Schistosoma haematobium.* An immunoblot was positive for *S haematobium* and negative for *Schistosoma mansoni* when the serum specimen was submitted to the CDC for blind retesting. In this example, the tests for schistosomiasis performed by the two laboratories were not equal in detecting the three *Schistosoma* species that infect humans. Situations such as these can lead to an inaccurate diagnosis.[43]

Because of the antigenic complexity of parasites, the antibody formed may not be detected because the assay uses a different antigen from the one to which the patient has formed the antibodies. Also, the sensitivity of an assay may vary, depending on the stage and type of the patient's disease. For example, antibodies against *Plasmodium* species (malaria) rise rapidly in an acute infection but decline if the parasite becomes latent or dormant in the liver as a hypnozoite. Similarly, because of the potential for cross-reactivity of the antibodies formed in a parasitic infection, a positive result should be interpreted with caution.

Although there are commercially available tests for the serological diagnosis of several parasitic agents, only a few commercial kits are available in the United States and they are not used by many clinical laboratories. In the United States, the majority of tests are performed by the following commercial laboratories: Focus Technologies, Mayo Medical Laboratories, Parasitic Disease Consultants, Quest Diagnostics, and Specialty Laboratories.[44] Most of the tests performed at the commercial laboratories and at the CDC are produced and evaluated in-house and use reagents that are not universally standardized. Because of the differences in the reagents used in the tests, discrepant results obtained by different laboratories are commonly observed.

Molecular-Based Diagnosis of Parasitic Disease

The microscopic detection and diagnosis of parasitic disease, although once considered to be the gold standard, is now recognized as having limitations in being able to detect agents responsible for parasitic infections. Decreased sensitivity associated with microscopic methods may be caused by intermittent shedding of the organism, as is seen with *Giardia lamblia,* or to low levels of the organism being present, as is observed with the bloodborne parasites such as the causative agents responsible for malaria or babesiosis. With the development of molecular-based assays, the diagnosis of parasitic infections using molecular-based technology is being used with increasing frequency. Although microscopy remains the means by which most blood and tissue parasites are detected, rapid real-time polymerase chain reaction (PCR) assays for babesiosis and malaria have recently been developed[45,46] and are available from commercial reference laboratories (e.g., Mayo Medical Laboratories in Rochester, Minnesota, and Focus Diagnostics in Cypress, California) and from the CDC. As of 2014, there are a limited number of FDA-approved molecular assays for the diagnosis and identification of gastrointestinal and blood and tissue parasites. Many laboratories have developed in-house assays for the identification and differentiation of parasites.[47] A number of new technologies, including nested multiplex PCR, have the potential for the detection and identification of gastrointestinal parasites.[48] Molecular tests are playing an increasingly important role as adjuncts to traditional diagnosis of parasitic infections and, in some cases, may even replace traditional methods as commercial companies develop molecular-based assays and seek FDA approval.

Fungal Immunology

Fungi represent a large heterogeneous group of eukaryotic organisms that are ubiquitous in the environment. Fungi can either be considered as parasites, deriving their nutrition from living matter, or more commonly as saprophytes, living off of dead and decaying matter. In the environment, fungi play an important role in maintaining the ecosystem with the decomposition of cellulose. The *Dictionary of Fungi* reported 97,330 species of described fungi in the "numbers of fungi" entry.[49] New organisms are being added as advances in molecular biology allow for the detection and differentiation of fungi. Despite the number and diversity of fungi, only about 300 fungal species are potentially pathogenic to humans and animals. Fewer than 50 of these species are responsible for approximately 90% of all fungal infections.[50]

When a human or animal has an infection or disease caused by a fungus, it is referred to as a **mycosis** (plural: **mycoses**). Mycoses are classified into four groups—superficial, cutaneous, subcutaneous, or systemic—based on the type and amount of tissue involvement and the host response to the pathogen. Mycotic diseases are of growing importance for a number of reasons:[51] (1) Fungi are widely distributed in nature and are able to maintain themselves in the environment, making them difficult to eradicate. (2) Many fungi are able to present with various clinical manifestations, ranging from invasive disease to localized infection, or allergic manifestations, as is observed with *Aspergillus.* (3) Diagnosis is difficult because of the varying clinical presentations. (4) Currently, vaccines are not available. (5) There are only a limited number of antifungal agents available for treating fungal infections. (6) Most fungal infections are opportunistic in nature and, increasingly, treatment of a primary disease puts individuals at risk for opportunistic infections.

Fungi do not possess a large array of virulence factors that allow for them to be true pathogens. However, fungal opportunistic infections have risen in recent years with the advent of AIDS and our increasing use of immunosuppressive therapies. Fungal diseases are often the first opportunistic diseases detected in patients with AIDS. One of the most common opportunistic diseases in AIDS patients is pneumonia caused by *Pneumocystis jiroveci* (previously *Pneumocystis carinii*). Although originally classified as a parasite, this organism now is designated a fungus because it has a greater gene sequence homology

with fungi than with parasites.[52] Many saprophytic fungi formerly dismissed as cultural contaminants are now reported as opportunistic pathogens.[53]

Characteristics of Fungi

Fungi are eukaryotic cells with nuclei and rigid cell walls composed primarily of chitin, glucans, mannans, and glycoproteins. Fungi can exist in two morphological states. They may exist as **yeast,** which are unicellular organisms, or as **mycelial fungi,** which are multicellular organisms. Yeast reproduce by budding, whereas the mycelial fungi reproduce through the production of spores or **conidia.** It should be noted that many individuals develop allergies to the spores and conidia produced by fungi. Although most fungi are monomorphic (existing in a single form), several fungi can exist as either yeast or mycelial fungi, depending on environmental conditions, and are referred to as dimorphic fungi.

Fungi grow more slowly that most bacteria (2 to 4 weeks versus 1 to 2 days) and have relatively simple nutritional requirements. Because most fungi are found in the environment, they prefer cooler temperatures to grow (25°C to 30°C).

Classification of Mycotic Infections (Mycoses)

Fungi can produce four types of clinical manifestations. Some individuals exhibit *hypersensitivity* to certain fungal agents. The hypersensitivity reaction is generally because of an allergic reaction to the spores. Hypersensitivity may occur only when an individual comes in contact with the specific spores he or she is allergic to, or it can occur as a chronic condition such as allergic bronchopulmonary aspergillosis (ABPA), which develops in response to the fungus *Aspergillus* (most commonly *Aspergillus fumigatus*). Fungi may also be responsible for *mycotoxicosis,* a poisoning of humans and animals by food products contaminated by fungi that produce toxins from a grain substrate. Major mycotoxins include aflatoxins, deoxynivalenol, fumonisins, zearalenone, T-2 toxin, ochratoxin, and certain ergot alkaloids. Mushrooms are classified as fungi. Thus, another type of disease that fungi can be responsible for is *mycetismus,* or mushroom poisoning, when the ingestion of toxin-producing mushrooms occurs. Potentially deadly mushrooms include *Amanita phalloides, Amanita verna, Amanita virosa,* and certain other species that contain neurotoxins.

The fourth type of mycotic disease is infection with clinical manifestations caused by the presence or growth of the fungi on or in the host tissue. Grouping the fungi according to their clinical manifestation is useful, both from a diagnostic standpoint and to facilitate identification of the agents in the laboratory. The *superficial* mycoses are fungal diseases that are restricted to the outer layers of the skin. These infections are cosmetic in nature and are not life threatening. *Cutaneous* mycoses are those infections that involve the keratinized body areas (skin, hair, nails) and rarely invade deeper tissue. Although not life threatening, these infections produce itchiness and cracking of the skin. Diseases of hair and nails are termed *dermatomycoses.* The fungi involved are called *dermatophytes* and can be transmitted through direct contact with an infected person or animal. The *subcutaneous* mycoses affect the subcutaneous tissue, where they usually form deep, ulcerated skin lesions. The causative agents are soil saprophytes and acquisition of the fungal agent is generally caused by trauma to the skin, usually the feet or legs. The *systemic* mycoses can involve the deep viscera and spread throughout the body. These infections originate in the lungs and then disseminate to other locations. Although considered to be pathogenic to man, many individuals infected with one of the causative agents are asymptomatic. Except for *Cryptococcus,* the causative agents in this group are dimorphic in nature. A number of fungi previously not considered to be pathogenic in humans are responsible for opportunistic infections in the immunosuppressed patient. Several agents have been long known as causing infections in immunosuppressed individuals, including *Candida, Aspergillus,* and the fungi causing Zygomycosis (*Rhizopus* species, *Mucor* species, *Cunninghamella bertholletiae,* and *Apophysomyces elegan*). **Table 22–4** lists the most frequent agents associated with the various classifications. Several fungi are considered to be commensalistic in nature, including *Candida albicans* and *Malassezia furfur;* however, these organisms may cause disease or clinical symptoms in some individuals.

Immune Responses to Fungi

As is the case with the immune responses to bacterial, viral, and parasitic agents, the immune defenses to fungal agents range from protective mechanisms that include innate immunity present early in the infection to specific adaptive mechanisms that are induced later. The first line of innate defense includes skin and the mucous membranes of the respiratory, gastrointestinal, and genitourinary tracts that provide physical barriers that separate the host from the environment. Fungi possess very few factors that allow them to overcome those physical barriers; because of the nutrients and environmental conditions needed for many fungi, those mechanisms have not evolved. For example, among the most common fungal infections are those caused by the dermatophytes (*Trichophyton* species, *Microsporum* species, and *Epidermophyton floccosum*). These fungi cause infection of the skin, hair, and nails because they require dead keratin for growth. Infections caused by these fungi are also sometimes known as "ringworm" or "tinea." They do not possess any invasive factors and the symptoms associated with these agents are caused by the inflammatory response of the host.

If the fungi penetrate the physical barriers, there are a variety of innate mechanisms for recognizing the organism. Innate immune cells express various pattern-recognition receptors (PRRs) that recognize specific structures and molecules present on bacteria and fungi. These structures and molecules of the organism, called *pathogen-associated molecular patterns (PAMPs)*, are conserved among microbial species. PRRs on the innate immune cells (phagocytes, macrophages, and dendritic cells [DCs]) initiate the immune response by sensing and recognizing the presence of PAMPs present on the bacteria, fungi, or viruses.

There are several classes of PRRs that can be either transmembrane proteins located on the cell surface or cytoplasmic

Table 22–4 Mycotic Infections Based on Site of Infection and Level of Tissue Involvement

CLASSIFICATION	REPRESENTATIVE AGENT	DISEASE
Superficial	*Malassezia furfur* *Phaeoanellomyces werneckii* *Piedraia hortae* *Trichosporon* species	Pityriasis versicolor (skin) Tinea nigra (skin) Black piedra (hair) White piedra (hair)
Cutaneous	*Trichophyton* species *Microsporum* species *Epidermophyton floccosum*	Tinea (e.g., ringworm, athlete's foot, jock itch)
Subcutaneous	*Sporothrix schenckii* *Fonsecaea pedrosoi* *Pseudallescheria boydii*	Sporotrichosis Chromoblastomycosis Eumycotic mycetoma
Systemic	*Histoplasma capsulatum* *Coccidioides immitis* *Paracoccidioides brasiliensis* *Blastomyces dermatitidis* *Penicillium marneffei* *Cryptococcus neoformans*	Histoplasmosis (endemic in Ohio and Mississippi river valleys) Coccidioidomycosis (endemic in the southwestern United States) Paracoccidioidomycosis (endemic in Central and South America, primarily Brazil) Blastomycosis (predominantly a veterinary pathogen in Ohio and Mississippi river valleys) *Penicilliosis marneffei* (predominantly seen in AIDS patients in Southeast Asia) Cryptococcosis (causes meningitis in immunosuppressed patients [e.g., AIDS])
Opportunistic	*Candida albicans* *Aspergillus* species *Rhizopus* species	Candidiasis Aspergillosis Zygomycosis
Commensalistic	*Candida albicans* *Malassezia furfur*	Urinary tract infections, vaginal yeast infections Tinea versicolor (pityriasis versicolor), dandruff, and seborrheic dermatitis

proteins contained within the cell. The four different classes include the **Toll-like receptors (TLRs)** and the **C-type lectin receptors (CLRs),** which are transmembrane proteins, and the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and the NOD-like receptors (NLRs), which are cytoplasmic proteins (see Chapter 3). The recognition of PAMPs by the PRRs upregulates the inflammatory response.[54] Of these, the TLRs play the most important role in defense against pathogenic microbial infection by stimulating production of inflammatory cytokines and type I interferons.[55,56] In addition to playing an important role in innate responses, TLRs are involved in shaping adaptive immunity.

To date, 10 different TLRs have been identified in humans and 13 TLRs have been discovered in mice.[57-60] Each TLR recognizes specific PAMPs found on viruses, bacteria, fungi, and parasitic-protozoa and helminths. Several fungal components are recognized by TLRs, including phospholipomannans and β-glucans, which are recognized by TLR2, and glucuronoxylomannans, which are recognized by CD14 and TLR4.[61]

Although TLRs play a role in the immunologic response to fungi, CLRs are central for fungal recognition and for the induction of the innate and adaptive immune responses to fungi. Fungal cell walls consist mainly of multiple layers of carbohydrates, including mannans (cell wall polysaccharide), β-glucan, and chitins (the main component of fungal cell walls). The CLR transmembrane protein, dectin 1, recognizes β-glucans. Dectin 2 recognizes high-mannose structures that are common to many fungi and binds hyphal forms with higher affinity than yeast forms.[62] Individuals with genetic deficiencies in CLRs are highly susceptible to fungal infections.[63-65]

There has been a debate as to the roles of cell-mediated immunity versus humoral immunity in host defense to fungal infections. It is now accepted that the cell-mediated immune response plays the most important role in the adaptive response to fungal infections.[66] Once the cells of the innate immune system have been activated, cytokines and chemokines are produced in response to the infection. Chemokines play a vital role in the recruitment of T cells to the site of the infection. Chemokines also aid in the formation of the adaptive immune response to fungi, specifically, those mediated by Th1 and Th2 cells. Th1 cells have been shown in mice to be vitally important to clearance of the organism in **cryptococcosis** and **histoplasmosis.**[67-69] Once the T cells have committed themselves in response to the fungi, they express an effector function through the release of cytokines, primarily IFN-α, TNF-α, and IL-17/22, contributing to protective immunity to pathogenic fungi.[70-72]

Although there is a humoral response to fungal infections, the evidence that antibodies contribute to effective defense

against fungal infections is not conclusive. One study has shown that humoral immunity can protect against experimental fungal infections if certain types of protective antibodies are present in sufficient quantities.[73] The main functions of antibodies in fungal infections include opsonization, ADCC, prevention of adherence, and toxin neutralization.[74] However, there is little evidence supporting the role of antibodies in host defenses in naturally acquired infections. The absence of an association between deficiencies in specific antibodies and susceptibility to fungal infections in patients with progressive fungal infections also provides evidence against a protective role of antibodies in fungal infections.[66]

Laboratory Diagnosis of Fungal Infections

Because of the increased numbers of individuals who are immunosuppressed, the incidence of invasive fungal infections associated with significant morbidity and mortality has risen dramatically in the past several decades.[75-79] The clinical diagnosis of fungal infections in many instances is difficult. The clinician must consider the patient's symptoms, history of other past or present infections, medical treatments, the patient's occupation, place of residence, and record of travel in evaluating the likely cause of a fungal infection. Available laboratory methods for diagnosing fungal infections include isolation of the organism in the laboratory, histopathological evidence of invasion, skin testing, and serological detection of antigens or antibodies. The traditional "gold standard" is the recovery of the organism in the laboratory. However, recovery is often difficult with uncommon fungi. In addition, once the organism is isolated, traditional identification methods may take several weeks because of the slow growth of fungi. Several nonculture laboratory methods, including antibody and antigen detection, are available for fungal pathogens that are commonly encountered in the clinical laboratory. These include *C albicans, Aspergillus* species, *Cryptococcus neoformans,* and the dimorphic fungi, *Histoplasma capsulatum* and *Coccidioides immitis.*

Although the humoral response to fungi is not the major defense against fungal infection, antibodies produced against the invading organisms may be readily detected and can be used to demonstrate current or past exposure to the agent. However, in that many fungal infections are opportunistic, serological diagnosis many times is of little value because of the fact that immunosuppressed individuals do not reliably produce antibodies during acute infections. In addition, in areas where a fungal agent is endemic to a geographic area, serological testing for antifungal antibodies is of little value because most people living in those areas will test positive for the antibodies.

Recently, molecular assays have been used in the detection and diagnosis of fungal infections.[80] Examples include peptide nucleic acid fluorescence in situ hybridization (PNA-FISH) for identification of *Candida* species in blood cultures, real-time PCR assays for detection of *Aspergillus* species and *P jirovecii* from bronchial lavage fluid, and multiplex PCR coupled with a bead probe fluid array for detection of numerous species of fungi in bronchial lavage fluid or blood. However, molecular-based diagnostic methods for fungal infections are in their infancy. In fact, none of the molecular assays are even included in the criteria of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Disease Mycoses Study Group (EORTC/MSG) for the definition of invasive fungal disease.[81]

Fungal Pathogens

The following discussion provides information on five of the fungal infections for which serological testing is used.

Aspergillus Species

Aspergillus is a ubiquitous fungus that is found worldwide in nature **(Fig. 22–5).** The genus has over 185 species and can be recovered from soil and plant material; its spores (conidia) may be recovered from the indoor air environment.[82] *A fumigatus* is the most commonly isolated species, with *Aspergillus flavus* and *Aspergillus niger* being the other frequently recovered species. Less commonly recovered isolates include *Aspergillus clavatus,* the *Aspergillus glaucus* group, *Aspergillus nidulans, Aspergillus oryzae, Aspergillus terreus,* and *Aspergillus versicolor.* In humans, *Aspergillus* is primarily an opportunistic pathogen causing a variety of infections. Infections in the immunocompetent individual include *Aspergillus otomycosis,* which is a superficial infection of the external auditory canal and auricle (otitis externa). Most of these infections are caused by *A niger.* Another infection is caused by growth of *Aspergillus* in the lung cavity, forming a solid mass of **hyphae,** which can develop into a fungus ball referred to as an *aspergilloma.* Pulmonary aspergilloma usually occurs in scarred lung tissue or in a preexisting

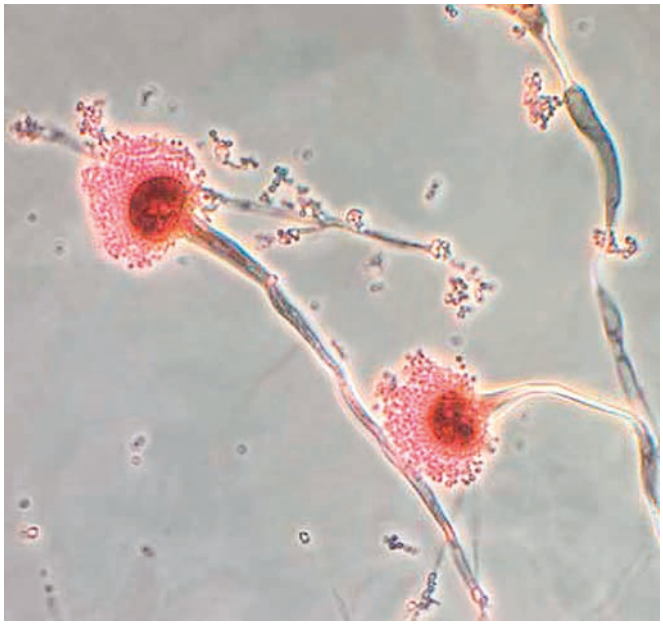

FIGURE 22–5 *Aspergillus fumigatus,* LPCB stain X 450. *(Courtesy of the CDC, Public Health Image Library.)*

lung cavity resulting from a previous infection (e.g., tuberculosis, sarcoidosis).[83] Some fungal antigens may elicit allergic responses to *Aspergillus,* causing ABPA, which results in a long-term allergic response. ABPA is thought to be present in 1% to 2% of patients with persistent asthma and in approximately 7% of patients with cystic fibrosis.[84,85]

Invasive forms of *Aspergillus* most frequently begin in the lung, resulting from inhalation of the conidia. The organism grows and spreads in the lung tissue. Invasive pulmonary *Aspergillosis* (IPA) may occur in the neutropenic patient because of immunosuppression. Disseminated aspergillosis may occur through hematogenous spread to distant sites or by contiguous extension from the lung.[86]

The diagnosis of **aspergillosis** generally requires a positive tissue biopsy demonstrating the hyphae or a positive culture for *Aspergillus.*[83] Nonculture methods may also be used to diagnose invasive aspergillosis. Serological diagnosis is of limited utility because the immunosuppressed patient will fail to mount an antibody response, even with invasive disease.[87] The detection of galactomannan in serum by EIA has increased the ability to diagnose invasive aspergillosis.[88] This assay is offered at larger or reference laboratories. Another assay to detect invasive fungal infections, including aspergillosis, is measuring β-D-glucan (BDG) in serum. BDG is a component of the cell wall of most fungi.[89] The assay is approved and included in the latest EORTC/MSG diagnostic criteria for the clinical diagnosis of invasive fungal infection.[81] Scientists have developed molecular diagnostics, including PCR, for *Aspergillus,* which appear to be more sensitive than other methods, including galactomannan,[90-93] and show promise in improving the diagnosis of invasive aspergillosis.[94]

Candida Species

Candida ssp. are yeast (unicellular fungi) that may exist as commensalistic organisms in the human host. They are commonly found on skin, the gastrointestinal (GI) tract, and in the female genital tract. Of the more than 150 species of *Candida,* only a small percentage is regarded as frequently pathogenic for humans. *C albicans* is the leading cause of human infections. Other members include *Candida guilliermondii, Candida krusei, Candida parapsilosis, Candida tropicalis, Candida pseudotropicalis, Candida lusitaniae, Candida dubliniensis,* and *Candida glabrata.* Following the introduction of antibiotics, *Candida* infections rose dramatically in the United States. In recent decades, *Candida* species have been the fourth most common organisms recovered from the blood of hospitalized patients in the United States.[95] The incidence of candidemia-related hospitalizations has risen by 52% between 2000 to 2005.[96] Increased use of immunosuppressive therapies, the use of advanced life support therapies, and the incidence of HIV-1 infection have all contributed to the increased incidence of *Candida* infections. Infections in the immunocompetent host include diaper rash, vaginitis and urinary tract infections in the female, and thrush (oral candidiasis) caused by the use of broad spectrum antibiotics. Individuals with thrush for no obvious reason should be evaluated for AIDS. The introduction of potent antiretroviral therapy has reduced the incidence of thrush in AIDS patients. *Candida* esophagitis, pneumonia, and septicemia may be observed in the neutropenic patient because of radiation and chemotherapy or use of broad spectrum antibiotics.

The diagnosis of *Candida* infections generally involves the recovery of the causative agent. The organisms grow well in routine culture media. Direct examination of the clinical material through the use of 10% potassium hydroxide (KOH) preparation may also be used to detect *Candida* species. The serological diagnosis of **candidiasis** is limited because of colonization by *Candida* species of the gastrointestinal tract or other body sites that can stimulate antibody production in uninfected individuals. In addition, immunocompromised patients may not mount detectable antibody responses. Current recommendations include the combined detection of mannan and anti-mannan antibodies for the specific identification of *Candida* species in serum samples.[97] These assays can be positive 6 days before blood cultures, on average. They also show a very high negative predictive value (greater than 85%).[98]

Cryptococcus Neoformans

The genus *Cryptococcus* includes 19 species of encapsulated yeasts. *C neoformans* is a major causative agent of human disease that went from being a rare human pathogen to a significant opportunistic pathogen as the population of immunocompromised patients increased. *C neoformans* is a *saprobe* in nature, obtaining its nutrition from dead or decaying organic matter.[100] The organism lives in certain trees and rotting wood and has frequently been isolated from soil contaminated by guano from birds (e.g., pigeons).[101,102] *C neoformans* enters the host mainly through the lungs and has a predilection for invading the CNS of the susceptible host. Although pulmonary infections are common, *Cryptococcal meningoencephalitis* represents the primary life-threatening infection caused by *C neoformans.* The pre-AIDS era had an overall incidence of 0.8 cases per million

Connections

Predictive Value

The predictive value of a laboratory test is the likelihood that the results it produces will lead to an accurate diagnosis. The predictive value depends on the sensitivity and specificity of the method, as well as the prevalence of the disease in the population undergoing testing (see Chapter 9). A negative predictive value is the likelihood that a negative test result is truly negative. Thus, an 85% negative predictive value means that there is an 85% chance that the patient does not have the disease in question. In general, negative predictive values are higher in populations with a low disease prevalence.

Over the years, tests for various serum antigens have been used to detect invasive *Candida* infections, including latex agglutination (LA), counterimmunoelectrophoresis, or ELISA-based assays; however, these have not proven to be useful. The use of molecular-based assays shows promise. Direct PCR of blood samples is a sensitive and specific method for the diagnosis of invasive candidiasis and may be used for early diagnosis of specific *Candida* species.[99]

persons per year. In 1992, during the peak of the AIDS epidemic, the rate in several large U.S. cities reached almost five cases of cryptococcosis per 100,000 persons per year. With the use of antiviral agents for the treatment of AIDS in developed countries, these numbers have declined.[103,104]

The most important feature contributing to the organism's virulence and pathogenicity is its capsule. The capsule consists of polysaccharide containing an unbranched chain of alpha-1,3-linked mannose units substituted with xylosyl and β-glucuronyl groups. The capsule has many effects on the host. These include acting as a barrier to phagocytosis, depleting complement, producing antibody unresponsiveness, dysregulating cytokine secretion, interfering with antigen presentation, and enhancing HIV replication.[105]

The two most common sites for infection with this encapsulated yeast are the lungs and CNS, with the respiratory tract being the most common portal of entry. The majority of cryptococcal infections are asymptomatic, as suggested by the fact that serological and hypersensitivity testing indicate a higher incidence of infections than the actual incidence of cryptococcosis.[106,107] The majority of cryptococcal infections are diagnosed in individuals with compromised immune systems. Respiratory symptoms range from asymptomatic colonization of the airway to life-threatening pneumonia with evidence of an acute respiratory distress syndrome.[108] Individuals with CNS involvement generally present with subacute meningitis or meningoencephalitis. Symptoms include headache, fever, lethargy, and memory loss over several weeks. Some patients may present with an acute onset of meningitis.[109]

The laboratory diagnosis of *Cryptococcus* infections may be done using direct microscopic examination of the clinical material, culturing for the organism, or performing serological tests. Microscopic examination of the organism can be performed by mixing the biological fluid (usually CSF) with India ink. The encapsulated yeast displaces the India ink, creating a halo around the yeast cell **(Fig. 22–6).** Although the test is easy to perform and interpret and has high specificity, it shows poor sensitivity (50% to 80%).[110] Detection of cryptococcal polysaccharide antigen in serum and CSF can be performed by LA and enzyme immunoassays (EIA). Both types of tests are more than 90% sensitive and specific.[111] Although the LA test is a rapid and reliable serological method for the diagnosis of cryptococcosis, false-positive LA test results can occur. False-positive results are thought to be caused by rheumatoid factor or other interference factors.[112-116] Pretreating the specimen with heat and pronase, or 2-Mercaptoethanol, destroys these factors and reduces the incidence of false-positive test results.[110]

Recently, a lateral flow immunochromatographic assay (LFA) (Immy, Inc., Norman, OK) was approved by the U.S. Food and Drug Administration for detection of cryptococcal antigen in CSF and serum **(Fig. 22–7).** The assay allows for the detection of cryptococcal antigen in fewer than 15 minutes. The assay has been shown to have a sensitivity of 100% and a specificity of 99.8% with serum samples,[117] as well as a sensitivity of 99.3% and a specificity of 99.1% with CSF.[118]

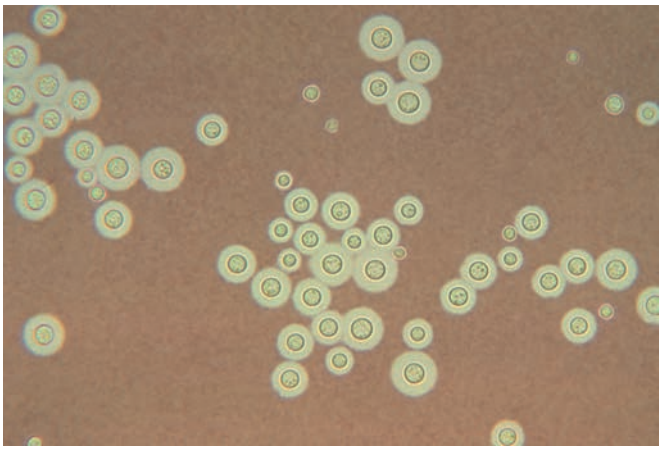

FIGURE 22–6 India ink prep showing *Cryptococcus neoformans* in CSF. The organism's large polysaccharide capsule displaces the India ink, allowing for visualization of the yeast. *(Courtesy of the CDC/Dr. Leanor Haley, Public Health Image Library.)*

FIGURE 22–7 IMMY CrAg® LFA (cryptococcal antigen lateral flow assay). (A) Kit components. The assay is a sandwich immunochromatographic dipstick assay for the qualitative and semiquantitative detection of cryptococcal antigen from CSF and serum. (B) Test results: The dipstick on the left shows one line for the control; the test sample is negative. The dipstick on the right shows two lines, indicating a valid control result and the presence of cryptococcal antigen in the test sample. *(Reprinted by permission Immuno-Mycologics, Inc. [IMMY], Norman, Oklahoma.)*

Measurement of cryptococcal antigen levels has been shown to have some clinical use. Serial polysaccharide antigen titers can be performed on serum or CSF. Initial high titers (1:1,024 or higher) indicate high levels of the yeast in the host and poor host immunity. These individuals have a greater chance of therapeutic failure. As is the case in many infectious diseases, increasing antigen titers are usually seen with worsening of the disease, whereas declining titers are usually associated with clinical improvement. Cryptococcal antigen may be detected even months after successful therapy, so cryptococcal antigen tests should not be used to indicate cure.

Histoplasma Capsulatum

H capsulatum is a common cause of infection in the Ohio and Mississippi river valleys. The organism is a dimorphic fungus, growing as a yeast at 35°C to 37°C and as a mycelial fungus at environmental temperatures. The organism is found and grows in soil under favorable conditions. The presence of bird and bat guano increases the likelihood of the organism being present in the soil. Whereas birds are not infected with the fungus, bats carry the fungus in their gastrointestinal tracts and shed it.[119] Histoplasmosis is acquired by inhalation of mycelial fragments and microconidia. A majority of the infections (more than 90%) are self-limiting and asymptomatic. The organism can cause a potentially lethal infection in patients with preexisting conditions. The organism causes opportunistic infection in patients with weakened immune systems (e.g., people with HIV infection). Of those that are symptomatic, pulmonary histoplasmosis, either acute or chronic, is the most frequent clinical presentation. In a primary infection, the organism disseminates to organs rich in mononuclear phagocytes. Disseminated disease in the immunocompromised patient is 100% fatal if untreated. Survival rates of the acute episode exceed 80% when treated.

The diagnosis of histoplasmosis can be made by culture of the organism. The organism is generally recovered within 3 weeks of culture, with 90% of samples exhibiting growth within 7 days. The ability to recover the organism is affected by the number of specimens submitted for culture, the type of specimen submitted (e.g., respiratory secretions, blood, or CSF), and the severity of the infection.[120] Complement fixation (CF) and precipitation have been the most common tests used for detection of *Histoplasma* antibodies in the clinical laboratory. For CF antibodies, a titer of 1:8 to yeast or mycelial antigen is considered positive and a titer of 1:32 may indicate histoplasmosis. Precipitin band testing looks for the presence of H and M antibodies in the serum of individuals. The H and M antigens are glycoproteins released by mycelial and yeast phase cultures. H antibodies are detected in fewer than 10% of patients but, when present, signify active infection. Antibodies to the M antigen are detected in up to 80% of individuals who have been exposed to the fungus. These antibodies may be found in individuals who have previously been exposed to the organism or who have active disease; thus, it is not useful in discriminating previous from current infection. Skin testing, similar to the Mantoux tuberculin skin test, may also determine if an individual has been exposed to the organism. The *H capsulatum* skin test becomes positive 2 to 4 weeks after infection and the majority of infected people are asymptomatic. Repeat testing will usually give positive results for the rest of a person's life. Thus, in areas where the fungus is endemic, the skin test is of limited utility.

Connections

"-emia" and "-uria"

The suffix "–emia" indicates the presence of a substance in the blood, whereas the suffix "–uria" indicates the presence of a substance in the urine. Thus, "antigenemia" means that a particular antigen (in this case, Histoplasma antigen) can be found in the blood, whereas "antigenuria" means that detectable antigen is present in the urine.

Laboratory diagnosis may be made by detecting the polysaccharide antigen in serum or urine by ELISA. Urinary antigen tests have been shown to have a high sensitivity and specificity for detecting *Histoplasma* infections.[121] *Histoplasma* antigen was detected in the urine in 95% of AIDS patients with disseminated disease and in the serum of 86% of these patients.[122] Ninety-two percent of patients without AIDS who have disseminated histoplasmosis demonstrate *antigenuria,* but only about 50% have *antigenemia.*[123,124] Molecular assays appear to have promise in the diagnosis of histoplasmosis; however, at this point, no molecular assays for routine use are commercially available.

Coccidioides Immitis

Similar to *H capsulatum, C immitis* is also a dimorphic fungus. For many years, **coccidioidomycosis** was thought to be a rare and nearly always fatal infection. In 1929, a medical student at Stanford University was accidentally exposed to the organism and experienced only a mild respiratory infection. His survival resulted in a reassessment of the coccidioidal infections. Coccidioidomycosis was initially recognized as a common respiratory condition in the San Joaquin Valley of California (valley fever).[125] Although originally thought to be only found in the dry, arid regions of the southwestern United States, coccidioidomycosis has become more prevalent throughout both nonendemic and endemic regions of the world.[126] The increased incidence of coccidioidomycosis can be mostly attributed to changes in demography. Populations at risk of exposure are greatly expanded. Regions in which *C immitis* is endemic have major metropolitan centers, such as Phoenix, Arizona. These areas were previously sparsely populated. Along with increased population growth in the southwestern United States, there has been increased tourism leading to movement of people into and out of endemic areas, increasing the numbers of people who are exposed to the organism and acquire coccidioidal infections.[127]

In a vast majority of the cases, infections are the result of inhaling arthroconidia. A single arthroconidium can be enough to produce a naturally acquired respiratory infection.[128] Up to two-thirds of cases caused by *C immitis* are either inapparent or so mild as to not prompt medical evaluation.[129] In those individuals who do become symptomatic, the clinical presentation is similar to a community-acquired pneumonia. *Coccidioides* was estimated to be responsible for approximately one-third

of all cases of community-acquired pneumonia in southern Arizona.[130]

Once inhaled, there is an incubation period of 1 to 3 weeks before the development of respiratory symptoms. Thus, travel history is important if the person has traveled to an endemic area. Once coccidioidomycosis is suspected, diagnosis may be established by identifying the fungus in, or recovering *C immitis* from, a clinical specimen or by detecting anticoccidioidal antibodies in serum, cerebrospinal fluid, or other body fluid. The most frequent way in which coccidioidomycosis is diagnosed is through serology. Although the organism can be cultured, obtaining a sputum specimen in individuals is not often possible because of the lack of sputum production in infected individuals. In addition, fungal culturing in an outpatient setting is not available. The use and limitations of skin testing for *Coccidioides* infection are similar to those for *Histoplasma.* Once positive, the skin test generally remains positive for the rest of one's life; therefore, it is of limited utility in individuals living in endemic areas.

Complement fixation, immunodiffusion (ID), and LA have been the most commonly used serological methods for detecting infection. One of the original methods for detecting infection was the tube precipitin test (TPT). The detection of IgM antibodies by this test has been used to demonstrate infection. The test involves overnight incubation of the patient's serum with coccidioidal antigen. Formation of a precipitin button at the bottom of a test tube demonstrates the presence of IgM antibodies. A polysaccharide from the fungal cell wall is used as the antigen in the test. Tube precipitin antibodies are detected in 90% of patients within the first 3 weeks of symptoms.[131] This test is often referred to as the "IgM test."

The other original test involved detection of coccidioidal antibodies using a CF assay. When mixed with a coccidioidal antigen, an immune complex is formed with the patient's antibodies, resulting in depletion of complement. When the complement is depleted, antibody-coated red blood cells (RBCs) added to the mixture fail to lyse. This test is often referred to as the "IgG test" because IgG is the immunoglobulin class usually involved in the formation of this type of immune complex. CF antibodies are detected later and for longer periods than tube precipitin-type antibodies. Immunodiffusion assays for detecting both IgG and IgM antibodies are available and have served as replacements for the earlier TPT and CF tests.

EIAs for coccidioidal antibodies are commercially available. The EIA assays allow for the specific detection of IgM or IgG antibodies. A positive EIA result is a highly sensitive indicator for coccidioidal infection.[132] Although the EIA tests for IgM and IgG are more sensitive than immunodiffusion tests, false positives have been reported.[133] At present, it is recommended that positive EIA results, particularly positive IgM results, should be confirmed with immunodiffusion tube precipitin or complement-fixing test results. Similar to *H capsulatum,* there are no commercially available molecular assays for the detection of *Coccidioides* infection.

SUMMARY

- Parasites are microorganisms that survive by living off of other organisms. The three major types of parasites are protozoa, helminths, and ectoparasites.
- Parasitic infections can result in eradication of the organism by the host's immune system, death of the host because of an ineffective immune response, or, in most cases, establishment of persistent infection because of the host's inability to completely eliminate the organism.
- Innate defenses (e.g., phagocytosis and cytokine release) and specific humoral and cell-mediated adaptive responses can be demonstrated against parasites. IgE antibodies and eosinophils can destroy some parasites by ADCC and many patients with parasitic infections have increased levels of IgE and eosinophil numbers in the blood.
- Because of their complex, multistage life cycles, the immune responses to the agents responsible for parasitic diseases are often ineffective.
- Parasites have also developed a number of strategies to avoid the immune system, including antigenic concealment, antigenic variation, antigenic shedding, antigenic mimicry, immunologic subversion, and immunologic diversion.
- Fungi are eukaryotic cells with nuclei and rigid cell walls. They can exist in two morphological states: unicellular yeasts or multicellular mycelia fungi.
- Fungal infections are known as mycoses. They can produce hypersensitivity, mycotoxicosis (a poisoning caused by fungal toxins), mycetismus (mushroom poisoning), skin infections, or systemic infections that disseminate from the lungs or skin to other sites of the body.
- Innate defenses including physical barriers such as the skin and attachment of immune cells to pattern-recognition receptors are an important first line of defense against fungal infections. Cell-mediated immunity is the most important adaptive immune response to fungal infections.
- The diagnosis of many parasitic and fungal infections relies on traditional laboratory techniques either by the direct observation of the parasite in clinical material (feces, duodenal fluid, small intestine biopsy specimen, or blood) or by culture and growth of the fungal agent responsible for the infection.
- Serological testing for parasitic and fungal diseases is less routine than serology for other infectious diseases because test availability is limited and assays exhibit variable performance with respect to sensitivity and specificity. Many of these tests are only offered by reference laboratories.
- Serology results should be used in conjunction with the patient's symptoms, history, and other clinical findings to make a diagnosis of a fungal or parasitic infection.

- Toxoplasmosis is an example of a parasitic infection for which serological testing is useful. Detection of IgG, IgM, and IgA antibodies to *T gondii* is helpful in determining whether the infection has been acquired recently or in the past and whether an infant has acquired a congenital infection with the organism.
- Fungal infections for which serological tests are useful include aspergillosis, candidiasis, cryptococcosis, histoplasmosis, and coccidioidomycosis. Serological testing can involve detection of fungal antibody or antigen, depending on the organism.
- To date, there are only a few commercial molecular tests that are available for the diagnosis of parasitic and invasive fungal infections, but advances in molecular technology will likely result in new diagnostic procedures in the future.

Study Guide: Escape Mechanisms of Parasites from Protective Host Responses

ESCAPE MECHANISM	NATURE OF RESPONSE	EXAMPLE(S)
Antigenic concealment	Intracellular survival within macrophages	*Leishmania donovani*
Antigenic variation	Random mutation Genetic recombination Gene switching Multistage parasitic life cycle	*Plasmodium* species *Plasmodium falciparum, Trypanosoma cruzi* *Trypanosoma gambiense, Trypanosoma rhodesiense* *Leishmania* species
Antigenic shedding	Shedding of surface antigens or components	*Entamoeba histolytica*
Antigenic mimicry	Incorporation of host "self" antigens into parasite surface	*Schistosoma* species
Immunologic subversion	Immunosuppression	*Schistosoma mansoni*
Immunologic diversion	Polyclonal B-cell activation	*Plasmodium* species

CASE STUDIES

1. An otherwise healthy infant developed a seizure 5 days following birth. The mother and baby returned to the hospital for evaluation. Upon examination of the infant, the physician found that the baby demonstrated chorioretinitis. Prescreening of the mother during the third trimester of pregnancy did not demonstrate the presence of antibodies against *T gondii*. At that time, she was advised to refrain from cleaning the litter boxes of the family's two cats. Upon questioning, the mother stated that she had been cleaning the litter boxes when other family members failed to do so. The physician suspected that the child may have congenital toxoplasmosis. Serological testing of the mother and the fetus gave the following results:

	Anti-*T gondii* IgM	**Anti-*T gondii* IgG**
Mother	1:256	1:512
Baby	Not done	1:256

Questions

a. Evaluate the baby's status related to *T gondii* infection.
b. What additional testing should be performed to confirm the baby's status?

2. A 60-year-old male with a medical history of chronic obstructive pulmonary disease (COPD), diabetes mellitus, and hepatitis C was seen in the emergency department. The patient admitted to using intravenous drugs in the past and has been receiving inhaled steroid therapy for his COPD. He complained of nausea and severe headaches, which interfered with his ability to carry out his normal activities. The patient also felt unbalanced and weak when standing. Head computed tomography (CT) and magnetic resonance imaging (MRI) revealed abnormalities in the cerebellum of the patient's brain. Cryptococcal meningitis was suspected. A lumbar puncture was performed and a CSF sample was sent for laboratory testing to confirm the suspected diagnosis.

Questions

a. What clinical presentations of the patient point to a diagnosis of cryptococcosis?
b. What laboratory tests should be performed to confirm the patient's diagnosis?

REVIEW QUESTIONS

1. Compared with a host's response to the mumps virus, overcoming a parasitic infection is more difficult for the host because of which of the following characteristics of parasites?
   a. Large size
   b. Complex antigenic structures
   c. Elaborate life cycle
   d. All of the above

2. Which of the following is indicative of a recent infection with *Toxoplasma gondii?*
   a. Anti-*Toxoplasma* IgM
   b. Anti-*Toxoplasma* IgE
   c. High avidity anti-*Toxoplasma* IgG
   d. Low avidity anti-*Toxoplasma* IgG

3. Parasites are able to evade host defenses by which of the following means?
   a. Production of antigens similar to host antigens
   b. Changing surface antigens
   c. Sequestering themselves within host cells
   d. All of the above

4. The chronic nature of parasitic infections is caused by the host's
   a. inability to eliminate the infective agent.
   b. type I hypersensitivity response to the infection.
   c. ability to form a granuloma around the parasite.
   d. tendency to form circulating immune complexes.

5. The presence of both IgM and IgG antibody in toxoplasmosis infections suggests that the infection
   a. occurred more than 2 years ago.
   b. occurred more recently than 18 months ago.
   c. is chronic.
   d. has resolved itself.

6. Which of the following is indicative of a parasitic infection?
   a. Increased IgA levels
   b. Increased IgE levels
   c. Increased IgG levels
   d. Increased IgM levels

7. In congenital toxoplasmosis, which class of antibodies is the most sensitive in detecting infection?
   a. IgA
   b. IgG
   c. IgM
   d. IgE

8. The most significant defense against fungal infections is
   a. cellular immunity.
   b. humoral immunity.
   c. phagocytosis.
   d. complement activation.

9. Clinical manifestations of fungal-related illness include
   a. hypersensitivity caused by fungal spores.
   b. poisoning caused by ingestion of mycotoxins.
   c. growth of fungi in or on tissue.
   d. all of the above.

10. Which of the following assay formats are increasingly being adopted by clinical laboratories for serological detection of fungal infections because of their ease of use?
    a. ELISA assays
    b. Lateral flow assays
    c. Radial immunodiffusion assays
    d. Indirect immunofluorescence assays

11. The presence of anti-H antibodies indicates which of the following?
    a. A previous infection with *Coccidioides immitis*
    b. A previous exposure to *Histoplasma capsulatum*
    c. An active infection with *Cryptococcus neoformans*
    d. An active infection with *Histoplasma capsulatum*

12. A limiting factor in reliably being able to detect antifungal antibodies in an acute infection is
    a. the lack of humoral response to fungal agents caused by immunosuppression.
    b. current assays lack specificity.
    c. antibodies are not normally formed against most fungi.
    d. antibodies tend to remain at low titer as a mycosis develops.

13. False positives may be observed in latex agglutination tests for the capsular antigen of *Cryptococcus neoformans* because of

a. the use of serum instead of CSF.
b. the presence of rheumatoid factor in the specimen.
c. cross-reactivity with other fungal antigens.
d. the low specificity of the assay.

14. A 27-year-old man from Ohio, diagnosed with AIDS, developed chest pains. After a short period of time he also developed severe headaches with dizziness. In his free time, his hobby was exploring caves (a spelunker). His physician ordered a sputum culture and spinal tap and both were positive for a yeastlike fungus. These findings are most consistent with infection by

a. *Candida albicans.*
b. *Coccidioides immitis.*
c. *Cryptococcus neoformans.*
d. *Histoplasma capsulatum.*

15. Which of the following serological tests detects the polysaccharide capsule antigen in serum and CSF of patients with suspected infection with *Cryptococcus neoformans?*

a. Complement fixation (CF)
b. India ink test
c. Latex agglutination (LA)
d. Hemagglutination test

16. Which of the following is a nondimorphic fungus that is found in concentrated bird droppings and can readily cause meningitis in immunocompromised individuals?

a. *Coccidioides immitis*
b. *Candida albicans*
c. *Cryptococcus neoformans*
d. *Histoplasma capsulatum*

23 Serology and Molecular Detection of Viral Infections

Linda E. Miller, PhD, I,MB^{CM}(ASCP)SI

LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Describe the immune defenses that are important in protecting humans from viral infections.
2. Discuss mechanisms by which viruses can escape host defenses.
3. Correlate the presence of viral IgM and IgG antibodies with their clinical significance in detecting current infections, congenital infections, or immunity to infections.
4. Discuss the role of molecular tests in diagnosing and monitoring patients with viral infections.
5. Differentiate between the different hepatitis viruses and their modes of transmission.
6. Correlate the various serological markers of hepatitis with their diagnostic significance.
7. Explain the laboratory methods that are most commonly used to screen for, confirm, or monitor hepatitis virus infections.
8. Associate the following viruses with the specific diseases they cause: Epstein-Barr virus (EBV), cytomegalovirus (CMV), varicella-zoster virus (VZV), rubella virus, rubeola virus, mumps virus, and the human T-cell lymphotropic virus type I.
9. Discuss the laboratory methods used to diagnose and monitor infections with the preceding viruses.
10. Correlate the heterophile antibody and EBV-specific antibodies with their clinical significance and describe the laboratory methods used to test for these antibodies.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Anti-HBe
Anti-HBs
Cytomegalovirus (CMV)
Epstein-Barr virus (EBV)
Hepatitis
Hepatitis A virus (HAV)
Hepatitis B surface antigen (HBsAg)
Hepatitis B virus (HBV)
Hepatitis Be antigen (HBeAg)
Hepatitis C virus (HCV)
Hepatitis D virus (HDV)
Hepatitis E virus (HEV)
Heterophile antibodies
Human T-cell lymphotropic virus type I (HTLV-I)
Human T-cell lymphotropic virus type II (HTLV-II)
IgM anti-HBc
Mumps virus
Parenteral
Rubella virus
Rubeola virus
Varicella-zoster virus (VZV)

Viruses are submicroscopic pathogens whose size is measured in nanometers. Their basic structure consists of a core of DNA or RNA packaged into a protein coat or capsid. In some viruses, the capsid is surrounded by an outer envelope of glycolipids and proteins derived from the host cell membrane **(Fig. 23–1)**. It is remarkable that these tiny particles are capable of causing severe, and sometimes lethal, disease in humans, ranging from childhood infections to inflammatory diseases with a predilection for a specific organ, disseminated disease in immunocompromised patients, cancer, and congenital abnormalities.

Viruses are obligate intracellular pathogens that rely on the host cell for their replication and survival. They infect their host cells by attaching to specific receptors on the cell surface; penetrating the host cell membrane; and releasing their nucleic acid, which then directs the host cell's machinery to produce more viral nucleic acid and proteins. These components assemble to form intact viruses that are released by lysis of the cell or by budding off the cell's surface **(Fig. 23–2)**. Replication can occur quickly in cytolytic viruses that produce acute infections, or slowly in viruses that result in chronic infections. The free virions that are generated can then infect neighboring host cells and begin new replication cycles that promote dissemination of the infection. Thus, viruses can be present in the host as both freely circulating particles and intracellular particles. This chapter briefly addresses the immunologic mechanisms required to attack the virus in its different states. Successful defense against viral infections requires a coordinated effort among innate, humoral, and cell-mediated immune responses **(Fig. 23–3)**. The remainder of the chapter discusses some of the most important viral infections detected by serology and molecular methods. These include the hepatitis viruses, herpes viruses, measles, mumps, rubella, and human T-cell lymphotropic viruses. Laboratory tests for the human immunodeficiency virus (HIV) are discussed separately in Chapter 24.

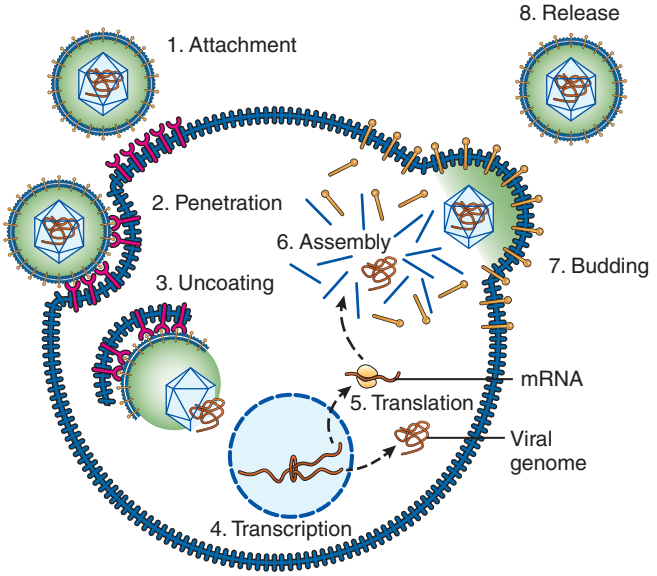

FIGURE 23–2 Basic steps of a virus life cycle. (1) Attachment of the virus to a receptor on the host cell surface. (2) Penetration, or entry of the virus into the host cell through endocytosis or other mechanisms. (3) Uncoating, or degradation of the viral capsid and subsequent release of viral nucleic acid. With some viruses, the nucleic acid integrates into the host cell genome. (4) Transcription to produce additional viral nucleic acid. (5) Translation of viral nucleic acid to produce viral proteins. (6) Assembly of the viral components to produce intact virions. (7) Budding off the host cell membrane or host cell lysis results in (8) release of viral progeny. Modifications of these steps can occur with different viruses.

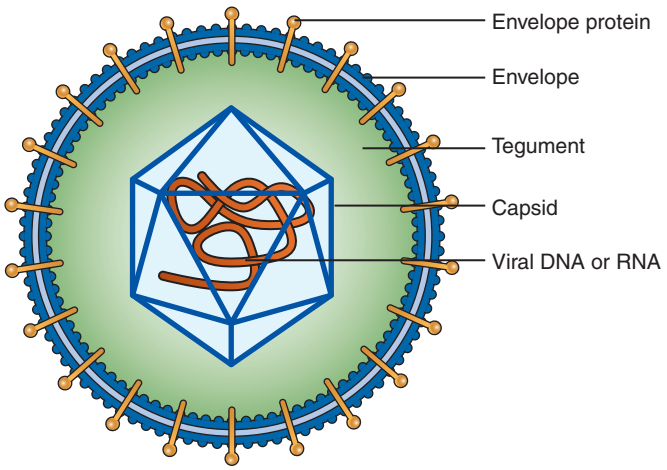

FIGURE 23–1 Basic structure of a virus.

Immune Defenses Against Viral Infections

Innate immunity provides the first line of protection against viral pathogens. Viruses first encounter naturally occurring barriers in the body such as the skin and mucous membranes. If they are able to invade these barriers, other innate defenses are activated when cells of the innate immune system recognize pathogen-associated molecular patterns (PAMPs) on the surface

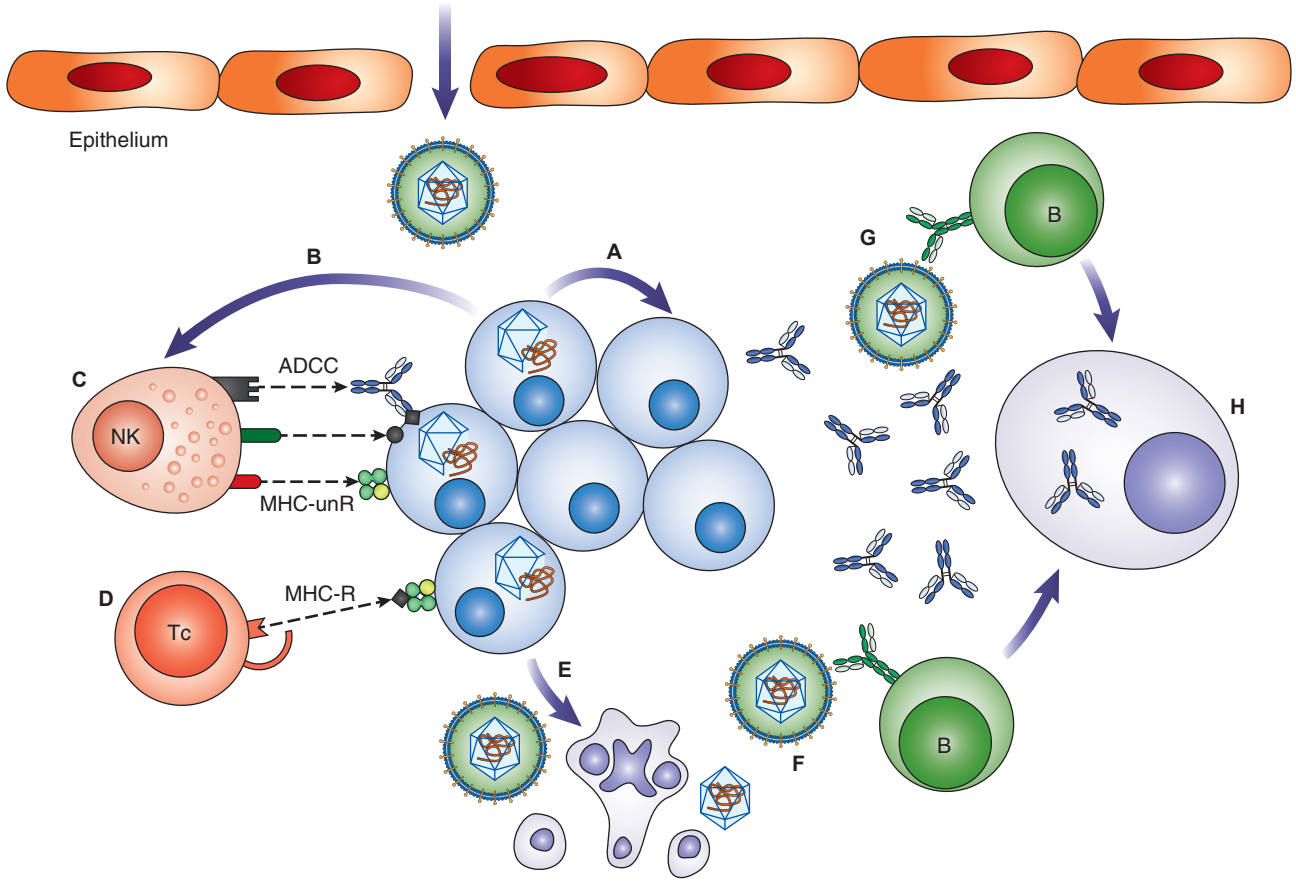

FIGURE 23–3 Innate defenses provide the initial barrier to viral infection. Infected cells release interferons α and β, which (A) inhibit viral replication in surrounding cells and (B) stimulate natural killer (NK) cells. Both NK and cytotoxic T (Tc) cells destroy virus-infected host cells (C, D), resulting in the release of free virions (E). Virus-specific B cells recognize these free virions (F), as well as virions that have penetrated the epithelium (G), leading to the production of antibodies (H) that bind free virions and mediate virus neutralization, opsonization, complement activation, and ADCC.

of or within virus-infected host cells. Two important nonspecific defenses against viruses involve type I interferons and natural killer (NK) cells.[1-3] Virus-infected cells are stimulated to produce IFN-α and IFN-β following recognition of viral RNA by toll-like receptors (TLR). Interferons inhibit viral replication by inducing the transcription of several genes that code for proteins with antiviral activity—for example, a ribonuclease enzyme that degrades viral RNA. IFN-α and IFN-β also enhance the activity of NK cells, which bind to virus-infected cells and release cytotoxic proteins such as perforin and granzymes, causing the cells to die and release the viruses. These cell-free virions are now accessible to antibody molecules.

When innate defenses are insufficient in preventing viral infection, specific humoral and cell-mediated defenses are activated.[1-3] Virus-specific antibodies are produced by B cells and plasma cells and can attack free virus particles in several ways. Antibodies play a key role in preventing the spread of a viral infection through neutralization. This process involves the production of antibodies that are specific for a component of the virus that binds to a receptor on the host cell membrane. When these neutralizing antibodies bind to the virus, they prevent it from attaching to and penetrating the host cell. Secretory IgA antibodies play an especially important role in this process because they neutralize viruses in the mucosal surfaces (e.g., respiratory and digestive tracts), which often serve as entryways for the pathogens. Meanwhile, IgM and IgG antibodies can bind to viruses in the bloodstream and inhibit dissemination of the infection. In addition, IgG antibodies promote phagocytosis of viruses through their opsonizing activity and promote destruction of viruses through antibody-dependent cell-mediated cytotoxicity (ADCC). IgG and IgM antibodies also activate complement, which can mediate opsonization via C3b or lyse enveloped viruses by inducing formation of the membrane attack complex. IgM antibodies may also inactivate viral particles by agglutinating them.

Although antibodies can attack viruses in many different ways, they cannot reach viruses that have already penetrated host cells. Elimination of intracellular viruses requires the action of cell-mediated immunity. Type 1 helper (Th1) cells and

Connections

Interferons

Interferons "interfere" with the ability of viruses to replicate by stimulating infected host cells to produce proteins that degrade viral nucleic acid and proteins (see Chapter 6). Interferons exert their effects not only on the original infected cell, but also on neighboring uninfected host cells.

cytotoxic T lymphocytes (CTL) play a key role in this mechanism of defense. Th1 cells produce interferon-γ, which induces an antiviral state within the virus-infected cells, and IL-2, which assists in the development of effector CTLs. In this process, CD8+ CTL become programmed to expand in number and attack the virus-infected cells.[4] To recognize the virus-infected host cell, the T-cell receptor (TCR) on the CTL must bind to a viral antigen complexed with class I major histocompatibility complex (MHC) on the surface of the infected cell (see Fig. 23–3). CD8 is a co-receptor in this interaction. Interaction of costimulatory molecules, such as B7 and CD28, provides secondary signals necessary for the CTL response. These molecular interactions stimulate the granules in the CTL to release a pore-forming protein called perforin, which produces pores in the membrane of the infected host cell, and proteases called granzymes, which enter the pores. These enzymes activate apoptosis in the host cell, interrupting the viral-replication cycle and resulting in release of assembled infectious virions. The free virions can then be bound by antibodies. The CTL response is powerful and involves a series of cell divisions that can produce up to 50,000 times the original number of cells in a period of 1 to 3 weeks.[4]

Viral Escape Mechanisms

Viruses can escape the host's defense mechanisms in several ways.[1-3] First, viruses are rapidly dividing agents that undergo frequent genetic mutations. These mutations result in the production of new viral antigens, which are not recognized by the initial immune response to the virus. For example, continual antigenic variation in the influenza virus results in the emergence of novel infectious strains that require development of new vaccines every year to protect the population. Antigenic variation is also seen in other viruses, including rhinoviruses, which cause the common cold, and HIV, which causes AIDS.

Second, some viruses can escape the action of components of the innate immune system such as interferons, complement proteins, or the lysosomal enzymes in phagocytic cells. For example, the hepatitis C virus can block interferon-mediated degradation of viral RNA and herpes simplex viruses (HSV) produce a protein that binds to the complement component, C3b, resulting in inhibition of the complement pathways.

Third, viruses can evade the host's defense by suppressing the adaptive immune system. Some viruses, such as the **cytomegalovirus (CMV)** and HIV, do this by reducing the expression of class I MHC molecules on the surface of virus-infected cells, making them less likely to be recognized by CTLs. Other viruses, such as rubeola, can cause decreased expression of class II MHC molecules, resulting in reduced Th cell activity. Some viruses can alter the function of certain cells of the immune system after directly infecting them. For example, the **Epstein-Barr virus (EBV)** causes polyclonal activation in B lymphocytes, whereas HIV suppresses the function of CD4 Th cells. EBV can also inhibit immune responses by producing a protein that can suppress Th1 cells because of its similarity to interleukin-10 (IL-10).

Finally, some viruses, such as CMV, **varicella-zoster virus (VZV),** and HIV, can remain in a latent state by integrating their nucleic acid into the genome of the infected host cells. In this situation, the virus is only stimulated to replicate again if the host is exposed to other infectious agents or if the host's immune defenses decline. Latent viruses can remain silent within host cells for years because they are hidden from the immune system, although reactivation can occur later in life.

By using these evasion mechanisms, viruses have established themselves as successful human pathogens that can cause a range of mild to life-threatening diseases. Rapid, reliable laboratory detection of these pathogens is essential for early patient diagnosis and treatment. Laboratory identification also leads to prompt implementation of measures to prevent further spread of the virus to other members of the population.

Laboratory Testing for Viral Infections

As our knowledge of viruses has increased, so has the development of laboratory assays to detect viral infections. Serological and molecular tests can be easily and rapidly performed by the clinical laboratory. Therefore, they play an essential role in helping physicians establish a presumptive diagnosis so that treatment can be initiated promptly. Serological tests are also important in monitoring the course of infection, detecting past infections, and assessing immune status, whereas molecular tests have enhanced our ability to detect active infection and are essential in guiding antiviral therapy.

In general, the presence of virus-specific IgM antibodies in patient serum indicates a current or recent viral infection, whereas IgG antibodies to a virus signify either a current or past infection and, in many cases, immunity. Virus-specific IgM antibody in the newborn's serum indicates a congenital infection because IgM is actively made during fetal life. In contrast, IgG antibodies in the infant's serum are mainly maternal antibodies that have crossed the placenta. Current infections in the adult or newborn may also be detected by immunoassays for viral antigens in serum or other clinical samples or by the presence of viral nucleic acids that can be detected by molecular methods.

Hepatitis Viruses

Hepatitis is a general term that means inflammation of the liver. It can be caused by several viruses and by noninfectious agents, including ionizing radiation, chemicals, and autoimmune processes. The primary hepatitis viruses affect mainly the liver. Other viruses, such as CMV, EBV, and HSV, can also produce liver inflammation, but it is secondary to other disease processes. This section will focus on the primary hepatitis viruses. The **hepatitis A virus (HAV)** and the **hepatitis E virus (HEV)** are transmitted primarily by the fecal–oral route, whereas the **hepatitis B virus (HBV),** the **hepatitis D virus (HDV),** and the **hepatitis C virus (HCV)** are transmitted mainly by the **parenteral** route (i.e., through contact with blood and other body fluids). All of the hepatitis viruses may produce similar clinical manifestations. The early, or acute, stages of hepatitis are characterized by general flu-like symptoms and mild to moderate pain

in the right upper quadrant (RUQ) of the abdomen.[5,6] Progression of the disease leads to liver enlargement (hepatomegaly) and tenderness, jaundice, dark urine, and light feces.

Initial laboratory findings typically include elevations in bilirubin and in the liver enzymes, most notably alanine aminotransferase (ALT).[5,6] These findings are nonspecific indicators of liver inflammation and must be followed by specific serological or molecular tests to identify the cause of hepatitis more definitively. The specific laboratory tests used to detect each type of hepatitis are listed in **Table 23–1.**

Hepatitis A

HAV is a nonenveloped, single-stranded ribonucleic acid (RNA) virus that belongs to the *Hepatovirus* genus of the *Picornaviridae* family.[6,7] Two major genotypes of the virus are associated with

Table 23–1 The Hepatitis Viruses and Their Associated Serological and Molecular Markers

HEPATITIS VIRUS	TYPE AND FAMILY	TRANSMISSION	PROGRESSION TO CHRONIC STATE	COMPLICATIONS	SEROLOGICAL AND MOLECULAR MARKERS	CLINICAL SIGNIFICANCE
Hepatitis A (HAV)	RNA *Picornaviridae*	Fecal-oral Blood transfusion (rare)	No	Low risk of fulminant liver disease	• IgM anti-HAV • Total anti-HAV • HAV RNA	• Acute hepatitis A • Immunity to hepatitis A • Detection of HAV in clinical, food, or water samples
Hepatitis B (HBV)	DNA *Hepadnaviridae*	Parenteral, sexual, perinatal	Yes	10% to 90% of cases may develop chronic hepatitis (depending on age), with increased risk for liver cirrhosis and hepatocellular carcinoma	• HBsAg	• Active hepatitis B infection
					• HBeAg	• Active hepatitis B with high degree of infectivity
					• IgM anti-HBc	• Current or recent acute hepatitis B
					• Total anti-HBc	• Current or past hepatitis B
					• Anti-HBe	• Recovery from hepatitis B
					• Anti-HBs	• Immunity to hepatitis B
					• HBV DNA	• Acute, atypical, or occult hepatitis B; viral load may be used to monitor effectiveness of therapy

Table 23–1 The Hepatitis Viruses and Their Associated Serological and Molecular Markers—cont'd

HEPATITIS VIRUS	TYPE AND FAMILY	TRANSMISSION	PROGRESSION TO CHRONIC STATE	COMPLICATIONS	SEROLOGICAL AND MOLECULAR MARKERS	CLINICAL SIGNIFICANCE
Hepatitis C (HCV)	RNA *Flaviviridae*	Parenteral, sexual, perinatal	Yes	Eighty-five percent develop chronic infection, with increased risk of cirrhosis, hepatocellular carcinoma, or autoimmune manifestations	• Anti-HCV	• Current or past hepatitis C infection
					• HCV RNA	• Current hepatitis C infection; viral load may be used to monitor effectiveness of therapy; also used to determine HCV genotype
Hepatitis D (HDV)	RNA Genus *Deltavirus*	Mostly parenteral, but also sexual, perinatal; HBV infection required	Yes	Increased risk of developing fulminant hepatitis, cirrhosis, or hepatocellular carcinoma	• IgM-anti-HDV	• Acute or chronic hepatitis D
					• IgG-anti-HDV	• Recovery from hepatitis D or chronic hepatitis D
					• HDV RNA	• Active HDV infection; viral load may be used to monitor effectiveness of therapy
Hepatitis E (HEV)	RNA *Hepeviridae*	Fecal-oral Blood transfusion	Yes, in immunocompromised individuals	Fulminant liver failure in pregnant women	• IgM anti-HEV	• Current hepatitis E infection
					• IgG anti-HEV	• Current or past hepatitis E infection
					• HEV RNA	• Current hepatitis E infection

human disease and both can be detected by the same serological assays (see the text that follows). Hepatitis A is a common infection responsible for an estimated 1.4 million cases of hepatitis worldwide.[8] HAV is transmitted primarily by the fecal–oral route, close person-to-person contact, or ingestion of contaminated food or water.[8,9] Conditions of poor personal hygiene, poor sanitation, and overcrowding facilitate transmission. Rarely, transmission through transfusion of contaminated blood has been reported and may occur during a short period within the acute stage of infection when a high number of viral particles can be found in the source blood.[9]

Following an average incubation period of 28 days, the virus produces symptoms of acute hepatitis in the majority of infected adults; however, most infections in children are asymptomatic.[8,9] The infection does not progress to a chronic state and is usually self-limiting, with symptoms typically resolving within 2 months. Treatment is mainly supportive, involving bed rest, nutritional support, and medication for fever, nausea, and diarrhea. Massive hepatic necrosis resulting in fulminant hepatitis and death is rare and occurs mainly in those patients with underlying liver disease or advanced age.[8,10]

HAV antigens are shed in the feces of infected individuals during the incubation period and the early acute stage of infection, but they usually decline to low levels shortly after symptoms appear and are not a clinically useful indicator of disease.[9] Therefore, serological tests for antibody are critical in establishing diagnosis of the infection. Hepatitis A antibodies are most commonly detected by automated, chemiluminescent microparticle immunoassays. Acute hepatitis A is routinely diagnosed in symptomatic patients by demonstrating the presence of IgM antibodies to HAV.[5,6,8,9] IgM anti-HAV is detectable at the onset of clinical symptoms and declines to undetectable levels within 6 months in the majority of infected individuals.[5,6,9] Because false-positive results can occur, the test should be reserved for symptomatic individuals.[8,9] Tests for total HAV antibodies also detect IgM, but predominantly detect IgG, which persists for life. Thus, a positive total anti-HAV test result in combination with a negative IgM anti-HAV indicates that the patient has developed immunity to the virus, either through natural infection or vaccination. Negative total anti-HAV tests can be used to identify nonimmune individuals who may have been exposed to the virus.[8]

Although IgM anti-HAV is the primary marker to detect acute hepatitis A, false-negative results can occur during the early phase of the infection.[11] Molecular methods to detect HAV RNA have been shown to be more sensitive in this situation.[11] The most common format of these methods is the reverse transcriptase polymerase chain reaction (RT-PCR).[11,12] Molecular methods can also be used to test samples of food or water suspected of transmitting the virus.[5,12] Multiplex real-time PCR (RT-PCR) methods that can simultaneously detect more than one type of hepatitis virus in clinical samples have also been developed.[11,13]

A vaccine consisting of formalin-killed HAV was licensed in the mid-1990s to prevent hepatitis A. Vaccination has resulted in a significant decrease in the number of HAV infections in the United States and other countries throughout the world.[7,9] To prevent infection in unimmunized individuals who have been exposed to the virus, prophylactic administration of the hepatitis A vaccine or injections of immune globulin are recommended.[8,10] The vaccine is the preferred treatment for persons aged 1 to 40 years, but intramuscular injection of immune globulin, a sterile preparation of pooled human plasma that contains antibodies to HAV, can be used to prevent infection in individuals of any age. To be effective, these treatments must be administered within 2 weeks of exposure.[8]

Connections

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

In RT-PCR, viral RNA is treated with the enzyme reverse transcriptase to generate a complementary DNA (cDNA) sequence. The cDNA is then amplified by the polymerase chain reaction to generate millions of copies that can be detected in the laboratory (see Chapter 12).

Hepatitis E

HEV is a nonenveloped, single-stranded RNA virus that belongs to the genus *Hepevirus,* in the family *Hepeviridae.*[7,14] HEV is a major cause of hepatitis worldwide: the World Health Organization (WHO) estimates that annually, the virus causes 20 million infections, over 3 million cases of acute hepatitis, and over 56,000 deaths.[15] Similar to the HAV, HEV is transmitted primarily by the fecal–oral route; however, person-to-person transmission is uncommon. The four genotypes of the virus differ in terms of their epidemiology and source of infection. Genotypes 1 and 2 are associated primarily with consumption of fecally-contaminated drinking water in developing regions of the world with poor sanitation, including parts of Africa, Asia, the Middle East, and Mexico.[14,16,17] Outbreaks commonly occur in times of natural disasters such as flooding and earthquakes and can affect thousands of individuals. Genotypes 3 and 4 have been increasingly recognized in developed parts of the world, including Europe, North America, and Japan. HEV3 and HEV4 are zoonotic infections in which pigs are the primary host.[14,16] These infections are thought to be transmitted mainly by consumption of infected pork and possibly by direct contact with infected animals or fecally-contaminated water.[14] HEV has also been detected in the blood supply in a number of countries and can be transmitted through blood transfusions.[14,16]

Although HEV infections are often silent, all genotypes are capable of causing an acute hepatitis with symptoms that are indistinguishable from other types of hepatitis. Following an incubation period of 2 to 6 weeks, HEV infection in most people causes a self-limiting illness with recovery occurring by 4 to 6 weeks.[14,16] However, the infection can have severe consequences. Some patients may experience extrahepatic symptoms, including neurological syndromes, renal injury, pancreatitis, and hematologic abnormalities.[14] Pregnant women infected with HEV1 or HEV2 have a mortality rate of 20% to 25% because of obstetric complications or development of fulminant hepatitis, which is associated with rapidly progressing

liver disease and failure.[14,16] The reason for this is unclear, but it may be caused by the hormonal and immunologic changes associated with pregnancy. HEV3 can result in chronic infection in immunocompromised individuals.[16] Chronic infection can progress to liver fibrosis, cirrhosis, and liver failure, which may require liver transplantation.[18]

Measures to prevent the infection include provision of clean drinking water, improvement of sanitation conditions in developing countries, and in the case of HEV3, avoidance of eating undercooked meat, especially pork.[14,15] A vaccine to prevent HEV1 infection has been licensed for use in the People's Republic of China.[19] The vaccine consists of viruslike particles that have been genetically modified to express a gene that codes for a key HEV protein.

Because HEV is not easily cultured, diagnosis relies on serology to detect antibodies to the virus and molecular methods to detect HEV nucleic acid. Antibodies to HEV are typically identified by sensitive enzyme immunoassays (EIAs) that use recombinant and synthetic HEV antigens. Rapid immunochromatographic assays have also been developed. Antibody tests for HEV can detect all four genotypes of the virus because there is only one viral serotype.[14,16] Acute infection is indicated by the presence of IgM anti-HEV, which is detectable at clinical onset, remains elevated for about 8 weeks, and becomes undetectable in most patients by 32 weeks.[14] HEV-specific IgG antibodies appear soon after IgM, reach peak levels about 4 weeks after symptoms develop, and persist for several years.[14,17] Immunoassays for IgG anti-HEV may be performed to detect patients in the later stages of infection, determine past exposure, and identify seroprevalence of the infection in a population.

Immunocompromised persons often yield negative antibody test results; molecular testing for HEV RNA is recommended in these patients.[14,18] HEV nucleic acid can be performed by real-time PCR or a loop-mediated isothermal amplification assay (LAMP), which is suitable for resource-limited settings because it is faster and does not require expensive equipment.[14] These assays can be performed on blood or stool samples. HEV RNA can be detected just before clinical symptoms. It becomes undetectable in the blood about 3 weeks after symptom onset; in the stool, it becomes undetectable at about 5 weeks.[14,16] Therefore, a negative result for HEV RNA does not exclude the possibility of a recent infection.

Hepatitis B

Hepatitis B is a major cause of morbidity and mortality throughout the world. The WHO estimates that HBV has infected 2 billion people worldwide, causing 240 million chronic infections and 780,000 deaths each year because of liver disease.[20,21] The virus is highly endemic in the Far East, parts of the Middle East, sub-Saharan Africa, and the Amazon areas. In the United States, which is considered a low-prevalence area, HBV is responsible for approximately 1.2 million chronic infections and 1,800 deaths annually.[22]

HBV is transmitted through the parenteral route by intimate contact with HBV-contaminated blood or other body fluids, most notably semen, vaginal secretions, and saliva.[6,23–25] Transmission has thus been associated with sexual contact, blood transfusions, sharing of needles and syringes by intravenous drug users, tattooing, and occupational needlestick injury. Inapparent transmission of HBV may occur through close personal contact of broken skin or mucous membranes with the virus. Transmission of HBV may also occur via the perinatal route, from infected mother to infant, most likely during delivery.

Several measures have been introduced to prevent HBV infection, including screening of blood donors, treating plasma-derived products to inactivate HBV, implementing infection-control measures, and, most importantly, immunizing with a hepatitis B vaccine.[24,25] The current vaccines, consisting of recombinant Hepatitis B surface antigen (HBsAg) produced from genetically engineered yeast or mammalian cells, are some of the most widely used vaccines throughout the world. Immunization has been highly successful, resulting in a significant decline in the incidence of acute hepatitis B in the United States since routine immunization was implemented in 1991.[24,26] Increasingly widespread use of the vaccine will likely continue to reduce the incidence of new HBV infections worldwide. The vaccine can also be administered to individuals thought to be exposed to the virus, along with HBIG (hepatitis B immune globulin), a preparation derived from donor plasma with high concentrations of antibodies to HBV that provides temporary protection.[24]

Despite the preventative measures that have been implemented, a substantial number of HBV infections continue to occur as previously discussed. Infection with HBV results in an incubation period of 30 to 180 days, followed by a clinical course that varies in different age groups.[6,21,25] Over 90% of newborns with perinatal HBV infection remain asymptomatic, whereas typical symptoms of acute hepatitis are observed in about 10% of children aged 1 to 5 years and in approximately one-third of adolescents and adults. Symptoms may last several weeks to several months and are usually managed through bed rest and other supportive treatment. Most HBV-infected adults recover within 6 months and develop immunity to the virus, but about 1% develop fulminant liver disease with hepatic necrosis. This highly fatal condition is treated with intensive life support, antiviral drugs, and, in some patients, liver transplantation.

Development of chronic HBV infection, in which the virus persists in the body for 6 months or more, occurs in about 90% of infected infants, 30% of young children, and 10% of infected adults. Chronic infection is also more likely to develop in persons who are immunosuppressed and those who have HIV.[21,25] Chronic infection with the virus results in inflammation and damage to the liver and places the patient at increased risk of developing cirrhosis or hepatocellular carcinoma.[27] Patients with chronic infection can be treated with antiviral drugs to reduce liver inflammation and the risk of developing liver complications.[26,28] Therapies consist of nucleoside analogues that inhibit the polymerase enzyme needed for viral replication and interferon alpha, which enhances the immune response against the virus.

The virus responsible for hepatitis B, HBV, is a DNA virus belonging to the *Hepadnaviridae* family.[6,23,27] Eight genotypes,

designated A through H, have been identified based on nucleotide sequence differences in their genomes. The genotypes vary in their geographic distribution, pathogenicity, and response to treatment, but can be identified by the same serological assays. The intact HBV virion is a 42 nm sphere consisting of a nucleocapsid core surrounded by an outer envelope of lipoprotein. The core of the virus contains circular, partially double-stranded DNA; a DNA-dependent DNA polymerase enzyme; and two proteins, the hepatitis B core antigen and the **hepatitis Be antigen (HBeAg).** A protein called the **hepatitis B surface antigen (HBsAg)** is found in the outer envelope of the virus. HBsAg is produced in excess and is found in noninfectious spherical and tubular particles that lack viral DNA and circulate freely in the blood.

These antigens, and antibodies to them, serve as serological markers for hepatitis B and have been used in differential diagnosis of HBV infection, monitoring the course of infection in patients, assessing immunity to the virus, and screening blood products for infectivity.[5,6,23,29,30] The levels of these markers vary with the amount of viral replication and the host's immune response. They are useful in establishing the initial diagnosis of hepatitis B and monitoring the course of infection. Serological markers for hepatitis B are listed in Table 23–1 and are described in the text that follows. Typical patterns of the markers during acute and chronic hepatitis B are shown in **Figures 23–4** and **23–5.** The HBsAg is the first marker to appear, becoming detectable 2 to 10 weeks after exposure to HBV. Its levels peak during the acute stages of infection, then gradually decline as the patient develops antibodies to the antigen and recovers. Serum HBsAg usually becomes undetectable by 4 to 6 months after the onset of symptoms in patients with acute hepatitis B. In patients with chronic HBV infection, HBsAg remains elevated for 6 months or more. Therefore, HBsAg is an indicator of active infection and is an important marker in detecting initial infection, monitoring the course of infection and progression to chronic disease, and screening of donor blood.

The HBeAg appears shortly after HBsAg and disappears shortly before HBsAg in recovering patients. It may be elevated during chronic infection. This marker is present during periods of active replication of the virus and indicates a high degree of infectivity. The HBeAg is not detectable in serum because the viral envelope masks it.

As the host develops an immune response to the virus, antibodies appear. First to appear is IgM antibody to the core antigen, or **IgM anti-HBc.** This antibody indicates current or recent acute infection. It typically appears 1 to 2 weeks after HBsAg during acute infection and persists in high titers for 4 to 6 months and then gradually declines. IgM anti-HBc is useful in detecting infection in cases in which HBsAg is undetectable—for example, just before the appearance of antibodies to the antigen (commonly referred to as the "core window" period), in neonatal infections, and in cases of fulminant hepatitis. Therefore, it is used in addition to HBsAg for the screening of donor blood. IgG antibodies to the core antigen are produced before IgM anti-HBc disappears and then persist for the individual's lifetime. They are the predominant antibodies detected in the test for total anti-HBc and can be used to indicate a past HBV infection.

The appearance of antibodies to the HBe antigen, or **anti-HBe,** occurs shortly after the disappearance of HBeAg and indicates that the patient is recovering from HBV infection.

Antibodies to HBsAg, or **anti-HBs,** also appear during the recovery period of acute hepatitis B, a few weeks after HBsAg disappears. These antibodies persist for years and provide protective immunity. Anti-HBs are also produced after immunization with the hepatitis B vaccine. Protective titers of the antibody in the serum are considered to be 10 mIU/mL or higher.[24,25] Anti-HBs is not produced during chronic HBV infection, in which immunity fails to develop.

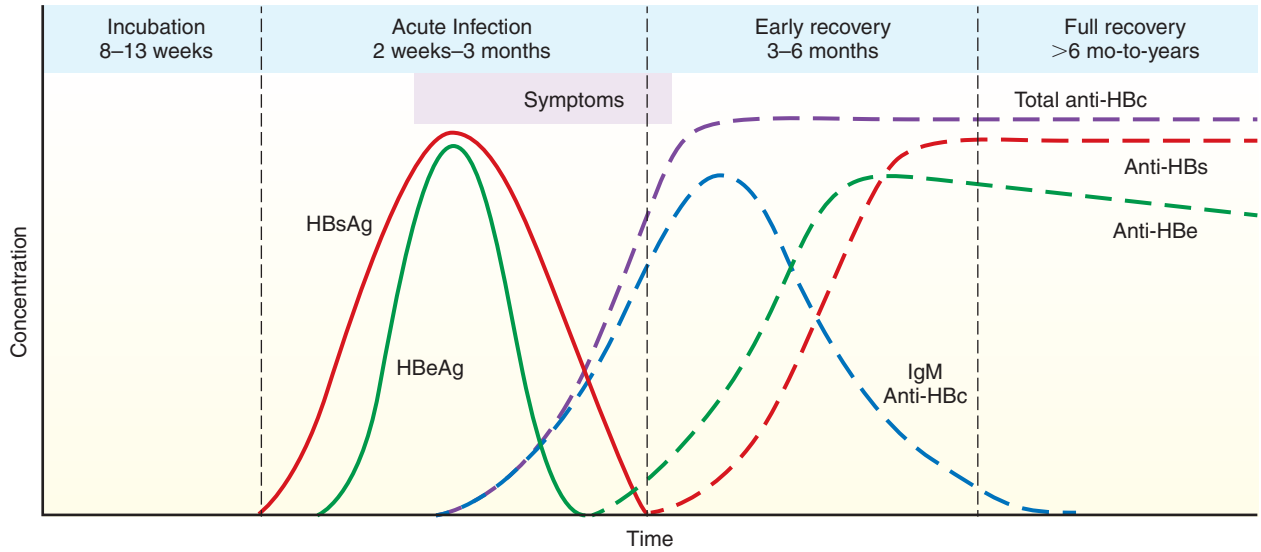

FIGURE 23–4 Typical serological markers in acute hepatitis B. Solid lines represent viral antigen concentrations, whereas dashed lines indicate antibody concentrations. Each antigen shares the same color with its associated antibody.

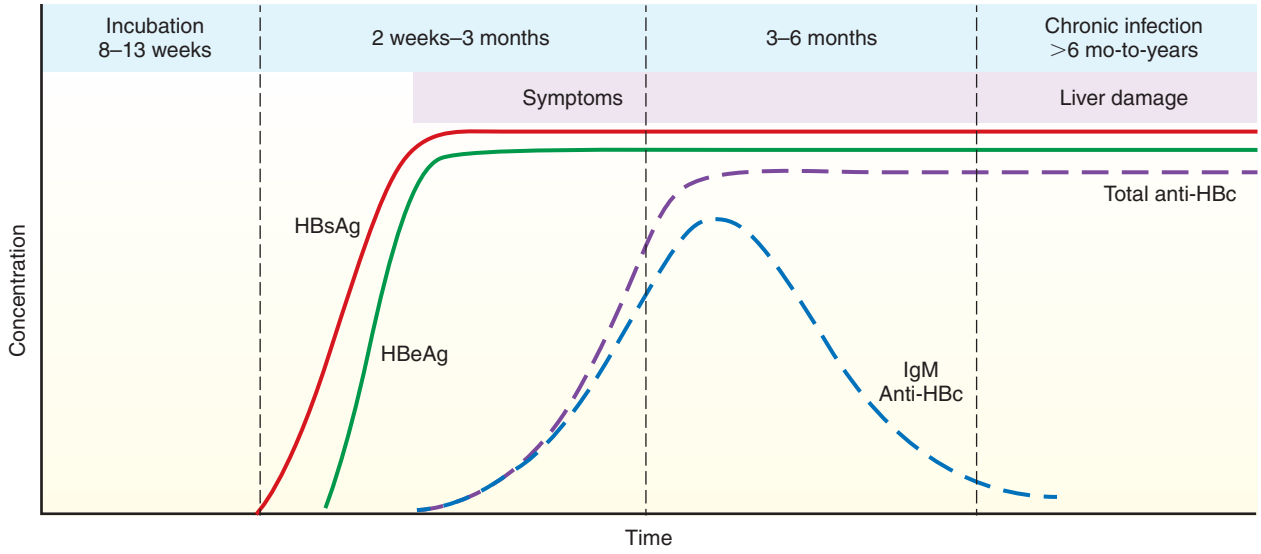

FIGURE 23–5 Typical serological markers in chronic hepatitis B.

Serological markers for hepatitis B are most commonly detected by commercial immunoassays. These are available in a variety of formats, such as EIA and chemiluminescent immunoassay (CLIA). They are typically automated to ease batch testing in the clinical laboratory and have excellent sensitivity and specificity.[5,23] An example of an immunoassay for detecting HBsAg is shown in **Figure 23–6.** Although these methods are highly sensitive and specific, false-positive and false-negative results can occur. Any initial positive results should be verified by repeated testing of the same specimen in duplicate, followed by confirmation with an additional assay, such as an HBsAg neutralization test or a molecular test that detects HBV DNA.

Several molecular methods have been developed to detect HBV DNA in serum or plasma and are mostly based on target amplification by traditional or real-time PCR or branched DNA (bDNA) signal amplification.[23,29,30] HBV DNA can be detected in the serum about 21 days before HBsAg and may be a useful adjunct in detecting early acute HBV infection in certain situations such as the screening of blood donors, assessing cases of occupational exposure, and evaluating patients with equivocal HBsAg test results.[23,30] HBV DNA testing is also used to evaluate the effectiveness of antiviral therapy in patients with chronic hepatitis B. Successful treatment is indicated by a $1\log_{10}$ reduction in HBV DNA levels by 6 months, whereas persistently elevated HBV DNA levels indicate possible drug resistance and a need to change therapy.[23] Molecular testing is also used to diagnose atypical cases of hepatitis B originating from mutations in the HBV genome that cause HBsAg tests to be negative.[23,30] Molecular methods to detect HBV genotypes and HBV mutations associated with antiviral drug resistance have also been developed.[23,29] These tests will

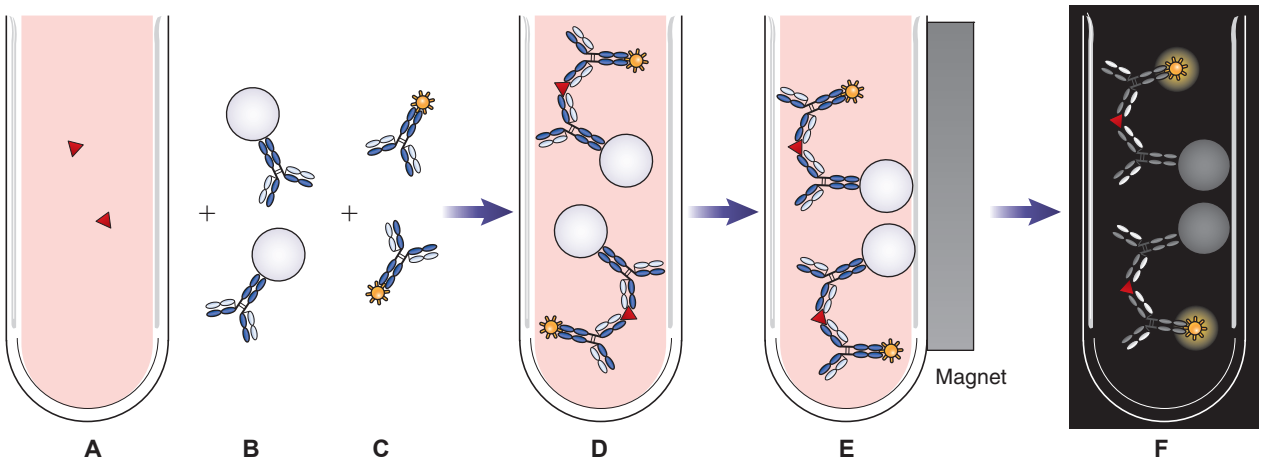

FIGURE 23–6 Detection of the HBs antigen by chemiluminescence microparticle immunoassay. Patient serum or plasma containing HBsAg (A) is mixed with magnetic microparticles coated with anti-HBs (B) and acridinium-labeled anti-HBs conjugate (C). During incubation complexes form, with the antigen sandwiched in between the antibodies (D). Application of a magnetic field holds the microparticles and bound reagents in the tube while unbound materials are washed away and chemiluminescent reagents are added (E). The magnitude of light produced is measured in a luminometer (F) and is proportional to the concentration of HBsAg in the sample.

likely be used more widely in the future to determine optimal patient therapy.

Hepatitis D

Hepatitis D, also known as *delta hepatitis,* is a parenterally transmitted infection that can occur only in the presence of hepatitis B. HDV is a defective virus that requires the help of HBV for its replication and expression. The only member within the *Deltavirus* genus, HDV consists of a circular RNA genome and a single structural protein called *hepatitis delta antigen* within its core, surrounded by a viral envelope that is of HBV origin and contains the HBsAg.[23,31] The eight known HDV genotypes vary in their geographical distribution, pathogenicity, and response to treatment. More than 15 million people around the world are believed to be infected with HDV, which is highly prevalent in Mediterranean Europe, the Middle East, the Amazon basin, central Africa, and parts of Asia.[32,33] The number of new infections appears to be increasing in certain parts of the world.

Similar to HBV, HDV is transmitted sexually in semen or vaginal secretions; through blood by intravenous drug use, needlestick injuries, or transfusions; or perinatally from mother to infant. Infection with the virus can occur in one of two ways: HDV can be transmitted simultaneously as a co-infection with HBV or HDV can be contracted as a superinfection of individuals who are already chronic HBV carriers. Clinically, most patients with co-infections experience an acute, self-limited hepatitis in which both viruses are cleared within a few months.[5,32,34] Some patients may experience more severe symptoms of acute hepatitis than those infected with HBV alone, but only about 2% of cases progress to a chronic state. In contrast, more than 70% of patients with superinfections develop chronic liver disease with an accelerated progression to cirrhosis and liver failure.[5,31,32,34] Combinations of interferon alpha and antiviral drugs can be administered to patients with chronic or severe hepatitis D in an attempt to eradicate the virus.[32,33]

Testing for hepatitis D should be performed in all patients who are HBsAg positive and involves detection of HDV antibodies and HDV RNA.[32] Antibodies are detected by immunoassays employing the hepatitis D antigen.[5,23,34] The presence of IgG anti-HDV antibodies indicates exposure to the virus and can signify an acute, chronic, or past hepatitis D infection. Although IgM anti-HDV is produced during acute hepatitis D infections, its appearance may be delayed, it may persist for only a short period of time, and it may be missed.[5,32] IgM antibodies to HDV can also persist during chronic infection.[32] Serology testing for hepatitis B can be used to help distinguish HBV and HDV co-infections from HBV and HDV superinfections, which, as previously discussed, have different clinical outcomes. In addition to being positive for HDV antibodies, patients with co-infections are positive for IgM anti-HBc, whereas patients with superinfections are positive for IgG anti-HBc.[33]

Detection of hepatitis D has been aided tremendously by the development of molecular methods to detect HDV RNA, a marker of active viral replication that is present in all types of active hepatitis D infections.[5] HDV RNA testing is routinely used to confirm a positive HDV antibody screen.[32] Molecular testing for serum HDV RNA is performed by sensitive, real-time RT-PCR assays.[5,35] These assays also provide quantitative results that can be used to monitor the response of patients to antiviral therapy.

Hepatitis C

Hepatitis C is a major public health problem, with an estimated 80 million people infected worldwide.[36] It is the most common bloodborne infection in the United States, affecting about 1.3% of the population.[36,37] Hepatitis C is also the most frequent cause of chronic liver infection and the leading indicator for liver transplantation in the United States.[38]

HCV, the virus that causes hepatitis C, is responsible for most of the infections previously classified as "nonA–nonB" before the discovery of the virus in 1989.[6] It is an enveloped, single-stranded, positive-sense RNA virus belonging to the family *Flaviviridae* and the genus *Hepacivirus.*[6,39,40] Scientists have discovered seven different genotypes of the virus, designated 1 through 7, and numerous subtypes for each, indicated by lowercase letters.[6,39,40,41] The genotypes differ in their geographic distribution, pathogenicity, and response to antiviral treatment. Genotype 1, the most common, is responsible for 46% of hepatitis C infections worldwide and over 70% of HCV infections in the United State.[36,39] Genotypes 1, 2, and 3 are predominant in North America, Europe, and Japan; genotypes 3 and 6 are found throughout south and southeast Asia; and genotypes 4, 5, and 7 are most common in parts of Africa.[39,41] The variability of HCV, along with its ability to undergo rapid mutations within its hosts, has created difficulty in developing an effective vaccine.

Hepatitis C is transmitted mainly by exposure to contaminated blood, with intravenous drug use being the main source of infection.[6,42] Blood transfusion was also a major vehicle of transmission before routine screening of blood donors for HCV antibody was implemented in 1992, but transmission by this means is rare today. Organ transplantation before 1992 was also a route of transmission. Other risk factors for acquiring hepatitis C include occupational exposures to contaminated blood, long-term hemodialysis, and unregulated body piercing or tattooing in environments such as correctional facilities where contaminated needles are likely to be used.[38,42] Sexual transmission of HCV is thought to be less common but is higher in those who have had multiple sex partners or a history of sexually transmitted diseases.[38,42] Perinatal transmission has been estimated to occur at a rate of about 6%.[43]

HCV has an average incubation period of 7 weeks (range is 2 to 30 weeks). The majority of infections are asymptomatic, with symptoms of acute hepatitis occurring in only about 20% of cases.[6,42,43] Asymptomatic infection is problematic because chronic infection develops in about 70% of infected persons and up to half of these individuals develop cirrhosis.[6,38,43] Cirrhosis occurs slowly over a 25- to 30-year period, causing damage to the liver and posing an increased risk of developing hepatocellular carcinoma. Patients with chronic HCV infection may also develop extrahepatic manifestations, including rheumatological

conditions; glomerulonephritis, vasculitis, or other autoimmune manifestations; neuropathy; ophthalmological symptoms; and dermatological symptoms.[38,43] Early detection would help prevent these complications, but HCV is often missed in its early stages because of the asymptomatic nature of the infection in most individuals.

Clearance of the infection may occur spontaneously or may require treatment with antiviral drugs. Until recently, the standard treatment involved a combination of pegylated interferon-α (PEG IFN-α) and ribavirin. Although this treatment has been successful in 80% of persons infected with genotypes 2 or 3, it has been effective in only half of those with genotype 1 and is associated with numerous side effects.[41,44] Increased understanding of the biology of HCV has led to the development of direct-acting antiviral drugs (DAAs) and host-targeted agents (HTAs) that inhibit specific steps of the viral replication cycle.[42-44] Combination therapies employing these agents are being evaluated at a rapid pace and are revolutionizing the way hepatitis C is being treated.

The laboratory plays an essential role in screening for hepatitis C, monitoring patients known to have HCV infection, and guiding therapy. Between 1998 and 1999, the CDC issued recommendations that screening for HCV infection be conducted in high-risk individuals, including those who received blood or blood products.[45,46] In 2012, the CDC extended these recommendations to include a one-time screening of all persons in the United States who were born between 1945 and 1965, regardless of risk factors.[47] In 2013, the U.S. Preventative Task Force endorsed this recommendation.[48] The rationale behind the latest recommendation was that about 75% of individuals living with HCV infection in the United States were born during this time period but are asymptomatic. Identification of these persons could lead to closer monitoring for disease progression and earlier administration of effective antiviral treatment.

Screening and diagnosis of hepatitis C begins with serological testing for HCV antibodies. Anti-HCV IgG is most commonly detected by sensitive EIAs or CLIAs that use recombinant and synthetic antigens developed from the conserved domains of the capsid core protein (C) and the nonstructural proteins, NS3, NS4, and NS5.[5,39,40] Alternatively, a rapid immunoblot assay can be used for point-of-care testing.[49] Antibodies become detectable 8 to 10 weeks after HCV exposure and can remain positive for a lifetime.[39] Thus, a reactive result can indicate the presence of a current HCV infection or a past HCV infection that has resolved.[37] In addition, despite the excellent specificity of these methods, false-positive results may occur because of cross-reactivity in persons with other viral infections or autoimmune disorders.[5,37] Therefore, any positive results from an anti-HCV screening test should be confirmed to distinguish between the various interpretations of these results. Current CDC guidelines recommend the use of nucleic acid testing (NAT) for HCV RNA for confirmation. If HCV RNA is detected, a current HCV infection is indicated. In contrast, if the NAT is nonreactive, this suggests a past HCV infection or false-positive antibody test result.[37] To distinguish between a true-positive and false-positive result, HCV antibody testing can be repeated using a different assay from the initial test because a biological false-positive result is unlikely to occur in two different methods.[37,50]

Molecular assays for HCV RNA can be classified as qualitative or quantitative. Qualitative tests distinguish between the presence or absence of HCV RNA in a clinical sample. These tests are used to confirm infection in HCV-antibody-positive patients (as previously mentioned), detect infection in antibody-negative patients who are suspected of having HCV, screen blood and organ donors for HCV, and detect perinatal infections in babies born to HCV-positive mothers.[51] Qualitative RT-PCR and transcription-mediated amplification (TMA) methods are commercially available.[39,51,52] These tests can detect as low as 5 International Units (IU) of HCV RNA per mL of serum (for TMA) or 50 IU/mL HCV RNA (for RT-PCR) and become positive within 1 to 3 weeks after infection.[39,51] They are generally positive at the onset of symptoms, but, in some patients, can transiently decrease to undetectable levels during the acute phase of the infection.[39]

Quantitative tests are performed by RT-PCR, real-time PCR, or bDNA amplification.[39,51,52] Commercial tests can detect a wide range of HCV concentrations, from about 10 IU/mL to 10 million IU/mL.[39] They are used to monitor the amount of HCV RNA, or "viral load," carried by patients before, during, and after antiviral therapy in chronically infected individuals. The ultimate goal of such therapy is to achieve a sustained virological response (SVR) in which the patient continuously tests negative for HCV RNA 12 or 24 weeks after therapy is completed.[42,51] The initial viral load level has also been used as a prognostic tool because those with a low initial viral load are most likely to achieve an SVR.[51]

Genotyping, to determine the exact genotype and subtype of the virus responsible for the infection, should be performed on all HCV-infected patients before antiviral therapy.[39,52] It is important to identify the patient's HCV genotype in order to determine the most effective treatment because HCV genotypes vary in their response to different antiviral drugs. For example, as previously mentioned, PEG IFN-α/ribavirin treatment is more effective in patients with genotypes 2 or 3 than in patients with genotype 1. Genotyping is also useful in epidemiological studies to determine the source of HCV infection in specific populations.[52]

Genotyping can be performed by PCR amplification and sequencing of the target gene, PCR followed by identification of the target gene with genotype-specific probes, or real-time PCR.[39,40] PCR/sequencing is the reference method because it provides precise information regarding the genomic variability of the virus in patients during the course of the disease. However, sequencing is primarily performed in research laboratories because of the specialized equipment and analysis software required, whereas clinical laboratories typically use real-time PCR methods or PCR/probe hybridization.[39,40]

Herpes Virus Infections

The herpes viruses are large, complex DNA viruses that are surrounded by a protein capsid, an amorphous tegument, and an outer envelope.[53] These viruses are all capable of establishing a latent infection with lifelong persistence in the host. The *Herpesviridae* family includes eight viruses that can

cause disease in humans: the herpes simplex viruses (HSV-1 and HSV-2); VZV; EBV; CMV; and the human herpes viruses HHV-6, HHV-7, and HHV-8, the latter of which has been associated with Kaposi sarcoma. This section presents the clinical manifestations and laboratory diagnosis of some of these viruses.

Epstein-Barr Virus

The EBV causes a wide spectrum of diseases, including infectious mononucleosis, lymphoproliferative disorders, and several malignancies.[54,55] EBV infections most commonly result from intimate contact with salivary secretions from an infected individual. Although transmission of the virus can occur by other means, including blood transfusions, bone marrow and solid organ transplants, sexual contact, and perinatal exposure, these routes appear to be much less frequent.[54,56]

In developing nations of the world and lower socioeconomic groups living under poor sanitation, EBV infections usually occur during early childhood, whereas in industrialized nations with higher hygiene standards, infections are typically delayed until adolescence or adulthood. However, by adulthood, more than 95% of individuals have been infected, as evidenced by the presence of EBV antibodies in their serum.[55,57]

Initial infection with EBV is believed to occur in the oropharynx, where the virus primarily infects epithelial cells and B lymphocytes.[54,55,58] EBV binds to β1 integrins on the surface of the epithelial cells, which take up the virus by endocytosis. Inside the oropharyngeal epithelial cells, EBV enters a lytic cycle, characterized by viral replication, lysis of host cells, and release of infectious virions, until the acute infection is resolved. The virions spread to adjacent structures, including the salivary glands and tonsils. There, EBV infects B lymphocytes, which spread the virus throughout the lymphoreticular system. EBV enters the B cells by binding to surface CD21, which is also the receptor for the C3d component of complement. The virus-infected B cells become polyclonally activated, proliferating and secreting a number of antibodies, including EBV-specific antibodies; heterophile antibodies; and autoantibodies such as cold agglutinins, rheumatoid factor, and antinuclear antibodies.[54,55,59] In healthy individuals, this process is kept in check by the immune response of NK cells and specific CTLs. However, EBV can persist in the body indefinitely in a small percentage of memory B cells, where it establishes a latent infection.[58] In the latent state, EBV nucleic acid exists as episomal DNA outside of the chromosomes; in these cases, active viral replication does not occur. Periodic reactivation results in re-entry of the virus into the lytic cycle, with viral shedding into the saliva and genital secretions, even in healthy, asymptomatic individuals.[54,55,59]

Several antigens have been identified in EBV-infected cells that are associated with different phases of the viral infection. Antibodies to these antigens have become an important diagnostic tool.[55,57,59] Antigens produced during the initial stages of viral replication in the lytic cycle are known as the *early antigens* (*EAs*). These antigens can be further classified into two groups based on their location within the cells: EA-D, which has a *diffuse* distribution in the nucleus and cytoplasm, and EA-R, which is *restricted* to the cytoplasm only. The late antigens of EBV are those that appear during the period of the lytic cycle following viral DNA synthesis. They include the viral capsid antigens (VCAs) in the protein capsid and the membrane antigens in the viral envelope. Antigens appearing during the latent phase include the EBV nuclear antigen (EBNA) proteins, EBNA-1, EBNA-2, EBNA-3 (or -3a), EBNA-4 (or -3b), EBNA-5 (or -LP), and EBNA-6 (or -3c), and the latent membrane proteins (LMPs), LMP-1, LMP-2A, and LMP-2B **(Table 23–2).**

The clinical manifestations of EBV vary with the host's age and immune status. Infections in infants and young children are generally asymptomatic or mild, whereas primary infections in healthy adolescents or adults commonly result in infectious mononucleosis **infectious mononucleosis** (IM).[54,55,59,60] More than half of patients with IM present with three classic symptoms: fever, lymphadenopathy, and sore throat. Symptoms usually last for 2 to 4 weeks, but fatigue, myalgias, and need for sleep can persist for months. Treatment is mainly directed at alleviating symptoms.[60] Although the associated symptoms are essential in diagnosing IM, they can also be caused by many other infectious agents, so laboratory testing plays an important role in differentiating IM from other infections.

Characteristic laboratory findings in patients with IM include an absolute lymphocytosis of greater than 50% of the total leukocytes and at least 20% atypical lymphocytes **(Fig. 23–7)**.[55,60] The atypical lymphocytes are predominantly activated cytotoxic T cells that are responding to the viral infection.[56,58] Serological findings include the presence of a heterophile antibody and antibodies to certain EBV antigens.

By definition, **heterophile antibodies** are antibodies that are capable of reacting with similar antigens from two or more unrelated species. The heterophile antibodies associated with IM are IgM antibodies produced as a result of polyclonal B-cell activation and are capable of reacting with horse red blood cells (RBCs), sheep RBCs, and bovine RBCs. These antibodies are produced by 40% of patients with IM during the first week of clinical illness and by 80% to 90% of patients by the third week.[54] They disappear in most patients by 3 months after the onset of symptoms but can be detected

Table 23–2 Epstein-Barr Virus Antigens

EARLY ACUTE PHASE	LATE PHASE	LATENT PHASE
EA-R (early antigen restricted) EA-D (early antigen diffuse)	VCA (viral capsid antigen) MA (membrane antigen)	EBNA (EBV nuclear antigens): EBNA-1, EBNA-2, EBNA-3 (3a), EBNA-4 (3b), EBNA-5 (LP), EBNA-6 (3c)
		Latent membrane proteins (LMP-1, LMP-2A, LMP-2B)

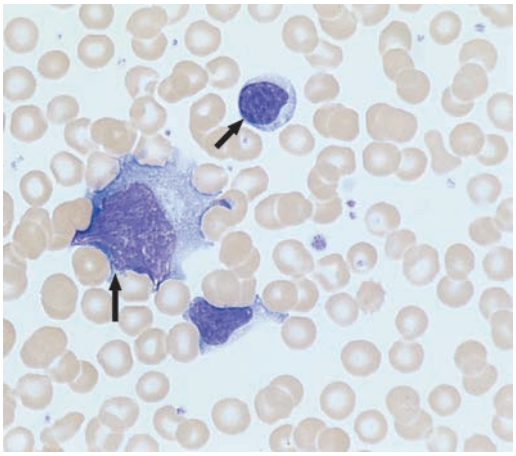

FIGURE 23–7 Atypical lymphocytes from a patient with infectious mononucleosis. Note the variation in size, nuclear:cytoplasmic ratio, and chromatin coarseness. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F. A. Davis; 2009.)*

in some patients for 1 to 2 years.[54,55] Because the heterophile antibody is present in most patients during the acute phase of illness, testing for this antibody has been typically performed to screen for IM in patients who present with symptoms of the disease.

For many years, the heterophile antibody of IM was detected by a rapid slide agglutination method called the "Monospot." In this test, serum premixed with guinea pig kidney antigen was still capable of agglutinating horse RBCs, whereas serum premixed with beef erythrocyte antigen could not agglutinate horse RBCs because the heterophile antibody was absorbed during the first step. The test was used to distinguish the heterophile antibody of IM from heterophile antibodies produced in other diseases, which had different reactivity. The antibody could then be titered by incubating serial dilutions of the patient's serum with sheep RBCs in the Paul–Bunnell test (see the Lab Exercise on Davis*Plus*). Today, these methods have been replaced by more sensitive, rapid agglutination tests or immunochromatographic assays using purified bovine RBC extract as the antigen. Although screening tests for the heterophile antibody are ideal for point-of-care testing, they are not as sensitive or specific as tests for antibodies to EBV, the direct cause of IM.[54] Negative heterophile antibody results occur in about 10% of adult patients with IM and up to 50% of children younger than 4 years old.[55] False-positive results, although uncommon, can occur in patients with lymphoma, viral hepatitis, malaria, and autoimmune disease, or can be caused by errors in result interpretation.[54,55]

Testing for EBV-specific antibodies can be performed to aid in the diagnosis of IM, especially in patients with a negative heterophile antibody screen, or to determine if individuals have had a past exposure to EBV.[54,55,60] These antibodies can be detected by indirect immunofluorescence assays (IFA) using EBV-infected cells, enzyme-linked immunosorbent assay (ELISA) or CLIA using recombinant or synthetic EBV proteins, or flow cytometric microbead immunoassays.[57,61,62] Although all of these methods have a high level of sensitivity (95% to 99%), IFA tests have a higher level of specificity and are considered the "gold standard" of EBV serology methods. However, many laboratories prefer ELISA or CLIA tests because they are less time consuming and easier to interpret.[57,62]

IgM antibody to the VCA is the most useful marker for acute IM because it usually appears at the onset of clinical symptoms and disappears by 3 months.[54,62] IgG anti-VCA is also present at the onset of IM but persists for life and can thus indicate a past infection. Antibodies to EA-D are also seen during acute IM, whereas anti-EBNA appears during convalescence.[54,55,58] Thus, acute primary infection is typically indicated by the presence of IgM anti-VCA and anti-EA-D, as well as the absence of anti-EBNA. A summary of serological responses during acute, convalescent, and post-IM is shown in **Table 23–3.**

Table 23–3 Serological Responses of Patients With Epstein-Barr Virus-Associated Diseases

	ANTI-VCA			ANTI-EA			
Condition	IgM	IgG	IgA	EA-D	EA-R	ANTI-EBNA	HETEROPHILE ANTIBODY (IgM)
Uninfected	–	–	–	–	–	–	–
Acute IM	+	++	±	+	–	–	±
Convalescent IM	–	+	–	–	±	+	±
Past infection IM	–	+	–	–	–	+	–
Chronic active infection IM	–	+++	±	+	++	±	–
Post-transplant lymphoproliferative disease	–	++	±	+	+	±	–
Burkitt's lymphoma	–	+++	–	±	++	+	–
Nasopharyngeal carcinoma	–	+++	+	++	±	+	–

EA-D = early antigen- diffuse; EA-R = early antigen- restricted; EBNA = EBV nuclear antigen; IM = infectious mononucleosis; VCA = viral capsid antigen.
Adapted from Straus SE, et al. Epstein-Barr virus infections: biology, pathogenesis, and management. Ann Intern Med. *1993;118:45, with permission.*

Some individuals develop chronic active EBV infection, with severe, often life-threatening IM-associated symptoms that persist or recur for more than 6 months after the acute illness.[56,58] In addition, EBV can sometimes integrate its DNA into the genome of the cells it infects and transform them into cancer cells. As a result, EBV has been associated with several malignancies, both hematologic (e.g., Burkitt's lymphoma and Hodgkin disease) and nonhematologic (e.g., nasopharyngeal carcinoma and gastric carcinoma).[54-56] EBV can also cause lymphoproliferative disorders in immunocompromised patients, including central nervous system (CNS) lymphomas in patients with AIDS, X-linked lymphoproliferative disease in males with a rare genetic mutation, and post-transplant lymphoproliferative disorders (PTLD) in patients who have received hematopoietic stem cell or solid organ transplants.[55,56,63] These disorders result from the inability of immunosuppressed patients to control primary EBV infection, leading to massive polyclonal expansion of the EBV-infected B cells and life-threatening illness with a high rate of mortality.

EBV-associated malignancies can be diagnosed with the help of serology tests for EBV antibodies and molecular methods to detect EBV DNA in blood and tissue samples.[57,58] Typical patterns of EBV antibodies seen in some of these disorders are shown in Table 23–2. Molecular tests may be more reliable than serology in immunocompromised patients who may not demonstrate a good humoral response. Quantitative real-time PCR is useful in monitoring viral load in transplant patients; a high or steadily increasing EBV viral load indicates the need to decrease immunosuppressive treatment and administer antiviral therapy.[58] Detection of EBERs (EBV-encoded RNA transcripts) by in situ hybridization is the method of choice for detecting EBV in tumor tissue.[57,58]

Cytomegalovirus

CMV is a ubiquitous virus with worldwide distribution. The prevalence of CMV ranges from 40% to 100%, depending on the population, and increases with age; however, crowded living conditions and poor personal hygiene facilitate spread earlier in life.[64,65] Transmission of the virus can occur in a variety of ways. CMV is spread through close, prolonged contact with infectious body secretions; intimate sexual contact; blood transfusions; solid organ transplants; and perinatal exposure from infected mother to infant. The virus has been isolated in many body fluids, including saliva, urine, stool, vaginal and cervical secretions, semen, breast milk, and blood.[64,65]

Primary, or initial, infections in healthy individuals are usually asymptomatic. However, some people experience a self-limiting, heterophile antibody-negative IM-like illness with fever, myalgias, and fatigue.[64,65] A small number of immunocompetent individuals who have other underlying disorders may develop severe CMV disease, which most commonly involves the gastrointestinal tract, CNS, and hematologic abnormalities.[66] An immune response against CMV is stimulated, but the virus persists in a latent state in monocytes, dendritic cells, myeloid progenitor cells, and peripheral blood leukocytes. It may be reactivated at a later time in the individual's life.[67]

The clinical consequences of CMV infection are much more serious in the immunocompromised host, most notably organ-transplant recipients and patients with HIV/AIDS. CMV is the most important infectious agent associated with organ transplantation, with infections resulting from reactivation of CMV in the recipient or transmission of CMV from the donor organ. CMV infection of a previously unexposed recipient is associated with increased risk for allograft failure or graft-versus-host disease (GVHD), and poses a high risk for a variety of syndromes, such as fever and leukopenia, hepatitis, pneumonia, gastrointestinal complications, CNS dysfunction, and retinitis.[64,65,68] Although combination antiretroviral therapy has reduced the incidence of CMV-related illness in patients with HIV infection, CMV remains a major opportunistic pathogen in patients with low CD4 T-cell counts.

Various measures can be undertaken to reduce the risk for CMV transmission and treat CMV infection in the immunocompromised host.[64,68] Serological testing can be performed to identify CMV-positive donors so that transplantation of their organs into CMV-negative recipients can be avoided. If a CMV infection has been established in a transplant patient, immunosuppressive treatment should be reduced to the lowest dose possible. In addition, a variety of antiviral drugs are currently used to treat CMV infection and may, in some instances, be given prophylactically (i.e., before organ transplantation).[64,68] Researchers are also investigating a vaccine design that involves the production of specific CMV antigens using genetic technologies.[69,70]

CMV is also the most common cause of congenital infections, occurring in 0.3% to 2.3% of all neonates.[71,72] Transmission of the virus may occur through the placenta, by passage of the infant through an infected birth canal, or by postnatal contact with breast milk or other maternal secretions. About 10% to 15% of infants with congenital CMV infection are symptomatic at birth.[72,73] Mothers who acquire primary CMV infection during their pregnancy have a significantly higher risk of giving birth to a symptomatic or severely affected infant than do women in whom CMV was reactivated during pregnancy. Symptomatic infants present with a multitude of symptoms that reflect platelet dysfunction and CNS involvement. Ten percent of infants who are asymptomatic at birth progressively develop sensorineural hearing loss.[73,74]

Several laboratory methods have been developed to detect CMV infection; the tests recommended for use depend on the clinical situation.[65] Assays for direct detection of the virus, such as viral culture, identification of CMV antigens, and molecular tests for CMV DNA, are necessary to detect a current CMV infection in individuals who are immunocompromised or in neonates suspected of being congenitally infected with CMV. Serology is most beneficial in determining a past exposure to the virus, for example, in pregnant women or in patients in need of a transplant.

Isolation of the virus in culture is the traditional method of direct viral detection. In this method, human fibroblast cell lines are inoculated with CMV-infected specimens, most commonly urine, respiratory secretions, or anticoagulated whole blood.[65] Presence of the virus is indicated by characteristic cytopathic effects (CPE) that produce enlarged, rounded, refractile cells.

Although conventional culture provides definitive results when positive, it is limited because CPE do not appear until a few days to several weeks after inoculation, depending on the viral titer. Implementation of the rapid centrifugation-enhanced (shell vial) method has reduced the time of detection to within 24 hours after inoculation.[65] In this assay, infected cells are grown on coverslips in shell vials and incubated with fluorescent-labeled monoclonal antibodies to CMV antigens produced early in the replication cycle. Fluorescent staining will appear in the nuclei of positive cells.

A widely used method for direct identification of CMV has been the CMV antigenemia assay, which uses immunocytochemical or immunofluorescent staining to detect the CMV lower matrix protein pp65 in infected leukocytes from peripheral blood or cerebral spinal fluid.[65,75] Following lysis of erythrocytes in the sample, the leukocytes are fixed onto a microscope slide, permeabilized, and stained with labeled monoclonal anti-pp65. Fluorescence appears in the nuclei of the infected cells, which can be counted to give quantitative results. The test can be completed in 2 to 4 hours, allowing for more rapid diagnosis and treatment of CMV infection in organ transplant patients and individuals infected with HIV.

Although the antigenemia assay and shell vial culture methods are sensitive, specific, and rapid, they are labor-intensive and require personnel with expertise in performing and interpreting these tests. For these reasons, they are progressively being replaced with molecular methods that detect CMV DNA or mRNA.[65,76] Real-time PCR is the most widely used molecular method because it is sensitive, simple to perform, and can provide quantitative results. PCR amplification of CMV DNA has been extremely useful for detecting CMV infections in HIV-infected hosts and establishing the diagnosis of CMV infection in transplant recipients.[65,76] PCR also provides a more sensitive alternative to culture in diagnosing congenital CMV infections. Identification of CMV or CMV DNA in amniotic fluid after the 20th week of gestation is considered the gold standard for confirmation of fetal infection. Neonatal infection is established by detecting CMV or CMV DNA in the urine of the infant during the first 10 days of life.[65,76] Quantitative PCR, which detects the CMV copy number in the peripheral blood, is used to monitor the effectiveness of antiviral treatment in immunocompromised hosts and to identify patients at risk for developing disseminated CMV disease.[76] In addition, increasing CMV DNA levels over time can be helpful in distinguishing an active infection from asymptomatic or latent infections.[65,76]

Although serology tests for CMV have been commercially available for many years, their clinical utility is limited. The serology methods performed most commonly are semi- or fully automated EIAs that employ microtiter plates or microparticle systems.[65,67] Assays for CMV IgG are most useful in documenting a past CMV infection and determining if an individual is at risk for future infection. For example, screening of blood and organ donors for CMV IgG is performed to identify those donors who are CMV-positive so that the risk of post-transfusion/post-transplant primary CMV infection in sero-negative recipients can be reduced. In addition, screening of pregnant women for CMV IgG can determine if they have been exposed to the infection in the past, or if they are susceptible to primary infection. In the latter case, the women could be educated on measures to reduce their chances of exposure while pregnant.[77]

Although a single positive CMV IgG result indicates past exposure to the virus, conversion from a negative antibody result to a positive antibody result over time indicates a recent CMV infection. However, serial assays for CMV IgG are not routinely performed. Assays for IgM CMV antibodies have been developed but are limited in value because of the potential for false-negative results in newborns and immunocompromised patients and for false-positive results caused by other infections or the presence of rheumatoid factor.[65,74,77] In addition, IgM antibodies may not necessarily indicate primary CMV infection because they can also be produced as a result of CMV reactivation and may persist for up to 18 months.[65,74,78] Serological methods that distinguish CMV antibody avidity appear to be more useful in distinguishing a past exposure from a current primary infection.[65,74,77] Low-avidity IgG antibodies indicate a recent infection, whereas high-avidity IgG antibodies reflect a past exposure because the avidity of the antibody increases during the course of the immune response. The presence of both IgM and low-avidity IgG antibodies can help identify pregnant women who have contracted a primary CMV infection. Because of the limitations of serology testing, direct methods of detecting CMV infection are essential.

Varicella-Zoster Virus

VZV is the cause of two distinct diseases: varicella, more commonly known as *chickenpox,* and herpes zoster, also known as *shingles.* The virus is transmitted primarily by inhalation of infected respiratory secretions or aerosols from skin lesions associated with the infection.[79-81] Transplacental transmission to the fetus may also occur.

Primary infection with VZV results in varicella, a highly contagious illness characterized by a blisterlike rash with intense itching and fever.[79-82] Historically, the majority of varicella cases have occurred during childhood. In a typical infection, vesicular lesions first appear on the face and trunk, and then spread to other areas of the body **(Fig. 23–8)**. The illness is usually mild and self-limiting in healthy children; however, in some cases it may produce complications, the most common of which are secondary bacterial skin infections caused by scratching of the lesions. CNS involvement may occur in some cases, but does not usually require hospitalization.[81] Primary

Connections

Rheumatoid Factor

Recall that rheumatoid factor (RF) is an antibody (usually of the IgM class) that is directed against the Fc portion of IgG. RF can cause a false-positive result in some IgM assays because it binds to IgG antibodies in the patient serum that are directed against the viral antigen bound to the solid phase (see Chapter 15).

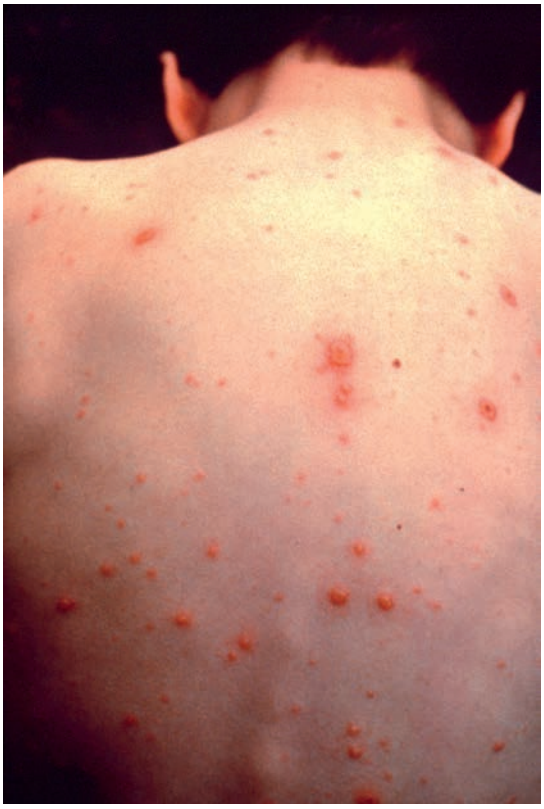

FIGURE 23–8 Vesicular lesions characteristic of chickenpox. These blisterlike lesions have a pus-filled center. *(Courtesy of the Centers for Disease Control and Prevention, Public Health Image Library.)*

infections in adults, neonates, or pregnant women tend to be more severe, with a larger number of lesions and a greater chance of developing other complications such as pneumonia. Varicella infection in pregnant women may also cause premature labor or congenital malformations if the infection is acquired during the first trimester of pregnancy or may cause severe neonatal infection if transmission of the virus occurs around the time of delivery.[79-82] Infections in immunocompromised patients are likely to result in disseminated disease, with extensive skin rash, neurological conditions (e.g., encephalitis), and other complications, including pneumonia, hepatitis, and nephritis.[79-82]

During the course of primary infection, VZV is thought to travel from the skin lesions and the blood to sensory neurons, where it deposits its DNA and establishes a lifelong latent state in the dorsal root, autonomic, and cranial ganglia.[82-84] The host's T-cell–mediated immune response is believed to keep the virus under control during this time.[84]

Reactivation of VZV, with active viral replication, occurs in 15% to 30% of persons with a history of varicella infection.[85] The number of cases increases with age or development of an immunocompromised condition, probably as a result of decreased cell-mediated immunity. During reactivation, the virus moves down the sensory nerve to the dermatome supplied by that nerve, resulting in eruption of a painful vesicular rash known as herpes zoster *herpes zoster*, or shingles, *shingles* in the affected area.[79,82,85] The rash may persist for weeks to months and is more severe in immunocompromised and elderly individuals. A significant number of patients with herpes zoster develop complications, the most common being postherpetic neuralgia, characterized by debilitating pain that persists for weeks, months, or even years after resolution of the infection.[79,84,85] Life-threatening complications such as herpes ophthalmicus that lead to blindness, pneumonia, and visceral involvement are more common in immunosuppressed persons.

Implementation of a vaccine consisting of a strain of live, attenuated varicella virus in 1995 has resulted in a significant decline in the incidence of chickenpox and its associated complications in the United States.[79,86] In 2005, a vaccine was licensed for use in healthy children that combines the varicella vaccine with that for measles, mumps, and rubella. In addition, a single-agent VZV vaccine was licensed in 2006 for prevention of herpes zoster in persons aged 60 or older, presumably by boosting T-cell immunity to the virus.[85,87] Because these vaccines all contain a live agent, they are not recommended for use in immunocompromised persons. A VZV subunit vaccine is being studied and may offer a viable alternative in the future.

Diagnosis of varicella and herpes zoster is usually based on identifying the characteristic vesicular lesions associated with the infection.[83,84] Laboratory testing is most important in the diagnosis of atypical cases, such as those in which the rash is absent or delayed, and in immunocompromised patients with disseminated disease.[83,88] Definitive diagnosis is based on identifying VSV or one of its products in skin lesions, vesicular fluids, or tissue. Older methods of identification involved cell culture and microscopy, but these have significant disadvantages. Culture of the virus and observation of characteristic CPE can be performed in a number of cell lines but is time consuming (4 days to 2 weeks) and may not yield productive results if clinical specimens do not contain sufficient amounts of the infectious virus.[84,89] Microscopic detection of multinucleated giant cells called *Tzanck cells* in stained smears made from material from the vesicles allowed for rapid identification of the virus, but this procedure could not distinguish between VZV and HSV.[84,89] Direct immunofluorescence staining of scrapings from vesicular lesions with monoclonal antibodies directed against VZV antigens provides a rapid, but more sensitive and specific means of detecting the virus.[84,89] Today, real-time PCR for VZV DNA is the laboratory method of choice for diagnosing varicella zoster infection because it is highly accurate, sensitive, and rapid.[83,84] Quantitative real-time PCR is also useful in monitoring the response of immunocompromised patients to antiviral drugs. PCR can be performed on a variety of samples, including vesicular fluid or scabs, skin swabs, throat swabs, cerebrospinal fluid, blood, saliva, and tissues from biopsies or autopsies.[83,84]

Serology testing is of limited use in detecting current infections because accurate detection requires demonstration of a four-fold rise in antibody titer between acute and convalescent samples, a process that takes 2 to 4 weeks to perform.[81,83,89] In addition, testing for VZV IgM is not performed routinely for several reasons: IgM antibodies to VZV may not be detectable until the convalescent stage of illness, they cannot distinguish between primary and reactivated infection, and they may not be free of IgG antibodies when serum is processed for testing.[89,90]

Serology is most useful in determining if immunity to VZV is present in certain individuals, such as health-care workers, pregnant women, and patients about to undergo organ transplantation.[84] Therefore, most serology tests detect total VZV antibody, which consists primarily of IgG. Several methods have been developed for this purpose.[83,84] The most sensitive and reliable method of detecting VZV antibody is a fluorescent test called fluorescent antibody to membrane antigen (FAMA) that detects antibody to the envelope glycoproteins of the virus.[83,89] Although FAMA is considered to be the reference method for VZV antibody, it requires live, virus-infected cells and is not suitable for large-scale routine testing. The most commonly used method to detect VZV antibodies in the clinical laboratory is the ELISA because it is automated, provides objective results, and does not require viral culture.[83,84] Although older ELISA methods that employ a whole antigen extract are less sensitive than FAMA, a newer ELISA that detects antibody to a highly purified VZV envelope glycoprotein has been shown to have a high level of sensitivity.[84,91] Despite this improvement, false-positive results can occur because the method can detect low levels of antibodies that do not confer long-term protection to varicella.[83,84]

Other Viral Infections

Rubella

The **rubella virus** is a single-stranded, enveloped RNA virus of the genus *Rubivirus,* belonging to the family *Togaviridae.*[92-94] It is transmitted through respiratory droplets or through transplacental infection of the fetus during pregnancy.

This virus is the cause of the typically benign, self-limited disease that is also known as *German measles.* Before widespread use of the rubella vaccine, this was mainly a disease of young children. However, today it occurs most often in young, unvaccinated adults.[95] Following an incubation period of 12 to 23 days, the virus replicates in the upper respiratory tract and cervical lymph nodes, then travels to the bloodstream. It produces a characteristic erythematous, maculopapular rash, which appears first on the face, then spreads to the trunk and extremities, and usually resolves in 3 to 5 days.[93,94] In adolescents and adults, this is usually preceded by a prodrome of low-grade fever, malaise, swollen glands, and upper respiratory infection lasting 1 to 5 days. However, up to 50% of rubella infections are asymptomatic.[93,94] The infection usually resolves without complications and no specific treatment is available. A significant number of infected adult women experience arthralgias and arthritis, but chronic arthritis is rare.[93,94]

Rubella infection during pregnancy may have severe consequences, including miscarriage, stillbirth, or congenital rubella syndrome (CRS).[93,94,96] The likelihood of severe consequences increases when infection occurs earlier in the pregnancy, especially during the first trimester. Infants born with CRS may present with a number of abnormalities, the most common of which are deafness; eye defects, including cataracts and glaucoma; cardiac abnormalities; mental retardation; and motor disabilities. In mild cases, symptoms may not be recognized until months to years after birth.

Scientists developed a vaccine consisting of live, attenuated rubella virus with the primary goal of preventing infection of pregnant women by reducing dissemination of the virus in the population as a whole.[93,95] The vaccine is part of the routine immunization schedule in infants and children and is usually given in combination with vaccines for measles and mumps (measles/mumps/rubella [MMR] vaccine) and sometimes with varicella (MMRV). Following licensure of the vaccine in 1969, the number of rubella infections and cases of CRS in the United States has dropped dramatically with only limited outbreaks occurring, mostly among unvaccinated young immigrants to this country. However, rubella and CRS are still important health problems in parts of the world where routine immunization against the virus is not established.[94,95]

Laboratory testing is helpful in confirming suspected cases of German measles because its symptoms may mimic those of other viral infections. It is essential in the diagnosis of CRS and in the determination of immune status in other individuals. Laboratory diagnosis of rubella infection can be accomplished through culture of the virus, demonstration of viral RNA, or detection of virus-specific antibodies. Rubella virus can be grown in a variety of cultures inoculated with throat swabs, nasopharyngeal secretions, or other clinical specimens and can be detected from almost all infected infants at the time of birth.[92] However, viral growth is slow and may not produce characteristic CPE upon primary isolation, requiring at least two successive subpassages.[92] In the absence of CPE, viral nucleic acid can be identified by RT-PCR or viral proteins can be detected by IFA or EIA.[92] Because culture is time consuming and labor intensive, it is increasingly being replaced by molecular methods that are more practical to perform in the clinical laboratory and provide more timely results.[97] The most widely used molecular method is RT-PCR. RT-PCR is a highly sensitive and specific aid in prenatal or postnatal diagnosis and can be used to detect rubella RNA in a variety of clinical samples, including chorionic villi, placenta, amniotic fluid, fetal blood, lens tissue, products of conception, pharyngeal swabs, spinal fluid, or brain tissue.[78,98]

Serology tests are the most common means of confirming a rubella diagnosis because they are rapid, cost effective, and practical in clinical laboratory settings.[93] Several methods have been developed to detect rubella antibodies, including hemagglutination inhibition (HI), latex agglutination, and immunoassays.[92] Although HI was once the standard technique for measuring rubella antibodies, the most commonly used method today is the ELISA because of its sensitivity, specificity, ease of performance, and adaptability to automation.[92,93] More specific solid-phase capture ELISAs can be used to detect IgM rubella antibodies. Automated chemiluminescence assays and a multiplex bead immunoassay that can simultaneously detect measles, mumps, rubella, and varicella are also available and demonstrate comparable performance with ELISAs.[99,100]

Primary rubella infection is indicated either by the presence of rubella-specific IgM antibodies or by a four-fold or greater rise in rubella-specific IgG antibody titers between acute- and convalescent samples collected at least 10 to 14 days apart.[93,94,97] The timing of serum collection is important because IgM antibodies to rubella do not appear in many patients until about 5 days after the onset of the rash, whereas IgG antibodies may not be detectable until 8 days after the rash.[92,97] Only about 50% of patients are positive for IgM antibodies on the day that the rash appears; thus, a false-negative result can occur if the sample is obtained too early. False-positive results can also occur. Although IgM antibodies generally decline by 4 to 6 weeks, they may persist in low levels for a year or more in some cases.[78,92] False-positive rubella IgM results have also been observed in individuals with parvovirus infections, heterophile antibodies, or rheumatoid factor.[78,93] It is therefore recommended that positive IgM results, particularly in pregnant women, be confirmed by a more specific test, such as an EIA that measures the avidity of rubella IgG antibodies, to distinguish between recent and past rubella infections.[78,101,102] In these assays, low antibody avidity indicates a recent infection (with a high risk for CRS), whereas high antibody avidity is seen in past infections, reflecting the normal change in avidity during the course of an immune response.

Laboratory diagnosis of congenital rubella infection begins with serological evaluation of the mother's antibodies and measurement of rubella-specific IgM antibodies in fetal blood, cord blood, or neonatal serum, depending on the age of the fetus or infant. To enhance the reliability of a CRS diagnosis, any positive IgM results should be confirmed by viral culture, RT-PCR–amplification of rubella nucleic acid, or demonstration of persistently high titers of rubella IgG antibodies after 3 to 6 months of age.[78]

Serology tests are also used to screen for immunity to rubella in populations such as pregnant women or health-care workers. IgG antibodies provide immunity and persist for life. Rubella-specific IgG antibodies are produced as a result of natural infection or immunization. An antibody level of 10 to 15 IU/mL is considered to be protective.[78,92]

Rubeola

The **rubeola virus** is a single-stranded RNA virus belonging to the genus *Morbillivirus* in the *Paramyxoviridae* family.[103] It is a highly contagious infection that is spread by direct contact with aerosolized droplets from the respiratory secretions of infected individuals. After initial infection of the epithelial cells in the upper respiratory tract, rubeola virus is disseminated through the blood to multiple sites in the body, such as the skin, lymph nodes, and liver.[104]

Rubeola virus infection is the cause of the disease commonly known as *measles*. Following an incubation period of about 10 to 12 days, the virus produces prodromal symptoms of fever, cough, coryza (runny nose), and conjunctivitis, which last 2 to 4 days.[93,103,105] During the prodromal period, characteristic areas known as *Koplik spots* appear on the mucous membranes of the inner cheeks or lips; these appear as gray-to-white lesions against a bright red background and persist for several days. The typical rash of measles appears about 14 days after exposure to the virus and is characterized by an erythematous, maculopapular eruption that begins on the hairline, then spreads to the face and neck, and gradually moves down the body to the trunk, arms, hands, legs, and feet **(Fig. 23–9).** The rash usually lasts 5 to 6 days.

Measles is a systemic infection that can result in complications, including diarrhea, otitis media, croup, bronchitis, pneumonia, and encephalitis.[93,103,104] Rarely, a fatal degenerative disease of the CNS, called *subacute sclerosing panencephalitis* (*SSPE*), can result from persistent replication of measles virus in the brain.[93,106] Measles infection during pregnancy can result in a higher risk of premature labor, spontaneous abortion, or low birth weight.[93]

The incidence of measles has been greatly reduced in developed nations of the world since the introduction of a live, attenuated measles virus vaccine in 1968. A vaccine consisting of killed rubeola virus was originally licensed in 1963 but was ultimately ineffective because recipients developed a case of atypical measles if they were subsequently infected with the measles virus.[93] The newer vaccine is used in the routine immunization schedule of infants and children, either in combination with rubella and mumps (MMR) or in combination with rubella, mumps, and varicella (MMRV).[93,95] Recommended administration of the vaccine is in two doses, the first between the ages of 12 and 15 months and the second between ages 4 and 6. Administration of the first dose before the age of 12 months may result in vaccine failure because the presence of maternal antibodies can interfere with the infant's immune response. The vaccine was considered to be so successful that the CDC and WHO declared measles to be eliminated from the United States in the year 2000 and from the Americas in 2002.[95,104] However, measles continues to be a global concern

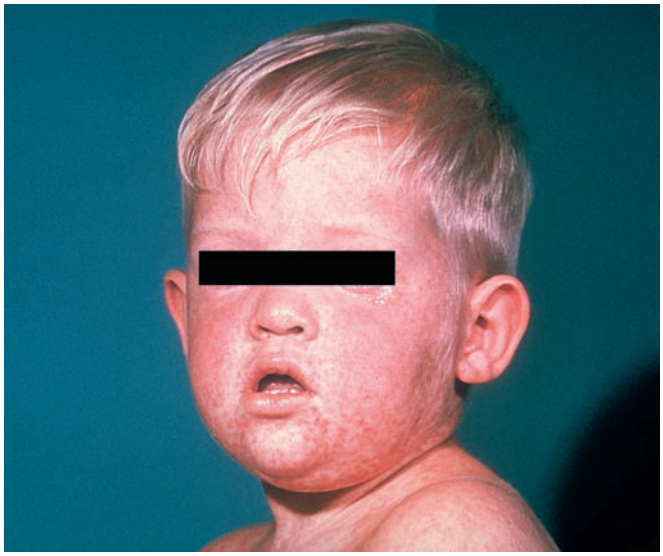

FIGURE 23–9 Characteristic rash of measles appearing on the face of a boy. *(Courtesy of the Centers for Disease Control and Prevention, Public Health Image Library.)*

and most cases in the United States and other industrialized nations are brought in by unvaccinated individuals from other countries.[93,95] Measles outbreaks have occurred in recent years in the United States because some people in the population refuse to become vaccinated or have their children vaccinated on the basis of religious reasons or unfounded fears of vaccine associations with disorders such as autism.

The diagnosis of measles has typically been based on clinical presentation of the patient. However, the success of the U.S. immunization program in reducing the number of measles cases has decreased the ability of some physicians to recognize the clinical features of measles.[93,104,105] In addition, atypical presentations of measles can occur in individuals who received the earlier form of the measles vaccine, who have low antibody titers, or who are immunocompromised.[93,103,105] Laboratory tests are therefore of value in ensuring rapid, accurate diagnosis of sporadic cases; in addition, they are important for epidemiological surveillance and control of community outbreaks.[93,105,107]

Isolation of rubeola virus in conventional cell cultures is technically difficult and slow and is not generally performed in the routine diagnosis of measles, but it may be useful in epidemiological surveillance of measles virus strains.[93,107] The optimal time to recover measles virus from nasopharyngeal aspirates, throat swabs, or blood is from the prodrome period of 3 to 4 days after rash onset. The virus may be isolated from urine up to 1 week after appearance of the rash.[93,106]

Serological testing provides the most practical and reliable means of confirming a measles diagnosis.[93,105,107] In conjunction with clinical symptoms, a diagnosis of measles is indicated by the presence of rubeola-specific IgM antibodies or by a fourfold rise in the rubeola-specific IgG antibody titer between serum samples collected soon after the onset of rash and 10 to 30 days later.[93] SSPE is associated with extremely high titers of rubeola antibodies.[103,107] IgM antibodies are preferentially detected by an IgM capture ELISA method, which is highly sensitive and has a low incidence of false-positive results.[92,93,105] IgM antibodies are detectable by 3 to 4 days after appearance of symptoms and persist for 1 to 2 months.[104,107] Samples collected before 72 hours may yield false-negative results and repeat testing is recommended in that situation.[93]

A variety of methods have been developed to detect IgG rubeola antibodies, but the most commonly used is ELISA.[92,93,107] IgG antibodies become detectable 7 to 10 days after the onset of symptoms and persist for life.[107] Presence of rubeola-specific IgG antibodies indicates immunity to measles because of past infection or immunization.[105,107] Testing for IgG antibodies is therefore routinely performed on serum samples of individuals such as health-care workers to determine their immune status.

Molecular methods to detect rubeola RNA can be used in cases in which serological tests are inconclusive or inconsistent and can be used to genotype the virus in epidemiological studies.[107-109] The preferred molecular technique is RT-PCR, performed by traditional or real-time PCR methodologies. These assays are sensitive, can be performed on a variety of clinical samples or on infected cell cultures, and can detect viral RNA within 3 days of rash appearance.[107]

Mumps

The **mumps virus,** similar to rubeola, is a single-stranded RNA virus that belongs to the *Paramyxoviridae* family (genus *Rubulavirus*). It is transmitted from person to person by infected respiratory droplets, saliva, and fomites and replicates initially in the nasopharynx and regional lymph nodes.[93,110,111] (Fomites are inanimate objects or substances that can transmit infectious organisms.) Following an average incubation period of 14 to 18 days, the virus spreads from the blood to various tissues, including the meninges of the brain, salivary glands, pancreas, testes, and ovaries, and produces inflammation at those sites.[93] Inflammation of the parotid glands, or *parotitis,* is the most common clinical manifestation of mumps, occurring in 30% to 40% of cases **(Fig. 23–10).**[93] The illness typically resolves in 7 to 10 days and does not require therapy other than supportive treatment to alleviate the symptoms.[93,110] Mumps infection in pregnant women results in increased risk for fetal death when it occurs in the first trimester of pregnancy, but it is not associated with congenital abnormalities.[95]

The number of mumps cases in the United States has declined significantly since the introduction of a live, attenuated mumps virus vaccine in 1967 and its routine use in childhood immunization schedules in 1977.[93,95] The vaccine is most commonly combined with the vaccines for rubella and mumps (MMR) or is used in combination with the rubella, mumps, and varicella vaccines (MMRV).

The diagnosis of mumps is usually made on the basis of clinical symptoms, especially parotitis, and does not require laboratory confirmation.[93,112] However, laboratory testing is very useful in cases in which parotitis is absent or when differentiation from other causes of parotitis is required. Culture of the mumps virus from clinical specimens is considered to be the gold standard for laboratory confirmation of acute infection.[112,113] Within the first few days of illness, the mumps virus can be isolated from saliva, urine, cerebrospinal fluid, or swabs

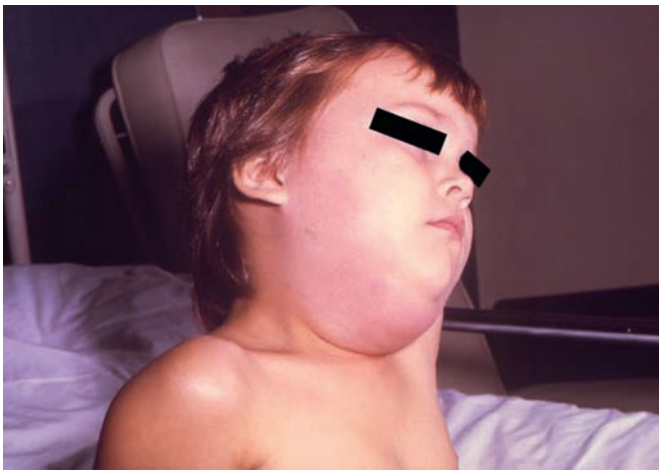

FIGURE 23–10 Parotitis characteristic of mumps. Note the swollen neck region caused by an enlargement of the boy's salivary glands. *(Courtesy of the Centers for Disease Control and Prevention, Public Health Image Library.)*

from the area around the excretory duct of the parotid gland. The preferred specimens are a buccal swab or saliva from the buccal cavity collected within 3 to 5 days of symptom onset.[114] The specimen can then be used to inoculate cell lines such as primary monkey kidney cells and Vero cells, which are grown in shell vial cultures and stained with fluorescein-labeled monoclonal antibodies to identify mumps antigens.[112,113] However, culture methods require experienced personnel and specialized reagents and are being increasingly replaced with molecular detection of viral nucleic acid.[97,110]

Standard and real-time RT-PCR methods have been developed to detect mumps virus RNA in specimens collected from the buccal cavity, throat, cerebral spinal fluid, or urine of patients with a suspected mumps infection.[112,113] In many laboratories, RT-PCR is recommended as the primary diagnostic test for mumps because it is more sensitive than serology.[114] As with culture, buccal swabs collected early in the illness provide the best results and false-negative results are frequent in clinical samples collected after 1 week of symptom onset.[110] Genotyping may be performed to track transmission of the virus during mumps outbreaks.[113,114]

When indicated, serological testing provides a simple means of confirming a mumps diagnosis, but it has some important limitations. ELISA is the most commonly used method to detect mumps antibodies because it is sensitive, specific, cost effective, and readily performed by the routine clinical laboratory.[93] Use of solid-phase IgM capture assays reduces the incidence of false-positive results because of rheumatoid factor. Current or recent infection is indicated by the presence of mumps-specific IgM antibody in a single serum sample or by at least a four-fold rise in specific IgG antibody between two specimens collected during the acute and convalescent phases of illness.[93,112] However, acute IgG titers are often high and a four-fold increase in titer may not be evident in the convalescent sample.[110,113,114] IgM antibodies can be detected within 3 to 4 days of illness and can persist for at least 8 to 12 weeks.[112] However, a negative IgM test does not rule out mumps because negative results can occur if the serum was collected too early or too late. In addition, individuals who received any doses of the mumps vaccine tend to have lower or absent IgM antibody.[110,112] IgG antibodies become detectable within 7 to 10 days and persist for years.[112] However, the presence of mumps IgG antibodies does not necessarily correlate with the presence of neutralizing antibodies, which would confer immunity to the virus.[113,114]

Human T-Cell Lymphotropic Viruses

Human T-cell lymphotropic virus type I (HTLV-I) and **human T-cell lymphotropic virus type II (HTLV-II)** are closely related retroviruses. Both viruses have three structural genes: *gag,* which codes for viral core proteins; *pol,* which codes for viral enzymes; and *env,* which encodes proteins in the viral envelope; as well as a region called pX, which encodes several regulatory proteins including Tax.[115,116] These viruses have RNA as their nucleic acid and the enzyme, reverse transcriptase, whose function is to transcribe the viral RNA into DNA. The DNA then becomes integrated into the host cell's genome as a provirus. The provirus can remain in a latent state within infected cells for a prolonged period of time. Upon activation of the host cell, the provirus can proceed to complete its replication cycle to produce more virions. However, HTLV-I and HTLV-II exist predominantly in the proviral state and are spread directly to uninfected cells through a viral synapse. Additional copies of the viral nucleic acid are produced when the infected host cells replicate.[116]

The human T-cell lymphotropic viruses preferentially infect CD4+ T lymphocytes, but can also infect CD8+ T cells, dendritic cells, and macrophages.[116,117] CD8+ CTLs effectively control proliferation of virus-infected cells in most individuals. However, inflammatory cytokines released during this immune response may contribute to the pathogenesis of HTLV-associated diseases. In addition, researchers have reported that HTLV-I infection of CD4+ T cells can increase production of proinflammatory cytokines, impair production of Th1 cytokines necessary for cell-mediated immunity, and induce differentiation of T regulatory (Treg) cells. The differentiated Treg cells can facilitate viral persistence by suppressing the host's immune response to the virus.[116] HTLV-I also transforms CD4+ T lymphocytes into malignant cells in a small percentage of individuals through mechanisms mediated by the Tax protein, which result in increased cell proliferation and accumulation of harmful genetic mutations.[116,117]

HTLV-I and HTLV-II can be transmitted by three major routes: bloodborne (mainly through transfusions containing cellular components or through intravenous drug abuse), sexual contact (most commonly from men to women), and mother-to-child (mainly through breastfeeding).[115,118] HTLV-I infection is endemic in southwestern Japan, the Caribbean islands, South and Central Africa, the Middle East, parts of South America, and Papua New Guinea. Between 5 million and 20 million people are thought to be infected with HTLV-I worldwide.[119] Infections in the United States and Europe have resulted mainly from immigrants from endemic areas. HTLV-II infections are highest in various Native Indian populations in the Americas, a few Pygmy tribes in Central Africa, and intravenous drug abusers in North America and Europe.[115,120]

HTLV-I is the cause of two diseases: adult T-cell leukemia/lymphoma (ATL) and HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP). ATL can be classified into four different subtypes based on clinical manifestations: acute, lymphomatous, chronic, and smoldering.[118,121] Over half of patients have the acute type, an aggressive variant with a median survival of 6 months.[118] All four types of ATL are characterized by a monoclonal proliferation of mature T cells that express the surface markers, CD3, CD4, and CD25. The malignant cells have lobulated, "flower-shaped" nuclei that contain proviral HTLV-I nucleic acid.[115,118] The lifetime risk of HTLV-I carriers for developing ATL is 3% to 5% and is highest in those who acquired the infection perinatally.[118,119] The disease typically appears after a latent period of 20 to 30 years following initial infection.

Individuals infected with HTLV-I also have a 4% lifetime risk of developing a progressive neurological disorder called HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[118] The risk is highest among those who contracted the infection through sexual transmission. The disease is characterized by slowly progressive weakness and stiffness of the legs, back pain, and urinary incontinence. HTLV-I has also been associated with a variety of autoimmune and inflammatory disorders, including uveitis (intraocular inflammation of the eyes), infective dermatitis, myositis (inflammation of the muscles), and arthropathy (inflammation of the joints); however, a causal relationship of the virus with these conditions has not been established.[115,118] It is unclear what factors influence the development and types of clinical manifestations of HTLV-I infection, but differences in viral strains, viral load, mode of transmission, HLA haplotypes, and immune responses mounted by the host may all play a role.[118]

The association of HTLV-II infection with disease is unclear and most individuals infected with the virus are asymptomatic. However, there is evidence that HTLV-II may rarely be associated with a neurological disease that is similar to HAM/TSP, as well as certain hematologic and dermatological diseases, and increased incidence of infections.[115,118,122]

Serological testing plays an important role in detecting HTLV-I and HTLV-II infections because culture of the viruses requires sophisticated techniques that cannot be performed in routine clinical laboratories. HTLV antibodies develop 30 to 90 days after exposure to the virus and persist for life.[123] Tests for HTLV-I and HTLV-II antibodies are used to detect HTLV infections in individuals and to screen blood donors. The tests most commonly used for screening are ELISA or CLIA methods that incorporate recombinant antigens or synthetic peptides from both HTLV-I and HTLV-II.[115,117] Particle agglutination tests have also been developed. Any sample producing a reactive result in the initial screen is retested by the same method and subsequently tested by a confirmatory method to reduce the incidence of false-positive results and to distinguish between HTLV-I and HTLV-II infection.

Commercially available Western blot assays are most commonly used for confirmation; line immunoassays (LIA) and IFA tests have also been developed.[115] Western blot and LIA identify antibodies to specific HTLV antigens. Specimens are considered positive if a particular band pattern representing antibodies to the gag and env proteins of HTLV-I or HTLV-II is obtained. According to criteria published by the WHO, for example, a sample is considered positive for HTLV-I antibodies if visible bands are produced for one of the env proteins (either gp46 or gp62/68) *and* one of the gag proteins (either p19, p24, or p 53).[124] A major problem with the Western blot and LIA methods is that indeterminate results can be obtained when a single band is observed or when multiple bands that do not meet the criteria for positivity are seen.[115,124] PCR can be performed to detect HTLV-I or HTLV-II DNA in provirus-carrying peripheral blood mononuclear cells to clarify repeatedly indeterminate results.[115,125] PCR methods can also be used to monitor the proviral load in patients with HTLV-associated diseases during therapy and to demonstrate the presence of the virus in cells from patients who are suspected of having HTLV-associated disease.[115,125]

SUMMARY

- Viruses are obligate intracellular pathogens that can produce a wide range of diseases in humans.
- Viruses can exist as either free infectious virions or intracellular particles in infected host cells. These different states require a combined effort of innate, humoral, and cell-mediated immune responses to successfully defend the host against viral infections.
- Innate defenses against viruses include the skin and mucous membranes, interferons to inhibit viral replication, and NK cells, which release cytotoxic proteins that destroy virus-infected host cells.
- Antibodies directed against specific viral antigens can prevent the spread of viral infection by neutralizing a virus and preventing it from binding to host cells, opsonizing a virus to make it more likely to be phagocytized, activating complement-mediated mechanisms of destruction, and agglutinating viruses.
- Cell-mediated immunity is needed to eliminate intracellular viruses. Virus-specific CTL bind to viral antigen complexed with class I MHC on the surface of infected host cells and release cytotoxic proteins that cause the cells to undergo apoptosis.
- Viruses have evolved in several ways to escape the host's defenses. These include frequent genetic mutations to produce new viral antigens; evading the action of interferons, complement, or other components of the immune system; or suppressing the immune system. Some viruses can establish a latent state by integrating their nucleic acid into the host's genome.
- Serological tests for viral antibodies can be used to indicate exposure to a viral pathogen. In general, the presence of IgM indicates a current or recent infection or a congenital infection, if present in infant serum. The presence of IgG antibodies indicates previous exposure to a virus or immunity as a result of vaccination.
- Culture, antigen detection, and molecular methods for viral nucleic acid can be used to directly identify viruses in clinical samples. Molecular methods have become increasingly important in the diagnosis of viral infections and can also be used to quantitate viral load to determine the effectiveness of antiviral therapy.
- The hepatitis viruses are those whose primary effect is inflammation of the liver. Hepatitis A and E are transmitted mainly by the fecal–oral route, whereas hepatitis B, C, and D are transmitted primarily by the parenteral route. Hepatitis B, C, and D can lead to chronic infections. Vaccines have been developed to prevent hepatitis A, hepatitis B, and hepatitis E.

- Serological markers of hepatitis infections consist of virus-specific antibodies and antigens that are commonly detected by automated immunoassays.
- IgM anti-HAV antibodies indicate current or recent hepatitis A infection, whereas IgG anti-HAV antibodies are developed later in the infection and indicate immunity to hepatitis A. Likewise, IgM anti-HEV antibodies are present during current or recent hepatitis E infection and IgG anti-HEV antibodies indicate later infection and immunity.
- Hepatitis B infection is indicated by the presence of the antigen HBsAg; HBeAg indicates high infectivity. IgM antibodies to hepatitis B core antigen are present in acute hepatitis B, whereas IgG anti-HBc is present during past or chronic hepatitis B infection. Antibodies to HBsAg (anti-HBs) indicate immunity and can be produced as a result of past hepatitis B infection or immunization with the hepatitis B vaccine.
- The presence of anti-HCV indicates exposure to the hepatitis C virus, but cannot distinguish between a current and a past infection. Molecular tests for HCV RNA are used to confirm antibody-positive results; if present, they indicate a current infection. Molecular tests for hepatitis C are also used to quantitate viral load to determine the effectiveness of antiviral therapy. A third application of molecular testing is genotyping of the virus to guide decisions about therapy.
- Hepatitis D occurs as a super- or co-infection with hepatitis B and is indicated by antibodies to hepatitis D or molecular tests to detect HDV RNA. Patients with co-infections are also positive for IgM-anti-HBc, whereas patients with superinfections are positive for IgG-anti-HBc.
- The Epstein-Barr virus (EBV) is the cause of infectious mononucleosis, Burkitt's lymphoma, Hodgkin disease, nasopharyngeal and gastric carcinomas, and lymphoproliferative disorders in immunosuppressed individuals.
- Most patients with infectious mononucleosis produce heterophile antibodies, which can react with antigens from bovine, horse, or sheep RBCs. These antibodies are routinely screened for by the "Monospot" test, which is performed by rapid immunochromatographic or agglutination methods to detect antibodies to bovine or horse erythrocyte antigens.
- ELISA or IFA tests for EBV-specific antibodies are used to confirm a diagnosis of infectious mononucleosis, detect heterophile-negative cases of infectious mononucleosis, and to diagnose other EBV-associated diseases. Acute infectious mononucleosis is indicated by the presence of IgM anti-VCA and anti-EA-D, as well as the absence of anti-EBNA. Molecular tests are useful in detecting EBV DNA in immunocompromised patients who may not develop a good antibody response and in monitoring viral load in patients with EBV-related malignancies during therapy.
- CMV (cytomegalovirus) infection is asymptomatic in most healthy individuals but may cause a mononucleosis-like syndrome, disseminated infection in organ transplant recipients and patients with HIV/AIDS, and congenital abnormalities in infants born to infected mothers.
- CMV infection is best detected by molecular assays for CMV DNA, CMV antigenemia assays for pp65 antigen, or shell vial culture. Quantitative PCR is useful in determining the CMV DNA copy number in immunocompromised hosts undergoing antiviral treatment. Serological assays for CMV antibody are most helpful in documenting a past infection in potential blood and organ donors.
- Primary infection with varicella virus causes chickenpox (varicella), whereas reactivation of the virus in nerve cells supplying the skin causes shingles (zoster). Diagnosis of current varicella virus infection is usually based on clinical findings, but detection of varicella virus DNA by PCR may be helpful in some clinical settings. Serological methods, most commonly ELISA, are used mainly to document immunity to varicella virus.
- Immunization programs have greatly reduced the incidence of three childhood infections: rubella, rubeola, and mumps. Rubella infection is the cause of German measles but can result in severe congenital abnormalities if it occurs during pregnancy. Rubeola viruses cause measles, a systemic infection that can cause complications in some individuals. Mumps virus is the cause of mumps, whose classic feature is swelling of the parotid glands, although other complications may occur.
- Although the diagnosis of rubella, measles, and mumps is usually based on clinical findings, laboratory testing may be helpful in confirmation. Current infections are indicated by the presence of IgM antibodies specific for the appropriate virus or by a four-fold rise in virus-specific IgG antibodies in two separate specimens collected during the acute and convalescent phases of disease. Testing for IgG antibodies is most commonly performed to screen for immunity to these viruses. RT-PCR is a useful adjunct to serology in detecting viral RNA in patients with inconclusive serology results, in epidemiological studies, and in the detection of congenital rubella infections.
- The human T-cell lymphotropic viruses, HTLV-I and HTLV-II, are retroviruses that infect CD4 T lymphocytes. HTLV-I is the cause of adult T-cell leukemia and lymphoma, HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and other inflammatory disorders. HTLV-II may be associated with HAM/TSP-like neurological disease as well as hematologic and skin disorders, but the disease associations of this virus are unclear. ELISA and CLIA tests are used routinely to screen blood donors for antibodies to HTLV-I and HTLV-II and to detect exposure to HTLV in other individuals. Positive results are confirmed by Western blot or line immunoassays. PCR for proviral DNA can be used to clarify indeterminate results and monitor viral load in patients undergoing therapy.

Study Guide: Immune Escape Mechanisms Commonly Used by Viruses

VIRAL ESCAPE MECHANISM	EXAMPLES
Acquisition of genetic mutations that result in new viral antigens	Influenza viruses, rhinoviruses, HIV
Inhibition of immunologic components	HCV blocks actions of interferons; HSV inhibits C3b
Suppression of the immune system	CMV and HIV reduce expression of class I MHC on the surface of virus-infected cells, reducing their recognition by CTLs; HIV destroys infected CD4 Th cells
Establishment of a latent state	CMV, VZV, and HIV integrate their nucleic acid into the host cell genome

CMV = cytomegalovirus; CTLs = cytotoxic T lymphocytes; HCV = hepatitis C virus; HIV = human immunodeficiency virus; HSV = herpes simplex viruses; CMV = cytomegalovirus: MHC = major histocompatibility complex; VZV = varicella zoster virus.

CASE STUDIES

1. A 25-year-old male had been experiencing flu-like symptoms, loss of appetite, nausea, and constipation for 2 weeks. His abdomen was tender and his urine was dark in color. Initial testing revealed elevations in his serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels.

Questions

a. What laboratory tests should be used to screen this patient for viral hepatitis?
b. If the patient tested positive for hepatitis B, which tests should be used to monitor his condition?
c. If the patient were to develop chronic hepatitis B, which markers would be present in his serum?

2. A 5-pound infant was born with microcephaly, purpuric rash, low platelet count, cardiovascular defects, and a cataract in the left eye. The infant's mother recalled experiencing flu-like symptoms and a mild skin rash early in her pregnancy. She had not sought medical attention at the time. The infant's physician ordered tests to investigate the cause of the newborn's symptoms.

Questions

a. What virus is the most likely cause of the infant's symptoms?
b. What laboratory tests would you suggest the doctor order on the mother to support your suggested diagnosis?
c. What tests should be performed on the infant's serum to support this diagnosis?

REVIEW QUESTIONS

1. The role of CTLs in immune responses against viruses is to
   a. neutralize viral activity.
   b. promote destruction of viruses by ADCC.
   c. destroy virus-infected host cells.
   d. attack free virions.

2. Viruses can escape immune defenses by
   a. undergoing frequent genetic mutations.
   b. suppressing the immune system.
   c. integrating their nucleic acid into the host genome.
   d. all of the above.

3. A patient who has developed immunity to a viral infection would be expected to have which of the following serology results?
   a. IgM+, IgG–
   b. IgM–, IgG+
   c. IgM+, IgG+
   d. IgM–, IgG–

4. A newborn suspected of having a congenital viral infection should be tested for virus-specific antibodies of which class(es)?
   a. IgM
   b. IgG
   c. IgA
   d. All of the above classes

5. Which of the following hepatitis viruses is transmitted by the fecal–oral route?
   a. Hepatitis B
   b. Hepatitis C
   c. Hepatitis D
   d. Hepatitis E

6. An individual with hepatomegaly, jaundice, and elevated liver enzymes has the following laboratory results: IgM anti-HAV (negative), HBsAg (positive), IgM anti-HBc (positive), and anti-HCV (negative). These findings support a diagnosis of
   a. hepatitis A.
   b. acute hepatitis B.
   c. chronic hepatitis B.
   d. hepatitis C.

7. The serum of an individual who received all doses of the hepatitis B vaccine should contain
   a. anti-HBs.
   b. anti-HBe.
   c. anti-HBc.
   d. all of the above.

8. Quantitative tests for HCV RNA are used to
   a. screen for hepatitis C.
   b. determine the HCV genotype.
   c. differentiate acute HCV infection from chronic HCV infection.
   d. monitor hepatitis C patients on antiviral therapy.

9. In the laboratory, heterophile antibodies are routinely detected by their reaction with
   a. B lymphocytes.
   b. bovine erythrocyte antigens.
   c. sheep erythrocyte antigens.
   d. Epstein-Barr virus antigens.

10. The presence of IgM anti-rubella antibodies in the serum from an infant born with a rash suggests
    a. a diagnosis of measles.
    b. a diagnosis of German measles.
    c. congenital infection with the rubella virus.
    d. passive transfer of maternal antibodies to the infant's serum.

11. A pregnant woman is exposed to a child with a rubella infection. She had no clinical symptoms but had a rubella titer performed. Her antibody titer was 1:8. Three weeks later, the test was repeated and her titer was 1:128. She still had no clinical symptoms. Was the laboratory finding indicative of rubella infection?
    a. No, the titer must be greater than 256 to be significant.
    b. No, the change in titer is not significant if no clinical signs are present.
    c. Yes, a greater than four-fold rise in titer indicates early infection.
    d. Yes, but clinical symptoms must also correlate with laboratory findings.

12. The cause of shingles is the
    a. cytomegalovirus.
    b. rubella virus.
    c. varicella-zoster virus.
    d. HTLV-I.

13. The method of choice for detecting VZV infection in immunocompromised hosts is
    a. serology to detect virus-specific IgM antibodies.
    b. serology to detect virus-specific IgG antibodies.
    c. viral culture.
    d. real-time PCR.

14. Which of the following is true regarding laboratory testing for mumps?
    a. RT-PCR is recommended as the primary diagnostic test.
    b. Serology is necessary for confirmation of a suspected clinical case.
    c. IgM tests for mumps are highly specific.
    d. An acute infection must be confirmed by a four-fold rise in IgG titer.

15. A positive result on a screening test for HTLV-I antibody should be
    a. considered highly specific for HTLV-I infection.
    b. followed by PCR.
    c. confirmed by Western blot.
    d. validated by viral culture.

24 Laboratory Diagnosis of HIV Infection

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LEARNING OUTCOMES

After finishing this chapter, you will be able to:

1. Describe the classification system used to identify HIV isolates.
2. Explain the conditions under which transmission of human immunodeficiency virus (HIV) can occur.
3. Describe the structure of the HIV particle, including pertinent antigens and the genes that encode them.
4. Depict the replication cycle of HIV, beginning with entry of the virus into host cells.
5. Describe the host's immune responses to HIV and the effects of HIV on the immune system.
6. Describe the clinical manifestations of HIV infection.
7. Explain the CDC classification system for HIV infection.
8. Discuss antiretroviral treatments and the impact they have had on HIV infection.
9. Discuss the 2014 CDC-recommended algorithm for screening for HIV infection, as well as its advantages compared with previous algorithms and its limitations.
10. Discuss the principle of enzyme-linked immunosorbent assay (ELISA) testing for HIV infection and contrast first-generation, second-generation, third-generation, and fourth-generation ELISA tests for HIV.
11. Discuss the underlying principle and clinical uses of rapid tests for HIV.
12. Describe the principle of the Western blot test, interpretation of the results, and limitations of the test.
13. Give reasons for false positives and false negatives in HIV antibody testing.
14. Discuss the principle and clinical utility of flow cytometric methods for CD4 T-cell enumeration.
15. Differentiate between reverse-transcriptase polymerase chain reaction (RT-PCR), quantitative (real-time) RT-PCR, and branched DNA (bDNA) testing for HIV nucleic acid.
16. Discuss the clinical utility of HIV viral load testing and drug-resistance testing.
17. Discuss the protocol for HIV testing of infants and children younger than 18 months of age.

CHAPTER OUTLINE

DavisPlus You can go to Davis*Plus* at **davisplus.fadavis.com** keyword Stevens for the laboratory exercises that accompany this text.

KEY TERMS

Acquired immunodeficiency syndrome (AIDS)
Amplicon
Antiretroviral therapy (ART)
Branched chain DNA
CD4 T cells
Combination antiretroviral therapy (CART)
ELISAs
Flow cytometry
Highly active antiretroviral therapy (HAART)
Human immunodeficiency virus (HIV)
Hybridization
p24 antigen
Reverse transcriptase
Viral load tests
Western blot test

Human immunodeficiency virus (HIV) is the etiologic agent of **acquired immunodeficiency syndrome,** or **AIDS,** a disease that has posed one of the greatest medical challenges worldwide. According to the World Health Organization (WHO), at the end of 2015 over 36 million people were living with HIV infection, 1.1 million people became newly infected, and 1.5 million people died of AIDS.[1] Since its discovery, the virus has claimed the lives of more than 39 million people worldwide. Although the majority of infected persons reside in developing countries, HIV infection has also created a significant problem in developed nations. For example, in the United States, over 1.1 million cases of AIDS and more than 673,000 AIDS-related deaths have been reported since 1981, when the first cases of AIDS were identified.[2]

The virus that is responsible for causing AIDS, HIV-1, was identified independently by the laboratories of Luc Montagnier of France and Robert Gallo and Jay Levy of the United States in 1983 and 1984, respectively.[3-5] Isolates of HIV-1 have been classified into four groups: group M (the main or major group), group O (the outlier group), and two newer groups, group N and group P.[6] Group M viruses are responsible for the majority of HIV-1 infections worldwide. This group contains nine subtypes or clades, designated A, B, C, D, F, G, H, J, and K. Subtype C is the most predominant subtype worldwide, whereas subtype B is the most prevalent subtype in the United States, Europe, and Australia. Groups N, O, and P occur much less frequently and are largely confined to West Africa.[6-8] An increasing number of circulating recombinant forms (CRFs) exist, which are produced as a result of genetic recombination between two subtypes that have infected a single individual.[9]

A related but genetically distinct virus, HIV-2, was discovered in 1986.[10] The majority of HIV-2 infections have occurred in West Africa, although the virus has also been identified in patients in other parts of the world.[7,8] HIV-2 is transmitted in the same manner as HIV-1 and may also cause AIDS, but it is less pathogenic and has a lower rate of transmission. Although this chapter discusses the differences between the two viruses, our focus is on HIV-1 because it is much more prevalent throughout the world. In this chapter, the term *HIV* is used to refer to HIV-1, and HIV-2 is so named.

Accurate diagnosis is essential for early intervention and halting the spread of HIV. This chapter will discuss characteristics of the virus, immunologic manifestations of the disease, and laboratory techniques used to diagnose and monitor HIV infection.

HIV Transmission

Transmission of HIV occurs through one of three major routes: intimate sexual contact, contact with blood or other body fluids, or perinatally (from infected mother to infant).[6,9] The majority of cases of HIV infection have occurred through sexual transmission involving either vaginal or anal intercourse. Worldwide, about 85% of cases of HIV infection can be attributed to heterosexual contact, whereas in the United States the largest number of cases has resulted from anal intercourse in homosexual males.[2,6,9] The presence of other sexually transmitted diseases such as syphilis, gonorrhea, or genital herpes increases the likelihood of transmission by disrupting protective mucous membranes and activating immunologic cells in the genital areas.[6,9,11]

The second route of transmission is through parenteral exposure to infected blood or body fluids, which occurs mainly through the sharing of contaminated needles by intravenous drug users. Less frequently, transmission can take place via blood transfusions, the use of clotting factors by hemophiliacs, occupational injuries with needlesticks or other sharp objects, or mucous membrane contact in health-care workers exposed to infectious fluids.[9,12,13] The virus has also been acquired by transplantation of infected tissue. Screening of blood and organ donors for HIV has dramatically decreased the incidence of infection in recipients of blood transfusions, clotting factors, and organ transplants. In the United States, it is estimated that one transmission occurs per 1 to 2 million blood donations, resulting in the release of about 11 infectious units each year.[9,14] Studies by the Centers for Disease Control and Prevention (CDC) have estimated the average risk of transmission to health-care workers to be approximately 0.3% after a percutaneous exposure to HIV-infected blood and about 0.09% after a mucous membrane exposure. Body fluids considered to be potentially infectious include blood, semen, vaginal secretions, cerebral spinal fluid, synovial fluid, pleural fluid, peritoneal fluid, pericardial fluid, amniotic fluid, saliva from dental procedures, other fluids containing visible blood, and any body fluid that cannot be differentiated. The risk of transmission is increased in exposures involving a large quantity of blood, hollow-bore needles placed directly into an artery or vein, or deep tissue injury. The risk of infection is also increased if the source patient is in the acute or advanced stages of HIV infection, when the amount of virus circulating in the bloodstream is high.[9,12,15]

The third route of transmission is perinatal, from infected mother to her fetus or infant. Transmission through this route can occur during pregnancy, by transfer of blood at the time of

delivery, or through breastfeeding.[9,11] Perinatal transmission has been markedly reduced through HIV screening during pregnancy, administration of antiretroviral drugs to HIV-positive pregnant women and their newborn babies, and use of infant formula by mothers who are infected with the virus. These measures have decreased the rate of perinatal transmission to less than 2%, as compared with rates of 15% to 35% in untreated mothers.[9,16]

Characteristics of HIV

Composition of the Virus

HIV belongs to the genus *Lentivirinae* of the virus family *Retroviridae*.[9,17] It is classified as a retrovirus because it contains ribonucleic acid (RNA) as its nucleic acid and a unique enzyme, called **reverse transcriptase**, which transcribes the viral RNA into DNA, a necessary step in the virus's life cycle. HIV is a spherical particle, 100 to 120 nm in diameter, which contains an inner core with two copies of single-stranded RNA surrounded by a protein coat or capsid and an outer envelope of glycoproteins embedded in a lipid bilayer.[9,17,18] The glycoproteins are knoblike structures that are involved in binding the virus to host cells during the infection. **Figure 24–1** shows the structure of the HIV virion.

Structural Genes

The genome of HIV includes three main structural genes—*gag, env,* and *pol*—and a number of regulatory genes. **Figure 24–2** shows the relative locations of the major HIV genes and indicates

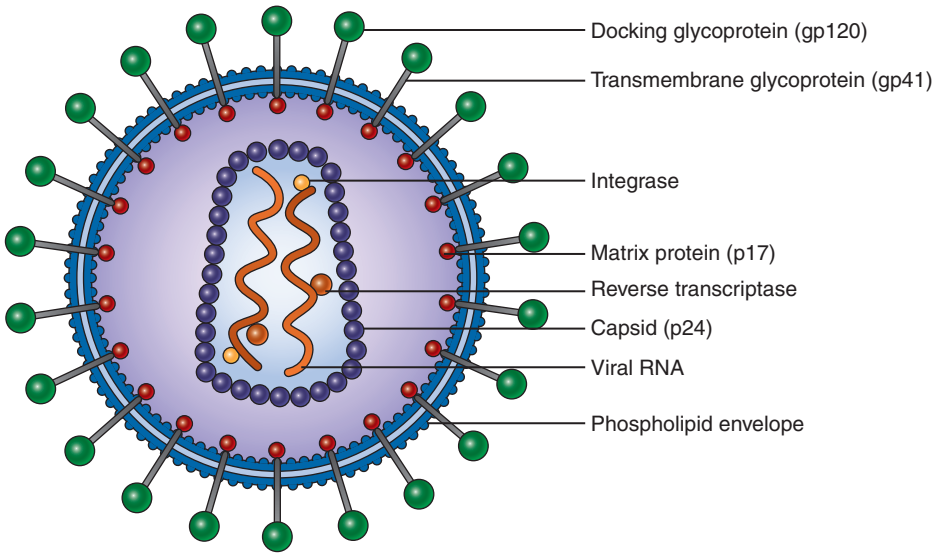

FIGURE 24–1 Structure of HIV virion, showing some of the major components. p = protein; gp = glycoprotein; the numbers refer to the molecular weights of the proteins in kilodaltons.

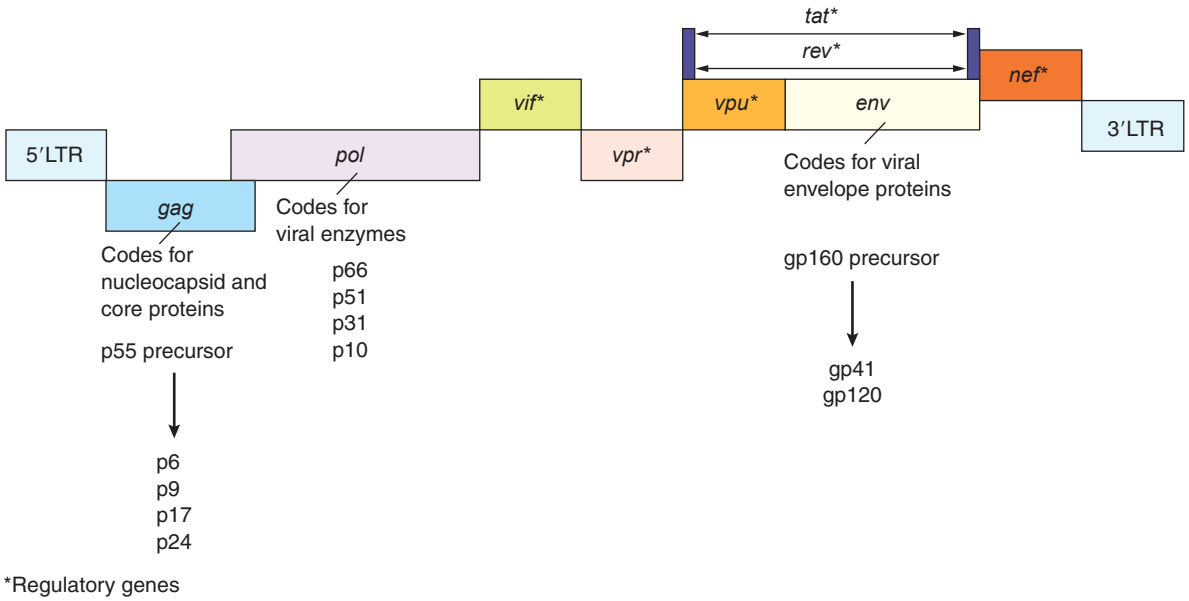

FIGURE 24–2 The HIV-1 genome. The relative locations of the major genes in the HIV-1 genome are indicated, as well as their gene products. * = regulatory genes

their gene products. The *gag* gene codes for p55, a precursor protein with a molecular weight of 55 kd, from which four core structural proteins are formed: p6, p9, p17, and p24.[19,20] All four are located in the nucleocapsid of the virus. The capsid that surrounds the internal nucleic acids contains p24, p6, and p9, whereas p17 lies in a layer between the protein core and the envelope, called the *matrix,* and is actually embedded in the internal portion of the envelope.[19]

The *env* gene codes for the glycoproteins gp160, gp120, and gp41, which are found in the viral envelope. Gp160 is a precursor protein that is cleaved to form gp120 and gp41. Gp120 forms the numerous knobs or spikes that protrude from the outer envelope, whereas gp41 is a transmembrane glycoprotein that spans the inner and outer membrane and attaches to gp120. Both gp120 and gp41 are involved in fusion and attachment of HIV to receptors on host cells.[17,20]

The third structural gene, *pol,* codes for enzymes necessary for HIV replication.[17,20] These include reverse transcriptase (p51); ribonuclease (RNase H; p66), an enzyme involved in the degradation of the original HIV RNA; integrase (p31), an enzyme which mediates the integration of viral DNA into the genome of infected host cells; and a protease (p10) that cleaves precursor proteins into smaller active units used to make the mature virions. These proteins are located in the core of the virus in association with HIV RNA.

Several other genes in the HIV genome code for products that have regulatory or accessory functions.[9,17,20] These genes include *tat* (transactivator), *rev* (regulator of expression of virion proteins), *nef* (negative effector), *Vpu* (viral protein "U"), *Vpr* (viral protein "R"), and *Vif* (viral infectivity factor). Although the products of these genes are not an integral part of the viral structure, they serve important functions in controlling viral replication and infectivity. The HIV gene sequence is surrounded by 5' and 3' long terminal repeat (LTR) regions, which also play a role in regulating the expression of the genes. **Table 24–1** summarizes the major HIV-1 genes, their products, and their functions.

HIV-2 has *gag, env, pol,* and regulatory or accessory genes that have similar functions to those seen in HIV-1. The homology between the genomes of the two viruses is approximately 50%.[9,19] The *gag* and *pol* regions are most similar, whereas the *env* region differs greatly. Thus, the viruses can most easily be distinguished on the basis of antigenic differences in their *env* proteins.

Table 24–1 Major HIV Genes and Their Products

GENE	PROTEIN PRODUCT	FUNCTION
gag	p17	Inner surface of envelope
	p24	Core coat for nucleic acids
	p9	Core-binding protein
	p6	Binds to genomic RNA
env	gp120	Binds to CD4 on T cells
	gp41	Transmembrane protein associated with gp120
pol	p66	Subunit of reverse transcriptase; degrades original HIV RNA
	p51	Subunit of reverse transcriptase
	p31	Integrase; mediates integration of HIV DNA into host genome
	p10	Protease that cleaves *gag* precursor
tat	p14	Activates transcription of HIV provirus
rev	p19	Transports viral mRNA to the cytoplasm of the host cell
nef	p27	Enhances HIV replication
vpu	p16	Viral assembly and budding
vpr	p15	Integration of HIV DNA into host genome
vif	p23	Infectivity factor

Viral Replication

The first step in the reproductive cycle of HIV occurs when the virus attaches to a susceptible host cell. This interaction is mediated through the host-cell CD4 antigen, which serves as a receptor for the virus by binding the gp120 glycoprotein on the outer envelope of HIV. T helper (Th) cells are the main target for HIV infection because they express high numbers of CD4 molecules on their surface and bind the virus with high affinity.[20] Other cells such as macrophages, monocytes, dendritic cells, Langerhans cells, and microglial brain cells can also be infected with HIV because they have some surface CD4. HIV viruses that preferentially infect T cells are known as *T-tropic* or *X4* strains, whereas those strains that can infect both macrophages and T cells are called *M-tropic* or *R5* strains.

Entry of HIV into the host cells to which it has attached requires an additional binding step, involving co-receptors that promote fusion of the HIV envelope with the plasma cell membrane. These co-receptors belong to a family of proteins known as chemokine receptors, whose main function is to direct white blood cells (WBCs) to sites of inflammation. The chemokine receptor, CXCR4, is required for HIV to enter T lymphocytes, whereas the chemokine receptor, CCR5, is required for entry into macrophages. In fact, individuals who have a genetic mutation in the CCR5 gene have been found to be resistant to HIV infection.[21,22] Binding of the co-receptors allows for HIV entry by inducing a conformational change in the gp41 glycoprotein, which mediates fusion of the virus to the cell membrane.[9,17]

After fusion occurs, the viral particle is taken into the cell and uncoating of the particle exposes the viral genome.[9,17,20] Action of the enzyme reverse transcriptase produces complementary DNA from the viral RNA. Double-stranded DNA is synthesized and, with the help of the HIV integrase enzyme, becomes integrated into the host cell's genome as a provirus **(Fig. 24–3).** The provirus can remain in a latent state for a long time, during which viral replication does not occur. Eventually, expression of the viral genes is induced when the infected host cell is activated by binding to antigen or by exposure to cytokines. Viral DNA within the cell nucleus is then transcribed into genomic RNA and messenger RNA (mRNA), which are transported to the cytoplasm. Translation of mRNA occurs, with production of viral precursor proteins and assembly of viral particles. The intact virions bud out from

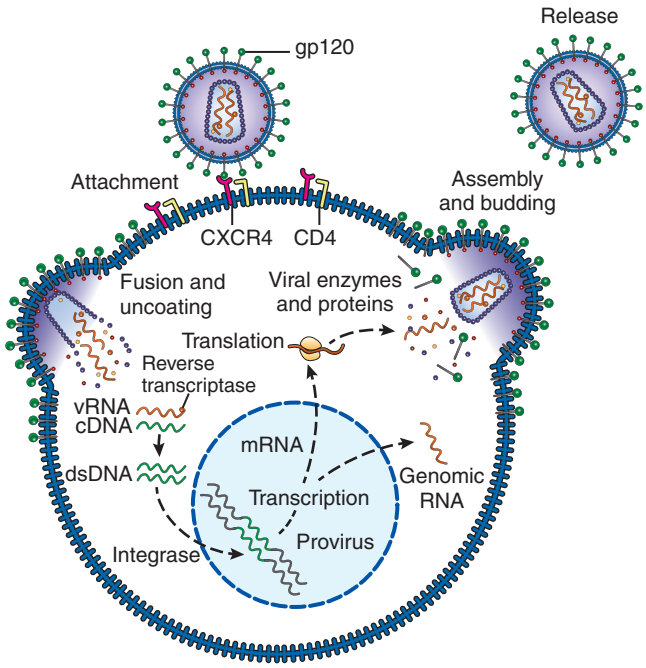

FIGURE 24–3 Replication cycle of HIV in a CD4+ T cell.

the host cell membrane and acquire their envelope during the process. The precursor proteins are cleaved by the viral protease enzyme in the mature virus particles. These viruses can proceed to infect additional host cells. Viral replication occurs to the greatest extent in antigen-activated Th cells.

Because viral replication occurs very rapidly and the reverse transcriptase enzyme lacks proofreading activity, genetic mutations occur at a high rate, producing distinct isolates that exhibit an extraordinary level of antigenic variation. In fact, the level of HIV diversity in a single individual is greater than the diversity of all the influenza virus isolates throughout the world in a given year![23] This tremendous genetic diversity of HIV hinders the ability of the host to mount an effective immune response.

Immunologic Manifestations

Immune Responses to HIV

When HIV infects a healthy individual, there is typically an initial burst of viral replication followed by a slowing down of virus production as the host's immune response develops and keeps the virus in check.[9,18,19,24] This initial viral replication can be detected in the laboratory by the presence of increased levels of **p24 antigen** and viral RNA in the host's bloodstream (see discussion in the text that follows). As the virus replicates, some of the viral proteins produced within host cells form complexes with class I major histocompatibility complex (MHC) antigens and are transported to the cell surface, where they stimulate lymphocyte responses. HIV-specific CD4+ Th cells are generated and assist both humoral and cell-mediated immune responses against the virus. However, the interactions between HIV and the immune system are complex and these responses are unable to eliminate HIV from the body,[8,9] as we will discuss in the text that follows.

B lymphocytes are stimulated to produce antibodies to HIV, which can usually be detected in the host's serum by 6 weeks after primary infection.[9,18] The first antibodies to be detected are directed against the gp41 transmembrane glycoprotein, followed by production of antibodies to the *gag* proteins such as p24, and finally production of antibodies to the *env, pol,* and regulatory proteins. The most immunogenic proteins are in the viral envelope and elicit the production of neutralizing antibodies. These antibodies usually appear 2 to 3 months after infection and prevent the virus from infecting neighboring cells.[24] Antibodies to the envelope proteins have also been shown to bind to Fc receptors on NK cells and participate in ADCC-mediated killing of HIV-infected cells. However, these antibodies may also participate in the pathogenesis of HIV infection by facilitating Fc-receptor mediated endocytosis of opsonized virus by uninfected cells. Furthermore, dense glycosylation of the *env* proteins can mask important epitopes, and the enormous diversity of the virus poses challenges to the host in producing a protective antibody response.[9,24]

T-cell–mediated immunity is thought to play an important role in the immune response to HIV, as it does in other viral infections.[9,18,19,24] CD8+ cytotoxic T lymphocytes, also known as cytolytic T cells (CTLs), appear within weeks of HIV infection and are associated with a decline in the amount of HIV in the blood during acute infection. Antibodies can attach only to virions circulating freely outside of host cells; in contrast, CTLs can attack host cells harboring viruses internally. This process involves the binding of CTLs containing HIV-specific antigen receptors to HIV proteins associated with class I MHC molecules on the surface of infected host cells. HIV-specific CTLs are stimulated to develop into mature, activated clones through the effects of cytokines released by activated CD4 Th cells, a process that is common to immune responses against other viruses (see Chapter 4 for details). After the CTLs bind to HIV-infected host cells, cytolytic enzymes are released from their granules and destroy the target cells. Free virions are released from the damaged cells and can be bound by antibodies. CTLs can also suppress replication and spreading of HIV by producing cytokines such as interferon-γ, which have antiviral activity.

Innate immune defenses may play a role in responding to HIV in the early stages of the infection.[24,25] In particular, NK cells become activated during acute HIV infection and can mediate cytolysis of host cells infected with the virus. In addition, dendritic cells recognize viral components through pattern-recognition receptors, resulting in release of proinflammatory cytokines that have antiviral effects and can activate other cells of the immune system. Future studies will likely clarify the role of these responses in early control of HIV infection.

Effects of HIV Infection on the Immune System

HIV has developed several mechanisms by which it can escape immune responses.[19,24,26,27] Although the humoral and cell-mediated immune responses of the host usually reduce the

level of HIV replication, they are generally not sufficient to completely eliminate the virus. CTL and antibody responses to HIV are hindered by the virus's ability to undergo rapid genetic mutations, generating escape mutants with altered antigens toward which the host's initial immune responses are ineffective. In addition, HIV can downregulate the production of class I MHC molecules on the surface of the host cells it infects, protecting them from CTL recognition. Numerous cells in the body can also harbor HIV as a silent provirus for long periods, including resting CD4 T cells, dendritic cells, cells of the monocyte and macrophage lineage, and microglial cells in the brain. In this proviral state, HIV is protected from attack by the immune system until cell activation stimulates the virus to multiply and display its viral antigens.

The ability of HIV to evade the immune response results in a persistent infection that can destroy the immune system. Because the virus's prime targets are the CD4 Th cells, these cells are most severely affected; a decrease in this cell population is the hallmark feature of HIV infection.[28] The gastrointestinal immune system (GALT), which is the largest immune organ in the body, is the most profoundly affected.[29] Early in the infection, HIV causes a rapid depletion of CCR5+ CD4+ memory T cells in the GALT. Th17 helper cells, which play an important role in the homeostasis of the epithelial cells lining the intestinal mucosa (enterocytes) as well as in the secretion of antimicrobial defensins, are also preferentially affected. This depletion results in damage to the intestinal epithelial barrier, with leakage of microbial products such as lipopolysaccharide (LPS) into the plasma, and a general state of immune activation.[6,29]

CD4 Th cells are thought to be killed or rendered nonfunctional by HIV through a variety of mechanisms such as loss of plasma membrane integrity because of viral budding, destruction by HIV-specific CTL, and viral induction of apoptosis.[19,27,30] Infected T cells turn over much more rapidly than they can be replaced, having a half-life of 12 to 36 hours.[27] There is only a partial recovery of the T cells lost early in the infection, which is followed by a progressive decrease in CD4 T cells during the natural course of untreated infections.[6] In addition to reducing T-cell numbers, HIV also causes abnormalities in Th cell function and impairment of memory Th cell responses.[9,19,20,28]

Because **CD4 T cells** play a central role in the immune system by regulating the activities of B and T lymphocytes (see Chapter 4), destruction of these cells results in decreased effectiveness of both antibody- and cell-mediated immune responses. These effects apply not only to the immune responses directed against HIV, but to the broad range of antigens that are encountered by the host. Dysregulated immune responses are also evident. HIV proteins actually stimulate polyclonal activation of B cells, resulting in maturational and functional defects with increased circulating immunoglobulin levels, immune complexes, and autoantibodies.[9,19,20,31] However, B cells in HIV-infected individuals have a reduced ability to mount antibody responses after exposure to specific antigens, caused by the decrease in T-cell help.[9,19,20]

Cell-mediated immunity is also affected by the reduction in Th activity, resulting in a decline in CTL activity and delayed-type hypersensitivity responses to specific antigens.[9,19,26,27] Altered production of cytokines and chemokines has also been seen, including increases in the levels of some cytokines during the early stages of disease, followed by declining levels of IL-2 and interferon-γ and a shift in the cytokine profile from Th1 to Th2 as the infection progresses toward development of AIDS.[19,20,27] Extensive damage to the lymphoid tissues is evident late in the infection, with loss of germinal centers and follicular dendritic cells resulting in an inability to activate T and B lymphocytes.[20] Other immunologic abnormalities, including defective antigen presentation and oxidative burst by monocytes and macrophages and decreased natural killer (NK) cell activity, have also been observed in AIDS patients.[9,19,27,28]

Clinical Symptoms of HIV Infection

HIV causes a chronic infection that is characterized by a progressive decline in the immune system. Although the manifestations of the disease vary in individual patients, the infection progresses through a clinical course that begins with primary, or acute, infection, followed by a period of clinical latency that eventually culminates in AIDS.[9,24,32] The acute, or early, stage of infection is characterized by a rapid burst of viral replication before the development of HIV-specific immune responses. In this stage, high levels of circulating virus, or *viremia,* can be seen in the blood of infected individuals; as a result, HIV begins to disseminate to the lymphoid organs. There is a reduction in the peripheral blood CD4 T-cell count, but this usually returns to slightly decreased or, sometimes, normal levels. As the immune system becomes activated, an acute retroviral syndrome may develop. This syndrome, which has been noted in 50% to 70% of patients with primary HIV infection, is characterized by flu-like or infectious mononucleosis-like symptoms.[9,19,24,32] Symptoms of the primary stage usually appear 3 to 6 weeks after the initial infection and resolve within 7 days to a few weeks. Many patients, however, are asymptomatic during this stage.

As HIV-specific immune responses develop, they begin to curtail replication of the virus and patients enter a period of clinical latency. This stage is characterized by a decrease in viremia as the virus is cleared from the circulation and clinical symptoms are subtle or absent.[9,19,24,32] However, studies have demonstrated that the virus is still present in the plasma, albeit at lower levels, and more so in the lymphoid tissues. The CD4 T-cell count remains stable for a variable period of time and then begins to progressively decline. A small proportion of HIV-infected individuals, termed *long-term nonprogressors (LTNP),* have normal or mildly depressed CD4 T-cell counts and low viral loads; they remain asymptomatic for more than 10 years in the absence of antiretroviral therapy.[9,33] The factors that influence this slower rate of progression are not completely understood, but appear to be associated with certain HLA types, non-HLA genes, and prevalence of the R5 strain of HIV.[9,24]

Untreated individuals will ultimately progress to AIDS, the final stage of HIV infection, which is characterized by profound immunosuppression with very low numbers of CD4 T cells, a resurgence of viremia, and life-threatening infections and malignancies. The rate at which individuals progress to the development of AIDS varies, but progression typically occurs in

untreated individuals within a median time of 10 years after the initial infection.[9,32] The rate of progression has dramatically decreased with the use of antiretroviral therapies (see *Treatment and Prevention* in the text that follows).

A list of the *opportunistic* illnesses considered to be indicative of AIDS is found in the Clinical Correlations box. These conditions appear in immunocompromised individuals but do not usually affect people with a healthy immune system. In addition to infections and malignancies, HIV-infected individuals often demonstrate neurological symptoms resulting from the ability of HIV to infect cells in the brain. In early HIV infection, these symptoms may manifest as forgetfulness, poor concentration, apathy, psychomotor retardation, and withdrawal, whereas progression to late disease may result in confusion, disorientation, seizures, dementia, gait disturbances, ataxia, or paraparesis.[9,34]

Clinical Correlations

Opportunistic Illnesses Indicative of AIDS (Stage 3 HIV Infection)

Bacterial infections, multiple or recurrent*
Candidiasis of bronchi, trachea, or lungs
Candidiasis of esophagus
Cervical cancer, invasive†
Coccidioidomycosis, disseminated or extrapulmonary
Cryptococcosis, extrapulmonary
Cryptosporidiosis, chronic intestinal (longer than 1 month's duration)
Cytomegalovirus disease (other than liver, spleen, or nodes), onset older than 1 month of age
Cytomegalovirus retinitis (with loss of vision)
Encephalopathy attributed to HIV
Herpes simplex: chronic ulcers (longer than 1 month's duration) or bronchitis, pneumonitis, or esophagitis (onset older than 1 month of age)
Histoplasmosis, disseminated or extrapulmonary
Isosporiasis, chronic intestinal (longer than 1 month's duration)
Kaposi sarcoma
Lymphoma, Burkitt (or equivalent term)
Lymphoma, immunoblastic (or equivalent term)
Lymphoma, primary, of brain
Mycobacterium avium complex or *Mycobacterium kansasii,* disseminated or extrapulmonary
Mycobacterium tuberculosis of any site, pulmonary†, disseminated, or extrapulmonary
Mycobacterium, other species or unidentified species, disseminated or extrapulmonary
Pneumocystis jirovecii (previously known as *"Pneumocystis carinii"*) pneumonia
Pneumonia, recurrent†
Progressive multifocal leukoencephalopathy
Salmonella septicemia, recurrent
Toxoplasmosis of brain, onset older than 1 month of age
Wasting syndrome attributed to HIV

*Only among children aged younger than 6 years.
†Only among adults, adolescents, and children aged 6 years or older.
Courtesy of Centers for Disease Control and Prevention . Revised surveillance case definition for HIV infection - United States, 2014. *MMWR* 2014;63(3):1-10.

Symptoms of AIDS in infants include failure to thrive, persistent oral candidiasis, hepatosplenomegaly, lymphadenopathy, recurrent diarrhea, or recurrent bacterial infections.[35,36] Abnormal neurological findings may be present. The rate by which HIV infection progresses in children varies and may be influenced by factors such as maturity of the immune system at the time of infection, the dose of virus to which the child was exposed, and the route of infection.[36]

The CDC first defined AIDS as "a disease, at least moderately predictive of a defect in cell mediated immunity, occurring in a person with no known cause for diminished resistance to that disease."[37] The definition has been revised several times over the years as more information has been acquired about HIV and additional laboratory tests for HIV have been developed.[38-41] The CDC also published a separate case definition for HIV infection in children.[41,42] The latest definition at the time of this writing was published in 2014.[43] It combines the case definitions for persons of all ages into a single definition that is intended to be used for surveillance of the disease. The 2014 definition bases a confirmed case of HIV infection on either laboratory criteria or clinical evidence, with laboratory results being the preferred criteria. Laboratory criteria consist of positive test results in multitest algorithms for HIV antibody or combination HIV antigen/antibody, whereas clinical evidence refers to physician documentation of HIV infection in the patient's medical record (see *Laboratory Testing for HIV Infection* in the text that follows).

HIV-positive patients are further classified into one of five stages (0, 1, 2, 3, or unknown).[43] The stage can change for an individual patient in either direction over time. Stage 0 includes those individuals with early HIV infection who had an initial confirmed HIV-positive laboratory result followed by a negative or indeterminate HIV test result within a 6-month period. These patients can be reclassified in one of the other categories 180 days or more after initial diagnosis. Patients are classified as being in stages 1, 2, or 3 based on their peripheral blood CD4 T-cell count or percentage; if this information is missing, they are classified in the "unknown" category. The CD4 T-cell parameters used in this classification system are shown in **Table 24–2.** Stage 3 is also indicated if any of the opportunistic illnesses listed in the Clinical Correlations box are present.

Treatment and Prevention

Treatment of HIV infection involves supportive care of the associated infections and malignancies as well as administration of **antiretroviral therapy (ART)** to suppress the virus's replication. Several classes of antiretroviral drugs have been developed to treat HIV infection: nucleoside analogue reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors, fusion inhibitors, and entry inhibitors (co-receptor antagonists).[1,9,44,45] These drugs block various steps of the HIV replication cycle. Their mechanisms of action are summarized in **Table 24–3.** New drugs continue to be developed as advances in this area are made. Updated guidelines on the use of these drugs are

Table 24–2 CD4 T-Cell Parameters Used in HIV Staging

	AGE ON DATE OF CD4+ T-LYMPHOCYTE TEST					
	YOUNGER THAN 1 YEAR		1 TO 5 YEARS		6 YEARS OR OLDER	
STAGE	CELLS/μl	PERCENT	CELLS/μl	PERCENT	CELLS/μl	PERCENT
1	≥1,500	≥34	≥1,000	≥30	≥500	≥26
2	750–1,499	26–33	500–999	22–29	200–499	14–25
3	<750	<26	<500	<22	<200	<14

The stage is based primarily on the CD4+ T-lymphocyte count; the percentage is considered only if the count is missing.
(Adapted from Centers for Disease Control and Prevention. Revised surveillance case definition for HIV infection—United States, 2014. MMWR *2014;63(3):1-10.)*

Table 24–3 Antiretroviral Drugs for HIV Therapy

ANTIRETROVIRAL DRUG CLASSIFICATION	MECHANISM OF ACTION	EXAMPLES
Nucleoside analogue reverse-transcriptase inhibitors (NRTI)	Similar in structure to nucleosides; incorporate into HIV nucleic acid and block synthesis of viral RNA	Zidovudine, lamivudine, didanosine, abacavir, tenofovir
Nonnucleoside reverse-transcriptase inhibitors (NNRTI)	Bind to and inactivate reverse transcriptase	Nevirapine, delavirdine, efavirenz, etravirine
Protease inhibitors	Prevent cleavage of precursor proteins needed for assembly of HIV virions	Saquinavir, indinavir, ritonavir, nelfinavir, fosamprenavir, lopinavir
Integrase inhibitors	Prevent integration of HIV DNA into the host genome	Raltegravir
Fusion inhibitors	Block viral infection by preventing fusion of HIV with target cells	Enfuvirtide
Entry inhibitors (co-receptor antagonists)	Block binding of HIV to the chemokine co-receptor CCR5	Maraviroc

available from the U.S. Department of Health and Human Services and the WHO.[44,46]

Studies have shown that treatment with multiple drugs is more effective in killing the virus and avoiding viral resistance than treatment with a single drug. Potent regimens involving a combination of drugs from at least two of the antiretroviral drug classes are the standard of treatment and are referred to as **combination antiretroviral therapy (CART)** or **highly active antiretroviral therapy (HAART).**[6,9,44,47] Currently preferred treatment protocols use combinations of two nucleoside reverse-transcriptase inhibitors and a nonnucleoside reverse-transcriptase inhibitor, a protease inhibitor, or an integrase inhibitor.[6,9,44] The goal of this therapy is to reduce the patient's HIV viral load to a level that is below the detectable limit of quantitative plasma viral load assays (see *Quantitative Nucleic Acid Tests (NATs)* in the text that follows).[48] This is most likely to be achieved if treatment is started early in the course of infection and the patient can adhere to the treatment as prescribed.[32,45]

CART has had a dramatic effect on the clinical course of HIV infection, as evidenced by a significant decline in the incidence of opportunistic infections, a delay in progression to AIDS, and decreased mortality in patients who have received this multidrug treatment.[9,44] Before the development of ART, the median survival time of AIDS patients was only 26 weeks from the time of diagnosis; currently, HIV-infected patients who are treated appropriately with CART can be expected to live 50 years or longer.[9] Because of this success, CART is recommended for all HIV-infected persons at the time of diagnosis.[44,46] Antiretroviral drugs have also had a significant impact in reducing perinatal transmission of HIV, as discussed previously in this chapter.[6,16] In 1994, investigators from the United States and France published the results of a large clinical trial demonstrating that the antiretroviral drug zidovudine, administered to HIV-positive women during pregnancy and labor and to the newborn during the first few weeks of life, reduced transmission of HIV to the infant by two-thirds.[49] Subsequently, CART and avoidance of breastfeeding by HIV-positive women have reduced the rate of perinatal transmission to 0.1% to 0.5% in developed countries of the world.[44]

Although antiretroviral drugs and CART have significantly improved morbidity and mortality in HIV-infected patients, they cannot be considered a cure for AIDS. Although blood levels of the virus are greatly reduced in patients treated with antiretroviral drugs, HIV is still harbored in lymphoid organs throughout the body and progressively destroys the immune

system.[9,19] Research is ongoing to develop additional drugs to target proviral HIV within infected cells.[50]

Several other approaches for dealing with HIV have been directed toward preventing initial infection. Community-based education aimed at high-risk groups such as homosexual males and intravenous drug users has provided beneficial information on reducing transmission of the virus. The CDC has published guidelines for the use of antiretroviral drugs for pre-exposure prophylaxis (PrEP) to prevent transmission to individuals who are HIV-negative but at a high risk of contracting the infection, such as those who inject illicit drugs or are sexually active with an HIV-infected partner.[51] In addition, the CDC and the Occupational Safety and Health Administration (OSHA) have published precautions to prevent transmission of HIV and other bloodborne pathogens in health-care workers.[52,53] Prophylactic therapy with antiretroviral drugs is also offered to health-care workers who may have been exposed to HIV through percutaneous or mucous membrane contact with potentially infected blood or body fluids, in hope that early treatment will prevent infection.[54] As a result of these measures, fewer than 60 documented cases of occupational HIV transmission to health-care workers have been reported to the CDC as of 2010.[55]

The ultimate means of preventing HIV infection would be the development of an effective vaccine. Much research has been directed in this area, but the task has been very difficult for many reasons.[9,18,56-59] For example, HIV can rapidly mutate and escape immune recognition and there is no ideal animal model in which to study vaccine effects. In addition, an effective vaccine would need to induce mucosal immunity because HIV is usually transmitted through mucosal surfaces. An effective vaccine should also stimulate potent CTL and broad neutralizing antibody responses that could detect many variants of the virus. If a vaccine that can prevent HIV infection in the traditional sense cannot be developed, it is possible that a less-than-perfect vaccine may provide some benefits by prolonging the disease-free period and lowering the level of viremia, thus reducing the risk of transmission to others.[18,60]

Laboratory Testing for HIV Infection

The laboratory plays a key role in establishing the initial diagnosis of HIV infection and in monitoring known patients for their response to antiretroviral therapy. In addition, the U.S. Preventative Task Force recommends routine HIV screening (referred to as "opt-out screening") for all consenting persons between 15 to 65 years of age, whereas the CDC recommends routine HIV testing for all persons 13 to 64 years old and annual testing for individuals in high-risk groups.[61-63] At the time of this writing, it is believed that only 20% or less of HIV-infected adults know their status.[47] Universal screening will hopefully identify more persons infected with HIV so they can begin early treatment with ART and be less likely to transmit the virus to others.

Several types of laboratory tests have been used to diagnose and monitor HIV infection, including HIV antibody detection, HIV antigen detection, viral nucleic acid testing (NAT), and CD4 T-cell enumeration. Principles of each of these methods are discussed in the text that follows along with their applications to the detection and monitoring of HIV infection. Although culturing the virus from patient samples is a definitive method of demonstrating HIV infection, it is not used in clinical settings because it is laborious, time consuming, costly, and potentially hazardous to laboratory personnel.

Screening and Diagnosis

Serological tests for HIV antibody are used in the initial diagnosis of HIV infection because most individuals develop antibody to the virus within 1 to 2 months after exposure.[64,65] Since 1985, these tests have played a critical role in screening the donor blood supply to prevent transmission of the virus through blood transfusions or administration of blood products. Serological tests are also used in epidemiology studies to provide health officials with information about the extent of the infection in high-risk populations. These groups can then be targeted for counseling, treatment, and vaccine trials, and their medical or social concerns can be addressed.

Different serological methods have been used to test for HIV. Standard screening methods for HIV antibody have involved enzyme-linked immunosorbent assay (ELISA) methodology (see Chapter 11). In addition, rapid tests have been developed that can detect HIV antibody within minutes, making them an attractive alternative to the ELISA in certain situations. Confirmatory tests are performed on samples that test positive on a screening test, to differentiate true-positive from false-positive results. The Western blot test (see section in the text that follows) was the standard confirmatory test for HIV for a number of years, but it has largely been replaced by newer methods. Serological testing for p24 antigen and nucleic acid testing for HIV RNA have been incorporated into the initial HIV testing scheme, providing for earlier and more accurate detection. The principles of these methods are discussed in the text that follows, along with their use in HIV testing algorithms.

Testing Algorithms

Over the years, the CDC and the Association of Public Health Laboratories have developed algorithms that use a combination of laboratory tests performed in sequence to screen for the presence of HIV infection and resolve any discrepant results. These algorithms have been revised as improvements in laboratory tests have been made. In 1989, for example, the standard diagnostic algorithm recommended that testing begin with a sensitive ELISA for HIV-1 antibody, with positive samples being retested and then confirmed with a more specific Western blot test for HIV-1 antibody (or less frequently, an HIV-specific indirect immunofluorescence assay (IFA).[66] In 1992, the algorithm was modified so that initial testing was performed with an HIV-1/HIV-2 antibody test in cases where HIV-2 was likely to be present.[67] In 2004, it was recommended that rapid HIV antibody tests could also be used for initial screening and that positive results should be confirmed by an HIV-1 Western blot or IFA.[68]

In 2014, the CDC recommended that initial screening be performed with a combination immunoassay that detects antibodies to HIV-1 and HIV-2 as well as the HIV-1 p24 antigen.[69] These recommendations apply to adults and children who are older than 24 months. There are separate recommendations for children younger than 2 years, as maternal antibodies may be present that are likely to confuse the interpretation of the test results (see the text that follows). All positive specimens should then undergo additional testing with a rapid immunoassay that discriminates between HIV-1 and HIV-2 antibodies. Any samples that are reactive in the initial test and nonreactive in the second test should then undergo nucleic acid testing (NAT) to resolve the discrepancy.

The 2014 algorithm is summarized in **Figure 24–4.** This testing scheme provides significant advantages over previous algorithms.[63,69] First, it allows for earlier detection of infections, as the time between exposure and detectable results on the HIV-1/2/p24 combo assay is typically between 15 and 17 days. Secondly, it overcomes the limitations associated with use of the Western blot test. The Western blot is a lengthy procedure that is typically performed only by specialized reference laboratories. In addition, Western blot testing is less sensitive than the initial ELISA used for screening; therefore, indeterminate results can occur, which can take as long as 3 to 6 months to resolve. The 2014 testing algorithm allows for more rapid and accurate identification of HIV infection. This makes it possible to begin appropriate medical care and ART sooner in infected individuals. The newer testing also helps to prevent additional infections by encouraging prompt initiation of counseling to reduce risky behaviors and earlier notification of sexual partners of diagnosed individuals.[63,69]

Although the 2014 algorithm is highly sensitive in the detection of HIV infection, it has some limitations, which will be discussed in the next section. The CDC will continue to evaluate its algorithm as additional tests become available for clinical use. The principles of the major laboratory tests used in testing for HIV infection are discussed in the text that follows.

Serological Test Principles

ELISA and CLIA

ELISAs have been the cornerstone of screening procedures for HIV because they are easy to perform, can be adapted to test a large number of samples, and are highly sensitive and specific.[9,64,65] They were first used to detect HIV antibody in the United States in 1985 in response to the need to screen donated blood. Several manufacturers have developed commercial kits that are useful in screening blood products and in diagnosing and monitoring patients. An updated list of kits approved for use in the United States is published by the FDA.[70]

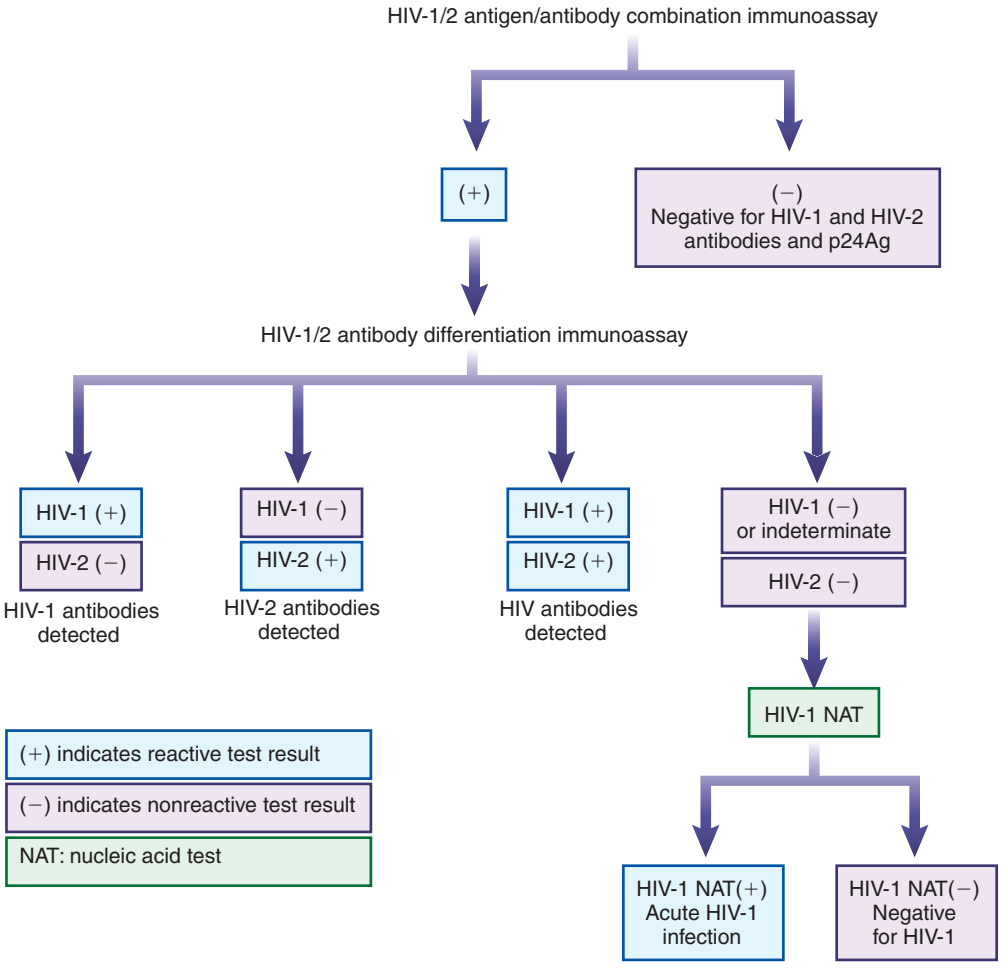

FIGURE 24–4 2014 Laboratory HIV testing algorithm recommended by the CDC. *(Adapted from Branson BM, Owen SM, Wesolowski LG, et al. Laboratory testing for the diagnosis of HIV infection: Updated recommendations. CDC Stacks. Web site. http://stacks.cdc.gov/view/cdc/23447. Updated 2014. Accessed December 18, 2014.)*

Over the years, five generations of ELISAs have been developed, resulting in improved sensitivity and specificity.

The first generation of ELISAs were based on a solid-phase, indirect-assay system that detected antibodies to only HIV-1.[64,65,69] (See Chapter 11 for general principles of ELISA.) In these tests, HIV antibodies in patient serum were detected after binding to a solid support coated with viral lysate antigens from HIV-1 cultured in human T-cell lines, followed by addition of an enzyme-labeled anti-human IgG conjugate and substrate. These first-generation assays were prone to false-positive results caused by reactions with HLA antigens or other components from the cells used to culture the virus, and they were unable to detect antibodies to HIV-2.[64,65]

The second-generation ELISAs, introduced in the late 1980s, were indirect binding assays that used highly purified recombinant (i.e., genetically engineered) or synthetic antigens from both HIV-1 and HIV-2, rather than crude cell lysates.[64,65,69] These assays demonstrated improved specificity and sensitivity overall and were able to detect antibodies to both HIV-1 and HIV-2. However, decreased sensitivity resulted when samples containing antibodies to certain subtypes of HIV that lacked the limited antigens used in the assays were tested.

Third-generation assays use the sandwich technique, based on the ability of antibody to bind with more than one antigen.[64,65,69] These assays are available in ELISA and chemiluminescent immunoassay (CLIA) formats. In these tests, antibodies in patient serum or plasma bind to recombinant HIV-1 and HIV-2 proteins coated onto a solid phase. After washing, enzyme- or chemiluminescent-labeled HIV-1 and HIV-2 antigens are added and bind to the already bound HIV-specific patient antibodies. Substrate (or trigger solution, if CLIA is used) is added next, and the color development (or release of light with CLIA) is proportional to the amount of antibody in the sample. This test format improves sensitivity by simultaneously detecting HIV antibodies of different immunoglobulin classes, including IgM. Enhancements in this method have increased sensitivity further by detecting low affinity antibodies and antibodies to group O HIV-1 as well as the more common group M. These enhancements resulted in a diagnostic sensitivity of 100% and diagnostic specificity of 99.9%.[71]

The most recent, fourth-generation assays can simultaneously detect HIV-1 antibodies, HIV-2 antibodies, and p24 antigen.[64,65,69] Previously, separate immunoassays were used to detect the p24 antigen from the core of the HIV-1 virion as a marker of early infection.[65] Recall that levels of p24 in the circulation are high in the initial weeks of infection during the early burst of viral replication, providing a marker that can be detected before the appearance of HIV antibody during the acute stage of infection.[69] The antigen becomes undetectable as antibody to p24 develops and binds the antigen in immune complexes; levels rise again during the later stages of infection when impairment of the immune system allows the virus to replicate.

The basic principle of the fourth-generation HIV-1 antibody/HIV-2 antibody/p24 antigen combination tests is illustrated in **Figure 24–5.** These tests employ a sandwich ELISA or CLIA in which patient serum is incubated with a solid phase onto which

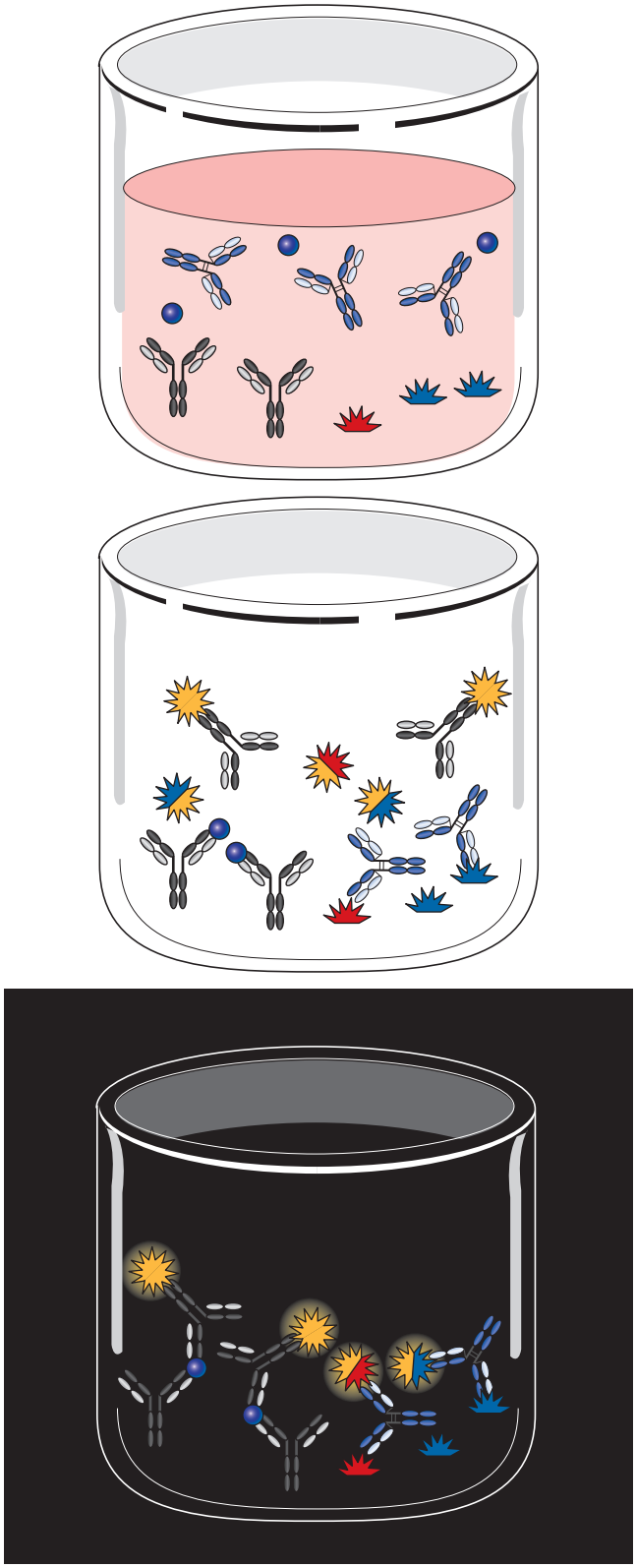

FIGURE 24–5 Principle of fourth-generation ELISA test for HIV. This test detects the HIV-1 p24 antigen as well as HIV-1 antibodies and HIV-2 antibodies.

synthetic or recombinant HIV-1 antigens, HIV-2 antigens, and a monoclonal antibody to HIV-1 p24 have been attached. If antibody to HIV is in the sample, it will bind to the HIV-1 or HIV-2 antigens (or both, if both infections are present); if p24 antigen is in the sample, it will bind to the anti-p24 on the solid phase. Following a wash step to remove excess sample, a conjugate containing chemiluminescent- or enzyme-labeled anti-p24 and HIV-1/HIV-2 antigens is added. After a second incubation and wash step, the appropriate trigger solution or substrate/stop solution is added and the relative light units released or optical absorbance is measured. As previously mentioned, these combination assays are used in the initial step of the 2014 laboratory testing algorithm recommended by the CDC.[69]

More recently, fifth-generation assays that provide more diagnostic information have been developed. One of these assays is a bead-based immunoassay that can detect both HIV antibodies and antigens, similar to the fourth-generation assays, but in addition can differentiate between HIV-1 and HIV-2 infection. In the future, implementation of assays such as these may change the testing algorithm for HIV.

Although the immunoassays used in HIV testing have a high level of sensitivity and specificity, they may sometimes give erroneous results. False-negative results for HIV antibody occur infrequently but may be caused by the collection of the test serum before the patient develops HIV antibodies (i.e., before *seroconversion*), administration of immunosuppressive therapy or replacement transfusion, conditions of defective antibody synthesis such as hypogammaglobulinemia, or technical errors attributed to improper handling of kit reagents.[65] False-negative results may also occur if the patient harbors a genetically diverse, recombinant strain of HIV, or an HIV-1 group O strain that is tested for by an assay that does not detect antibody to the group O virus. The likelihood of false negatives occurring has been reduced by implementation of fourth-generation tests that can identify HIV infection several days earlier than third-generation tests because they detect p24 antigen in addition to HIV antibodies.[69,72,73] This makes it possible for the infection to be diagnosed within 2 to 3 weeks after exposure.[63] However, there are rare situations in which a patient can persistently test negative for HIV antibody despite the presence of HIV RNA.[69]

False-positive results may also occur in these assays. These can result from several factors, including heat inactivation of serum before testing, repeated freezing and thawing of specimens, the presence of autoreactive antibodies, history of multiple pregnancies, severe hepatic disease, passive immunoglobulin administration, recent exposure to certain vaccines, and certain malignancies.[65] False positives can also result from specimen mix-up or mislabeling.[69,72] In addition, combination immunoassays cannot distinguish between HIV-1 and HIV-2 infection. Any positive results obtained from the initial ELISA or CLIA screen must therefore be confirmed by additional testing that can differentiate the two viruses.

Clinical Correlations

Seroconversion

By definition, seroconversion is the change of a serological test result from antibody negative to antibody positive. This occurs over time when the immune system is responding to HIV infection. Seroconversion can be detected by comparing the test results from two samples collected from the patient, the first soon after exposure to the virus and the second a few weeks later.

Rapid Tests for HIV Antibodies

Although ELISAs are ideal tests for the detection of HIV antibodies in clinical laboratories that perform large-volume batch testing, they require expensive instrumentation and skilled personnel with technical expertise and may have a turnaround time of a few days. To overcome these limitations and to encourage more patients to be tested, simple, rapid methods to screen for HIV antibody have been developed. These tests are available for use around the world and provide results within 30 minutes.

Several commercially available rapid tests have been approved by the FDA.[70] These kits detect antibodies to HIV-1 alone or to both HIV-1 and HIV-2; they can be used on serum, plasma, whole blood samples obtained by venipuncture or fingerstick, or, for some kits, oral fluid. Although each test has unique features, all are lateral flow or flow-through immunoassays that produce a colorimetric reaction in the case of a positive result. The flow-through assays require multiple steps in which the sample and reagents are added to a solid support encased in a plastic device, whereas the lateral flow assays involve a one-step procedure in which the patient sample migrates along the test strip by capillary action. With either procedure, the patient's sample is applied to a test strip or membrane containing HIV antigens. The antigen–antibody complexes bind to an enzyme-labeled conjugate or an antibody-binding (protein A) colloidal gold conjugate and are detected by a colorimetric reaction that produces a colored line or dot in the case of a positive result.[64,65,74,75] Interpretation of the results is made through visual observation of the test device and does not require instrumentation.

A primary use for rapid tests has been to screen for HIV infection; these are especially suitable in certain circumstances.[65,75] Rapid tests are ideal for use in resource-limited settings around the world that do not have access to expensive equipment and highly trained personnel. They are also beneficial in situations in which fast notification of test results is desired. For example, rapid results are important in guiding decisions to begin prophylactic therapy with antiretroviral drugs following occupational exposures because this therapy appears to be most effective when administered soon after exposure. Other situations in which rapid tests are advantageous include testing women whose HIV status is unknown during labor and delivery and testing patients in sexually transmitted disease clinics or emergency departments who are unlikely to return for their test results.

Although rapid tests are highly sensitive and specific, false positives can occur.[75] In addition, a negative test result does

not rule out early acute HIV infection.[76] For these reasons, rapid testing should be followed by the current recommended testing algorithm whenever possible.[63,69] Rapid tests that detect p24 antigen as well as HIV-1 and HIV-2 antibodies are under evaluation for use in HIV screening.[76]

Some rapid HIV kits are available through the Internet or over the counter for home testing; the FDA has recently approved one such kit for the testing of oral fluid.[70] These tests offer the advantages of convenience, privacy, and anonymity, and have the potential of encouraging more widespread screening among high-risk individuals.[77] However, false-negative results can occur because home tests are not as sensitive as conventional testing, and there is concern that some people who test positive might not seek confirmatory testing and the appropriate medical care.[77]

As we previously discussed, rapid immunoassays that differentiate between HIV-1 and HIV-2 have also been recommended by the CDC as confirmatory tests for samples that test positive in the HIV-1 antibody/HIV-2 antibody/p24 antigen combo screen.[69] These assays have several advantages over the previously recommended Western blot test (see the text that follows). Specifically, they detect HIV antibodies earlier, reduce the incidence of indeterminate results, have a shorter result turnaround time, are less costly, and can detect HIV-2 infections in addition to those caused by HIV-1.[69,72,73]

Western Blot

Recall that because of the possibility of obtaining false-positive results, all positive samples from HIV screening tests must be referred for testing with a more specific confirmatory method. The **Western blot test,** or immunoblot, for HIV antibodies was introduced in 1984 and was the most common method used for systematic confirmation of positive ELISA results from 1985 to 2014. This technique is more technically demanding than ELISA but can provide an antibody profile of the patient sample that reveals the specificities to individual HIV antigens. Several commercial kits are available for this type of testing and can provide results within a few hours.[64,74,78]

Western blot kits are prepared commercially as nitrocellulose or nylon strips containing individual HIV proteins that have been separated by polyacrylamide gel electrophoresis and blotted onto the test membrane. The protein antigens are derived from HIV virus grown in cell culture. Antigens with low molecular weight migrate most rapidly and are therefore positioned toward the bottom of the test strip, whereas antigens of high molecular weight remain toward the top of the membrane.

The testing laboratory then applies patient serum to the test strip. During the incubation period, any HIV antibodies present in the sample will bind to their corresponding antigens on the test membrane. Unbound antibody is then removed by washing. Next, an anti-human immunoglobulin with an enzyme label (i.e., the conjugate) is added directly to the test strip and binds to specific HIV antibodies from the patient sample. Unbound conjugate is removed by washing, whereas bound conjugate is detected after adding the appropriate substrate, which produces a chromogenic reaction. Colored bands appear in the positions where antigen-specific HIV antibodies are present. Separate HIV-1- and HIV-2-specific Western blot tests must be used to test for antibodies to each virus.[64,65,78]

In HIV-1 infection, antibodies to the *gag* proteins p24 and p55 appear relatively early after exposure to the virus, but tend to decrease or become undetectable as clinical symptoms of AIDS appear.[66] Antibodies to the envelope proteins gp41, gp120, and gp160 appear slightly later but remain throughout all disease stages in an HIV-infected individual, making them a more reliable indicator of the presence of HIV.[79] Other antibodies commonly detected by this method are those directed against *pol* proteins p51 and p66, whereas antibodies against the regulatory gene products are usually not detectable by conventional methods.[65,66] The bands produced by the test sample are examined visually for the number and types of antibodies present. Densitometry can also be performed to quantitate the intensity of the bands, which would reflect the amount of each antibody produced. Patients can be followed over time to determine whether there is a change in the antibody pattern.

Because Western blot testing is highly dependent on the laboratorian's technical skill and subjective interpretation, it is generally performed only in specialized reference laboratories that have an adequate proficiency testing program. Positive and negative control sera must be included in the test run to provide quality control. For the test to be valid, the negative control should produce no bands and the positive control should be reactive with p17, p24, p31, gp41, p51, p55, p66, and gp120/160. A negative test result for the patient sample is reported if either no bands are present or if none of the bands present correspond to the molecular weights of any of the known viral proteins.[65]

Criteria for determining a positive test result have been published by the Association of State and Territorial Public Health Laboratory Directors and CDC, the Consortium for Retrovirus Serology Standardization, the American Red Cross, and the FDA.[65,66,78,79] According to these criteria, a result should be reported as positive if at least two of the following three bands are present: p24, gp41, and gp120/gp160 **(Fig. 24–6).**

Specimens that have some of the characteristic bands present but do not meet the criteria for a positive test result are considered to be *indeterminate.* This result may be produced if the test serum is collected in the early phase of seroconversion or if the serum contains antibodies that cross-react with some of the immunoblot antigens, producing false-positive results. False positives may be caused by antibodies to contaminants from the cells used to culture HIV to prepare the antigens for the test; to autoantibodies, including those directed against HLA, nuclear, mitochondrial, or T-cell antigens; or to antibodies produced after vaccinations.[64,65]

The use of recombinant antigens instead of viral lysates has reduced the incidence of false-positive results. If an indeterminate test result is obtained, it is recommended that the test be repeated with the same or a fresh specimen; if the test is still indeterminate, testing may be performed with a new specimen obtained 4 to 6 weeks later. If the pattern converts to positive, it can be concluded that the first specimen was obtained during the early phase of seroconversion. Failure of an indeterminate

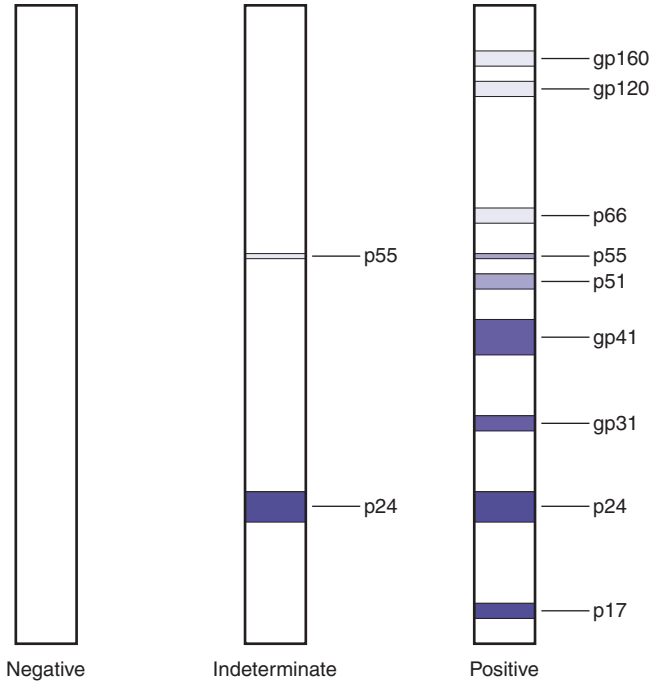

FIGURE 24–6 Western blot, showing results from a negative sample, an indeterminate sample, and a positive sample.

test pattern to convert to positive after a few weeks strongly suggests that the pattern is caused by a false-positive test rather than HIV infection.[9,65] During this period, HIV nucleic acid testing can be performed to provide more conclusive results (see the text that follows).

Because of its relative insensitivity, level of technical difficulty, and long turnaround time to obtain results, the Western blot test is inappropriate for use as an initial screen for HIV infection.[65] For these same reasons, as we previously discussed, the CDC has recommended that the Western blot be replaced with rapid HIV-1/HIV-2 antibody tests as the standard method for confirmation of positive screening results.[69] Comparison studies have shown that rapid tests are more suitable confirmatory tests because they can be performed more quickly, detect infection earlier, reduce the incidence of indeterminate results, are less expensive, and can detect HIV-1 and HIV-2 infections simultaneously.[69,72,73] Although other confirmatory methods have been developed, including IFA, radioimmunoprecipitation assay (RIPA), and line immunoassays, they have not been widely used in clinical settings.[64,65]

Qualitative Nucleic Acid Tests (NATs)

Qualitative nucleic acid tests (NATs) are used to determine whether or not a detectable level of HIV nucleic acid is present in human plasma. These tests can be used to screen for infection or make an initial patient diagnosis. They are particularly beneficial in cases where serological results are inconclusive such as in very early infection or in the diagnosis of infants, where presence of maternal antibodies can confuse test results.

As previously mentioned, they are an integral part of the 2014 CDC recommendations for laboratory testing for HIV. The tests are used to resolve discrepancies between a positive result in the initial antigen–antibody combo assay and the follow-up HIV-1/HIV-2 antibody differentiation assay.[69] If the HIV-1 antibody/HIV-2 antibody/p24 antigen test is nonreactive, but the NAT is reactive, then this result is considered evidence for acute HIV-1 infection. If the HIV-1/HIV-2 antibody test is indeterminate and the NAT is reactive, this suggests that HIV-1 antibodies were indeed present. In contrast, if the NAT is nonreactive and the HIV-1/HIV-2 antibody test is reactive or indeterminate, a false-positive result on the initial HIV antigen–antibody combo assay is indicated.

HIV nucleic acid testing is highly sensitive; it is able to detect HIV RNA about 10 to 12 days after infection.[20,63] Methods approved by the FDA include a qualitative polymerase chain reaction (PCR)-based assay to screen donors of whole blood, blood components, or organs, and a transcription-mediated amplification (TMA) assay for diagnosis.[70]

Disease Monitoring

Once a diagnosis of HIV infection has been established, it is essential to monitor patients over time to evaluate the effectiveness of their antiretroviral therapy. This way, signs of disease progression can be detected early and guide decisions about further treatments. Two laboratory markers are routinely used to monitor patients with HIV infection for disease progression and guide their treatments: (1) the peripheral blood CD4 T-cell count, which is considered to be the best indicator of immune function in HIV-infected individuals, and (2) the HIV-1 RNA level, or "viral load," which reflects patient responses to ART.[9,44,80] Each of these laboratory markers will be discussed in detail in the text that follows.

CD4 T-Cell Enumeration

Destruction of the CD4 T lymphocytes is central to the immunopathogenesis of HIV infection and CD4 lymphopenia has long been recognized as the hallmark feature of AIDS. Therefore, enumeration of CD4 T cells in the peripheral blood has played a central role in evaluating the degree of immune suppression in HIV-infected patients for many years. Normally, the peripheral blood CD4 T-cell count ranges from 450 to 1,500 cells/μL (average, 1,000 cells/μL).[81] In untreated patients, there is a progressive decline in the number of CD4 T cells during the course of infection **(Fig. 24–7).** The rate of decline varies among patients and can be rapid or gradual. As we previously discussed, the CDC classification system uses CD4 T-cell counts to place patients into various stages of HIV infection, with those whose counts are below 200/μL being categorized as having stage 3 infection.[69]

Another important clinical application of CD4 T-cell counts is to monitor the effectiveness of antiretroviral therapy. Physicians use CD4 T-cell values to help determine whether a change in ART is necessary and if prophylactic drugs for certain opportunistic infections should be administered. According to guidelines published by the U.S. Department of Health and Human Services, a baseline CD4 T-cell measurement should

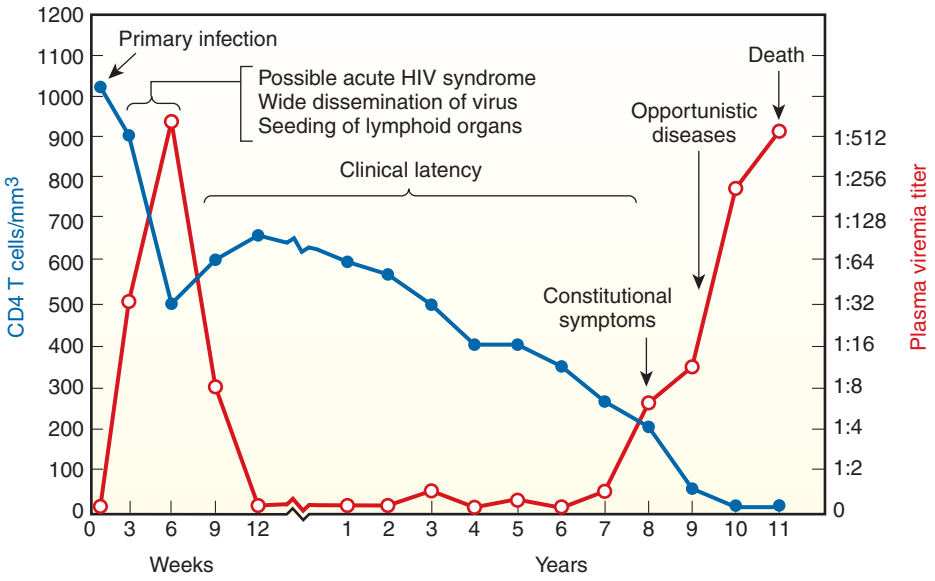

FIGURE 24–7 Typical CD4 T-cell numbers and plasma viremia during the natural course of HIV infection. *(Adapted from Pantaleo G, Graziosi C, Fauci AS.* The immunopathogenesis of human immunodeficiency virus infection. N Engl J Med. *1993;328:327–335.)*

be performed before the initiation of ART, then every 3 to 6 months for the first 2 years. Thereafter, annual measurements can be performed in patients whose CD4 T-cell counts have consistently remained between 300 and 500 cells/μL.[44] More frequent testing should be performed if there is a change in the patient's clinical status. If there is a decline in the CD4 T-cell count of greater than 25%, the physician may change the CART.[9] Patients with a CD4 T-cell count below 200/μL are placed on prophylactic therapy for *Pneumocystis jiroveci* pneumonia, whereas those with a count of less than 50/μL are given prophylactic treatment for *Mycobacterium avium* complex.[9,44]

The gold standard for enumerating CD4 T cells is immunophenotyping with data analysis by **flow cytometry** (see Chapter 13). The CDC has published guidelines to standardize the performance of CD4 T-cell determinations by flow cytometry.[81-83] Early guidelines referred to a dual-platform technology in which both a flow cytometer and a hematology analyzer were required to make CD4 T-cell measurements. According to this protocol, the percentage of CD4 T cells in a sample is determined by dividing the number of lymphocytes positive for the CD4 marker by the total number of lymphocytes counted by the flow cytometer, according to the following equation:

$$\%\ \text{CD4 T Cells} = \frac{\#\ \text{CD4 Lymphocytes}}{\text{Total \# Lymphocytes}} \times 100$$

The percentage of CD4 T cells obtained for the patient sample is compared with a reference range established by the laboratory performing the test.

In the dual-platform approach, absolute numbers of CD4 T cells were calculated by multiplying the absolute number of lymphocytes (determined by the complete blood cell [CBC] count and differential from a hematology analyzer) by the percentage of CD4 T cells in the sample, according to the following equation:

$$\text{Absolute \# CD4 T Cells} = \text{WBC Count} \times \%\ \text{Lymphocytes} \times \%\ \text{CD4 T Cells}$$

The absolute CD4 T-cell count is then compared with the reference range, which is typically from 450 to 1,500 cells/μL peripheral blood.[81]

Current technologies employ multicolor monoclonal antibody panels in a single-platform approach, which allows CD4 T-cell percentages and absolute numbers to be obtained from one tube using a single instrument, the flow cytometer.[81,82] This is made possible by counting CD4+ T cells in a precisely measured blood volume or by incubating the sample with a known number of commercially available fluorescent microbeads, which function as an internal calibrator. The counts can then be determined by specific flow cytometry software, according to the following equation:[82]

$$\frac{\#\ \text{of Events in the Bright CD45 Region}}{\#\ \text{of Events in the Microfluorosphere Region}} \times \frac{\text{Total \# Microspheres Added}}{\text{Volume of Blood Added}}$$

As with the dual-platform technology, lymphocytes are selected for analysis (or "gated") on the basis of their low-side scatter and ability to stain brightly with CD45 antibody.

Connections

Flow Cytometry

In flow cytometry, histograms display the patterns of light scatter and fluorescence emitted by individual cell populations. Four-color immunofluorescence assays are used in which the lymphocytes are differentiated from other cell types in the sample on the basis of their low-side scatter and their ability to stain brightly with fluorescent-labeled antibody to the CD45 marker, which is present on all WBCs. The side scatter represents the amount of cell complexity as determined by properties such as granularity and membrane irregularity. Because lymphocytes are more round and less granular than other types of WBCs, they reflect the laser light to a smaller degree and can be characterized by their low light scatter.

In addition to CD4+ T-cell percentages and absolute numbers, the ratio of CD4 T cells to CD8 T cells may be reported to assess the dynamics between the two T-cell populations. In HIV-infected patients, particularly those with AIDS, the large decrease in the number of CD4+ T cells, along with a possible increase in CD8+ cytotoxic T cells, results in an inverted ratio, or a ratio that is less than 1:1.

Although flow cytometry is the accepted gold standard for enumeration of CD4 T cells, it is a costly method that requires the need for highly skilled personnel, a stable electricity supply, refrigeration of reagents, and regular instrument maintenance.[84,85] These conditions are not available in many areas of the world. As a result, there has been much interest in developing simpler, less expensive methods to determine CD4 T-cell measurements. This need has led to the manufacture of miniaturized flow cytometers that can be easily operated by smaller laboratories.[81,86] These instruments are dedicated for CD3/CD4 measurements and use simple procedures that require a small volume of reagents.

Another major advance has been development of point-of-care devices that can be easily transported to remote regions.[84,86] These devices can use blood obtained by fingerstick or venipuncture and contain stable, dried reagents that can withstand hot, humid temperatures. Disposable test cassettes are used to capture CD4+ cells with labeled antibodies, and the results are analyzed by small, portable instruments that can be powered by a rechargeable battery. These methods are particularly suitable for resource-limited settings in developing countries, where the incidence of HIV infection is significant. Although the performance of these less complex methods is still under evaluation, preliminary studies show that there is reasonable agreement with conventional flow cytometry and they have already made an impact in providing wider access to a laboratory test that is an integral part of patient care for HIV-infected individuals.[84,85]

Quantitative Viral Load Assays

Quantitative **viral load tests** measure the amount of circulating HIV nucleic acid and play an essential role in helping physicians predict disease progression, monitor patient response to antiretroviral therapy, and guide treatment decisions.[44,78] The amount of HIV RNA, or viral load, in a patient's plasma reflects the natural history of HIV infection in that individual.[9] HIV RNA levels become detectable about 11 days after infection and rise to very high levels shortly thereafter, during the initial burst of viral replication. Typically, over a period of a few months, the viral load drops as the individual's immune system clears viral particles from the circulation and a stable level of plasma HIV RNA, known as the "set point," is achieved (see Fig. 24–7). In untreated individuals, this level can persist for a long time and then rise again later as the immune system deteriorates and the patient progresses to AIDS. In contrast, successful therapy with antiretroviral drugs will result in a drop in the viral load to an undetectable level, usually by 8 to 24 weeks after initiation of ART.[9,44]

Studies performed by the Multicenter AIDS Cohort and other groups have demonstrated that information obtained from viral load tests has prognostic value.[44,80] These studies have shown that baseline plasma viral load values obtained in patients before the start of antiretroviral therapy are an important predictor of disease progression because a higher number of HIV RNA copies/mL of plasma are associated with more rapid development of an AIDS-defining illness or AIDS-related death.

Viral load tests are used routinely to monitor the effectiveness of antiretroviral therapy in HIV-infected patients and play an essential role in the clinical management of these individuals. Patients who attain a lower number of HIV-RNA copies/mL of plasma are more likely to achieve a longer treatment response.[44,80] The optimal goal of therapy is to reach undetectable levels of HIV RNA (i.e., < 20 to 75 copies/mL, depending on the assay).[44,78,87] Patients who have a persistently elevated viral load should undergo resistance testing to determine if an alternative drug regimen is needed (see *Drug Resistance Testing* in the text that follows).[44,46]

The U.S. Department of Health and Human Services recommends that plasma HIV RNA testing be performed before antiretroviral therapy begins to obtain a baseline value; testing should be performed periodically thereafter to determine the effectiveness of the therapy.[44] To obtain an accurate assessment of viral load dynamics in a single patient, it is recommended that the same assay be used for sequential viral load measurements because values may differ between different molecular tests.[78] A change in viral load is considered to be significant if there is at least a threefold or 0.5 $\log_{10}$ increase or decrease in the number of copies/mL.[44,78] A change in the antiretroviral therapy protocol (or initiation of therapy for individuals who have not received ART) is recommended for patients whose HIV RNA levels and CD4 T-cell counts reach the critical values established by the AIDS Clinical Trial Group or the WHO.[44,46]

Methods

Viral load assays are based on amplification methods that increase the number of HIV RNA copies (or their derivatives) in test samples to detectable levels. Several amplification methods have been developed for this purpose, including PCR; the **branched chain DNA** assay (bDNA); and nucleic acid sequence-based amplification (NASBA), which amplifies HIV RNA. The basic principles of PCR and bDNA are discussed briefly here and are covered in more detail in Chapter 12. NASBA, which is based on the amplification of HIV RNA, is a technically complex method that is not suitable for clinical laboratory settings.

Polymerase Chain Reaction (PCR). Two kinds of PCR methods have been developed to detect HIV nucleic acid: the RT-PCR and. more recently, quantitative (real-time) PCR, also known as qPCR. A commercial RT-PCR was the first assay to be licensed by the FDA for quantitative measurement of circulating HIV nucleic acid. The basic principle of this test is to amplify a DNA sequence that is complementary to a portion of the HIV RNA genome.[78,88] In this assay, HIV RNA is isolated

from patient plasma by lysis of the virions and precipitation with alcohol. The RNA is treated with a thermostable DNA polymerase enzyme that has both reverse-transcriptase activity and the ability to initiate DNA synthesis in the presence of the appropriate reagents. The reverse-transcriptase activity of the enzyme transcribes the HIV RNA into complementary DNA (cDNA). The cDNA is then amplified by standard PCR methodology (see Chapter 12).

Although the development of standard RT-PCR methods was a revolution in molecular testing for HIV infection, they have several disadvantages, including a limited dynamic range (i.e., the range of HIV RNA copies per mL that can be detected). In addition, they are highly susceptible to cross-contamination with extraneous nucleic acid. For these reasons, these assays have largely been replaced with qPCR assays that can detect and quantify the PCR products as they are being produced (see Chapter 12).[78,88] This is accomplished by adding a fluorescent probe that binds to the **amplicon** during the reaction. The PCR amplification primers target highly conserved regions of the HIV-1 *gag* or *pol* genes. An internal control consisting of a different nucleic acid sequence is simultaneously amplified with each sample to compensate for the effects of inhibition and allow for more accurate quantitation. qPCR is highly sensitive and can detect a broad range of RNA copies, from 20 copies/mL to 10 million copies/mL.[78] Commercially available assays can also detect all subtypes of groups M, N, O, and recombinant strains of HIV.[78]

Branched-Chain DNA Assay (bDNA). In contrast to RT-PCR, which involves amplification of the HIV target sequence, the bDNA method is based on amplifying the detection signal generated in the reaction. This is accomplished by using a solid-phase sandwich **hybridization** assay that incorporates multiple sets of oligonucleotide probes and hybridization steps to create a series of "branched" molecules.[78,88] First, RNA isolated from lysed virions in patient plasma is captured on wells of a microtiter plate coated with a number of probes. The captured RNA is then hybridized with branched amplifier probes and incubated with an enzyme-labeled probe that will bind to the DNA branches. Finally, a chemiluminescent substrate is added and color change is measured with a luminometer. Quantitative results are generated from a standard curve.

The bDNA test can detect 75 to 500,000 copies of HIV RNA/mL of plasma. As compared with real-time PCR, the bDNA has higher reproducibility and higher throughput (rate of producing results). However, it requires a larger sample volume, lacks an internal control, and has a lower specificity. This method is most conducive for laboratories with high testing volumes.[78,88]

Alternatives to Conventional Viral Load Tests

Although conventional HIV viral load tests have had a large impact on patient care, they pose many difficulties for resource-limited areas of the world, including the need for a continuous power supply, refrigeration, costly instrumentation, skilled personnel to perform the testing, and adequate processing time to obtain results.[76,84] Lack of accessibility to adequate testing has resulted in higher rates of failed responses to ART and development of viral resistance in these settings.[46,84] As a result, there has been much interest in the development of simpler technologies to determine viral load. These include point-of-care devices such as closed cartridges that can provide semiquantitative analysis with simpler instrumentation in a shorter period of time. Many of these technologies, including loop-mediated and helicase-dependent amplification, are based on isothermal reactions in which the temperature remains constant.[76] In addition, the use of dried blood spots could eliminate the need for venous blood draw. Costs could be further reduced by pooling of blood samples; if a pool tests negative, then no further testing would be needed. Reactive pools could be further subdivided to identify positive individuals.[84] Further development and implementation of these innovative methods into resource-limited settings could allow clinicians to better monitor the effectiveness of ART in individuals living in these regions and have a profound impact on combating HIV infection throughout the world.

Drug-Resistance Testing

As we previously discussed, HIV is a rapidly replicating virus that has an intrinsically high rate of mutation. Because of these properties, it is possible for drug-resistant subpopulations of the virus to emerge during the course of antiretroviral therapy. Drug resistance has been a major reason for the failure of ART in many people. As a result, laboratory tests have been developed to assess drug resistance patterns; these tests have had a major impact on guiding the selection of optimal ARTs for individual patients.

Two types of laboratory methods can be used to test for drug resistance: genotype resistance assays and phenotype resistance assays.[44,78,88] Genotype resistance assays are performed more frequently than phenotype resistance assays because they are less expensive, more widely available, and have a shorter turnaround time.

Genotype resistance assays detect mutations in the reverse-transcriptase and protease genes of HIV. These tests are available commercially and can be performed in clinical laboratory settings. In these tests, RNA is isolated from patient plasma, the desired genes are amplified by RT-PCR, and the products are analyzed for mutations associated with drug resistance by automated DNA sequencing.[78,88] The nucleotide sequences of the genes of interest are identified and entered into a database, where they are compared with the corresponding sequences in wild-type HIV. The results are analyzed by commercially available software and reported qualitatively as "resistance," "possible resistance," or "no evidence of resistance" for each drug tested. Results can generally be obtained in 1 to 2 weeks. Although genotyping tests have many advantages as compared with phenotypic methods, they can identify only known mutations and cannot assess the effects of combinations of individual mutations on drug resistance.[78]

Phenotype resistance assays determine the ability of clinical isolates of HIV to grow in the presence of antiretroviral drugs.[44,78,88] In these assays, recombinant viruses are created by inserting the reverse-transcriptase, protease, integrase, or *env* gene sequences from HIV RNA in the patient's plasma into a laboratory reference strain of HIV and transfecting the recombinant virus into mammalian cells. Varying concentrations of antiviral drugs are incubated with the transfected cells and the IC_{50} values, or drug concentrations needed to suppress the replication of the patient's viral isolate by 50%, are calculated. These values are then compared with the IC_{50} values of cells transfected with a reference strain of HIV in order to determine drug resistance. The major advantage of phenotypic assays is that they measure drug susceptibility directly, on the basis of all mutations present in the patient's isolate. However, these assays are expensive and have a longer turnaround time than genotypic assays (2 to 3 weeks). In addition, phenotypic assays involve sophisticated technologies and are only performed by a few highly specialized reference laboratories.[78]

Both genotypic and phenotypic assays require that a viral load of at least 500 to 1,000 copies of HIV RNA/mL be present in the test sample and for the resistant virus to constitute more than 25% of the total viral population in the patient to produce detectable results.[78,88] Despite these limitations, studies have shown that patients undergoing drug-resistance testing, particularly by genotyping methods, have a better chance of receiving antiretroviral therapy regimens that are more likely to result in greater reductions in viral load.[78,89,90] Therefore, the U.S. Department of Health and Human Services recommends that drug-resistance testing be performed in individuals before initiating antiretroviral therapy, in patients in whom CART has failed as evidenced by viral load values that have not been optimally reduced, and in all HIV-positive pregnant women.[44,78] Genotypic testing is recommended for patients upon entry into medical care for HIV or after one to two unsuccessful treatment regimens, because of its lower cost, faster turnaround time, and greater sensitivity for detecting wild-type/resistant virus mixtures. The addition of phenotypic testing is recommended when patients are thought to harbor HIV mutants with complex drug-resistance patterns, especially to protease inhibitors.[44]

Specialized tests have been developed in addition to the standard tests. These include genotypic tests for resistance to integrase inhibitors, fusion inhibitors, or *env* mutations; genotypic or phenotypic assays for co-receptor tropism; and typing for the HLA-B*27 allele.[78,91] Analysis of the co-receptor tropism is used to determine whether a patient is eligible for treatment with the drug maraviroc, which inhibits entry of HIV strains that use the CCR5 co-receptor to bind to host cells. Early tests for CCR5 tropism were phenotypic assays, but a more rapid genotypic assay that sequences the third variable loop of the HIV-1 *env* gene is now available.[78,92] Pharmacogenetic screening for HLA-B*5701 is helpful for patients who are being considered for treatment with the nucleoside reverse-transcriptase inhibitor abacavir because this allele has been associated with development of hypersensitivity to the drug. The test is typically performed by PCR amplification of the allele, followed by hybridization with sequence-specific oligonucleotide probes.[91]

Testing of Infants Younger Than 18 Months

Serological tests are not reliable in detecting HIV infection in children younger than 18 months of age because of placental passage of IgG antibodies from an infected mother to her child. These maternal antibodies persist in the bloodstream of the infant during the first year of life (or longer in a small proportion of infants) and can confuse the interpretation of serological results from infant samples.[93,94] Thus, a child born to an HIV-positive mother may test positive for HIV antibody during the first 18 months of life even though the child is not infected.

Because of the difficulties with serological testing, HIV infection in infants is best diagnosed using molecular methods.[44] A qualitative HIV-1 DNA PCR test is the preferred method for this purpose. This test, which detects proviral DNA within the infants' peripheral blood mononuclear cells, has a sensitivity of 55% at birth, increasing to greater than 90% at 2 to 4 weeks, and 100% by 3 to 6 months of age; and a specificity of 99.8% at birth and 100% in infants older than 1 month.[44] Alternatively, quantitative HIV RNA assays may be used to diagnose HIV infection in infants. Although they are less sensitive than DNA assays, RNA tests can be used to provide a baseline viral load measurement and are more likely to detect infections with strains other than subtype B.[44,94] They can also be used as a confirmatory test for infants who initially had a positive HIV DNA test.

It is recommended that nucleic acid tests for HIV be performed in infants with known perinatal exposure at the ages of 14 to 21 days, 1 to 2 months, and 4 to 6 months, and possibly at birth for infants at high risk for HIV infection.[44] A positive test result should be confirmed by repeat testing on a second specimen because false positives can occur. Two or more negative test results (the first at greater than 1 month and the second at greater than 4 months) provide evidence for the absence of HIV infection and may be confirmed by serological tests at 12 to 18 months of age. The HIV status of breastfed infants, who are continually exposed to the virus, cannot be determined accurately until breastfeeding is stopped.[94]

Increased emphasis on screening pregnant women for HIV infection should also help in the identification of HIV-positive infants.[95] Rapid tests for HIV antibody should be performed on women whose HIV status is unknown and on their newborn infants soon after birth.[94] Prompt detection of HIV infection in newborns is important because infected infants have a better prognosis when CART is started early and can benefit from treatment with prophylactic drugs for opportunistic infections.[16,44,46]

SUMMARY

- Human immunodeficiency virus type 1 (HIV-1) is responsible for the majority of AIDS cases throughout the world. A related virus, HIV-2, may also cause AIDS but is generally less pathogenic.
- Transmission of HIV occurs by three major routes: (1) intimate sexual contact, (2) contact with contaminated blood or body fluids, or (3) vertical transmission from infected mother to her fetus or infant.
- HIV belongs to the retrovirus family, which contains RNA as the genetic material from which DNA is transcribed. HIV has three main structural genes: *gag,* which codes for the core proteins of the virus such as p24; *pol,* which codes for the enzymes reverse transcriptase, integrase, and protease; and *env,* which encodes the envelope proteins gp120 and gp41.
- The primary target cells for HIV are CD4 T lymphocytes and macrophages, which possess some surface CD4. The CD4 molecule acts as a receptor for attachment of the virus by binding to the gp120 envelope protein. Following attachment, entry of the virus into the host cells is mediated by the chemokine co-receptors, CXCR4 and CCR5.
- A burst of viral replication occurs after initial infection followed by a period of latency during which viral DNA becomes integrated into the host genome as a provirus.
- Viral production slows down as the host's immune response develops and keeps the virus in check. The host produces neutralizing antibodies, which prevent the virus from infecting neighboring cells and develops HIV-specific CTLs that lyse virus-infected target cells. Innate defenses are also activated.
- Although the immune responses of the host reduce the level of HIV replication, they are usually not sufficient to completely eliminate the virus. HIV can escape these responses by undergoing rapid genetic mutations that generate altered antigens, downregulating production of class I MHC molecules on the surface of the infected target cells, and existing in a latent proviral state.
- The hallmark feature of HIV infection is a decline in the number of CD4 Th cells during the natural course of infection. This decline results in an immunodeficiency that affects both cell-mediated and humoral antibody responses to a variety of antigens.
- The clinical course of untreated HIV infection begins with an acute phase in which patients may experience flu-like symptoms. This is followed by a latent, asymptomatic period that lasts an average of 10 years. The infection culminates in AIDS, which is characterized by profound immunosuppression with life-threatening opportunistic infections and malignancies.
- The 2014 CDC case definition of HIV infection is based on laboratory criteria or clinical evidence and classifies patients into one of five stages based on CD4 T-cell measurements and the presence of opportunistic illnesses.
- Treatment with antiretroviral therapy (ART) is recommended for all HIV-infected persons and has resulted in a significant delay in disease progression, decreased mortality, and reduction in perinatal transmission.
- Several classes of antiretroviral drugs have been developed—nucleoside analogue reverse-transcriptase inhibitors, nonnucleoside reverse-transcriptase inhibitors, protease inhibitors, integrase inhibitors, and entry inhibitors. These drugs are most effective when administered in combinations known as CART (combination antiretroviral therapy) or HAART (highly active antiretroviral therapy).
- The algorithm recommended by the CDC and the Association of Public Health Laboratories in 2014 to screen for HIV infection consists of a sequence of laboratory tests. The initial test is a fourth-generation ELISA that simultaneously detects HIV-1 antibody, HIV-2 antibody, and p24 antigen. Positive test results must be confirmed by a rapid test that discriminates between HIV-1 antibody and HIV-2 antibody. Any samples that give discrepant results should undergo nucleic acid testing.
- Rapid screening tests for HIV antibodies are typically sensitive, lateral flow assays that can provide results in fewer than 30 minutes. These tests are especially suitable for use in certain situations, including resource-limited settings, occupational exposures, labor and delivery, and clinics or emergency departments where patients are unlikely to make a return visit.
- The Western blot, which was used for many years to confirm positive HIV antibody screening test results, detects antibody specificities to individual HIV antigens. It is no longer recommended for initial screening and diagnosis of HIV because it is labor intensive, relatively insensitive, and has a long result turnaround time.
- HIV-infected patients are routinely monitored using two laboratory measurements: CD4 T-cell enumeration and HIV viral load.
- Peripheral blood CD4 T-cell counts and percentages are an excellent indicator of immune function and are routinely measured by multicolor immunofluorescence staining followed by analysis with flow cytometry. These measurements are used to stage HIV-infected patients and to monitor patients undergoing ART. Declining numbers can indicate if there is a need to change antiretroviral therapy or initiate prophylactic therapy for opportunistic infections.
- Qualitative nucleic acid tests can be used to screen for HIV infection or make an initial patient diagnosis. Quantitative tests, which measure the amount of HIV nucleic acid circulating in patient plasma, are known as *viral load tests.* These tests have had an important impact on the clinical management of HIV-infected patients by allowing physicians to predict disease progression, monitor patient response to antiretroviral therapy, and guide treatment decisions.
- Nucleic acid tests are performed by one of three molecular methods: reverse-transcriptase polymerase chain reaction (RT-PCR), a method that converts HIV RNA into cDNA and then amplifies the cDNA generated; qPCR, a quantitative

real-time RT-PCR method, and the branched chain DNA assay (bDNA), which amplifies a labeled signal bound to a test plate.
- Drug-resistance testing can be performed by genotypic assays that use molecular methods or by phenotypic assays in which HIV replication in clinical isolates is assessed in the presence of varying concentrations of antiretroviral drugs.
- Diagnosis of HIV in neonates is more complex than testing in adults. The presence of maternally acquired antibody in newborns makes tests for HIV antibody unreliable until a child is over 18 months old.
- Nucleic acid testing is recommended for diagnosis of HIV infection in infants younger than 18 months. The preferred method is a qualitative PCR that detects HIV proviral DNA in the infant's peripheral blood mononuclear cells. Careful monitoring of HIV-infected mothers and early testing of infants at risk is recommended to facilitate prompt medical intervention.

CASE STUDIES

1. A young woman recently discovered that her boyfriend tested HIV-positive. She was concerned that she may have also contracted the infection because she had experienced flu-like symptoms 1 month ago. She decided to visit her physician for a medical evaluation.

Questions

a. What initial laboratory test should be performed on the young woman to determine if she has been exposed to HIV?
b. If the woman tests positive in the initial evaluation, what follow-up testing should be performed to confirm the results?
c. If the woman's test results are confirmed to be positive, what tests should be done to monitor her over time?

2. A pregnant woman had used intravenous drugs in the past and recently discovered that she was HIV-positive. She was concerned that her baby would also contract HIV infection and discussed this with her physician.

Questions

a. How is HIV infection transmitted from mother to infant and what measures should be taken to reduce the risk of HIV infection to the infant?
b. Should testing for HIV antibody be performed to determine if the infant is HIV-positive after birth? Explain your answer.
c. What type of laboratory testing would be best to evaluate the infant for HIV infection after birth?

REVIEW QUESTIONS

1. All of the following describe HIV *except*

a. it possesses an outer envelope.
b. it contains an inner core with p24 antigen.
c. it contains DNA as its nucleic acid.
d. it is a member of the retrovirus family.

2. HIV virions bind to host T cells through which receptors?

a. CD4 and CD8
b. CD4 and the IL-2 receptor
c. CD4 and CCR5
d. CD8 and CCR2

3. Antibodies to which of the following viral antigens are usually the first to be detected in HIV infection?

a. gp120
b. gp160
c. gp41
d. p24

4. Which of the following is typical of the latent stage of HIV infection?

a. Proviral DNA is attached to cellular DNA.
b. Large numbers of viral particles are synthesized.
c. A large amount of viral RNA is synthesized.
d. Viral particles with no envelope are produced.

5. The decrease in T-cell numbers in HIV-infected individuals is caused by

a. lysis of host T cells by replicating virus.
b. fusion of the T cells to form syncytia.
c. killing of the T cells by HIV-specific cytotoxic T cells.
d. all of the above.

6. The most common means of HIV transmission worldwide is through

a. blood transfusions.
b. intimate sexual contact.
c. sharing of needles in intravenous drug use.
d. transplacental passage of the virus.

7. The drug zidovudine is an example of a
   - **a.** nucleoside analogue reverse-transcriptase inhibitor.
   - **b.** nonnucleoside reverse-transcriptase inhibitor.
   - **c.** protease inhibitor.
   - **d.** fusion inhibitor.

8. False-negative test results in a laboratory test for HIV antibody may occur because of
   - **a.** heat inactivation of the serum before testing.
   - **b.** collection of the test sample before seroconversion.
   - **c.** interference by autoantibodies.
   - **d.** recent exposure to certain vaccines.

9. Which of the following combinations of bands would represent a positive Western blot for HIV antibody?
   - **a.** p24 and p55
   - **b.** p24 and p31
   - **c.** gp41 and gp120
   - **d.** p31 and p55

10. The fourth-generation ELISA tests for HIV detect
    - **a.** HIV-1 and HIV-2 antigens.
    - **b.** HIV-1 and HIV-2 antibodies.
    - **c.** p24 antigen.
    - **d.** HIV-1 antibodies, HIV-2 antibodies, and p24 antigen.

11. The conjugate used in the fourth-generation ELISA tests for HIV consists of enzyme-labeled
    - **a.** anti-human immunoglobulin.
    - **b.** HIV-1- and HIV-2-specific antibodies.
    - **c.** HIV-1- and HIV-2-specific antigens.
    - **d.** HIV-1- and HIV-2-specific antigens plus antibody to p24.

12. The characteristic laboratory finding in HIV infection is
    - **a.** decreased numbers of CD4 T cells.
    - **b.** decreased numbers of CD8 T cells.
    - **c.** decreased numbers of CD20 B cells.
    - **d.** decreased immunoglobulins.

13. Which of the following tests is currently recommended by the CDC to confirm a positive screening test result for HIV infection?
    - **a.** Rapid test for HIV-1 and HIV-2 antibodies
    - **b.** Western blot
    - **c.** Molecular testing for HIV RNA
    - **d.** HIV viral culture

14. Which of the following tests would give the *least* reliable results in a 2-month-old infant?
    - **a.** CD4 T-cell count
    - **b.** ELISA for HIV antibody
    - **c.** PCR for HIV proviral DNA
    - **d.** p24 antigen

15. Which of the following measurements are routinely used to monitor patients with HIV infection who are undergoing antiretroviral therapy?
    - **a.** HIV antibody titer
    - **b.** p24 antigen levels
    - **c.** CD4 T-cell and CD8 T-cell counts
    - **d.** CD4 T-cell count and HIV RNA copy number

25 Immunization and Vaccines

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LEARNING OUTCOMES

After finishing this chapter, you should be able to:

1. Differentiate between active immunity, passive immunity, and adoptive immunity.
2. Recognize examples of active immunity, passive immunity, and adoptive immunity.
3. Discuss the historical evolution of vaccines from the early contributions of Edward Jenner through modern approaches to producing next generation vaccines.
4. Define *vaccine, toxoid, attenuation, adjuvant,* and *recombinant protein vaccine*.
5. Describe the composition of live attenuated vaccines, inactivated vaccines, and subunit vaccines, and contrast their advantages and limitations. Provide examples of each type of vaccine.
6. Explain how factors that influence the immune response to vaccines determine the ways in which vaccines are administered.
7. Recognize examples of adjuvants and explain the mechanisms by which they enhance the immune response to vaccines.
8. Contrast the benefits and adverse effects associated with vaccines.
9. Differentiate between standard human immune serum globulin and specific human immune serum globulin and their clinical applications.
10. Differentiate between monoclonal antibodies, chimeric antibodies, humanized antibodies, and fully human antibodies in terms of their structure and nomenclature.
11. Discuss some of the clinical applications of monoclonal antibody therapy and immunosuppressive therapy with gamma globulins.
12. Provide examples of clinical applications of adoptive immunotherapy in the areas of cancer treatment and transplantation.

CHAPTER OUTLINE

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KEY TERMS

Active immunity
Adjuvant
Adoptive immunity
Adoptive immunotherapy
Antitoxin
Attenuation
Cross-reactivity
Human immune serum globulin (HISG)
Immunization
Immunoprophylaxis
Immunotherapy
Passive immunity
Passive immunotherapy
Recombinant protein vaccine
Serotype
Toxoids
Tumor-infiltrating lymphocytes (TILs)
Vaccine

As we discussed in Chapter 1, *immunity* can be defined as the condition of being resistant to disease, most notably to infections. The process by which this state of protection is acquired is called **immunization.** There are three types of immunity that can be acquired through immunization: active, passive, and adoptive.

Active immunity results from immunization with a specific antigen by natural exposure to infection or administration of a vaccine. Adaptive, or antigen-specific, immune responses to bacteria, viruses, fungi, and parasites can all result in active immunity. For example, production of antibodies to a specific strain of Group A streptococci bacteria following a streptococcal sore throat protects the individual from future infection with that strain of bacteria. Active immunity is also stimulated through the administration of vaccines (see sections that follow). For example, after receiving all doses of the measles vaccine, most people develop immunity to the measles virus. In active immunity, the individual's own immune system is stimulated to mount an adaptive immune response to an antigen. The advantage of active immunization over other types of immunization is that it results in long-term memory to an antigen, providing potentially lifelong protection against the harmful effects of a pathogenic organism.

Passive immunity results from the transfer of antibodies from immunized hosts to a nonimmune individual. This state of immunity can occur naturally, from transfer of a mother's antibodies to her fetus or infant, or artificially, through passive immunization of an individual with commercial preparations of antibodies formed by other hosts to prevent or treat a disease. The latter application is also known as **passive immunotherapy.** Use of pooled human antibodies to protect a person who has an immunodeficiency disease is an example of passive immunotherapy. The main advantage of passive immunization is that it provides immediate protection to an individual who has not developed immunity to a particular antigen.

Adoptive immunity results from the transfer of cells of the immune system, usually lymphocytes, from an immunized host to a nonimmune individual. **Adoptive immunotherapy** involves the administration of these cells to treat patients with conditions such as immunodeficiency diseases or cancer. This type of therapy can be beneficial if the cells transferred are able to successfully establish themselves in the recipient. An example of adoptive immunotherapy is the transplantation of hematopoietic stem cells into leukemia patients who have undergone treatment with high doses of irradiation and chemotherapy to destroy the malignant cell population.

The primary difference between the immunity gained by active, passive, and adoptive immunization is that in active immunity, an individual's own immune system is responding to an antigen. In passive and adoptive immunity, immunity is provided to an individual through the transfer of antibodies or cells from another source to provide immediate protection.

This chapter discusses the mechanisms by which active, passive, and adoptive immunity occur in the context of their advantages and limitations. Clinical examples under each category are described so that the student can develop a better understanding of how knowledge of the basic principles of the immune system can be translated into therapies that can benefit humankind. A special focus is placed on the topic of vaccines because their use has significantly improved the health of populations throughout the world.

Vaccines

A **vaccine** is an antigen suspension derived from a pathogen.[1] Vaccines are routinely administered to healthy individuals to stimulate an immune response to an infectious disease. Vaccination therefore is a form of **immunoprophylaxis,** or the prevention of disease through immunization. Vaccines have had a tremendous impact on public health by significantly reducing the incidence of illness and death from numerous diseases that had devastating effects on civilization. In addition, experimental "vaccines" for cancer have been developed as **immunotherapy** for patients who already have the disease (see Chapter 19). In this section, we will discuss the historical evolution of vaccines, the different forms of vaccines available, how they are routinely administered, factors that affect their efficacy, and vaccine design for the future.

Historical Evolution of Vaccines

As was previously mentioned in Chapter 1, the science of immunology was born out of early observations of immunity and studies involving vaccination. The motivation behind these studies was the desire to eliminate the death and suffering caused by infectious disease. Advances in science and technology during the 20th century and beyond have led to the creation of safer, more effective vaccines and remarkable success toward that goal.

Early Discoveries

One of the most feared diseases in ancient times was smallpox, a highly fatal illness characterized by a high fever and pustular rash.[2] Those who survived the disease were usually left with disfiguring scars or blindness. However, it was noted in Greece as early as 430 BC that people who were fortunate enough to

survive became immune to this "speckled monster"; they were then asked to care for others afflicted with the deadly disease.[3] These observations led to the procedure of *variolation,* in which fresh material taken from a skin lesion of a person recovering from smallpox was subcutaneously injected with a lancet into the arm or leg of a nonimmune person. Recall from Chapter 1 that in an older form of the procedure practiced in ancient China, the material was dried into a powder and inhaled by the nonimmune person. The practice of variolation usually resulted in milder disease and recovery and was performed for centuries in Africa, India, and China. The practice was introduced to Europe in the 18th century. At that time, over 400,000 people in Europe died each year from smallpox, so the procedure became popular very quickly.[3] However, variolation was not without risks; 1% to 2% of the recipients developed smallpox and died, whereas others contracted infectious diseases such as syphilis or tuberculosis from the injected material.[4]

These observations led to the search for a safer procedure. Farmers such as Benjamin Jesty observed that milkmaids who had a similar but milder disease called cowpox were protected from contracting the deadlier smallpox. The farmers, therefore, used cellular material from the cowpox lesions of milkmaids to variolate others and observed the same protective effects without the danger of contracting smallpox **(Fig. 25–1).** The English physician Edward Jenner brought fame to this procedure when, in 1796, he injected fluid from the cowpox lesions of milkmaid Sarah Nelmes into an 8-year-old boy named James Phipps.[4,5] When Jenner subsequently inoculated the boy with smallpox, James did not develop the disease, showing that the method was a success. Jenner called this procedure "vaccination" after the Latin word *vacca*, which means "cow."[3] The protective effects induced by the vaccination were caused by the phenomenon of **cross-reactivity,** or antigenic similarity between the viruses that caused cowpox and smallpox. This early vaccine for smallpox led to the development of the modern smallpox vaccine, which is still derived from the *vaccinia* virus that causes cowpox **(Fig. 25–2).** The vaccine was so successful that smallpox has been eradicated from the world, and the vaccine is no longer routinely administered.[3,4]

Despite the early development of the smallpox vaccine, many years passed before scientists understood that microbes were the underlying cause of infectious diseases and that components of these pathogens could be used to produce protective vaccines. Thus, it was not until 80 years later that the next vaccine was developed by Louis Pasteur against chicken cholera.[6] Pasteur later went on to develop vaccines against anthrax and rabies. These vaccines were all based on the principle of attenuation. **Attenuation** involves the use of bacteria or viruses that have been weakened through exposure to modifying conditions such as chemical treatment, elevated or cold temperatures, or repeated in vitro *passage* in cell culture (a technique in which some of the cells are periodically transferred to a flask containing fresh nutrient medium). These weakened microorganisms do not cause disease in healthy individuals, but are able to stimulate the immune response because they contain many of the same antigens as their pathogenic counterpart.[5] It is thought that Pasteur discovered the effect of attenuation by accident during his studies of chicken cholera.[7] After returning from a summer vacation in 1881, he noticed that he had left a culture of the bacteria that cause chicken cholera, now known as *Pasteurella multocida,* on his laboratory bench. Instead of disposing of the aged culture, he decided to use it to inoculate chickens. The chickens did not develop the disease; furthermore, when Pasteur later inoculated them with a fresh culture

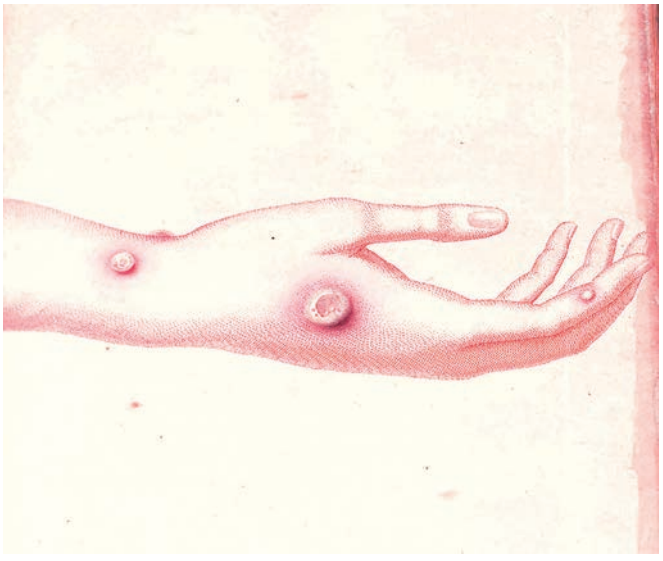


FIGURE 25–1 Color etching of a hand and wrist with cowpox lesions, from Edward Jenner's "Inquiry Into the Causes and Effects of the Variolae Vaccinae." The etching shows several stages of cowpox, from early blistering to its later dimpled rupture. *(Courtesy of the National Library of Medicine, National Institutes of Health & Human Services. Bethesda, MD.)*

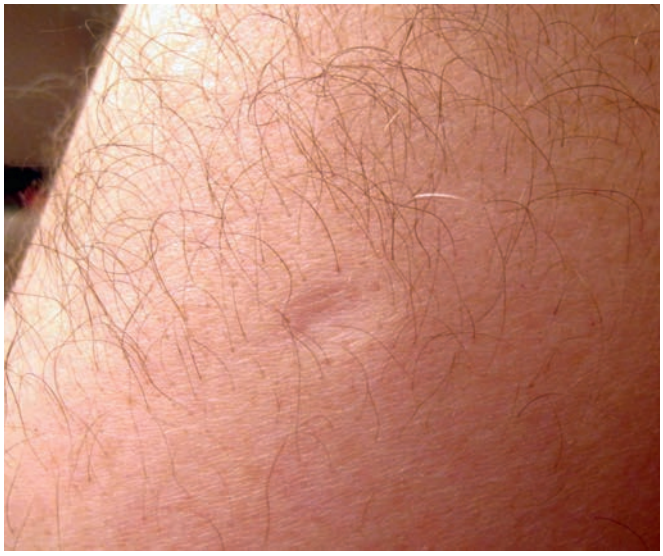


FIGURE 25–2 Evidence of smallpox vaccination. Individuals who have received the smallpox vaccine develop a blister, which dries up and forms a scab within the first 2 weeks. They can be easily identified by the scar that forms at the site of injection once the scab falls off. (Linda Miller.)

of the bacteria, they proved to be resistant to cholera. This series of events exemplified Pasteur's philosophy for science, that "success favors the prepared mind."[7] Within the next year, he developed an attenuated bacterial vaccine to protect sheep against anthrax.

The rabies vaccine, for which Pasteur is most famous, was developed in 1885. Pasteur did not know the causative agent of rabies, but recognized that it affected the central nervous system (CNS). He prepared an attenuated vaccine by repeatedly infecting rabbits with material contaminated with rabies, recovering the rabbits' spinal cords, and exposing them to dry air. In 1885, after testing the vaccine on dogs, he was convinced to administer his vaccine preparation to a 9-year-old boy, Joseph Meister, who had been severely bitten by a rabid dog.[8] The boy received a series of subcutaneous injections of the material over a period of 10 days and never developed rabies **(Fig. 25–3).**[9] Pasteur received worldwide honors for this treatment and used the proceeds to build the famous Pasteur Institute in Paris, where vaccine studies and other biomedical research is conducted today. He was the first person to use the word *vaccination* in reference to all immunization procedures.[9,10]

Pasteur believed that only live, attenuated organisms could be used for effective immunization and these remain the basis for many vaccines that are used today. However, in the late 1800s, Salomen and Smith showed that a killed suspension of *Vibrio cholerae* could provide pigeons with protection against cholera. A few years later, vaccines against human cholera, typhoid fever, and plague were developed using whole killed organisms.[9]

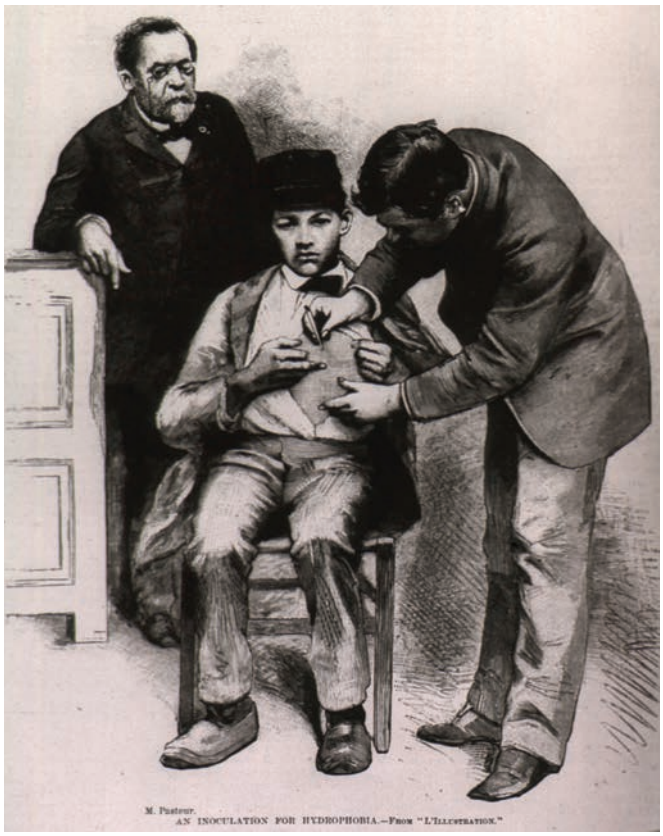


FIGURE 25–3 Louis Pasteur observes as a young boy receives an inoculation for hydrophobia (a symptom of rabies). *(Courtesy of the National Library of Medicine, National Institutes of Health & Human Services. Bethesda, MD.)*

Twentieth Century Vaccines

The 20th century witnessed a tremendous expansion in the development of vaccines as a result of advances in scientific research, techniques, and laboratory technology.[7,10] New methods of attenuating microorganisms by repeated culture passage in special media resulted in the development of live, attenuated vaccines against tuberculosis (TB) and typhoid fever. Production of live, attenuated vaccines for yellow fever and influenza A was made possible in the 1930s, following Goodpasture's development of techniques that permitted viral growth in embryonic eggs.[9,11]

Another major advance of the 20th century was the use of inactivated bacterial toxins in vaccine preparations. These preparations, referred to as **toxoids,** are made by chemically treating bacterially derived toxins known to cause pathogenesis so that they cannot cause harm to the host, but retain their ability to stimulate an immune response. Using a formalin treatment, inactivated toxoid vaccines were developed against diphtheria by Glenny in 1923 and by Ramon and Zoeller against tetanus in 1926.[9] Inactivated toxoids still serve as the basis for the diphtheria and tetanus vaccines in use today.

The second half of the 20th century has been referred to as the "golden age of vaccine development."[6,9] During this period, researchers developed revolutionary techniques that enabled successful growth of viruses in cell culture. These important advances led to the development of numerous attenuated viral vaccines from 1950 to 1980, including those targeted against polio, measles, mumps, rubella, and varicella.[6,7,9]

An important development in the last part of the 20th century was the use of purified polysaccharides to treat bacterial infections.[9,10] These vaccine preparations were developed in the 1970s and 1980s to prevent meningococcal meningitis, pneumococcal pneumonia, and *Haemophilus influenzae* type b (Hib). In the late 1980s, these vaccines were made more effective by increasing the immunogenicity of the polysaccharides. This was accomplished by forming *glycoconjugates* consisting of polysaccharides linked to a protein that can be recognized by T cells.

The last part of the 20th century also saw the first applications of genetic technologies to vaccine development.[6,9,10] The first **recombinant** (i.e., genetically engineered) **protein vaccine** was produced in 1986 against hepatitis B. This vaccine consists of purified hepatitis B surface antigen (HBsAg) made by genetically modified yeast cells that have incorporated the gene for HBsAg. It replaced an older form of the vaccine that used a less purified preparation of HBsAg derived from the plasma of patients who were infected with hepatitis B. Genetic engineering was also used to develop recombinant vaccines to prevent other diseases, including pertussis and Lyme disease.

Beyond the 20th Century

The beginning of the 21st century has seen continued use and refinements in the vaccine technologies developed in the

1900s. The first decade of the 21st century witnessed the licensure of live, attenuated vaccines for influenza, rotavirus, and herpes zoster, as well as multivalent, glycoconjugate vaccines for pneumococcus and meningococcus. A vaccine to help prevent cervical cancer, based on production of genetically recombinant antigens from the human papilloma virus (HPV), was also introduced.[6,9]

Currently, 27 infectious diseases are preventable by vaccines licensed in the United States. However, many diseases still present a challenge to the medical profession because they cannot be prevented through immunization. Future vaccines will likely be based on attempts to target these diseases through application of advanced genetic technologies and stimulation of innate defenses as well as humoral antibody production and cellular components of the adaptive immune system.[6,7,10,12,13] Some of the strategies that are likely to be used in the future development of vaccines will be discussed later in this chapter.

Conventional Vaccines

As we previously mentioned, vaccines are antigen preparations that are administered to prevent infectious diseases. Conventional vaccines in use today consist of live, attenuated (nonpathogenic) microorganisms, inactivated (killed) microorganisms, or antigenic components of microorganisms, known as subunit vaccines. The main features of each of these vaccine forms will be discussed in more detail in the sections that follow. Newer strategies for vaccine development, which incorporate modern genetic technologies and approaches to enhance immune responses, will be introduced in a later section.

Live, Attenuated Vaccines

Live, attenuated vaccines have been in routine use since Jenner's discovery of the smallpox vaccine. As we previously discussed, this vaccine was based on the concept of cross-reactivity, in which material from the cowpox virus was used to develop immunity against the antigenically similar, but highly pathogenic, smallpox virus. However, most pathogens do not have an immunologically similar, but less pathogenic, counterpart.

A number of laboratory techniques are currently used to modify bacteria or viruses so that they lose their pathogenic properties but are still capable of stimulating a good immune response. The techniques used to prepare conventional vaccines involve culture of the microorganism under conditions that are different from those present in the host and unfavorable to its growth.[13] As we previously discussed, Pasteur used these principles to develop nonpathogenic strains of the bacteria that cause chicken cholera from aged cultures and attenuated rabies virus from dried spinal cords of infected rabbits. Another example of an attenuated preparation is the vaccine for tuberculosis developed by Albert Calmette and Camille Guerin at the Pasteur Institute in 1927. The vaccine, referred to as BCG *(Bacillus Calmette Guerin),* uses an attenuated strain of *Mycobacterium bovis* developed by growing the bacteria on culture media containing increasing concentrations of bile. After several years, the bacteria had adapted to growing in media containing a high bile content and were therefore suitable for use in the human body. This is because the body's lower bile concentration is not conducive to the pathogenic growth of the bacteria.[14] Researchers have also developed a live, attenuated bacterial vaccine against typhoid fever, which consists of a mutated strain of *Salmonella typhi* packaged into capsules that are ingested orally.[15] To produce this vaccine, the bacteria were chemically treated to induce genetic mutations that weakened them so they were no longer pathogenic.

Attenuated vaccines are more easily prepared against viral infections than bacterial infections.[9] A major viral disease for which an attenuated vaccine was developed is polio. Polio is a serious disease that causes aseptic meningitis and leaves its victims with disabling paralysis. In the early 1960s, Albert Sabin developed an oral polio vaccine from live, attenuated strains of poliovirus cultured in monkey kidney cells.[16] The vaccine contained the three serotypes of poliovirus that are capable of causing the disease. Each **serotype** is a form of the virus that can be distinguished by the presence of specific antigens that can be identified by serological typing. Sabin's vaccine was the main poliovirus vaccine used in the United States from 1963 to 1997, before a potent inactivated polio vaccine was licensed. Although the oral polio vaccine is no longer available in the United States because of its potential for adverse effects (see *Benefits and Adverse Effects of Vaccines* in the text that follows), it is used commonly in underdeveloped areas of the world because it is easier to administer than the attenuated vaccine, which requires injections. The vaccines for measles, mumps, rubella, and varicella also consist of live strains of viruses that have been attenuated through repeated passage in cultured cells. The measles and mumps viral strains are produced in chick embryo cells, whereas the rubella strain is produced in human diploid cells.[17] The vaccines to prevent chickenpox consist of live, attenuated Oka strains of varicella virus, which were originally isolated from a Japanese child with chickenpox and sequentially propagated in cultures of human or embryonic guinea pig cells.[18] These strains are used to produce an individual varicella vaccine or a combination vaccine that also contains the vaccines for measles, mumps, and rubella (MMR or MMRV). A more potent formulation of the attenuated Oka varicella strain is used to prevent herpes zoster (shingles) in adults.

A live, attenuated vaccine has also been developed to prevent influenza. The vaccine is administered intranasally. The main antigens targeted by the vaccine are two surface glycoproteins of the influenza virus called hemagglutinin (H) and neuraminidase (N); these antigens are also used to classify the viruses on the basis of their serotype. Similar to the inactivated influenza vaccine discussed later, a new attenuated influenza vaccine must be prepared each year because of the high mutation rate of the influenza viruses, which results in the synthesis of new antigens. The vaccine consists of a quadrivalent suspension containing the most common circulating antigenic strains of influenza virus from four common virus types: influenza A (H3N2), influenza A (H1N1), and two influenza B strains.[19] The viral strains have been cultivated in chicken eggs and attenuated for adaptation to colder temperatures, so that they grow optimally at 25°C rather than body temperature.[9,20]

The primary advantage of live, attenuated vaccines is that they are able to replicate at a low level in the host and are

therefore capable of inducing both humoral and cell-mediated immune responses.[5,9] This is especially important for viral infections because cytotoxic T cells are required in order to attack viruses during the intracellular phase of the viral life cycle. Because of the broad immunity induced by live, attenuated vaccines, they generally induce an effective immune response after just a single dose.[5]

Despite these advantages, live, attenuated vaccines also have some significant limitations.[5] It is important *not* to administer vaccines containing live organisms to immunocompromised individuals. Although the organisms are attenuated, they may cause severe, disseminated, and potentially fatal infections in patients with immunodeficiency diseases or patients receiving immunosuppressive treatments. Live vaccines may also not be recommended for use in pregnant women. On rare occasions, mutations may occur in the vaccine organism, causing it to lose its attenuation and revert to the pathogenic form. This unfortunately occurred during use of the live, attenuated (Sabin) vaccine for polio, which led to the vaccine's replacement with an inactivated polio vaccine in industrialized countries. Use of genomic techniques to design live, attenuated strains that lack genes required for pathogenicity will hopefully prevent such an occurrence in the future. Another limitation of live, attenuated vaccines is potential interference with replication of the organism in infants by maternal antibodies, necessitating a delay in the dosing schedule (see *Factors Influencing Immunogenicity* in the text that follows). Finally, careful handling and storage of attenuated vaccines is very important because exposure to heat and light may destroy the live organisms, causing the vaccines to be ineffective. This requirement can pose a major problem in developing countries of the world, where refrigeration is not readily available.

Inactivated Vaccines

Inactivated vaccines consist of intact, killed viruses or bacteria. The microorganisms are killed by heat or chemical treatment so that they are not pathogenic but retain their antigenic properties. Chemicals such as formaldehyde or β-propiolactone are used more frequently than heat treatment because they are less likely to alter the chemical structure of the surface epitopes.[14] Examples of inactivated vaccines are the intramuscular vaccine for polio, the classic influenza vaccine, and the hepatitis A vaccine.

In the early 1950s, Dr. Jonas Salk developed the first effective inactivated vaccine for polio. The vaccine consisted of the three disease-related serotypes of polio virus killed by formaldehyde treatment. A more potent inactivated polio vaccine with greater antigenic content was developed in 1978.[16] This form of the vaccine is used routinely today in developed countries of the world because of its effectiveness and safety.

Similar to the live, attenuated influenza vaccine, the inactivated influenza vaccines contain one influenza A (H3N2) virus strain, one influenza A (H1N1) virus strain, and one or two influenza B virus strains grown in embryonated hen eggs.[19,21] However, these viruses have been killed by treatment with formaldehyde or β-propiolactone and the vaccine is administered by intramuscular injection.[9,20] Researchers have also developed new influenza vaccines that use virus grown in cell culture systems or recombinant hemagglutinin antigens.[19] These vaccines avoid or minimize the use of eggs, allowing for faster production and eliminating or reducing the likelihood of hypersensitivity to egg proteins.

The hepatitis A vaccine consists of purified hepatitis A virus (HAV) cultured in human fibroblasts and inactivated by formalin-treatment.[22] Although this vaccine was initially only given to individuals at high risk for contracting hepatitis A, it has been incorporated into routine childhood immunization programs to reduce the incidence of this common infection.

A major advantage of inactivated vaccines is that they can safely be given to immunocompromised people because the organisms have been killed and cannot replicate in the host.[5,14] However, this property makes it necessary to provide a larger amount of antigen in order to stimulate an effective immune response and may require two or more booster doses administered over time to produce protective immunity. Because the inactivated organisms do not infect host cells, these vaccines predominantly induce a humoral immune response, with little or no cell-mediated immunity.[5,14]

Subunit Vaccines

Subunit vaccines consist of one or more purified components of a pathogen that are capable of stimulating an immune response. The forms of subunit vaccines that are routinely used are toxoids, capsular polysaccharides, purified proteins, and recombinant protein antigens.

Toxoid Vaccines. The pathology of some bacterial diseases, such as diphtheria and tetanus, is caused by a single exotoxin. Diphtheria is a contagious, life-threatening disease of the upper respiratory tract characterized by formation of a thick membrane that can cover the back of the pharynx, making it difficult to breathe. Tetanus is a serious bacterial infection that affects the nervous system, causing painful, prolonged muscle contractions and, sometimes, difficulty breathing.

As we previously mentioned, toxoids are bacterial exotoxins that have been chemically inactivated so they cannot cause harm to the host, but retain their ability to stimulate an immune response. Toxoids are used in vaccines to induce the production of antibodies that can bind to exotoxins and neutralize their effects.[9,14] The first toxoid vaccine was developed in 1923 against diphtheria and consisted of a formalin-inactivated toxin from the causative organism, *Corynebacterium diphtheriae.* In 1926, scientists developed a toxoid vaccine against tetanus, which consisted of a formalin-inactivated toxin from the causative organism, *Clostridium tetani.* Toxoids are still used in the composition of today's vaccines for diphtheria and tetanus. Another commonly used toxoid, which consists of inactivated toxin from the bacterium *Bordetella pertussis,* is part of the acellular pertussis vaccine (see *Purified Protein Vaccines* in the text that follows). The vaccines for diphtheria, tetanus, and pertussis are available singly or in combinations known as DTaP, TdaP, DT, or Td, depending on the amounts of diphtheria, tetanus, and acellular pertussis components present.[23]

Polysaccharide Vaccines. Another virulence factor possessed by some bacteria is the presence of a hydrophilic polysaccharide capsule, which covers the bacterial outer membrane. The

capsule allows these bacteria to resist phagocytosis and other immune defenses by masking components of the membrane that might otherwise be targets of the immune response. However, if antibodies to the capsular polysaccharides are present, they can facilitate clearance of the bacteria by inducing opsonization or complement-mediated lysis.[9,14] Therefore, vaccines against encapsulated bacteria contain purified capsular polysaccharides from specific bacterial strains that stimulate the production of antibodies. Because the structure of these capsular antigens varies with different bacterial serotypes, these vaccines contain multiple polysaccharide types to ensure an immune response that provides broad protection.[9]

The first polysaccharide vaccine was developed against *Streptococcus pneumoniae,* the cause of pneumococcal pneumonia. The vaccines in use today contain polysaccharides from 13 or 23 different *S pneumoniae* serotypes.[24] Another polysaccharide vaccine has been developed against *H influenzae* type b (Hib), which was a major cause of pneumonia and meningitis in infants and young children before the vaccine was implemented. The vaccine is composed of the polyribosylribitol phosphate component of the Hib capsule conjugated to a protein carrier.[25] A polysaccharide vaccine has also been developed against *Neisseria meningitidis,* an important cause of bacterial meningitis, especially in young individuals living in close quarters such as dormitory buildings. The vaccine consists of four purified bacterial capsular polysaccharides (A, C, Y, W-135) from *N meningitidis.*[26]

A problem with polysaccharide antigens is that they do not induce a good immune response, especially in infants and the elderly, two populations that are at high risk for severe consequences of encapsulated bacterial infections. This is because polysaccharides are T-independent antigens that stimulate IgM production with no immunoglobulin class-switching or long-term memory response. This problem has been circumvented through vaccines composed of *glycoconjugates,* in which the polysaccharide antigens are linked to a carrier protein such as tetanus toxoid or diphtheria toxoid. These conjugates are able to induce a more effective immune response by activating T helper (Th) cells, resulting in immunoglobulin class switching, with production of polysaccharide-specific IgG antibodies and generation of memory cells.[9,14]

Purified Protein Vaccines. Vaccines can also be composed of proteins from a pathogen. One such vaccine protects against pertussis, a serious respiratory disease also known as "whooping cough" because of the characteristic whooping sound patients make while trying to breathe during violent coughing fits. The first vaccine against pertussis was composed of whole killed *B pertussis* bacteria and was thought to be associated with rare, but serious, neurological effects such as encephalitis or encephalopathy and convulsions, especially in children with neurological disorders.[27] Today's pertussis vaccines are less frequently associated with side effects because they are composed of two to five purified proteins from *B pertussis* rather than whole bacterial cells.[23] One of these proteins is a toxoid derived from the pertussis toxin (see *Toxoid Vaccines* in the previous text).

Recombinant Protein Vaccines. Recombinant DNA technology has made it possible to develop even more highly purified protein vaccines. In these methods, the gene coding for a specific protein antigen from a pathogenic microorganism is isolated and incorporated into the genome of nonpathogenic bacteria, yeast, or other cells. The genetically modified cells are cultured in large quantities and produce the desired antigen, which can then be purified by conventional biochemical methods.[14] The first *recombinant protein vaccine* was developed in 1986 for hepatitis B and is widely used today. It is safer than the previous hepatitis B vaccine, which consisted of HBsAg isolated from pooled plasma of infected patients. The recombinant hepatitis B vaccine is produced by cloning the gene for HBsAg in yeast cells, then harvesting and purifying the HBsAg protein.[28] The protein spontaneously assembles into viruslike particles that are not infectious but induce an effective immune response because they are similar in structure to the actual hepatitis B virus (HBV).[9]

Recombinant DNA technology is also the basis for vaccines developed against the HPV, which can cause cervical cancer, anal cancer, and other genital cancers. These vaccines contain recombinant L1 major capsid proteins from 2 to 9 HPV virus types (including types 16 and 18), which are highly associated with anal–genital cancers.[29,30] The genes for the L1 proteins are cloned in yeast or insect cell lines infected with baculovirus, a type of virus that infects invertebrate cells. The isolated proteins then combine into viruslike particles that induce an effective immune response. HPV vaccination is recommended for adolescent girls and boys to confer protection before they become sexually active. The vaccine is also recommended for young men who have sex with men and immunocompromised individuals, including those infected with HIV.

Factors Influencing Immunogenicity

There are many factors that affect the quality of the immune response to a vaccine antigen. Important factors include the age of the recipient, the individual's immune status, and the nature of the vaccine.[31] All of these factors are considered by immunization experts when deciding how a vaccine should be administered to achieve an optimal immune response.

Age

Age is an important factor in determining how a vaccine should be provided to individuals in a population. Recommendations for the age at which a vaccine should be routinely administered are based on age-specific risks for contracting the disease and developing associated complications, as well as age-related ability to respond to the vaccine. In general, it is recommended that vaccines be administered to the youngest individuals at risk for the vaccine's targeted disease, as long as effectiveness and safety of the vaccine have been demonstrated in that age group.[31]

For example, in the United States, vaccination schedules recommended by the Centers for Disease Control and Prevention's Advisory Committee on Immunization Practices (ACIP) are categorized according to age. Routine immunization against hepatitis B should begin at birth because the hepatitis B virus may have been transferred through the placenta, whereas vaccines for diphtheria, pertussis, tetanus, rotavirus, *H influenza* type b, polio, and streptococcal pneumonia should begin at

2 months of age. Multiple inoculations of these vaccines, administered at specific time intervals through the first 18 months of life, are necessary to achieve optimal immunity because the young infant's immune system is immature. Additional doses of some of these vaccines are also recommended during childhood, adolescence, or adulthood to maintain high antibody titers.

Some vaccines, such as the live, attenuated vaccine for measles, mumps, and rubella, are not started until 12 to 15 months of age because administration before that age does not result in an effective immune response. The response is less than optimal because passively acquired maternal antibodies present in the younger infant's serum limit replication of the attenuated viruses. Other vaccines, such as those for meningococcal meningitis and HPV, are not administered until 11 to 12 years of age because the risk for contracting these infections is greater during adolescence. Still other vaccines, such as those for varicella zoster (the cause of shingles) and pneumococcal pneumonia, are not administered until later adulthood because the natural decline of immune function in older individuals makes them more susceptible to developing these infections. A few vaccines, such as those for typhoid fever or yellow fever, are recommended only for individuals traveling to areas of the world where there is a high incidence of these diseases.

Vaccination schedules are revised annually by the ACIP in consultation with the American Academy of Pediatrics (AAP) and the American Academy of Family Physicians (AAFP).[5,31] The 2016 immunization schedules for children, adolescents, and adults are shown in **Figures 25–4** and **25–5**. Up-to-date schedules can be accessed from the Centers for Disease Control and Prevention (CDC) at www.cdc.gov/vaccines.[32]

Nature of the Vaccines

The nature of the vaccine is another important factor influencing the quality of the immune response. In general, the most immunogenic vaccines consist of live, attenuated organisms that are able to replicate in the host; the least immunogenic vaccines consist of purified components (subunits) derived from the pathogen. Vaccine antigens with a low level of immunogenicity require an **adjuvant,** a substance that is co-administered with a vaccine antigen to produce an enhanced immune response. The term *adjuvant,* coined by Ramon in 1926, is derived from the Latin word *adjuvare,* which means "to help."[6,33]

Connections

Principles of Immunologic Memory

Memory B and T lymphocytes are generated as a result of active immunity. These memory cells can be activated quickly if the individual is exposed to the same antigen at a later time, reducing the lag period before antibody production. Antibody titers rise quickly and reach higher levels than those produced after the first exposure to the antigen. Antibody concentrations remain high for a long period and provide long-lasting immunity. The protection provided by the memory response serves as the basis for repeated vaccine injections during routine immunization schedules. It also provides the host with lifelong immunity after recovery from a natural infection with a pathogen.

A diverse group of molecules can function as vaccine adjuvants, including emulsions, mineral salts, microbial products, small molecules, microparticles, and liposomes.[9] These molecules are thought to activate pathways of the innate immune system, inducing the release of cytokines that promote inflammation and stimulate cells of the adaptive immune system.[9] Adjuvants can be classified as *antigen delivery systems,* which enhance the uptake of antigens by antigen-presenting cells (APCs) or *immunopotentiators,* which activate dendritic cells to present antigens to T cells in humoral or cell-mediated immune responses.[9] In some vaccine formulations, the two types of adjuvants have been combined together.

The ultimate purpose for using adjuvants in vaccines is to increase antibody titers and, for some vaccines, to induce cell-mediated immunity as well. Effective adjuvants can potentially reduce the dose of antigen needed in a vaccine, decrease the number of inoculations required, and increase the speed and duration of the immune response.[9,33] They are capable of enhancing immunity in both young and elderly persons.

Despite the number of adjuvants that have been discovered, only a few have been licensed, based on their safety and efficacy. The most widely used adjuvant, and the only one licensed in the United States, is "alum," which consists of aluminum hydroxide and aluminum phosphate.[9] The adjuvant activity of alum was discovered by Glenny in 1926, in his experimentation with diphtheria toxoid.[34] Alum is routinely used today in vaccine formulations against HAV, HBV, HPV, diphtheria, tetanus, meningococcus, pneumococcal conjugates, tick-borne encephalitis, and anthrax, which are adsorbed to aluminum salts.[9]

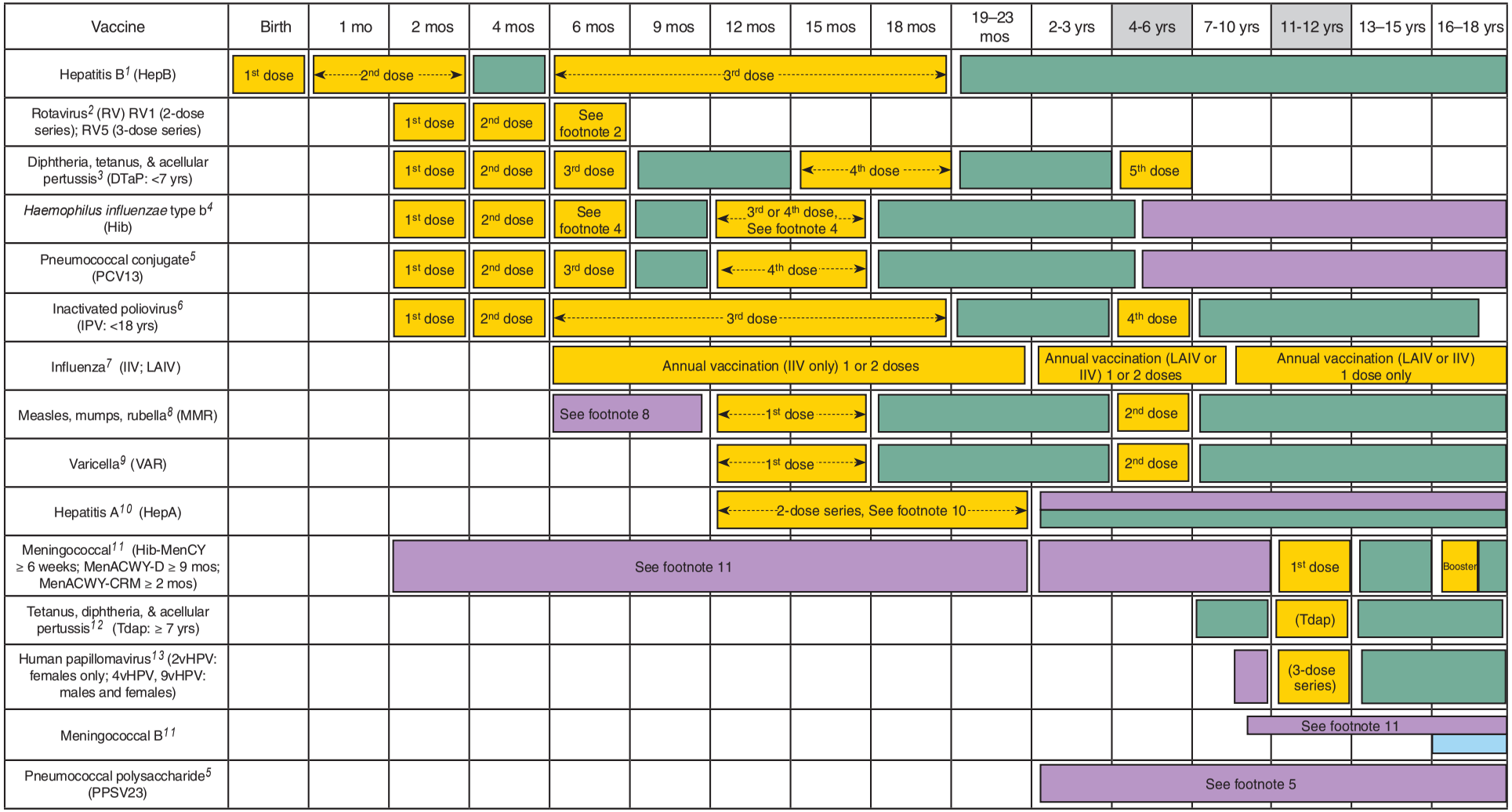
Other commonly used adjuvants are oil-in-water emulsions, composed of liquid dispersions of oil droplets stabilized with surfactants.[9] Oil-in-water emulsions are mixed with vaccine antigens and are believed to stimulate the immune response by inducing release of chemokines and enhancing antigen uptake and migration of APCs.[9] The first such adjuvant was discovered by Freund in the 1930s and is known as Freund's complete adjuvant (FCA). FCA is a powerful adjuvant containing killed mycobacteria. It has been used in animal studies, but is not suitable for use in humans because it produces abscesses and scar formation at the site of inoculation. Freund's incomplete adjuvant (FIA), a water-in-oil emulsion without mycobacteria, has been used in some human vaccines because it is less toxic.[33] Two squalene-based water-in-oil emulsions, MF59 and AS03, have been licensed in Europe for use in influenza vaccines.[33] Additional water-in-oil emulsions and other forms of adjuvants are under investigation.

Next Generation Vaccines

Although conventional forms of vaccines have been highly effective in preventing many infections, we still have no vaccines for many diseases that are major causes of illness and death in the world. These diseases are caused by viruses, bacteria, and parasites that have complex mechanisms of pathogenesis. They may display variability through genetic mutations or multistage life cycles, or have developed other methods to escape attack by the immune system.[35] For example, the ability

Figure 1. Recommended immunization schedule for persons aged 0 through 18 years – United States, 2016.
(FOR THOSE WHO FALL BEHIND OR START LATE, SEE THE CATCH-UP SCHEDULE [FIGURE 2]).

These recommendations must be read with the footnotes that follow. For those who fall behind or start late, provide catch-up vaccination at the earliest opportunity as indicated by the green bars in Figure 1. To determine minimum intervals between doses, see the catch-up schedule (Figure 2). School entry and adolescent vaccine age groups are shaded.



This schedule includes recommendations in effect as of January 1, 2016. Any dose not administered at the recommended age should be administered at a subsequent visit, when indicated and feasible. The use of a combination vaccine generally is preferred over separate injections of its equivalent component vaccines. Vaccination providers should consult the relevant Advisory Committee on Immunization Practices (ACIP) statement for detailed recommendations, available online at http://www.cdc.gov/vaccines/hcp/acip-recs/index.html. Clinically significant adverse events that follow vaccination should be reported to the Vaccine Adverse Event Reporting System (VAERS) online (http://www.vaers.hhs.gov) or by telephone (800-822-7967). Suspected cases of vaccine-preventable diseases should be reported to the state or local health department. Additional information, including precautions and contraindications for vaccination, is available from CDC online (http://www.cdc.gov/vaccines/recs/vac-admin/contraindications.htm) or by telephone (800-CDC-INFO [800-232-4636]).This schedule is approved by the Advisory Committee on Immunization Practices (http//www.cdc.gov/vaccines/acip), the American Academy of Pediatrics (http://www.aap.org), the American Academy of Family Physicians (http://www.aafp.org), and the American College of Obstetricians and Gynecologists (http://www.acog.org).

NOTE: The above recommendations must be read along with the footnotes of this schedule.

FIGURE 25–4 Recommended immunization schedule for persons aged 0 through 18 years, 2016. *(Source: Centers for Disease Control and Prevention.)*

Recommended Adult Immunization Schedule—United States - 2016

Note: These recommendations must be read with the footnotes that follow containing number of doses, intervals between doses, and other important information.

Figure 1. Recommended immunization schedule for adults aged 19 years or older, by vaccine and age group[1]

VACCINE ▼ / AGE GROUP ▶	19-21 years	22-26 years	27-49 years	50-59 years	60-64 years	≥ 65 years
Influenza*,[2]	1 dose annually					
Tetanus, diphtheria, pertussis (Td/Tdap)*,[3]	Substitute Tdap for Td once, then Td booster every 10 yrs					
Varicella*,[4]	2 doses					
Human papillomavirus (HPV) Female*,[5]	3 doses					
Human papillomavirus (HPV) Male*,[5]	3 doses					
Zoster[6]					1 dose	
Measles, mumps, rubella (MMR)*,[7]	1 or 2 doses depending on indication					
Pneumococcal 13-valent conjugate (PCV13)*,[8]	1 dose					
Pneumococcal 23-valent polysaccharide (PPSV23)[8]	1 or 2 doses depending on indication					1 dose
Hepatitis A*,[9]	2 or 3 doses depending on vaccine					
Hepatitis B*,[10]	3 doses					
Meningococcal 4-valent conjugate (MenACWY) or polysaccharide (MPSV4)*,[11]	1 or more doses depending on indication					
Meningococcal B (MenB)[11]	2 or 3 doses depending on vaccine					
Haemophilus influenzae type b (Hib)*,[12]	1 or 3 doses depending on indication					

*Covered by the Vaccine Injury Compensation Program

Recommended for all persons who meet the age requirement, lack documentation of vaccination, or lack evidence of past infection; zoster vaccine is recommended regardless of past episode of zoster

Recommended for persons with a risk factor (medical, occupational, lifestyle, or other indication)

No recommendation

Report all clinically significant postvaccination reactions to the Vaccine Adverse Event Reporting System (VAERS). Reporting forms and instructions on filing a VAERS report are available at www.vaers.hhs.gov or by telephone, 800-822-7967.

Information on how to file a Vaccine Injury Compensation Program claim is available at www.hrsa.gov/vaccinecompensation or by telephone, 800-338-2382. To file a claim for vaccine injury, contact the U.S. Court of Federal Claims, 717 Madison Place, N.W., Washington, D.C. 20005; telephone, 202-357-6400.

Additional information about the vaccines in this schedule, extent of available data, and contraindications for vaccination is also available at www.cdc.gov/vaccines or from the CDC-INFO Contact Center at 800-CDC-INFO (800-232-4636) in English and Spanish, 8:00 a.m. - 8:00 p.m. Eastern Time, Monday–Friday, excluding holidays.

Use of trade names and commercial sources is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services. The recommendations in this schedule were approved by the Centers for Disease Control and Prevention's (CDC) Advisory Committee on Immunization Practices (ACIP), the American Academy of Family Physicians (AAFP), the America College of Physicians (ACP), the American College of Obstetricians and Gynecologists (ACOG) and the American College of Nurse-Midwives (ACNM).

FIGURE 25–5 Recommended adult immunization schedule—United States, 2016. *(Courtesy of the Centers for Disease Control and Prevention.)*

of the immune system to eliminate HIV is hampered by the virus' capacity to infect and kill CD4+ T cells, integrate into the host genome, and rapidly mutate (see Chapter 24). *Plasmodium falciparum,* the cause of malaria, has posed a challenge for vaccine development through its ability to alter its surface antigens in the different stages of its complex life cycle.[36] The vaccine for tuberculosis, BCG, is not optimally effective because mycobacteria can establish a carrier state and become reactivated during periods of immune suppression. Other infections that have posed a global challenge for effective vaccine development include hepatitis C, respiratory syncytial virus, Epstein-Barr virus, cytomegalovirus, herpes simplex, rhinovirus, leishmaniasis, and dengue fever.[35]

New vaccine designs, adjuvants, and methods of delivery need to be developed to conquer these diseases. The potential for these to be successful will be aided by technical advances in multiple disciplines, including molecular genetics, structural biology, bioinformatics, systems biology, nanotechnology, formulation techniques, and immune response monitoring.[35]

New Approaches to Vaccine Design

The first step in producing an effective vaccine is the discovery of antigens from a pathogen that will elicit an effective immune response. This step can now be facilitated by *reverse vaccinology.* In this process, computer analysis is used to screen the entire genome of a pathogen to identify genes that code for proteins that would make good vaccine targets.[35,37] The process reverses the order of conventional methods for antigen detection, in which culture of the organism is followed by isolation of potential antigens, and finally bioinformatic analysis of the genes that code for the antigens. Reverse vaccinology allows for fast identification of candidate vaccine antigens and facilitates development of vaccines that have been difficult to produce. This new approach has been applied successfully to meningococcus and is being studied in additional organisms, including group A streptococcus, *S pneumoniae, Staphylococcus aureus,* and *Chlamydia.*[35] Vaccine antigen detection is also being facilitated by other technologies, including screening of gene libraries for immunogenic proteins and identification of microbial antigens by mass spectrometry.[35]

Once potential vaccine antigens are identified, it must be determined which antigens will induce the most protective immune response. For pathogens that show a high degree of variability, such as the HIV and influenza viruses, finding antigens that can induce broadly neutralizing antibodies will be desirable.[12,35,38] These antibodies are directed against epitopes that are highly conserved among different strains of the microorganism. The identification of antigens that can induce broadly neutralizing antibodies may allow for development of a universal vaccine, which could provide long-lasting cross-protection against multiple strains of a pathogen.[38] Advanced technologies also allow for synthesis of "mosaic" antigens, composed of fragments of natural peptides from a pathogen combined to produce novel proteins that are highly immunogenic.[35]

For those pathogens that have both intracellular and extracellular phases in their life cycles, antigen combinations that induce cell-mediated responses as well as humoral antibody responses are needed.[35,39] One way of stimulating both arms of the adaptive immune response is to use a "prime-boost" strategy, in which the host is initially "primed" through administration of an antigen-carrying vector that induces T-cell responses (see the text that follows), followed by a "boost" with a subunit vaccine to stimulate antibody production.[12]

New Adjuvants

As we previously discussed, several vaccines require administration with an adjuvant to induce an optimal immune response. The need for good adjuvants is becoming increasingly important as new, highly specific vaccine antigens are discovered. To this end, scientists are searching for new, effective adjuvants. There is also ongoing research as to whether these new substances can be used in combination with each other or with traditional adjuvants. Novel adjuvants targeting pattern-recognition receptors, such as the Toll-like receptors (TLRs), Rig-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (see Chapter 3), are being studied because of their ability to stimulate innate immunity and the release of cytokines that affect the adaptive immune responses.[9,12,40] Examples of such adjuvants are poly-IC, a synthetic analog of double-stranded RNA that activates TLR3; monophosphoryl lipid A (MPL) and its derivatives, which bind to TLR4; bacterial flagellin, which activates TLR5; and CpG (cytosine-phosphate-guanine) oligodeoxynucleotides, which bind to TLR9.[33,40] Saponin-based adjuvants are also under study. These are plant-derived glycosides that are combined with antigen in nanoparticles called immunostimulatory complexes (ISCOMs). They are thought to stimulate strong antibody and cell-mediated responses by increasing antigen uptake and activation of dendritic cells.[40] Various cytokines, chemokines, and inactivated bacterial toxins are also being studied for their ability to act as immunopotentiators.[33] In order to induce an optimal immune response, it will be important to combine these immunopotentiators with efficient antigen delivery systems. A number of novel vaccine delivery methods are under investigation.[35,38,39,41] These include viral vectors such as vaccinia virus, adenoviruses, and baculoviruses that have been genetically modified so that they carry the antigen of interest, but are incapable of causing disease. Another delivery vehicle being studied consists of noninfectious viruslike particles formed from viral proteins that self-assemble at the plasma membrane when recombinant viral vectors are used to infect cultured cells. A third vaccine form under study is delivery of "naked" DNA plasmids, encoding the antigen of interest under control of a mammalian promoter gene sequence. Naked DNA can be administered through a needleless gene gun that uses pressurized gas. Synthetic delivery systems, consisting of nanoparticles, copolymers, DNA nanostructures, or liposomes loaded or coated with the antigens of interest, are also being investigated.[39] Entrapment of antigens within microparticles has been shown to protect the antigen from degradation in the environment and increase uptake by APCs such as dendritic cells and macrophages to enhance the immune response.[41]

Different Routes of Vaccine Delivery

Nonparenteral routes of antigen delivery such as oral, intranasal, aerosol, transcutaneous, intradermal, and rectal are

also being studied.[10,41] Licensed oral vaccines are available already for rotavirus and typhoid fever along with an intranasal vaccine for influenza.[31] Additional needle-free methods will be particularly attractive for developing areas of the world because they reduce the risk of transmitting bloodborne diseases and do not require sterile equipment or highly trained personnel. They will also avoid the pain associated with administration by injection. Research is being conducted with oral vaccines composed of edible transgenic plants such as bananas or tomatoes that express the gene for the vaccine antigen of interest. Oral vaccines have an additional advantage in that they can potentially stimulate mucosal immunity as well as humoral antibody production and cell-mediated responses.[41] However, these systems need to be refined so that effective immune responses, rather than immunologic tolerance, is induced.

New Methods to Assess the Immune Response

Finally, in order to evaluate the effectiveness of a vaccine, more sophisticated methods will also be needed to assess the immune response. Antibody titers have traditionally been used as indicators of vaccine-induced immunity.[42] However, by using techniques such as DNA microarray analysis, multiplexed flow cytometry, and intracellular staining, the phenotype and activation status of individual cells of the immune system can be analyzed, potentially generating a signature profile of markers that more specifically represents the immune response to a vaccine.[35,37] These techniques can also be used to monitor cells in the tissues, where interaction with the antigen takes place, as well as the blood.[35] Used in conjunction with advances in vaccine antigen discovery and improved delivery mechanisms, immune monitoring methods will surely accelerate the development of new, effective vaccines against major global diseases in the next generation.

Benefits and Adverse Effects of Vaccines

Vaccines have been cited as one of the 20th century's greatest medical achievements.[43] Because of routine immunization, smallpox has been eradicated worldwide and poliomyelitis has been eliminated from the Western world. Infectious diseases that were once leading causes of illness and death in the beginning of the 20th century, such as diphtheria and measles, have a greatly reduced incidence today, especially in developed nations. A study conducted by the CDC in 2007 found that the overall incidence of diseases for which vaccines had been developed before 1980 decreased by over 92%, and mortality from these diseases decreased by more than 99%.[43,44] For example, before the development of the DTP vaccine, an estimated 176,000 cases of diphtheria, 1,300 cases of tetanus, and 147,000 cases of pertussis were reported annually in the United States. In 2013, these numbers had decreased to 0, 26, and 28,639 cases, respectively.[45] Annual cases of measles in the United States dropped from over 500,000 before 1963 to fewer than 200 cases in 2013, and the number of German measles/congenital rubella cases in the United States decreased from 48,000 before 1969 to 9 cases in 2013. *H influenzae* type b, which was the leading cause of bacterial meningitis and invasive pneumonia before 1985, has been virtually eliminated from the United States and Canada because of immunization.[43] The success of vaccination continues in the 21st century as immunization programs have expanded to countries throughout the world, preventing about 2.5 million deaths each year in children aged 5 and under.[43] The mission of the Global Vaccine Action Plan endorsed by the World Health Assembly is to continue this expansion so that access to immunization will be universal by the year 2020.[46]

An important feature of immunization is that it not only benefits the individuals receiving the vaccine, but also reduces the risk of nearby persons, who have not been vaccinated, of contracting the infectious disease. When a sufficient proportion of individuals in a population have been immunized, unvaccinated individuals, such as newborns and immunocompromised patients, are offered some protection because there is little chance for the disease to spread in the community. This concept of extending protection to others in the population is known as *community immunity* or *herd immunity* and is of great importance to public health **(Fig. 25–6).**[47]

Persons who have altered immunocompetence are at risk from certain immunizations and require special consideration. These individuals have decreased levels of humoral or cell-mediated immunity because of inherited primary immunodeficiency diseases or acquired deficiencies secondary to other conditions, such as HIV infection, hematologic malignancies, or treatment with immunosuppressive drugs or radiation (see Chapter 17). The administration of live vaccines is contraindicated in immunodeficient individuals because they are highly susceptible to contracting infections; therefore they should be immunized with inactivated or subunit vaccines.[31] Although the organisms contained in live vaccines are attenuated and usually will not cause pathology in healthy individuals, they have the potential for uncontrolled replication and may cause disseminated disease in immunodeficient persons. For example, the vaccine for smallpox was known to cause a highly fatal condition in infants with severe combined immunodeficiency (SCID), involving progressive spread of necrotic lesions from the site of vaccine injection to adjacent areas of the skin, bone, and internal organs. Administration of the BCG vaccine for tuberculosis to infants with SCID or HIV infection has also resulted in disseminated, life-threatening infections.[5] The CDC publishes specific recommendations for vaccination of persons with altered immunocompetence.[31]

Vaccines can also produce adverse effects in previously healthy individuals, but fortunately, most of these are not severe. A local inflammatory response at the site of injection is frequently reported because of stimulation of TLRs by the vaccine antigen or adjuvant. Systemic inflammatory reactions are also common. These manifest with fever, irritability, nausea, vomiting, and myalgia 24 to 48 hours after injection of killed vaccines, or 14 to 21 days after receipt of a live vaccine, and generally resolve within 72 hours.[48] Other adverse effects of vaccines include hypersensitivity reactions and effects related to the vaccine antigen or its administration.[5]

Hypersensitivity reactions to vaccines may be local or systemic and can be immediate or delayed (see Connections box). IgE-mediated, type I hypersensitivity (anaphylactic) is usually

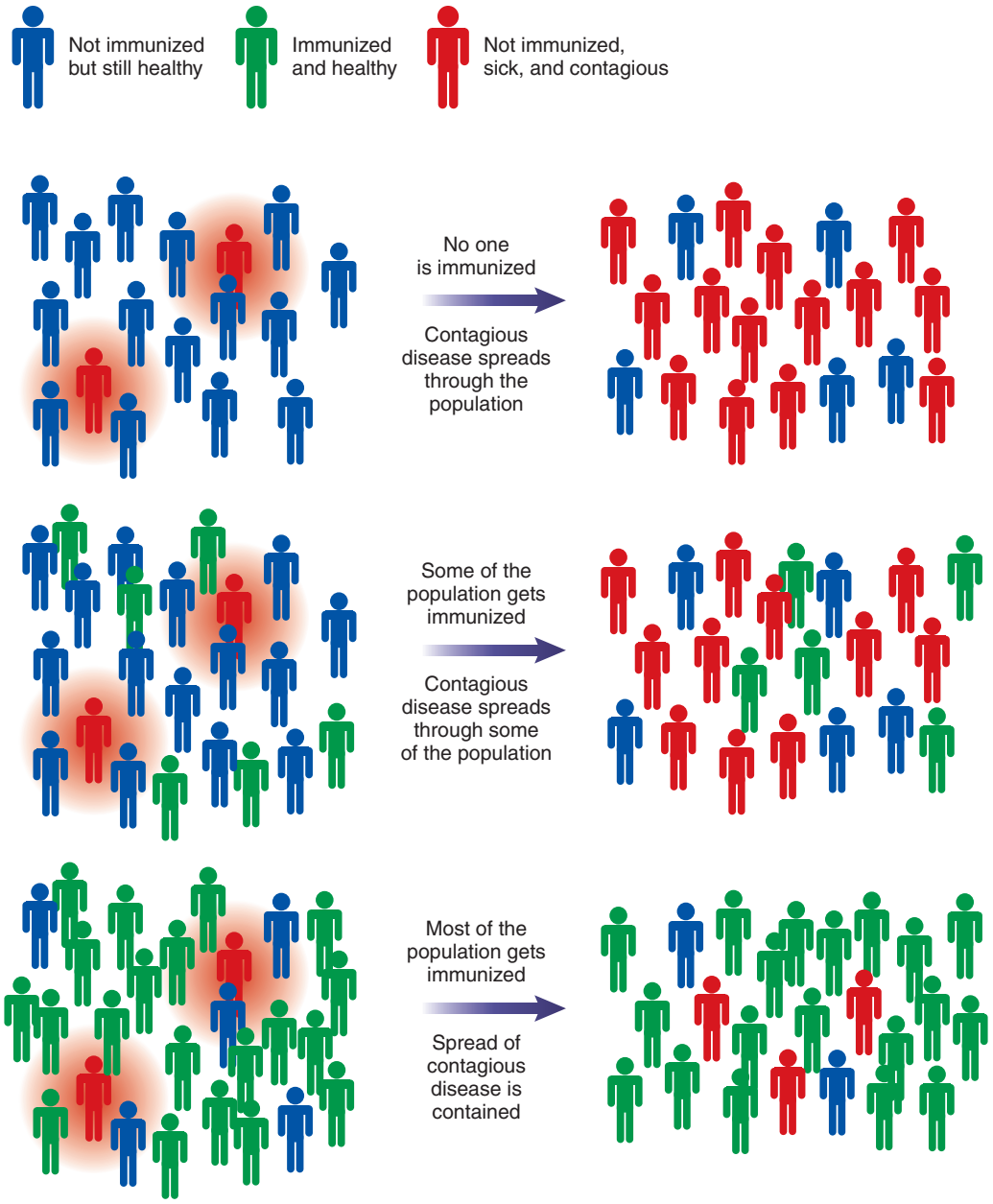


FIGURE 25–6 Community immunity ("herd immunity"). *(Adapted from the National Institute of Allergy and Infectious Diseases. http://www.niaid.nih.gov/topics/pages/communityimmunity.aspx Accessed November 30, 2015.)*

triggered by vaccine additives such as gelatin (a stabilizer) or neomycin (an antibiotic used to prevent bacterial contamination). Anaphylactic reactions are rare, occurring in 0.65 cases per 1 million vaccine doses, on average.[48] Development of an Arthus skin reaction because of local immune complex formation (type III hypersensitivity) has been reported in individuals who have received a booster shot of the tetanus vaccine and who possess residual antibodies from previous tetanus immunization (see Chapter 14).[48] Contact dermatitis, a delayed (type IV) hypersensitivity reaction that appears 48 hours after vaccination, is considered harmless. It has been reported in some individuals in response to vaccine additives, including adjuvants such as alum, preservatives such as thimerosol and 2-phenoxyethanol, the toxin-inactivating agent formaldehyde, and antibiotics such as neomycin.[49] Hypersensitivity develops infrequently in response to vaccines and human serum preparations and is more common when serum from animal sources is used for passive immunization.

Fortunately, other adverse effects associated with vaccines are rare. Interested readers can learn about effects associated with specific vaccines from vaccine information sheets and written inserts that accompany vaccine preparations and from the CDC website on vaccines and immunizations.[32] The most dramatic example of such an effect was the development of paralytic poliomyelitis after use of the live, attenuated oral (Sabin) vaccine for polio. Occurring at a rate of approximately 1 case per 2.7 million doses of the vaccine, this event was caused by reversion of the Sabin polio virus type 3 to a

Connections

Hypersensitivity Reactions

Hypersensitivity to vaccine components can be in the form of type I, II, or III reactions. Recall the mechanisms of these reactions from Chapter 14. In sum:

Type I reactions: Individuals produce high levels of IgE antibody, which binds to mast cells and basophils. Binding of allergen to adjacent cell-bound IgE antibodies triggers the granules in these cells to release chemical mediators, which rapidly induce inflammatory reactions and smooth muscle contractions.

Type III reactions: Persons develop IgG and IgM antibodies that bind to vaccine or serum components. Complement binds to these complexes, activating the classical pathway and release of inflammatory mediators. Neutrophils are attracted to the areas of immune complex deposition, where they release lysosomal enzymes that destroy surrounding tissues.

Type IV reactions: Some individuals may develop a delayed response to a vaccine component. This is a cell-mediated reaction in which Th1 cells are stimulated to release cytokines that attract macrophages and cause inflammation.

neurovirulent strain.[4] To avoid this tragic consequence, industrialized countries have stopped using the Sabin vaccine and replaced it with the injectable, killed Salk-type polio vaccine in their routine immunization schedules.[50] Another example of a potentially serious vaccine consequence was the possible association of a vaccine for Lyme disease with development of chronic arthritis that was resistant to treatment. This condition developed in individuals with the HLA type DR-4, possibly because of a cross-reactive autoimmune response.[51] Although the association of the vaccine with arthritis could not be definitively demonstrated, wide media coverage generated fear from the public and the vaccine was withdrawn from the market in 2002, just 3 years after its licensure.

In 1998, public concern arose from a study conducted in England by Dr. Andrew Wakefield, who proposed a linkage between the MMR vaccine and development of a form of autism. Wakefield and his colleagues hypothesized that the vaccine caused intestinal inflammation and damage to the intestinal barrier, allowing pathogenic proteins to enter into the bloodstream and cause damage to the brain.[52] It was not until 2010 that research studies using valid scientific designs proved Wakefield's findings to be unsubstantiated; his early papers were then retracted.[52] Unfortunately, wide media coverage of Wakefield's claims had stirred up fears in the public and many parents refused to get their children vaccinated.

Fears such as these, as well as religious or personal objections against immunization, have caused some individuals to delay or refuse vaccination for themselves or their children. Anti-vaccine public sentiments can result in lower than optimal vaccine coverage, leaving a significant number of individuals in the population unprotected against serious diseases. This situation, coupled with importation of diseases by unvaccinated individuals immigrating into a country, can lead to outbreaks of diseases that would normally be preventable. For example, from 2001 to 2011, 63 outbreaks of measles, resulting in 911 cases of the infection, were reported in the United States, mostly in people who had not been vaccinated against the disease or who had unknown vaccination status.[53] Pertussis presents another challenge, because immunity wanes 5 to 10 years after vaccination, requiring multiple boosters at various ages to maintain adequate protection.[54] Failure to maintain immunity with up-to-date immunization has resulted in frequent outbreaks, with over 48,000 cases of pertussis reported in the United States in the year 2012.[55] High vaccine coverage is essential to preventing such outbreaks and maintaining a healthy population.

Passive Immunization

As we previously discussed, passive immunity results from the transfer of preformed antibodies to an unimmunized host. Antibodies can be transferred naturally to a mother's fetus or infant in two ways: (1) A pregnant woman's IgG antibodies pass through the placenta to her unborn fetus or (2) maternal IgA antibodies in breast milk and colostrum are ingested by the infant during the nursing process (see Chapter 5). These antibodies provide protection to the newborn against pathogens to which the mother has developed immunity, either through natural infection or through vaccination. IgG antibodies protect the infant during its first few months of life, a time when the baby's own immune system is immature and has not yet encountered many antigens. IgA antibodies provide mucosal immunity, an important mechanism in attacking pathogens at their portals of entry into the body. Antibodies can also be passively transferred to a host as a means of immunoprophylaxis or immunotherapy.

Passive Immunization as Therapy for Infectious Diseases

The benefits of passive immunization were first discovered in the late 1800s by von Behring and Kitasto. The two scientists first developed an antibody preparation against diphtheria and tetanus by injecting rabbits with small doses of the toxins responsible for these diseases. They went on to demonstrate that injection of serum from these rabbits into mice could protect the mice from infection with virulent forms of the bacteria that caused the two diseases.[14,56,57] These historical experiments showed that protective substances (now known to be antibodies) could be generated in the blood and passively transfer their immune properties when injected into nonimmune individuals.

Today, human serum preparations are used to provide passive immunity to individuals who have been exposed to a pathogen but have not been vaccinated or developed immunity through natural infection. Two types of preparations are available: Standard **human immune serum globulin** (also known as **HISG** or *gamma globulin*) and specific human immune serum globulins. Standard HISG is a sterile preparation of concentrated antibodies made from pooled serum of several thousands of donors.[58] In the United States, only donors who test negative for hepatitis B and HIV are used. The plasma from these individuals is enriched for immunoglobulins by a cold

ethanol precipitation procedure, known as Cohn's alcohol fractionation.[59] This is followed by depletion of blood coagulation factors, removal of IgG aggregates, and several virus inactivation steps to further ensure safety of the preparation.[58] HISG consists predominantly of IgG; IgM and IgA are found in insignificant quantities because of their lower serum concentrations and rapid half-lives (see Chapter 5).[59] HISG has been administered for more than 60 years as a prophylactic treatment to prevent infections in immunodeficient patients who are unable to produce sufficient amounts of antibodies (see Chapter 17).[58] HISG contains antibodies specific for numerous antigens to provide generalized humoral protection against a variety of pathogens.

Antigen-specific immune globulins, also known as *hyperimmune globulins,* are prepared from pooled serum of human donors who have developed immunity against a particular pathogen through a recent natural infection or vaccination. These preparations contain a high concentration of antibody against the pathogen or its product and are used to treat individuals who have been potentially exposed to the pathogen but have not been immunized. The potency of the preparation is ensured by determining the antibody titer through laboratory testing. Specific HISGs have been developed for a variety of infectious diseases, including hepatitis A, hepatitis B (hepatitis B immune globulin; HBIG), varicella zoster, rabies, tetanus, and respiratory syncytial virus.[5,59,60]

Specific immune globulins for some antigens have also been prepared from animal sera, usually horse serum. Examples of these include antitoxins for tetanus, diphtheria, and botulism, as well as antisera against snake venoms. **Antitoxins** are antibodies that specifically bind to epitopes on bacterial toxins. They protect against the harmful effects of these toxins by neutralizing their activity.

Advantages and Limitations of Passive Immunization

As we previously mentioned in this chapter, the main advantage of passive immunization is that it provides immediate immunity to the host. This is because the antibodies are already present in the serum that is being transferred and the host does not have to experience the lag period required for its own immune system to be activated by the antigen (see Chapter 5). This immediate protection can be especially beneficial in situations in which unimmunized individuals have been exposed to a harmful antigen; they would develop disease symptoms and possibly die if they had to wait for an immune response to occur. For example, disease could be avoided in an unvaccinated person who had contact with soil that was potentially contaminated with tetanus-causing bacteria or who had an accidental needlestick involving blood from a hepatitis B patient. Hepatitis A could be prevented in customers who dined at a restaurant in which a food handler was found to have the infection, and death could be prevented in people who have been bitten by a poisonous snake or an animal with rabies. When a mother naturally transfers antibodies to her infant through the placenta or breast milk, her child is provided with immediate protection to a variety of pathogens until its own immune system can mature.

However, passive immunity is not long-lasting. The length of the immunity is limited by the biological half-life of the immunoglobulins (23 days for IgG, the predominant immunoglobulin in human serum). Therefore, patients with immunodeficiency diseases require repeated, periodic injections or intravenous administration of HISG to be adequately protected. In addition, no memory lymphocytes are generated, so an individual will not be protected if exposure to the same antigen occurs at a later time in life. Another disadvantage of passive immunization is that hypersensitivity reactions, although rare with HISG, can occur frequently after therapy with animal serum. These reactions involve type I hypersensitivity (anaphylaxis) or type III hypersensitivity (serum sickness) (see Chapter 14).

Immunosuppressive Effects of Passive Immunization

In addition to its protective effects, passive immunization of gamma globulins can also have immunosuppressive effects in certain situations. For example, in Chapter 14, we discussed how the administration of Rhogam can prevent hemolytic disease of the newborn. Rhogam inhibits the production of anti-Rh antibodies in an Rh-negative mother toward paternally-derived Rh antigens on her fetus. In addition, it has been found that intravenous immunoglobulin therapy can modulate the proinflammatory activities of IgG antibodies in patients with autoimmune diseases. Over 30 years ago it was discovered that intravenous infusion of HISG results in an immediate increase in platelet counts and improvement of symptoms in patients with immune thrombocytopenia (ITP).[58] Similar results have been found in patients with other inflammatory disorders. Intravenous immunoglobulin therapy is approved by the Food and Drug Administration (FDA) for treatment of ITP, chronic inflammatory demyelinating polyneuropathy (CIDP), Kawasaki's disease, and Guillain-Barre syndrome. Its effects are also being studied in other chronic inflammatory disorders, including multiple sclerosis, systemic vasculitis, rheumatoid arthritis, SLE, autoimmune hemolytic anemia, autoimmune skin blistering diseases, graft-vs.-host disease (GVHD), and sepsis.[58]

The way in which intravenous immunoglobulins inhibit the inflammatory response is unclear, but several mechanisms have been proposed.[5,58] The antibodies in the HISG preparation contain many antigen specificities and may mediate killing of target cells by antibody-dependent cellular cytotoxicity (ADCC), prevent interactions of ligands with cell surface receptors, inhibit cytokines, neutralize autoantibodies, or bind to activated complement components such as C3a and C5a, blocking their activity. Other immunomodulating effects of HISG may be mediated through the Fc portion of the immunoglobulins, including saturation of Fc receptors on cells of the immune system, limiting the access of immune complexes to Fc receptors on phagocytic cells, enhancement of T regulatory (Treg) cell activity, modulation of dendritic cell activation, or inhibition of B-cell function.

Monoclonal Antibodies

Monoclonal antibodies, as we discussed in Chapter 5, are derived from a single clone of B cells; therefore, they have exquisite specificity for a particular epitope of an antigen. This specificity is being harnessed in the use of monoclonal antibodies as agents of passive immunization, most notably for the treatment of cancer and autoimmune diseases.[61,62] Numerous monoclonal antibodies have been approved by the FDA for treatment of patients with hematologic malignancies, solid tumors, autoimmune disorders, and other miscellaneous conditions.[61] Examples of monoclonal antibodies that have been widely used as therapeutic agents include rituximab, directed against the CD20 antigen on B cells, for treatment of non-Hodgkin lymphoma; trastuzumab (Herceptin), directed against Her2/neu, for treatment of certain breast cancers; and adalimumab (Humira), directed against tumor necrosis factor-α (TNF-α), used for reduction of inflammation in patients with rheumatoid arthritis. These and other examples of monoclonal antibodies approved by the FDA for therapeutic use in patients are listed in **Table 25–1.**

As was previously discussed in Chapter 5, monoclonal antibodies were originally developed using mouse B cells to produce hybridomas that secreted antibody to the desired antigen. Mouse monoclonal antibodies can be identified by the suffix, "*–omab*." When these monoclonal antibodies were used as therapeutic agents in humans, immune responses were likely to occur to the foreign epitopes on the mouse immunoglobulins, resulting in the production of *human anti-mouse antibodies (HAMA)*. HAMA significantly limited the usefulness of the monoclonal antibody therapy because they caused type I (anaphylactic) or type III (immune complex) hypersensitivity reactions.

Over the years, the development of recombinant DNA technology has allowed us to produce monoclonal antibodies that have an increasingly larger human component, making them less likely to trigger an adverse immune reaction. Initially, chimeric antibodies were developed, which consisted of a mouse-derived immunoglobulin variable region combined with a human-derived constant region **(Fig. 25–7).** Chimeric antibodies can be identified by the suffix "*–ximab*." This was followed by the production of humanized antibodies, which contain all human sequences except for the antigen-binding complementarity determining regions; the latter are mouse derived. Humanized antibodies are denoted by the suffix, "*–zumab*." Today, it is possible to produce fully human

Connections

Monoclonal Antibodies and Mice

Monoclonal antibodies were originally produced from mice, by injecting the mice with the desired antigen, isolating B cells from the immunized animals, and fusing them with cultured mouse myeloma cells to produce immortal hybrid cell lines known as "hybridomas" (see Chapter 5). Cell culture techniques were used to isolate the hybridomas that produced the desired antibody. The monoclonal antibodies purified from these cultures consisted entirely of mouse protein.

Table 25–1 Examples of FDA-Approved Monoclonal Antibodies Used for Immunotherapy

NAME*	TYPE	SPECIFICITY	DISEASE INDICATION(S)
Adalimumab (Humira)	Human	TNF-α	Rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, plaque psoriasis, juvenile idiopathic arthritis
Alemtuzumab (Campath)	Humanized	CD52	Chronic lymphocytic leukemia
Basiliximab (Simulect)	Chimeric	CD25	Kidney transplant rejection
Bevacizumab (Avastin)	Humanized	Vascular endothelial growth factor (VEGF)	Colorectal cancer, non-small cell lung carcinoma, renal cell carcinomas, glioblastoma
Cetuximab (Erbitux)	Chimeric	Epidermal growth factor receptor (EGFR)	Colorectal cancer, squamous cell carcinoma of the head and neck
Infliximab (Remicade)	Chimeric	TNF-α	Rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, plaque psoriasis, ulcerative colitis
Ipilimumab (Yervoy)	Human	CTLA-4	Melanoma
Omalizumab (Xolair)	Humanized	IgE	Allergic asthma
Rituximab (Rituxan)	Chimeric	CD20	Non-Hodgkin lymphoma, chronic lymphocytic leukemia, rheumatoid arthritis, Wegener's granulomatosis, microscopic polyangitis
Trastuzumab (Herceptin)	Humanized	HER2/neu	Breast cancer, gastric adenocarcinoma, gastroesophageal junction adenocarcinoma

**Brand names are in parentheses.*

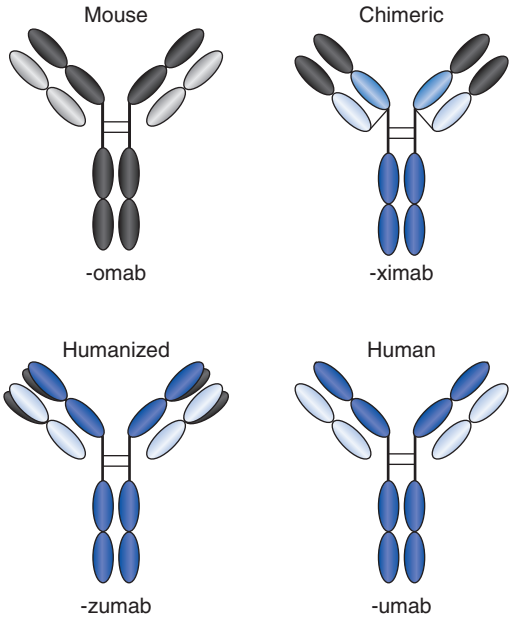


FIGURE 25–7 Monoclonal antibodies used as therapeutic agents can be made in mice, but humans may react to the foreign protein. The antigen-binding portion of the molecule can be grafted onto the constant regions of human antibodies to reduce such reactivity. It is also possible to humanize such antibodies so that only variable regions are incorporated into human antibody molecules.

monoclonal antibodies by phage display, a technique in which genes coding for a specific antibody molecule are cloned in bacteriophages or by transgenic mouse technologies involving incorporation of the immunoglobulin genes of interest into genetically modified mice.[61] Fully human antibodies are indicated by the suffix, "*–umab*." These antibodies have a low potential for immunogenicity and are becoming more widely available for therapeutic use.[61,63]

The development of humanized and fully human antibodies has especially had an impact in the area of cancer treatment. Monoclonal antibodies are able to mediate killing of tumor cells by a variety of mechanisms (see Chapter 17).[61,63] They can directly kill tumor cells by binding to cell-surface receptors or other membrane-bound proteins, causing apoptosis or inhibition of signaling and cell proliferation. They can also bind to the tumor cells and facilitate their destruction through opsonization and phagocytosis, ADCC, activation of the complement cascade, or activation of T cells. Some monoclonal antibodies have been conjugated to drugs or toxins, allowing other therapeutic agents to be specifically delivered to the tumor cells. Because of their ability to specifically target a diverse range of molecules, monoclonal antibodies are considered to be one of the most successful cancer therapies in the last 20 years.[63]

Adoptive Immunotherapy

Although passive immunization is aimed at providing the humoral component of the immune response, adoptive immunotherapy involves transferring cells of the immune system to increase the cell-mediated immune response of the recipient. The cells transferred can be derived from a donor individual or from the recipients themselves. In the case of *autologous* transfers, the cells are removed from the patient, treated in vitro to become more immunologically reactive, and infused back into the same patient. The cells transferred in adoptive immunotherapy can be naturally derived or genetically engineered to target a particular antigen. Heterogeneous cell types can be transferred, although most applications have focused on the transfer of T-cell–mediated activity.

Human studies involving adoptive cell transfer have led to the development of some exciting immunotherapies for cancer patients and transplant recipients. Cancer immunotherapy with adoptively transferred cells is based on the observation of immune responses against tumor cells in animal studies. In the 1960s, researchers discovered that lymph node cells from mice immunized with chemically or virally induced tumors were able to protect against growth of the same tumor type when they were transferred to genetically identical recipients.[64,65] They found that this immune response was enhanced in the presence of the cytokine IL-2.[66] Scientists later began to look for the ability of lymphoid cells actually found within a tumor mass to mount an anti-tumor response. Through their work, they identified a heterogeneous population of T cells that can demonstrate cytotoxic effects against the tumor. These cells came to be known as **tumor-infiltrating lymphocytes (TILs).**[67] TILs appear to be ineffective in eliminating the tumor at their natural site, possibly because they are not present in adequate numbers, they become anergic as a result of chronic activation, or they are surrounded by Treg cells and myeloid-derived suppressor cells.[66,67] However, Rosenberg and his colleagues at the National Institutes of Health (NIH) found that they could enrich for and expand the TIL population in vitro by incubating cells from the tumor in the presence of IL-2.

In 1986, Rosenberg's group demonstrated protective effects of autologous TILs against metastases in mice. In 1988, the researchers applied a similar approach to humans. In their landmark publication, the scientists showed that TILs isolated from patients with metastatic melanoma and expanded in vitro could mediate tumor regression when given back to the same patients.[68] In this adoptive immunotherapy procedure, which has been improved over the years, tumors are surgically removed, dissected into fragments, and incubated in culture in the presence of IL-2 for a few weeks. During the incubation period, the TILs proliferate and kill the residual tumor cells. T cells showing potent anti-tumor activity are isolated from the mixture and further expanded in the presence of IL-2, anti-CD3 antibody, and irradiated mononuclear cells derived from normal peripheral blood.[66] These purified, potent TILs are then infused into the same patient from which the tumor fragments were derived. The expanded TILs migrate back to the tumor, where they mediate its destruction (see Fig 17-8).

This therapy has been cited as the most effective treatment for patients with metastatic melanoma.[66] In a study conducted in 2011, adoptive transfer of autologous TILs along with IL-2 into patients with stage IV melanoma resulted in objective clinical response rates in up to 72% of the patients. Complete

tumor regression was seen in 22% of the patients; the majority of these patients were free of the disease at the time of follow-up, which ranged from 57 months to over 8 years.[67] The success of this treatment is thought to be to the result of the high level of immunogenicity of melanoma tumors and pretreatment of patients with total body irradiation, which is thought to deplete regulatory and suppressor cells within the host that could inhibit the anti-tumor effects of the TILs.[66,67] Adoptive TIL therapy is also being investigated in patients with other types of tumors who have exhausted other treatment options. Efforts are underway to improve techniques to reduce the cost of this expensive, personalized treatment so that it can be available to a larger number of patients.[67] Research with genetically engineered T cells expressing high-affinity T-cell receptors for target tumor antigens is also underway.[67]

Hematopoietic stem-cell transplantation is another area in which medical practitioners are using adoptive immunotherapy. These stem cells are routinely used as a treatment for certain immunodeficiency disorders (see Chapter 19).[69] Autologous or allogeneic transplantation of hematopoietic stem cells can also be used to restore the immune system in patients who have been treated with chemotherapy and a high dose of total body irradiation. The treatment has been used most commonly in patients with multiple myeloma, non-Hodgkin lymphoma, or acute leukemias.[69]

Clinical trials are also underway in other exciting areas related to transplant immunology. For example, clinical trials involving adoptive transfer of Treg cells to transplant patients are being conducted in an attempt to prevent graft rejection or GVHD.[70] In addition, medical scientists are adoptively transferring cytotoxic T cells to transplant recipients to treat opportunistic infections with viruses such as CMV, EBV, and adenovirus and their associated complications, which can be life threatening in these immunosuppressed patients.[71]

SUMMARY

- Immunity is the condition of resistance to a disease. Immunization is the process by which immunity is acquired. There are three types of immunization—active, passive, and adoptive.
- Active immunization involves stimulation of an individual's own immune system to mount an adaptive immune response to an antigen, as a result of either natural infection or receipt of a vaccine.
- Passive immunization involves transfer of premade antibodies from an immune host to a nonimmune host. This type of immunity can occur naturally, by transfer of maternal IgG antibodies through the placenta to the fetus, or by transfer of IgA antibodies from a mother to her infant through breast milk. Passive immunity can also be transferred artificially through commercial antibody preparations such as human immune serum globulin.
- Adoptive immunotherapy is achieved by transferring cells of the immune system to a nonimmune host. The cells can be derived either from a different (allogeneic) host or they can be stimulated in vitro before re-introduction into the same individual (autologous).
- Active immunity takes time to develop, but provides long-lasting protection to the host because of the production of immunologic memory. Passive immunity provides immediate protection, but is short lived because antibody titers decline at a rate that is determined by the biological half-life of the immunoglobulins. Adoptive immunity provides long-lasting protection if the cells become established in the host.
- A traditional vaccine is a microbially derived antigen suspension that is administered to a healthy host in order to stimulate an immune response that will prevent the host from developing an infectious disease. Thus, vaccines are used for immunoprophylaxis.
- In 1796, Dr. Edward Jenner was the first to publicize the procedure of vaccination by using material from cowpox skin lesions to immunize against smallpox. In the 1800s, Louis Pasteur used the principle of attenuation, or weakened microorganisms, to produce vaccines against chicken cholera, anthrax, and rabies.
- In the 20th century, vaccine development grew exponentially as a result of new methods to attenuate microorganisms, production of toxoids, and technologies that allowed viruses to be grown in cell culture. In the latter part of the 20th century, the first recombinant vaccine, produced by genetic engineering, was developed.
- Conventional vaccines may be composed of live, attenuated (weakened, nonpathogenic) microorganisms; inactivated (killed) microorganisms; or antigenic subunits such as recombinant antigens or toxoids. Toxoids are bacterial toxins that have been inactivated so that they are no longer pathogenic, but are still immunogenic.
- Adjuvants are substances that are often included in vaccines in order to increase the immune response to the target antigens. Examples of adjuvants include alum (aluminum hydroxide and aluminum phosphate) and oil-in-water emulsions. They are believed to enhance the immune response by promoting migration and antigen uptake by APCs and inducing the release of proinflammatory cytokines and chemokines.
- In the future, scientists will develop next generation vaccines that will identify vaccine target antigens more efficiently by using genetic technologies. In developing these vaccines, researchers will attempt to induce broadly neutralizing antibodies and cell-mediated responses, employ novel adjuvants, and investigate the use of nonparenteral routes of delivery.
- The effectiveness of a vaccine is not only influenced by the nature of the vaccine, but also by the age and immune status of the host. To obtain an optimal immune response, vaccines must be administered according to recommended schedules.

- Vaccines protect not only the individuals who have received the vaccines, but also their contacts, a phenomenon known as "community immunity" or "herd immunity."
- Vaccines are considered to be one of the greatest achievements of medicine. The benefits of vaccines greatly outweigh their risks. Rarely, vaccines can cause side effects such as hypersensitivity reactions. Live vaccines should not be administered to patients with immunodeficiency disorders because they can replicate uncontrollably and cause disseminated disease.
- Passive immunization with preformed antibody preparations is administered in order to provide immediate humoral immunity to unimmunized persons.
- Patients with humoral immunodeficiencies are routinely treated with standard human immune serum globulin, prepared from pooled serum of many donors, to provide protection against a variety of pathogens. Specific human immune serum globulins containing antibody against a particular pathogen can be used to treat nonimmune, healthy individuals who have had contact with the pathogen to provide immediate protection.
- Antitoxins are antibodies directed against toxins from pathogenic bacteria. They can be isolated from the serum of laboratory animals that have been injected with the toxin and used to prevent diseases such as tetanus, diphtheria, and botulism.
- Passive immunization of immunoglobulins can also be used as immunosuppressive therapy for certain autoimmune and chronic inflammatory disorders, as well as to prevent hemolytic disease of the newborn.
- Monoclonal antibodies have specificity for a particular antigenic epitope. They are being used as therapeutic agents for a variety of cancers and autoimmune diseases. These antibodies were originally 100% mouse derived because they were produced by hybridoma technology. Advancement in genetic technologies has allowed scientists to synthesize hybrid antibodies that consist partially of mouse protein and partially of human protein. These antibodies can be characterized as chimeric, humanized, or fully human, depending on the amount of the human component. Administration of these antibodies reduces the chance that the recipients will produce human-anti-mouse antibodies (HAMA) and develop hypersensitivity reactions.
- Adoptive immunotherapy involves the transfer of cells of the immune system to provide immunity. Examples include the treatment of melanoma patients with autologous, IL-2 activated tumor-infiltrating lymphocytes (TIL) and the administration of hematopoietic stem cells to patients with hematologic malignancies who have been treated with high doses of chemotherapy and irradiation.

Study Guide: Major Features of Active Immunity, Passive Immunity, and Adoptive Immunity

	MECHANISM	EXAMPLES	ADVANTAGES	LIMITATIONS
Active Immunity	Activation of humoral and cell-mediated responses in an individual's own immune system by exposure to an antigen	Natural infection with pathogen Immunization with a vaccine	Long-term immunologic memory to the antigen is generated	Delay in initiation of immune response
Passive Immunity	Transfer of antibodies from immunized host(s) to nonimmune individuals	Passage of IgG through the placenta, from pregnant woman to her fetus Passage of IgA through breast milk, from mother to infant Standard human immune serum globulin Specific human immune serum globulin Antitoxins Rhogam Monoclonal antibodies	Provides immediate protection to the recipient	Immunity is temporary, declining with the half-life of the antibodies Immunologic memory is not generated Hypersensitivity can develop, especially when sera of animal origin are used
Adoptive Immunity	Transfer of cells of the immune system to nonimmune individuals	Adoptive immunotherapy with activated TILs to cancer patients Hematopoietic stem cell transplantation	Can transfer cell-mediated immunity	Patient's own immune cells must be depleted to increase chance of successful therapy Allogeneic cells may be rejected

Study Guide: Characteristics of Conventional Vaccines

	COMPOSITION	EXAMPLES	ADVANTAGES	LIMITATIONS
Attenuated	Live pathogens that have been weakened by growth under modified culture conditions	BCG Typhoid fever Oral polio (Sabin) Measles (Rubeola) Mumps German measles (Rubella) Chickenpox (Varicella) Shingles (Zoster) Influenza (nasal mist) Rotavirus	Induce both humoral and cell-mediated immunity Effective in inducing immunity after a single dose	Cannot be administered to immunocompromised individuals Rare potential to mutate to a pathogenic form Maternal antibodies can interfere with immune response to the vaccine in infants Require careful handling and storage
Inactivated	Killed microorganisms	Intramuscular polio (Salk) Influenza (intramuscular or intradermal) Hepatitis A	Can safely be given to immunocompromised individuals	Stimulates humoral immunity but little or no cell-mediated immunity May require two or more booster doses to produce protective immunity
Subunit	One or more purified components of a pathogen	Toxoids Purified proteins Polysaccharides Recombinant antigens	Induces an immune response to the pathogenic component(s) of a microorganism Safer than administration of an intact organism	Requires two or more booster doses to produce protective immunity Requires an adjuvant to increase immunogenicity Must be multivalent if a broad immune response is desired
a. Toxoids	Bacterial toxins that have been chemically inactivated so that they are not pathogenic	Diphtheria Tetanus	See information for Subunit vaccines in this table	Requires two or more booster doses to produce protective immunity Requires an adjuvant to increase immunogenicity
b. Purified Components	Biochemically purified components of a microorganism	Pertussis (whooping cough)	See information for Subunit vaccines in this table Produces fewer side effects than whole bacteria	See information for Subunit vaccines in this table
c. Polysaccharides	Biochemically purified polysaccharide from bacterial capsule	Streptococcal pneumonia *Haemophilus influenzae* type b Neisserial *meningitis*	See information for Subunit vaccines in this table	See information for Subunit vaccines in this table Requires conjugation to a carrier protein to induce IgG production and long-term immunity
d. Recombinant Antigen	Protein produced by genetically modified nonpathogenic bacteria, yeast, or other cells	Hepatitis B Human papilloma virus (cervical, anal, genital cancers)	Highly purified protein that is safer than administration of intact organism	See information for Subunit vaccines in this table Cannot be used to produce antigens other than proteins

CASE STUDIES

1. A 2-year-old boy has made numerous visits to his doctor because he has suffered from recurring respiratory and ear infections. An immunologic workup revealed that the child had a low number of B cells and decreased immunoglobulin concentrations. Based on these results, the boy was diagnosed with an antibody immunodeficiency disease.

Questions

a. What childhood vaccines could safely be administered to this child? What is the composition of these vaccines?
b. What childhood vaccines should not be administered to this child? Why?
c. How can the child be protected against the diseases for which he is unable to receive vaccination?

2. Suppose you ate lunch at a popular restaurant a few days ago. Your local health department puts out a notice that several of the customers who dined at the restaurant recently have confirmed cases of hepatitis A and that the source of infection was traced to a supply of green onions that had been used in the salads. Public health officials advise anyone who has eaten at the restaurant in the last 2 weeks to visit the local county health department to receive an injection to prevent the infection.

Questions

a. What would the injection consist of, and why would it be the treatment of choice?
b. If you received this treatment and did not develop hepatitis A, would you be immune to this virus 10 years from now? Why or why not?

REVIEW QUESTIONS

1. Suppose an individual develops antibodies in response to a streptococcal pharyngitis infection. This is an example of
   a. active immunity.
   b. passive immunity.
   c. adoptive immunity.
   d. immunoprophylaxis.

2. Which of the following illustrates passive immunity?
   a. Development of high antibody titers in a healthy person after receipt of the hepatitis B vaccine
   b. Recovery of a patient from a hepatitis A infection
   c. Passage of IgG antibodies through the placenta of a pregnant woman to her fetus
   d. Transfer of tumor-infiltrating lymphocytes to a cancer patient

3. Which of the following is *not* a characteristic of passive immunity?
   a. Transfer of antibodies
   b. Occurs naturally or as a result of therapy
   c. Provision of immediate protection
   d. Development of long-term memory

4. What was one of the major contributions of Louis Pasteur to vaccine development?
   a. Development of the smallpox vaccine
   b. Use of attenuated microorganisms in vaccines
   c. Inactivation of bacterial toxins for vaccines
   d. Discovery of recombinant vaccine antigens

5. The antigenic component of the hepatitis B vaccine differs from those of many of the conventional vaccines in that it consists of a
   a. live, attenuated virus.
   b. inactivated virus.
   c. cryptic antigen.
   d. recombinant antigen.

6. Which of the following describes the properties of a toxoid?
   a. Both pathogenic and immunogenic
   b. Pathogenic but not immunogenic
   c. Not pathogenic but immunogenic
   d. Neither pathogenic nor immunogenic

7. Suppose a vaccine was available in two forms: attenuated and inactivated. What is an advantage of the attenuated form?
   a. It can be used in immunocompromised patients.
   b. It induces both humoral and cell-mediated immunity.
   c. There is no interference of the immune response in infants by maternal antibodies.
   d. It does not require special handling and storage to maintain its effectiveness.

8. What factor(s) influence the effectiveness of a person's immune response to a vaccine?
   a. Age of the recipient
   b. The individual's immune status
   c. The nature of the vaccine
   d. All of the above

9. An oral vaccine may be advantageous over an injectable vaccine for a pathogen because it
   - **a.** reduces the risk of transmitting bloodborne pathogens in developing areas of the world.
   - **b.** avoids the pain associated with injections.
   - **c.** induces mucosal immunity.
   - **d.** all of the above.

10. When one individual becomes immunized by receiving a series of vaccine injections according to schedule, the resulting protection extends to that individual's nearby contacts. This concept is known as
   - **a.** immunologic memory.
   - **b.** neighborhood immunity.
   - **c.** herd immunity.
   - **d.** contagious immunity.

11. Which preparation would you recommend for treatment of a patient with an antibody deficiency?
   - **a.** Monoclonal antibody
   - **b.** Specific human immune serum globulin
   - **c.** Standard human immune serum globulin
   - **d.** Animal serum antitoxins

12. Immunoglobulins consisting of a mouse-derived variable region combined with a human-derived constant region are known as
   - **a.** monoclonal antibodies.
   - **b.** chimeric antibodies.
   - **c.** humanized antibodies.
   - **d.** fully human antibodies.

13. HAMA are
   - **a.** mouse-derived antibodies that have been used for therapy.
   - **b.** monoclonal antibodies with therapeutic benefits.
   - **c.** human antibodies that are produced against mouse proteins.
   - **d.** antitoxins that can provide immediate immunity.

14. What is a major characteristic of adoptive immunotherapy?
   - **a.** It involves the transfer of cells to deliver immunity.
   - **b.** It involves the transfer of cytokines to deliver immunity.
   - **c.** It can only occur in the presence of autologous cells.
   - **d.** Its purpose is to increase the humoral immune response.

15. Infusion of TILs into a cancer patient is an example of
   - **a.** active immunity.
   - **b.** adoptive immunity.
   - **c.** passive immunity.
   - **d.** natural immunity.

Glossary

Accelerated rejection: A form of graft rejection that occurs within 1 to 5 days after second exposure to tissue antigens based on reactivation of B- and T-cell responses.

Accuracy: The ability of a test to actually measure what it claims to measure.

Acquired immunodeficiency syndrome (AIDS): A disease affecting the immune system caused by the human immunodeficiency virus (HIV).

Activation unit: The combination of complement components C1, C4b, and C2b that form the enzyme C3 convertase, whose substrate is C3.

Active immunity: Immunity resulting from natural exposure to an infectious agent or administration of a vaccine.

Acute graft-versus-host disease (GVHD): Graft-versus-host disease, which occurs shortly after immunocompetent cells are transplanted into a recipient. It is characterized by skin rashes, diarrhea, and increased susceptibility to infection.

Acute-phase reactants: Normal serum proteins that increase rapidly as a result of infection, injury, or trauma to the tissues.

Acute rejection (AR): A type of rejection that occurs days to weeks after transplantation as the result of cellular mechanisms and antibody formation.

Acute rheumatic fever: A disease that develops as a sequel to group A streptococcal pharyngitis, characterized by the presence of antibodies that cross-react with heart tissue.

Adaptive immunity: A type of resistance that is characterized by specificity for each individual pathogen, or microbial agent, and the ability to remember a prior exposure, which results in an increased response to that pathogen upon repeated exposure.

Adaptive T regulatory 1 (Tr1) cells: CD4+ T cells induced from antigen-activated naïve T cells under the influence of interleukin-10. They exert suppressive activities.

Adjuvant: A substance administered with an immunogen that enhances and potentiates the immune response.

Adoptive immunity: Immunity resulting from the transfer of cells of the immune system (usually lymphocytes) from an immunized host to a nonimmune individual.

Adoptive immunotherapy: Administration of immune cells to treat patients with conditions such as immunodeficiency diseases or cancer.

Affinity: The initial force of attraction that exists between a Fab site on an antibody and one epitope or a determinant site on the corresponding antigen.

Agammaglobulinemias: Immunodeficiency diseases in which antibody levels in the blood are significantly decreased.

Agglutination: The process by which particulate antigens such as cells aggregate to form large complexes when a specific antibody is present.

Agglutination inhibition: An agglutination reaction based on competition between antigen-coated particles and soluble patient antigens for a limited number of antibody-combining sites. Lack of agglutination is a positive test result.

Agglutinin: An antibody that causes clumping or agglutination of the cells that triggered its formation.

Allele: An alternate form of a gene that codes for a slightly different form of the same product.

Allelic exclusion: The selection of an allele on one chromosome only.

Allergen: An antigen that triggers a type I hypersensitivity response (i.e., an allergy).

Allergy immunotherapy (AIT): Therapy involving administration of increasing doses of an allergen over time with the goal of inducing immune tolerance to the allergen.

Alloantigen: An antigen that is found in another member of the host's species and that is capable of eliciting an immune response in the host.

Allograft: Tissue transferred from an individual of one species into another individual of the same species.

Allotype: A minor variation in amino acid sequence in a particular class of immunoglobulin molecule that is inherited in Mendelian fashion.

Alpha-fetoprotein (AFP): A tumor marker that is commonly elevated in patients with primary hepatocellular carcinoma and nonseminomatous testicular cancer.

Alpha$_1$-antitrypsin (AAT): An acute-phase protein that acts as an inhibitor of proteases released from white blood cells (WBCs).

Alternative pathway: A means of activating complement proteins without an antigen–antibody combination. This pathway is triggered by constituents of microorganisms.

Amplicon: A copy of a select portion of DNA that is obtained by the polymerase chain reaction (PCR).

Amplification: Copying of nucleic acids to increase the amount available for testing.

Analyte: The substance being measured in an immunoassay.

Analytic sensitivity: The lowest measureable amount of an analyte.

Analytic specificity: An assay's ability to generate a negative result when the analyte is not present.

Anaphylatoxin: A small peptide formed during complement activation that causes increased vascular permeability, contraction of smooth muscle, and release of histamine from basophils and mast cells.

Anaphylaxis: A life-threatening response to an allergen characterized by the systemic release of histamine.

Anergy: A state of immune unresponsiveness to a specific antigen.

Antagonism: When the action of one cytokine counteracts the activity of another cytokine.

Antibodies: Glycoproteins produced by B lymphocytes and plasma cells in response to foreign substance exposure. Antibodies are also known as *immunoglobulins*.

Antibody array: A multiplex assay that uses antibody-coated beads to identify target antigens, such as tumor antigens.

Antibody-dependent cell cytotoxicity (ADCC): The process of destroying antibody-coated target cells by natural killer cells, monocytes, macrophages, and neutrophils, all of which have specific receptors for an antibody.

Antibody–drug conjugates: Antibody that is attached to toxins or radioisotopes to help specifically destroy cancer cells.

Anticentromere antibodies: Autoantibodies that bind to proteins in the centromere (the middle region of a chromosome where the sister chromatids are joined); associated with the CREST syndrome.

Anticyclic citrullinated peptide (anti-CCP or ACPA): Autoantibodies to proteins that contain an atypical amino acid called citrulline (a modified arginine), produced by granulocytes and monocytes. This antibody is highly specific for rheumatoid arthritis.

Anti-DNase B: An antibody directed against DNase B, which is secreted by group A streptococci.

Antigen: Macromolecule that is capable of eliciting formation of immunoglobulins (antibodies) or sensitized cells in an immunocompetent host.

Antigen-dependent phase: The final phase of B-cell development, which occurs when a B cell is stimulated by an antigen and undergoes transformation to a blast stage, resulting in the formation of memory cells and antibody-secreting plasma cells.

Antigen-independent phase: The first phase of B-cell development in the bone marrow, which results in mature B cells that have not yet been exposed to antigen.

Antigen presentation: The process by which degraded peptides within cells are transported to the plasma membrane with MHC molecules so T cells can then recognize them.

Antigen switching: A protecting mechanism used by parasites that involves varying synthesis of surface antigens to evade an immune response by the host.

Antigenic concealment: Means by which parasites conceal their antigens from the host by remaining inside of the host's cells without their antigens being displayed.

Antigenic mimicry: A mechanism by which parasites express epitopes that are similar or identical to host molecules, thus protecting the parasite from being recognized and eliminated by the immune system.

Antigenic shedding: A mechanism by which parasites can evade the immune system by shedding surface antigens that bind to the host's antibodies and cells.

Antigenic variation: Result of the process of antigen switching.

Anti-HBe: Antibody to a hepatitis B capsid antigen.

Anti-HBs: Antibody to hepatitis B surface antigen.

Antihistone antibodies: Autoantibodies to histones, which are nucleoproteins found in chromatin. Elevated levels are associated with drug-induced lupus.

Antineutrophil cytoplasmic antibody (ANCA): Autoantibodies produced against proteins in the neutrophil granules; these antibodies are strongly associated with vascular inflammatory syndromes.

Antinuclear antibody (ANA): Antibody produced to different components of the nucleus during the course of several autoimmune diseases. Examples include anti-DNA, antideoxyribonucleoprotein, and antiribonuclear protein antibodies, all of which occur in systemic lupus erythematosus.

Antiphospholipid antibodies: A heterogeneous group of autoantibodies that bind to phospholipids or phospholipid-protein complexes.

Antiretroviral therapy (ART): Therapeutic drugs that suppress the replication of a retrovirus such as HIV.

Anti-RNP antibody: Autoantibodies directed against ribonucleoprotein (RNP), which consists of several nonhistone proteins complexed to a small nuclear RNA called U1-nRNP. These antibodies are found in some patients with autoimmune rheumatic diseases.

Antitoxin: Antibody used in passive immunization for the purpose of neutralizing a bacterial toxin.

Apoptosis: Programmed cell death.

Arthus reaction: A type III hypersensitivity skin reaction that occurs when an animal has a large amount of circulating antibody and is exposed to the antigen intradermally, resulting in localized deposition of immune complexes.

ASO titer: A test for the diagnosis of poststreptococcal sequelae, based on the neutralization of streptolysin 0 by antistreptolysin 0 found in patient serum.

Aspergillosis: An opportunistic fungal infection predominantly caused by *Aspergillus fumigatus*.

Ataxia-telangiectasia (AT): An autosomal recessive syndrome that results in a combined defect of both cellular and humoral immunity. The defect is in a gene responsible for recombination of immunoglobulin superfamily genes.

Atopy: An inherited tendency to respond to naturally occurring allergens; it results in the continual production of IgE.

Attenuation: A process of producing nonpathogenic bacteria or viruses for use in vaccines. These organisms have been weakened by treatment with a chemical, exposure to elevated or cold temperatures, or repeated in vitro passage in cell culture.

Autoantibody: An antibody produced against an antigen found on an individual's own cells, tissues, or organs.

Autoantigen: An antigen that belongs to the host and is not capable of eliciting an immune response under normal circumstances.

Autocrine: Effect produced by a cell that stimulates the same cell to grow.

Autograft: Tissues removed from one area of an individual's body and reintroduced in another area of the same individual.

Autoimmune disease: A condition in which damage to body organs results from the presence of autoantibodies or autoreactive cells.

Autoimmune hemolytic anemia: An autoimmune disorder in which patients form antibodies that destroy their own red blood cells (RBCs).

Autoimmune hepatitis (AIH): Autoimmune disease in which patients produce antibodies that damage the liver; formerly known as chronic active hepatitis.

Autoimmune liver disease: An autoimmune disease that mainly affects the liver, including autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC).

Autoimmune thyroid disease (AITD): An autoimmune disease that affects the function of the thyroid gland, caused by the formation of antibodies or sensitized cells; includes Hashimoto's disease and Graves disease.

Automatic sampling: Automatic pipetting of a sample that is programmed into an instrument for testing of that sample.

Avidity: The strength with which a multivalent antibody binds a multivalent antigen.

Basophil: A type of white blood cell (WBC) found in peripheral blood, containing granules that release substances that are involved in allergic reactions.

Batch analyzer: An instrument that permits analysis of several different samples at the same time.

Bence Jones proteins: Monoclonal immunoglobulin light chains found in the urine of patients with multiple myeloma.

Benign: Tissue that is not malignant.

Biohazardous material: Patient specimens that may contain potentially harmful infectious agents.

Biomarker profiling: The use of proteomic methods to identify unique patterns of proteins that are associated with a disease, such as a specific type of cancer.

Blowout pipette: A type of pipette in which the last drop of liquid must be forced out using a pipetting bulb or other device to deliver an accurate volume.

Bone marrow: The largest tissue in the body, located in the long bones. Its role is the generation of hematopoietic cells.

Borrelia burgdorferi: A spirochete bacterium that is the causative agent of Lyme disease.

Borrelia miyamotoi: A spirochete bacterium that causes relapsing fever.

Branched DNA (bDNA): A technique used to detect a small amount of DNA via several hybridization steps that create a branching effect with several nucleic acid probes.

Bruton's tyrosine kinase (Btk) deficiency: An X-linked recessive immunodeficiency disease that results in a lack of mature B lymphocytes and immunoglobulins of all classes.

Bystander lysis: A phenomenon that occurs in complement activation when C3b becomes deposited on host cells, making them a target for destruction by phagocytic cells.

C1 inhibitor (C1-INH): A glycoprotein that acts to dissociate Clr and C1s from C1q, thus inhibiting the first active enzyme formed in the classical complement cascade.

C3 glomerulopathies (C3G): Diseases involving the glomeruli of the kidneys.

C4-binding protein (C4BP): A protein in the complement system that serves as a cofactor for factor 1 in the inactivation of C4b.

Cancer: A disease characterized by the presence of a malignant tumor.

Cancer antigen 125 (CA 125): A glycosylated protein that is used clinically as a marker for ovarian cancer.

Cancer vaccines: Vaccines that have been developed for the purpose of preventing or treating cancer.

Candidiasis: An opportunistic fungal infection caused by *Candida albicans* and other *Candida* species.

Capture assay: An enzyme immunoassay using two antibodies: The first binds the antigen to a solid phase, and the second contains the enzyme label and acts as an indicator.

Carcinoembryonic antigen (CEA): An oncofetal protein that may be elevated in patients with cancers of the breast, gastrointestinal tract, pancreas, or lung.

Carcinogenesis: The process by which a cell is transformed into a malignant tumor.

Carcinoma: Malignant tumor derived from the skin or epithelial linings of internal organs or glands.

Cascade induction: When a cytokine secreted by a specific type of cell activates target cells to produce additional cytokines.

CD4 T cell: Type of lymphocyte that provides help to B cells to initiate antibody production.

CD45: A leukocyte marker present on all white blood cells; used to identify WBC populations in flow cytometry analyses.

Celiac disease: An autoimmune disease affecting the small intestine and other organs.

Cell flow cytometry: An automated system for identifying cells based on the scattering of light as cells flow single file through a laser beam.

Cell-mediated immunity: A type of immunity in which T cells produce cytokines that help to regulate both the innate and adaptive immune response.

Central tolerance: Destruction of potentially self-reactive T and B cells as they mature in either the thymus or the bone marrow.

Ceruloplasmin: An acute-phase reactant that acts as the principal copper-transporting protein in human plasma.

Chain of infection: A continuous link between three elements—a source, a method of transmission, and a susceptible host.

Chain termination sequencing: A modification of the DNA replication process, which utilizes modified nucleotide bases called dideoxynucleotide triphosphates (ddNTP). When these are incorporated into the growing DNA chain, synthesis stops.

Chancre: The initial lesion that develops on the external genitalia in syphilis.

Chemical Hygiene Plan: A plan that identifies appropriate work practices, standard operating procedures, and safety considerations in regard to the use of chemicals in the laboratory.

Chemiluminescence: The production of light energy by a chemical reaction.

Chemiluminescent immunoassay: A technique that employs a chemical attached to either an antigen or antibody. Light is emitted because of a chemical reaction and indicates an antigen–antibody combination has taken place.

Chemokines: A large family of homologous cytokines that promote migration of white blood cells through chemotaxis.

Chemotaxin: A protein or other substance that acts as a chemical messenger to produce chemotaxis.

Chemotaxis: The migration of cells in the direction of a chemical messenger.

Chronic granulomatous disease (CGD): An immunodeficiency inherited in either an X-linked or autosomal recessive fashion that results in an inability of the neutrophils to produce the reactive forms of oxygen necessary for normal bacterial killing.

Chronic rejection: Rejection of a graft that usually occurs after the first year and results from progressive fibrosis of blood vessels in the grafted tissue.

Class I MHC (HLA) molecules: Proteins coded for by genes at three loci (A, B, C) in the major histocompatibility complex. They are expressed on all nucleated cells and are important to consider in the transplantation of tissues.

Class II MHC (HLA) molecules: Proteins coded for by the DR, DP, and DQ loci of the major histocompatibility complex. They are found on B cells, macrophages, activated T cells, monocytes, dendritic cells, and endothelium, and are important to consider in the transplantation of tissues.

Class switching: The production of immunoglobulins other than IgM by daughter cells of antigen-exposed B lymphocytes.

Classical pathway: A means of activating complement that begins with antigen–antibody combination.

Clinical and Laboratory Standards Institute (CLSI): An organization that develops clinical laboratory testing standards based on input from industry, government, and health-care professionals.

Clinical Laboratory Improvement Amendments (CLIA): Federal regulatory standards that apply to all clinical laboratory testing in the United States.

Clonal deletion: The process of elimination of clones of lymphocytes that would be capable of an autoimmune response.

Clonal selection theory: A theory postulated to explain the specificity of antibody formation, based on the premise that each lymphocyte is genetically programmed to produce a specific type of antibody and is selected by contact with an antigen.

Clusters of differentiation (CD): Antigenic features of leukocytes that are identified by groups of monoclonal antibodies expressing common or overlapping activity.

Coccidioidomycosis: A fungal disease caused by *Coccidioides immitis* that is endemic to the southwestern United States and may be characterized by primary pulmonary infection.

Coefficient of variation (CV): The average distance each data point in a normal distribution is from the mean. It is expressed as a percentage of the mean.

Cold agglutinins: Antibodies that react below 30°C, typically formed in response to diseases such as *Mycoplasma* pneumonia and certain viral infections.

Colony stimulating factor (CSF): A protein in human serum that promotes monocyte differentiation.

Combination antiretroviral therapy (CART): A combination of antiretroviral drugs used to treat individuals with HIV infection (see also *highly active antiretroviral therapy*).

Commensalistic: A relationship between two species of organisms in which there is no benefit or harm to either organism.

Common variable immunodeficiency (CVI): A heterogeneous group of immunodeficiency disorders that usually appears in patients between the ages of 20 and 30 years. It is characterized by a deficiency of one or more classes of immunoglobulins.

Competitive immunoassay: An immunoassay in which unlabeled and labeled antigen compete for a limited number of binding sites on reagent antibody.

Complement: A series of proteins which are normally present in serum and whose overall functions are mediation of inflammation and destruction of foreign cells.

Complement-dependent cytotoxicity (CDC): Killing of cells that results from attachment of antibody with activation of complement.

Complement receptor type 1 (CR1): A cell-bound regulator of complement activation. It assists in degrading C3b and C4b and mediates transport of C3b-coated immune complexes to the liver and spleen.

Complementary or copy DNA (cDNA): DNA made from RNA using the enzyme reverse transcriptase.

Conformational epitope: Key antigenic site that results from the folding of one chain or multiple chains, bringing certain amino acids from different segments of a linear sequence or sequences into close proximity with each other so they can be recognized together.

Congenital syphilis: The transfer of syphilis from an infected mother to the fetus during pregnancy.

Conidia: Asexual reproductive structures produced by fungi at the tip of hyphae; also known as *spores*.

Constant region: The carboxy-terminal segment of antibody molecules (half of immunoglobulin light chains or three-quarters of heavy chains) that consists of a polypeptide sequence found in all chains of that type.

Contact dermatitis: A delayed hypersensitivity reaction caused by T-cell sensitization to low molecular weight compounds, such as nickel and rubber, that come in contact with the skin.

Control mean: The average of all data points.

C-reactive protein (CRP): A trace constituent of serum that increases rapidly following infection or trauma to the body and acts as an opsonin to enhance phagocytosis.

CREST syndrome: A subset of scleroderma named after its five major features: calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia.

Crossmatch: Incubation of donor lymphocytes with recipient serum to determine the presence of antibodies, which would indicate rejection of a potential transplant.

Cross-reactivity: A phenomenon that occurs when an antibody reacts with an antigen that is structurally similar to the original antigen that induced antibody production.

Cryoglobulins: Immunoglobulins of the IgM class that precipitate at cold temperatures, causing occlusion of blood vessels in the extremities if a patient is exposed to the cold.

Cryptococcosis: A fungal disease caused by *Cryptococcus neoformans* and characterized as a pulmonary infection that may spread to the central nervous system and the brain.

C-type lectin receptors (CLRs): Plasma membrane receptors found on white blood cells (WBCs) that bind to mannan and β-glucans in fungal cell walls.

Cyst: Inactive form of a parasite that can transmit infection.

Cytogenetics: The branch of genetics devoted to the study of chromosomes.

Cytokine: Chemical messenger produced by stimulated cells that affects the function or activity of other cells.

Cytomegalovirus (CMV): A virus in the herpes family that is responsible for infection, ranging from a mononucleosislike syndrome to a life-threatening illness in immunocompromised patients.

Cytotoxic T cell: T cells that bear the CD8 marker. They kill virus-infected cells and tumor cells by triggering apoptosis.

Decay-accelerating factor (DAF): A glycoprotein found on peripheral red blood cells (RBCs), endothelial cells, fibroblasts, and epithelial cell surfaces that is capable of dissociating C3 convertases formed by both the classical and alternative pathways of complement.

Delayed hypersensitivity: Type IV or T-cell–mediated hypersensitivity; so named because its manifestations are not seen until 24 to 48 hours after exposure to the inducing antigen.

Delta check: A QA procedure that compares a patient's test results with the previous results.

Denaturation: Treating double-stranded DNA to separate it into single strands.

Dendritic cell: Tissue cells covered with long membranous extensions that make them resemble nerve cell dendrites.

Deoxyribonucleic acid (DNA): The nucleic acid whose sugar is deoxyribose. It is the primary genetic material of all cellular organisms and DNA viruses.

Diapedesis: The process by which cells are capable of moving from the circulating blood to the tissues by squeezing through the wall of a blood vessel.

DiGeorge anomaly: A congenital defect of the third and fourth pharyngeal pouches that affects thymic development, leading to a T-cell deficiency.

Diluent: One of two entities needed for making up a solution. It is the medium into which the solute is added.

Direct agglutination: An antigen–antibody reaction that occurs when antigens are naturally found on a particle.

Direct allorecognition: Pathway in which recipient T cells recognize intact HLA molecules on donor cells.

Direct antiglobulin test (DAT): A technique to determine in vivo attachment of antibody or complement to red blood cells (RBCs), using anti-human globulin to cause a visible agglutination reaction.

Direct immunofluorescent assay: A technique to identify a specific antigen using an antibody that has a fluorescent tag attached.

Domain: Region of an antibody molecule that consists of approximately 110 amino acids.

Double-negative (DN) thymocyte: Stage in the development of T cells when neither CD4 nor CD8 is expressed.

Double-positive (DP) thymocyte: Stage in the development of T cells when both CD4 and CD8 antigens are expressed.

Double-stranded DNA (dsDNA) antibodies: Autoantibodies produced against double-stranded DNA; they are diagnostic for SLE.

Dual-parameter dot plot: Grouping of cells based on two different characteristics, one of which is plotted on the x-axis and the other on the y-axis.

Ectoparasite: Multi-celled parasitic organism that lives on the skin of the host.

Electropherogram: Sequencing result from capillary gel electrophoresis that appears as a series of fluorescent peaks.

Electrophoresis: The separation of molecules in an electrical field based on differences in charge and size.

ELISA: See *enzyme-linked immunosorbent assay.*

Endocrine: Internal secretion of substances such as hormones or cytokines directly into the bloodstream that causes systemic effects.

Endogenous pyrogen: A substance produced by the body that causes fever. Interleukin-1 is an example.

Endotoxin: A component of the cell walls of gram-negative bacteria, which consists of the portion of lipopolysaccharide (LPS) called lipid A. Endotoxin is a potent stimulator of cytokine release that can lead to septic shock.

End-point method: A radial immunodiffusion technique in which antigen is allowed to diffuse out until the point of equivalence is reached.

Enzyme-linked immunosorbent assay (ELISA): An immunoassay that employs an enzyme label on one of the reactants.

Eosinophil: A white blood cell (WBC) that contains reddish-orange granules and is involved in allergic reactions.

Epigenetics: The study of modifications in gene expression that are not caused by changes in the nucleotide sequence of DNA.

Epitope: The key portion of the immunogen against which the immune response is directed; also known as the *determinant site*.

Epitope spreading: An expansion of an immune response to unrelated antigens, which may be involved in the development of autoimmunity.

Epstein-Barr virus (EBV): A DNA virus of the herpesvirus family.

Erythropoietin (EPO): A colony stimulating factor that increases red blood cell (RBC) production in the bone marrow.

Examination variable: A variable that includes reagent and test performance, instrument calibration and maintenance, personnel requirements, and technical competence.

Exotoxin: Potent protein released from living bacteria (mostly gram-positive) that causes harm to the host by binding to a specific cellular receptor.

External defense system: Structural barriers that prevent most infectious agents from entering the body.

External quality assessment (EQA): The testing of unknown samples received from an outside agency.

Extractable nuclear antigen (ENA): A member of a family of small nuclear proteins associated with uridine-rich RNA that can stimulate the production of autoantibodies; examples are Sm and RNP.

Extrinsic parameter: A parameter that is not an inherent part of the cell. These specific cell surface proteins are analyzed through attachment of fluorescent antibodies.

F(ab')$_2$: Fragment of an immunoglobulin molecule obtained by pepsin cleavage that consists of two light chains and two heavy-chain halves held together by disulfide bonding. This piece has two antigen-binding sites.

Fab fragment: Fragment of an immunoglobulin molecule obtained by papain cleavage that consists of a light chain and one-half of a heavy chain held together by disulfide bonding.

Factor H: A control protein in the complement system. It acts as a cofactor with factor I to break down C3b formed during complement activation.

Factor I: A serine protease that cleaves C3b and C4b formed during complement activation. A different cofactor is required for each of these reactions.

Fc fragment: Fragment of an immunoglobulin molecule obtained by papain cleavage that consists of the carboxy-terminal halves of two heavy chains. These two halves are held together by disulfide bonds. This fragment spontaneously crystallizes at 4°C.

Fibrinogen: An acute-phase reactant that changes to fibrin and forms clots in the bloodstream.

Flanking: Placement on either side of the region of the template DNA to be copied.

Flocculation: The formation of downy masses of precipitate that occurs over a narrow range of antigen concentration.

Flow cytometer: An automated system in which single cells in a fluid are analyzed in terms of intrinsic light-scattering characteristics as well as extrinsic properties.

Flow cytometry: See *cell flow cytometry*.

Fluorescence in situ hybridization (FISH): A technique used to identify a specific region of DNA in a chromosome through binding of fluorescent-tagged complementary DNA probes.

Fluorescence polarization immunoassay (FPIA): An immunoassay based on the change in polarization of fluorescent light emitted from a labeled molecule when it is bound by antibody.

Fluorescent antinuclear antibody (FANA) testing: Testing to identify the presence of antibody to nuclear antigens, using animal cells and a fluorescent-labeled anti-human immunoglobulin.

Fluorescent treponemal antibody absorption (FTA-ABS) test: Fluorescent treponemal antibody absorption test, a confirmatory test for syphilis, which detects antibodies to *Treponema pallidum* by using anti-human immunoglobulin with a fluorescent label.

Fluorochrome: A molecule that absorbs light across a spectrum of wavelengths and emits light of lower energy across a spectrum of longer wavelengths.

Forward-angle light scatter (FSC): Light scattered at an angle of less than 90 degrees, which indicates the size of a cell.

Fungi: Organisms made up of eukaryotic cells with rigid walls composed of chitin, mannan, and sometimes cellulose.

Gate: A set of filters placed around a population of interest to analyze various parameters (extrinsic and intrinsic) of the cells contained within the selected region.

Gel electrophoresis: Method of separating either proteins or DNA based on their size and electrical charge. Samples are placed in wells on the gel and exposed to an electrical current.

Genetic code: A set of 3 nucleotides that code for one amino acid.

Germinal center: The interior of a secondary follicle where blast transformation of B cells takes place.

Goodpasture's syndrome: An autoimmune disease characterized by the presence of an autoantibody to collagen in the glomerular (kidney) or alveolar (lung) basement membranes.

Graduated pipette: A pipette that has markings all along its length to allow for varying amounts of liquid to be measured.

Graft-versus-host disease (GVHD): A condition that results from transplantation of immunocompetent cells into an immunodeficient host. The transfused cells attack the tissues of the recipient within the first 100 days posttransplant.

Granulocyte colony stimulating factor (G-CSF): A cytokine produced by fibroblasts and epithelial cells that enhances the production of neutrophils.

Granulocyte macrophage colony stimulating factor (GM-CSF): A cytokine produced by T cells and other cell lines that stimulates an increased supply of granulocytic cells and macrophages.

Granuloma: An organized cluster of inflammatory cells formed in some type IV hypersensitivity responses.

Granulomatosis with polyangiitis (PGA): An autoimmune disease involving inflammation of the small- to medium-sized blood vessels and the production of ANCA.

Graves disease: An autoimmune disease characterized by hyperthyroidism caused by the presence of antibody to thyroid-stimulating hormone receptors. Antigen–antibody combination results in continual release of thyroid hormones.

Group A streptococci: Gram-positive, catalase-negative cocci often found in pairs or chains that are responsible for diseases ranging from pharyngitis to necrotizing fasciitis.

Gummas: Localized areas of granulomatous inflammation on bones, skin, and subcutaneous tissue caused by tertiary syphilis.

Hairy cell leukemia: Chronic leukemia characterized by the formation of large mononuclear cells with irregular cytoplasmic projections found in bone marrow.

Haplotype: A set of genes that are located close together on a chromosome and are usually inherited as a single unit.

Hapten: A simple chemical group that can bind to antibody once it is formed but that cannot stimulate antibody formation unless tied to a larger carrier molecule.

Haptoglobin: An acute-phase reactant that binds irreversibly to free hemoglobin released by intravascular hemolysis.

Hashimoto's thyroiditis: An autoimmune disease that results in hypothyroidism caused by the presence of antithyroglobulin and antimicrosomal antibodies, which progressively destroy the thyroid gland.

Heavy (H) chain: One of the polypeptide units that makes up an immunoglobulin molecule. Each immunoglobulin monomer consists of two heavy chains paired with two light chains.

Heavy-chain diseases: B-cell lymphomas characterized by the production of monoclonal immunoglobulin heavy chains that are not attached to light chains.

Helicobacter pylori: A gram-negative spiral bacterium that is a major cause of gastric and duodenal ulcers.

Helminth: Parasitic worms, including flatworms, tapeworms, and roundworms.

Hemagglutination: An antigen–antibody reaction that results in the clumping of red blood cells (RBCs).

Hemagglutination inhibition: A test for detecting antibodies to certain viruses, based on lack of agglutination as a result of antibody neutralizing the virus.

Hematopoietic growth factors: Cytokines or other factors in the blood that stimulate formation and differentiation of blood cells.

Hemolytic disease of the newborn (HDN): A cytotoxic reaction that destroys an infant's red blood cells (RBCs) because of placental transfer of maternal antibodies to Rh antigens.

Hemolytic titration (CH_{50}) assay: An assay that measures complement-activating ability by determining the amount of patient serum required to lyse 50% of a standardized concentration of antibody-sensitized sheep erythrocytes.

Hemolytic uremic syndrome (HUS): A condition characterized by hemolytic anemia, low platelet count, and acute renal failure caused by either a Shiga toxin related to an infection or complement dysregulation.

Hepatitis: Inflammation of the liver that can be caused by several viruses as well as noninfectious factors, such as radiation, exposure to chemicals, or autoimmune diseases.

Hepatitis A virus (HAV): An RNA virus that can cause hepatitis; transmitted by the fecal–oral route, close person-to-person contact, or ingestion of contaminated food or water.

Hepatitis B surface antigen (HBsAg): The surface antigen of hepatitis B virus, the first marker to appear in hepatitis B infection.

Hepatitis B virus (HBV): A DNA virus that can cause hepatitis; it is transmitted by a parenteral route, through sexual contact, or acquired perinatally.

Hepatitis Be antigen (HBeAg): Antigen associated with the capsid of hepatitis B virus.

Hepatitis C virus (HCV): An RNA virus that can cause hepatitis; it is transmitted sexually or through contaminated blood or needles.

Hepatitis D virus (HDV): An RNA virus that can cause hepatitis; it requires the presence of HBV for its replication and expression.

Hepatitis E virus (HEV): An RNA virus that can cause hepatitis; it is transmitted by the fecal–oral route or ingestion of contaminated food or water.

Hereditary angioedema (HAE): A disease characterized by swelling of the extremities, the skin, the gastrointestinal tract, and other mucosal surfaces as a result of a deficiency in the complement inhibitor C1NH.

Heteroantigen: An antigen of a species different from that of the host, such as other animals, plants, or microorganisms.

Heterogeneous enzyme immunoassay: Immunoassay in which enzyme is used as a label and which requires a separation step to separate free from bound analyte.

Heterophile antibody: Antibody that is capable of reacting with similar antigens from two or more unrelated species; commonly found in patients with infectious mononucleosis.

Heterophile antigen: An antigen that exists in unrelated plants or animals but is either identical or closely related, so that antibody to one will cross-react with antibody to the other.

High-dose hook effect: Limitation of antibody-based assays caused by massive amounts of tumor marker antigens present.

Highly active antiretroviral therapy (HAART): A multidrug regimen that is the standard of treatment for HIV infection (see also *combination antiretroviral therapy*).

Hinge region: The flexible portion of the heavy chain of an immunoglobulin molecule that is located between the first and second constant regions. This allows the molecule to bend to let the two antigen-binding sites operate independently.

Histamine: A vasoactive amine released from mast cells and basophils during an allergic reaction.

Histoplasmosis: An infection caused by the fungus *H capsulatum.*

HLA antibody screen: Detection of HLA antigens in candidates and recipients of solid-organ transplants by performance of a cross-match test.

HLA genotype: Actual alleles, for HLA antigens, that are inherited.

HLA matching: The pairing up of donor and recipient in a transplant on the basis of similar HLA antigens.

HLA phenotype: The expression of HLA genes that actually appear as proteins on cells.

HLA typing: Laboratory testing used to identify the HLA antigens or genes in a transplant candidate or donor.

Hodgkin lymphoma (HL): A malignant disease that typically begins in one lymph node and is characterized by the presence of Reed-Sternberg cells, giant multinucleated cells that are usually transformed B lymphocytes.

Homogeneous enzyme immunoassay: An immunoassay in which no separation step is necessary. It is based on the principle of a decrease in enzyme activity when specific antigen– antibody combination occurs.

Human chorionic gonadotropin (hCG): A hormone that is synthesized by trophoblasts and used as a marker for tumors of the ovaries, testes, and trophoblast cells.

Human epithelial growth factor receptor 2 (HER2): A transmembrane receptor that binds human epidermal growth factor; is overexpressed in a certain type of breast cancer.

Human immune serum globulin (HISG): A sterile preparation of concentrated antibodies made from pooled serum of thousands of donors; used as a prophylactic treatment in patients with antibody immunodeficiencies.

Human immunodeficiency virus (HIV): A retrovirus that is the etiologic agent of AIDS.

Human leukocyte antigen (HLA): Protein coded for by the human MHC genes that has essential roles in the immune response and the rejection of foreign transplants.

Human T-cell lymphotropic virus type I (HTLV-I): An RNA retrovirus that causes adult T-cell leukemia or lymphoma and HTLV-associated myelopathy/tropical spastic paraparesis.

Human T-cell lymphotropic virus type II (HTLV-II): An RNA retrovirus that is thought to be associated with neurological disease, certain hematologic and dermatological diseases, and an increased incidence of infections.

Humoral immunity: Protection from disease resulting from substances in the serum (e.g., antibodies).

Hybridization: Specific binding of two single-stranded DNA segments, as in binding of a probe with a known nucleic acid sequence to an unknown piece of DNA.

Hybridoma: A cell line resulting from the fusion of a myeloma cell and a plasma cell. These can be maintained in tissue culture indefinitely and can produce a very specific type of antibody known as *a monoclonal antibody.*

Hyperacute rejection: Rejection of tissue that occurs within minutes or hours following transplantation, because of antibodies to ABO and HLA antigens which are already present in the graft recipient.

Hypercytokinemia: Dysregulation of cytokines, producing hyperstimulation of the immune response.

Hypersensitivity: A heightened state of immune responsiveness.

Hyphae: Filamentous tubular branching structures characteristic of some fungi.

Idiotype: The variable portion of light and heavy immunoglobulin chains that is unique to a particular immunoglobulin molecule. This region constitutes the antigen-binding site.

IgM anti-HBc: Antibody that is the first to appear in hepatitis B infection. It is of the IgM class and is directed against core antigen on the virus particle.

Immature B cell: A phase in the growth of B cells characterized by the appearance of complete IgM antibody molecules on the cell surface.

Immediate hypersensitivity: Reaction to an allergen that occurs in minutes and can be life-threatening.

Immune adherence: The ability of phagocytic cells to bind complement-coated particles.

Immunity: The condition of being resistant to infection.

Immunization: The process by which immunity is acquired.

Immunoblotting: A technique used to identify antibodies to complex antigens. It consists of electrophoresis of the antigen mix followed by transfer of the pattern to nitrocellulose paper for reaction with patient serum.

Immunochromatography: A rapid technique in which the analyte is applied at one end of a strip and migrates toward the distal end, where the results can be visualized in minutes.

Immunodeficiencies: Inherited or acquired disorders in which a part of the body's immune system is missing or dysfunctional.

Immunoediting: The ability of tumor cells to escape immune surveillance through suppression of immunogenicity.

Immunofixation electrophoresis (IFE): A semiquantitative gel precipitation technique in which proteins are first separated by electrophoresis, then incubated with antibodies to individual proteins that are added directly to the gel surface; used commonly to identify monoclonal immunoglobulins.

Immunofluorescent assay (IFA): Identification of antigens on cells using an antibody with a fluorescent tag.

Immunogen: Any substance that is capable of inducing an immune response.

Immunogenetics: The analysis of gene mutations and polymorphisms that affect immune function.

Immunogenicity: The ability of an immunogen to stimulate a host response.

Immunoglobulin (Ig): Glycoproteins in the serum portion of the blood that are considered part of humoral immunity.

Immunohistochemistry: The use of labeled antibodies to directly detect tumor markers in tissue.

Immunologic diversion: A mechanism by which parasites enhance their survival in the host by inducing the production of proteins that divert the attention of the immune system.

Immunologic subversion: A mechanism by which parasites can avoid the effector mechanisms of the immune response by producing proteins that act as homologues of various components of the immune system.

Immunologic tolerance: A state of immune unresponsiveness directed against a specific antigen.

Immunology: The study of the reactions of a host when foreign substances are introduced into the body.

Immunophenotyping: Identifying cells according to their surface antigen expression.

Immunoprophylaxis: The use of immunization to prevent disease.

Immunosubtraction (immunotyping): A procedure that uses capillary electrophoresis to identify monoclonal immunoglobulin components.

Immunosuppressive agent: An agent used to suppress an antigraft immune response to transplanted tissue.

Immunosurveillance: The mechanisms by which the immune system patrols the body for the presence of cancerous or precancerous cells and eliminates them before they become clinically evident.

Immunotherapy: Treatment which uses the ability of the immune system to destroy tumor cells.

Immunotoxins: Antibodies conjugated to toxins to help destroy cancer cells.

Impetigo: A skin infection caused by bacteria such as Group A streptococci.

Indigenous microbiota: Symbiotic microorganisms that reside on and colonize the surfaces of an individual, also known as "normal flora."

Indirect allorecognition: Pathway by which the immune system recognizes foreign HLA proteins on a donor graft; involves the uptake, processing, and presentation of foreign HLA proteins by the recipient's APCs to recipient T cells to produce antibodies and cell-mediated responses against the graft.

Indirect immunofluorescent assay: A technique to identify antigen by using two antibodies: one that is specific to the antigen and a second that is an anti-human immunoglobulin with a fluorescent tag.

Infection control: Procedures used to control and monitor infections occurring within health-care facilities.

Infectivity: An organism's ability to establish an infection; the proportion of individuals exposed to a pathogen through horizontal transmission (i.e., person-to-person contact) who will become infected.

Inflammasome: A protein oligomer that contains caspase enzymes and other proteins associated with apoptosis; may be defective in some autoinflammatory disorders.

Inflammation: Cellular and humoral mechanisms involved in the overall reaction of the body to injury or invasion by an infectious agent.

Innate (natural) immunity: The ability of the individual to resist infection by means of normally present body functions.

Integrins: Molecules on certain leukocytes that cause adhesion to endothelial cells.

Interferons (IFN): Cytokines produced by T cells and other cell lines that inhibit viral synthesis or act as immune regulators.

Interleukins (IL): Cytokines or chemical messengers produced by leukocytes that affect the inflammatory process through an increase in soluble factors or cells.

Internal amplification control: A control used in PCR that is a gene target always present at a constant level.

Internal defense system: Defense mechanism inside the body in which both cells and soluble factors play essential parts.

Intrinsic parameter: Light-scattering properties that are a part of the cell, such as size and granularity.

Invariant chain: A protein that associates with HLA class II antigens shortly after they are synthesized to prevent interaction of their binding sites with any endogenous peptides in the endoplasmic reticulum.

Isograft (syngeneic graft): Graft that involves the transfer of tissue between two genetically identical members of the same species.

Isohemagglutinin: Antibody that agglutinates red blood cells (RBCs) of other individuals of the same species.

Isothermal: An amplification process using a reaction that proceeds at a single temperature.

Isotype: A unique amino acid sequence that is common to all immunoglobulin molecules of a given class in a given species.

Joining (J) chain: A glycoprotein with a molecular weight of 15,000 that serves to link immunoglobulin monomers together. These are found only in IgM and secretory IgA molecules.

Joint Commission (JC): An independent body that certifies and accredits health-care organizations in the United States,

Kappa (κ) chain: One of two types of immunoglobulin light chains that are present in approximately two-thirds of all immunoglobulin molecules.

Karyotype analysis: A test used to examine the chromosomes in a cell sample for numerical or structural abnormalities.

Lambda (λ) chain: One of two types of immunoglobulin light chains that are present in approximately one-third of all immunoglobulin molecules.

Lancefield group: A means of classifying streptococci on the basis of differences in the cell wall carbohydrate.

Lateral flow immunochromatographic assay (LFA): Immunochromatographic assay for rapid antigen detection.

Lattice: The combination of antibody and multivalent antigen to produce a stable network that results in a visible reaction.

Law of mass action: A law used to mathematically describe the equilibrium relationship between soluble reactants and insoluble products. It can be applied to antigen–antibody relationships.

Lean system: A system used in the laboratory that focuses on the elimination of waste to allow a facility to do more with less and at the same time increase customer and employee satisfaction.

Lectin pathway: A pathway for the activation of complement based on binding of mannose-binding protein to constituents on bacterial cell walls.

Leukemia: A progressive malignant disease of blood-forming organs, characterized by proliferation of leukocytes and their precursors in the bone marrow.

Leukocytes: White blood cells (WBCs).

Leukotrienes (LT): A class of secondary mediators released from mast cells and basophils during type I hypersensitivity reactions.

Light (L) chain: Small chain in an immunoglobulin molecule that is bound to the larger chain by disulfide bonds. The two types of light chains are called *kappa* and *lambda.*

Linear epitope: Amino acids following one another on a single chain that act as a key antigenic site.

Lyme disease: A disease caused by infection with the spirochete bacteria, *Borrelia burgdorferi.*

Lymph node: A secondary lymphoid organ that is located along a lymphatic duct and whose purpose is to filter lymphatic fluid from the tissues and act as a site for processing of foreign antigen.

Lymphocyte: The key white blood cell (WBC) involved in the adaptive immune response.

Lymphoma: Cancer of the lymphoid cells that tends to proliferate as a solid tumor.

Macrophage: A white blood cell (WBC) that kills microbes and presents antigen to T and B cells.

Macrophage colony stimulating factor (M-CSF): A cytokine that induces growth of hematopoietic cells destined to become monocytes and macrophages.

Major histocompatibility complex (MHC): The genes that control expression of a large group of proteins originally identified on leukocytes but now known to be found on all nucleated cells in the body. These proteins regulate the immune response and play a role in graft rejection.

Malignant: A descriptive term for cancerous tumors that can circulate to other parts of the body and invade nearby organs.

Mannose-binding lectin (MBL): Normally present protein in the blood that binds to mannose on bacterial cells and initiates the lectin pathway for complement activation.

Mast cell: A tissue cell that plays a role in allergic reactions and also functions as an antigen-presenting cell.

Membrane attack complex (MAC): The combination of complement components C5b, C6, C7, CS, and C9 that becomes inserted into the target cell membrane, causing lysis.

Membrane cofactor protein (MCP): A protein found on all epithelial and endothelial cells that helps to control complement-mediated lysis by acting as a cofactor for Factor I–mediated cleavage of C3b.

Memory cell: Progeny of an antigen-activated B or T cell that is able to respond to antigen more quickly than the parent cell.

Metastasis: Process of malignant cells traveling through the body, thereby causing new foci of malignancy.

MHC restriction: The selection of thymocytes that will only interact with the MHC antigens found on host cells.

Microarray: A technology that enables simultaneous analysis of thousands of genes in a sample by hybridization with a panel of molecular probes that are complementary to portions of specific genes or chromosome regions; the probes are spotted onto separate locations on a small glass slide or nylon membrane.

Microbiome: The collection of microorganisms that exists on the body, including bacteria, viruses, yeast, and fungi.

Micropipette: Mechanical pipettes that deliver volumes in the microliter (μL) range; used when very small volumes are needed.

Minor histocompatibility antigens (mHAs): Non-HLA proteins that can induce a weak graft rejection response when introduced into an individual possessing a different polymorphic variant.

Mitogen: A substance that stimulates mitosis in all T cells or all B cells, regardless of antigen specificity.

Mixed lymphocyte reaction (MLR): A means of measuring the proliferation of responder CD8+ T cells to nonself antigens in a potential transplant.

Molecular mimicry: The similarity between an infectious agent and a self-antigen that causes antibody formed in response to the former to cross-react with the latter.

Monoclonal antibody: Very specific antibody derived from a single antibody-producing cell that has been cloned or duplicated.

Monoclonal gammopathy: A clone of lymphoid cells that causes overproduction of a single immunoglobulin component called a *paraprotein.*

Monoclonal gammopathy of undetermined significance (MGUS): A premalignant plasma cell disorder characterized by the presence of monoclonal immunoglobulin in the serum, a plasma bone marrow count of less than 10%, and absence of clinical features. Some cases may progress to multiple myeloma over time.

Monocyte: The largest white blood cell (WBC) in peripheral blood. It migrates to the tissues to become a macrophage.

Multiple myeloma: A malignancy of mature plasma cells that results in a monoclonal increase in an immunoglobulin component. The most common component increased is IgG.

Multiple sclerosis (MS): An autoimmune disease in which the myelin sheath of axons becomes progressively destroyed by antibodies to myelin proteins.

Mumps virus: A single-stranded RNA virus that is the causative agent of mumps, a disease characterized by swelling of the parotid glands.

Mutation: A permanent change in the nucleotide sequence within a gene or chromosome.

Mutualistic: A relationship between a human host and microbial species in which both organisms benefit.

Myasthenia gravis (MG): An autoimmune disease characterized by progressive muscle weakness caused by formation of antibody to acetylcholine receptors.

Mycelium: A dense mat formed by some fungi that is made up of intertwined hyphae.

Mycoplasma pneumoniae: A small gram-negative bacterium that lacks a cell wall and is the cause of upper respiratory infections.

Mycoses: Diseases produced by fungi.

Natural killer (NK) cell: A type of lymphocyte that has the ability to kill target cells without prior exposure to them.

Negative predictive value: The probability that a person with a negative screening test does not have the disease.

Negative selection: The process by which T cells that can respond to self-antigen are destroyed in the thymus.

Neoplasm: An abnormal cell mass; a tumor.

Nephelometry: A technique for determining the concentration of particles in a solution by measuring the light scattered at a particular angle from the incident beam as it passes through the solution.

Neutrophil: A white blood cell (WBC) with a multilobed nucleus and a large number of neutral staining granules. Its main function is phagocytosis.

Next generation sequencing (NGS): A technique that is able to sequence large numbers of templates simultaneously (massively parallel sequencing), yielding hundreds of thousands of short sequences in a single run.

Nick: An amplification technique that starts with breaking one phosphodiester bond on one strand of a double-stranded DNA molecule.

Noncompetitive immunoassay: An assay that allows any patient antigen present to be captured. The amount of label measured is directly proportional to the amount of patient antigen present.

Non-Hodgkin or lymphocytic lymphoma (NHL): A wide range of cancers of the lymphoid tissue, of which B-cell lymphomas represent the majority.

Nontreponemal tests: Serological tests for syphilis that detect antibody to cardiolipin and not specific antitreponemal antibody.

Nucleic acid sequence: A specific ordering of nucleic acid bases that identifies a particular target.

Nucleolus: A prominent structure within the nucleus where transcription and processing of rRNA and assembly of the ribosomes takes place.

Nucleosome antibodies: Autoantibodies against DNA-histone complexes [also known as nucleosomes or deoxyribonucleoprotein (DNP)].

Nucleotide: A nucleotide is a unit of DNA or RNA composed of a phosphorylated ribose or deoxyribose sugar and a nitrogen base.

Occupational Safety and Health Administration (OSHA): Monitors and enforces safety regulations for workers.

Oncofetal antigens: Antigens that are expressed in the developing fetus and in rapidly dividing tissue, such as that associated with tumors, but that are absent in normal adult tissue.

Oncogene: Gene that encodes a protein capable of inducing cellular transformation.

Opsonins: Serum proteins that attach to a foreign substance and enhance phagocytosis (from the Greek word meaning "to prepare for eating").

Ouchterlony double diffusion: A qualitative gel precipitation technique in which both antigen and antibody diffuse out from wells cut in the gel. The pattern obtained indicates whether or not antigens are identical.

Oxidative burst: An increase in oxygen consumption in phagocytic cells, which generate oxygen radicals used to kill engulfed microorganisms.

p24 antigen: A structural core antigen that is part of the human immunodeficiency virus (HIV).

Palindromic sites: Nucleotide sequences that read the same 5′ to 3′ on both strands of the DNA.

Paracrine: Secretions such as cytokines that affect only target cells in close proximity.

Paraprotein (M protein): A single immunoglobulin component produced by a malignant clone of lymphoid cells in lymphoproliferative diseases.

Parasite: Microorganism that survives by living off of another organism.

Parasitic: Relationship in which an organism causes harm to its host.

Parenteral: Mode of transmission other than through the intestinal tract, most notably bloodborne transmission.

Paroxysmal cold hemoglobinuria: A condition in which patients produce a biphasic autoantibody that binds to RBCs at cold temperatures and activates complement at 37°C to produce an intermittent hemolysis.

Paroxysmal nocturnal hemoglobinuria (PNH): A disease characterized by complement-mediated hemolysis of erythrocytes resulting from a deficiency of decay-accelerating factor on the red blood cells (RBCs).

Particle-counting immunoassay (PACIA): A technique for measuring residual nonagglutinating particles in a specimen using nephelometry to determine the amount of forward light scatter. Antigen–antibody combination decreases light scatter so that the amount of patient antigen present is indirectly proportional to the amount of light scattered.

Passive agglutination: A reaction in which particles coated with antigens not normally found on their surfaces clump together because of their combination with antibody.

Passive immunity: A type of immunity that results from the transfer of antibodies from immunized hosts to a nonimmune individual.

Passive immunodiffusion: A precipitation reaction in a gel in which antigen–antibody combination occurs by means of diffusion.

Passive immunotherapy: Passive immunization of an individual with commercial preparations of antibodies formed by other hosts to prevent or treat a disease.

Pathogen-associated molecular patterns (PAMPs): Structural patterns of carbohydrates, nucleic acids, or bacterial peptides on microorganisms that are recognized by pathogen recognition receptors (PRRs) on the cells of the innate immune system.

Pathogen recognition receptors (PRRs): Receptors on cells of the innate immune system that bind to PAMPs on pathogenic microorganisms.

Pathogenicity: The inherent capacity of an organism to cause disease.

Percent panel reactive antibody (%PRA): The proportion of lymphocytes with known HLA phenotypes in a test panel that are killed by antibodies in the serum of a transplant patient.

Periarteriolar lymphoid sheath (PALS): White pulp of splenic tissue, which is made up of lymphocytes, macrophages, plasma cells, and granulocytes. It surrounds the central arterioles.

Peripheral tolerance: Destruction or repression of lymphocytes in the peripheral lymphoid organs that could respond to self-antigens.

Personal protective equipment (PPE): Items such as gowns, masks, gloves, and face shields used to protect the body from infectious agents.

Phagocytosis: From the Greek word *phagein,* meaning "cell eating," the engulfment of cells or particulate matter by leukocytes, macrophages, or other cells.

Phagolysosome: The structure formed by the fusion of cytoplasmic granules and a phagosome during the process of phagocytosis.

Phagosome: A vacuole formed within a phagocytic cell as pseudopodia surround a particle during the process of phagocytosis.

Plasma cell: A differentiated B cell that actively secretes antibody.

Plasma cell dyscrasias: Immunoproliferative diseases characterized by overproduction of a single immunoglobulin component by a clone of lymphoid cells.

Plasmid: A self-replicating genetic element located in the cytoplasm of a bacterium, which has a limited number of genes that can be transferred between bacteria.

Pleiotropy: Many different actions of a single cytokine. The cytokine may affect the activities of more than one kind of cell and have more than one kind of effect on the same cell.

Polyclonal: Derived from many clones of cells. Polyclonal antibodies are derived from many clones of B cells or plasma cells, and are therefore diverse in terms of their antigen specificity.

Polymerase chain reaction (PCR): A means of amplifying tiny quantities of nucleic acid using a heat-stable polymerase enzyme and a primer that is specific for the DNA sequence desired.

Polymorphism: The presence of two or more different genetic compositions (e.g., HLA genes) among individuals in a population.

Positive predictive value: The percentage of all positives in a serological test that are true positives.

Positive selection: The process of selecting immature T lymphocytes for survival on the basis of expression of high levels of CD3 and the ability to respond to self-MHC antigens.

Postexamination variable: A process that affects the reporting of results and correct interpretation of data.

Postexposure prophylaxis (PEP): Course of preventative treatment provided after exposure to potentially infectious organisms.

Poststreptococcal glomerulonephritis: A condition that damages the glomeruli of the kidney, caused by an initial immune response to a streptococcal infection.

Postzone phenomenon: Lack of a visible reaction in an antigen–antibody reaction, caused by an excess of antigen.

Pre-B cell: The stage of development of a B cell where the heavy-chain part of the antibody molecule is present.

Precipitation: The combination of soluble antigen with soluble antibody to produce visible insoluble complexes.

Precision: The ability to consistently reproduce the same result upon repeated testing of the same sample.

Preexamination variable: A variable that occurs before the actual testing of the specimen and includes test requests, patient preparation, timing, specimen collection, handling, and storage.

Primary biliary cirrhosis (PBC): An autoimmune disease that involves progressive destruction of the intrahepatic bile ducts.

Primary follicle: A cluster of B cells that have not yet been stimulated by antigen.

Primary immunodeficiency (PID): Inherited diseases in which part of the immune system is absent or dysfunctional.

Primary lymphoid organs: The organs in which lymphocytes mature: these are the bone marrow and the thymus.

Primary response: The initial response to a foreign antigen, characterized by a long lag phase, a slow rise in antibody, and consisting of mostly of IgM.

Primer: Short sequences of DNA, usually 20 to 30 nucleotides long, used to hybridize specifically to a particular target DNA to help initiate replication of the DNA.

Pro-B cell: A stage in B-cell development in which rearrangement of the genes that code for the heavy-chain region of an antibody molecule occur.

Probe: A nucleic acid, several hundred to a few thousand bases in length, with a known sequence that is used to identify the presence of a complementary DNA or RNA sequence in an unknown sample.

Proficiency testing: The testing of unknown samples received from an outside agency.

Properdin: A protein that stabilized the C3 convertase generated in the alternative complement pathway.

Prostate-specific antigen (PSA): A glycoprotein that is produced specifically by epithelial cells in the prostate gland; PSA is a widely used marker for prostate cancer.

Proteomics: The field of study that involves identification and quantification of the array of proteins present in a sample.

Proto-oncogenes: Regulatory genes that promote cell division.

Protozoa: A diverse group of single-celled organisms that can live and multiply inside of human hosts.

Prozone phenomenon: Lack of a visible reaction in antigen–antibody combination caused by the presence of excess antibody. This may result in a false-negative reaction.

Purine-nucleoside phosphorylase (PNP) deficiency: Lack of the enzyme purine nucleoside phosphorylase. The deficiency is inherited as an autosomal recessive trait. Accumulation of a purine metabolite is toxic to T cells, leading to a defect in cell-mediated immunity.

Quality assessment (QA): The overall process of guaranteeing quality patient care. It involves the continual monitoring of the entire test process from test ordering and specimen collection through reporting and interpreting results.

Quality control (QC): The materials, procedures, and techniques that monitor the accuracy, precision, and reliability of a laboratory test.

Quality indicator: Measurements developed by each laboratory to determine if the quality system essentials are being met.

Quality management (QM): The overall process of guaranteeing quality patient care.

Quality management system (QMS): A system that incorporates the objectives of total quality management and continuous quality improvement to ensure quality results, staff competence, and efficiency within an organization.

Quality system essentials (QSEs): Methods to meet the requirements of regulatory, accreditation, and standard-setting organizations.

Quantitative PCR (qPCR): Accumulation of a PCR product in real time during amplification.

Radial immunodiffusion (RID): A precipitation technique in which antibody is uniformly distributed in the support gel, and antigen

is applied to a well cut into the gel. As the antigen diffuses out from the well, an antigen–antibody combination occurs until the zone of equivalence is reached.

Radioimmunoassay (RIA): A technique used to measure small concentrations of an analyte, using a radioactive label on one of the immunologic reactants.

Random access analyzer: An analyzer that can run multiple tests on multiple samples using multiple analytes.

Rapid antigen detection system (RDTS): See *lateral flow immunochromatographic assay.*

Rapid plasma reagin (RPR) test: A slide flocculation test for syphilis that detects antibody to cardiolipin.

Rate nephelometry: A technique that measures the rate of light scattering after the reagent antibody is added to a sample containing patient antigen. The rate change is directly related to antigen concentration if the concentration of antibody is kept constant.

Reagin: An antibody formed during the course of syphilis that is directed against cardiolipin and not against *Treponema pallidum* itself.

Recognition unit: The complement component that consists of the C1qrs complex. This must bind to at least two Fc regions to initiate the classical complement cascade.

Recombinant protein vaccine: A vaccine produced by cloning the gene coding for the vaccine antigen into the genome of bacteria, yeast, or cultured cells.

Redundancy: A phenomenon that occurs when different cytokines have the same effect.

Reference interval: The range of values found in healthy individuals who do not have the condition detected by the assay.

Reliability: The ability to maintain both precision and accuracy in laboratory testing.

Reportable range: The range of values that will generate a positive result for the specimens assayed by the test procedure.

Restriction endonucleases: Enzymes that cleave DNA at specific recognition sites that are typically 4 to 6 base pairs long.

Restriction fragment length polymorphisms (RFLPs): Variations in nucleotides within DNA that change where restriction enzymes cleave the DNA. Where mutations occur, different-size pieces of DNA are obtained, resulting in an altered electrophoretic pattern.

Reverse passive agglutination: A reaction in which carrier particles coated with antibody clump together because of a combination with antigen.

Reverse transcriptase: An enzyme produced by certain RNA viruses to convert viral RNA into DNA.

Rheumatoid arthritis (RA): An autoimmune disease that affects the synovial membrane of multiple joints. It is characterized by the presence of the autoantibodies, *anti-CCP* and *rheumatoid factor.*

Rheumatoid factor (RF): An antibody of the IgM class produced by patients with rheumatoid arthritis that is directed against IgG.

Ribonucleic acid (RNA): The nucleic acid containing the sugar ribose. It is the primary genetic material of RNA viruses and plays a role in the transcribing of genetic information in cells.

Rickettsiae: Small gram-negative fastidious bacteria that are obligate intracellular parasites and are responsible for diseases such as Rocky Mountain spotted fever and typhus.

Rocky Mountain spotted fever (RMSF): A disease caused by infection with *R rickettsii.*

Rubella virus: An RNA virus that causes German measles and congenital infection.

Rubeola virus: A single-stranded RNA virus that causes measles.

S protein: A control protein in the complement cascade that interferes with binding of the C5b67 complex to a cell membrane, thus preventing lysis.

Safety data sheet (SDS): An SDS contains information on physical and chemical characteristics, fire, explosion reactivity, health hazards, primary routes of entry, exposure limits and carcinogenic potential, precautions for safe handling, spill clean-up, and emergency first aid information.

Sandwich immunoassays: Immunoassays based on the ability of antibody to bind with more than one antigen.

Sarcoma: A type of cancer derived from bone or soft tissues such as fat, muscles, tendons, cartilage, nerves, and blood vessels.

Scarlet fever: An illness with a characteristic rash and fever that is caused by the erythrogenic toxins released from group A streptococcal bacteria.

Secondary follicle: A cluster of B cells that are proliferating in response to a specific antigen.

Secondary immunodeficiency: An immunodeficiency that is acquired secondary to other conditions, such as certain infections, malignancies, autoimmune disorders, and immunosuppressive therapies.

Secondary lymphoid organs: Organs that include the spleen, lymph nodes, appendix, tonsils, and other mucosal-associated lymphoid tissue where the main contact with foreign antigens takes place.

Secondary response: A second or memory response to an antigen, characterized by a shortened lag period, a more rapid rise in antibody, and higher serum levels for a longer period of time.

Secretory component (SC): A protein with a molecular weight of 70,000 that is synthesized in epithelial cells and added to IgA to facilitate transport of IgA to mucosal surfaces.

Self-tolerance: The ability of the immune system to accept self-antigens and not initiate a response against them.

Sensitivity: The lowest amount of an analyte that can be measured.

Sensitization: (1) The combination of antibody with a single antigenic determinant on the surface of a cell without agglutination. (2) Induction of an immune response.

Serial dilution: A method of decreasing the strength of an antibody solution by using the same dilution factor for each step.

Serological pipette: A graduated or measuring pipette that has marks all along its length all the way down to the tip.

Serology: The study of the noncellular portion of the blood known as *serum.*

Serotype: A group of related bacteria or viruses that share specific antigens that can be identified by serological testing.

Serum: The liquid portion of the blood minus the clotting factors.

Serum amyloid A (SAA): An acute-phase protein that acts as a chemical messenger to activate monocytes and macrophages in order to increase inflammation.

Serum sickness: A type III hypersensitivity reaction that results from the buildup of antibodies to animal serum used in passive immunization.

Severe combined immunodeficiency (SCID): An inherited deficiency of both cell-mediated and antibody-mediated immunity. It results in death in infancy caused by overwhelming infections.

Shift: An abrupt change in the mean that may be caused by a malfunction of the instrument or a new lot number of reagents.

Side (right angle) scatter: Light scattered at 90 degrees in a flow cytometer that indicates the granularity of a cell.

Single-parameter histogram: Plot of a chosen parameter or measurement on the x-axis against the number of events on the y-axis.

Six Sigma: A method employed by health-care organizations to reduce variables and decrease errors.

Sm antigen: An extractable nuclear antigen; autoantibodies to Sm are specific for lupus.

Solute: One of the two entities needed for making a dilution.

Southern blot: Technique for the identification of specific DNA sequences in which DNA is cleaved into fragments by enzymes, separated electrophoretically, denatured, transferred to a nitrocellulose membrane, and incubated with a labeled probe that is specific for the sequence of interest.

Specificity: The proportion of people who do not have the disease or condition and who have a negative test.

Spleen: The largest secondary lymphoid organ in the body, located in the upper left quadrant of the abdomen. Its function is to filter out old cells and foreign antigens.

SS-A/Ro: An extractable nuclear antigen consisting of small, uridine-rich RNA complexed to cellular protein; found in a significant percentage of patients with Sjögren's syndrome.

SS-B/La: An extractable nuclear antigen consisting of small, uridine-rich RNA complexed to cellular protein; found in a significant percentage of patients with Sjögren's syndrome.

Standard deviation (SD): A measurement statistic that describes the average distance each data point in a normal distribution is from the mean.

Standard of care: The attention, caution, and prudence that a reasonable person in the same circumstances would exercise in performing laboratory testing.

Standard precautions: Guidelines describing personnel protection that should be used for the care of all patients, including hand washing, gloves, mask, eye protection, face shield, gown, patient-care equipment, environmental control, linens, taking care to prevent injuries, and patient placement.

Strand cleavage: Breaking the phosphodiester bonds that connect nucleotides in the DNA chain by using physical or enzymatic methods.

Strand displacement amplification (SDA): A method for amplifying DNA by using a DNA primer that is nicked by an endonuclease, allowing for displacement of the amplified strands.

Streptococcus pyogenes: Group A Streptococci; gram-positive cocci that have a number of clinical manifestations, including pharyngitis, impetigo, scarlet fever, acute rheumatic fever, and post-streptococcal glomerulonephritis.

Streptolysin O: A protein capable of lysing red blood cells (RBCs) and white blood cells (WBCs), which is produced by some groups of streptococci as they grow.

Streptozyme: A serological test for infection with group A streptococci that detects five different antibodies to streptococcal products.

Stringency: Conditions that affect the ability of a probe to correctly bind to a specific target DNA sequence. These include temperature, salt concentration, and concentration of formamide or urea.

Superantigens: Microbial proteins that can act as potent T-cell mitogens because they bind to both class II MHC molecules and T-cell receptors, regardless of antigen specificity.

Surrogate light chain: Two short polypeptide chains noncovalently associated with each other that appear before actual light chains are formed by a developing B cell.

Symbiotic: A relationship in which two species live together, often maintaining a long-term, but not necessarily a beneficial, interaction (e.g., a bacterium and a human).

Synergistic: Cytokines whose effects complement and enhance each other.

Syngeneic graft: The transfer of tissue or organs between genetically identical individuals such as identical twins.

Syphilis: A sexually transmitted disease caused by the spirochete bacterium *Treponema pallidum.*

Systemic lupus erythematosus (SLE): A chronic inflammatory autoimmune disease characterized by the presence of antinuclear antibodies. Symptoms may include swelling of the joints, an erythematous rash, and deposition of immune complexes in the kidneys.

T-dependent antigen: An antigen that requires T-cell help in order for B cells to respond.

T helper (Th) cells: Lymphocytes that express the CD4 antigen. Their function is to provide help to B cells in recognizing foreign antigen and producing antibody to it.

T helper 1 (Th1) cells: T cells that are developed through the expression of IL-12 by dendritic cells, and which are primarily responsible for cell-mediated immunity.

T helper 2 (Th2) cells: T cells which are developmentally regulated by IL-4, and whose main function is to drive antibody-mediated immunity.

T helper 17 (Th17) cells: A subset of T cells that play an important role in host defense against bacterial and fungal infections at mucosal surfaces. They secrete IL-17 which attracts neutrophils to the site of infection.

***T pallidum* particle agglutination (TP-PA) test:** A particle agglutination test that detects antibodies to *Treponema pallidum* to aid in the diagnosis of syphilis.

T regulatory (Treg) cell: A subpopulation of T cells that play an important role in suppressing the immune response to self-antigens.

Tertiary syphilis: The last stage of syphilis that appears months to years after secondary infection. It is characterized by granulomatous inflammation, cardiovascular disease, and central nervous system involvement.

Tetrapeptide: The basic four-chain unit common to all immunoglobulin molecules, consisting of two large heavy chains and two smaller light chains.

Threshold cycle: The cycle at which the sample fluorescence reaches a certain optimal level as determined by an instrument performing a polymerase chain reaction (PCR).

Thymocyte: Immature lymphocyte, found in the thymus, that undergoes differentiation to become a mature T cell.

Thymus: A small, flat, bilobed organ found in the thorax of humans, which serves as the site for differentiation of T cells.

Thyroglobulin (Tg): A large iodinated glycoprotein from which the active thyroid hormone triiodothyronine (T3) and its precursor, thyroxine (T4), are synthesized.

Thyroid peroxidase (TPO): An enzyme that oxidizes iodine ions to form the iodine atoms that are incorporated into thyroglobulin to facilitate the synthesis of the thyroid hormones, T3 and T4.

Thyroid-stimulating hormone (TSH): A hormone produced by the thyroid gland that binds to specific receptors, causing thyroglobulin to be broken down into secretable T3 and T4.

Thyroid-stimulating hormone receptor antibody (TRAb): An antibody that is directed against the receptor for thyroid-stimulating hormone. It is associated with Graves disease and results in overstimulation of the thyroid gland.

Thyrotoxicosis: A condition caused by overproduction of thyroid hormones, as seen in Graves disease.

Thyrotropin-releasing hormone (TRH): A hormone secreted by the hypothalamus that acts on the pituitary gland to induce the release of thyroid-stimulating hormone (TSH).

T-independent antigens: Antigens that are able to elicit antibody formation in the absence of T cells.

Tissue transglutaminase (tTG): An intestinal enzyme that converts the glutamine residues in gliadin to glutamic acid; autoantibodies to tTG are commonly produced in patients with celiac disease.

Titer: A figure that represents the relative strength of an antibody. It is the reciprocal of the highest dilution in which a positive reaction occurs.

Toll-like receptors (TLRs): Receptors found on human leukocytes and other cell types that recognize microorganisms and aid in their destruction.

Toxoid: A chemically inactivated bacterial toxin used in some vaccines.

Toxoplasma gondii: The protozoal organism that causes toxoplasmosis, an infection that can have severe consequences in immunocompromised individuals and congenitally infected infants.

TP-PA test: A particle agglutination test that detects antibodies to *Treponema pallidum* to aid in the diagnosis of syphilis.

Transcription: The process of generating a messenger RNA strand from DNA. This is used to code for protein.

Transcription-mediated amplification (TMA): Method of increasing target DNA through the use of two enzymes, an RNA polymerase and a reverse transcriptase, to make new strands of DNA.

Transforming growth factor-β (TGF-β): A cytokine that induces antiproliferative activity in a variety of cell types and downregulation of the inflammatory response.

Transient hypogammaglobulinemia: A condition characterized by low immunoglobulin levels that occurs in infants around 2 to 3 months of age. It is believed to be caused by delayed maturation of one or more components of the immune system and usually corrects itself spontaneously.

Translation: The process by which messenger RNA is used to make functional proteins.

Transporters associated with antigen processing (TAP 1 and TAP 2): Proteins that are responsible for the ATP-dependent transport of newly synthesized short peptides from the cytoplasm to the lumen of the endoplasmic reticulum for binding to class I HLA antigens.

Trend: A gradual change in the mean in one direction that may be caused by a gradual deterioration of reagents or deterioration of instrument performance.

Treponema pallidum: A spirochete that is the causative agent of syphilis.

Treponemal tests: Serological tests for syphilis that detect antibodies directed against *Treponema pallidum* itself.

Tumor: An abnormal cell mass that can either be benign or malignant.

Tumor-associated antigens (TAA): Antigens that are expressed by normal cells as well as tumor cells.

Tumor-infiltrating lymphocyte (TIL): Lymphocytes within a tumor mass that are able to react with antigens on tumor cells to help destroy them.

Tumor marker: Biological substances found in increased amounts in the blood, body fluids, or tissues of patients with a specific type of cancer.

Tumor necrosis factor (TNF): A major mediator of the innate defense against gram-negative bacteria.

Tumor-specific antigen (TSA): Antigens that are unique to the tumor of an individual patient or shared by a limited number of patients with the same type of tumor.

Tumor suppressor genes: Genes that inhibit the growth of tumors.

Turbidimetry: A technique for determining the concentration of particles in a solution based on the change in absorbance, caused by the scattering of light that occurs when an incident beam is passed through the solution.

Turnaround time (TAT): The amount of time required between the point at which a test is ordered by the health-care provider and the results are reported to the health-care provider.

Type 1 diabetes mellitus (T1D): A chronic autoimmune disease characterized by insufficient insulin production, caused by progressive destruction of the beta cells of the pancreas.

Type I hypersensitivity: An allergic reaction in which antigen-specific IgE antibody binds to mast cells and basophils, triggering degranulation and the release of chemical mediators; also known as *anaphylactic hypersensitivity.*

Type II hypersensitivity: An immune reaction in which IgG or IgM antibodies are produced to cell surface receptors, causing damage to the cells, dysfunction of the cells, or overstimulation of the function of the cells; also known as *antibody-mediated cytotoxic hypersensitivity*.

Type III hypersensitivity: An immune reaction in which IgG or IgM antibodies react with soluble antigens to form small complexes that precipitate in the tissues and activate complement to induce inflammation; also known as *complex-mediated hypersensitivity.*

Type IV hypersensitivity: A cell-mediated response involving the release of cytokines that induce inflammation and tissue damage 24 to 72 hours after contact with an antigen.

Typhus: A group of *Rickettsiae* that causes endemic and epidemic typhus, diseases characterized by fever, rash, and a cough.

Urease: An enzyme that breaks down urea to form ammonia and bicarbonate. Presence of urease is used as an indicator of *Helicobacter pylori*.

Vaccine: An antigen preparation derived from a pathogen that is administered to healthy individuals in order to produce immunity to an infectious disease.

Variable: Anything that can be changed or altered in laboratory testing.

Variable region: The amino-terminal region of an immunoglobulin molecule (half of a light chain or quarter of a heavy chain) that has a unique amino acid sequence for each different immunoglobulin molecule. This part is responsible for the specificity of a particular immunoglobulin molecule.

Variants: Changes in a nucleotide sequence.

Varicella-zoster virus (VZV): A herpes virus that is responsible for chickenpox and zoster, or shingles.

Venereal Disease Research Laboratory (VDRL) test: A flocculation test for the cardiolipin antibody produced in syphilis patients; an example of a nontreponemal test.

Viral load tests: Quantitative tests for nucleic acid from viruses such as HIV. These tests are used to predict disease progression and to monitor the effects of antiretroviral therapy.

Virulence: A quantitative trait of an organism that refers to the extent of pathology it can cause when it infects a host.

Virulence factors: Characteristics of a microorganism that can increase its pathogenicity by contributing to its ability to establish itself in the host, invade or damage host tissue, or evade the host immune response.

Volumetric pipette: A pipette that is marked and calibrated to deliver only one volume of the specified liquid.

Waldenström macroglobulinemia: An immunoproliferative disease caused by a malignancy of lymphocytes that results in production of` IgM paraproteins.

Wegener's granulomatosis (WG): See *granulomatosis with polyangiitis.*

Western blot test: A confirmatory test for HIV based on separation of HIV antigens by electrophoresis followed by transfer or blotting of the antigen pattern to a supporting medium for reaction with test serum.

Wiskott-Aldrich syndrome (WAS): A rare X-linked recessive syndrome characterized by immunodeficiency, eczema, and thrombocytopenia.

Xenograft: The transfer of tissue from an individual of one species to an individual of another species, such as animal tissue transplanted to a human.

Yeast: A unicellular form of certain fungi that reproduces asexually by budding, in which the parent cell divides into two unequal parts.

Zone of equivalence: The point in an antigen–antibody reaction at which the number of multivalent sites of antigen and antibody are approximately equal, resulting in optimal precipitation.

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Answer Key

Chapter 1 Introduction to Immunity and the Immune System

Answers to Case Studies

1. a. Because the swelling has occurred within 2 days, it is most likely caused by an innate immune response. The adaptive immune response takes longer to develop because it depends upon lymphocytes recognizing a specific antigen. Swelling and redness in the tissue is caused by neutrophils leaving the bloodstream by means of diapedesis in response to the presence of bacteria. **b.** There may be macrophages, neutrophils, and dendritic cells present.

2. a. The adaptive immune system is characterized by specificity and memory. When exposed to the same foreign substance numerous times, the response is increased each time. Thus, with a serious disease such as tetanus, getting a booster shot of a similar but harmless substance will stimulate the immune system each time a booster is given. Restimulating the adaptive immune response provides greater protection than the innate immune system on its own, which could possibly be overwhelmed by pathogens such as the bacteria that cause tetanus.

Answers to Review Questions

1. c	2. d	3. a	4. a	5. d	6. b	7. a
8. d	9. c	10. a	11. b	12. a	13. b	14. c
15. a	16. b	17. c	18. d	19. c	20. b	

Chapter 2 Nature of Antigens and the Major Histocompatibility Complex

Answers to Case Study

1. a. Because every child inherits one haplotype (set of genes) from the mother and one from the father, 50% of the HLA antigens would match the mother and 50% would match the father. It would never be more than that unless the mother and father have at least one antigen in common. **b.** According to the law of independent assortment, there would be a 1:4 chance that the sister would be an exact match, a 1:2 chance that a sister would share half of the same alleles, and a 1:4 chance that a sister would share no alleles, having received the opposite haplotype from each parent. **c.** It is possible that a cadaver kidney may actually be a better match, if neither sister is an exact match. The most important alleles to match are HLA A, B, and DR. If a cadaver match has more than one allele in common with the recipient at each of these loci, then it would be a closer match.

Answers to Review Questions

1. d	2. b	3. a	4. c	5. d	6. a	7. b
8. a	9. a	10. c	11. b	12. c	13. a	14. b
15. d	16. c	17. b.	18. a			

Chapter 3 Innate Immunity

Answers to Case Studies

1. a. Although the cholesterol levels were within normal limits for both HDL and total cholesterol, recent studies indicate that an increase in CRP has been associated with a greater risk of a future heart attack. Higher fibrinogen levels are also associated with an increased risk for a future cardiovascular event, although increased fibrinogen is not as great a risk factor as increased CRP. A rise in both of these acute phase reactants indicates an underlying inflammatory process. Such a process is associated with atherosclerosis, a condition that damages coronary blood vessels. Rick's wife should encourage him to follow a healthy diet and to lose weight through exercise.

2. a. CRP is one of the first indicators of a possible infection. Levels also rise in the case of a malignancy, heart attack, or trauma to the body. If the infection was bacterial, an increase in the white blood cell (WBC) count should have been seen. This increase would mainly be because of recruitment of neutrophils to help fight the invading organism. However, if an infection is caused by a virus, there is typically no increase in the WBC count. As an acute phase reactant, CRP levels increase dramatically within 24 hours, long before specific antibody can be detected. **b.** An increase in CRP would likely be seen if the student had infectious mononucleosis, a viral infection. However, CRP doesn't specifically indicate which type of viral infection may be present. The symptoms are consistent with the possibility of mono, but other conditions can't be ruled out. Repeating the mono test in a few days will allow enough time for a detectable level of antibody to form, and the diagnosis could be confirmed.

Answers to Review Questions

1. a	2. c	3. c	4. a	5. d	6. b	7. c
8. b	9. d	10. c	11. a	12. d	13. a	14. b
15. b	16. d					

Chapter 4 Adaptive Immunity

Answers to Case Studies

1. a. The normal CD19+ cell count indicates that there is not a lack of B cells, which are presumably capable of responding to antigen and producing antibodies. The low CD4+ T cell count indicates that there is a decrease in T helper (Th) cells. T helper (Th) cells are necessary for a response to T-dependent antigens. The decreased CD4+ count means that B cells are not activated by Th cells and class switching to IgG does not occur. Memory B cells are not produced either. **b.** Lack of Th cells severely limits the B-cell response, resulting in increased bacterial infections.

2. a. CD8+ T cells are responsible for the destruction of cancer cells as well as any intracellular pathogens, such as viruses. Although antibodies may be produced, they are not very effective against cancerous cells or virally infected cells. Thus, an important arm of the adaptive immune system would not be working and an individual would be more susceptible to certain kinds of infections, or to cancer.

Answers to Review Questions

1. b	2. d	3. a	4. a	5. c	6. b	7. d
8. c	9. a	10. b	11. b	12. d	13. c	14. d
15. c	16. b	17. a	18. d			

Chapter 5 Antibody Structure and Function

Answers to Case Studies

1. a. The presence of IgM only is an indicator of an early acute infection. IgM is the first antibody to appear, followed by IgG. In a reactivated case of mono, a small amount of IgM might be present, but IgG would also be present. Thus, the patient is encountering the virus for the first time. **b.** The memory cells triggered by the first exposure to the virus would cause production of IgG in a much shorter time and there would be a greater increase in IgG compared with the amount of IgM present.

2. a. The increase in IgE is an indicator that the cold symptoms may actually be caused by an allergy. This is especially evident in the springtime when pollen levels are high. The child should be tested for specific allergies to determine the cause of the symptoms. Treatment with antihistamine and avoidance of the allergen will help to relieve the symptoms. **b.** Chronic respiratory infections may be caused by a decrease or lack of IgA, but this is not the case here. Normal levels of IgG, IgM, and IgA indicate that this child is not immunocompromised.

Answers to Review Questions

1. a	2. a	3. b	4. d	5. b	6. c	7. a
8. d	9. a	10. a	11. c	12. b	13. a	14. c
15. d	16. b	17. b	18. c	19. b	20. d	21. a
22. c						

Chapter 6 Cytokines

Answers to Case Study

1. a. G-CSF. **b.** IFN-gamma and IL-2. **c.** IL-4 and IL-10.

Answers to Review Questions

1. b	2. a	3. d	4. d	5. c	6. a	7. d
8. b	9. c	10. d	11. b	12. b	13. a	14. c
15. b	16. d					

Chapter 7 Complement System

Answers to Case Studies

1. a. A decreased CH50 indicates a problem with the classical pathway. The decreased AH50 indicates a problem with the alternative pathway as well. **b.** Levels of C3 and C4 are normal, indicating that a deficiency of one or more of the membrane attack components is involved. Although a lack of C1q or C2 cannot absolutely be ruled out, the fact that the alternative pathway is also affected is an additional indicator that the components common to both pathways, C5 through C9, are the ones involved. Because defense against encapsulated bacteria such as meningococci is reduced if there is a decrease in C5 through C9, the patient's symptoms are in accord with this conclusion. **c.** In order to confirm the actual deficiency, testing for the individual components C5 through C9 should be performed. Because this type of deficiency reduces the overall functioning of the complement system, patients should receive prompt therapy when signs of infection are noted.

2. a. Although the abdominal pain and vomiting could be caused by several infectious agents, the normal white blood cell count decreases the likelihood of a bacterial infection. The accompanying swelling of the hands and legs may be an indicator of a possible inflammatory problem associated with continuous activation of the complement system. Because this has been a recurring problem, the likelihood of an immune problem is increased. Because total serum protein is within the normal range, it is unlikely that the deficiency is from lack of antibody production. A decrease of one complement component would not be apparent on a total protein determination. **b.** Reduced levels of both C4 and C2 could be from inheritance of defective genes for both components. However, the possibility of that is extremely rare. A more plausible explanation is that the deficiency of both C2 and C4 is caused by overconsumption rather than a lack of production. **c.** A lack of C1-INH would result in overconsumption of C4 and C2. As this is the most common deficiency of the complement system, this represents a likely explanation for the symptoms. This can be verified by testing for this particular component.

Answers to Review Questions

1. b	2. a	3. d	4. c	5. c	6. a	7. d
8. a	9. c	10. a	11. b	12. b	13. b	14. a
15. c	16. d	17. d	18. b	19. c	20. b	

Chapter 8 Safety and Quality Management

Answer to Case Studies

1. a. Gloves should never be removed when working with patient specimens. When they are, hands should be washed right away using the correct procedure. Any contamination of the lab bench should be treated with sodium hypochlorite and the paper towels should be disposed of in the regulated medical waste container. Because the supervisor's lab coat was disposable and became contaminated, it should be discarded in the regulated medical waste container and replaced with a new coat. Pipetting should have been done behind a Plexiglass shield because this would have prevented the spill onto the lab coat.

2. a. Take corrective action. **b.** Take corrective action. **c.** Accept. **d.** Take corrective action.

Answers to Review Questions

1. c 2. a 3. a 4. c 5. c 6. c 7. b
8. d 9. a 10. c 11. b 12. d 13. a 14. d
15. a 16. a 17. d 18. c 19. b
20. 2, 1, 2, 3, 2, 2

Chapter 9 Principles of Serological Testing

Answer to Case Study

1. a. The serological pipette must be emptied completely to obtain the correct volume because it is marked to contain (TC) rather than to deliver (TD). Therefore, it should have been blown out to obtain the last bit, or the measurement should have been from point to point, as in filling the pipette up to the 0.8 mL mark and then letting it drain to the 0.9 mL mark. **b.** The 1.9 diluent was not correct. In order to make a 1:40 dilution with 0.1 mL of serum, the calculations are as follows:

$$1/40 = 0.1/X$$

X = 4.0 mL (This represents the total volume.)

4.0 − 0.1 = 3.9 mL of diluent to make a 1:40 dilution.

Answers to Review Questions

1. b 2. a 3. c 4. a 5. c 6. d 7. b
8. a 9. b 10. a 11. d 12. c 13. b 14. a
15. d 16. c 17. d 18. a

Chapter 10 Precipitation and Agglutination Reactions

Answers to Case Studies

1. a. The results indicate normal levels of IgG and IgM, but there is a decreased level of IgA. This most likely indicates a selective IgA deficiency, the most common genetic immunodeficiency. Selective IgA deficiency occurs in approximately 1:1,000 individuals. **b.** A decrease in serum IgA most likely indicates a decrease in secretory IgA, the immunoglobulin that is found on mucosal surfaces. Individuals with a selective IgA deficiency are more prone to respiratory tract and gastrointestinal tract infections because IgA represents the first line of defense against pathogens that invade mucosal surfaces. **c.** Nephelometry is a more sensitive method for measuring immunoglobulin levels. It is able to detect small quantities of immunoglobulin present. Results are obtained faster in comparison to RID; because the process is automated, it is not subject to human error in reading the results. Other errors that may occur with RID include overfilling or underfilling of wells, nicking of wells, and inaccurate incubation time or temperature. Therefore, nephelometry has largely replaced RID for the measurement of immunoglobulin levels.

2. a. A positive test on an undiluted patient specimen indicates immunity to the virus if the patient was tested immediately after exposure to the disease. Testing 2 days after exposure would not give enough time for antibody to be formed if this is a first exposure to the virus. **b.** The presence of antibody indicates that the patient has immunity because of her vaccination and likely will not be re-infected with the virus. Therefore, she does not have to be concerned about possible consequences for the fetus. If there is any further question about her immunity, further testing to determine the class of antibody present could be done. The presence of IgG antibody indicates a previous exposure, whereas IgM would indicate a recent infection.

Answers to Review Questions

1. c 2. d 3. b 4. d 5. b 6. a 7. b
8. a 9. c 10. d 11. a 12. c 13. a 14. c
15. b 16. c 17. b 18. d 19. a 20. c

Chapter 11 Labeled Immunoassays

Answers to Case Study

1. a. A negative finding only means that no parasites were observed for that particular specimen at that particular time. It does not rule out the possibility that parasites may actually be present. **b.** Capture enzyme immunoassays that are specific for parasites such as *Giardia* and *Cryptosporidium* are available. Typically, a solid phase such as microtiter wells is coated with specific antibody, and very small amounts of antigen can be detected. If a parasite is suspected and the traditional results are negative, this would be the next step. **c.** Capture enzyme immunoassays are very sensitive and are capable of detecting minute amounts of parasitic antigens that may be present. This is important in testing a stool culture because large amounts of antigen may not be present at any one time. Many organisms, such as *Giardia* and *Cryptosporidium,* are extremely small and may not be easily found on a stained slide preparation. **d.** In addition to the increased sensitivity, enzyme immunoassays are simple to perform and less time consuming than traditional tests for parasites. Because instrumentation is usually used, the results are more easily interpreted with less subjectivity than stained smears.

Answers to Review Questions

1. c 2. a 3. c 4. b 5. a 6. c 7. b
8. a 9. b 10. d 11. d 12. b 13. c 14. c
15. b 16. b

Chapter 12 Molecular Diagnostic Techniques

Answers to Case Studies

1. a. Controls would include a reagent blank to test for contamination and a negative control for the mutation (a DNA template known not to have the mutation) to demonstrate that the restriction enzyme will cut the normal product. A positive control for the mutation (a DNA template known to have the mutation) will demonstrate that the restriction enzyme will not cut the mutant product. An internal control for the restriction enzyme activity—ideally, a negative control—should be cut in the same reaction as the test sample to demonstrate that the enzyme is active in that reaction. **b.** It is not necessary to see the 11 bp product. The presence of the 80 bp and 59 bp products indicate the activity of the enzyme and the 139 bp product shows the presence of the mutation that prevents the enzyme

from cutting. **c.** The cutting of the normal control indicates that the enzyme is active and confirms the interpretation of a positive result (presence of mutation). **d.** If the internal control was not digested by the restriction enzyme, then the restriction enzyme was not active. Therefore, no interpretation can be made about the patient's DNA.

2. a. The amplification control should always be present to demonstrate that the PCR is working and to avoid false negatives. A reagent blank (no template) control is included to detect contamination. In a true negative, the amplification control would be positive, whereas the test target is negative. The previous results don't necessarily predict the current test results should be positive. Because the amplification control did not work, the current results are not interpretable. **b.** Because the amplification control is positive, the PCR is working and the result is a true negative. The reagent blank (no template) control, not the amplification control, is used to detect contamination. In a true negative, the amplification control would be positive, whereas the test target is negative. The previous results may be reviewed for interpretation of the result, but do not predict a positive result in the current sample. **c.** No. The test sensitivity goes to 50 copies/mL, meaning that there may be fewer than 50 copies/mL present that will not be detected by the test method. Although the previous results don't predict a positive result because there is a history of the presence of a virus, a residual low level of viral copies could be present. The results should be reported as fewer than 50 copies/mL to indicate the test's inability to detect a low-level presence of HIV.

Answers to Review Questions

1. b	2. a	3. a	4. b	5. a	6. a	7. c
8. d	9. c	10. c	11. b	12. a	13. a	14. d
15. d	16. b	17. d	18. b	19. c	20. b	21. c

Chapter 13 Flow Cytometry and Laboratory Automation

Answers to Case Studies

1. a. The result may represent an error of specificity given that the newer instrument is getting positive results on specimens that were negative by the older method. However, the newer instrument could be more sensitive than the older one, so these could actually be positive samples. **b.** To resolve this discrepancy, known positive and negative controls should be run. The positive controls need to include those at the lower limit of detection, as well as more highly positive samples. This would help to determine if the new instrument is actually more sensitive rather than lacking in specificity.

2. a. The flow pattern in A indicates that the majority of lymphocytes are B cells because they are CD19+. The population most affected appears to be CD3+, which are T cells. Pattern B indicates that of the CD3+ lymphocytes, the majority are CD8+, or cytotoxic T cells. The CD4+ count is very low. **b.** T helper cells are necessary to provide help to B cells so they can respond by making antibody. Thus, the child is unable to make IgG in response to potential pathogens she might encounter in the environment. **c.** This child should be tested for HIV. That would explain the decrease in CD4+ T cells.

Answers to Review Questions

1. a	2. c	3. b	4. c	5. a	6. b	7. d
8. c	9. a	10. d	11. c	12. c	13. c	14. d
15. d	16. b	17. d	18. b	19. b	20. d	

Chapter 14 Hypersensitivity

Answers to Case Studies

1. a. An increase in eosinophils is typically found in allergic individuals. Interleukins released by stimulated Th1 cells are involved in the recruitment of eosinophils from the bone marrow. Although there are other causes of eosinophilia, such as a parasitic infection, an increased number most often indicates an allergic reaction. **b.** The patient can have a skin prick test performed to determine which allergens he is sensitized to. The patient would know his results immediately because a positive test would be indicated by formation of wheal-and-flare reactions within 20 minutes at the site(s) of injection. If he is unable to discontinue any antihistamines he might be taking, or if a clear area of skin in his forearm or back could not be found, a solid-phase immunoassay for allergen-specific IgE could be performed. **c.** A solid-phase immunoassay for total IgE could be performed to monitor the patient's response to allergen immunotherapy. If the therapy is successful, the IgE concentration in the patient's serum should decrease to a level within the reference age for patients his age.

2. a. A positive DAT indicates that the red blood cells (RBCs) are coated with either antibody or complement components. The destruction of some RBCs is the reason for the man's symptoms. **b.** The most likely cause of the positive DAT is the presence of an antibody of the IgM class. It might be an anti-I, triggered by *Mycoplasma pneumonia*. This is a cold-reacting antibody. **c.** A DAT that is only positive with anti-C3d indicates that only complement products are present on the RBCs. This is a further indication that the antibody is an IgM antibody because it does not remain on the cells at 37°C but does trigger complement activation, which can cause the cell destruction.

Answers to Review Questions

1. c	2. b	3. d	4. b	5. a	6. b	7. d
8. b	9. c	10. c	11. a	12. b	13. d	14. d
15. c						

Chapter 15 Autoimmunity

Answers to Case Studies

1. a. In systemic lupus erythematosus, a low titer of rheumatoid factor is often present. Conversely, a low titer of antinuclear antibodies can be associated with rheumatoid arthritis. Thus, these two conditions cannot be differentiated on the basis of the rapid RF and ANA test results alone. **b.** The decreased red blood cell (RBC) count may be because of the presence of a

low-level autoantibody directed against RBCs, often associated with lupus. **c.** A fluorescent antinuclear antibody (FANA) test is a good screening tool to help distinguish between these two conditions. A homogeneous pattern or a peripheral pattern would be indicative of lupus, whereas a speckled pattern can sometimes be found in rheumatoid arthritis or lupus. Therefore, if a speckled pattern is obtained, more specific testing for ENA antibodies should be done. The presence of anti-Sm antibody would be diagnostic for lupus. This is what was found in this case. Testing for anti-CCP could also be performed, as this antibody is highly specific for rheumatoid arthritis.

2. a. The low T4 level, enlarged thyroid gland, and presence of antithyroglobulin antibody are all indicators of Hashimoto's thyroiditis. **b.** Antithyroglobulin antibodies progressively destroy thyroglobulin produced by the thyroid. Thyroglobulin is normally cleaved in the thyroid to produce the secretable hormones triiodothyronine (T3) and thyroxine (T4). The presence of antithyroglobulin antibodies causes enlargement of the thyroid because of the immune response, and hypothyroidism results, characterized by fatigue and weight gain. **c.** Graves disease is also an autoimmune illness that affects the thyroid, but it is characterized by hyperthyroidism. In this disease, antibodies to thyroid-stimulating hormone receptors are produced, sending a signal to the thyroid to constantly produce T3 and T4; consequently, these hormones are elevated in the blood. Symptoms include nervousness, insomnia, restlessness, and weight loss, exactly opposite of the characteristics of Hashimoto's thyroiditis.

Answers to Review Questions

1. a 2. d 3. d 4. a 5. d 6. b 7. a
8. c 9. d 10. c 11. a 12. b 13. d 14. a
15. c

Chapter 16 Transplantation Immunology

Answers to Case Studies

1. a. The most compatible donor for this patient would be Friend 2. Sibling 1 has the B35 antigen for which the patient possesses HLA antibody. Sibling 2 also has the B35 antigen and is also ABO incompatible. Friend 1 is ABO incompatible. Friend 2 is ABO identical and does not express the HLA-B35 antigen and is thus the most appropriate donor.

2. a. Maybe, for an unrelated donor, one can't be sure that they have the same alleles at each locus even if they have the same low resolution type. **b.** The physician requested high-resolution HLA in order to determine if the donor and recipient had the same alleles at each locus. Serological typing (phenotyping) provides low-resolution results as indicated. The best outcomes for transplant occur if the recipient and donor are HLA allele level matched. High-resolution typing of the donor was performed. The donor's B locus typing indicated he or she had the alleles HLA-A*02:05 and HLA-B*44:03. Thus, they were actually mismatched for two alleles. Based on this finding, this donor was declined and an additional search was conducted.

Answers to Review Questions

1. b 2. c 3. d 4. d 5. b 6. b 7. a
8. d 9. a 10. b 11. b 12. c 13. a 14. c
15. b

Chapter 17 Tumor Immunology

Answers to Case Studies

1. a. If no further CA 15-3 is being produced by tumor tissue, levels will decrease at the rate of biological half-life for the molecule. Because CA 15-3 levels are not decreasing at this rate, residual tumor is suspected. **b.** HER-2 overexpression indicates that therapy with the monoclonal antibody Herceptin may be successful. **c.** Because the tumor lacks estrogen and progesterone receptors, hormone-suppressing therapy is unlikely to improve prognosis.

2. a. No other tissues in men are known to produce PSA, so another source is extremely unlikely. **b.** PSA velocity is the rate of PSA increase between determinations. Because PSA increases with age and prostatic enlargement, examining PSA velocity is an attempt to separate benign and malignant conditions, as velocity is higher in malignancy. Current recommendations for biopsy are for PSA velocities that exceed 0.35 ng/mL per year. **c.** Although increased to above the reference interval, the proportion of free PSA remains within the interval associated with benign disease. Furthermore, his PSA velocity did not exceed 0.35 ng/mL per year, and the digital rectal exam did not detect any obvious sign of malignancy. Given the man's age, benign prostatic hypertrophy is likely, and further PSA testing after a waiting period may be warranted in lieu of a biopsy. **d.** Once a man's life expectancy is fewer than 10 years, PSA testing is no longer recommended.

Answers to Review Questions

1. d 2. b 3. a 4. c 5. d 6. d 7. a
8. c 9. d 10. d 11. a 12. a 13. b 14. a
15. c.

Chapter 18 Immunoproliferative Diseases

Answers to Case Studies

1. a. The patient has evidence of anemia and pneumonia. The elevated erythrocytic sedimentation rate (ESR) is a nonspecific indicator of inflammation or elevated serum proteins. Based upon these findings, the physician requested the measurement of serum immunoglobulins. Elevated serum immunoglobulins can produce an elevated ESR. The extremely high IgG levels indicate that a monoclonal gammopathy is probably present. The patient is most likely suffering from multiple myeloma. Infiltration of cancerous myeloma cells into the bone marrow is likely to be responsible for the patient's anemia; despite having pneumonia, the white blood cell count is only slightly elevated. The back pain could also be caused by infiltration of myeloma cells into the vertebra.

The age of the patient is appropriate for the diagnosis of multiple myeloma. **b.** The diagnosis could be confirmed by performing a bone marrow biopsy because having more than 10% plasma cells in the bone marrow is one of the diagnostic criteria for multiple myeloma. Radiographs could reveal the presence of lytic bone lesions responsible for the patient's back pain. In addition, serum protein electrophoresis (SPE) could be used to detect a monoclonal band and serum immunofixation electrophoresis (IFE) would be used to identify the suspected monoclonal IgG and possibly detect Bence Jones proteins in the patient's urine. Free light chain assays can be used to determine the concentration of monoclonal light chains in the serum and the κ/λ ratio. A serum monoclonal protein concentration of greater than 3 g/dL and a urinary monoclonal protein greater than 200 mg/day indicate the presence of multiple myeloma.

2. a. Although hairy cell leukemia (HCL) cells are not generally seen in the bone marrow, the hematologic bone marrow studies describe malignant cells characteristic of HCL. Splenomegaly is often seen in patients with HCL. **b.** Malignant HCL cells often express CD20 and CD25, the markers found in this patient. In addition, CD103 is a sensitive and specific marker for this disease. Although not tested for in this patient, CD123 is also a specific marker for HCL.

Answers to Review Questions

1. c 2. c 3. a 4. c 5. d 6. a 7. d
8. b 9. a 10. d 11. b 12. a 13. c 14. d
15. a

Chapter 19 Immunodeficiency Diseases

Answers to Case Studies

1. a. The constant bacterial infections coupled with laboratory results indicate an immunodeficiency disease, likely Bruton's tyrosine kinase deficiency or severe combined immunodeficiency syndrome (SCID). **b.** In both conditions, an X-linked recessive gene can be inherited, which affects males almost exclusively. **c.** To differentiate between the two immunodeficiency states, several types of testing are recommended. Measurement of serum IgA, IgM, and IgG levels should be performed to determine if, in fact, all classes of antibody are absent. Enumeration of classes of lymphocytes should also be determined by flow cytometry. In SCID, both T- and B-cell development is affected and both lymphocyte populations would be deficient, whereas in Bruton's tyrosine kinase deficiency, only B-cell development is affected. Because the differential indicates that some lymphocytes are present, this would point to Bruton's tyrosine kinase deficiency. Flow cytometry findings confirming the presence of T cells only validate this diagnosis.

2. a. The patient's specimen is seen in region 4. Note the faint, diffuse IgG and light chain bands. No IgA or IgM bands are visible. Specimen 1 is a normal control. Specimen 2 contains a monoclonal IgG kappa protein. Specimen 3 is a concentrated 24-hour urine specimen that contains albumin. **b.** Her history and the SPE results indicate that she is immunocompromised and producing very little antibody at all. The faint IgG band would confirm this.

Answers to Review Questions

1. c 2. c 3. d 4. a 5. b 6. a 7. d
8. d 9. a 10. b 11. d 12. a 13. c 14. d
15. b

Chapter 20 Serological and Molecular Detection of Bacterial Infections

Answers to Case Studies

1. a. Poststreptococcal glomerulonephritis. **b.** *Streptococcus pyogenes* (Group A streptococci). **c.** Streptococcal antigen–antibody complexes may deposit in the glomeruli of the kidneys or antibody formed against the organisms cross-reacts with antigens in the glomeruli. These immune responses stimulate an inflammatory response that causes damage to the glomeruli, leading to renal impairment and function. The rapid GAS test was negative because the organism is no longer present in the throat and the patient did not present with pharyngitis. **d.** A urinalysis is helpful because microscopic hematuria is typically present in children with acute poststreptococcal glomerulonephritis. The proteinuria rarely exceeds 3+ by dipstick; however, massive proteinuria and a nephrotic picture may be observed in a small percentage of patients. **e.** The streptozyme test measures antibodies against five extracellular streptococcal antigens: anti-streptolysin (ASO), anti-hyaluronidase (AHase), anti-streptokinase (ASKase), anti-nicotinamide-adenine dinucleotidase (anti-NAD), and anti-DNAse B. The streptozyme test is positive in 95% of patients with acute poststreptococcal glomerulonephritis caused by GAS pharyngitis.

2. a. *Mycoplasma pneumoniae*. **b.** The patient has only been ill for several days and has not had time to mount a serological response to the causative agent. IgG levels suggest that the patient had a previous exposure to the organism and the level may represent residual immunoglobulin G (IgG) from an earlier exposure. **c.** Definitive diagnosis of *M pneumoniae* requires documented seroconversion by paired specimens obtained 2 to 4 weeks apart, measuring both IgM and IgG. A four-fold rise in IgG levels is considered diagnostic. **d.** Cold agglutinin titers used for the diagnosis of *M pneumoniae* infections are not very specific or very sensitive. Testing for cold agglutinins is no longer recommended for the detection of *M pneumoniae* infections because the development of cold agglutinins occurs in other conditions, including some viral infections and collagen vascular diseases. Although not specific for *M pneumoniae* infection, a high cold agglutinin titer in a patient with community-acquired pneumonia symptoms (>1:64) is likely to be caused by *M pneumoniae*.

Answers to Review Questions

1. d 2. c 3. b 4. b 5. a 6. a 7. b
8. d 9. c 10. c 11. a 12. b 13. c 14. c
15. b

Chapter 21 Spirochete Diseases

Answers to Case Studies

1. a. Almost 25% of individuals with Lyme disease do not exhibit the characteristic rash; therefore, its absence does not rule out the possibility of the disease. **b.** There are several conditions that can cause false-positive results in EIA testing, including syphilis, other treponemal diseases, infectious mononucleosis, and autoimmune diseases such as rheumatoid arthritis. Thus, low levels of antibody might indicate one of these other diseases. However, false-negative results in Lyme disease are also possible because of a low level of antibody production. Therefore, an indeterminate test neither rules out nor confirms the presence of Lyme disease. **c.** If there is a history of tick bite and patient symptoms are consistent with Lyme disease, then a confirmatory Western blot should be performed. The Western blot is fairly specific for Lyme disease. If 5 of 10 protein bands specific for *Borrelia burgdorferi* IgG antibodies are positive, this confirms the presence of Lyme disease.

2. a. Although it is possible that the mother's positive RPR test could be a false positive, it is also likely that the mother is in the latent stage of syphilis, with no obvious signs of the disease. Although syphilis is not sexually transmitted during this stage, it can be transmitted from a mother to her unborn child. Many infants do not exhibit clinical signs of the disease at birth; however, if infected and untreated, a large percentage of babies develop later symptoms, including neurological deficits such as blindness and mental retardation. **b.** A positive RPR on cord blood could be from transplacental passage of the mother's IgG antibodies. A titer should be performed on the cord blood and a serum sample obtained from the infant in several weeks. If infection is present in the infant, the titer will remain the same or increase. An IgM capture assay could also be performed. The presence of specific anti-treponemal IgM would indicate that the infant had been exposed to *Treponema pallidum* because IgM antibodies do not cross the placenta. **c.** Because there is a good chance that the infant is at risk for congenital syphilis, immediate treatment with penicillin can prevent any further neurological consequences.

Answers to Review Questions

1. d 2. c 3. b 4. d 5. b 6. c 7. c
8. d 9. b 10. d 11. d 12. b 13. c 14. c
15. a

Chapter 22 Serological and Molecular Diagnosis of Parasitic and Fungal Infections

Answers to Case Studies

1. a. It cannot be determined by the test results available whether the baby has congenital toxoplasmosis. The IgG antibodies in the newborn may reflect maternal antibodies that crossed the placenta. The presence of IgG antibodies in the newborn may reflect either past or recent infection in the mother. IgM antibodies may persist for up to 18 months after infection with *T gondii*. Thus, the greatest value of testing for IgM antibodies is in determining whether a woman has had a recent infection. If no IgM antibodies are detected and only IgG is detected, this excludes a recent infection before pregnancy. The presence of IgG and IgM in the mother may indicate a recent infection. **b.** Tests for IgA and IgM antibodies are commonly used for the diagnosis of infection in the newborn. If IgG, IgM, and IgA are detected, a diagnosis of congenital toxoplasmosis is established. If IgG antibodies are detected but serological test results for IgM and IgA antibodies are negative, follow-up serological testing in suspected cases is indicated. Maternally transferred antibodies usually decrease and disappear within 6 to 12 months. Established infection in the mother can also be indicated by the presence of high avidity IgG antibodies.

2. a. The majority of patients with symptomatic cryptococcosis have an identified underlying immunocompromised condition. These include acquired immunodeficiency syndrome (AIDS), prolonged treatment with corticosteroids, organ transplantation, advanced malignancy, diabetes, and sarcoidosis. The clinical symptoms and outcome of cryptococcal meningitis vary, in part because of the related underlying medical conditions and the immune status of the host. The most common symptoms are headache, altered mental status, personality changes, confusion, lethargy, and coma. Nausea and vomiting are also common and are caused by increased intracranial pressure. Onset of the disease is often subacute and worsens over several weeks. The patient in this case was immunosuppressed because of his long-term steroid use and presented with severe headaches, gait instability, and weakness upon standing. **b.** The simplest diagnostic test is an India ink test on the patient's CSF. Because of the large polysaccharide capsule produced by the organism, the India ink is displaced, allowing for visualization of the yeast. The serological tests for cryptococcal antigen in serum and CSF are highly sensitive and specific for the diagnosis of invasive disease. New immunochromatographic assays may also be used for the detection of the capsular antigen in serum and CSF.

Answers to Review Questions

1. d 2. d 3. d 4. a 5. b 6. b 7. a
8. a 9. d 10. b 11. d 12. a 13. b 14. d
15. c 16. c

Chapter 23 Serology and Molecular Detection of Viral Infections

Answers to Case Studies

1. a. The patient's clinical symptoms and increased liver function enzymes indicate inflammation of the liver. To determine whether this inflammation is the result of viral hepatitis and to identify the cause, the following tests should be ordered: (1) IgM anti-HAV to screen for hepatitis A, (2) HBsAg to screen for hepatitis B, (3) IgM anti-HBc to screen for hepatitis B in the core window period when HBsAg is absent, and (4) anti-HCV to screen for hepatitis C. **b.** To monitor hepatitis B infection, testing for HBsAg and HBeAg should be performed periodically

to determine how long the infection is persisting and the relative infectivity of the patient. Tests for anti-HBe and anti-HBs are performed to indicate whether the infection has resolved and whether immunity has been established, respectively. **c.** In chronic hepatitis B, HBsAg persists in the serum for more than 6 months. Total anti-HBc is also present; HBeAg may or may not be present, depending on the degree of disease progression. Anti-HBe and anti-HBs are usually not present, but may have a delayed appearance in those individuals who eventually recover.

2. a. Many viruses can produce congenital abnormalities in an infant born to a mother infected during pregnancy. These include cytomegalovirus, rubella virus, and varicella zoster virus. The infant's symptoms and mother's history suggest infection with rubella virus. **b.** Ideally, the mother would have been tested at the time of her illness during her pregnancy for rubella antibodies. Demonstration of rubella-specific IgM antibody, seroconversion from negative to positive for rubella antibody, or a four-fold rise in antibody titer would have indicated an active rubella infection. However, because this was not done, the mother could be tested for rubella-specific IgG antibody, which would indicate if rubella exposure occurred. Tests that measure the avidity of the IgG antibody can be performed to distinguish between a recent infection (low avidity) and a past infection (high avidity). **c.** The infant's serum should be tested for rubella-specific IgM antibody, preferably with an IgM antibody capture enzyme immunoassay. IgM antibodies, which cannot pass through the placenta, would have been produced by the fetus as a result of active rubella infection. IgG antibodies, on the other hand, are derived mainly from the mother's serum as a result of passive transfer through the placenta. Viral culture or RT-PCR should be used to confirm positive IgM results.

Answers to Review Questions

1. c 2. d 3. b 4. a 5. d 6. b 7. a
8. d 9. b 10. c 11. c 12. c 13. d 14. a
15. c

Chapter 24 Laboratory Diagnosis of HIV Infection

Answers to Case Studies

1. a. The physician could perform a rapid EIA for HIV antibody. If the test is positive, the physician should order a fourth-generation ELISA, which simultaneously detects antibodies to HIV-1, antibodies to HIV-2, and p24 antigen, to verify the result. **b.** If the fourth-generation ELISA was positive, a rapid test for HIV-1 and HIV-2 antibodies should be performed to confirm the results and distinguish between infection by the two viruses. **c.** If it is determined that the woman truly has HIV infection, her CD4 T-cell counts should be monitored periodically to assess the effects of the virus on her immune system. In addition, she should be placed on antiretroviral therapy and the effectiveness of the therapy should be evaluated by periodically performing viral load assays to monitor the amount of HIV RNA in her plasma.

2. a. HIV infection is transmitted from mother to infant via three routes: (1) passage through the placenta during pregnancy, (2) exposure to maternal blood during the delivery process, or (3) through breast milk. To reduce the risk of transmission, antiretroviral therapy should be administered to the mother during her pregnancy and to the infant after birth. In addition, the mother should be advised not to breastfeed her baby. **b.** Testing the infant's serum for HIV antibody would yield confusing results. This is because the IgG HIV antibodies in the mother's serum would pass through the placenta during the pregnancy and be detectable in the infant's serum. The result would be a false positive if the infant was not HIV-infected. **c.** Because of the problems associated with antibody testing, molecular methods are preferred to make a diagnosis of HIV infection in infants younger than 18 months of age. The preferred molecular test is a PCR that detects the presence of proviral HIV DNA in the infant's peripheral blood mononuclear cells. Alternately, a quantitative HIV RNA test could be performed on the infant's plasma.

Answers to Review Questions

1. c 2. c 3. d 4. a 5. d 6. b 7. a
8. b 9. c 10. d 11. d 12. a 13. a 14. b
15. d

Chapter 25 Immunization and Vaccines

Answers to Case Studies

1. a. The child could safely receive vaccines that do not contain a live component. These include the vaccines for hepatitis B (a recombinant antigen); diphtheria and tetanus (toxoids); pertussis, *Haemophilus* influenza b, and pneumococcus (subunit vaccines); and polio, hepatitis A, and influenza (the preparations containing inactivated virus). **b.** The child could not receive any live, attenuated vaccines because the organisms in these vaccines, although weakened, could not be controlled by the child's immune system and could cause serious, disseminated infections. Such vaccines include those against the viral diseases: measles, mumps, rubella, varicella, and rotavirus. The nasal mist form of the influenza vaccine should also not be administered because it contains live, attenuated virus. **c.** The child could be protected against these infections by receiving regular injections of human serum immunoglobulin (gamma globulin), a preparation that has been pooled from the serum of other individuals, and which contains numerous pre-made antibodies. In addition, family members and close friends of the child should make sure that they are up-to-date on their own immunizations. By preventing development of these diseases in themselves, they are ensuring that they cannot pass the pathogen along to the child. This concept, whereby protection against infectious diseases is extended to others in a population, is known as "community immunity" or "herd immunity."

2. a. Hepatitis A-specific human immune serum globulin, consisting of a high concentration of antibody to the hepatitis A virus, should be used to prevent the infection in anyone

who has dined at the restaurant recently. Because this preparation is derived from individuals who have previously been exposed to hepatitis A, the antibodies are premade and provide immediate protection when they are administered within 2 weeks after exposure to the pathogen. **b.** Although human immune serum globulin provides immediate protection, that protection is only temporary because the antibody titers decline over time, according to the half-life of the immunoglobulin molecules. The half-life for IgG, which comprises the majority of the preparation, is 23 days. To achieve long-term immunity without actually acquiring the infection, you would need to be immunized with the hepatitis A vaccine. The vaccine, consisting of inactivated hepatitis A virus, would stimulate an immune response against the viral antigens and the generation of memory lymphocytes that could quickly be reactivated in case of a later exposure.

Answers to Review Questions

1. a 2. c 3. d 4. b 5. d 6. c 7. b
8. d 9. d 10. c 11. c 12. b 13. c 14. a
15. b

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