Manual of Immunoperoxidase Techniques

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Preface

The idea for this manual originated from our teaching at workshops sponsored by the American Society of Clinical Pathologists. These workshops were concerned with basic immunology and immunoperoxidase techniques. After talking with numerous participants, it soon became obvious that an easy-to-follow manual dealing with light microscopic immunoperoxidase techniques was needed. This manual is an attempt to fill that need.

The purpose of this manual is to give the histopathology technician, medical technologist, researcher, and pathologist a ready reference to common techniques using peroxidase histochemistry. The manual begins with a brief introduction to immunology and to the use of antibodies as laboratory reagents. The remainder of the manual deals with various aspects of immunoperoxidase histochemistry. The manual is not intended to be an all-inclusive research book but rather a vehicle for a rapid introduction to this growing field.

We appreciate all the encouragement given to us by the workshop participants. Without their questions and enthusiasm, this manual would not have been conceived. It is our sincere hope that this manual will be of use to all who endeavor to improve the quality of life by using these advanced diagnostic and research techniques.

The authors wish to acknowledge with grateful thanks the invaluable assistance of Barbara Wordinger.

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Introduction to Immunology

THE IMMUNE SYSTEM

Immunology is of utmost importance to modern medicine. It is a relatively old field of study whose concepts originated from the study of resistance to infection.

As a young medical student, Edward Jenner made the unprecedented observation that milkmaids who had contracted cowpox were resistant to infection with smallpox. He further discovered that inoculation with cowpox crusts protected man from smallpox. With this most important finding, the future of immunobiology was assured.

Immunology is defined simply as the study of immune responses. One, if not the most sophisticated, characteristic of the immune system is the acquired capacity not only to distinguish self from nonself, but to do so with memory and specificity. In a specific immune response, components of the immune system are concerned with the recognition and ultimate disposal of foreign substances in a highly sophisticated and discriminatory fashion.

The function of the immune system is host defense; and to carry out this function, a collection of cells, the lymphoreticular system, is distributed through the tissues of the body. These cells are housed primarily in the bone marrow, peripheral blood, thymus, lymph nodes, and spleen.

A substance recognized by the immune system as foreign initiates

the immune response. This foreign substance is usually a large protein, polysaccharide, or lipoprotein complex. Whatever the foreign substance, its chemical composition makes it unique and enables it to initiate a specific immune response. Such a substance is called an *antigen*.

To be antigenic, a compound usually must have a high molecular weight (5,000 or greater) and also exhibit certain chemical characteristics. The bodies of bacteria and viruses frequently are antigenic. Also, almost all bacterial toxins have antigenic properties. Tissues, cells, and proteins from humans and animals contain numerous antigens that will elicit immune responses when transplanted into other persons or animals.

Lymphocytes and the body's lymphoid tissues primarily are responsible for acquired immunity. Lymphoid tissue is distributed throughout the body in such a fashion that foreign invaders can be destroyed before they actually become widespread. Lymph nodes of various sizes are distributed throughout the body. In many areas (eg, axillary, inguinal), large groups of nodes exist. Lymphocytes "traffic" through the body, stopping to populate lymph nodes, spleen, thymus, and bone marrow.

Morphologically, all lymphocytes are alike; however, these cells, which are morphologically nondescript, can be divided into two large populations, *T cells* and *B cells*. They are set apart by differentiation patterns, antigen-binding surface receptors, cell-surface antigens, distribution in lymphoid tissues, and, of course, function. In a very general sense, T cells are responsible for forming sensitized lymphocytes that provide *cell-mediated immunity*, and B cells are responsible for producing antibodies that provide *humoral immunity*.

All lymphocytes arise from stem cells in the bone marrow. In birds, B-cell precursors traffic to the bursa of Fabricius, a primary lymphoid organ, where these cells become immunologically committed or programmed to react with specific antigens. In mammals, the analog to the bursa of Fabricius is believed to be the bone marrow or spleen. Upon leaving the primary lymphoid organ, antigen-sensitive B cells traffic to secondary lymphoid tissues (lymph nodes, spleen, Peyer's patches), where they react with the antigens for which they have been

programmed. Antibody molecules are secreted by large lymphocytes and especially by plasma cells (end-stage B cells).

T-cell precursors also arise from stem cells in the bone marrow. They then traffic to the thymus, a primary lymphoid organ, where they become antigen sensitive. In the thymus, these cells divide rapidly, and many of the cells die before leaving. The mature T cells, which are the cells that survive and become immunologically programmed, leave the thymus to circulate through peripheral lymphoid tissue, where they function according to their previous commitment.

In the thymus, these cells not only acquire the ability to regulate B-cell responses to antigen, but also become immunologically committed to act as effector cells for cell-mediated immune responses, such as destruction of tumor cells and rejection of allografts. T cells also act as effector cells to regulate macrophages, lymphoreticular cells that also arise from bone marrow stem cells.

Lymphocytes first circulate freely in the blood and gradually filter into tissues, where they then enter lymphatic channels and are carried into peripheral lymphatic tissue. The cells may be captured in lymphatic tissue by a meshy network of reticuloendothelial (RE) cells for some time. They subsequently may leave the tissue by efferent lymph channels and go to the thoracic duct, where they can then recirculate. In normal situations, the majority of circulatory cells are T cells.

T LYMPHOCYTES: CELLULAR IMMUNITY

Sensitized T lymphocytes are products of the contact between antigen-sensitive T cells and antigen. These T lymphocytes are responsible for host defense through the cell-mediated response (see Figure 1–1). This type of protection or immunity is often referred to as "long-lived immunity," since sensitized T lymphocytes have been shown to have a long life span. The cell-mediated immunity of the acquired response is active in resisting slow-developing bacterial diseases such as tuberculosis rejecting transplanted tissue, mediating delayed-type hypersensitivity, fighting fungal and viral infections, and destroying tumor cells.

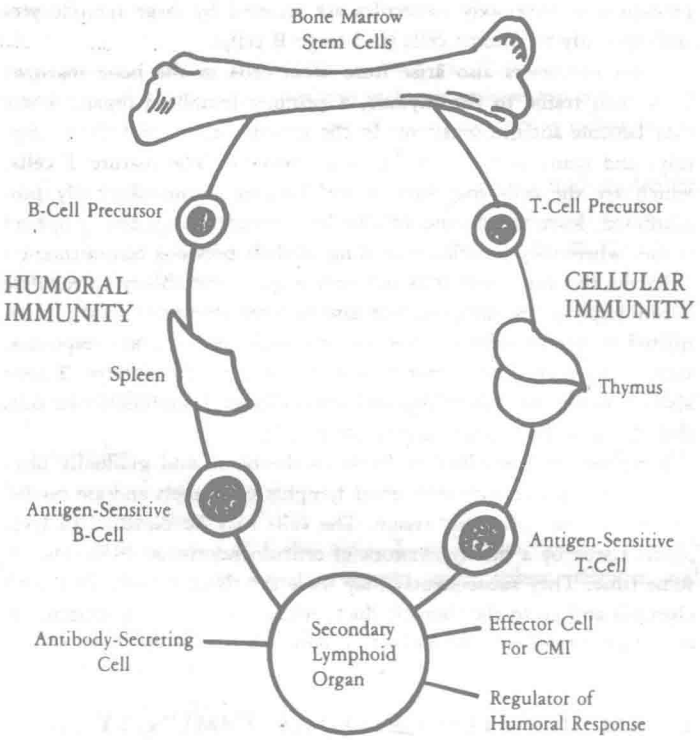


Figure 1–1. Cell-mediated immunity and humoral immunity.

To perform their function, sensitized T lymphocytes may work either alone or in concert with other cells of the lymphoreticular system, particularly macrophages. When the attack on the foreign substance is direct (sensitized cells working alone), the sensitized cells themselves become bound to antigens in the cell membrane of the foreign substance. The sensitized lymphocyte swells and releases a destructive, probably lysozomal enzyme into the intruder. On the other hand, the indirect attack brings into play ancillary cells to aid in the destructive mechanism.

In addition to their role as effector cells of cell-mediated immunity, T lymphocytes serve as regulators of the humoral immune response. Subsets of T lymphocytes function either as helpers or suppressors in the production of antibody by B cells and plasma cells.

ANTIBODIES: HUMORAL IMMUNITY

Antibodies are members of a large family of proteins known as *immunoglobulins (Ig)*. The variability in the structural organization of immunoglobulins has been the object of numerous investigations for many years, and at this time remains an issue of controversy. While a detailed explanation of immunoglobulin structure is not necessary here, it is important to appreciate that the variability in antibody structure is the basis for antibody diversity, and this diversity enables an antibody to recognize a specific antigen.

The specificity of antibodies, their stability, and their ease of production in response to challenge with specific antigen have resulted in the use of these proteins not only for immunologic studies, but as laboratory reagents. In the laboratory, these reagents are used for the detection of antigens in cells, tissues, and body fluids.

Immunoglobulin Classes

The family of immunoglobulins in humans consists of five different classes. Each class demonstrates a distinct chemical structure and exhibits specific biologic activity. These classes are designated IgG, IgA, IgM, IgD, and IgE.

IgG is the immunoglobulin found most abundantly in serum, since the bulk of antibodies that is produced as a consequence of antigen belong to the G class of immunoglobulins. IgG has a long half-life (23 days), crosses the placenta, activates complement, and affords protection against bacteria, viruses, parasites, and some fungi.

IgA is known as the secretory immunoglobulin because of its high concentration in human secretions (tears, saliva). Also, it is the second most abundant Ig in serum. It is capable of crossing the placenta but affords protection to the newborn via colostrum.

The largest of the immunoglobulins is *IgM*. This is the first Ig to peak in response to antigenic challenge and therefore is an important protector immediately after exposure to antigen.

The biologic role of *IgD* remains uncertain. This immunoglobulin appears on the surface of young (neonatal) lymphocytes. It also appears in serum but in only trace amounts. It has been postulated to play a role as a specialized cell receptor.

IgE appears in only trace amounts in serum. This Ig is capable of attaching to skin, basophils in blood, and mast cells in tissue. After binding with these cells, IgE acts as a receptor for specific antigen. Upon antigen-antibody contact, the cells degranulate, release histamine into the circulation, and initiate a hypersensitivity reaction.

Immunoglobulin Structure

All Ig's have a similar structural configuration. However, they make up an immensely diversified family arranged in groups and subgroups determined by antigenic properties and the chemical structure (amino acid sequence) of the molecule itself.

Functionally, all Ig's have two basic characteristics: (1) they are capable of specific binding with antigen, and (2) they participate in effector reactions. It is important to note that these functions reflect different parts of the Ig molecule.

Most Ig molecules consist of four polypeptide chains: two identical *light chains (L)* and two identical *heavy chains (H)*. Each of the five classes of human immunoglobulins has similar sets of L chains, but the H chains are antigenically distinct and are named with Greek letters according to the specific Ig: γ chains in IgG, μ chains in IgM, α chains in IgA, δ chains in IgD, and ϵ chains in IgE. There are two types of L chains found in all five Ig classes. These L chains are termed kappa (κ) and lambda (λ). The κ and λ chains differ in their amino acid sequences, and one or the other type is present in every Ig molecule regardless of the H-chain class. Therefore, any Ig can be named according to its H-L chain composition (eg, IgG can be $\gamma_2\kappa_2$, or $\gamma_2\lambda_2$). These Ig components that designate class and subclass are called isotypes and are present in all persons.

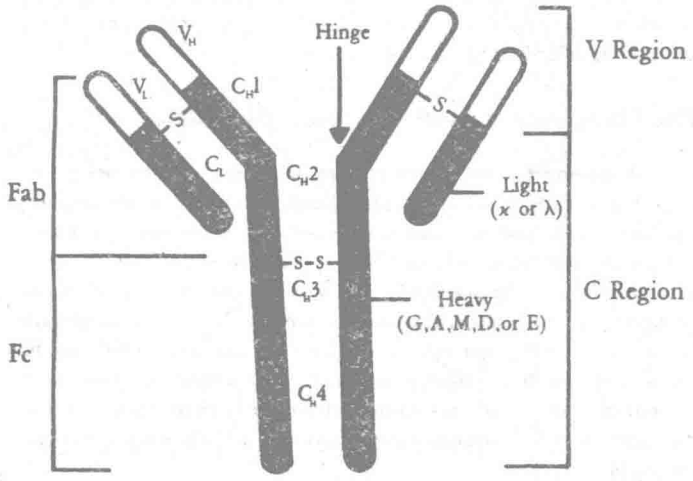
The basic four-chain structural unit is the configuration for IgG.

IgD and IgE also have this four-chain configuration, with differences in H-chain amino acids. IgM is pentameric, having five groups of four chains each. IgA has one to five groups of four chains each.

In the four-chain configuration (see Figure 1–2), two identical L and two identical H chains are held together by disulfide bonds. IgG is an especially stable Ig because of the large number of disulfide bonds binding the chains one to another.

Each polypeptide chain is divided into a *variable (V) region* and a *constant (C) region*. Separate genes code for the C and V regions. A large number of genes code for the V region, and a small number code for the C region. In the V region, the amino acid sequence is variable. This variability is the basis for Ig diversity. The combining site for specific antigen also is in the V region, and it is this site that affords antibody its specificity. Within the C region of each chain, the sequences are identical except for genetic variances (allotypes), which occur in the H chains and the κ chain.

Figure 1–2. Immunoglobulin structure.



The polypeptide chains are actually three-dimensional chains of amino acids that are extensively folded with disulfide bonds to form areas called *domains*. In H chains, the domains are V_H, C_H1, C_H2, C_H3, and C_H4; and in L chains, they are V_L and C_L.

The entire immunoglobulin molecule consists of two portions, the *antigen-binding fragment (Fab)*, which contains the combining sites for antigen; and the *crystallizable fragment (Fc)*, which regulates biologic activity. Enzymatic digestion of IgG molecule with papain yields two Fab fragments and one Fc fragment. The Fab fragment remains capable of binding one antigen, and the Fc portion retains most of its biologic activity, that is, complement fixation, cutaneous anaphylaxis, placental crossing, etc. Other enzymes and chemicals may be used to split Ig molecules, producing slightly different fragments of the Fab portion. The hinge region is located in the constant region of the H chains between domains C_H1 and C_H2. This area is more flexible, or more exposed to enzymes, and thus, papain acts at this point to produce Fab and Fc fragments. In polymeric Ig's (IgM and IgA), there is a small glycopeptide chain that facilitates polymerization of the basic Ig units.

In secretory IgA is found a single polypeptide chain, a secretory component, synthesized by epithelial cells whose function may be to transport IgA across mucosal tissues.

The Complement System

In most mammals, there exists a series of at least 15 circulating proteins that mediate many of the physiologic effects of antigen-antibody reactions. These proteins may react with each other, with antibody, or with components of cell membranes. This series of mediating proteins is known as the *complement (C) system*. When this system of distinct proteins is activated, the biologic activity that can result is quite varied. The complement system can mediate the destruction, via lysis, of many kinds of cells. In addition, upon activation, this system of proteins can induce histamine release from mast cells, mediate phagocytosis, aid in inflammatory responses, and influence direct migration of leucocytes.

Under normal conditions, these proteins circulate in an inactive

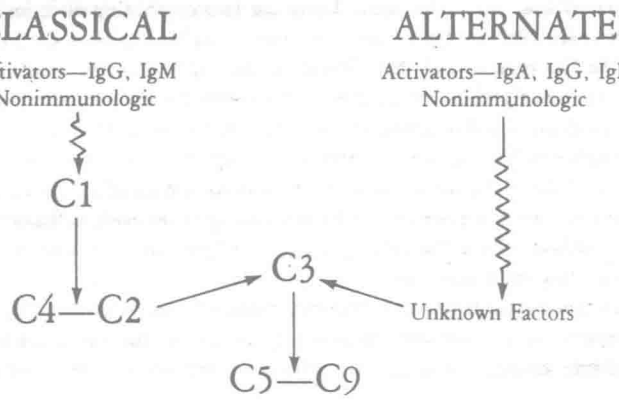
form. In order for the system to perform its functions, each component must be activated sequentially. Therefore, the "turning on" of the system is not represented by a single event, but rather by a series of events taking place in a cascade-like fashion. The classical components of the system are numerically named (C1 through C9). These classical components may in turn consist of different protein molecules (eg, Clq, Clr, and Cls subunits of C1). Therefore, the classical components plus their subdivisions collectively compose the complement system.

There are two pathways by which this system can be activated, the *classical pathway* and the *alternate (properdin) pathway* (see Figure 1–3). Although these pathways are triggered by different substances, the reaction sequence of the end components (C5–C9) is common to both pathways.

The classical pathway can be activated by immunoglobulins (IgG and IgM), antigen-antibody complexes, or nonimmunologic substances (eg, C-reactive protein). The alternate pathway also can be triggered by either immunologic substances (IgA, IgE, and some IgG) or nonimmunologic substances (lipopolysaccharides).

For cells to become damaged as a result of complement activation, the end components (C5–C9) of the system must become membrane

Figure 1–3. Complement pathways.



bound. The intact complex of C5, C6, C7, C8, and C9, whether generated through the classical or the alternate pathway, can cause cellular cytotoxicity or lysis when bound to cell-surface membranes.

Several human cell types, such as B lymphocytes, erythrocytes, and macrophages, possess receptors on their cell surface for C3 and C4. The biologic significance of these receptors remains unclear, but it has been speculated that these receptors may play a role in the induction of immune responses. Fragments of components of C3 and C5 have been shown to induce chemotactic activity, release of histamine from mast cells, and release of lysosomal enzymes (C5 only).

Congenital defects in certain complement components are associated with predisposition to disease, illustrating the role of complement in maintaining health. For example, persons with systemic lupus erythematosus may be characterized by having deficiencies in Clq, Clr, Cls, C2, C4, or C5. Persons with deficiencies in C3 suffer from repeated, life-threatening infections.

Antigen-Antibody Reactions

When an antibody is formed in response to a particular and specific antigen, it is different from antibodies produced in response to any other antigen. Such a reaction exemplifies the specificity and diversity of the immune response.

The antigen-binding site of an Ig molecule is that part that combines with a specific antigen. There are two combining sites for each four-chain (2L and 2H) molecule. These combining sites are located in the Fab region, and there is one combining site per Fab region.

When antigen meets antibody, the union can be expressed in different degrees of "tightness of fit." The tighter or more exact the fit, the more stable the antigen-antibody complex. When a precise fit is accomplished, the antibody is referred to as a high-affinity antibody. Likewise, when a poor fit results from antigen-antibody combination, the antibody has a low affinity for the antigen, and the antigen-antibody complex is unstable.

In the clinical laboratory, there are numerous serologic/immunologic tests that assay, via antigen-antibody reactions, for the quantity of antibody present in serum. In this way, the activity of a patient's

immune system can be monitored. There are many types of test systems designed to monitor antibody levels (titer) in serum. Among the most common are precipitation reactions, radial immunodiffusion (also precipitation phenomena), immunoelectrophoresis, agglutination reactions, and complement-fixation reactions. In addition to these test systems, immunocytochemical techniques are used for assaying antibody in serum and also for identification of antigens in cells, tissues, and body fluids.

In a *precipitation reaction,* a soluble antigen (proteins or polysaccharides) is used. The combination of antigen with antibody forms lattices or aggregates, which, when large enough, become visible. In assays of this nature, the antibody concentration (dilution of serum) varies while the antigen concentration remains constant. The highest dilution of serum in which aggregates are visible is reported as the antibody titer.

Radial immunodiffusion (RID) in gels works according to the same theory as precipitation reactions in the test tube. However, in RID, antigen and antibody diffuse through a gel matrix and form aggregates within the gel.

Immunoelectrophoresis (IEP), a variation of RID, employs an electric current to cause antigens to migrate in a gel. Antibody is placed in a trough cut in the agar gel and the diffusion of antigen and antibody then takes place.

In contrast to precipitation reactions, in which the antigen is soluble, *agglutination reactions* require a particulate or insoluble antigen (eg, RBCs). Erythrocytes may themselves be agglutinated by specific antisera, or they may serve as indicators onto which antigen has been coated. For example, RBCs are coated with IgG (IgG is the antigen) and then reacted with anti-IgG (anti-antibody); an antigen-antibody reaction occurs and a visible end product is seen because of the agglutinated antigen-coated RBCs.

Complement-fixation test systems, frequently used by serologists, employ the fixation of complement to antigen-antibody complexes as an indication of circulating antibody in serum.

A complement-fixation system includes a test system (known antigen and unknown antibody), an indicator system (known antigen [sheep red blood cells] and known antibody [anti-SRBCs]), and com-