Christine Dorresteyn Stevens
Linda E. Miller

Clinical Immunology and Serology A Laboratory Perspective

FOURTH EDITION



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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

IMMUNITY AND IMMUNIZATION
INNATE VERSUS ADAPTIVE IMMUNITY
CELLS OF THE INNATE IMMUNE SYSTEM
- Leukocytes in Peripheral Blood
- Tissue Cells

CELLS OF THE ADAPTIVE IMMUNE SYSTEM
- B Cells
- T Cells
- Natural Killer (NK) Cells

ORGANS OF THE IMMUNE SYSTEM
- Primary Lymphoid Organs
- Secondary Lymphoid Organs

SUMMARY
CASE STUDIES
REVIEW QUESTIONS

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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.

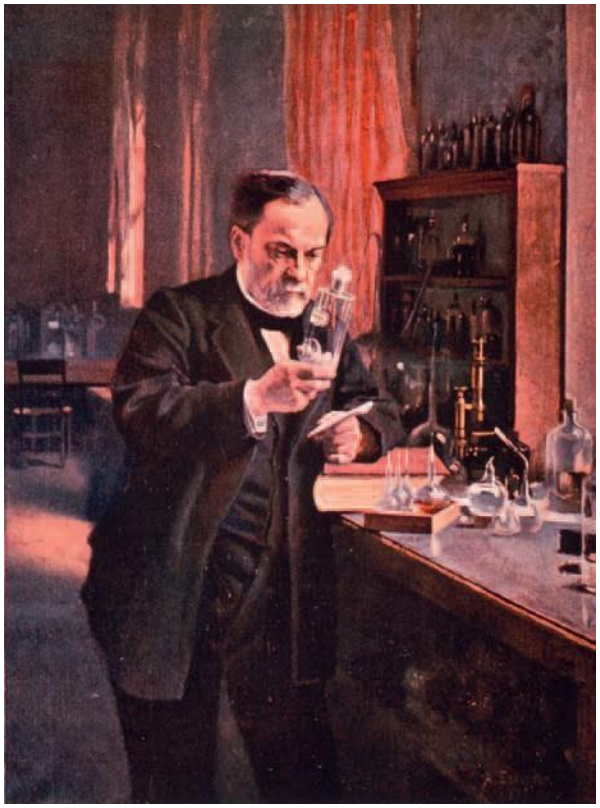


FIGURE 1–1 Louis Pasteur. *(Courtesy of the National Library of Medicine.)*

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]

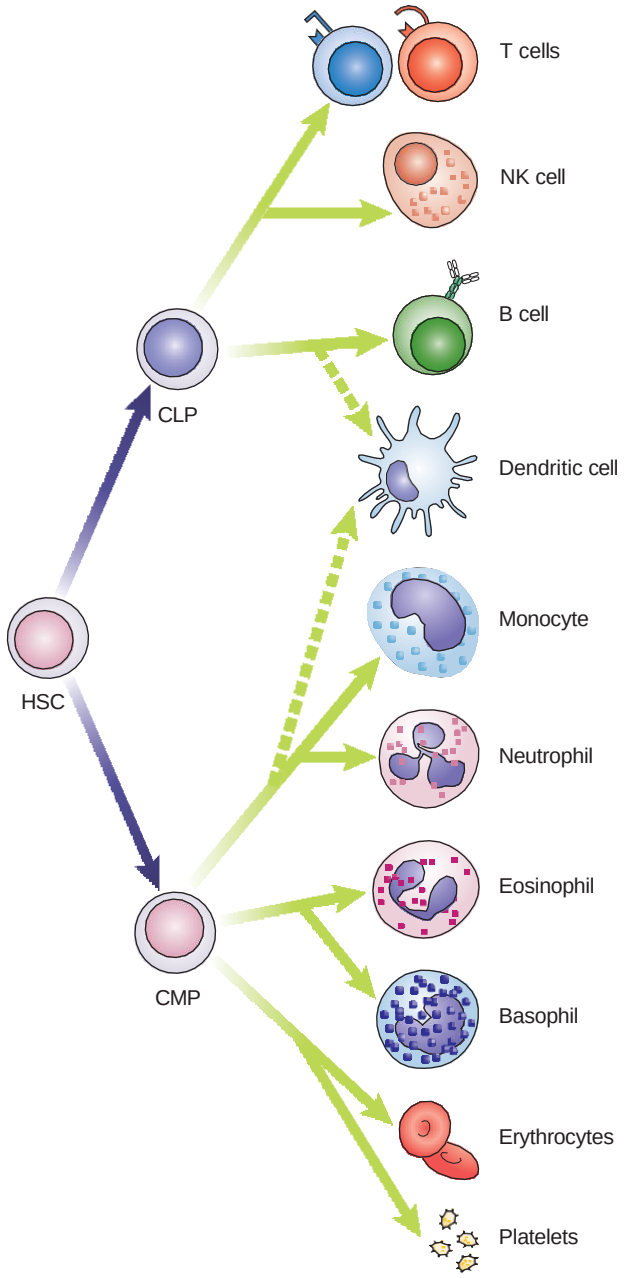


FIGURE 1–2 Simplified scheme of hematopoiesis. In the marrow, hematopoietic stem cells (HSC) give rise to two different lines—a common lymphoid precursor (CLP) and a common myeloid precursor (CMP). CLPs give rise to T/NK progenitors, which differentiate into T and NK cells, and to B-cell progenitors,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

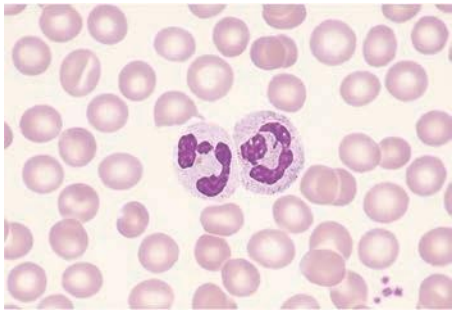


FIGURE 1–3 Neutrophils. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009. Fig. 1–4.)*

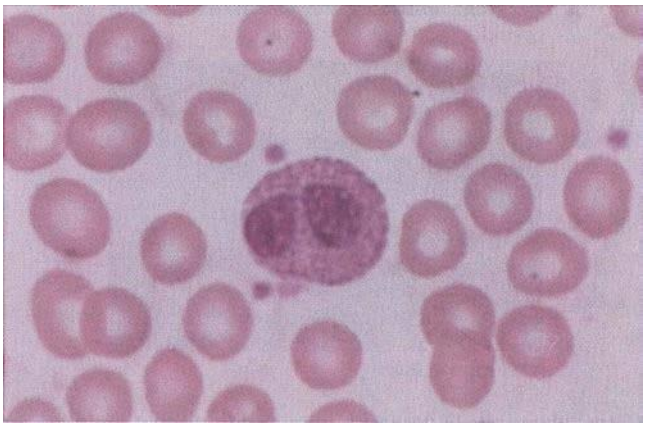


FIGURE 1–4 Eosinophil. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009. Fig. 1–6.)*

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

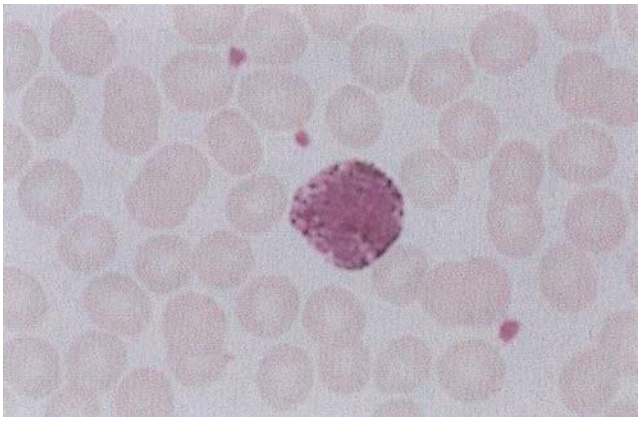


FIGURE 1–5 Basophil. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009. Fig. 1–7.)*

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]

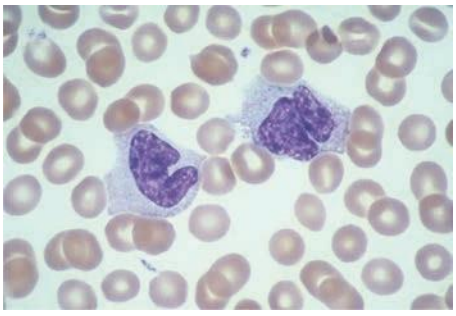


FIGURE 1–6 Two monocytes. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009. Fig. 1–13.)*

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

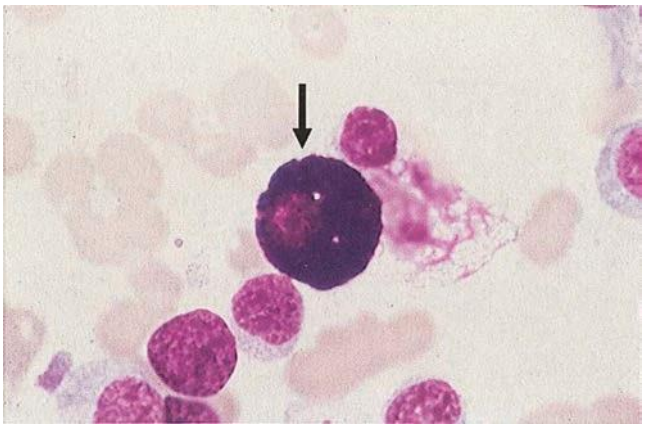


FIGURE 1–7 Mast cell. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009. Fig. 1–44.)*

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]

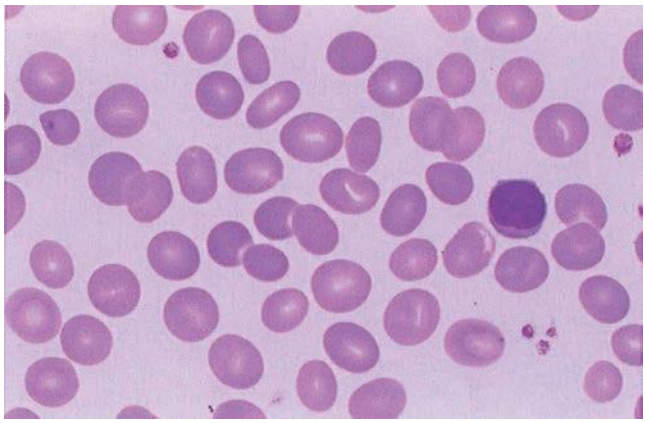


FIGURE 1–8 Typical lymphocyte found in peripheral blood. *(From Harr R.* Clinical Laboratory Science Review. *4th ed. Philadelphia, PA: F.A. Davis; 2013. Color Plate 31.)*

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	>12	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

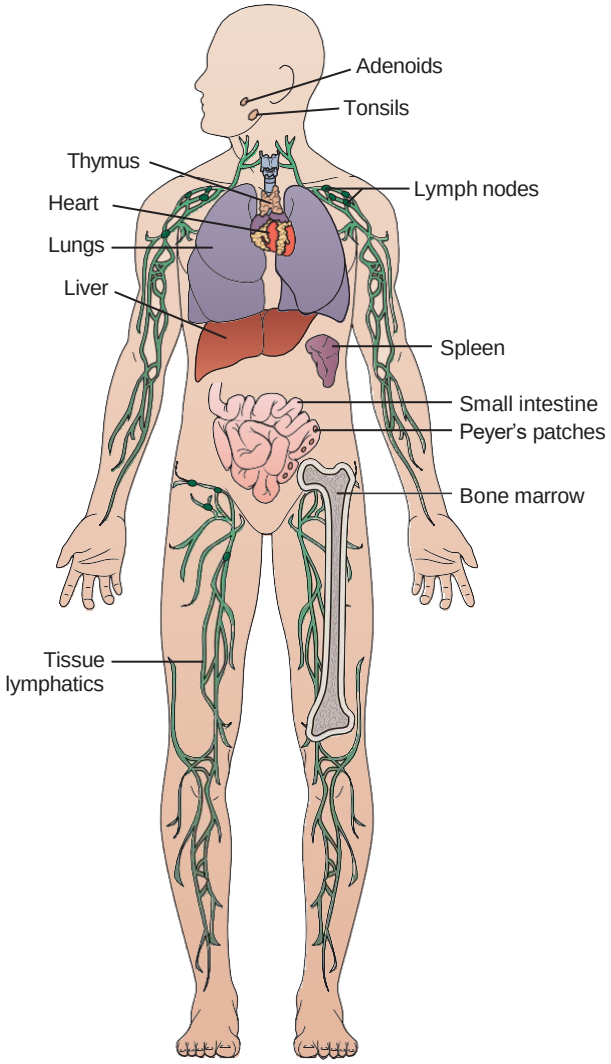


FIGURE 1–9 Sites of lymphoreticular tissue. Primary organs include the bone marrow and the thymus. Secondary organs are distributed throughout the body and include the spleen, lymph nodes, and mucosal-associated lymphoid tissue (MALT). The spleen filters antigens in the blood, whereas the lymphatic system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

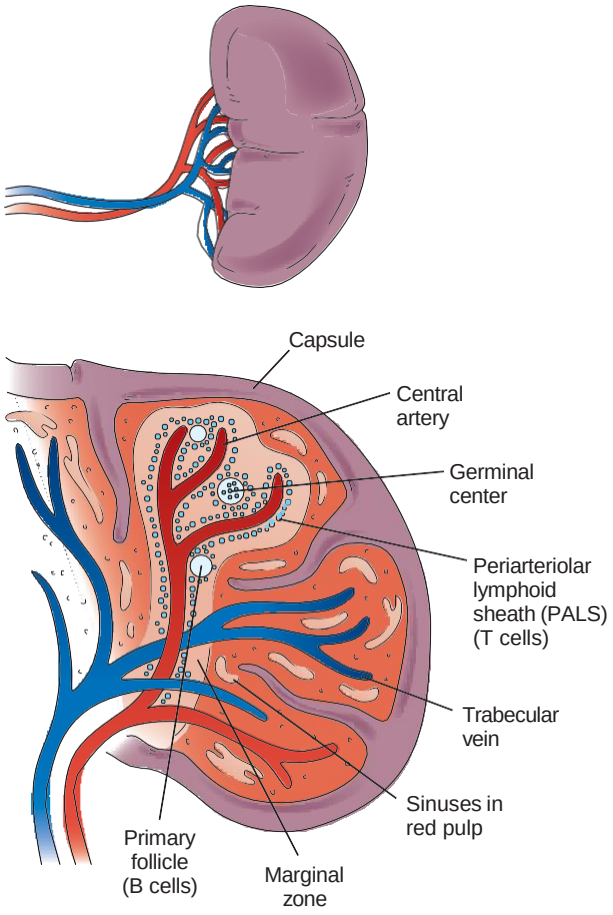


FIGURE 1–10 Cross-section of the spleen showing organization of the lymphoid tissue. T cells surround arterioles in the PALS. B cells are just beyond in follicles. When stimulated by antigens, the B cells form germinal centers. All of the lymphoid tissue is referred to as the *white pulp*.

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

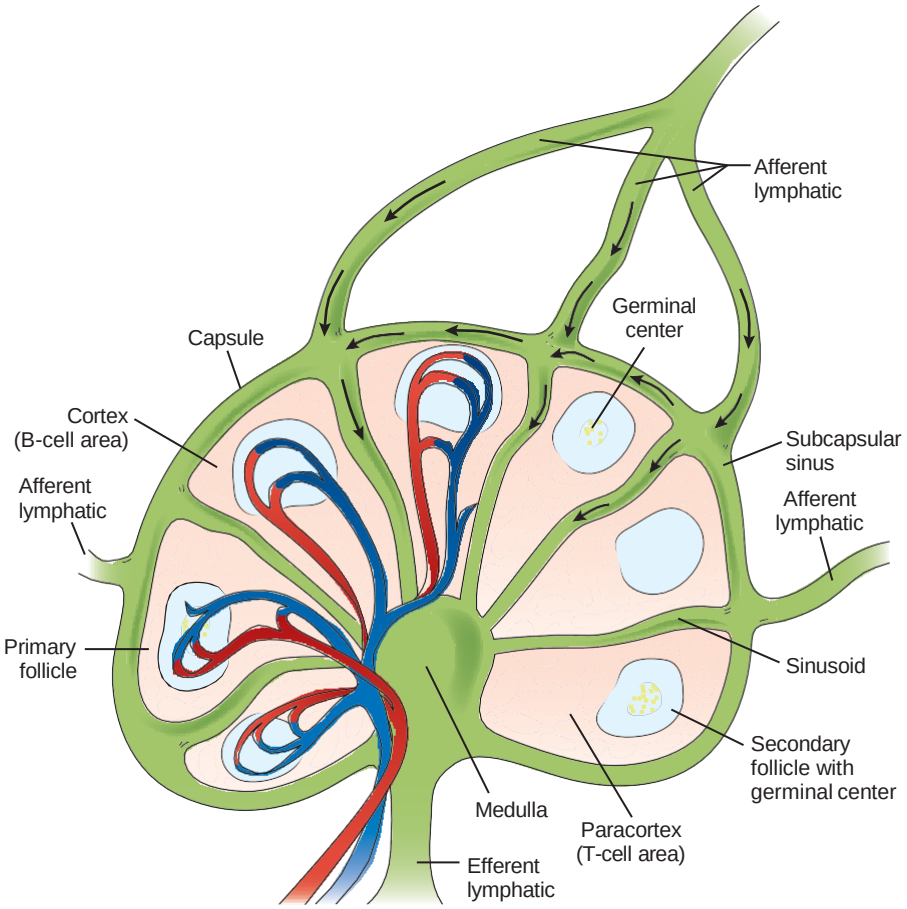


FIGURE 1–11 Structure of a lymph node. A lymph node is surrounded by a tough outer capsule. Right underneath is the subcapsular sinus, where lymph fluid drains from afferent lymphatic vessels. The outer cortex contains collections of B cells in primary follicles. When stimulated by antigens, secondary 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.

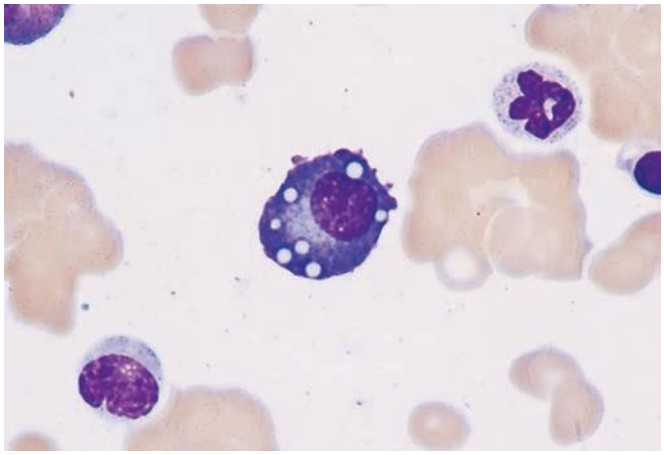


FIGURE 1–12 A typical plasma cell. *(From Harmening D.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis 2009. Fig. 1–47.)*

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).

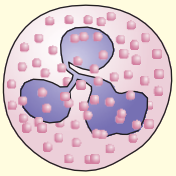
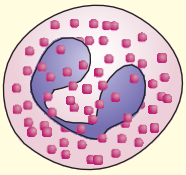
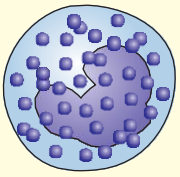
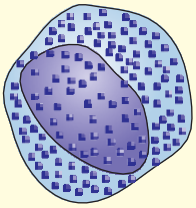
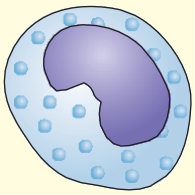
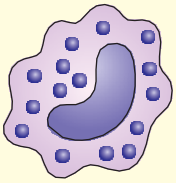
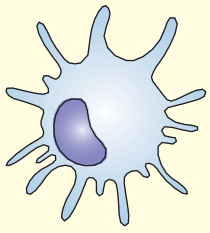
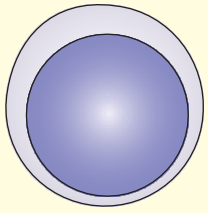
Study Guide: Comparison of T, B, and NK Cells

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 sec–ondary 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

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

FACTORS INFLUENCING THE IMMUNE RESPONSE
TRAITS OF IMMUNOGENS
EPITOPES
HAPTENS
ADJUVANTS
RELATIONSHIP OF ANTIGENS TO THE HOST
MAJOR HISTOCOMPATIBILITY COMPLEX
- Genes Coding for MHC Molecules (HLA Antigens)
- Structure of Class I and II MHC Molecules
- Role of Class I and II Molecules in the Immune Response
- Clinical Significance of MHC

SUMMARY
CASE STUDY
REVIEW QUESTIONS

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.

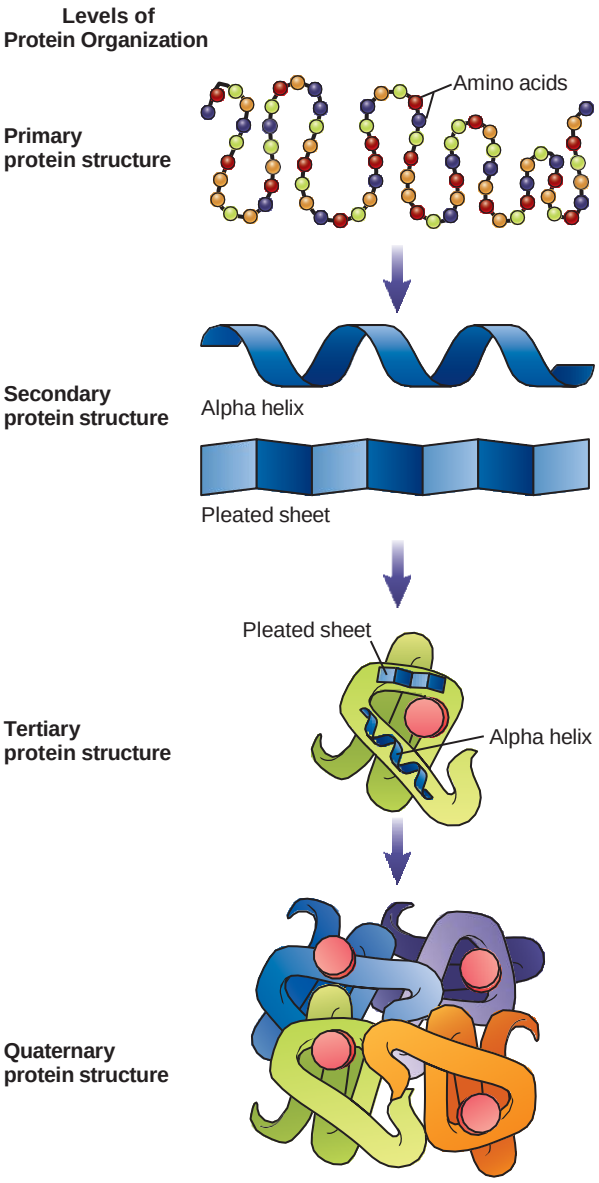


FIGURE 2–1 Levels of protein organization.

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

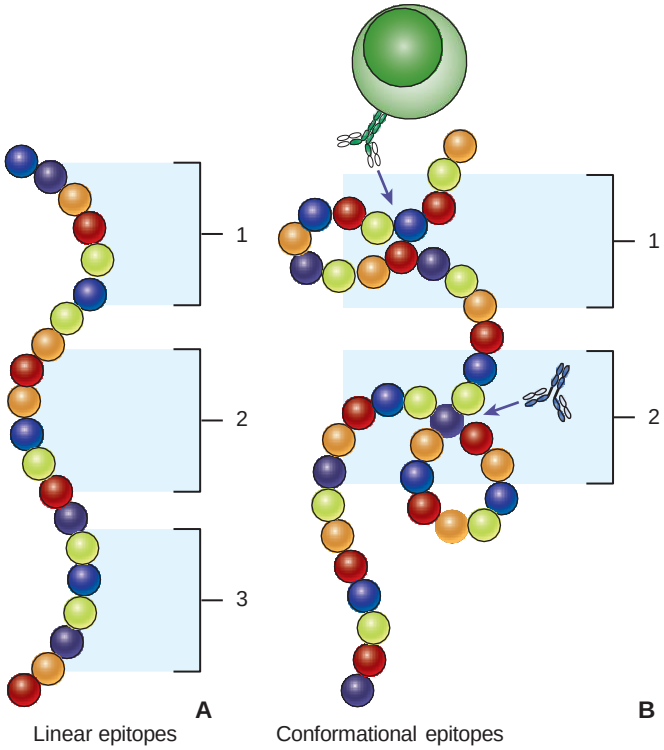


FIGURE 2–3 Linear versus conformational epitopes. (A) Linear epitopes consist of sequential amino acids on a single polypeptide chain. There may be several different types on one chain. (B) Conformational epitopes result from the folding of a polypeptide chain or chains, and nonsequential amino acids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

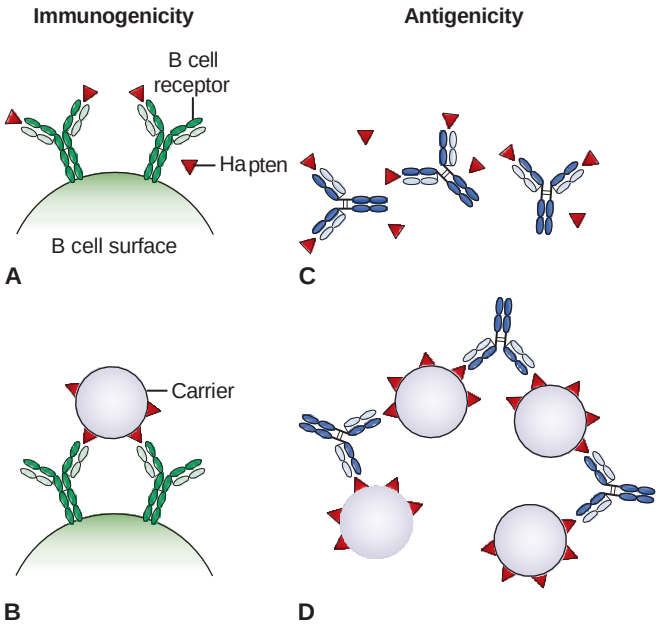


FIGURE 2–4 Characteristics of haptens. (A) Haptens bind B-cell receptors, but the receptors remain independent: no activation (not immunogenic). (B) Hapten/carrier complex cross-links receptors: B cells are activated and begin producing antibodies (immunogenic). (C) Although haptens can bind to the antibody 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

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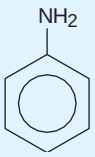
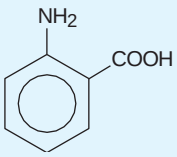
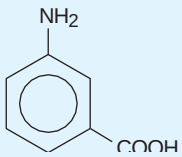
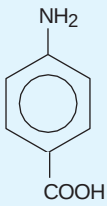
				
Antiserum against	Aminobenzene (aniline)	*o*-Aminobenzoic acid	*m*-Aminobenzoic acid	*p*-Aminobenzoic acid
Aminobenzene	+ + +	0	0	0
o-Aminobenzoic acid	0	+ + +	0	0
m-Aminobenzoic acid	0	0	+ + + +	0
p-Aminobenzoic acid	0	0	0	+ + +/+ + + +

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

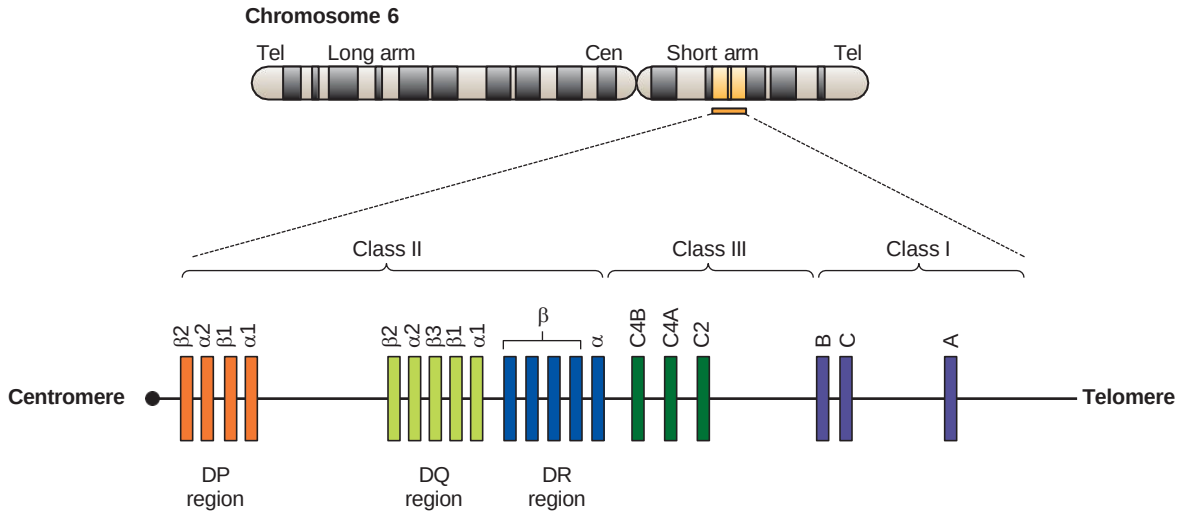


FIGURE 2–6 The major histocompatibility complex. Location of the class I, II, and III genes on chromosome 6. Class I consists of loci A, B, and C, whereas class II has at least three loci: DR, DQ, and DP.

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

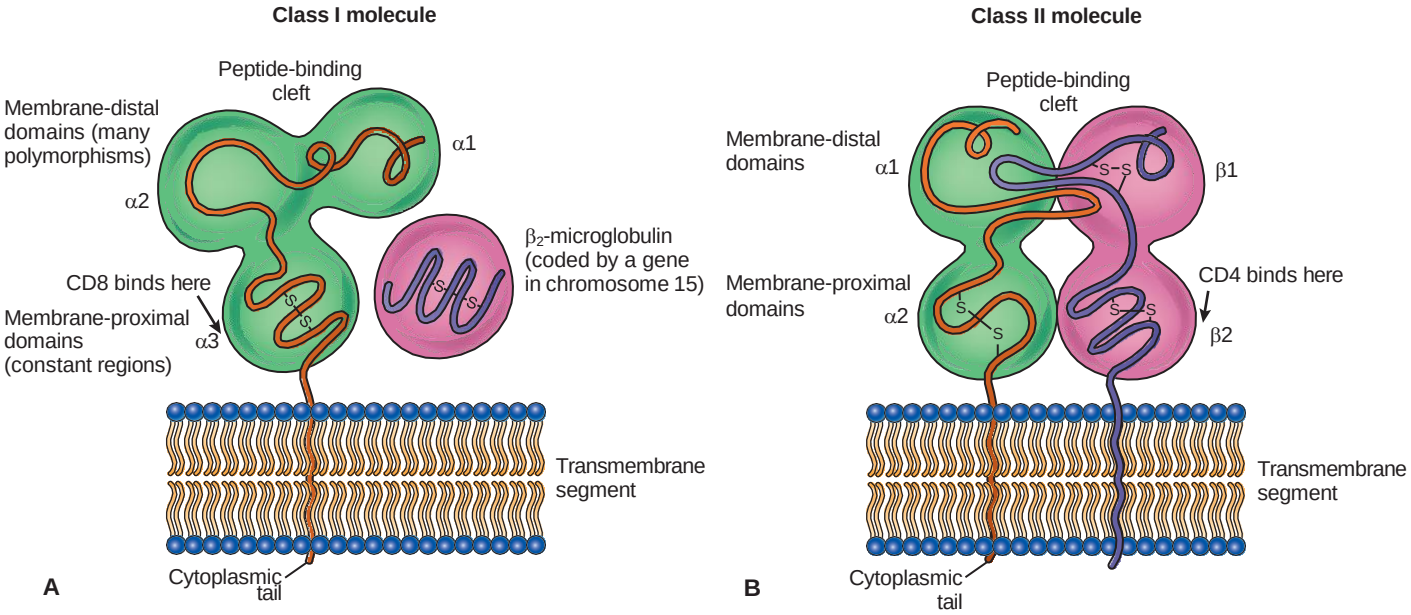


FIGURE 2–7 Structure of (A) class I and (B) class II MHC products. Class I MHC molecules consist of an α chain with three domains and a shorter chain, β2–microglobulin, common to all class I molecules. Class II MHC molecules consist of an α chain and a β chain, each of which has two domains.

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

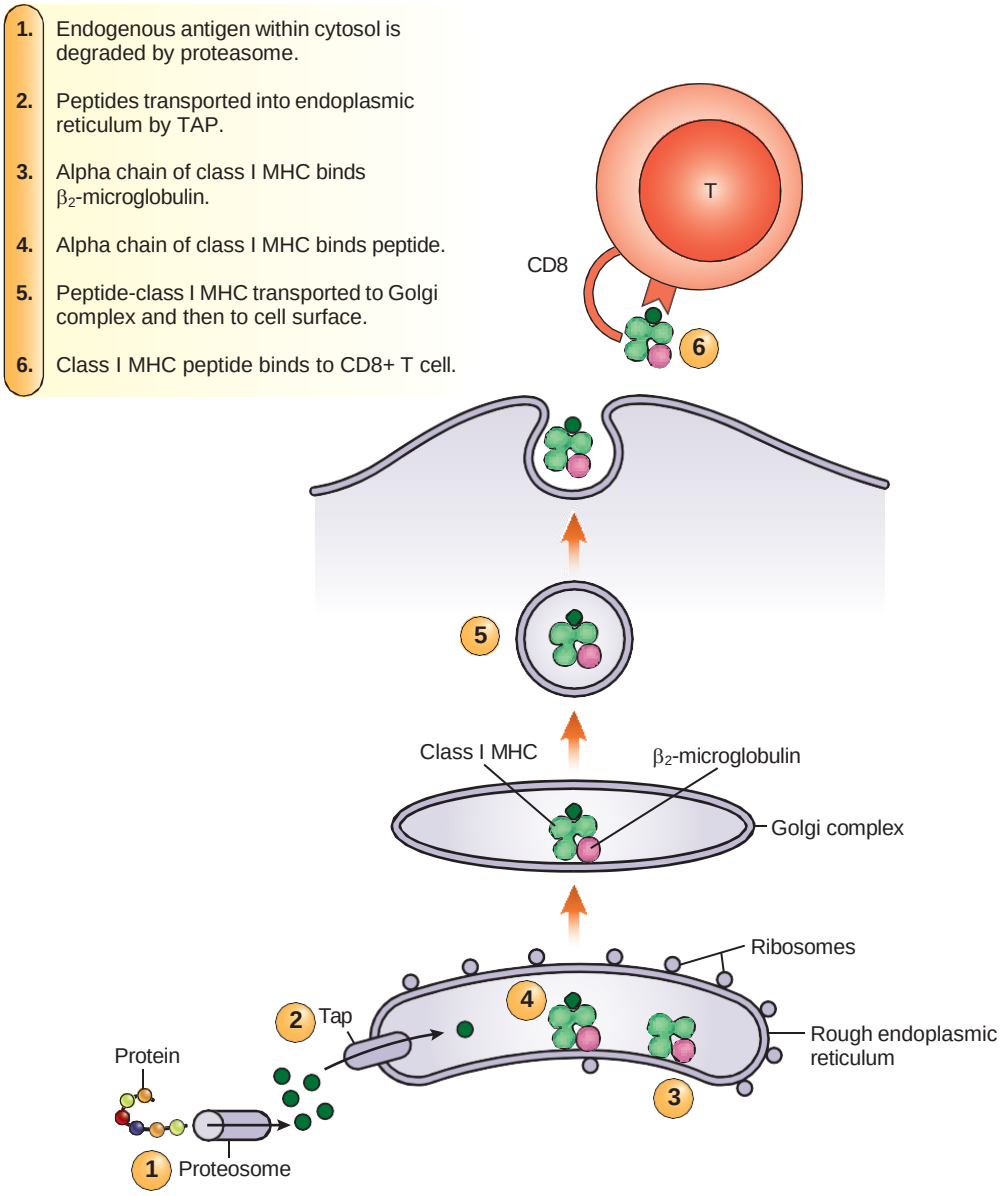


FIGURE 2–8 Antigen-processing pathway for endogenous antigens.

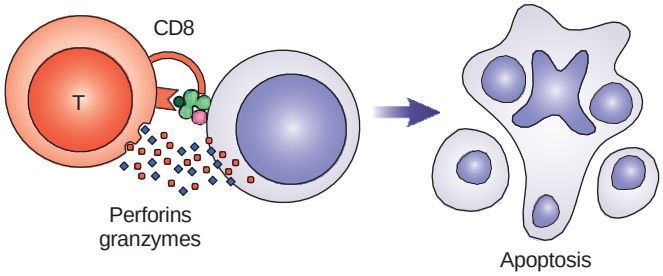


FIGURE 2–9 The CD8+ T cell recognizes antigen in association with class I MHC. If the antigen is recognized as being foreign, cytokines are released, causing destruction of the target cell.

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

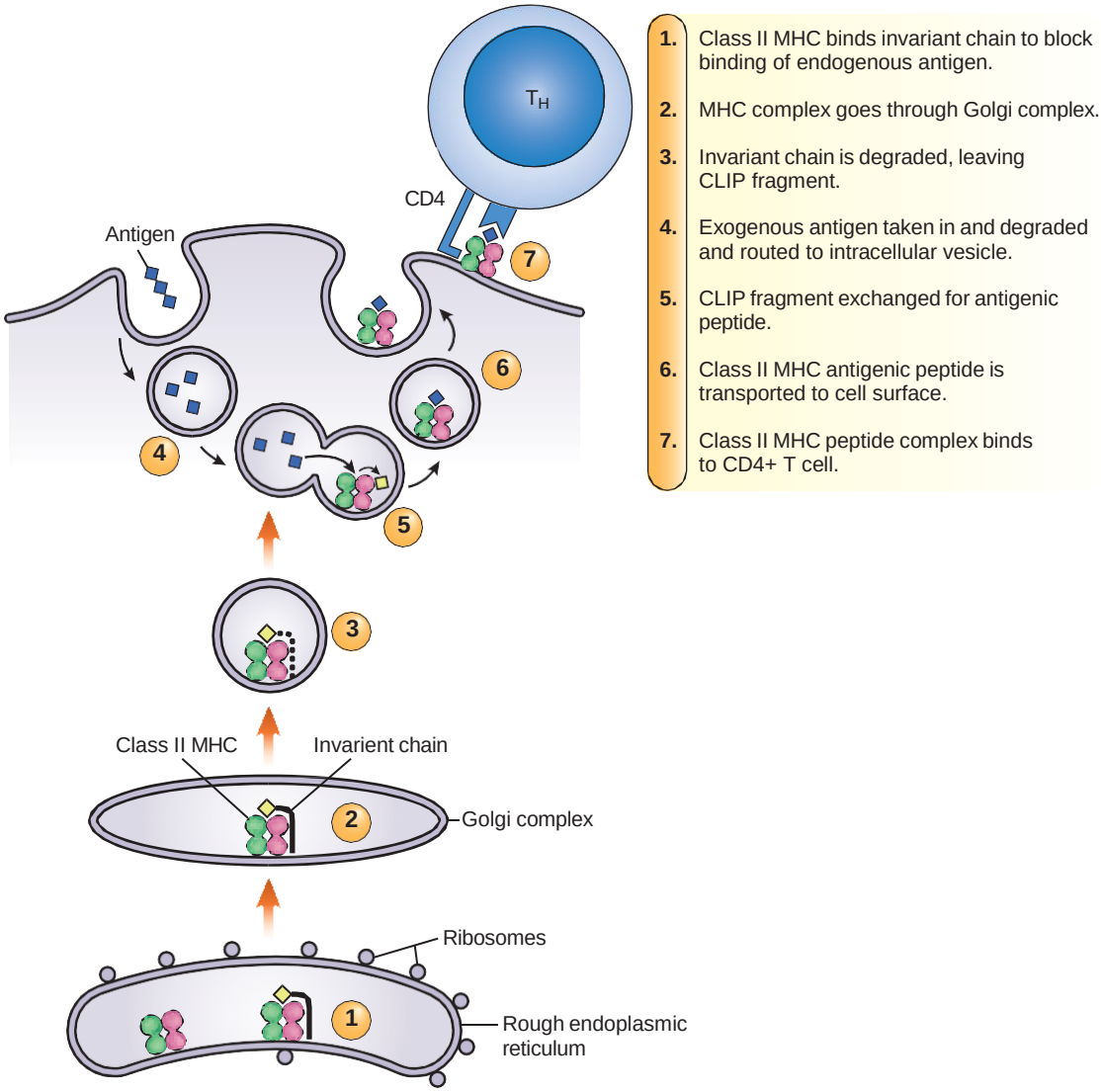


FIGURE 2–10 Antigen-processing pathway for exogenous antigen. The binding site of class II MHC molecules is first occupied by an invariant chain (Ii). This is degraded and exchanged for short exogenous peptides in an endosomal compartment. The exogenous peptide class II MHC complex is then transported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

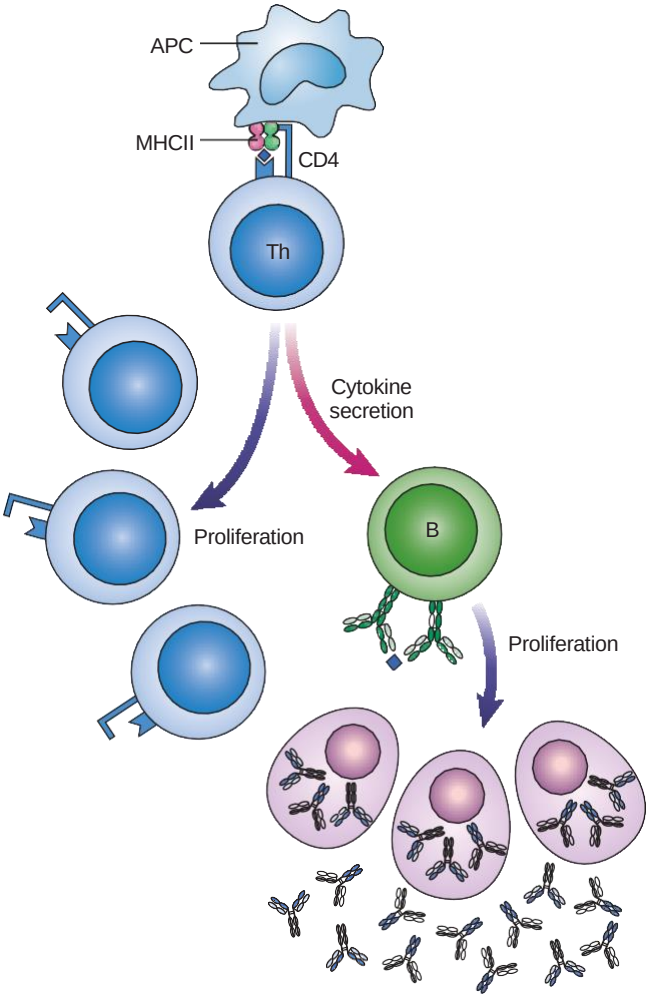


FIGURE 2–11 CD4+ T cells recognize exogenous antigen on phagocytic antigen-presenting cells along with class II MHC. CD4+ helper T cells are stimulated by contact with antigen and clonal expansion takes place. These cells secrete cytokines that cause an antigen-activated B cell to proliferate and produce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

EXTERNAL DEFENSE SYSTEM
INTERNAL DEFENSE SYSTEM
- Pathogen Recognition Receptors
- Acute-Phase Reactants
- Inflammation
- Phagocytosis
- Action of Natural Killer Cells

SUMMARY
CASE STUDIES
REVIEW QUESTIONS

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

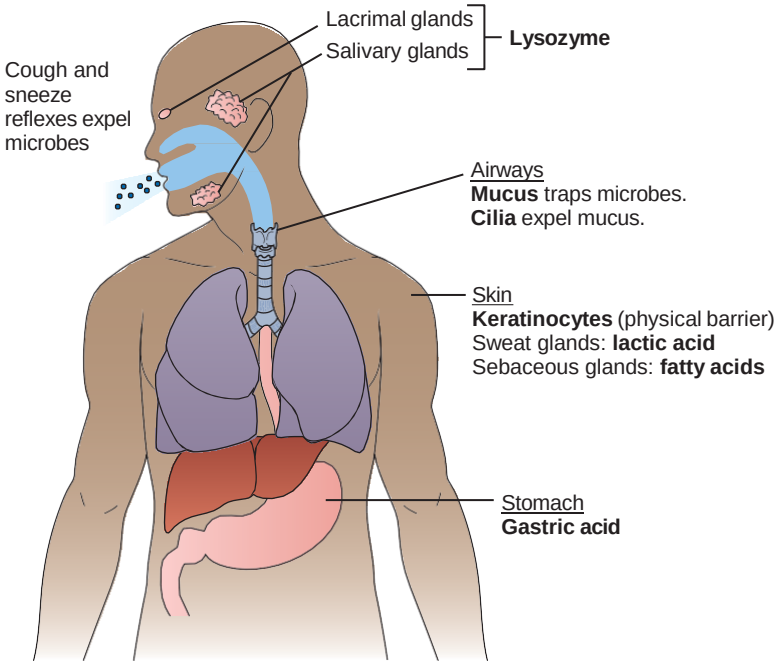


FIGURE 3–1 The external defense system.

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

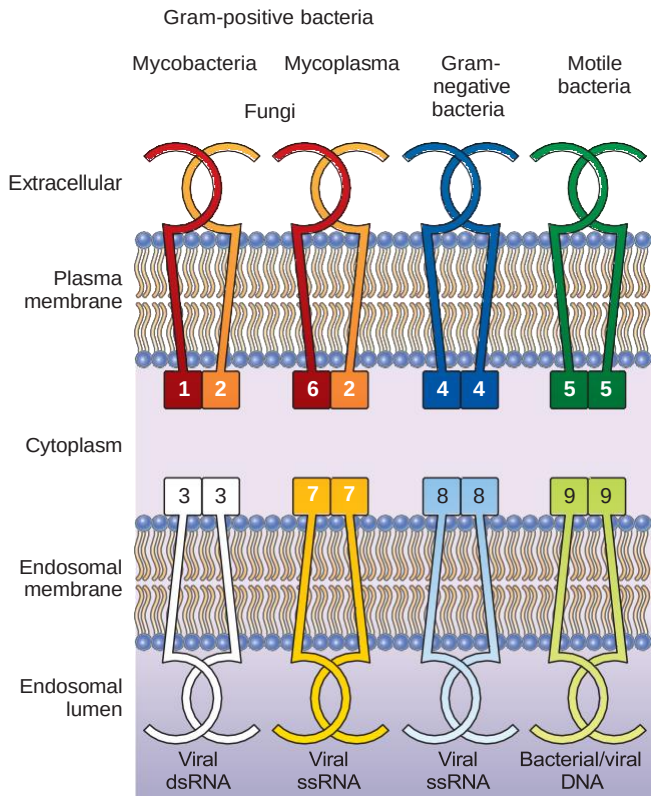


FIGURE 3–2 Toll-like receptors on a WBC membrane. Each of the 10 different TLRs recognizes a different pathogenic product. TLRs found on the cell surface tend to form dimers to increase chances of binding to a foreign substance.

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

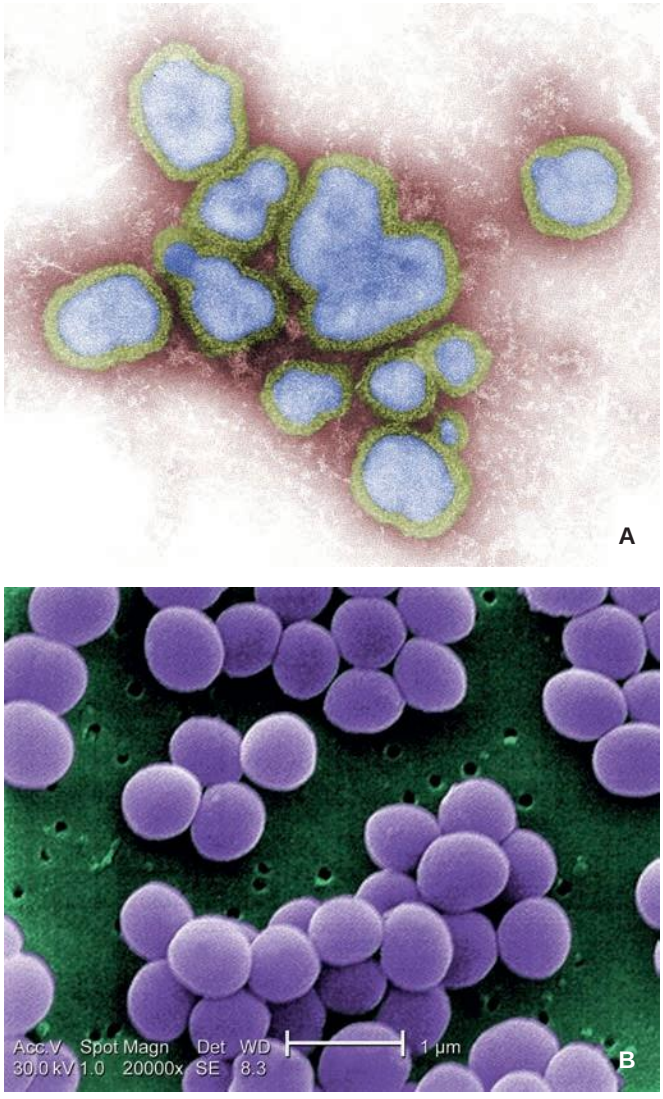


FIGURE 3–3 A. Digitally colored and negative stained transmission electron micrograph of the influenza A virus, an RNA virus that TLR3, TLR7, and TLR8 recognize as foreign. B. Scanning electron micrograph of *Staphylococcus aureus;* gram-positive bacteria that are recognized by the TLR2 receptor. *(A. 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

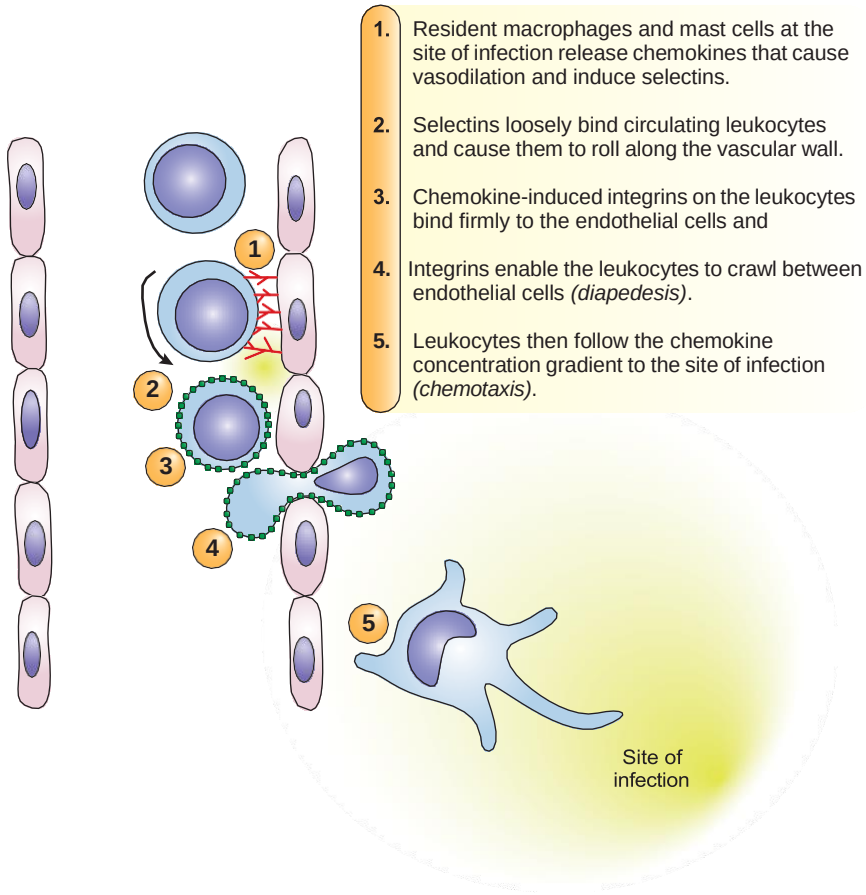


FIGURE 3–4 Local inflammatory events.

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

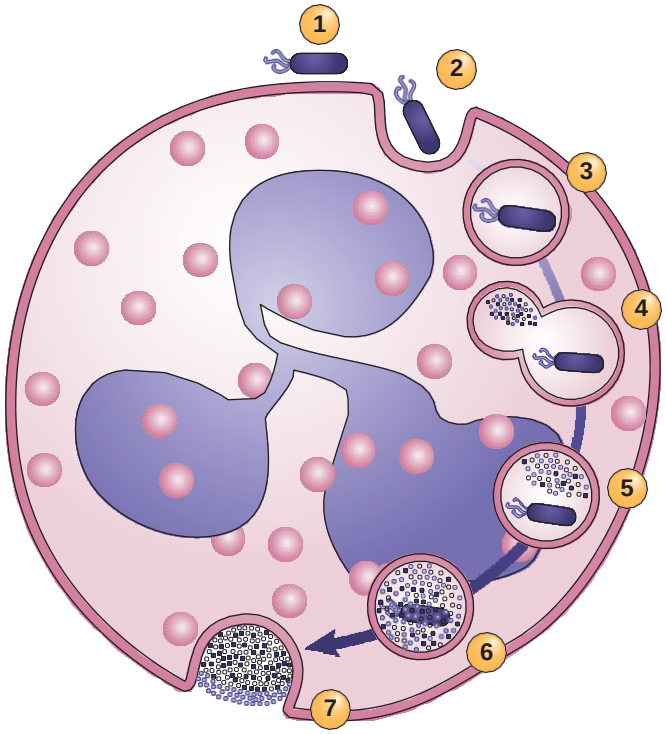
Once the WBCs are attracted to the area, the actual process of phagocytosis consists of seven main steps **(Fig. 3–5):**

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

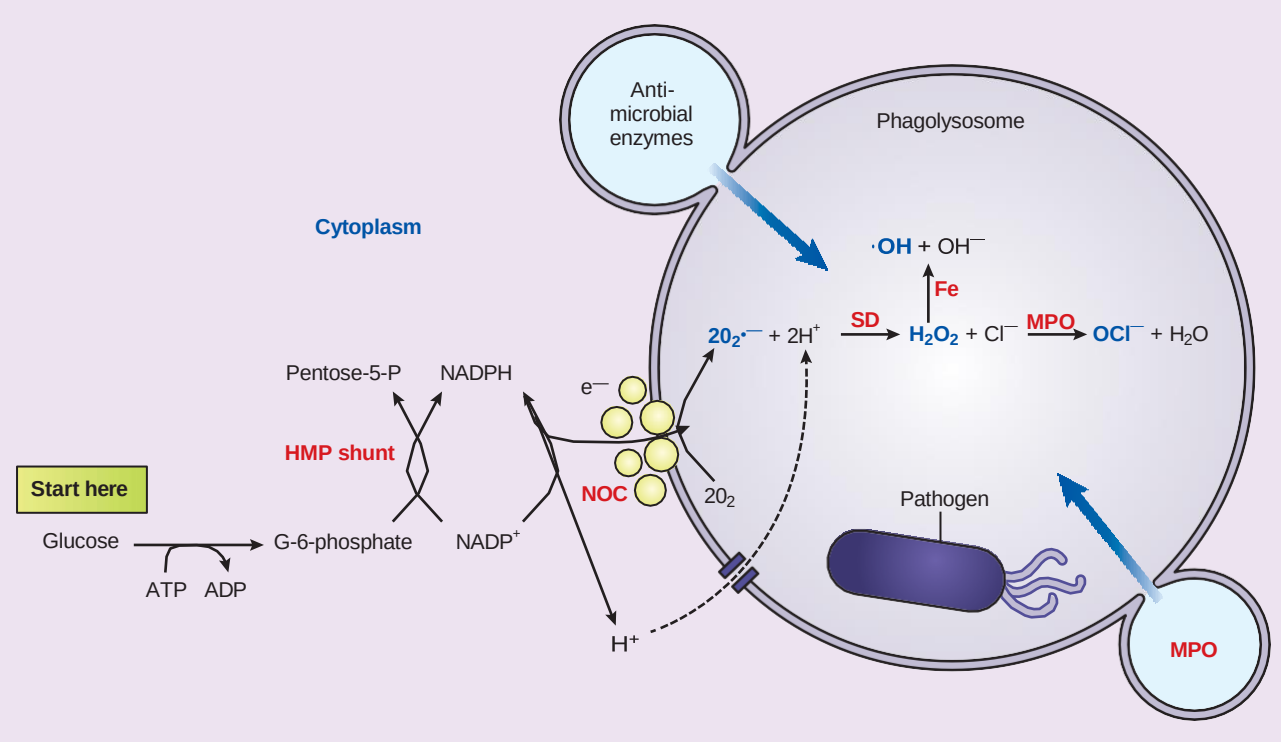


FIGURE 3–6 Creation of oxygen rad icals in the phagocytic cell. The hexose monophosphate (HMP) shunt reduces NADP to NADPH. NADPH reduces oxygen to superoxide ($2O_2^{\bullet-}$) when the NADPH oxidase complex (NOC) is assembled in the membrane of the phagolysosome. Superoxide dismutase (S OD) catalyzes 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

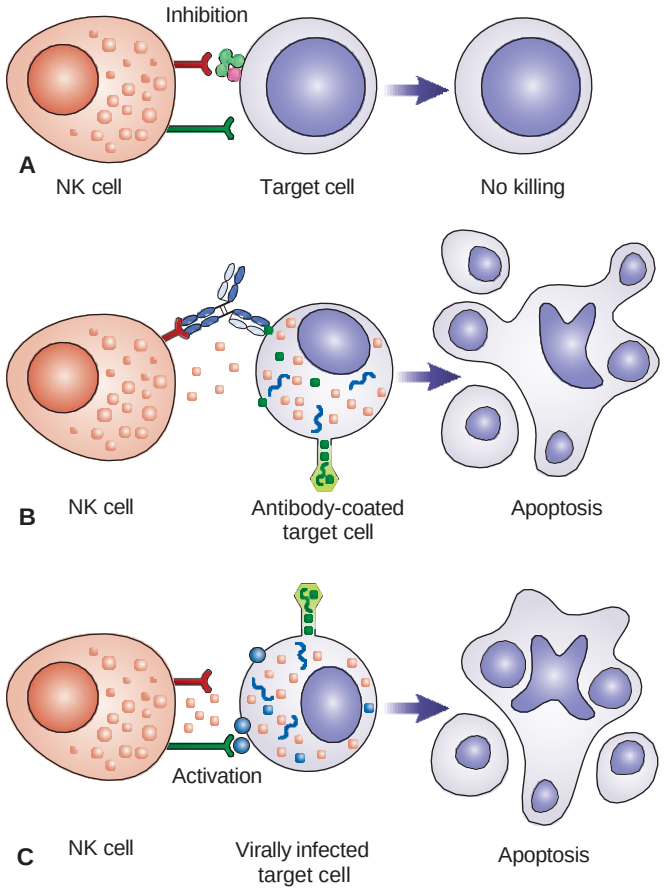


FIGURE 3–7 Actions of NK cells. NK cells are constantly surveying cells. (A) If class I MHC protein is present and there are no foreign or stress proteins, then an inhibitory signal is sent to the NK cell, no killing occurs, and the normal cell is released. (B) Alternatively, infected cells may express 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

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

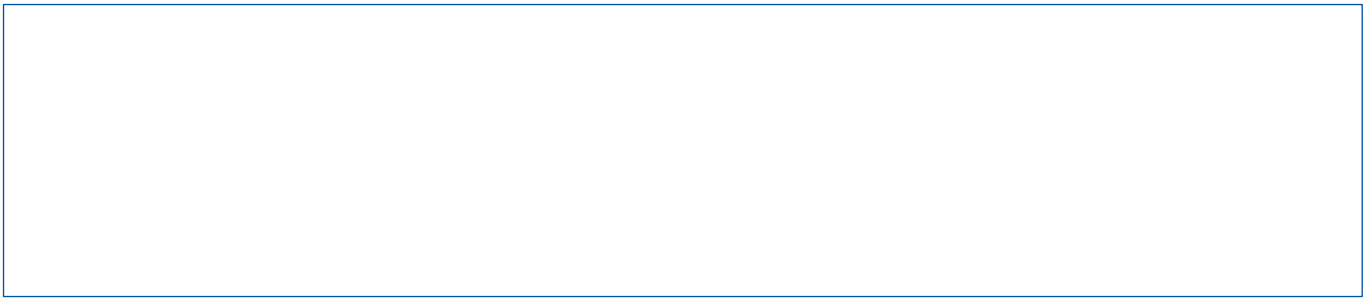
10. Which is the most significant agent formed in the phagolysosome for the elimination of microorganisms?
- **a.** Proteolytic enzymes
- **b.** Hydrogen ions
- **c.** Hypochlorite ions
- **d.** Superoxides

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.



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

T□CELL DIFFERENTIATION
- Double-Negative Stage
- Double-Positive Stage
- Mature T Cells

STAGES IN B□CELL DIFFERENTIATION
- Pro-B Cells
- Pre-B Cells
- Immature B Cells
- Mature B Cells
- Plasma Cells

THE ROLE OF T CELLS IN THE ADAPTIVE IMMUNE RESPONSE
- Action of T Helper Cells
- Action of Cytotoxic T Cells

THE ROLE OF B CELLS IN THE ADAPTIVE IMMUNE RESPONSE
- Response to T-Dependent Antigens
- Response to T-Independent Antigens

LABORATORY IDENTIFICATION OF LYMPHOCYTES

SUMMARY

CASE STUDIES

REVIEW QUESTIONS

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

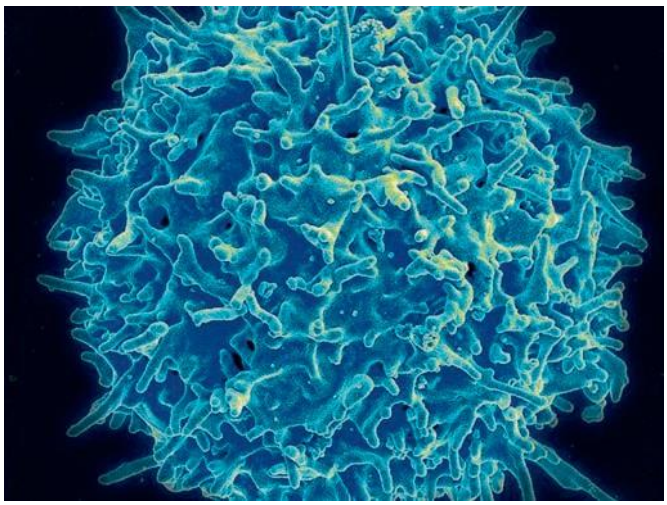


FIGURE 4–1 Scanning electron micrograph of a typical T cell. *(Courtesy of National Institute of Allergy and Infectious Diseases [(NIAID].)*

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

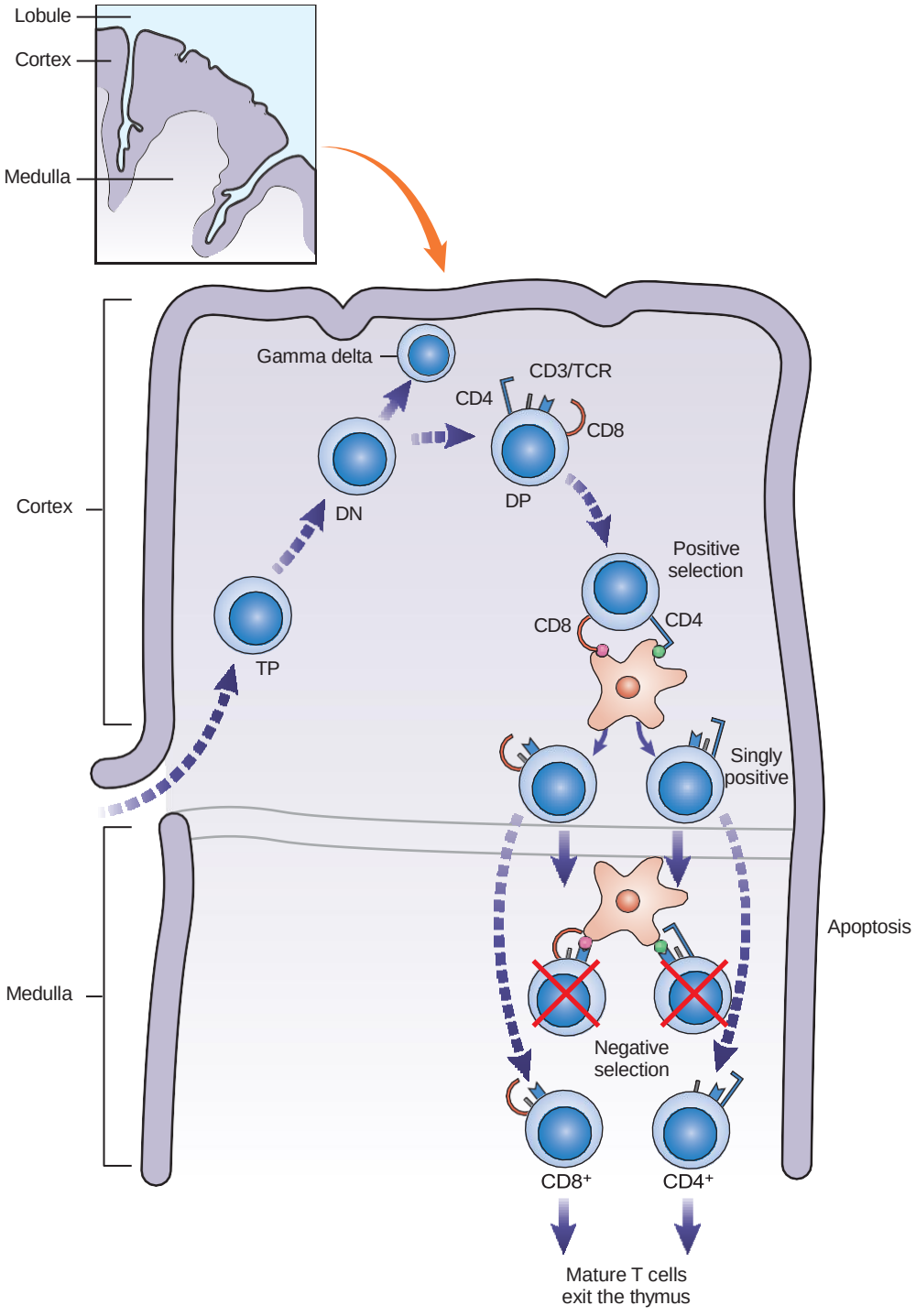


FIGURE 4–2 T-cell maturation in the thymus. T-lymphocyte precursors (TP) enter the thymus at the corticomedullary junction. They migrate upward in the cortex and begin development of the T-cell receptor. A small percentage of precursors develop gamma-delta chains, whereas the majority develop alpha-beta 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.

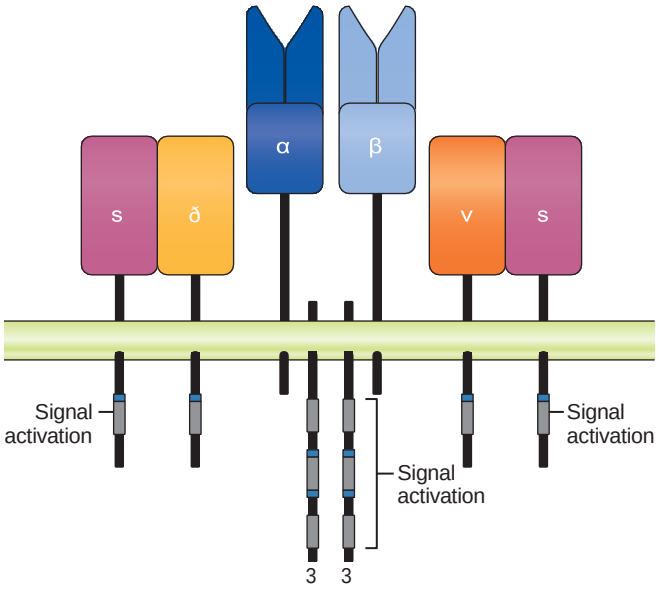


FIGURE 4–3 The CD3:T-cell receptor complex. The TCR that recognizes antigen consists of two chains, α and β, which have constant and variable regions. Four other types of chains are collectively known as CD3. These are ε, γ, δ, and ζ. They take part in signaling to the interior of the cell when antigen 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,

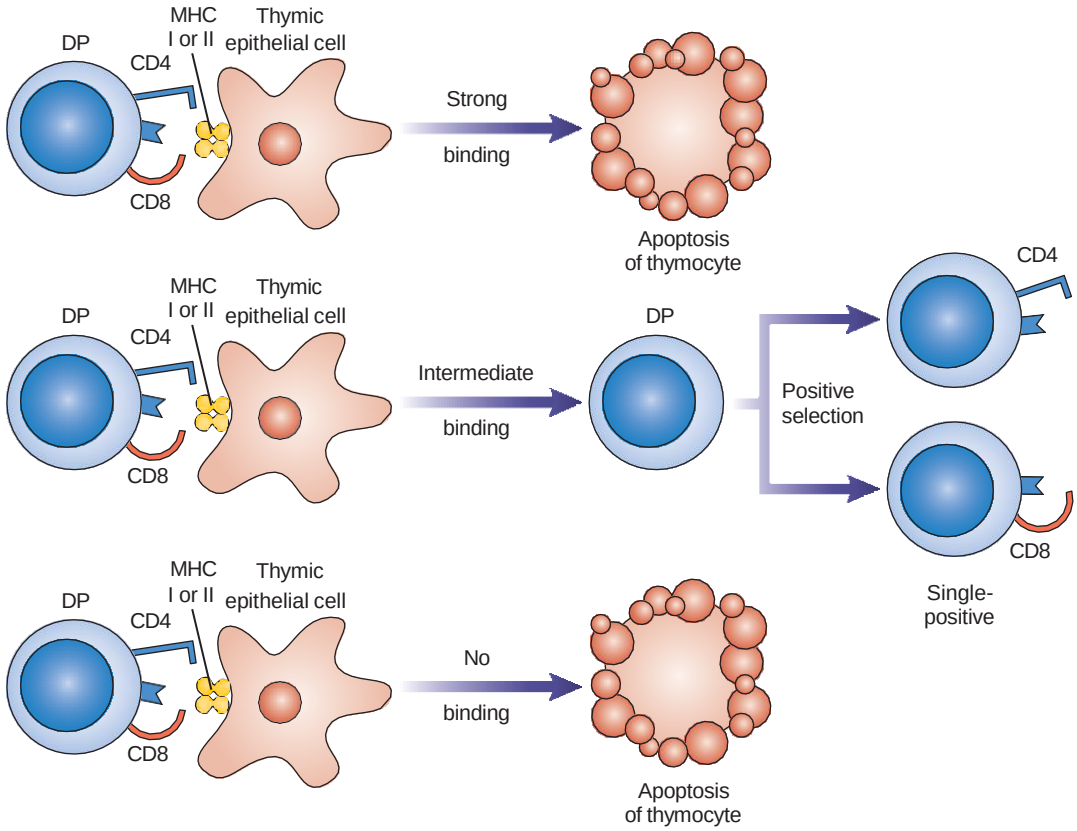


FIGURE 4–4 Positive selection of thymocytes in the cortex. Double-positive (CD4+ and CD8+) thymocytes interact with thymic epithelial cells. If very strong bonding occurs, cells are eliminated by apoptosis. If very weak or no bonding occurs, cells are also eliminated.

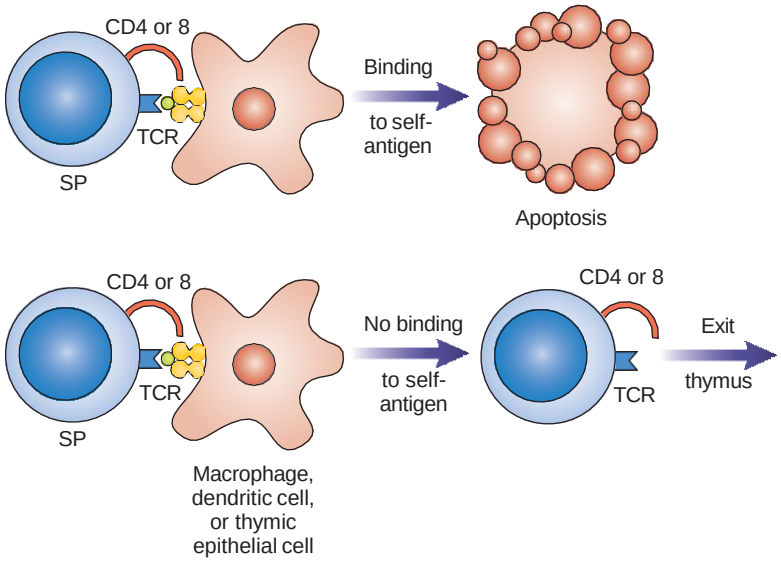


FIGURE 4–5 Negative selection of thymocytes in the medulla. When self-antigen is presented by a macrophage, dendritic cell, or thymic epithelial cell to a thymocyte, if the T-cell receptor (TCR) binds, the thymocyte is eliminated by apoptosis.

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

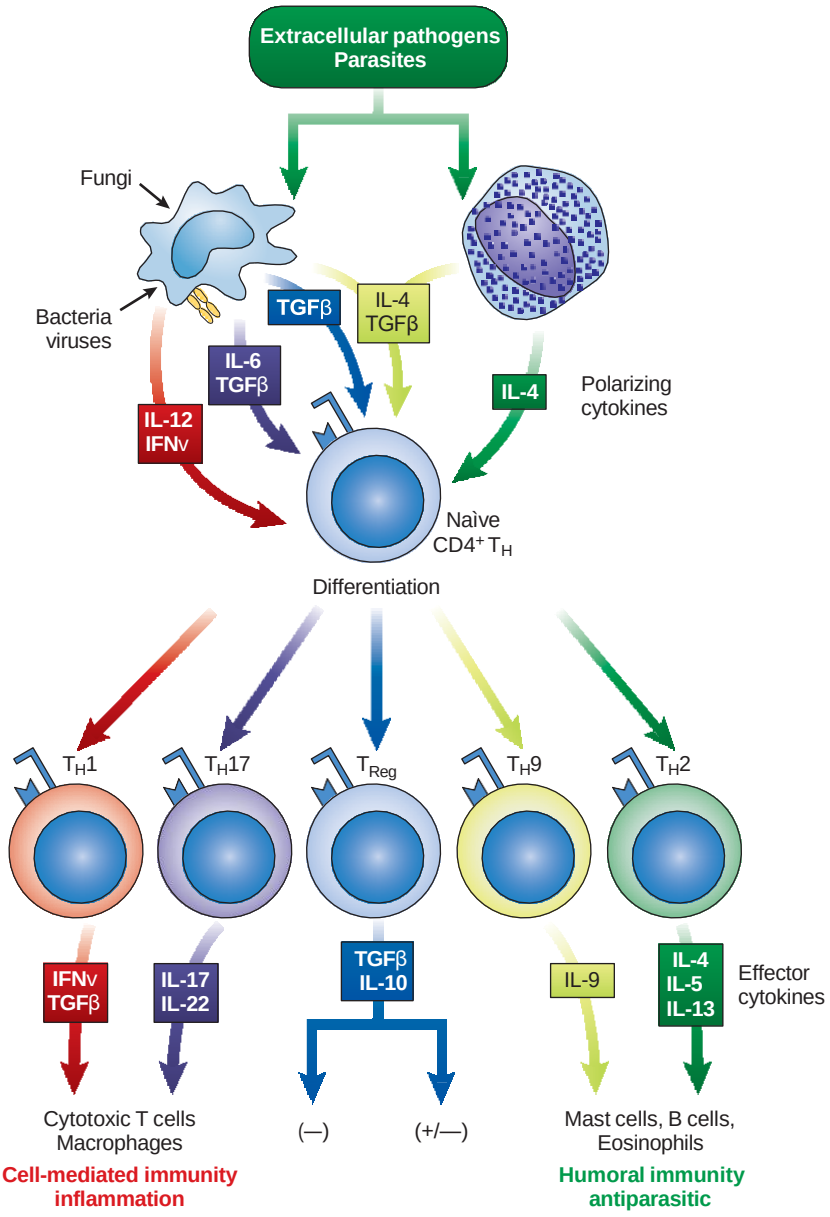


FIGURE 4–6 T helper (Th) subsets. Depending upon the type of pathogen encountered, antigen-presenting cells (APCs) secrete a specific combination of polarizing cytokines that direct naïve CD4+ Th cells to further differentiate into one of five subsets: Th1, Th2, Treg, Th9, or Th17. These specialized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

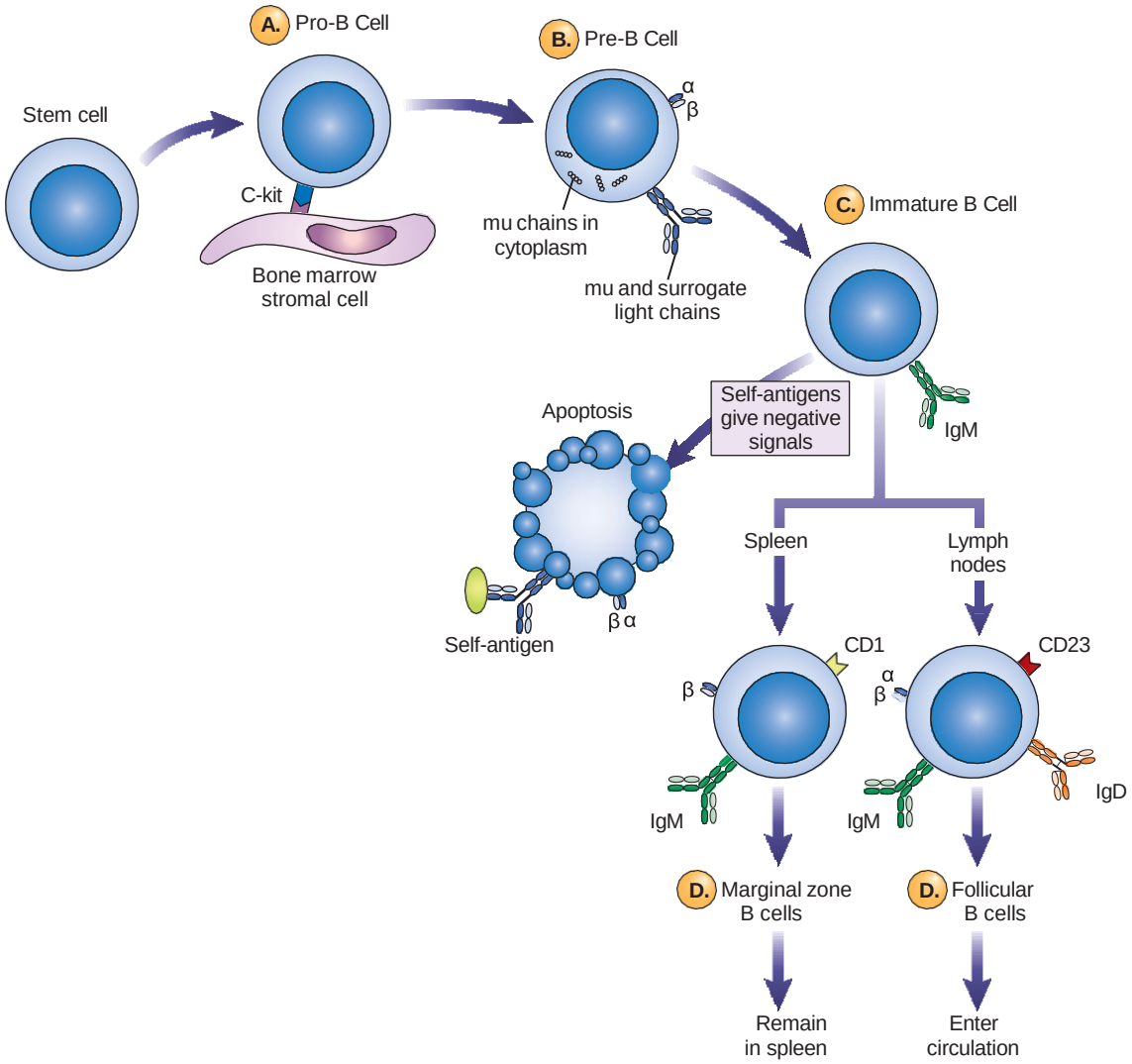


FIGURE 4–7 B-cell development in the bone marrow. Selected markers are shown for the various stages in the differentiation of B cells. Stages up to the formation of mature B cells occur in the bone marrow. (A) Pro-B cell. (B) Pre-B cell. (C) Immature B cell. (D) Mature B cell.

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

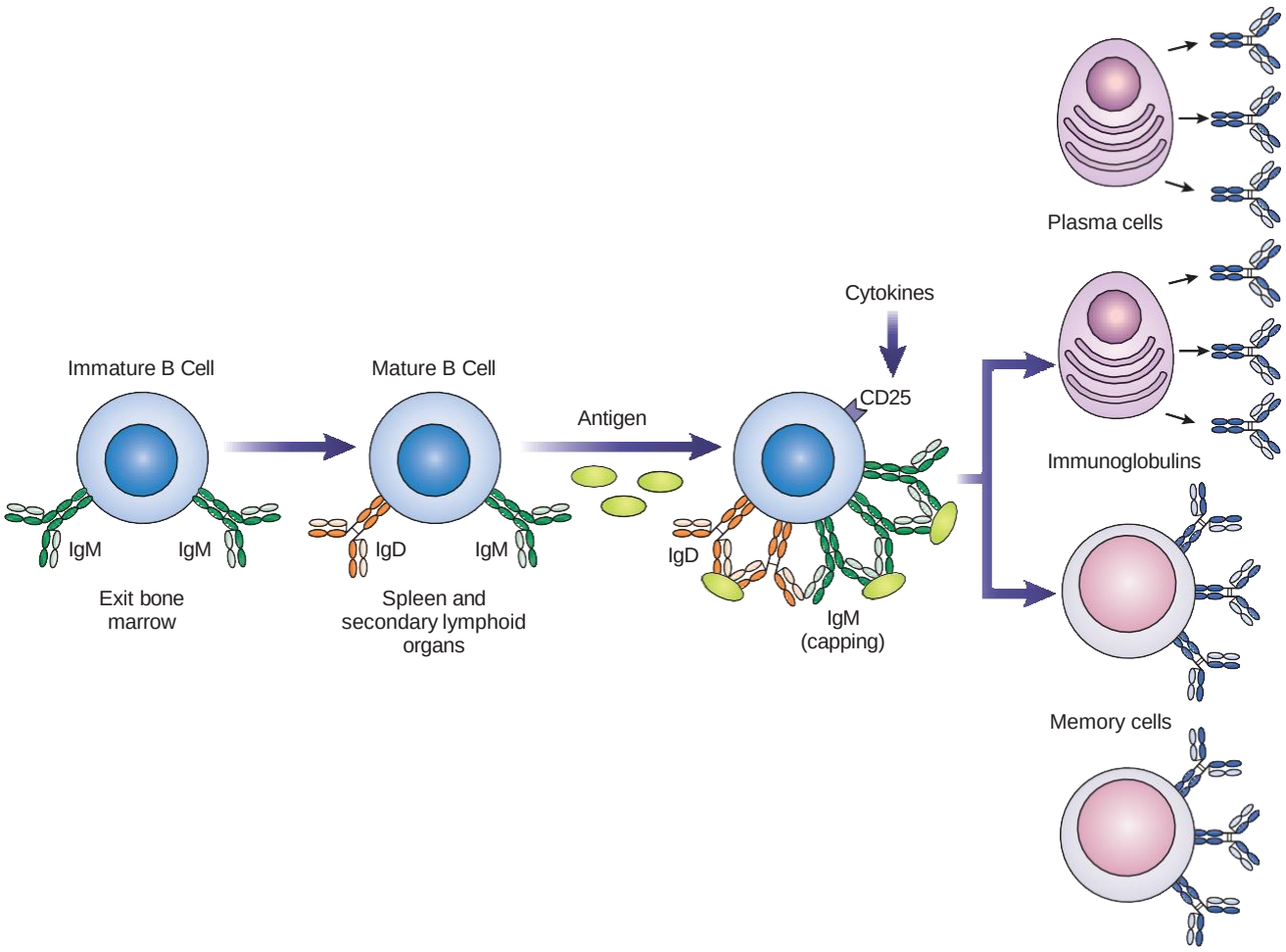


FIGURE 4–8 B-cell activation in peripheral lymph nodes. B cells capture specific antigen by means of immunoglobulin receptors. The activity of cytokines produced by Th cells produces transformation of naïve B cells into antibody-producing plasma cells and memory cells.

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]

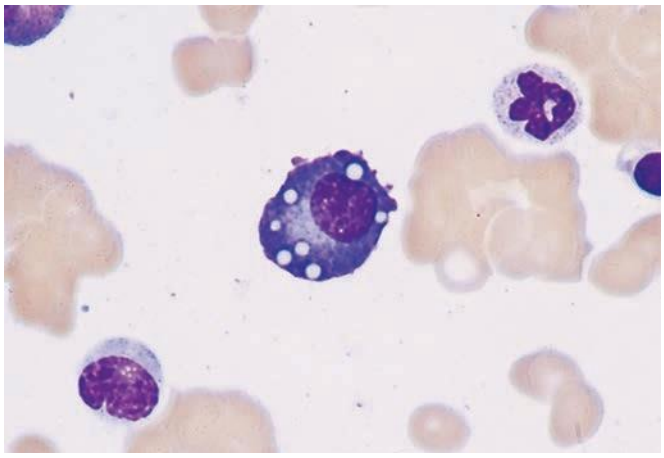


FIGURE 4–9 A typical plasma cell. *(From Harmening DM.* Clinical Hematology and Fundamentals of Hemostasis. *5th ed. Philadelphia, PA: F.A. Davis; 2009.)*

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-ß, 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]

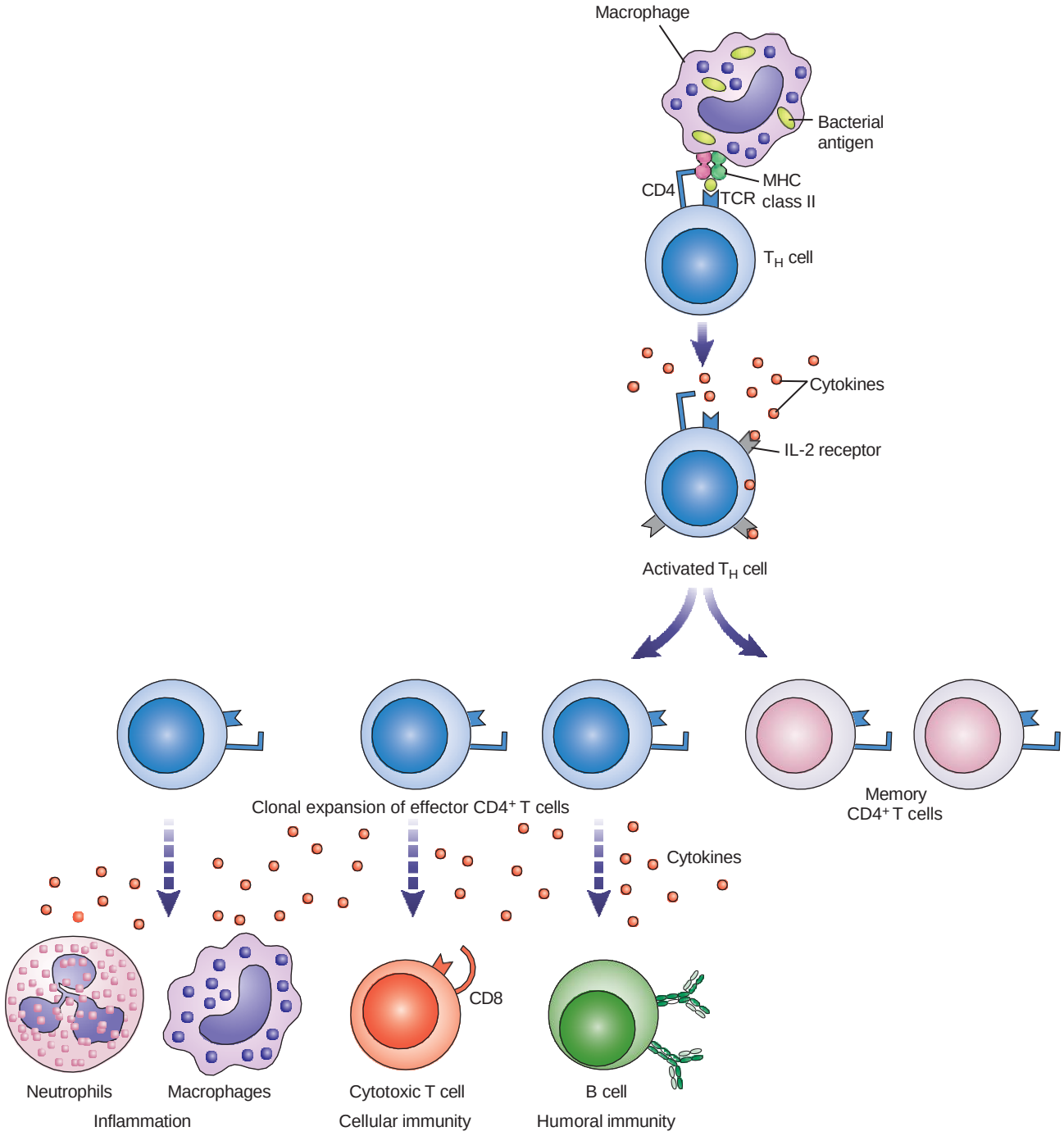


FIGURE 4–10 Activation of Th cells. Exposure to antigen presented by macrophages causes production of CD25 receptors for interleukin-2 (IL-2). IL-2 causes sensitized CD4+ T cells to secrete cytokines, resulting in CD4+ effector cells that have various functions. Some CD4+ cells secrete interleukins that 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.

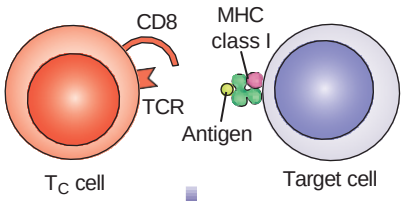
Response to T-Dependent Antigens

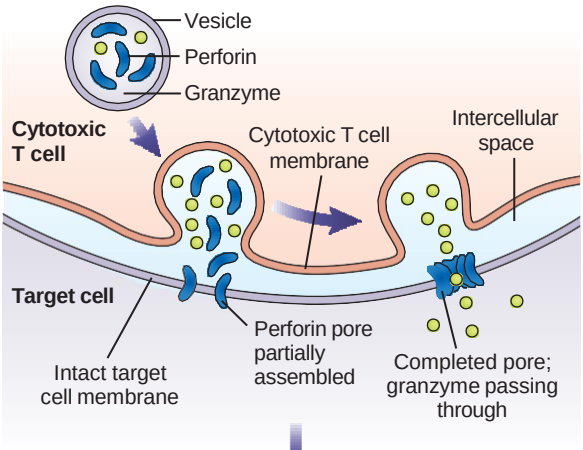
When B cells respond to T-dependent antigens, the B cells require two signals to be activated. The first occurs when antigen binds to membrane immunoglobulin receptors on the B-cell surface and cross-links them. Cross-linking leads to activation of intracellular signaling pathways that allow stimulated 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).**

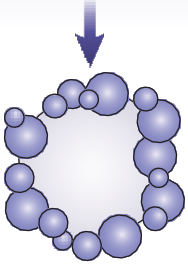
Connections

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.







Target cell death

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.

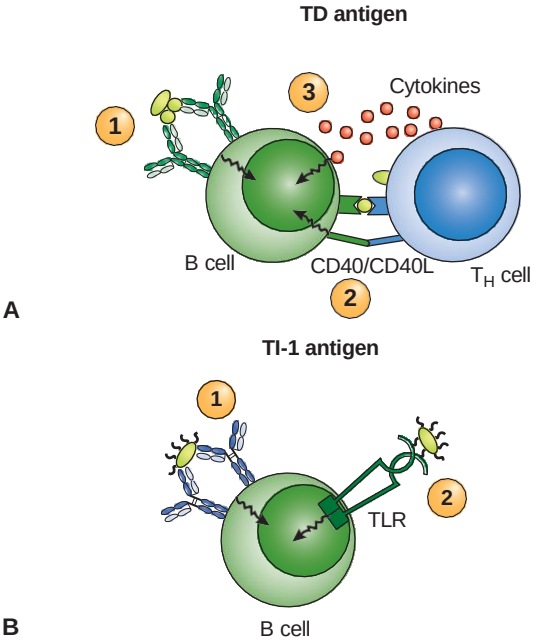


FIGURE 4–12 Activating signals for T-dependent and T-independent antigens. (A) T-dependent antigens bind to immunoglobulin receptors on B cells. The antigen is processed and delivered to CD4+ T cells. The Th cell binds by means of its CD3-TCR complex and delivers further activating signals through binding 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

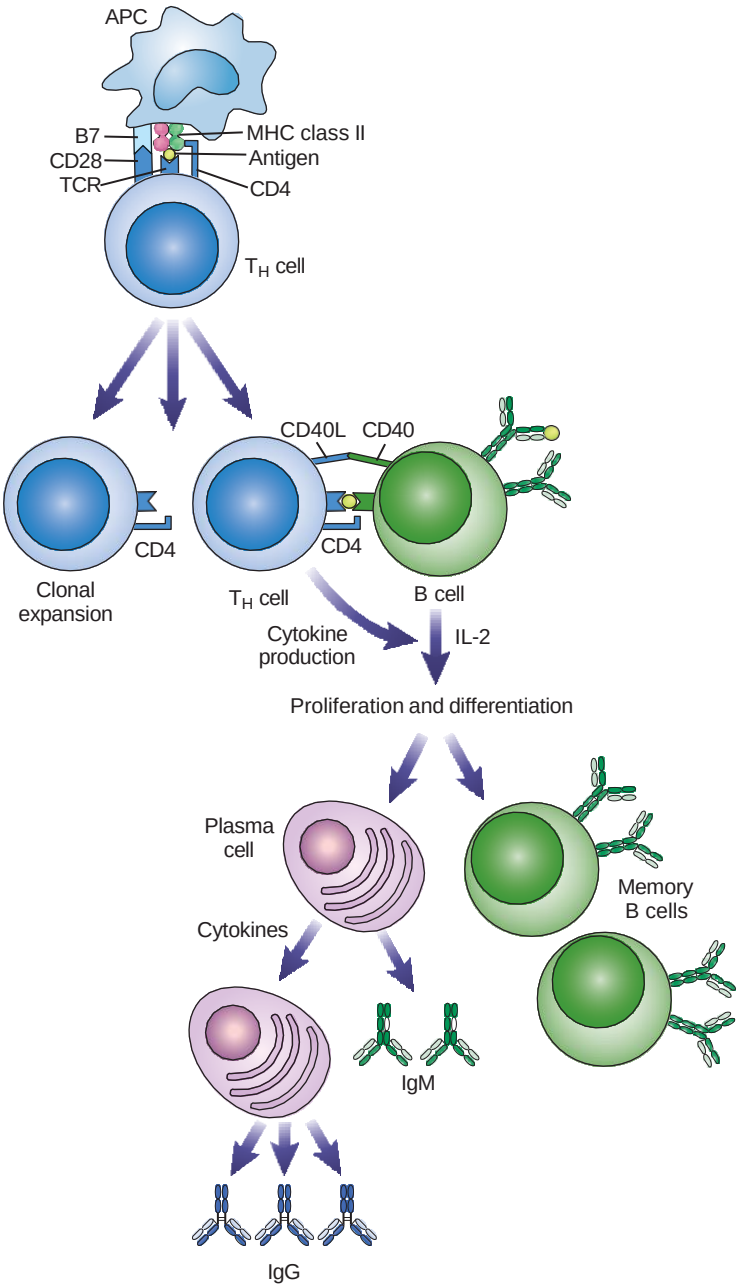


FIGURE 4–13 T- and B-cell cooperation in the immune response. CD4+ T cells recognize exogenous antigen on a macrophage along with class II MHC. Binding between CD28 and B7 enhances interaction between the cells. Th cells go through clonal expansion and produce cytokines, including interleukin-2 (IL-2). 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

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

Christine Dorresteyn Stevens, EdD, MT(ASCP)

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

TETRAPEPTIDE STRUCTURE OF IMMUNOGLOBULINS
THE NATURE OF LIGHT CHAINS
HEAVY□CHAIN SEQUENCING
HINGE REGION
THREE□DIMENSIONAL STRUCTURE OF ANTIBODIES
- Immunoglobulin G (IgG)
- Immunoglobulin M (IgM)
- Immunoglobulin A (IgA)
- Immunoglobulin D (IgD)
- Immunoglobulin E (IgE)

THEORIES TO EXPLAIN ANTIBODY DIVERSITY
- Ehrlich's Side-Chain Theory
- Clonal Selection Hypothesis

GENES CODING FOR IMMUNOGLOBULINS
- Rearrangement of Heavy-Chain Genes
- Light Chain Rearrangement

MONOCLONAL ANTIBODY
- Hybridomas
- Clinical Applications

SUMMARY
CASE STUDIES
REVIEW QUESTIONS

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

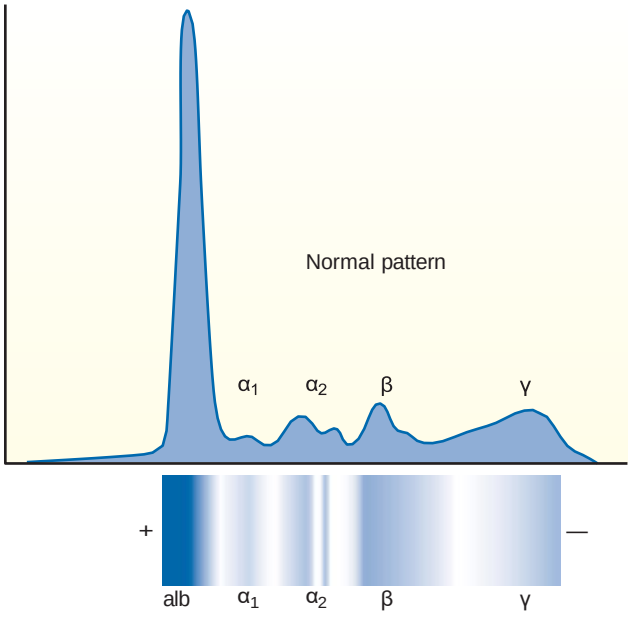


FIGURE 5–1 Serum electrophoresis. Serum is subjected to an electrical charge, and proteins are separated out on the basis of size and charge. Antibodies are found in the gamma region because they are the slowest proteins and have a charge that is close to neutral. Hence, they do not move far from the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

involving over- or underproduction of antibodies, so it will be discussed in later chapters in relation to specific diseases.

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

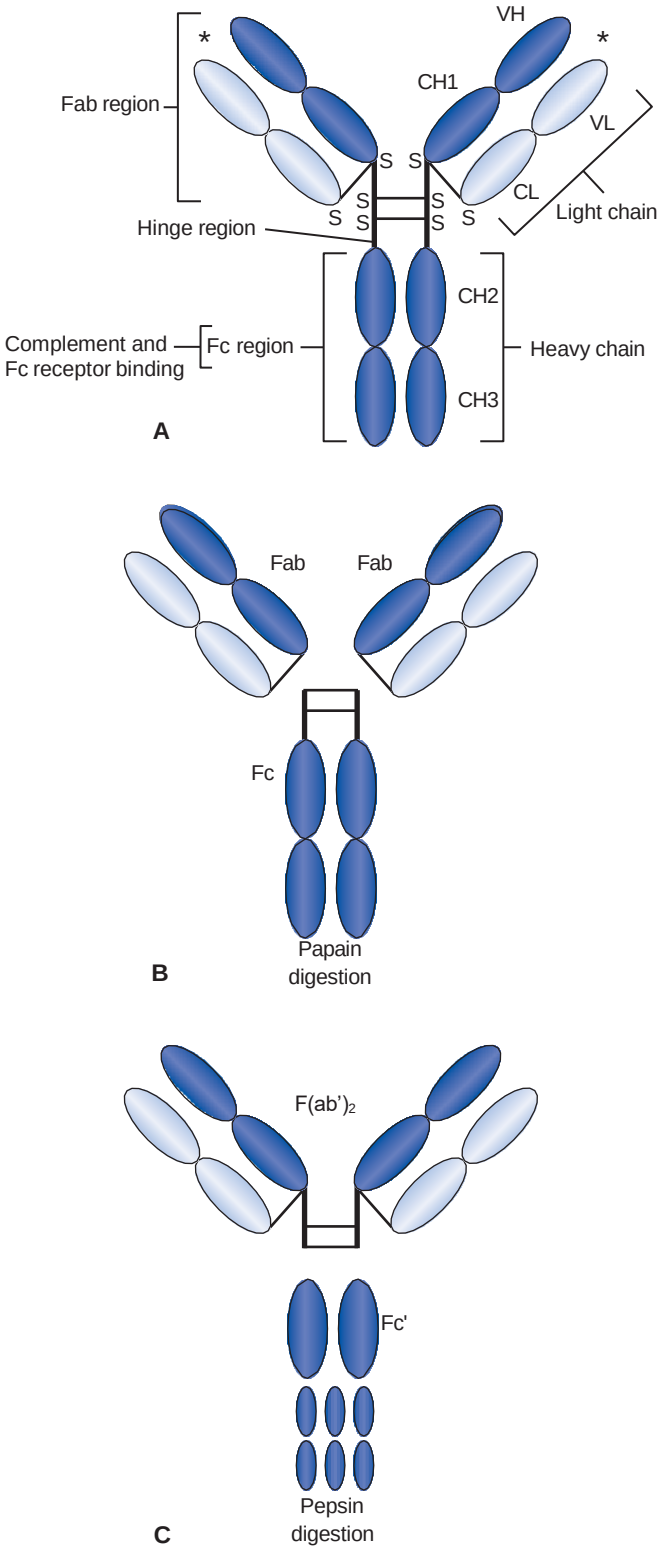


FIGURE 5–2 Basic structure of an immunoglobulin molecule, as discovered by Gerald Edelman. (A) Immunoglobulin G is made up of two H chains (50,000 MW each) and two L chains (22,000 MW each), held together by disulfide bonds. Intrachain disulfide bonds create looped regions or domains. The amino-terminal 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

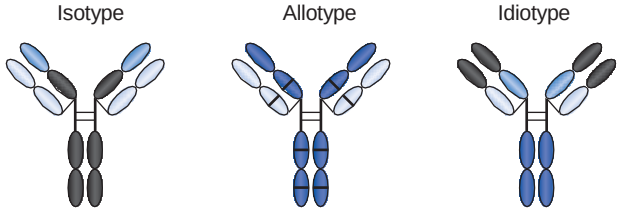


FIGURE 5–3 Antibody variations (shown in black). (A) Isotype—the H chain that is unique to each immunoglobulin class. (B) Allotype—genetic variations in the constant regions. (C) Idiotype—variations in variable regions that give individual antibody molecules specificity.

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.*

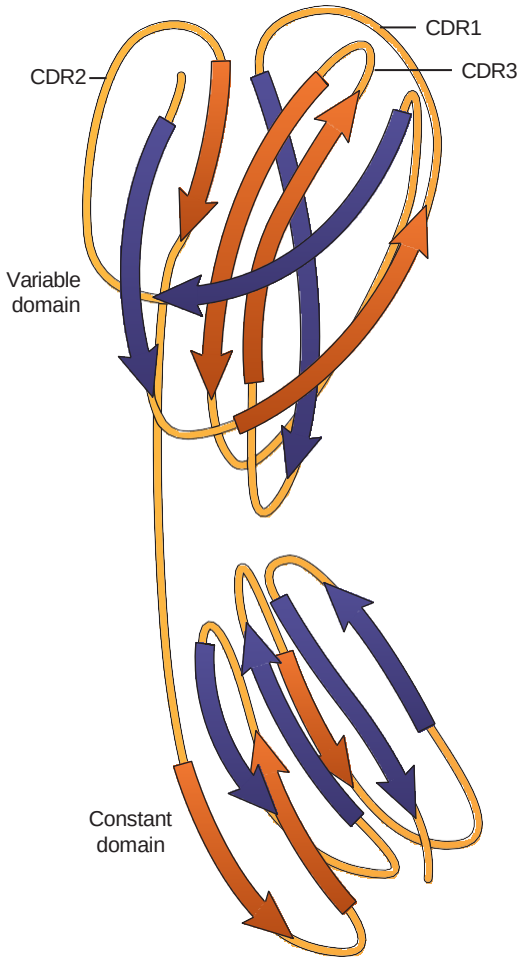


FIGURE 5–4 Three-dimensional structure of an L chain. In this ribbon diagram tracing the polypeptide backbone, β strands (polypeptide chains) are shown as wide ribbons, with other regions as narrow strings. Each of the two globular domains consists of a barrel-shaped assembly of seven to nine antiparallel 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

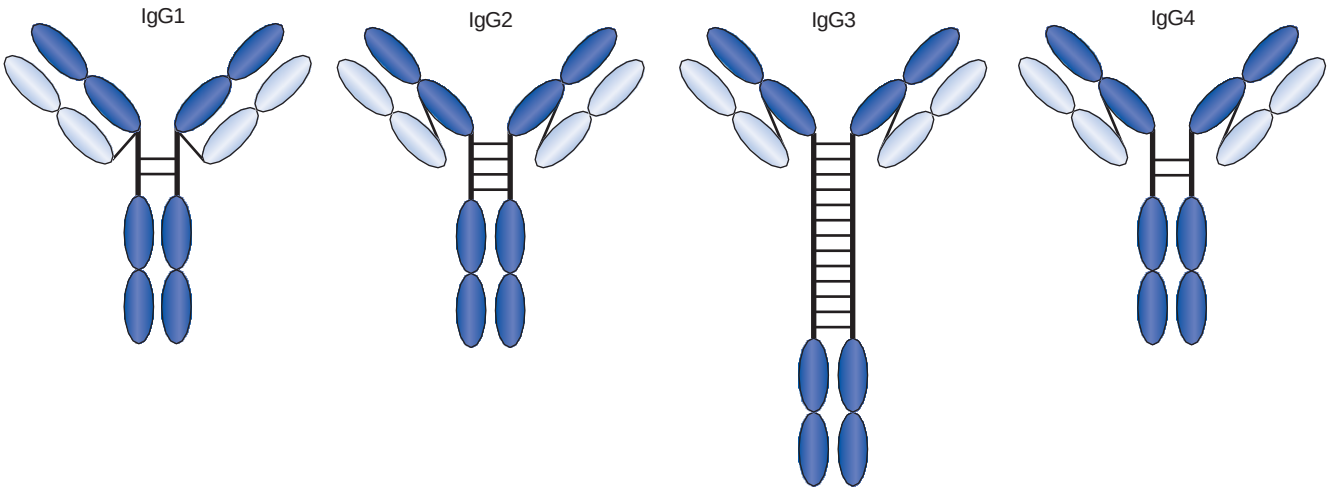


FIGURE 5–5 The four IgG subclasses are IgG1, IgG2, IgG3, and IgG4. These differ in the number and linkages of the disulfide bonds.

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.

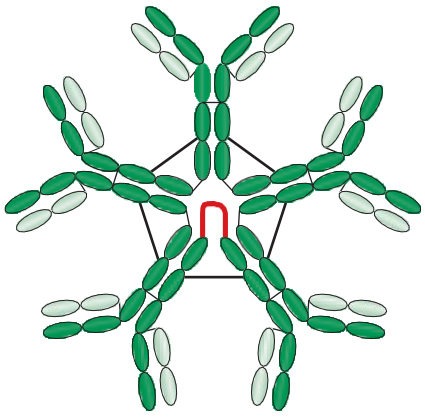


FIGURE 5–6 The pentameric structure of IgM is linked by a J chain (shown in red). Each arm can bend out of the plane to capture antigen.

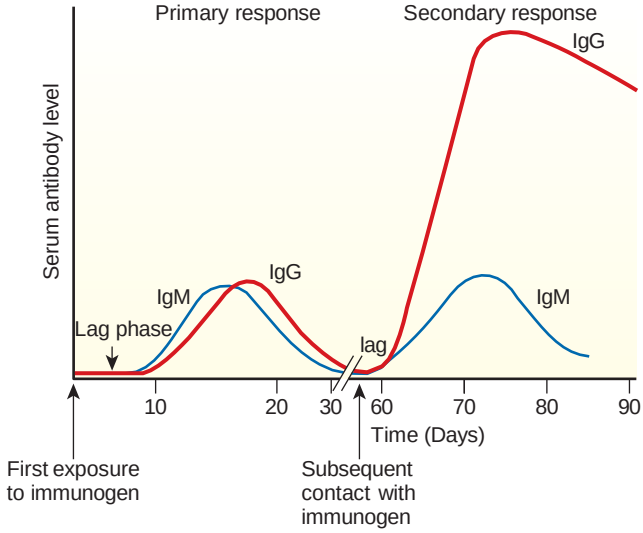


FIGURE 5–7 A comparison of the primary and secondary responses to an immunogen. The primary response is characterized by a long lag phase, whereas the secondary or anamnestic response has a shortened lag phase and a much more rapid increase in antibody titer, mainly IgG.

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.)

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]

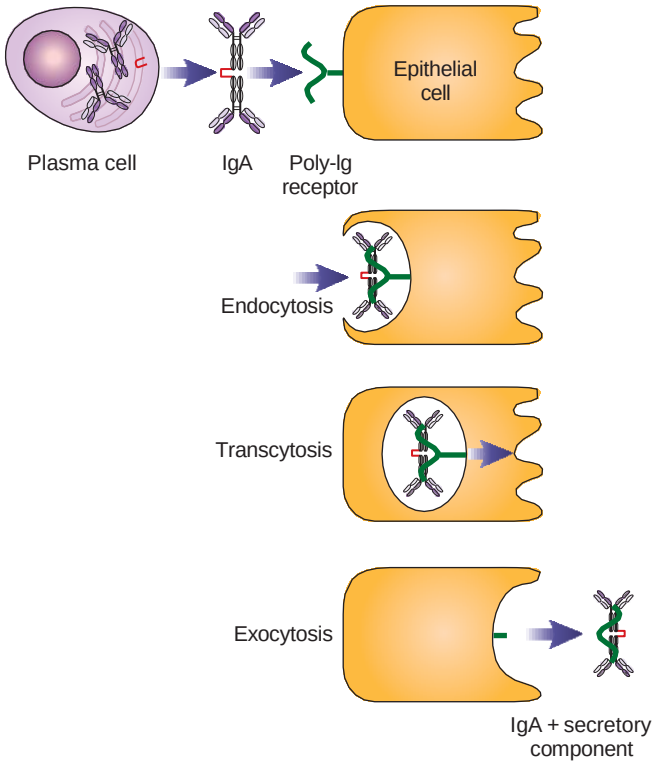


FIGURE 5–8 Formation of secretory IgA. IgA is secreted as a dimer from plasma cells and is captured by specific receptors on epithelial cells. The receptor is actually an SC, which binds to IgA and exits the cell along with it.

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

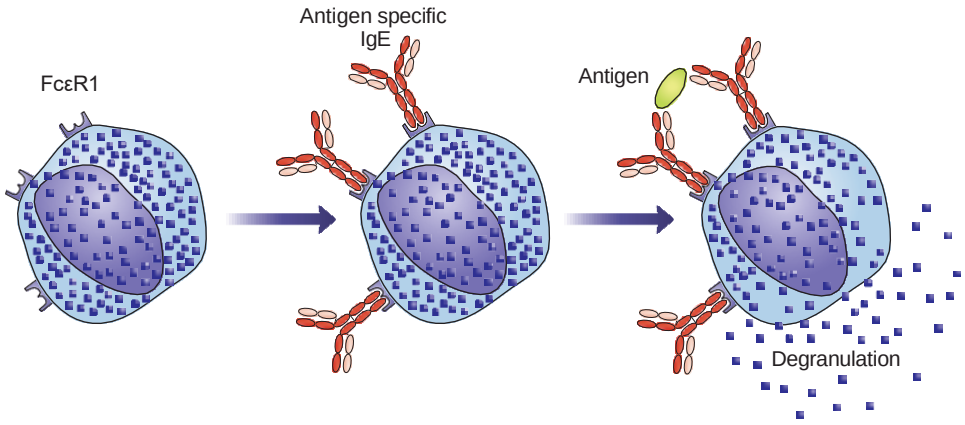


FIGURE 5–9 Action of IgE on mast cells. (A) IgE binds to specific ε receptors on mast cells. (B) When antigen bridges two nearby IgE molecules, the membrane is disturbed and degranulation results. Chemical mediators are released.

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

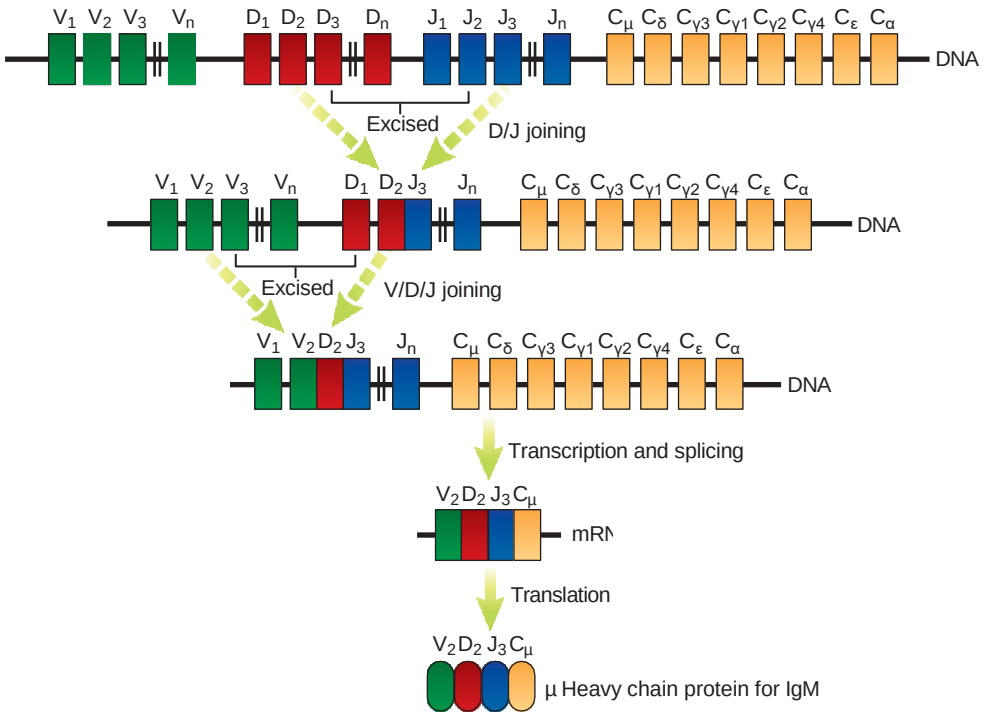


FIGURE 5–10 Coding for immunoglobulin H chains. Four separate regions on chromosome 14 code for H chains. DJ regions are spliced first, and then this segment is joined to a variable region. When RNA synthesis occurs, one constant region is attached to the VDJ combination. μ H chains are made first, but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

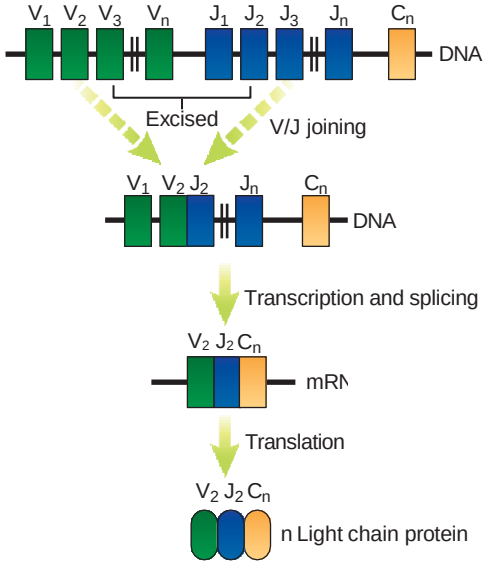


FIGURE 5–11 Assembly and expression of the κ L chain locus. A DNA rearrangement fuses one V segment to one J segment. The VJ segment is then transcribed along with a unique C region to form mature κ mRNA. Unarranged J segments are removed during RNA splicing.

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

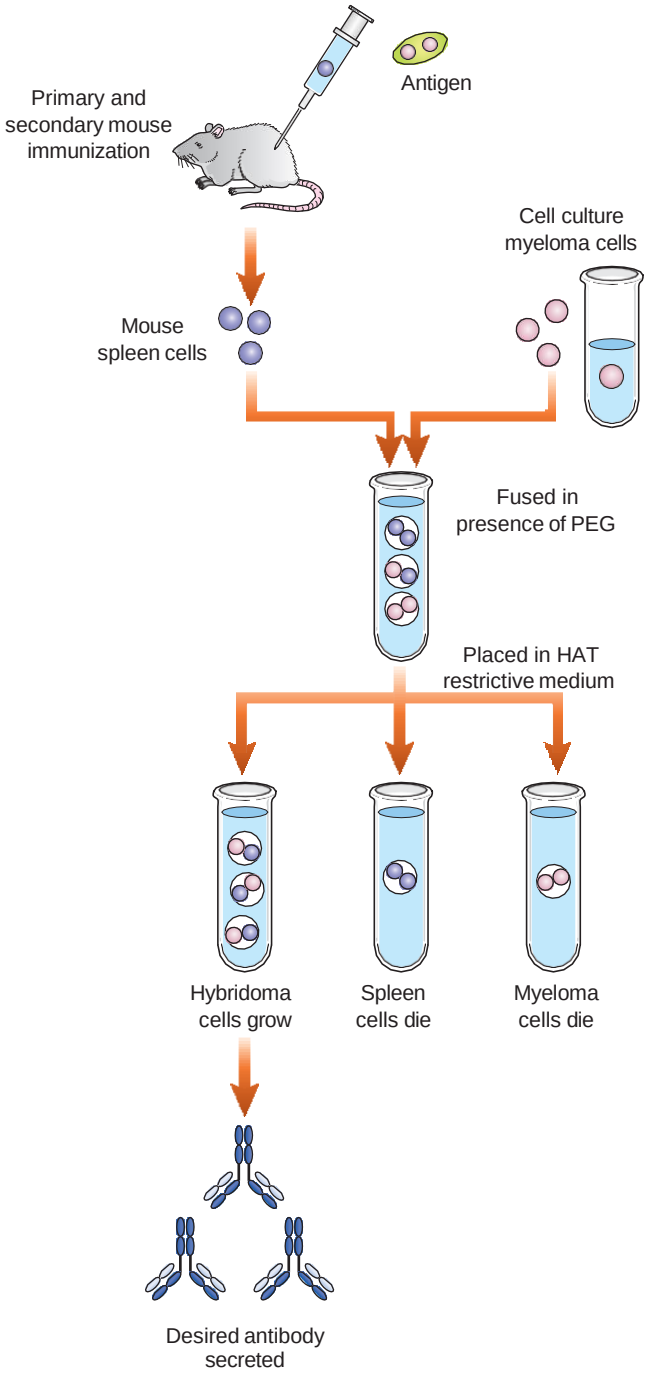


FIGURE 5–12 Formation of a hybridoma in monoclonal antibody production. A mouse is immunized and spleen cells are *removed.* These cells are fused with nonsecreting myeloma cells and then plated in a restrictive medium. Only the hybridoma cells will grow in this medium where they synthesize and secrete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

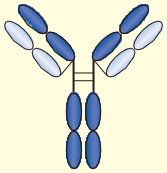
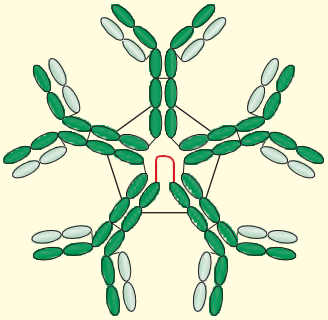
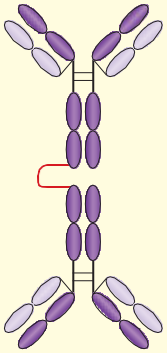
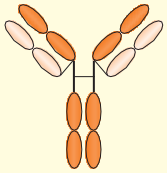
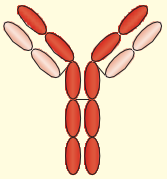
Use of monoclonal antibodies in several areas of medicine continues to grow. This represents a developing area of research in pharmacology; as it rapidly expands, it is likely to change future treatment options for numerous diseases. (See Chapter 25 for additional information about the use of monoclonal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

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

Rita M. Heuertz, PhD, MT(ASCP),
Uthayashanker R. Ezekiel, PhD, MB(ASCP)CM

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

INTRODUCTION TO CYTOKINES
CYTOKINES IN THE INNATE IMMUNE RESPONSE
- Interleukin-1 (IL-1)
- Tumor Necrosis Factors
- Interleukin-6
- Chemokines
- Transforming Growth Factor-β
- Interferon-α and Interferon-β

CYTOKINES IN THE ADAPTIVE IMMUNE RESPONSE
- Th1 Cytokines
- Th2 Cytokines
- Cytokines Associated With T Regulatory Cells

TH17 CYTOKINES IN INNATE AND ADAPTIVE IMMUNE RESPONSES
HEMATOPOIETIC GROWTH FACTORS
CYTOKINE AND ANTICYTOKINE THERAPIES
CLINICAL ASSAYS FOR CYTOKINES
SUMMARY
CASE STUDY
REVIEW QUESTIONS

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.

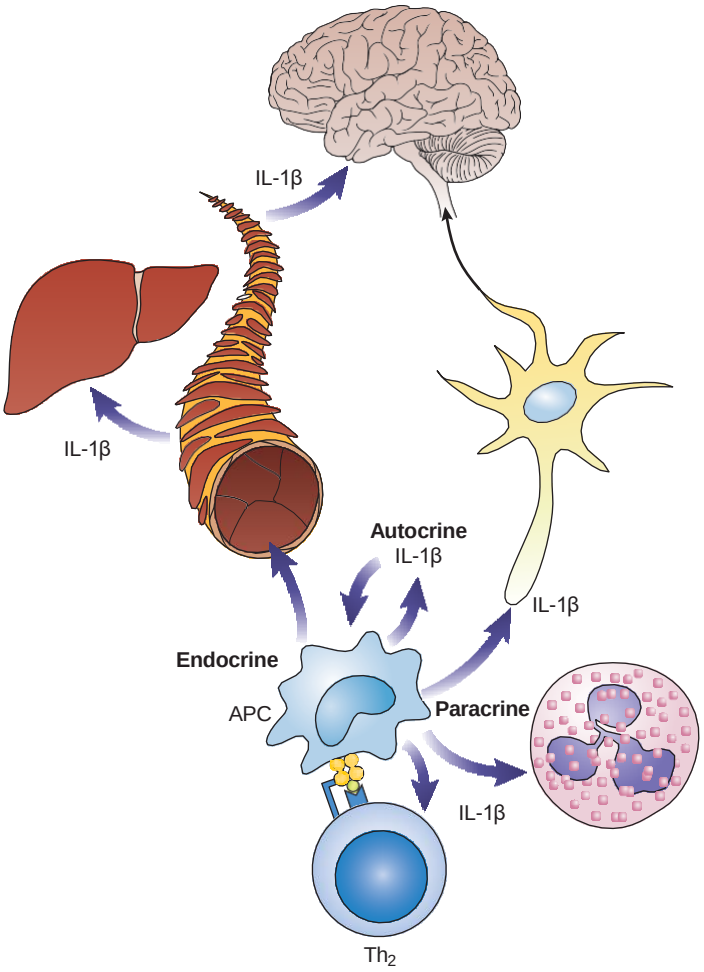


FIGURE 6–1 Range of cytokine actions. Autocrine: Cytokine acts on the cell that secreted it (e.g., IL-1β increases activation of APC). Paracrine: Cytokine acts on nearby cells (e.g., IL-1β stimulates Th cells, activates neutrophils, depolarizes neurons). Endocrine: Cytokine travels through blood vessels 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

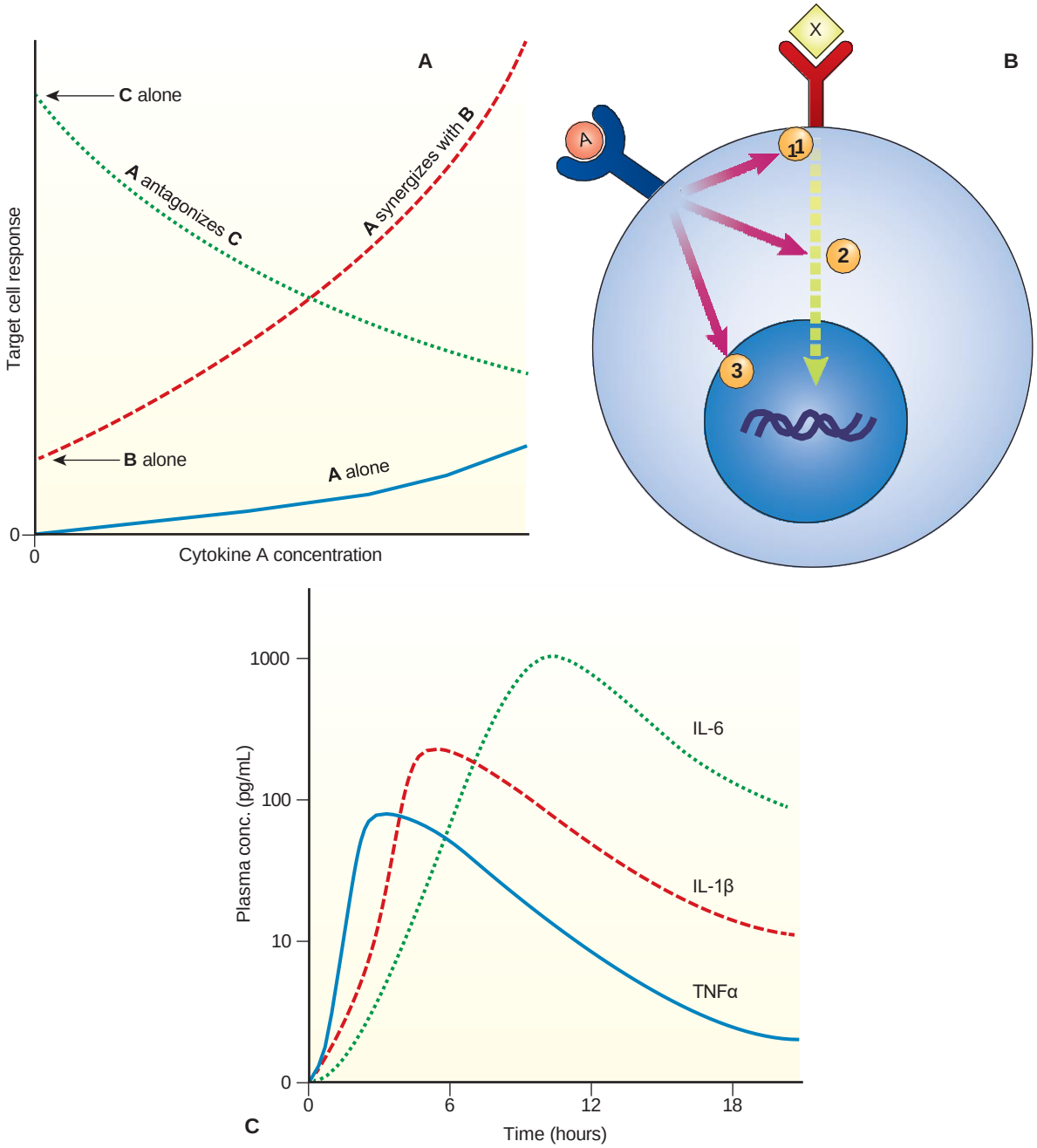


FIGURE 6–2 (A) Cytokine characteristics of synergy, antagonism, and cascade induction. Synergy: Neither cytokine A nor B induce a strong response individually; however, when combined, the net response is much greater than the sum of the individual responses. Antagonism: individually, cytokine C induces 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-1ß. 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.

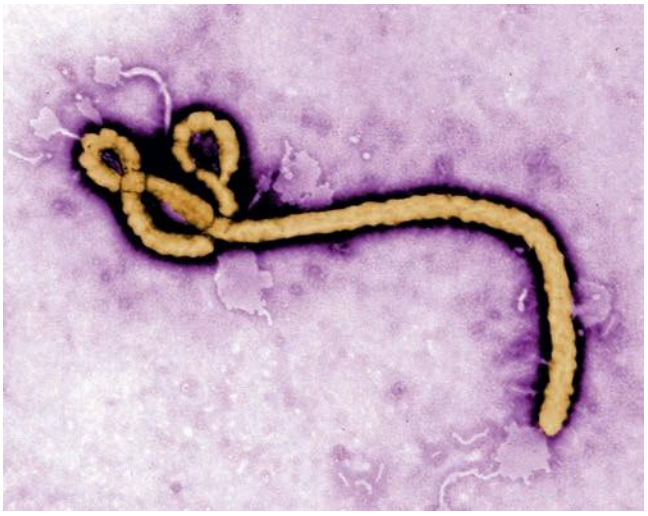


FIGURE 6–3 False-colored micrograph of the Ebola virus virion. *(Courtesy of the CDC/Frederick A. Murphy, Public Health Image Library.)*

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]

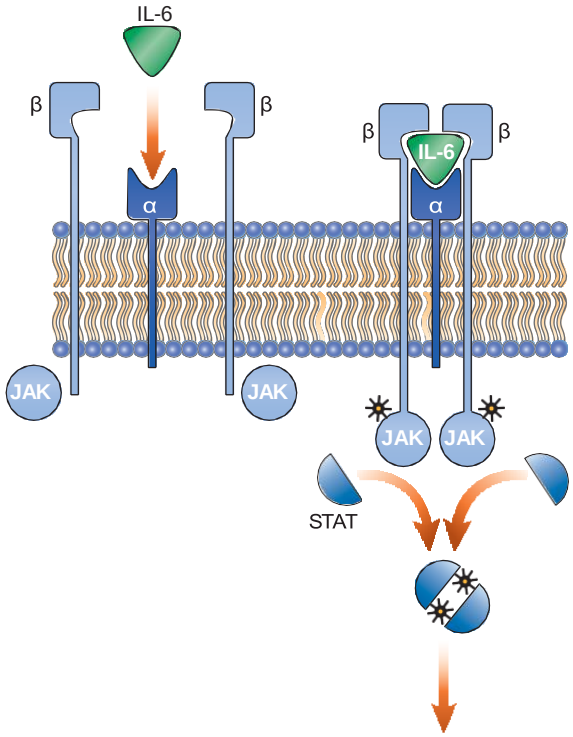


FIGURE 6–4 IL-6 binding to the IL-6R subunit recruits two gp130 subunits to the receptor complex. Signal transduction takes place through tyrosine phosphorylation of the gp130 subunits by one of the Janus kinases (J).

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

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

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.

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

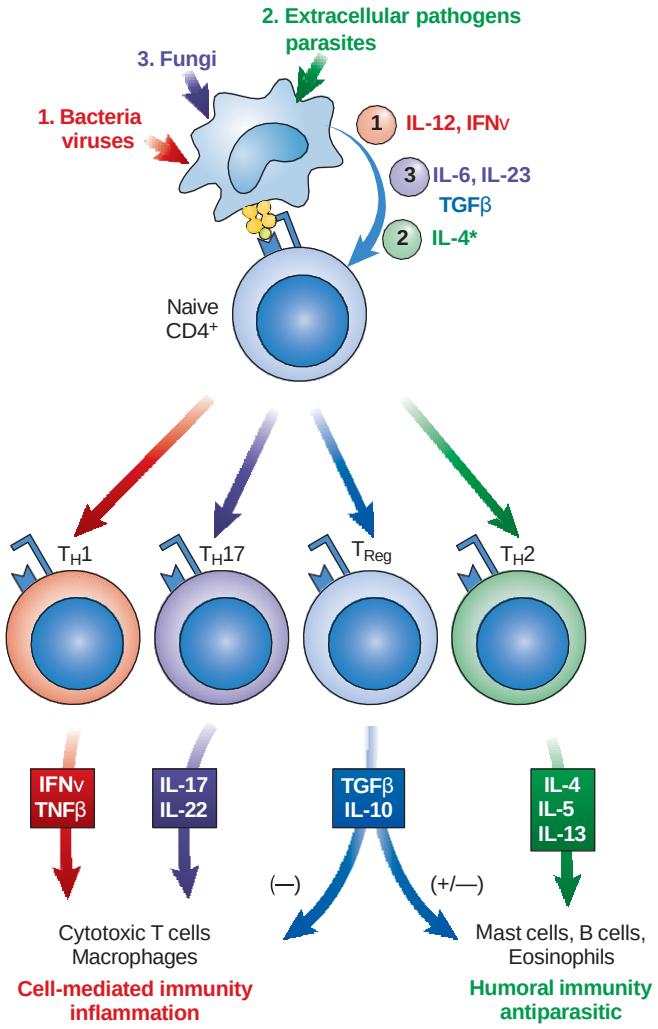


FIGURE 6–5 Subpopulations of T cells. Depending upon the pathogen encountered, antigen-presenting cells (APCs) secrete a specific combination of polarizing cytokines that direct naïve T helper (Th) cells to differentiate into specialized subsets. Each T-cell subpopulation exerts different types of immune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

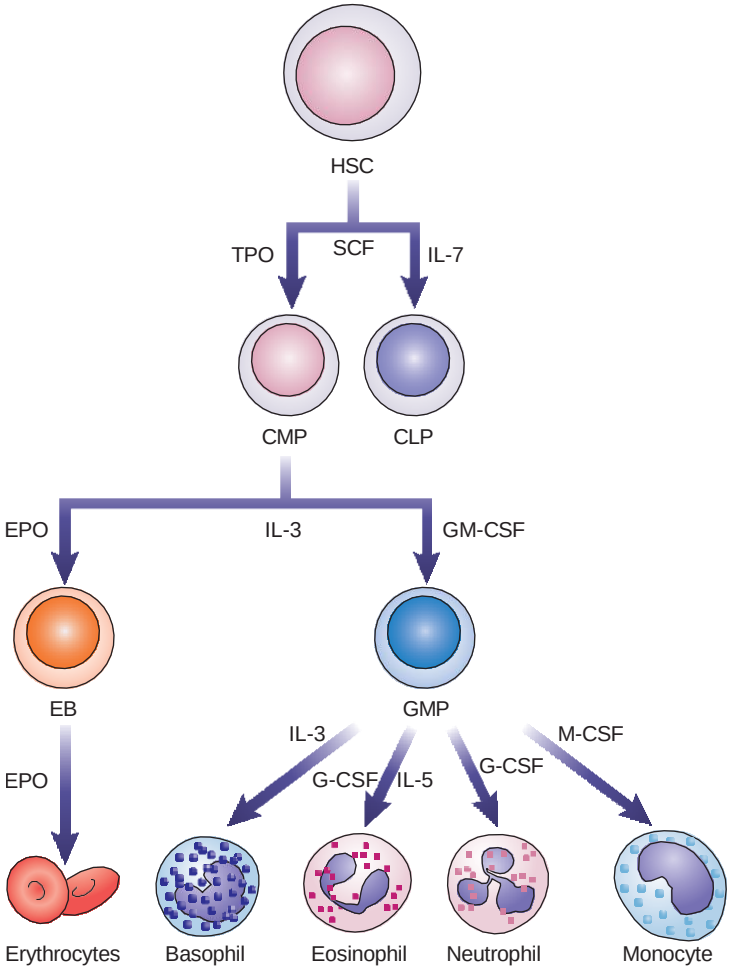


FIGURE 6–6 Influence of colony stimulating factors on growth and differentiation of blood cells. Growth of hematopoietic stem cells (HSC) requires stem cell factor (SCF) with differentiation determined by IL-7 or thrombopoietin (TPO). Growth of common myeloid progenitors (CMP) depends upon IL-3. Differentiation 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

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

Ashley Frazer-Able, PhD,
and Christine Dorresteyn Stevens, EdD, MT(ASCP)

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

PATHWAYS OF THE COMPLEMENT SYSTEM
- The Classical Pathway
- The Lectin Pathway
- The Alternative Pathway

SYSTEM CONTROLS
- Regulation of the Classical and Lectin Pathways
- Regulation of the Alternative Pathway
- Regulation of Terminal Components

COMPLEMENT RECEPTORS AND THEIR BIOLOGICAL ROLES

BIOLOGICAL MANIFESTATIONS OF COMPLEMENT ACTIVATION

COMPLEMENT AND DISEASE STATES

COMPLEMENT DEFICIENCIES
- Major Pathway Components
- Regulatory Factor Components

LABORATORY DETECTION OF COMPLEMENT ABNORMALITIES
- Immunologic Assays of Individual Components
- Assays for the Classical Pathway
- Alternative Pathway Assays
- Interpretation of Laboratory Findings

SUMMARY

CASE STUDIES

REVIEW QUESTIONS

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.

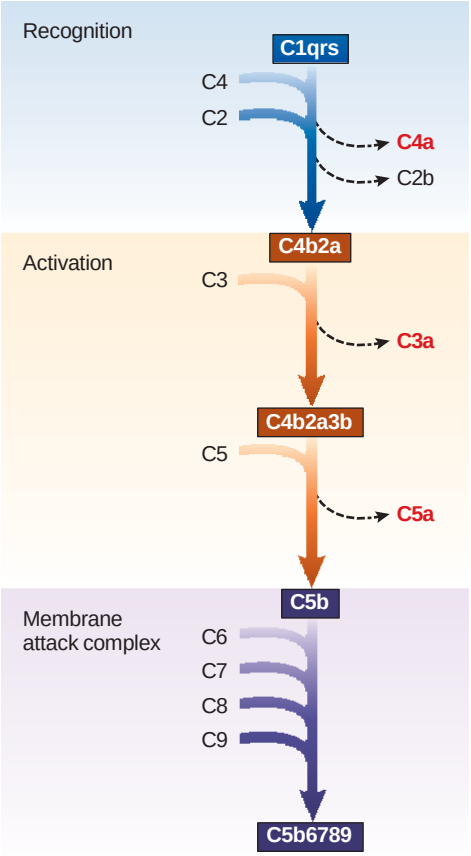


FIGURE 7–1 The classical complement cascade. C1qrs is the recognition unit that binds to the FC portion of two antibody molecules. C1s is activated and cleaves C4 and C2 to form C4b2a, also known as C3 convertase. C3 convertase cleaves C3 to form C4b2a3b, known as C5 convertase. The combination of C4b2a3b 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.

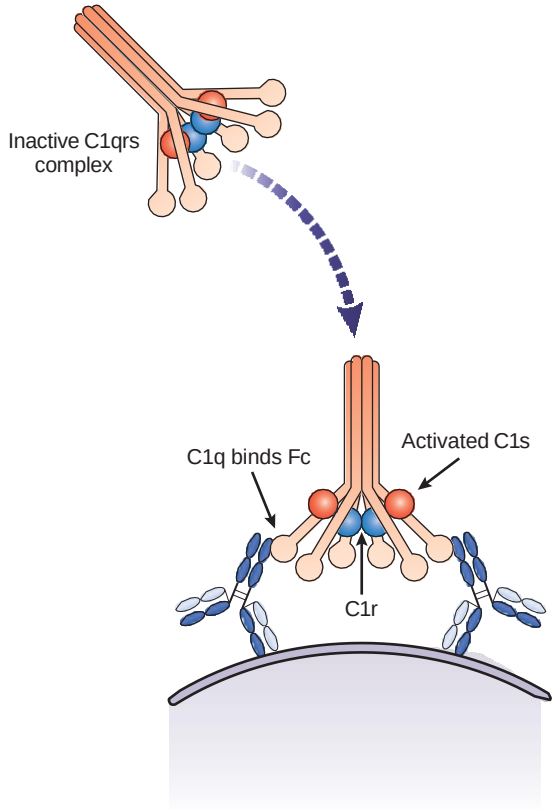


FIGURE 7–2 Structure of C1qrs. When two or more globular heads of C1q attach to bound immunoglobulin molecules, the collagen-like stalks change their configuration. The resulting shape change causes C1r to become a serine protease, which cleaves a small fragment of C1s, uncovering the C1s protease, whose 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 $\overline{C4b2a}$ 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

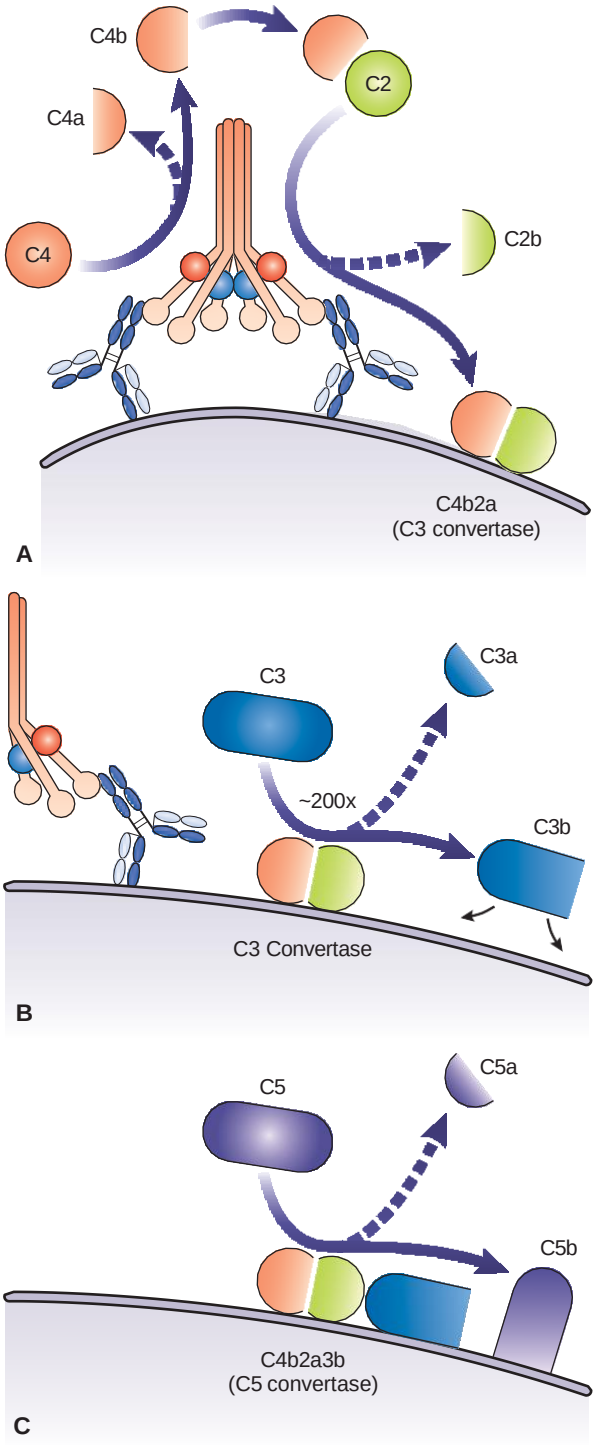


FIGURE 7–3 Formation of the activation unit. (A) Activated C1qrs cleaves C4 and C2, with the larger pieces, C4b and C2a, binding to the target cell surface and forming the enzyme C3 convertase. (B) Each C3 convertase cleaves ~200 C3 molecules into C3a and C3b. C3b is a powerful opsonin that binds to 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 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

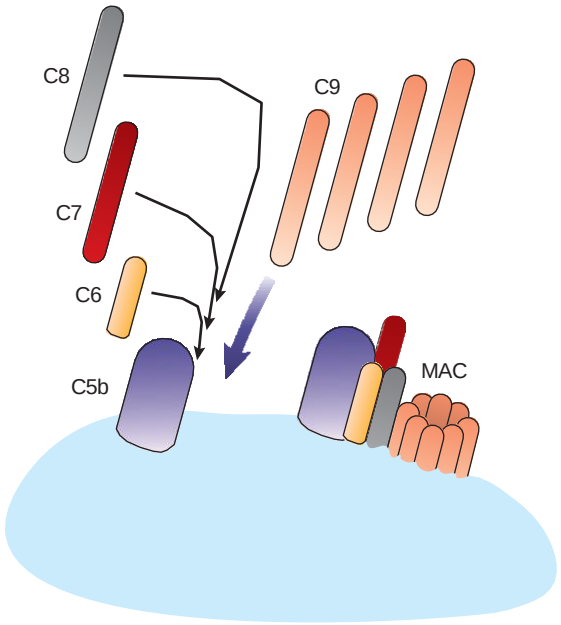


FIGURE 7–4 Formation of the membrane attack unit. C5b binds to the target cell, whereas C6 and C7 attach to it. C8 binds to these associated molecules and begins (along with C7) to penetrate the cell membrane. Multiple C9 molecules bind to C5b678 and polymerize to form a transmembrane channel, the membrane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

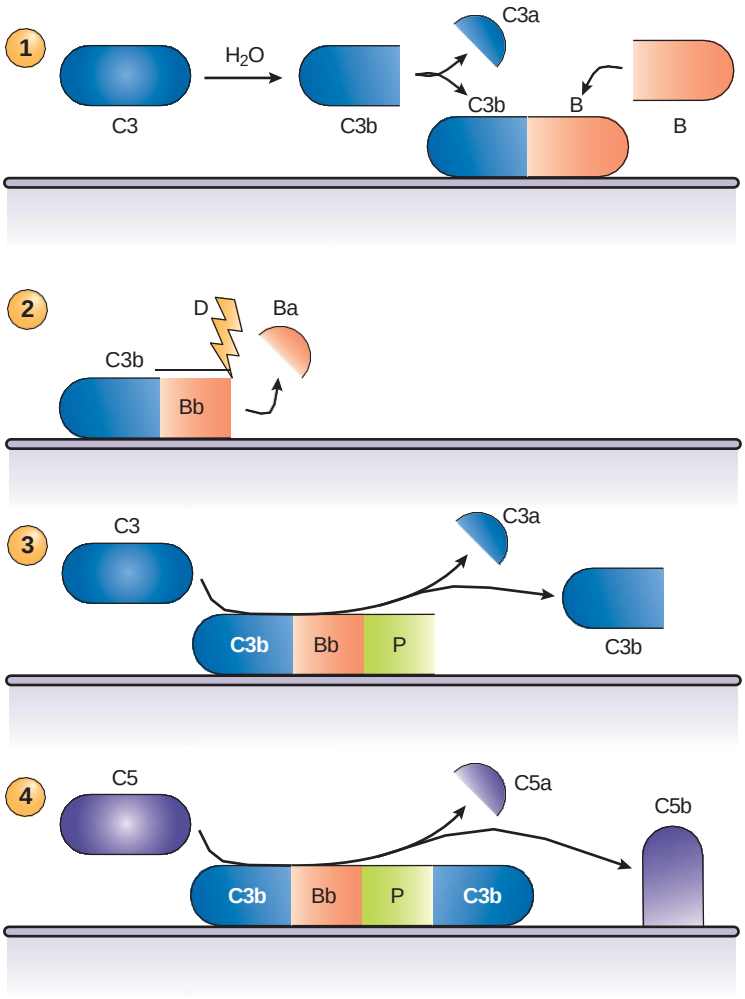
First described by Pillemer and his associates in the early 1950s, the alternative pathway was originally named for the protein **properdin,** a constituent of normal serum with a concentration of approximately 5 to 15 μg/mL.[5] Although the alternative pathway can be activated on its own, it appears 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 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, 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

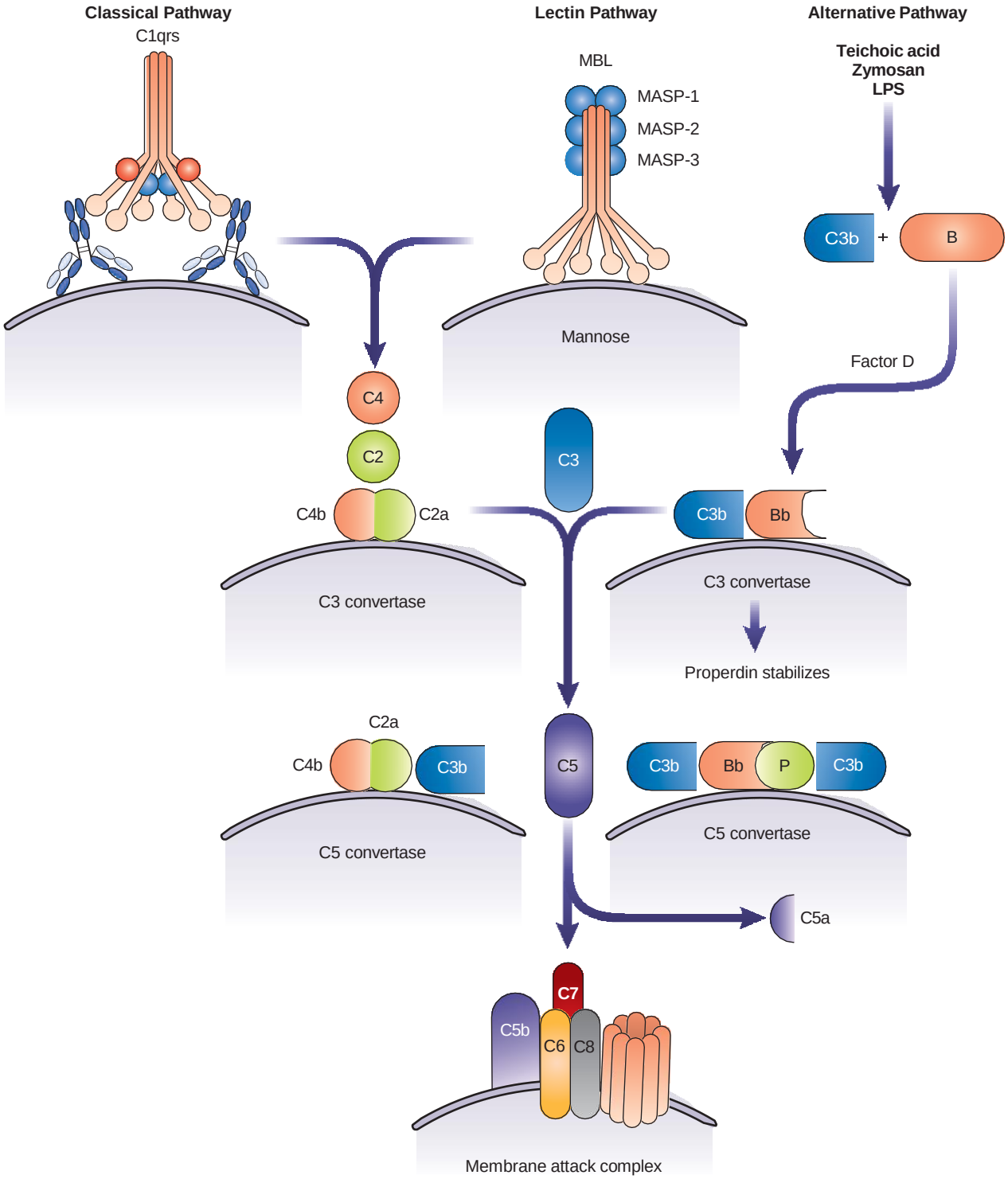


FIGURE 7–6 Convergence of the classical, alternative, and lectin pathways. The binding of C1qrs to two antibody molecules activates the classical pathway, whereas the alternative pathway is started by hydrolysis of C3. The lectin pathway is triggered by binding of MBP to mannose on bacterial cell walls. 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).

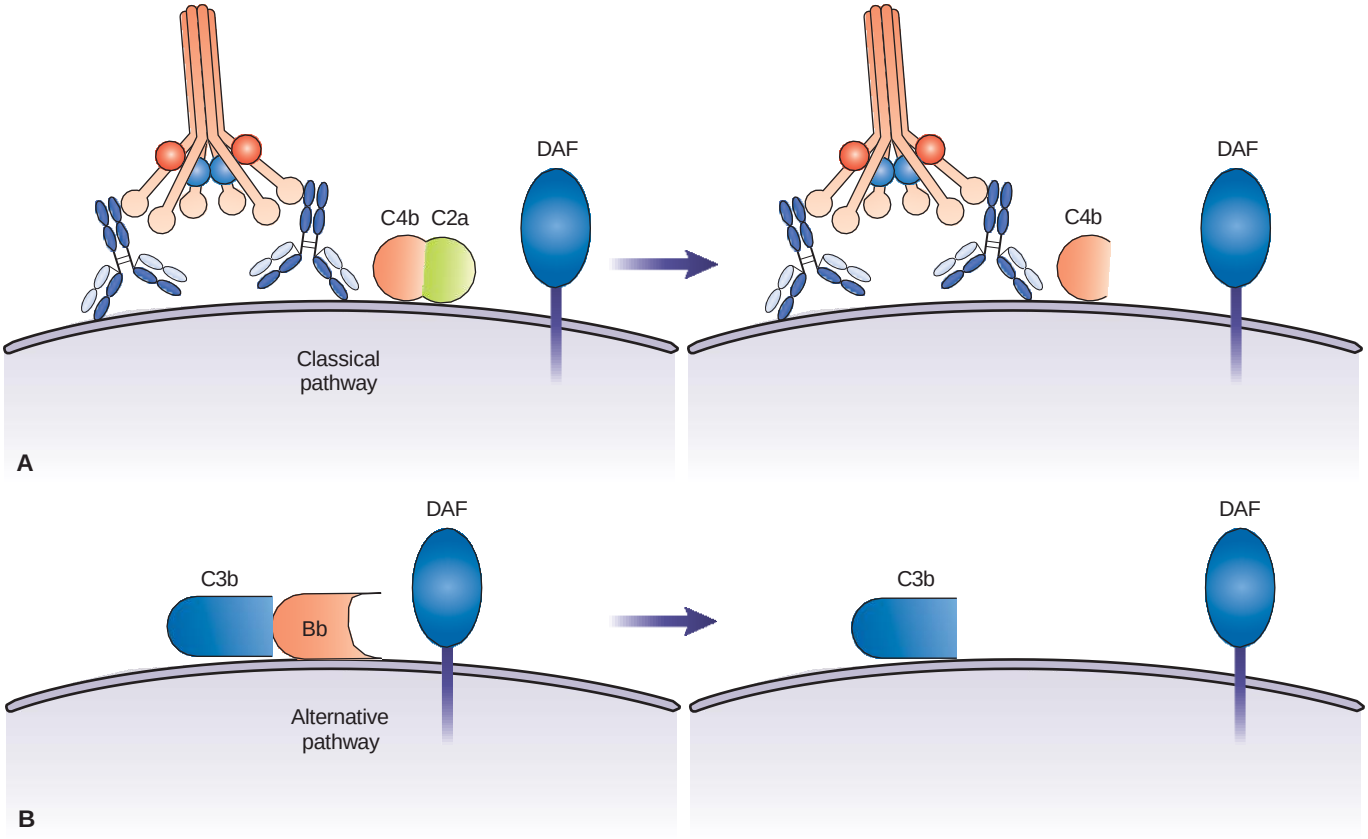


FIGURE 7–7 A. In the classical pathway, DAF dissociates C2a from C4b. B. Inhibitory effects of DAF. In the alternative pathway, when C3b binds to cell surfaces that have DAF present, DAF helps dissociate Bb from binding to C3b.

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]

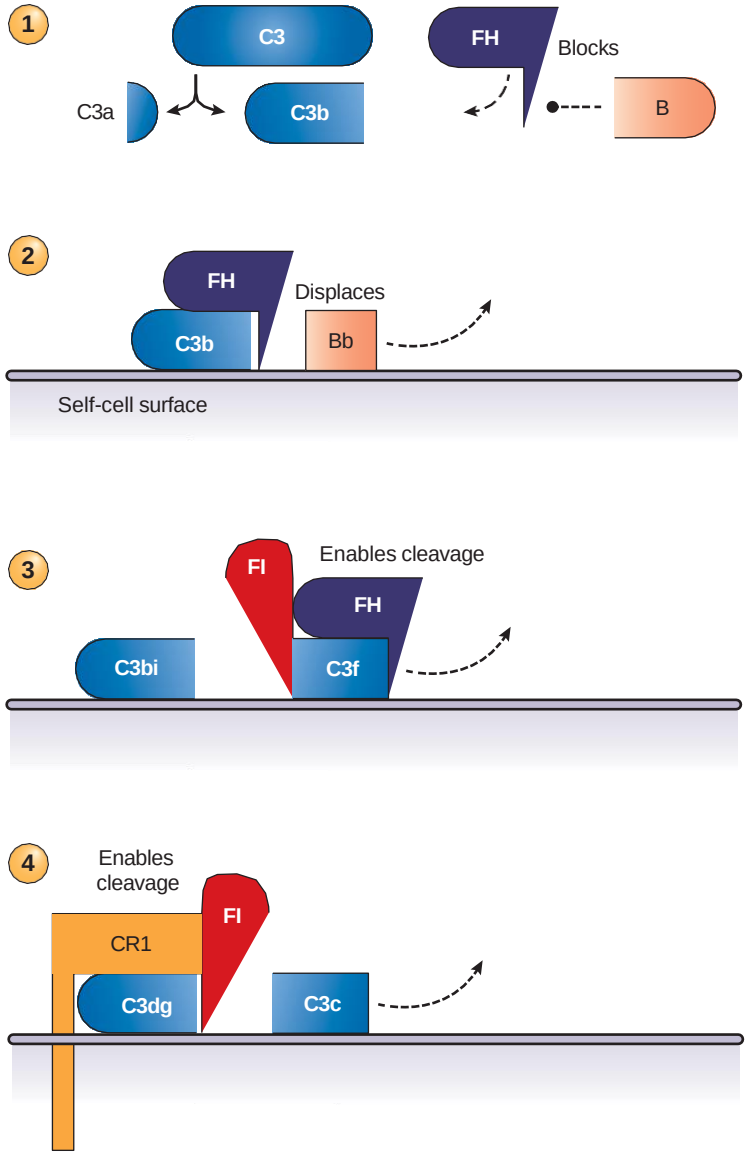
A receptor, known by various terms, including membrane inhibitor of reactive lysis (MIRL) or CD59, also acts to block formation of the MAC. MIRL is widely distributed on the cell membranes of all circulating blood cells, including RBCs, and on endothelial, epithelial, and many other types of cells.[8,10]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

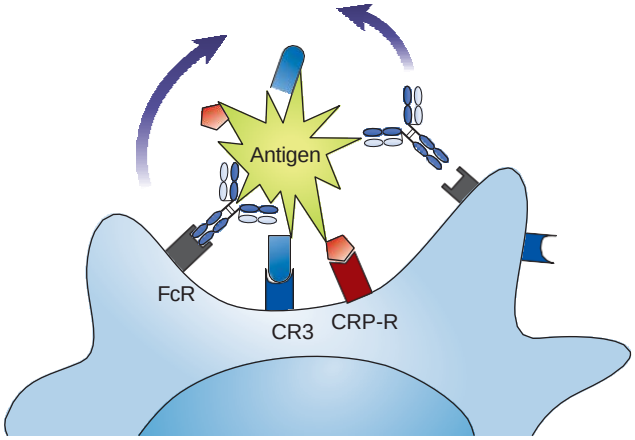


FIGURE 7–9 Role of C3b in opsonization. C3b, antibody, and C-reactive protein are all important opsonins that coat antigens and accelerate phagocytosis. Receptors for C3b (CR3), antibody (FcR), and C-reactive protein (CRP-R) on the phagocytic cell membrane bind the opsonins and pull the membrane around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

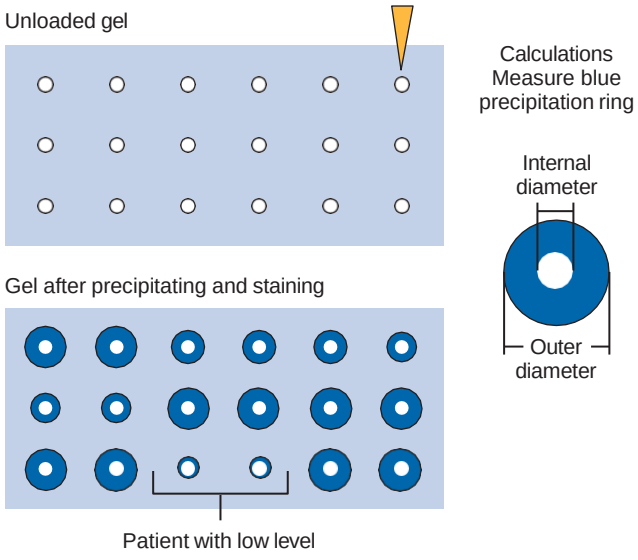


FIGURE 7–10 Radioimmunodiffusion for measurement of complement component C5.

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.

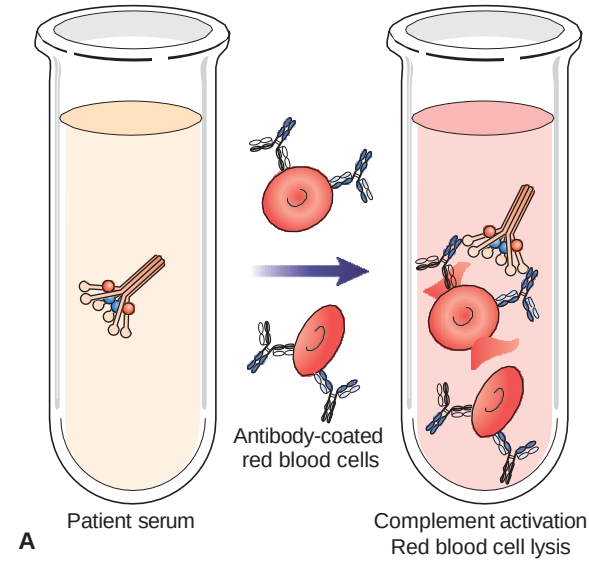


FIGURE 7–11 CH50 testing. (A) Antibody-coated RBCs are added to patient serum. This activates the complement in the sample and the RBCs are lysed. Although only C1qrs is shown for simplicity, this test requires C1 through C9 to be present in order for lysis to occur. The degree of lysis indicates the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

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

LABORATORY HAZARDS
- Biological Hazards
- Sharps Hazards
- Chemical Hazards
- Radioactive Hazards
- Electrical Hazards
- Fire and Explosive Hazards
- Physical Hazards

QUALITY MANAGEMENT
- Procedure Manual
- Preexamination Variables
- Examination Variables
- Postexamination Variables

REGULATORY ISSUES
- Clinical Laboratory Improvement Amendments (CLIA)
- Clinical and Laboratory Standards Institute (CLSI)
- The Joint Commission (TJC)
- College of American Pathologists (CAP)

QUALITY MANAGEMENT SYSTEMS
- Quality System Essentials
- The Lean System
- Six Sigma

SUMMARY

CASE STUDIES

REVIEW QUESTIONS

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

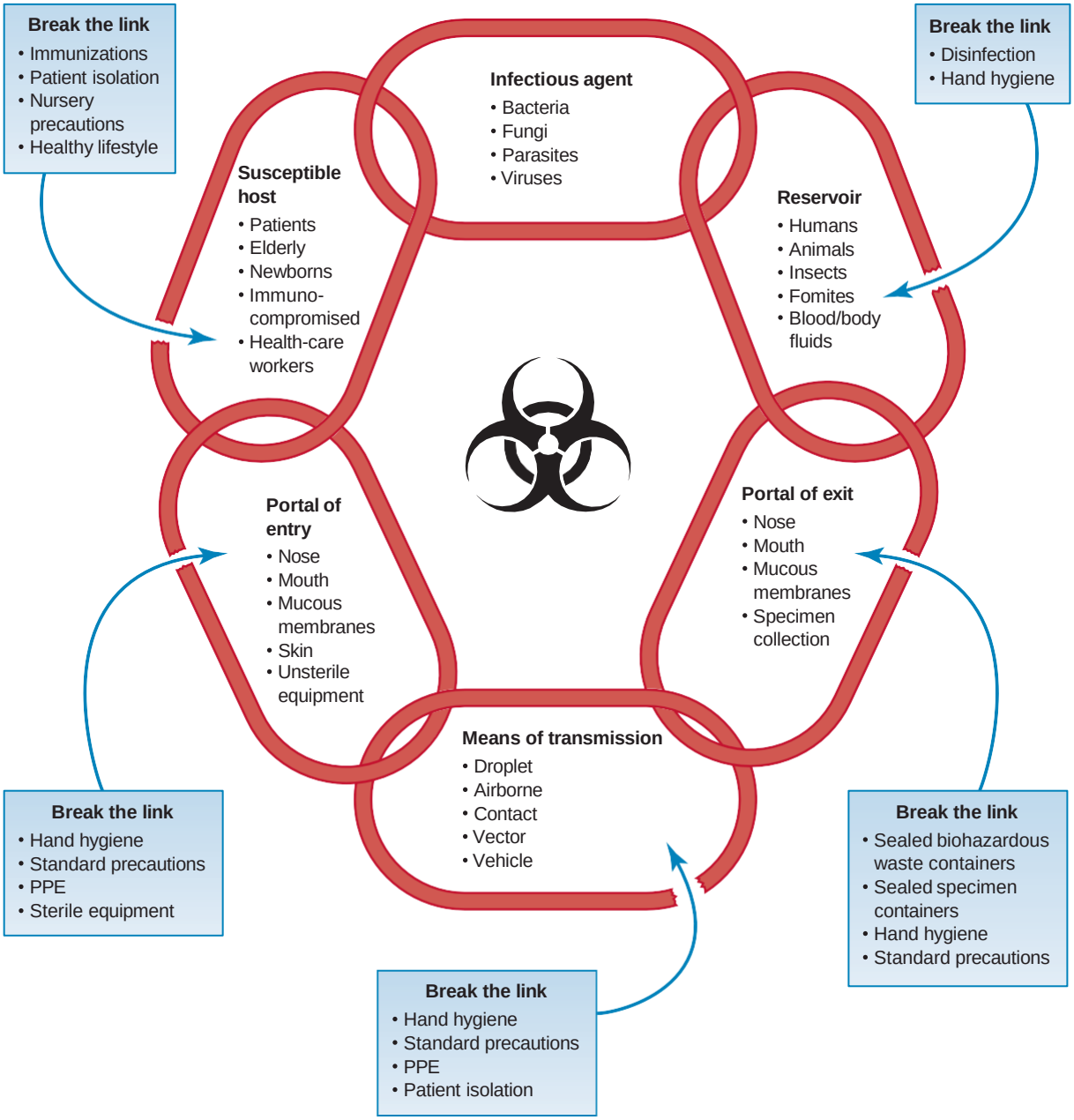


FIGURE 8–1 Chain of infection and safety practices related to the biohazard symbol. *(From Strasinger SK, DiLorenzo MS.* The Phlebotomy Textbook. *3rd ed. Philadelphia, PA: F.A. Davis, Philadelphia; 2011, with permission.)*

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

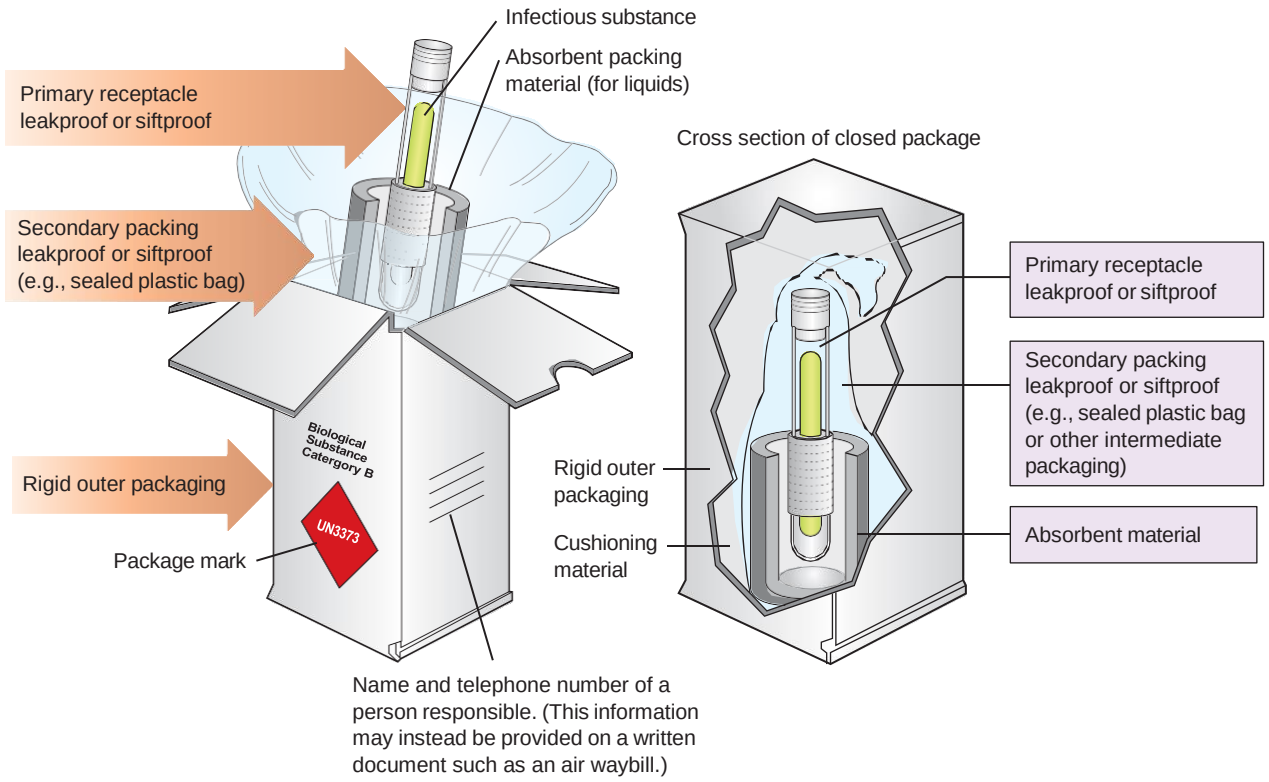


FIGURE 8–4 Packing and labeling of category B infectious substances. If multiple fragile primary receptacles are placed in a single secondary package, they must be either individually wrapped or separated to prevent contact. *(From U.S. Department of Transportation. Pipeline and Hazardous Materials Safety 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.**

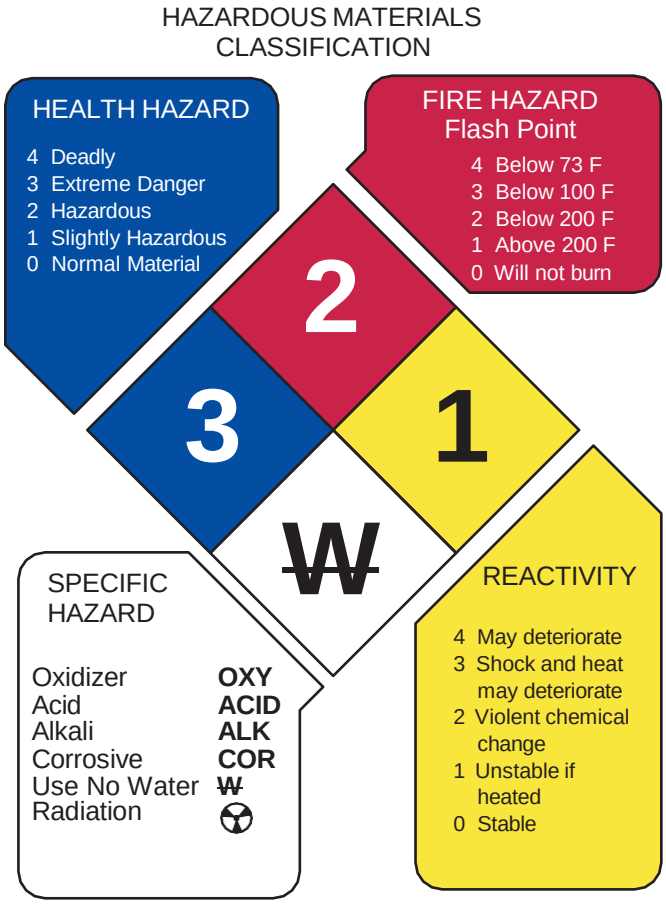


FIGURE 8–8 NFPA hazardous material symbol and classification. *(From Strasinger SK, DiLorenzo MS.* The Phlebotomy Textbook. *3rd ed. Philadelphia, PA: F.A. Davis; 2014.)*

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. Health-care 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.

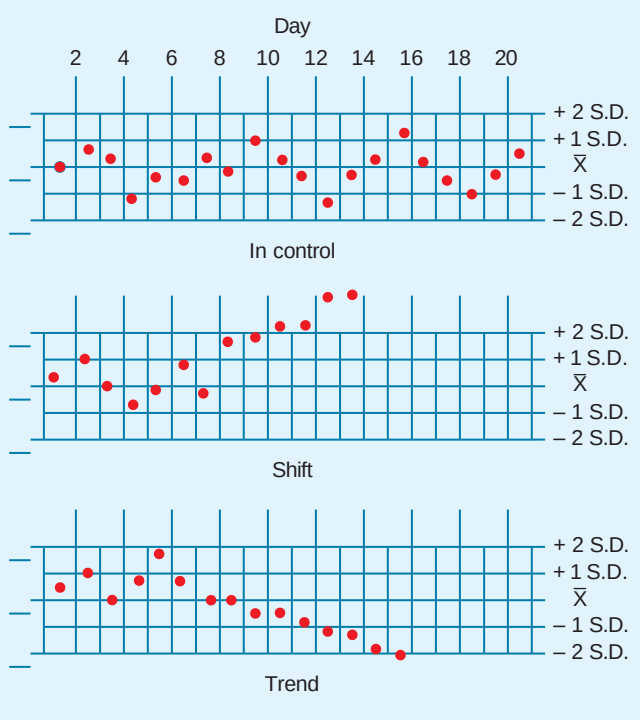


FIGURE 8–9 Levey–Jennings charts showing in–control, shift, and trend results. *(From Strasinger SK, DiLorenzo MS.* Urinalysis and Body Fluids. *6th ed. Philadelphia, PA: F.A. Davis; 2014.)*

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.

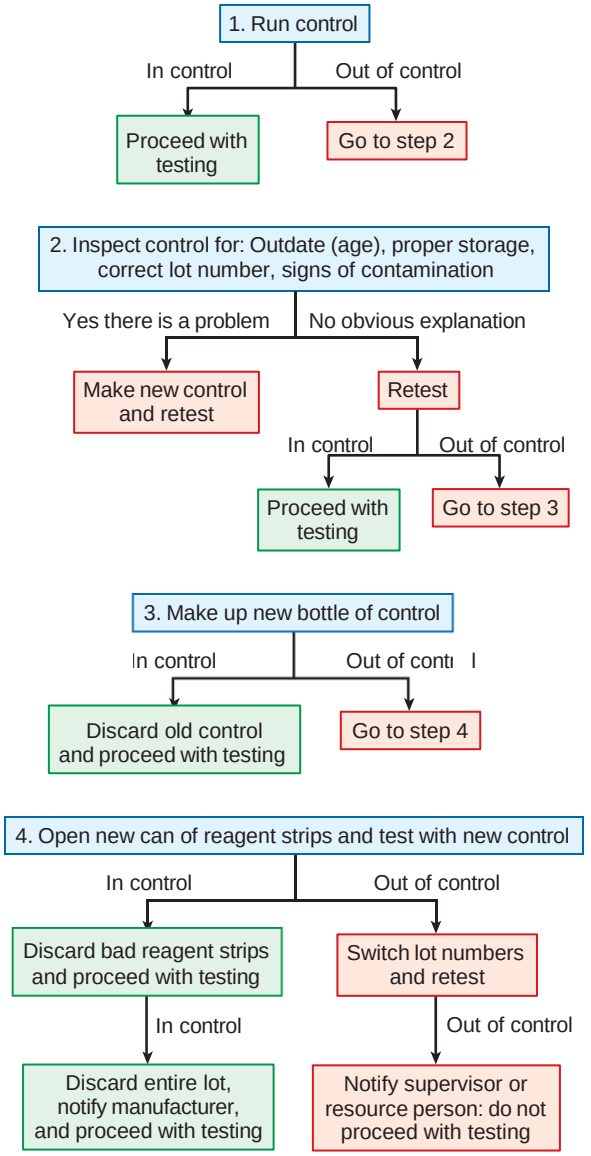


FIGURE 8–10 Procedure for "out of control" results. *(Adapted from Schweitzer SC, Schumann JL, Schumann GB. Quality assurance guidelines for the urinalysis laboratory.* J Med Technol. *1986; 3(11):567–572.*

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