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## 15

# Clinical Laboratory Methods for Detection of Antigens & Antibodies

Clifford Lowell MD, PhD

This chapter discusses the tests used for the detection of **antibodies** and **antigens**. The presence of an antibody to a defined protein or compound depends on the immune response of the patient; hence antibody detection is used to quantitatively and qualitatively evaluate normal and abnormal immune responses. The increasing use of molecularly cloned antigens has dramatically improved the precision of antibody detection. In contrast, antigen detection is usually used to determine the presence of foreign proteins or compounds (eg, infectious agents or drugs). Antigen detection as the characterization of the cell surface phenotype (identification of molecules expressed on the cell surface) is a fundamental method for the analysis of cells, particularly hematopoietic cells. The widespread use of **monoclonal antibodies (mAbs)** as specific reagents for defined antigens (either cellular proteins or components of pathogenic organisms) has dramatically improved methods of antigen detection.

Although the technologies for detecting antigens and antibodies have become increasingly automated, the scientific principles that underlie these methodologies remain the same. Fundamentally, detection of antibodies and antigens depends on the formation of antibody-antigen complexes. One of the binding partners (the antibody or the antigen) is defined, often labeled, and is used as a probe to search for the other partner. Hence, we begin this chapter by reviewing the principles of antibody-antigen binding and then provide an overview of the specific methods that depend on these principles.

## ANTIGEN-ANTIBODY BINDING

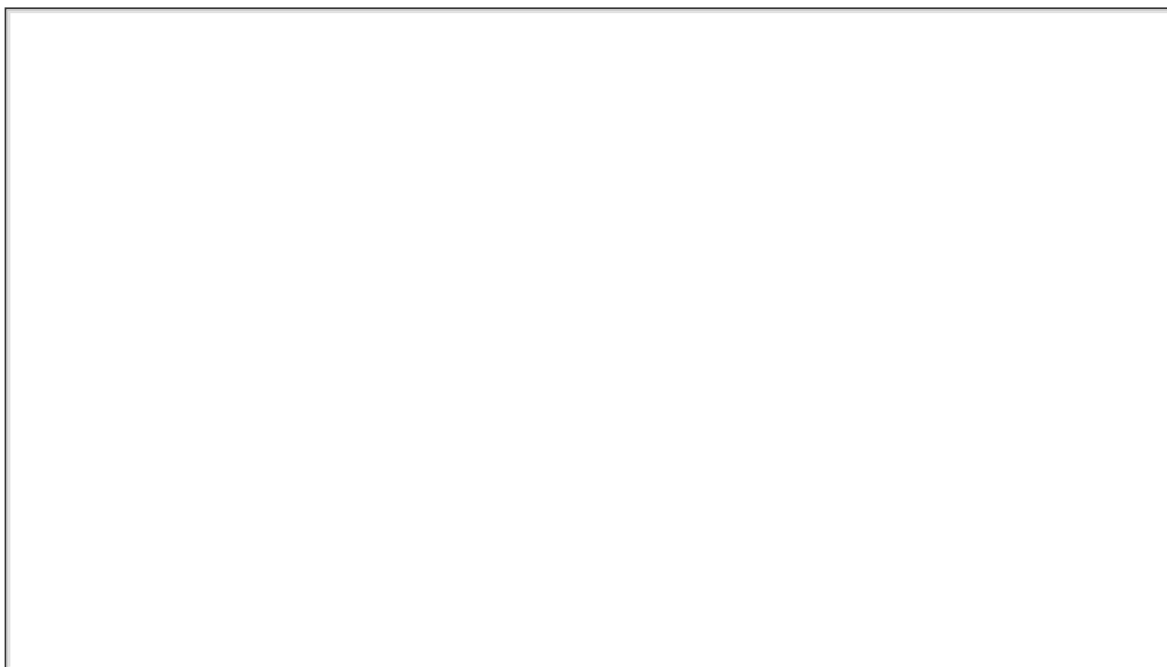
The process of antibody binding to antigen (the formation of immune complexes) underlies much of immunologic testing. In its simplest form, a specific mAb is used to look for a single antigenic epitope, and formation of the complex is monitored by precipitation of the complex or by the presence of a tag (fluorescent, radioactive, or enzymatic) on the antibody. Reciprocally, use of molecularly cloned proteins as model antigens can be used to look for specific antibodies that recognize this protein; furthermore, one can determine the

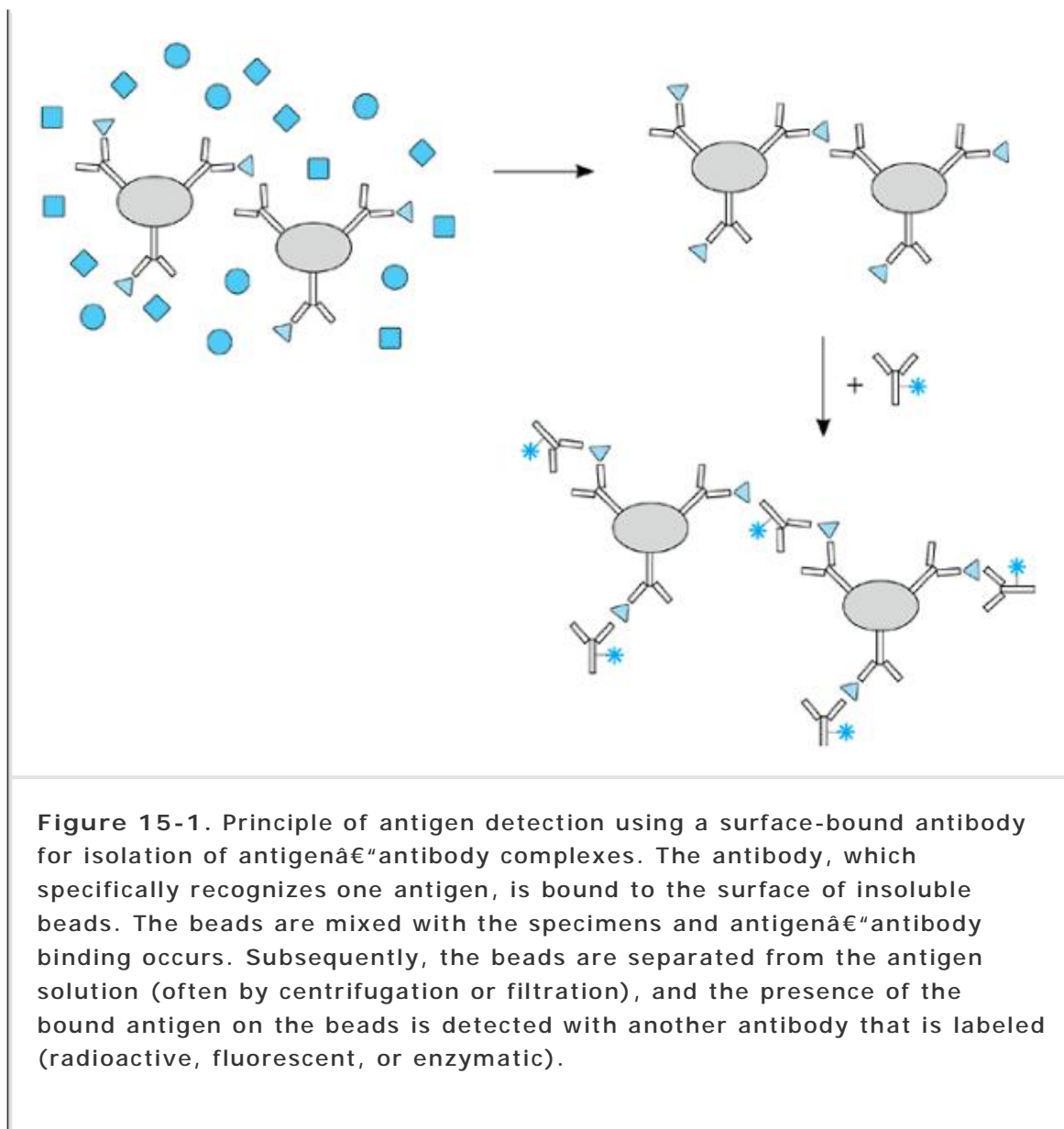
isotype of the reacting antibodies (IgG, IgM, or IgE). More complex mixtures of antibodies-antigens can also be used. For example, the antibodies for a specific test may be the polyclonal sera from an animal immunized with the antigen (or sera from a patient with a known disease or condition), and the antigens may be a complex mixture of proteins, carbohydrates, and nucleic acids from an infectious agent. Often the antibody or antigen is fixed to a solid support that allows the immune complex to be separated from other components of the binding mixture (Figure 15-1). This process, often referred to as immunoprecipitation, is usually used when the antibody or antigen on the solid support is present in vast excess in the mixture. Immunoprecipitation requires some process or method for separation of the immune complexes, such as centrifugation or filtration. The amount of the complex is then determined by a second binding reaction with a labeled reagent.

When the antibodies and antigens are present in equimolar ratios, they form insoluble complexes that precipitate naturally. Light scattering (or formation of turbidity) can be used to monitor accumulation of precipitate in the solution. In this circumstance, the relative concentrations of the antigen and antibody are the most important determinants for formation of the complexes (Figure 15-2). Maximum precipitation occurs when the antibody-antigen concentrations are equivalent (**zone of equivalence**) and decreasing amounts of precipitate (or very small complexes) are formed in zones of antigen or antibody excess. Thus

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formation of insoluble immune complexes can be used to quantify the amount of antigens if a known concentration of antibody is used (or vice versa). The **prozone** phenomenon occurs when antibody or antigen is in vast excess and suboptimal immune complexes form. This phenomenon can lead to misinterpretation of tests when large amounts of antibody are present (eg, in multiple myeloma or polyclonal gammopathies) or when antigens are improperly diluted (eg, in agglutination reactions-see later section).





## *Testing Methods That Depend on Formation of Immune Complexes*

### **Immunodiffusion**

Immunodiffusion is the simple technique by which antigens and antibodies are placed in separate wells within a semisolid support (eg, agar) then allowed to mix through the support by diffusion. When a zone of equivalence is reached, a line of precipitation occurs, which is visible when light is passed through the gel (Figure 15-3). This simple technique launched the field of serology in the first part of the 20th century and remains in use today. Double diffusion in agar, often referred to as **Ouchterlony** analysis, characterizes the relationship between different antigens. In this methodology, antigens are placed in wells of

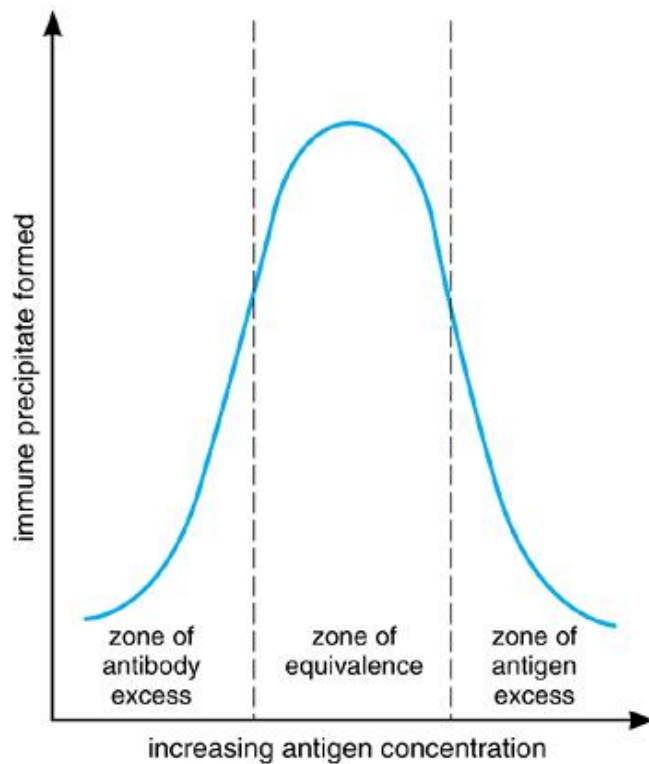
agar (poured in small dishes or on glass slides), and antibody is placed in a center well. The reactants are allowed to diffuse together, and the nature of the precipitation lines between the wells is characterized (Figure 15-4). Although simple, immunodiffusion methods are limited by insensitivity and by the requirement for relatively large amounts of precipitating antigens or antibodies. Also the rate of diffusion can make the test time-consuming. This latter problem is often solved by placing the agar matrix in an electric field, which drives the antigens and antibodies together—a technique called **countercurrent immunoelectrophoresis (CIE)**. CIE also increases the efficiency of antigen–antibody complex formation and often increases the sensitivity of the assay. Table 15-1 lists examples of antibodies and antigens that are still assayed by immunodiffusion methods.

## Nephelometry

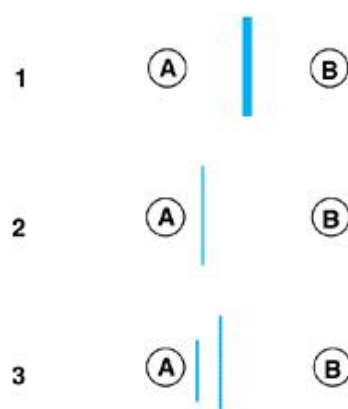
In nephelometry, the formation of immune complexes in solution is monitored by spectrometry. Scattering of an incident light is used to detect

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complexes in dilute solutions of antigens and antibodies. In more concentrated mixtures of reactants, the immune complexes turn the solution cloudy, which can be measured by light absorption or turbidimetry. Nephelometric determination of antigens is performed by addition of constant amounts of highly purified and optically clear specific antisera to varying amounts of antigen. Mixing is performed in a cuvette within a light beam, and the progressive formation of immune complexes is measured in a photoelectric cell as the optical density (Figure 15-5). Accurate measurement of antigens can be made only in the ascending limb of the precipitation curve (see Figure 15-2) where there is a direct linear relationship between antigen concentration and optical density. Thus, samples with high concentrations of antigens may require dilution for accurate measurements. The amount of optical density can be measured at a single instant following addition of antibody to the antigen—the so-called endpoint determination. This method is hampered, however, by the fact that many components in serum samples, such as lipids or preformed immune complexes, can contribute substantially to background light scattering. To avoid this problem, modern nephelometers subtract background light scatter prior to addition of antisera and then measure formation of immune complexes continuously. The determination of the kinetics of immune complex formation, a process called **rate nephelometry**, provides a more accurate quantitation of antigen levels. In this method, the amount of antigen is proportional to the peak rate of immune complex formation as long as the reaction occurs on the ascending limb of the precipitation curve (or at slight antibody excess). Automated nephelometers confirm that the reaction is in antibody excess by adding known amounts of each antigen being analyzed (so-called calibrators) to the reaction and confirming increased immune complex formation. Nephelometry is used to measure the levels of a variety of serum antigens and proteins (Table 15-2).



**Figure 15-2.** Antigen-antibody precipitin curve. Typical precipitin curve resulting from titration of increasing antigen concentration plotted against amount of immune precipitate formed. The amount of antibody is kept constant throughout.

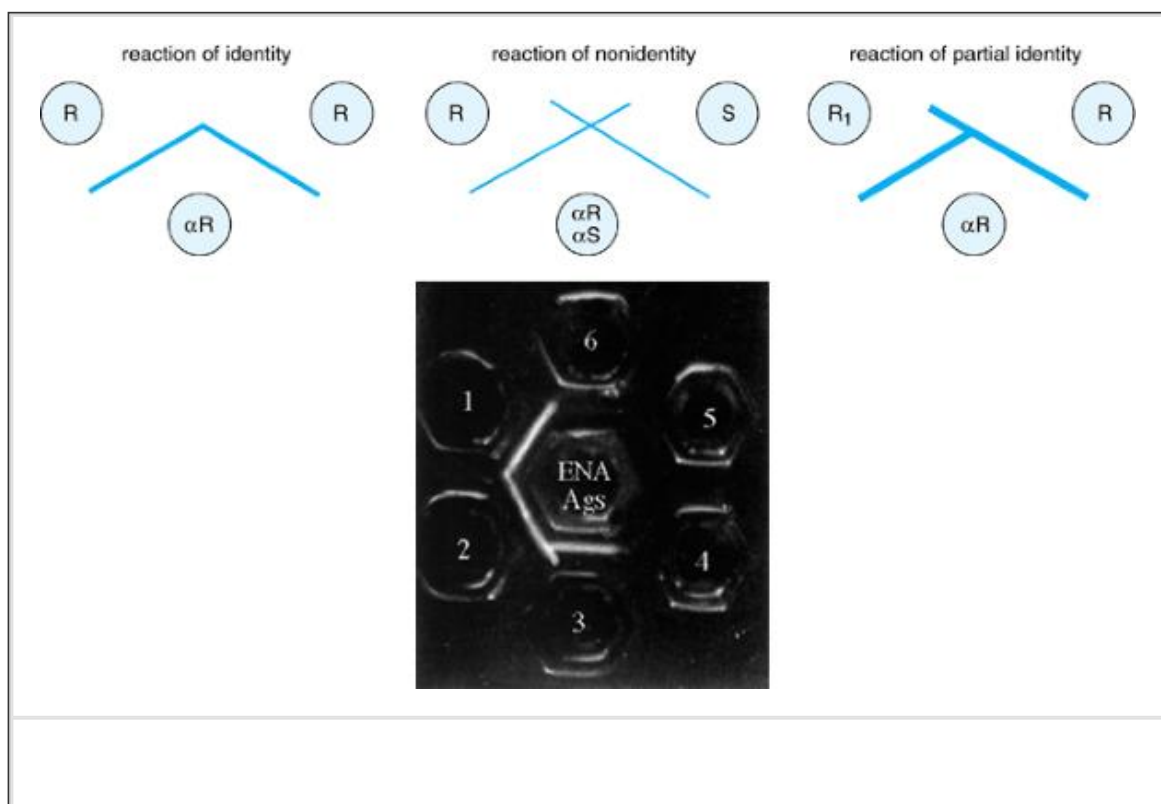


**Figure 15-3.** Reactions in simple double diffusion. In (1) antigen A and antibody B react equidistantly and intensely at equivalence. In (2) antigen A

is present in reduced concentration or has not diffused as rapidly owing to size or charge, forming a precipitin line closer to the antigen well. In (3) a contaminant or impurity present in antigen A is reacting with antibody B.

## Complement Fixation

The formation of immune complexes in solution can also be monitored by the ability of these complexes to fix and consume complement proteins. Because of their relative ease and low cost, complement fixation (CF) assays are widely used to detect immune responses to infectious agents (eg, coccidioidomycosis, histoplasmosis, and others [Table 15-3]). Many CF assays have been replaced by more sensitive, enzyme-based methods (see following section) and are now usually used as confirmatory assays. The CF assay is a two-stage reaction: In the first stage the antigen is mixed with patient sera with a known amount of complement. Formation of immune complexes leads to fixation of complement. In the second stage of the reaction, the amount of residual hemolytic complement activity is determined (Figure 15-6). Results are expressed as the dilution of patient sera in which complement consumption is lost. CF assays usually reflect IgG titers against the antigen and are best used to diagnose disseminated disease. Since CF assays work by measuring functional activity (RBC lysis) of complement, they can be complicated by the presence of any anticomplement activity in the patient serum, such as preformed immune complexes, heparin, or chelating agents.



**Figure 15-4. Top:** Reaction patterns in angular double immunodiffusion (Ouchterlony). Antigens are shown as R, R<sub>1</sub>, and S, and the reactive antibodies are  $\hat{I} \pm R$ , and  $\hat{I} \pm S$ . As the antibodies and antigens diffuse outward from their spots in the gel, precipitation reactions occur. If the antigens are the same (the reaction of identity), the precipitin lines fail to cross because all the reactive antibody will precipitate. However, if the antigens are unrelated (the reaction of nonidentity) and the antibody mixture contains immunoglobulins that react with both antigens, the precipitin lines cross because the reactions occur independently of one another. If antigenic determinants are partially shared between antigens, in the reaction of partial identity, the precipitin lines cross in only one direction. In the case shown, antigen R<sub>1</sub> shares some antigenic determinants with antigen R, but not all. **Bottom:** An example of a screening immunodiffusion assay used to detect the presence of antibodies against extractable nuclear antigens (ENA) in various patients. Patients 1 and 2 have lines of identity with the ENA; patient #3 has only a partial reaction to some of the ENA. Other patients are nonreactive.

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## Cryoglobulins

Cryoglobulins are serum immunoglobulins (Igs) that precipitate at temperatures less than 37°C. The presence of cryoglobulins in the blood is determined by incubating serum samples at 4°C for several hours, then looking for the formation of a precipitate. The precipitated proteins are then isolated by centrifugation, solubilized in 37°C buffer, and assayed for the presence of immunoglobulins by nephelometry or immunofixation electrophoresis. The three types of cryoglobulins are

**Table 15-1. Antibodies routinely assayed by immunodiffusion or electroimmunodiffusion.**

Antibodies to extractable nuclear antigens (ENA)-anti-sn RNP, anti-Sm, anti-SSA (Ro), anti-SSB (La), anti-Scl-70 (topoisomerase I)

Antifungal antibodies (Coccidiomycosis, Aspergillus, Histoplasmosis)

Anti-*Entamoeba histiolytica*

Type I: Cryoprecipitable monoclonal Ig or light chains.

Type II: Monoclonal Igs, most often of the IgM isotype, that bind to normal polyclonal IgG. Antibodies that recognize normal IgG are called **rheumatoid factors (RFs)**; hence type II cryoglobulins are cold-precipitable monoclonal RFs bound to their antigen (polyclonal IgG).

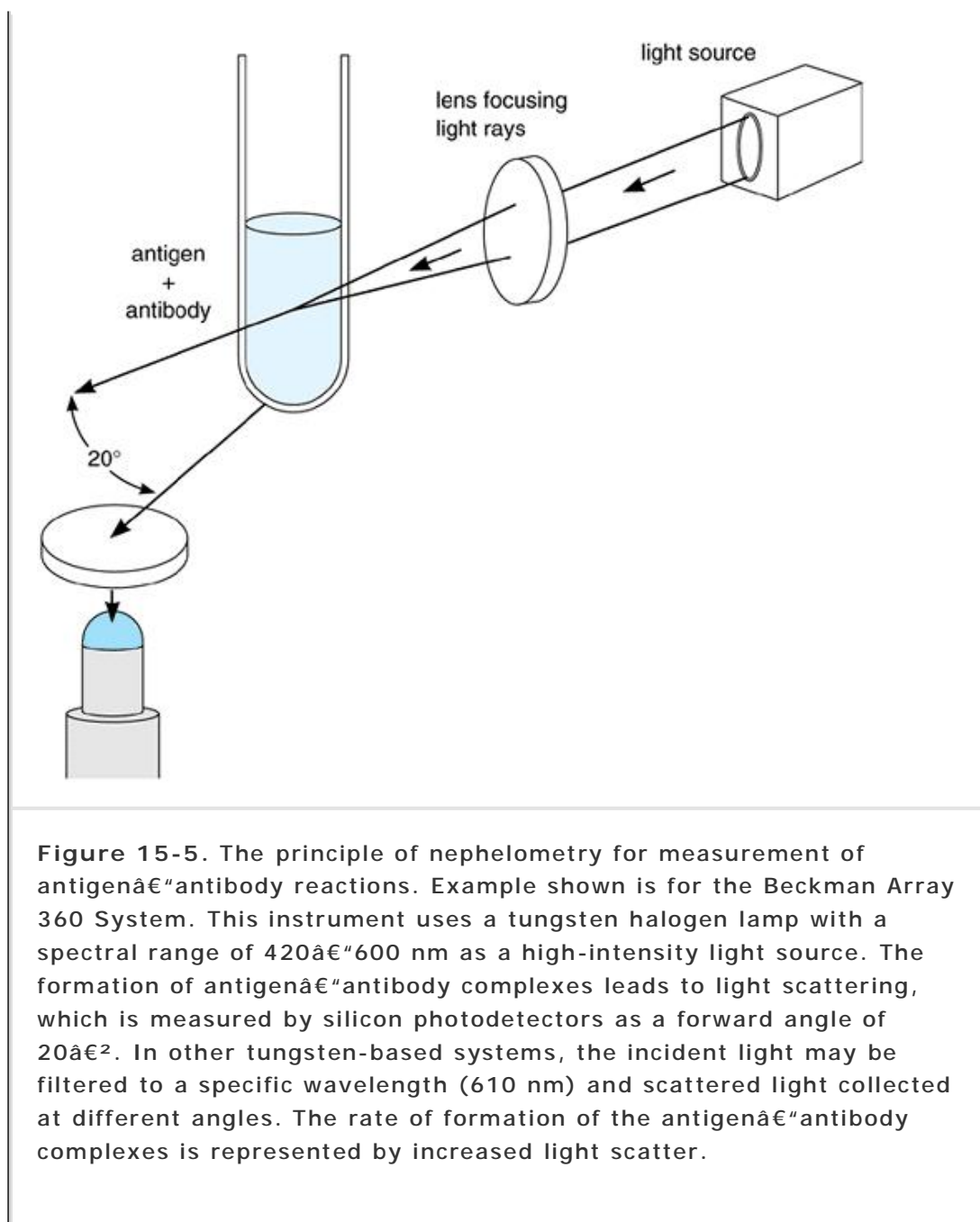
Type III: Polyclonal RFs (usually IgM or IgA isotypes) bound to polyclonal IgG.

Type I cryoglobulins usually are associated with malignancy and often are found in high concentrations in the serum (greater than 5 mg/mL). These can cryoprecipitate at physiologically relevant temperatures and, therefore, can present with cold-induced symptoms, such as cold-induced digital ischemia. Type II cryoglobulins are associated with chronic infections, most notably hepatitis C. They generally do not precipitate at physiologic temperatures and usually present as immune complex disease (eg, cutaneous vasculitis, glomerulonephritis). Type III cryoglobulins

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occur in autoimmune disorders, such as systemic lupus, and a variety of chronic viral, bacterial, and parasitic infections. Type III cryoglobulins typically are found in low concentrations in the serum (less than 1 mg/mL) and basically are circulating immune complexes.





## ***Testing Methods in Which the Antigen or Antibody Is Fixed to a Solid Surface***

### **Agglutination Assays**

Agglutination (or aggregation) of antigen-coated particles by reactive antibodies is among the most time-honored of immuno-assays. Much of this testing technology has now been replaced by more sensitive methods for antibody detection; however, agglutination-based assays are still routinely used in blood

bank testing to classify red blood cell (RBC) types and to look for autoimmune anti-RBC antibodies (reviewed in Chapter 17). Although very simple to perform, all agglutination assays suffer from the fact that they are semiquantitative.

**Table 15-2. Antigens and antibodies routinely assayed by nephelometry.**

Complement proteins (C3 and C4)  
Immunoglobulins (IgM, IgG, IgA)  
Rheumatoid factor  
 $\hat{I}\pm_1$ -Antitrypsin  
Ceruloplasmin  
Microalbumin  
Prealbumin

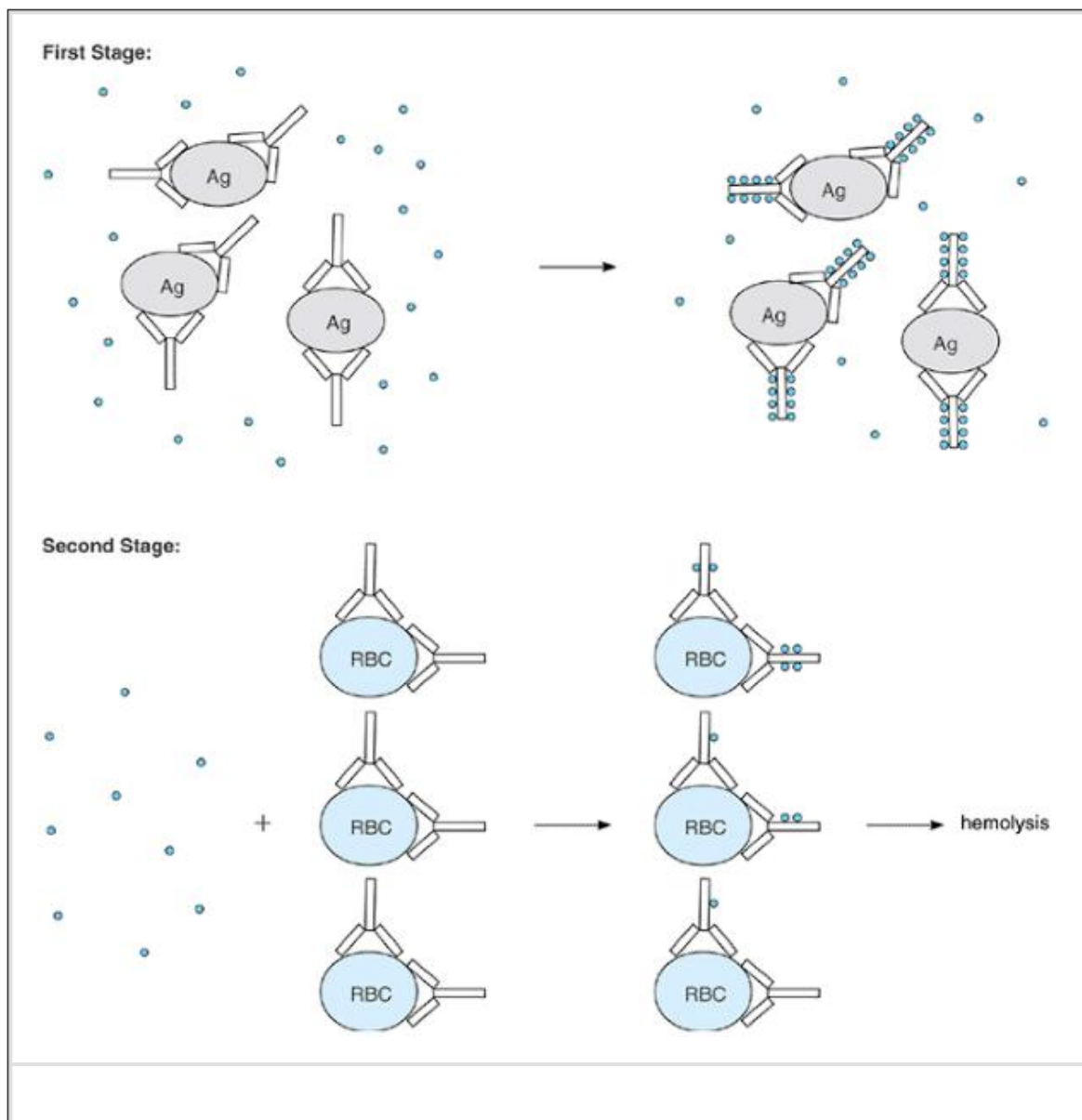
Agglutination assays that test for the presence of an antibody depend on the availability of a particle that is coated with the appropriate antigen. The particle can be an RBC displaying its natural blood group antigens or a synthetic particle (eg, a latex bead) which is artificially coated with antigen. In the presence

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of specific antibody, the particles aggregate. The formation of aggregates can be seen visually in a tube, in a microtiter well, or even on a simple glass slide (Figure 15-7). This process can be reversed and used to detect antigens. In this case, the particle is coated with specific antibody to look for antigens that are capable of binding and agglutinating the particles. A special category of agglutination involves spontaneous agglutination of RBCs by certain viruses, such as influenza virus. These viruses contain surface proteins that bind to RBC proteins and aggregate the RBCs. One can block this reaction with antiviral antisera that adsorbs to the viral surface and prevents interaction with the RBCs. This inhibition of viral hemagglutination can be used to titer the antiviral antibody activity of patient sera.

**Table 15-3. Examples of antibodies routinely assayed by complement fixation (most often as a confirmatory test).**

Antifungal antibodies (Coccidiomycosis, Histoplasmosis)  
Antiviral antibodies (adenovirus, herpes virus, influenza)  
Anti*Mycoplasma pneumoniae*  
Antirickettsial antibodies

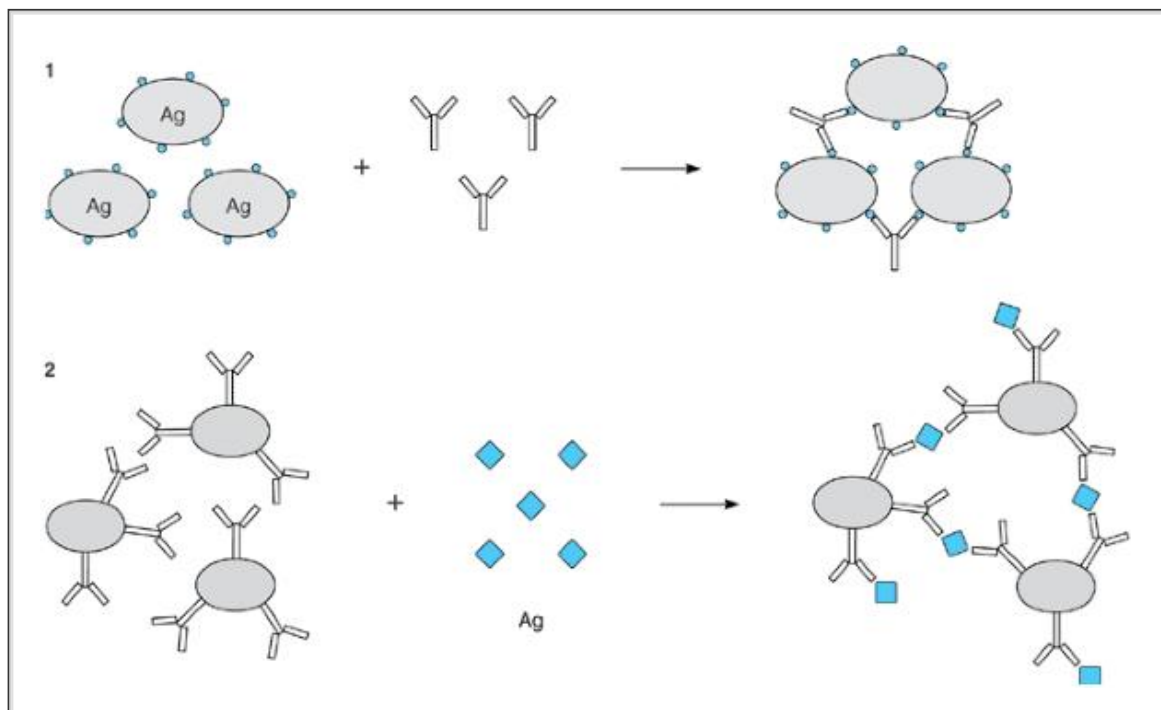


**Figure 15-6.** The principle of complement fixation assays. In the first stage, antigen and antibody react in the presence of complement (shown as dots). The antigen-antibody complexes fix complement proteins, resulting in the consumption of some but not all of the available complement components. In the second stage, the activity of the residual complement is determined by adding an excess of sensitized sheep red blood cells (RBCs); these fix the residual complement and undergo hemolysis. Thus, a reciprocal relationship exists between the amount of antigen in the first stage and the residual complement left over for the second stage.

A frequently used hemagglutination assay is the **heterophile antibody test** for acute infectious mononucleosis (often referred to as the **monospot test**). Heterophile antibodies are IgM antibodies that, probably, result from the cross reaction between antigens on the agent of infectious mononucleosis (the Epstein-Barr virus) and equine RBC antigens. Incubation of horse RBCs with sera from patients with infectious mononucleosis

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leads to agglutination—the presence of anti-EBV antibodies in the patient can then be confirmed by more specific bead-based or immunofluorescent methods.



**Figure 15-7.** Examples of agglutination reactions. In (1) the particles are coated with antigens; for example, latex beads that are artificially coated with antigen or RBCs that are displaying their own natural surface antigens. Following addition of antibodies, the particles are aggregated by bridging

antibody reactions. In (2) particles are coated with the test antibody and exposed to the antigen mixtures. If the antigens have multiple epitopes, antibodies on different particles will react with the same antigen molecule and become aggregated.

In **latex agglutination** assays latex beads are coated with either antigen (to look for specific antibody) or a defined antibody (to assay for antigen). Although widely used in the past, most have been replaced by more sensitive, automated methods. Examples include particles coated with human immunodeficiency virus, hepatitis B virus, or various fungal antigens to assay patient sera for evidence of prior infection (as reflected by the presence of specific antibodies). The classic pregnancy test is a latex agglutination assay in which beads coated with antibodies to human chorionic gonadotropin are mixed with patient urine to detect the presence of the hormone.

In response to some infectious agents (most commonly *Mycoplasma pneumoniae*) or during autoimmune reactions, patients produce antibodies that have the particular ability to agglutinate RBCs at 4Å°C. These antibodies, referred to as **cold agglutinins**, are assayed by incubation of serial dilutions of patient sera with a 1% RBC solution at 4Å°C overnight and then examined for agglutination. True cold agglutinins go back into solution at 37Å°C, and therefore reincubation of the sample at 37Å°C should result in deagglutination of the RBCs. This simple assay is widely used as a surrogate marker for immune response to *M pneumoniae* infection; indeed, 50â€“80% of acutely infected patients produce cold agglutinins, which are typically IgM antibodies.

Screening assays to look for antibodies against rare bacterial pathogens continue to be performed using agglutination reactions. Examples include assays for brucellosis or *Francisella* infections. In these cases, the particle is the organism itselfâ€”incubation of whole, fixed bacteria with patient sera leads to agglutination of the organisms if the patient is mounting an immune response against the agent. These assays suffer from low sensitivity and cross-reactivity (eg, patients infected with *Tularensis* can have a false-positive test for *Brucella* antibody). They are extremely easy to perform, however, and are very inexpensive; hence they remain in common use today. Positive tests are confirmed by other methods (see later section).

## Enzyme-Linked Immunoabsorbent Assays (ELISA)

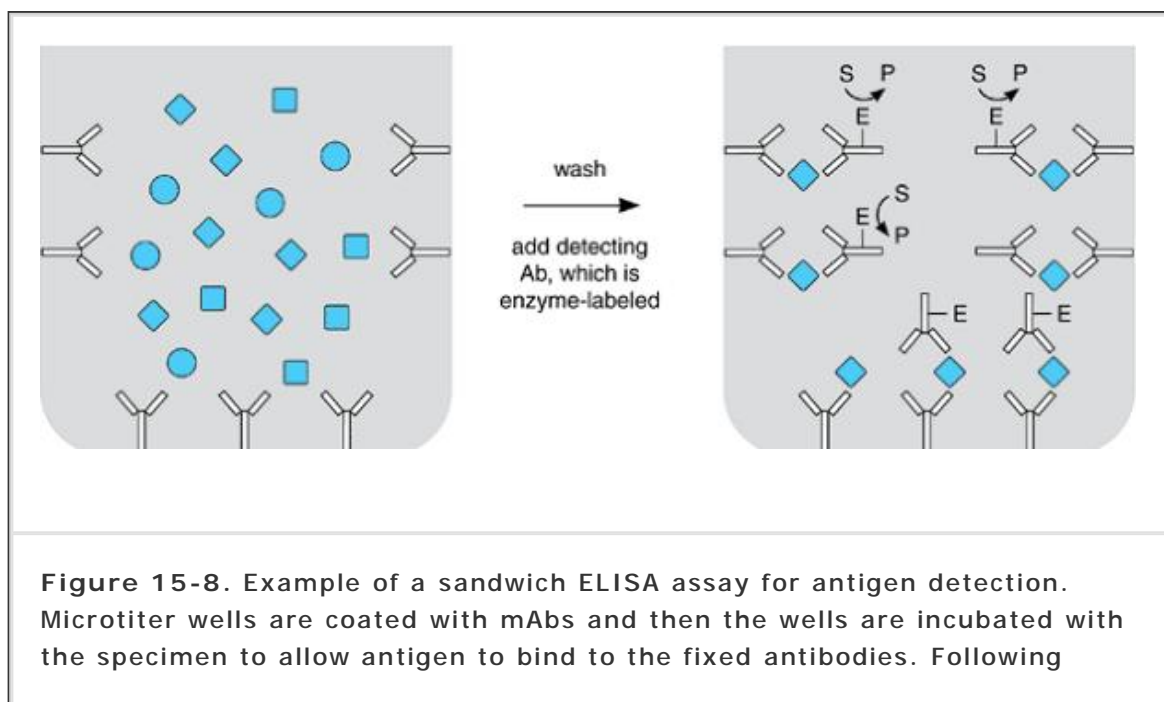
In ELISA, the antibody (or antigen) is fixed to a surface, such as a well of a microtiter plate or a plastic bead. The test sample is applied and bound material is detected by a secondary, enzymatically labeled antibody. These assays are rapid, simple, and easily adaptable to automated analyzers. They require

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highly purified reagents; use of mAbs and recombinant antigens have greatly facilitated the widespread use of ELISA.

The most common version of the ELISA is the **sandwich assay** (Figure 15-8). A mAb to a specific antigen is fixed to microtiter plates (small plastic plates, treated to maximize protein binding, that contain 96 wells with a volume of 200  $\mu$ L each). The wells are incubated with serial dilutions of the patient sample to allow binding of the antigen to the surface-bound antibody, then washed. Bound antigen is detected with a secondary antibody that is enzymatically labeled. After another washing, the wells are incubated with a substrate for the enzyme and the enzymatic reaction (appearance of product) is determined. This basic ELISA method can be modified in a variety of ways: Wells can be coated with antigens to detect specific antibodies in patient sera (the isotype of the antibody can be determined by using either anti-IgM or anti-IgG as the second-step reagent). An alternative approach to measuring antibodies to specific antigens is the **antibody capture assay**. Wells are coated with anti-IgM and anti-IgG, resulting in capture of all patient IgM or IgG. The wells are then incubated with a known antigen, followed by a mAb specific for that antigen (Figure 15-9).

Common enzymes used in the detection step are horseradish peroxidase and alkaline phosphatase; these enzymes can be covalently coupled to mAbs without affecting either the antigen-binding capacity of the antibody or inhibiting the activity of the enzyme. A variety of substrates can be incubated with these enzymes to produce colored products that can be quantitated using microtiter plate spectrophotometers. Because the last step of the assay is enzymatic, these assays have the advantage of being extremely sensitive. Many modern autoanalyzers use horseradish peroxidase substrates that produce **chemiluminescent** products, further enhancing sensitivity. Measuring the rate of the reaction, rather than simply the extent of the reaction at a single fixed instant, allows ELISA to be accurate quantitatively. The amount of antigen is determined by comparison to a standard curve generated with known amounts of antigen.



**Figure 15-8.** Example of a sandwich ELISA assay for antigen detection. Microtiter wells are coated with mAbs and then the wells are incubated with the specimen to allow antigen to bind to the fixed antibodies. Following

washing, an enzyme-labeled (shown as E) detecting antibody is used to reveal antigen bound to the surface. Plates are washed free of unbound detecting antibody and substrate (S) is added to the wells. The formation of colored (or fluorescent) product (P) is monitored in a microtiter plate spectrophotometer.

In many situations, the sensitivity of these assays can be further enhanced by use of additional steps in the reaction. Most commonly, this is done by use of secondary antibodies labeled with the vitamin biotin; wells are then incubated with enzymatically labeled avidin (a protein component of egg white), which binds biotin with extremely high affinity ( $K_D$  of  $10^{-15}$  M) and specificity (Figure 15-10). **Biotin/avidin-enhanced immunoassays** allow for antigen detection using extremely small samples. Biotin/avidin-enhanced assays are also used in immunofluorescent assays (see later discussion).

The popularity of the **enzyme-linked immunoassays (EIA)** in clinical practice reflects their easy adaptation to automated analyzers that allow substantially increased throughput in the laboratory. Automated machines can handle microtiter plates, using robotic arms to automatically fill and wash the wells and to move the plate to a coupled spectrophotometer.

## Microparticle Enzyme Immunoassays

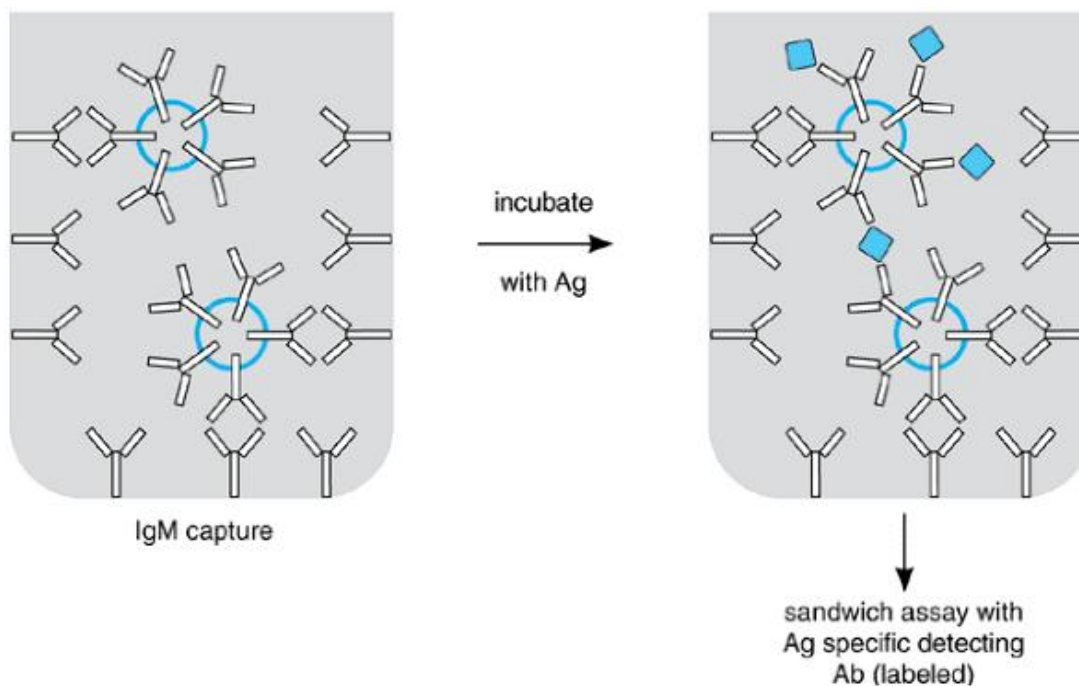
A variation of the ELISA uses small beads (1 mm in size) coated with the appropriate antigen or antibody. The microparticle enzyme immunoassay (MEIA) is an extension of the bead assay. In this case, submicron-sized particles are coated with antibody or antigen. The advantage of these tiny particles is that their relatively large surface area leads to higher concentrations

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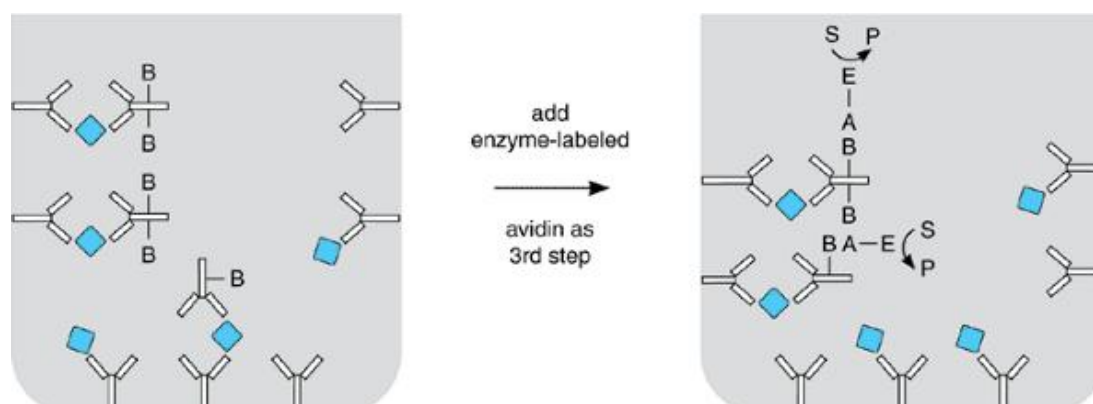
of antibody or antigen. As a result, binding reactions can be completed in a very short (15–30 minutes) time. The assay proceeds as a standard sandwich assay, but performed in suspension. The particles then are separated from the unbound reagents by filtration through glass fiber filters, which irreversibly bind the microparticles. The filters are exposed to the appropriate substrate (depending on the enzyme label used), and the extent of the enzymatic reaction is measured using automated analyzers. Table 15-4 provides a partial list of antibodies and antigens measured by MEIA. A number of tests traditionally based on immunodiffusion, CF, or agglutination reactions are now determined by MEIA technology on automated analyzers. In addition to the advantage of automation, these new methods can

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offer a tenfold to 1000-fold increase in sensitivity of detection compared with the older manual methods.



**Figure 15-9.** Example of antibody capture method. In this method, microtiter plate surfaces are coated with antibodies that bind specifically to patient IgM (shown as pentameric structures) or IgG (not shown). Thus, the entire pool of patient IgM or IgG is immobilized in the well. The wells are washed and mixed with antigens, following which a standard sandwich ELISA is performed. The amount of antigen bound is proportional to the amount of reactive IgM or IgG in the patient sera.



**Figure 15-10.** Example of biotin/avidin-enhanced ELISA sandwich assay.



After the second step of the assay, in which the detecting antibody is labeled with biotin (B), a third step is added in which enzyme-labeled avidin (A) is added. Subsequently, the binding of avidin is monitored by conversion of substrate (S) to product (P). This enhances the signal because of the extremely high affinity and specificity of biotin–avidin interaction. Also, the detecting antibodies can be labeled with many biotin molecules, so that many avidin–enzyme conjugates can bind.

**Table 15-4. Examples of antigens and antibodies routinely assayed for by MEIA-based assays (partial list).**

Antiviral antibodies (CMV, HBV, HAV, rubella)  
 Ferritin, B<sub>12</sub>, folate  
 IgE  
 Antitoxoplasma  
 Tumor markers (PSA, CA-125, CEA, AFP)  
 Thyroid hormones (TSH, T<sub>3</sub>, T<sub>4</sub>)  
 Therapeutic drug monitoring (digoxin, quinidine)  
 Detection of drugs of abuse (cocaine, barbiturates, THC)

*Abbreviations:* CMV = cytomegalovirus; HBV = hepatitis B virus; HAV = hepatitis A virus; PSA = prostate-specific antigen; CEA = carcinoembryonic antigen; AFP = a-fetoprotein; TSH = thyroid-stimulating hormone; T<sub>3</sub> = triiodothyronine; T<sub>4</sub> = tetraiodothyronine; THC = tetrahydrocannabinol.

## ***Electrophoresis Methods***

The separation of serum proteins in electric fields has been used for generations to characterize human immune responses and disease states. There are two basic procedures: **zone electrophoresis**, which separates proteins based on surface electric charge, and **denaturing electrophoresis**, which separates proteins based on molecular weight. In both methods, serum samples are placed in support media and subjected to an electric field to induce protein migration. Commonly used support media include agarose gels strips, cellulose acetate, or polyacrylamide gel. In clinical applications for routine separation of serum proteins, cellulose acetate strips are most commonly used because they are optically clear, allowing microquantities of proteins to be used and detected by

chemical (or immunochemical) staining methods.

## Serum Protein Electrophoresis (SPEP)

In this assay, a small amount of patient serum (or other biologic fluid) is placed in the center of a shallow well within a strip of cellulose acetate. The film is subjected to an electric field, the proteins migrate based on their charge, and then the film is stained to localize the protein bands (Figure 15-11). The stained film can be scanned by a densitometer to provide an analytical representation of the electrophoretic pattern. Normal human sera is separated into five major bands: albumin,  $\hat{I}^{\pm_1}$ -globulin,  $\hat{I}^{\pm_2}$ -globulin,  $\hat{I}^2$ -globulin and  $\hat{I}^3$ -globulin, which represent mainly IgG. SPEP is useful in the diagnosis of human paraprotein disorders such as multiple myeloma and Waldenström's macroglobulinemia (Figure 15-12). In these disorders, an electrophoretically restricted protein spike usually occurs in the  $\hat{I}^3$ -globulin region. The spike represents accumulation of a single type of Ig that has a defined surface charge, versus the normal pattern of multiple different types of Igs that have varying charges (and hence produce a smear in the  $\hat{I}^3$ -globulin region of the gel). A marked decrease in serum  $\hat{I}^3$ -globulin concentration can sometimes also be detected by this technique. Electrophoresis of urine samples (UPEP) analyzes proteins excreted by the kidney. Free immunoglobulin light chains are readily detectable in urine when present in increased amounts, as in the Bence Jones proteinuria of myeloma (Figure 15-13). Zone electrophoresis in agarose gels has also been useful in the diagnosis of

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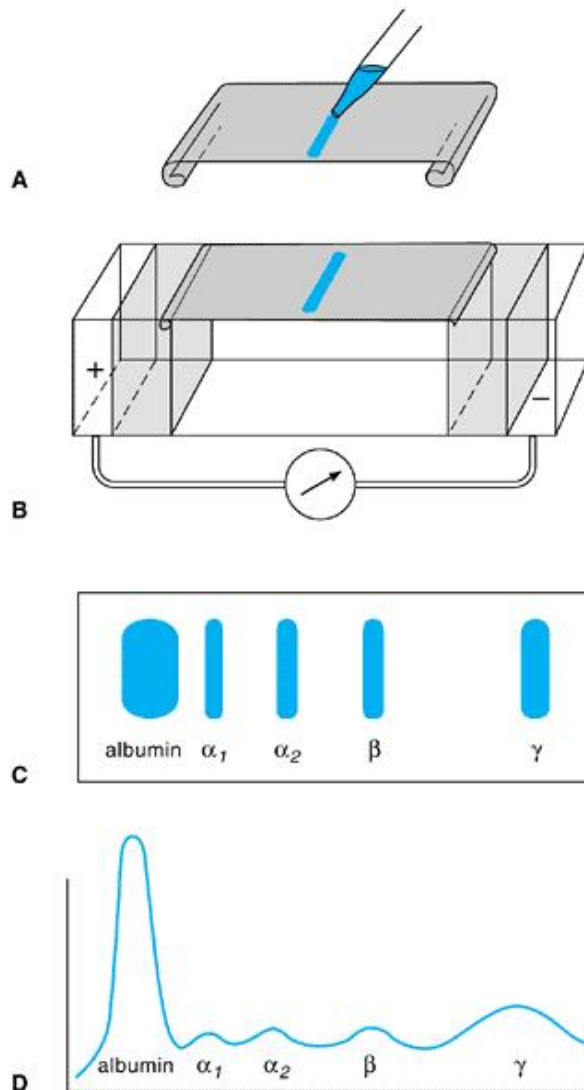
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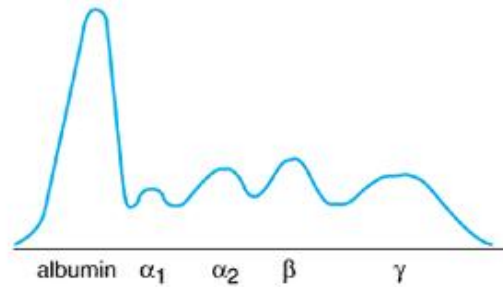
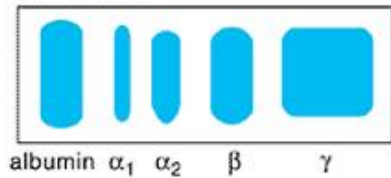
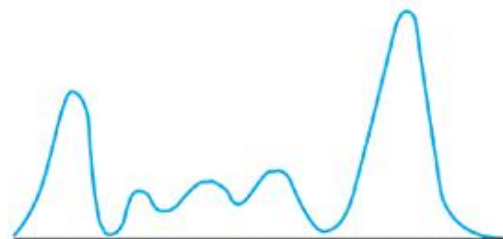
certain central nervous system diseases, such as multiple sclerosis, with alterations in cerebrospinal fluid (CSF) proteins (Figure 15-14). Abnormalities in levels of serum proteins other than Igs may also be detected by SPEP. Although very easy to perform, SPEP is considered a screening assay for determination of protein abnormalities; more quantitative or specific assays are performed on samples with an abnormal pattern.



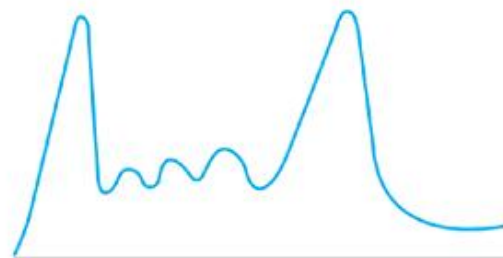


**Figure 15-11.** Technique of cellulose acetate zone electrophoresis. **A:** Small amount of serum or other fluid is applied to cellulose acetate strip. **B:** Electrophoresis of sample is performed in electrolyte buffer. **C:** Separated protein bands are visualized in characteristic position after being stained. **D:** Densitometer scanning from cellulose acetate strip converts bands to characteristic peaks of albumin,  $\hat{\Gamma}_{\pm 1}$ -globulin,  $\hat{\Gamma}_{\pm 2}$ -globulin,  $\hat{\Gamma}^2$ -globulin, and  $\hat{\Gamma}^3$ -globulin.

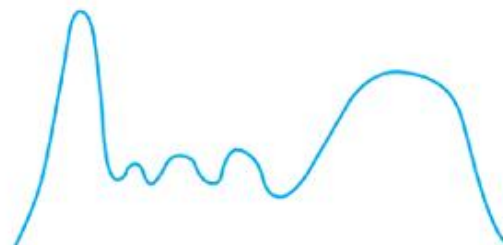
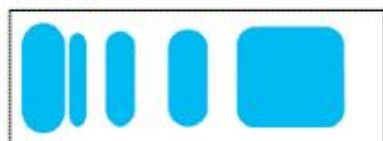
normal serum

IgG myeloma with  $\gamma$  spike and reduced albumin

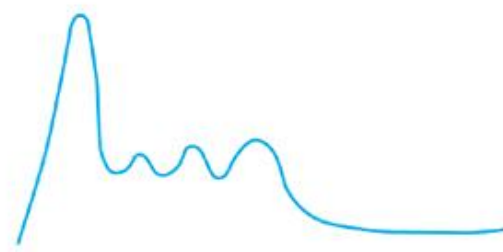
Waldenström's macroglobulinemia with IgM spike



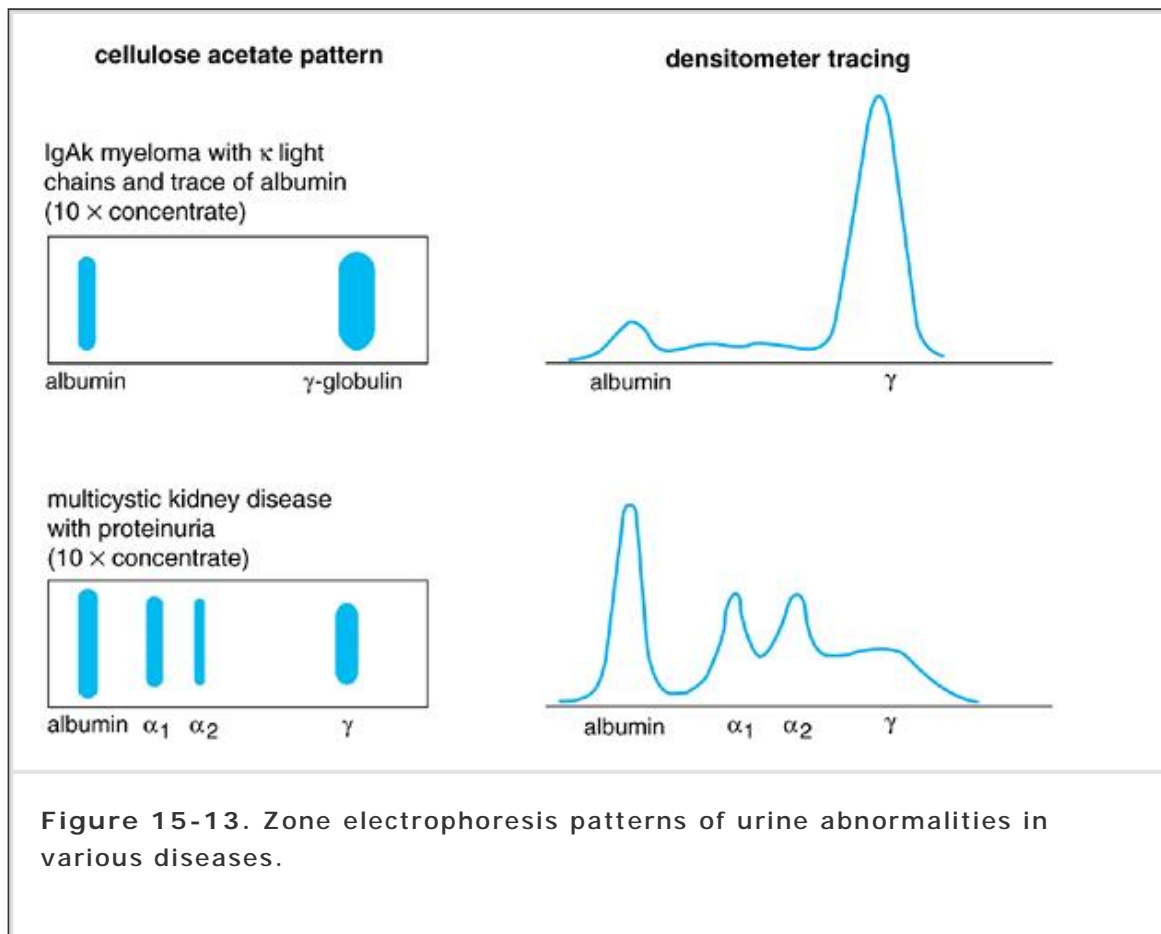
polyclonal hypergammaglobulinemia



hypogammaglobulinemia



**Figure 15-12.** Zone electrophoresis patterns of serum immunoglobulin abnormalities in various diseases.



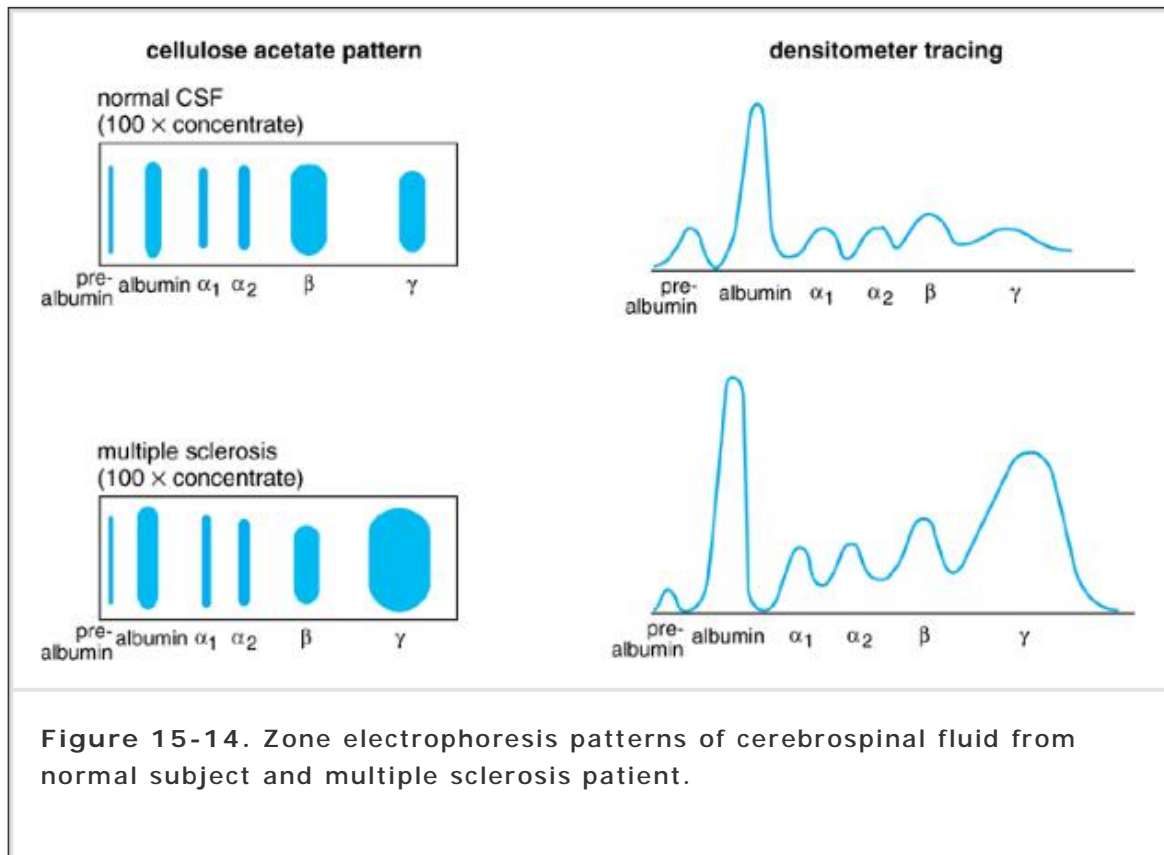
## Immuno-electrophoresis

This assay combines electrophoretic separation of serum proteins followed by immunologic detection of particular proteins using specific antisera. This assay is widely used to characterize and quantitate monoclonal paraproteins.

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Past methods have used agarose gels as supports for electrophoresis of serum, urine, or CSF proteins, followed by immunodiffusion within the agarose to look for precipitin arcs. This technology has been replaced by **immunofixation electrophoresis** in which the sample proteins are separated in a buffered agarose gel. After electrophoresis, antiserum against antibody heavy or light chains is overlaid directly onto the gel surface along the axis of the electrophoretic migration, and immunoprecipitation in situ is allowed to occur. The resulting antigen-antibody complexes become trapped in the gels pore structure. The gel is then processed to removed excess soluble proteins, dried,

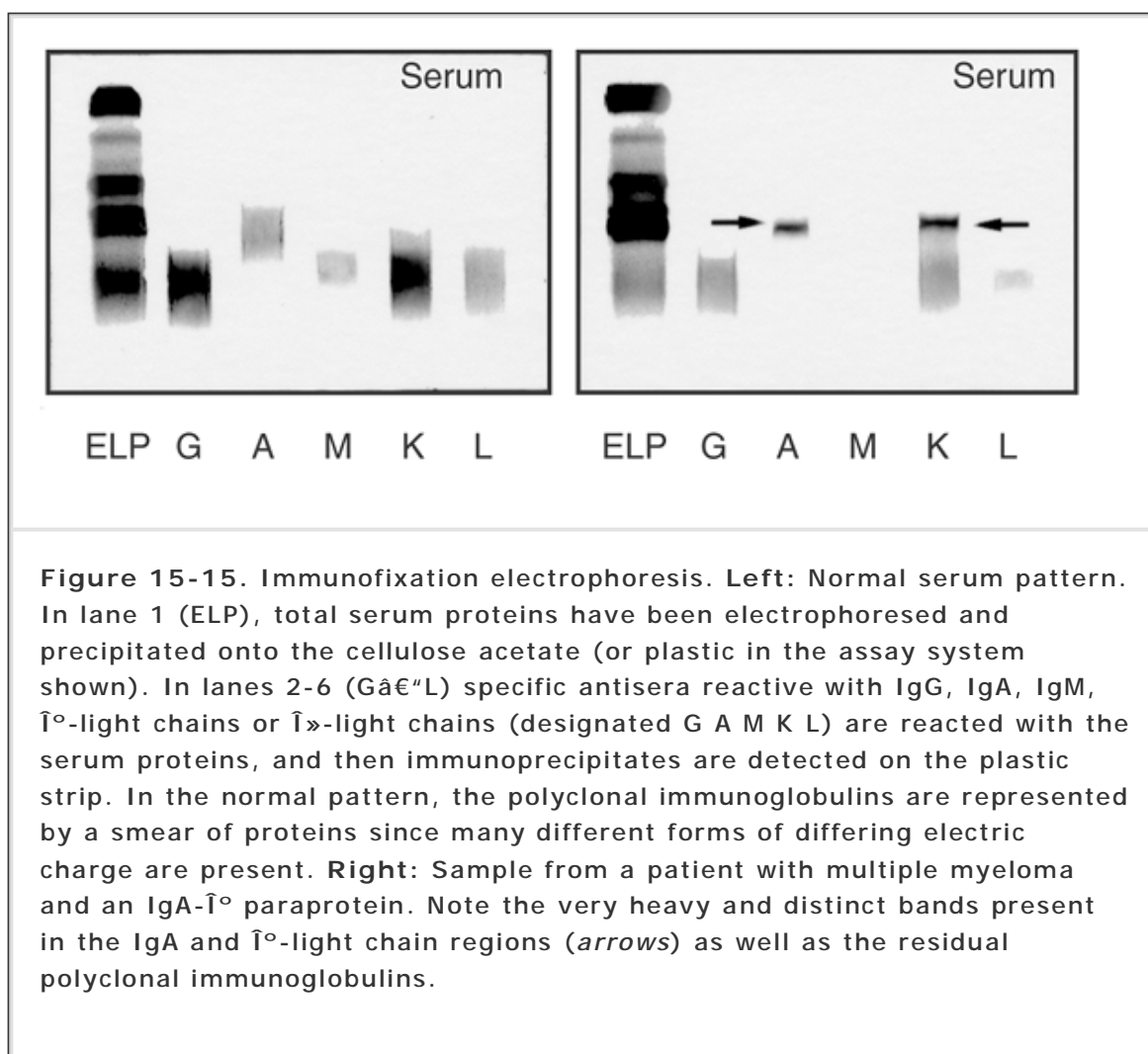
and stained with a protein-specific stain to reveal the precipitin bands. Interpretation is made by visually comparing the specific protein bands with the reference protein electrophoretic pattern (Figure 15-15); normal Igs appear as a smear, and monoclonal proteins appear as a specific band. This method is especially helpful in the detection of paraproteins of the IgM and IgA isotypes, which may be undetectable in the excess of normal IgG.



## Denaturing Gel Electrophoresis and Western Blotting

In this technique, proteins are denatured by incubation with an ionic detergent (sodium dodecyl sulfate, or SDS) at 100°C, followed by electrophoresis in polyacrylamide gels (SDS-PAGE). Because the detergent uniformly coats all the proteins, rendering the surface charge of the protein negative, the proteins migrate within the electric field based on the amount of SDS molecules bound to them, which in turn is influenced by the size of the protein. This method therefore separates proteins by molecular weight. SDS-PAGE is often combined with immunoblotting to specifically identify particular proteins in a given sample. Following electrophoresis by SDS-PAGE, the proteins are electrophoretically transferred to a piece of filter paper (often nylon or nitrocellulose) to which they adhere by nonpolar interactions. The filter paper can then be incubated with specific antisera to reveal the reactive proteins. This entire process is referred to as Western blotting or immunoblotting. This procedure is used extensively in research laboratories to define specific proteins in biologic samples. A widespread clinical use of immunoblotting is to define the pattern of antibody

reactivity of individual patients to certain pathogens, such as human immunodeficiency virus (HIV). In the **HIV Western blot**, viral proteins (isolated by in vitro culture of virus) are separated on SDS-PAGE and transferred to a filter. The filter serves as a solid antigen support for a typical sandwich type ELISA assay. The filter is incubated with patient sera, washed, and then incubated with anti-IgG (which is enzymatically labeled) in order to reveal the presence of patient antibodies to specific HIV proteins. Because the filter can be stored indefinitely, commercial vendors are able to produce and sell these strips with electrophoretically separated HIV viral antigens. This assay is widely used as a confirmatory assay for the presence of HIV infection. Patients are confirmed to have been infected by HIV if they make antibodies that recognize at least two specific viral proteins (Figure 15-16).



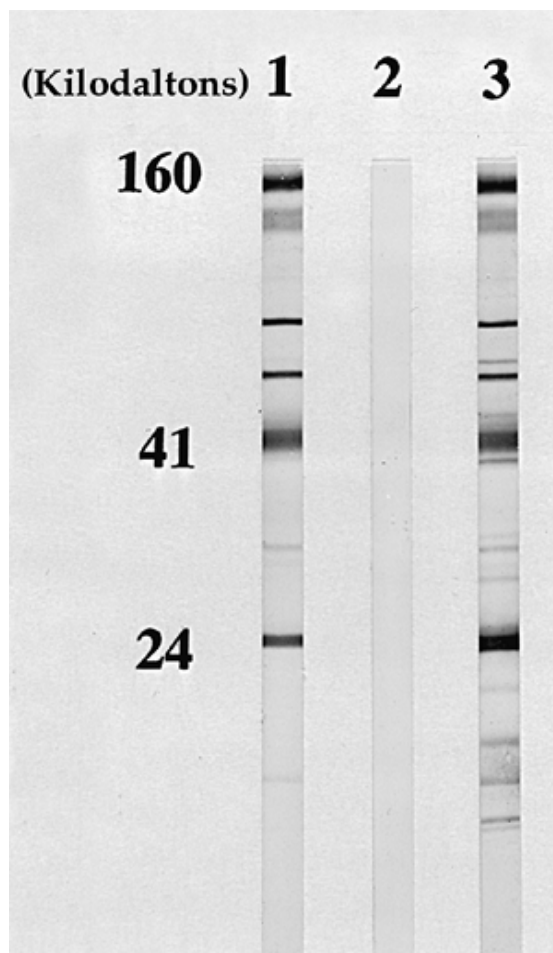
## SERUM VISCOSITY

The measurement of serum viscosity is a simple and valuable tool for evaluating the likelihood of complications with paraproteinemia. Normally, the formed elements of the blood contribute more significantly to whole-blood viscosity than

do plasma proteins. In diseases with elevated concentrations of serum proteins, however, particularly the Igs, the serum viscosity may reach very high levels and result in a characteristic symptom complex, the **hyperviscosity syndrome**. This syndrome is characterized by

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very slow flow of blood through the microvasculature, resulting in tissue ischemia. This can be directly visualized in the retina as aggregates of RBCs in the small vessels where blood flow has nearly stopped (referred to as "boxcar" lesions). Serum viscosity is determined by a variety of factors, including protein concentration; the size, shape, and deformability of serum molecules; and the molecular charge or temperature sensitivity of the proteins.



**Figure 15-16.** HIV Western blot analysis of serum from a patient who tested repeatedly positive on HIV ELISA screening assays. Lane 1 shows the reaction of a known positive control serum sample that contains antibodies that recognize a host of viral proteins—the most clinically significant are p24, p41, and p160 (which are named based on their molecular weights and are viral core, or in the case of p160, envelope proteins). Lane 2 shows the lack of reaction with negative control sera. Lane 3 shows the test patient



sera; the patient has a spectrum of antibodies that recognize the same viral structural proteins as the control sample. Hence, this patient is confirmed to be infected with HIV (ie, HIV-positive).

In clinical practice, serum viscosity is measured in an Ostwald viscosimeter. A few milliliters of serum are warmed to 37°C and allowed to descend through a narrow-bore capillary; the rate of the descent of the serum through the capillary is compared with the rate at which distilled water moves. The ratio of these two numbers provides a measure of the relative serum viscosity. Normal values range from 1.4 to 1.9.

Serum viscosity measurements are primarily of use in evaluating patients with Waldenström's macroglobulinemia, multiple myeloma, and cryoglobulinemia. In myeloma, aggregation or polymerization of the paraprotein in vivo often results in hyperviscosity. The correlation between levels of relative serum viscosity and clinical symptoms is, however, not direct, and thus it can be difficult to predict at what point clinical symptomatology will result. Increased serum viscosity may interfere with various laboratory tests that employ flow-through devices, such as hematology counters and analyzers in clinical chemistry.

## IMMUNOHISTOCHEMICAL METHODS

### *Immunofluorescence Assays (IFA)*

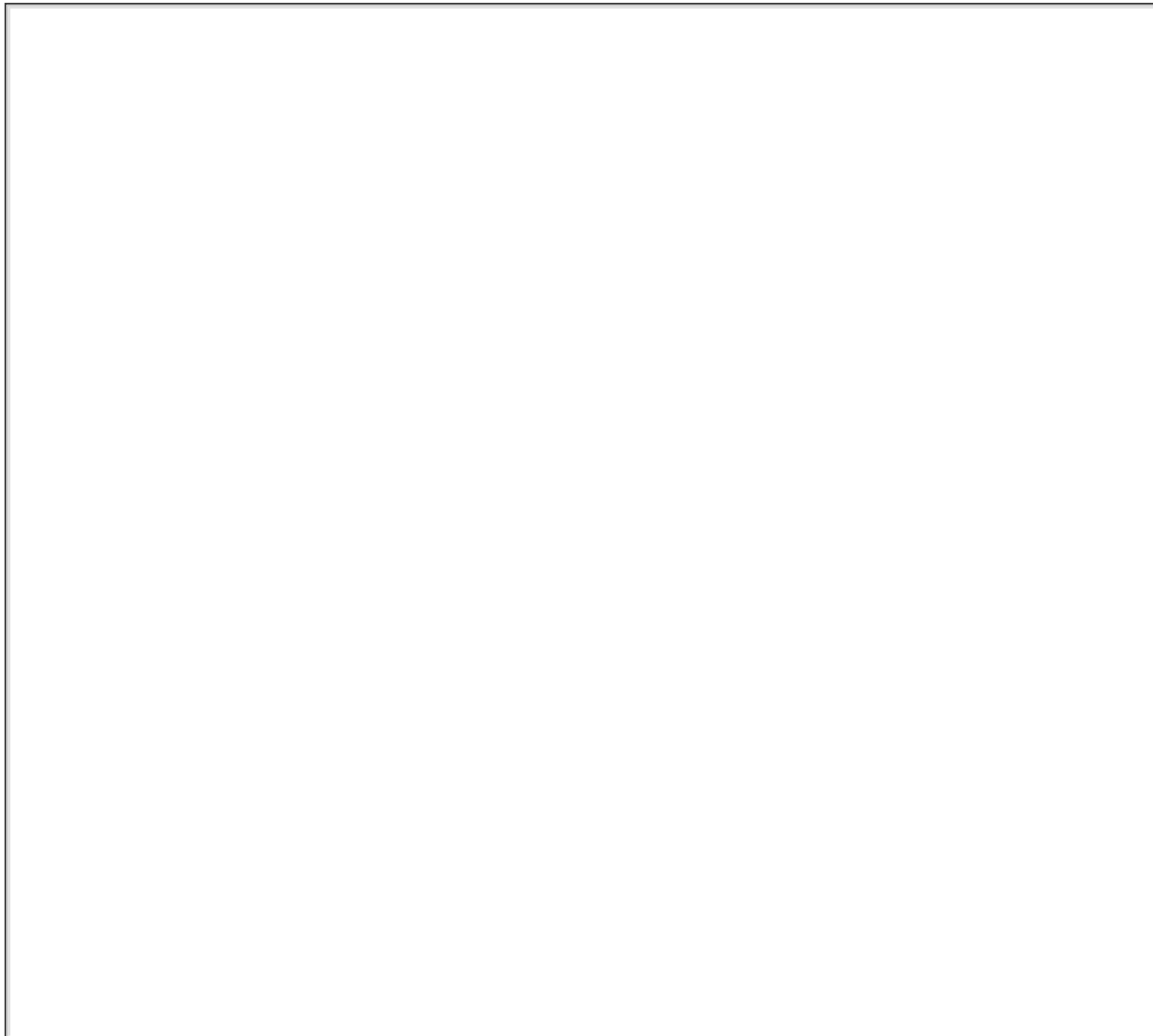
In this method, specific antibodies (usually mAbs) conjugated with fluorescent labels are used as probes for the detection of antigens in samples of patient tissues or on patient cells. In each case, the binding of the antibodies to the tissues or cells is visualized directly using a fluorescence microscope. The latter contains a high-intensity light source, excitation filters to produce a wavelength capable of causing fluorescence activation, and a barrier filter to remove interfering wavelengths of light (Figure 15-17). When observed in the fluorescence microscope against a dark background, fluorescent antibodies bound specifically to antigens can be visualized by their bright color. The advantage of this assay is that it allows visualization of the antigen within specific cell types in a tissue or even within specific subcellular compartments of cells. For example, using this method, cytoplasmic antigens can be easily distinguished from nuclear antigens. Use of a fluorescent antibody to detect cellular or tissue antigens is referred to as **direct IFA** (Figure 15-18)

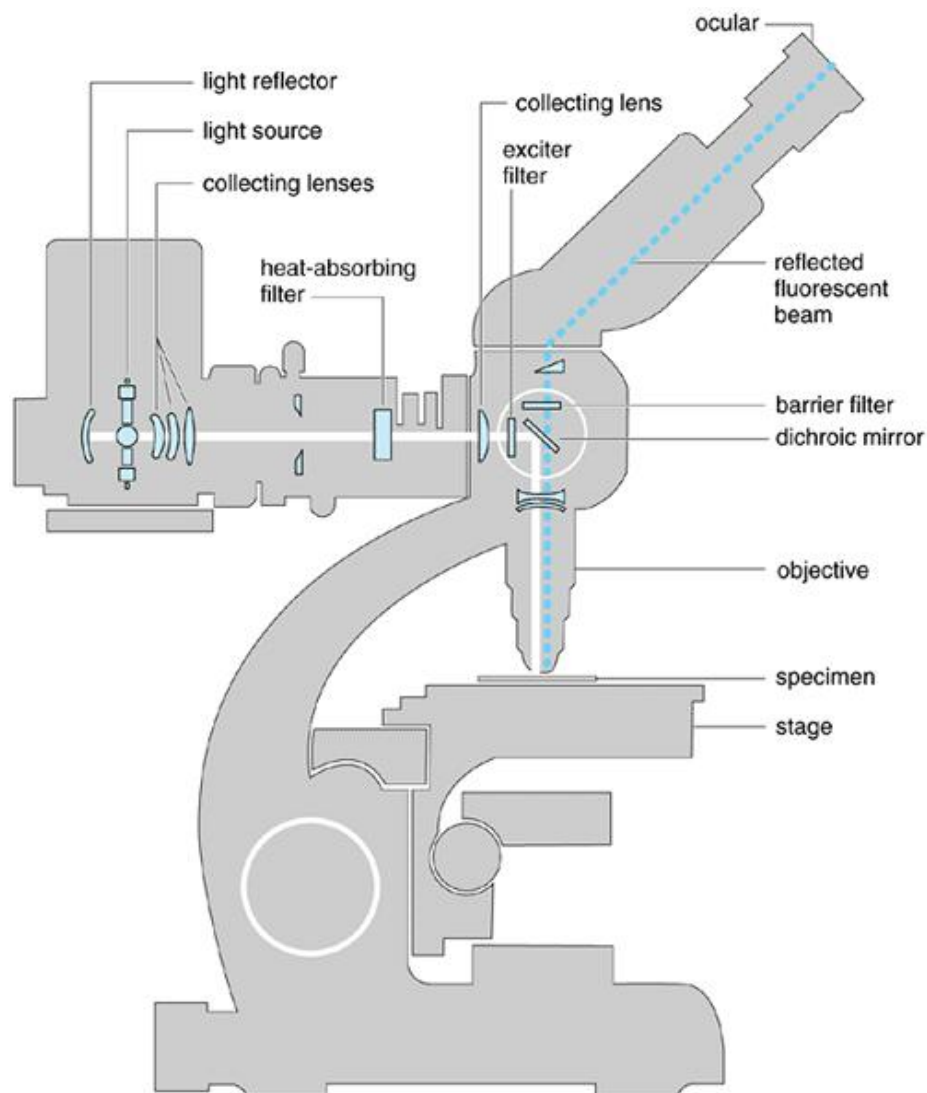
Alternatively, this basic process can be used to detect antibodies within patient sera that are reactive to a specific pathogen or that are cross-reactive to specific tissue antigens. Dilutions of patient sera are incubated with cells or tissues known to be infected with the pathogen. Unbound antibodies are removed by washing, and the specifically bound antibodies are visualized with fluorescently labeled anti-Ig antisera. This method remains in use for detection of antibodies against herpes simplex virus or Epstein-Barr virus (although it is being

replaced by MEIA-based detection methods). The detection of reactive antibodies in patient sera using a secondary labeled anti-Ig

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is referred to as an **indirect IFA** (see Figure 15-18). Indirect IFA assays are commonly used to assay for the presence of autoantibodies that react inappropriately to specific cell types or subcellular structures. The classic **antinuclear antibody (ANA)** test is an example (Figure 15-19). In this test, dilutions of patient sera are incubated with tissue culture cells (a human cell line called HepG2 is used), and specifically bound antibodies (of either IgM or IgG isotypes) are detected with labeled secondary antibodies. The pattern of binding of patient antibodies to the nuclear antigens in this cell line (homogenous, speckled, rim pattern, nucleolar) correlate with fine specificity of the ANA and with the presence of specific autoimmune disorders, such as systemic lupus erythematosus (SLE) (see Chapter 31). IFA assays can also take advantage of the biotin-avidin amplification method used in ELISA assays. In this case, the primary antibody is labeled with biotin (for indirect assays the detecting antibody is biotinoylated), and fluorescently labeled avidin is used for detection. Because of the high affinity and specificity of the biotin-avidin interaction, this method significantly improves the sensitivity of IFA assays.





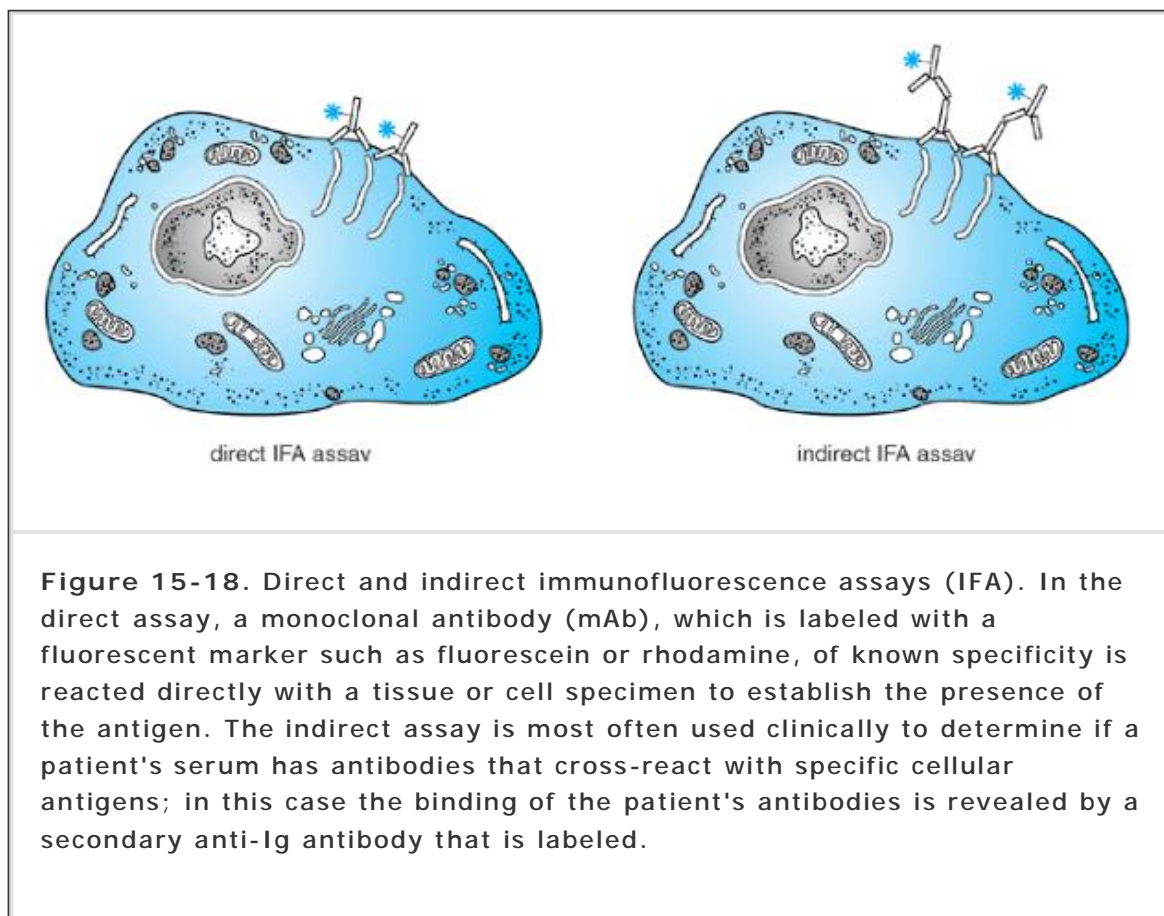
**Figure 15-17.** Fluorescence microscope with epillumination. The light beam is directed through the exciter filter and down onto the specimen. A dichroic mirror allows passage of selected wavelengths in one direction but not another. After reaching the specimen, the light is reflected through the dichroic mirror and emitted fluorescent light is visualized at the ocular.

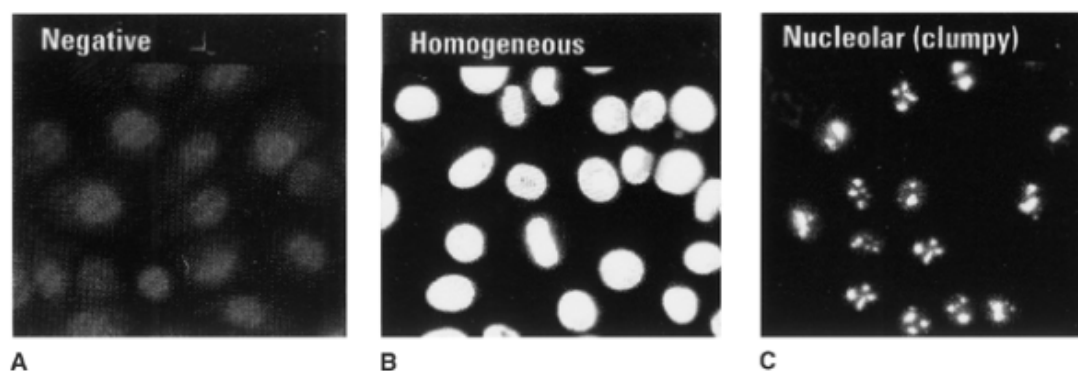
**Fluorescence** is the emission of light of one color (wavelength) while a substance is irradiated with light of a different color. Several different fluorochromes are used in the clinical laboratory for IFA assays. These same reagents are also heavily used in flow cytometry (fluorescence-activated cell sorter,

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FACS) assays (see Chapter 16). The classic fluorochromes are fluorescein and

rhodamine. These compounds are linked to isothiocyanate to form reactive agents (called fluorescein isothiocyanate [FITC] or tetramethyl-rhodamine isothiocyanate, respectively) that readily form covalent bonds with the  $\epsilon$ -amino residues of lysine and terminal amino groups. Incubation of these agents with proper concentrations of antibodies results in the covalent labeling of the antibody without affecting the ability of the antibody to bind to antigen. When stimulated with light at approximately 480–490 nm, FITC-conjugated antibodies emit at roughly 530 nm (green), whereas rhodamine-labeled mAbs emit at approximately 580 nm (red). Using a microscope with filters that distinguish these wavelengths, one can use differently labeled antibodies simultaneously to detect different antigens in the same sample or to examine the relationship of different subcellular proteins. The simultaneous use of differently labeled sets of mAbs for antigen detection is routinely used in flow cytometry.





**Figure 15-19.** Examples of antinuclear antibody (ANA) staining patterns. In these indirect immunofluorescence assays, patient sera are incubated with HepG2 cells, and the binding of patient antibodies is revealed using a fluorescein secondary antibody. Examples of negative control, homogeneous staining, and nucleolar staining patterns are shown. The particular staining pattern for ANA is correlated with the particular nuclear antigen to which the patient makes autoantibodies. In many cases, different ANA patterns reflect different disease states in the patient—homogenous staining is seen in 60–80% of patients with systemic lupus erythematosus, whereas nucleolar staining is more commonly seen in patient with scleroderma.

### ***Immunohistochemical Assays***

In this method, the primary antibodies used are labeled with enzymes, and their binding is detected by the presence of enzymatic activity. These antibodies can be the same reagents used in ELISA assays. In these cases, the samples are incubated with enzymatic substrates that produce a product that precipitates directly on the tissue section (usually producing a brown- or red-stained product).

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This method is useful because binding of the antibody to samples can be visualized directly with a normal light microscope. To enhance signal production, indirect assays often use a horseradish peroxidase-labeled secondary antibody or a biotin/avidin-based antibody to reveal an unlabeled primary antibody. Immunohistochemical staining is used widely to detect tumor antigens or to classify lymphocyte cell types in surgical pathology sections.

### **COMPLEMENT ASSAYS**

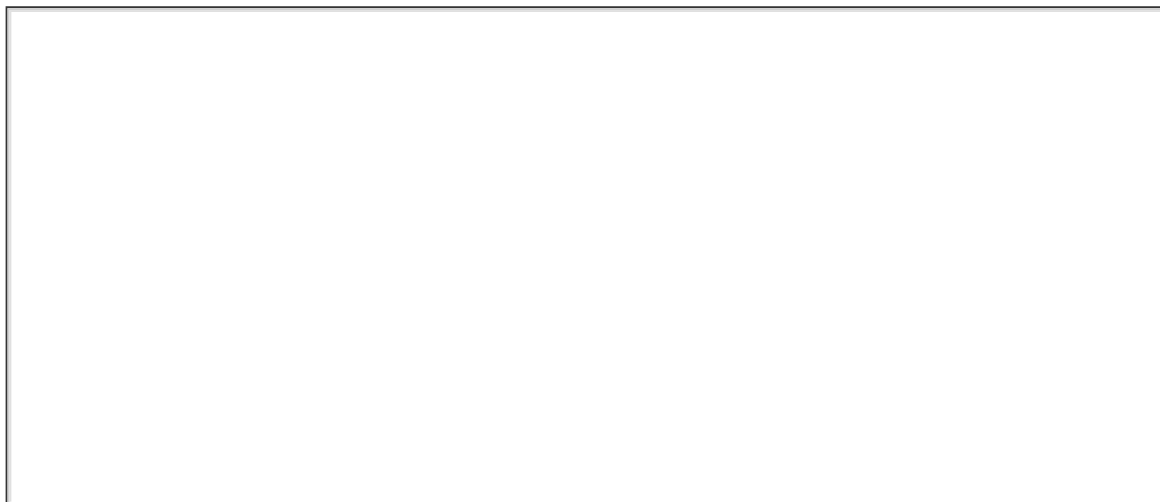
Complement is one of the effector mechanisms of immune complex-induced tissue damage. Clinical disorders of complement function have been recognized

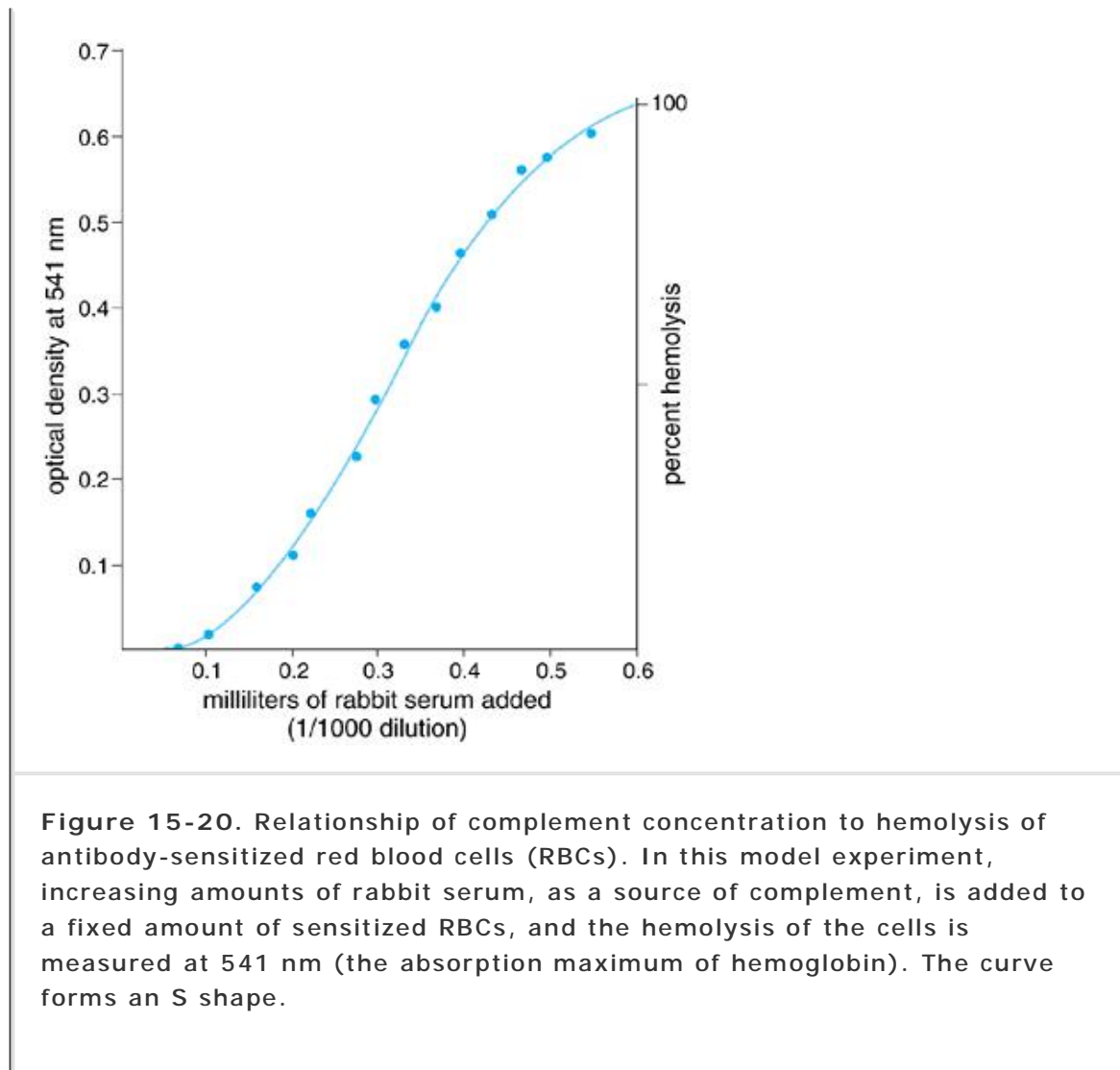
for many decades. The nine major complement components of the classic pathway (C1 to C9), several from the alternative pathway, and various inhibitors can be measured in human serum. Clinically useful assays of complement include those that test pathway function (the total hemolytic assays;  $CH_{50}$  and  $AH_{50}$ ) and tests of the quantities and functions of individual components.

It is worth emphasizing that the collection and storage of serum samples for functional or immunochemical complement assays present special problems as a result of the remarkable lability of some of the complement components. Rapid removal of serum from clotted specimens and storage at temperatures of  $-70^{\circ}\text{C}$  or lower is required for preservation of maximal activity. An important source of error in complement determination is poor sample handling.

## ***Hemolytic Assays***

The hemolysis of RBCs by antibodies in vitro depends on complement. This requirement forms the basis for widely used assays of complement activity. The hemolytic assay for the classical complement pathway employs sheep RBCs, rabbit antibodies against sheep RBCs, and patient sera as a source of complement. The sheep RBCs are opsonized with the rabbit antibody (at a subagglutinating dose of antibody) and then mixed with dilutions of patient sera; hemolysis is measured spectrophotometrically. The amount of lysis in a standardized system describes an S-shaped curve (Figure 15-20) when plotted against increasing amount of complement (or patient sera). In the midregion of the curve, near 50% hemolysis, a linear relationship exists between the degree of hemolysis and the amount of complement activity present. For clinical purposes, measurement of total hemolytic activity of serum is taken at the 50% hemolysis level; this is called the  $CH_{50}$  unit.  $CH_{50}$  units are standardized by using a defined amount of sheep RBCs, antish sheep RBC antibody and guinea pig serum as a source of complement. Standard curves are set up with the known reagents and used to compare with patient samples. Variables that can influence the degree of hemolysis include RBC concentration, fragility of the RBCs, amount of antibody used for sensitization, the nature of the antibody (IgM versus IgG), and the presence of preformed immune complexes or anticomplement factors in patient sera.





**Figure 15-20.** Relationship of complement concentration to hemolysis of antibody-sensitized red blood cells (RBCs). In this model experiment, increasing amounts of rabbit serum, as a source of complement, is added to a fixed amount of sensitized RBCs, and the hemolysis of the cells is measured at 541 nm (the absorption maximum of hemoglobin). The curve forms an S shape.

The value  $CH_{50}$  units in human serum may be determined by converting the S-shaped curve to a linear curve using the von Krogh equation:

$$X = K(Y/1 - Y)^{1/n}$$

where  $X$  = number of milliliters of diluted serum used;  $Y$  = percentage of RBC lysis;  $K$  = constant; and  $n = 0.2 \pm 10\%$  under standard conditions.

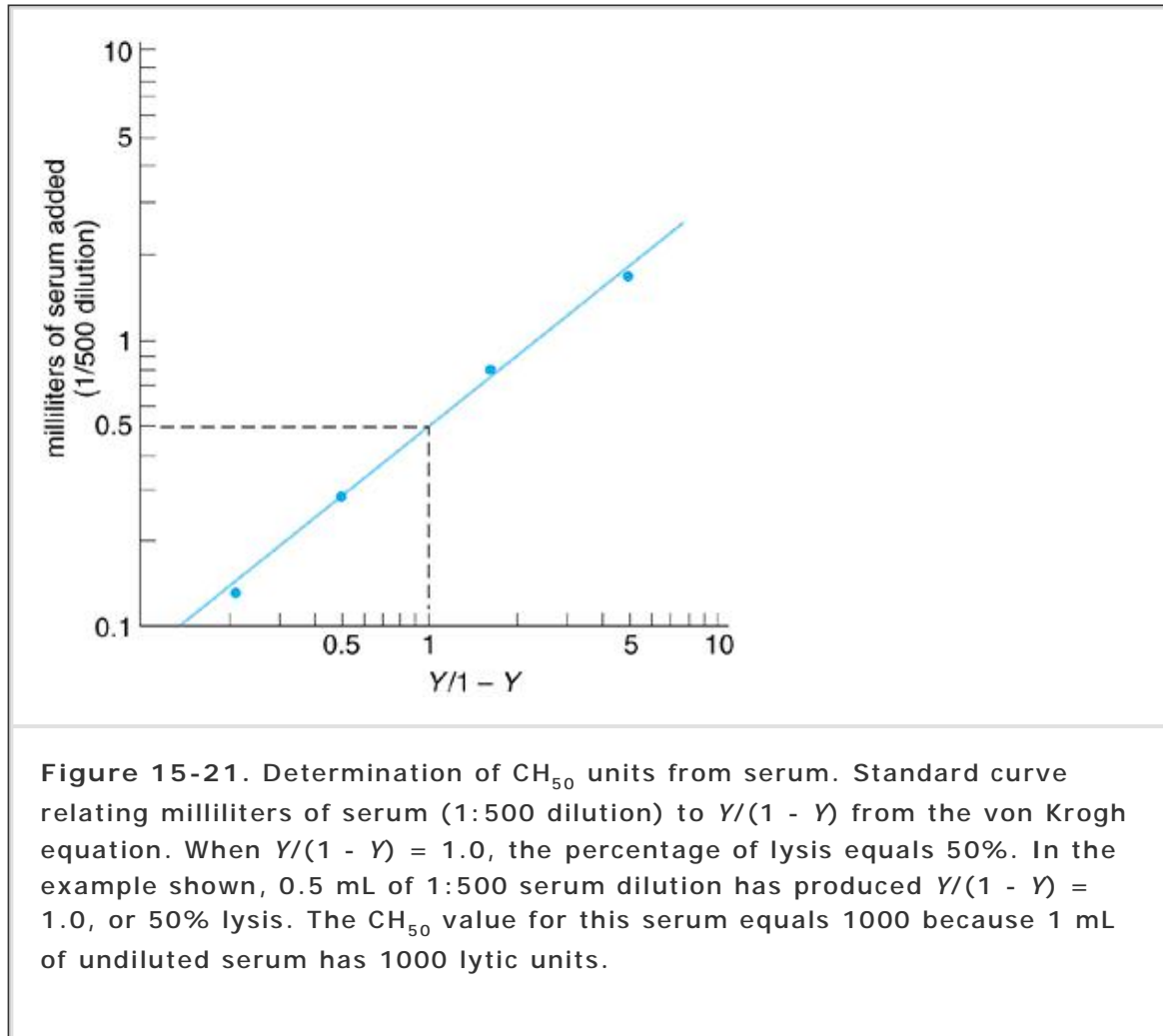
Converting this equation to log form allows one to graph the results on a log-log plot, where the values of  $Y/(1 - Y)$  are plotted against the serum dilutions  $X$ . The reciprocal of the dilution of serum that intersects the curve at the value  $Y/(1 - Y) = 1$  is the  $CH_{50}$  unit (Figure 15-21). The values for  $CH_{50}$  units can vary significantly between different laboratories unless standardized reagents are used.

The alternative pathway of complement activation shares the terminal components (C3 and C5-C9) with the classical pathway but has several unique components (D, B, and P). To measure the function of this pathway, the  $AH_{50}$  assay is used. This assay depends on the ability of complement to lyse

### unsensitized sheep RBCs by activation of the initial components

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of the alternative pathway (to ensure that the classical pathway is not activated, these assays are done with ethylene glycol tetraacetic acid (EGTA) to chelate  $\text{Ca}^{2+}$  ions—the classical pathway is calcium-dependent). Because the alternative pathway and the classical pathway share common terminal components, deficiencies in these proteins result in loss of both  $\text{CH}_{50}$  and  $\text{AH}_{50}$  activity. Normal  $\text{CH}_{50}$  activity with reduced  $\text{AH}_{50}$  values results from defects in alternative complement components.



The  $\text{CH}_{50}/\text{AH}_{50}$  assays are relatively insensitive assays for total complement activity but are excellent screens to rule out genetic deficiency of complement components. Homozygous deficiency of an individual component totally abrogates hemolytic activity for the pathway tested. For example, patients with homozygous deficiency of C2 have undetectable  $\text{CH}_{50}$ . Reduced serum complement activity occurs in a variety of acquired disease states (Table 15-5). Reduction in serum complement activity can be due to any one or a combination of (1) complement consumption by in vivo formation of antigen–antibody complexes, (2) decreased synthesis of complement, (3) increased catabolism of



complement, or (4) formation of inhibitors (usually autoantibodies). In all cases, however, a significant (80%–90%) reduction in one component must occur in order to affect total hemolytic activity. In principle, the activity of each individual component of the complement cascade can be determined using variations of the standard  $CH_{50}/AH_{50}$  assay. These assays are done by providing an excess of all the components except for the one tested and then adding serial dilutions of the patient serum until 50% hemolysis is observed. The easiest way to do this is to use serum that is genetically deficient in a specific component (eg, C4- deficient guinea pig serum or C6-deficient rabbit serum) or that is depleted of a given component by chemical means. Sensitized RBCs are mixed with the deficient sera and serial dilutions of the patient sera to determine the point at which 50% hemolysis is observed.

### **Table 15-5. Diseases associated with reduced hemolytic complement activity.**

Systemic lupus erythematosus with glomerulonephritis  
Acute glomerulonephritis  
Immune complex diseases  
Infective endocarditis with immune complexes and glomerulonephritis  
Disseminated intravascular coagulation  
Hereditary complement deficiencies  
Mixed cryoglobulinemia  
Advanced cirrhosis of the liver

### ***Immunochemical Assays for Complement Components***

Because functional assays reflect only major changes in the levels or activity of complement proteins, many laboratories test for the level of individual complement proteins using a number of the standard methods for antigen detection described earlier. The method most commonly used to measure C3, C4, C1-inhibitor, and factor B is rate nephelometry; the other components can be measured by immunodiffusion or ELISA. Although these tests can pick up more subtle changes in complement protein levels than functional assays, improper handling of specimens can also result in erroneous values in these assays. For example, in storage, C3 spontaneously converts to C3c, which has a smaller molecular size than native C3.

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## 16

# Clinical Laboratory Methods for Detection of Cellular Immunity

Clifford Lowell MD, PhD

The immune system in humans has been divided into two major parts: **humoral immunity** (antibody and complement) and **cellular immunity**. In practical terms, such a division allows one to separate B-lymphocyte function (antibody production) from the activities of T cells and innate immune cells (monocytes and granulocytes). In many ways this separation is artificial; production of antibodies by B cells, for example, is critically dependent on helper T-cell function. Nevertheless, this division of the immune system provides a practical framework for the laboratory evaluation of immunity in clinical practice.

In this chapter we focus on methods to evaluate aspects of immune effector cells not directly related to antibody production. A variety of assays assess the function of these cells, but, in contrast to the evaluation of antibodies, these assays are beset by difficulties in test standardization, biologic variability, imprecision, complexity, and cost. Hence, only a limited number of them are used in routine clinical practice. The most consistent and reproducible of the methods for evaluating cellular immunity employ immunochemical means for detecting cellular antigens or markers. The advent of **monoclonal antibodies (mAbs)** for detecting various leukocyte subsets has provided us with panels of reagents to enumerate and characterize cells of the immune system. As our understanding of the cellular immune system improves, many of the methods used in the research laboratory will make their way into routine clinical use.

The present chapter reviews the tests that have medical application in the detection of immune cell types and their corresponding functions. The intention is to familiarize the reader with the principles, applications, and interpretations of assays in routine clinical use. The topics discussed include (1) assays for leukocyte phenotyping, (2) delayed-type hypersensitivity skin testing, (3) lymphocyte activation assays, (4) assessment of monocyte/macrophage function, and (5) determination of granulocyte function.

## LEUKOCYTE PHENOTYPING

Determination of the number and types of cell surface molecules (often referred

to as markers) is a widely used method for assessing the cells of the immune system. This method allows us quantify B cells, T cells, monocytes, and granulocytes within different sites in the body (bone marrow, spleen, blood, lymph nodes). In addition, we can enumerate subsets of these cell types. For example, T-lymphocyte subsets that differ in their functional properties can be distinguished phenotypically. Moreover, as immune cells develop from precursor cells or respond to external stimuli, they express characteristic patterns of surface molecules. The surface phenotype can also therefore provide clues as to the differentiation state of the cell.

Panels of mAbs have been developed that recognize defined antigenic determinants on the leukocyte surface. The mAbs are conjugated with either fluorescent dyes or enzymes and then are used to stain leukocytes in tissue sections or in fresh cell suspensions. The cell types that are recognized by the mAbs can be counted by immunohistochemical techniques, fluorescence microscopy (Chapter 15) or by flow cytometry (see next section). Precise quantitation of T and B cells in human peripheral blood has made important contributions to our understanding of immunodeficiency disorders, autoimmune diseases, tumor immunity, and immunity to infections. It should be emphasized, however, that the numbers of T or B cells do not necessarily correlate with the functional capacity of these cells. These assays provide a nosologic classification of immunocompetent cells; further evaluation of lymphocyte function may be required to assess immunologic competence in clinical practice.

In 1983, the First International Workshop on Human Leukocyte Differentiation Antigens met and established a new nomenclature for immunologically defined cellular types and subtypes. They defined a series of **clusters of differentiation (CD) antigens** that are expressed on specific cell types of the hematopoietic

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system. The number of recognized CD antigens has increased steadily over the years and now numbers well over 150 (see Appendix). Of note, CD antigens are not necessarily leukocyte-specific; many are expressed on nonhematopoietic cell types as well. Moreover, the definition of leukocyte surface phenotypes is a work in progress; the list of CD antigens is incomplete.

## ***Flow Cytometry for the Detection of Leukocyte Antigens***

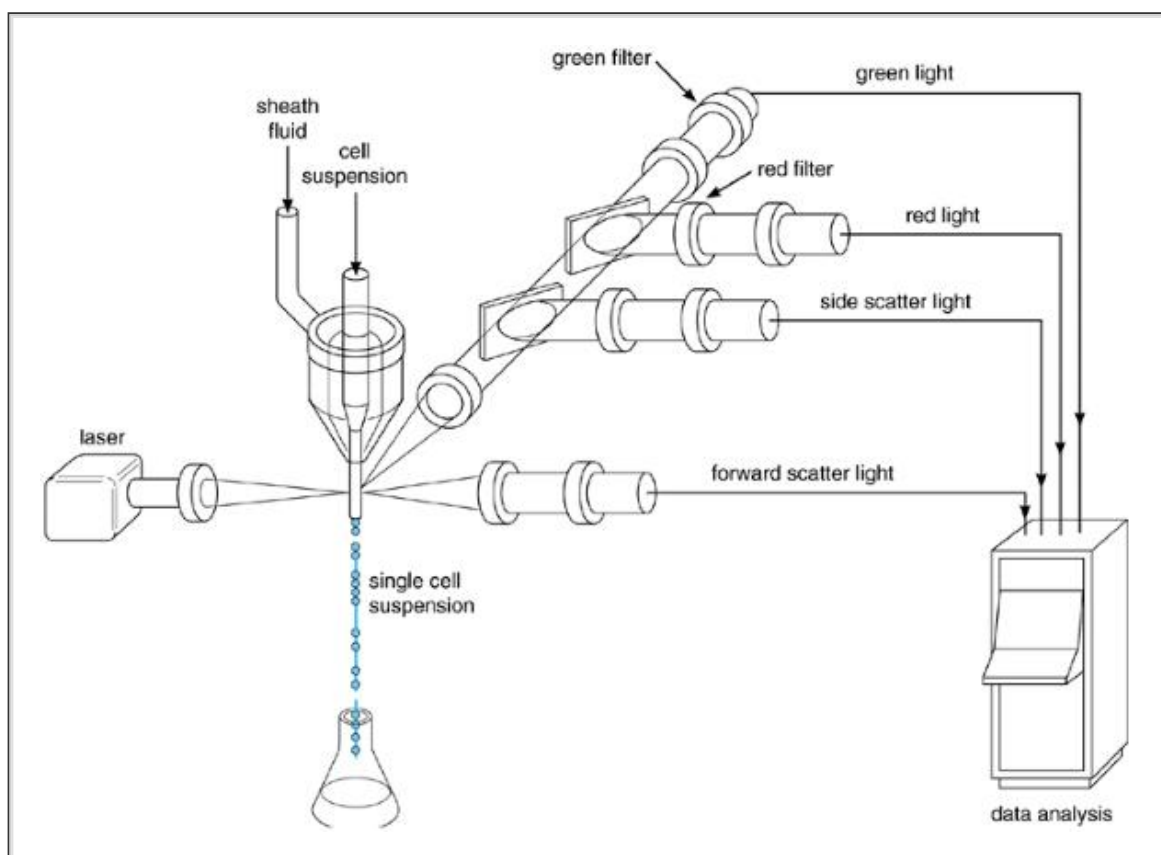
### **Flow Cytometers**

The most widely used method for detecting the binding of mAbs to leukocyte surfaces is flow cytometry. The engineering of flow cytometers is extremely complex, and hence only the basic concepts will be reviewed here. In general, a flow cytometer is an instrument capable of analyzing single cells as they pass through an orifice at high velocity. The flow cytometer measures the properties of light scattering by the cells and the emission of light from fluorescently labeled mAb bound to the surface of the cell (Figure 16-1). The light-scattering properties of cells are related to their size and intracellular content or complexity. In general, larger cells produce more forward light scatter

(conceptually similar to a shadow), and cells with more intracellular complexity (ie, granules, vesicles, mitochondria, etc) produce more side light scatter (Figure 16-2). Light-scattering properties can be used to calculate cell volume and have been used by a variety of analyzers to count and characterize different blood cells (neutrophils, basophils, lymphocytes, etc). The flow cytometer also detects fluorescently emitted light of different wavelengths from each cell. Using mAb that are conjugated with different fluorochromes (eg, fluorescein isothiocyanate [FITC] for green light emission, rhodamine for red/orange light emission—see Chapter 15), one can detect the emission of light from single cells that bind these mAbs. More complex flow cytometers have multiple filters and detection systems that allow for analysis of as many as seven different photochrome-conjugated mAbs that

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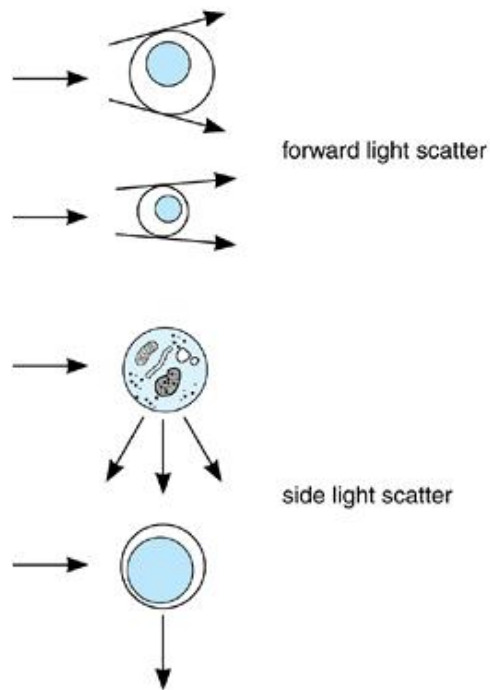
emit light at different wavelengths. Hence, multiple markers on the same cell can be analyzed simultaneously. Modern flow cytometers analyze as many as 2000–4000 cells per second; hence one is able to rapidly collect a large amount of data on different cell populations within a single sample.



**Figure 16-1.** Conceptualized components of a simple “four-parameter” flow cytometer. Laser light is scattered by single cells in suspension and collected at approximately 180° and 90° to the incident beam. The reflected light is passed through filters and detected by photomultiplier tubes (PMTs), which convert the light signal to an electronic



signal. A computer analyzes the data. Increasingly complex flow cytometers use more than one laser and multiple light filters and PMTs to enable the collection of fluorescent light emitted at a variety of different wavelengths.



**Figure 16-2.** Light scattering of cells in the flow cytometer. Forward scatter is proportional to size; larger cells have greater scatter. Side scatter is proportional to intracellular complexity; cells with more granules (neutrophils) have more scatter than cells with a simple cytoplasm (lymphocytes).

## Sample Preparation

Cells must be in a single cell suspension for analysis by flow cytometry. Aggregates or clumps of cells, which are often found during isolation of solid tissue, are not amenable to flow cytometry. In contrast, single-cell suspensions produced from bone marrow samples, lymph node biopsies, tissue samples from lymphoid malignancies (made by teasing the tissue apart in a buffered saline solution), or simply from peripheral blood are optimal for flow cytometric analysis. The mononuclear cells from a particular tissue sample can be purified further by density gradient centrifugation on Ficoll-Hypaque. This method results in a yield of 70%–90% mononuclear cells with a high degree of purity but may also result in the loss of some lymphocyte subtypes or even of tumor cells. As an

alternative, many labs simply stain samples of peripheral blood with labeled mAbs, lyse the red blood cells with commercial lysing reagents, and then analyze the sample on the flow cytometer. By using the ability of the cytometer to electronically gate individual populations of cells, one can determine the cell surface staining of different cell types within a mixed population of cells (see following section). This “whole blood lysis” method is widely used to analyze leukocyte types.

## Data Collection and Analysis

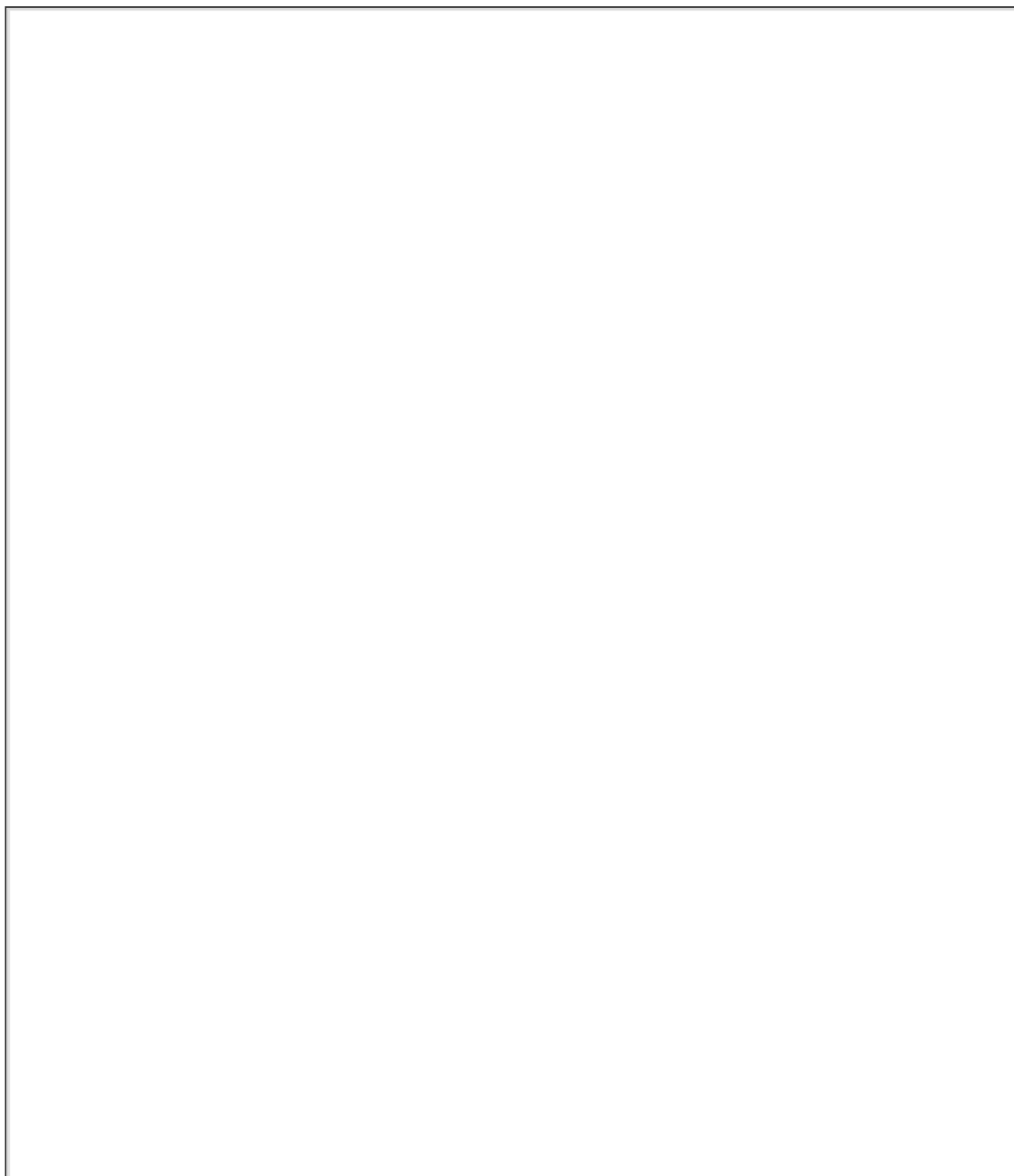
The simple type of flow cytometer illustrated in Figure 16-1 collects four pieces of data for every cell that passes through the laser: (1) forward light scatter, (2) side light scatter, (3) green fluorescence, (4) red/orange fluorescence. More complex machines are able to separate red and orange fluorescence. These data are fed into a computer, and the operator can display them in a variety of fashions. Most commonly used are *X/Y* plots in which light scattering or emitting properties are shown on the different axes. One can then estimate the number of leukocytes that stain for one, both, or neither of the markers.

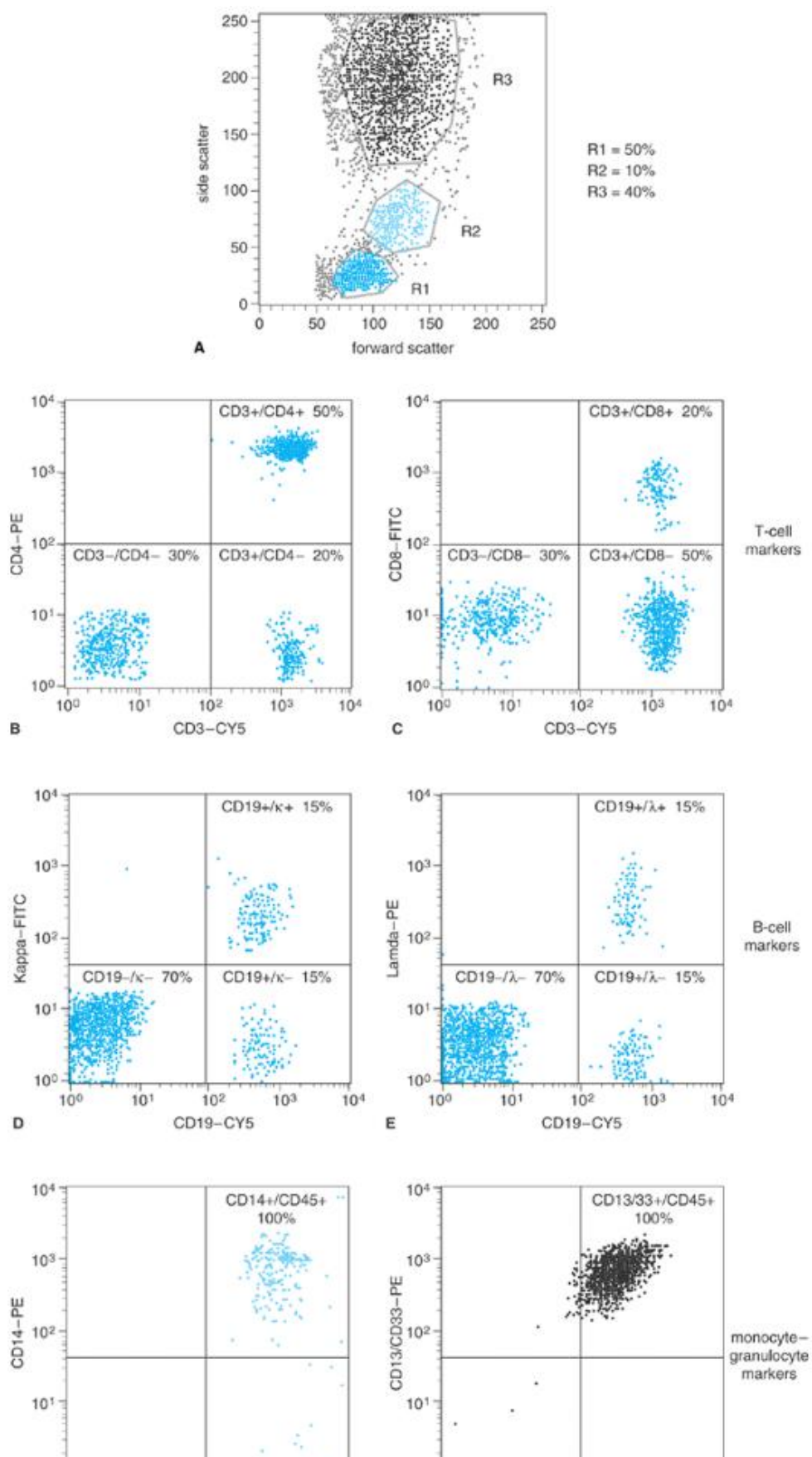
Figure 16-3 shows a typical example of lymphocyte staining of a normal peripheral blood sample. Based on their forward/side scatter light properties, one can define the lymphocytes (low forward/low side scatter, R1 area in Figure 16-3), the monocytes (medium forward/medium side scatter, R2 area), and the granulocytes (medium forward scatter/high side scatter, R3 area). Using standard flow cytometry analysis, one can “draw” an electronic region (or gate) around each group of these cells (as shown by the regions in Figure 16-3) and then display the fluorescent properties of these based on the mAbs with which the cells were stained. In the sample shown in Figure 16-3, 50% of total white blood cells are lymphocytes (in R1), 10% are monocytes (in R2) and 40% are granulocytes (R3). The T-cell/B-cell marker analysis of the lymphocytes reveals that 70% of the cells are T cells as defined by the CD3 marker, the other 30% of the lymphocytes are B cells as defined by the CD19 marker. Of the T cells, 71% are CD4+ and 29% are CD8+, whereas the B cells are evenly divided into 50% with the  $\kappa$ -light chain immunoglobulin and 50% with  $\lambda$ -light chain. In R2 all the cells are monocytes as defined by the CD14 marker, and in R3 all the cells are granulocytes as defined by the staining with a cocktail of the CD13/33 mAbs. Both the monocytes and granulocytes (and the lymphocytes as well, but not shown) stain with CD45 mAb. By comparison with many other individuals, we would find that the numbers and proportions of T-cell subsets, B-cell subsets, monocytes, and granulocytes in this patient would fall into the normal range. Hence, this patient has no quantitative defects in immune cell development that would result in immunodeficiency (of course, he may have defects in cellular function).

In contrast, the peripheral blood sample shown in Figure 16-4 is from a patient with a severe aberration in his immune system. As can be seen in the forward/side scatter plot, this patient has cells only within the lymphocyte region (R1) and has virtually no monocytes or granulocytes (in R2 or R3, respectively).

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When his cells are stained with a mixture of CD5 and CD19 mAbs, we find that 85% of all his lymphocytes coexpress CD5 and CD19, which is extremely abnormal as CD5 is only found on a small subset of B cells. Costaining with mAbs that recognize the  $\hat{I}^+$  and  $\hat{I}^\circ$  Ig-light chains reveals that 85% of all the lymphocytes (or 100% of all the B cells) express only the  $\hat{I}^\circ$ -light chain. Hence, this patient has a monoclonal proliferation of B cells. Analyses with other mAbs reveals that the  $\hat{I}^\circ$ -restricted cells stain for a variety of B-cell markers in a pattern consistent with the disease chronic lymphocytic leukemia. The remaining 15% of non-B cells are normal T cells. Hence, the patient has a hematopoietic malignancy with a severe defect in the cellular arm of his immune system.





**Figure 16-3.** Monoclonal antibody staining and flow cytometric analysis of normal peripheral blood. The top panel shows an xy plot of the forward versus side light scatter of the blood elements, which reflect their size and complexity, respectively. Under the top graph are six panels representing analysis of the cells in the various gated region (T lymphocytes in the first two panels, B lymphocytes in the middle two panels, and monocytes/granulocytes in the lower two panels). The percentage number of each cell type within each quadrant (ie, positive for neither, one or two of the mAbs shown) are shown as a function of the number of cells within the region (R1, R2, or R3) not the total number of leukocytes. To calculate the total number of cells in a given subset one would need to multiply the number of cells in that subset times the total number of cells in that region. For example, CD4 T cells make up a total of 25% of this patient's peripheral leukocytes (50% of the lymphocytes are CD4+ and the lymphocytes in R1 are 50% of all the cells). Shown next to each marker is the type of fluorochrome used to label that mAb: fluorescein isothiocyanate (FITC) for green fluorescence, phycoerythrin (PE) for orange color, and CY5 for red color.

As can be seen from these simple cases, flow cytometry has the overwhelming advantage of being a rapid and objective method for the enumeration of the different subsets of various hematopoietic cells. With the continued development of mAbs that recognize an increasing array of cell surface markers, our ability to subclassify blood cells will continue to improve.

## Fluorescence-Activated Cell Sorters

Flow sorter machines are more complex versions of standard flow cytometers that can not only detect the fluorescence from surface-bound mAbs but can also separate the differentially stained cells (Figure 16-5). The machines direct the flow of single cells into different collection tubes using electric fields that are triggered by the fluorescence of the cells. This results in rapid, accurate, and highly reproducible separation of cells based on their differential staining with mAbs. Viability and sterility can be maintained, so that separated cell populations can be cultured *in vitro* and used for functional assays. Cell sorters are used extensively in the research setting, but less so in the clinical laboratory.

## *Clinical Uses of Flow Cytometry*

Some of the many clinical applications of flow cytometry are listed in Table 16-1. The most widespread use of the technique is for enumeration of lymphocyte subsets in patients with immune defects (most commonly AIDS—see Chapter 46) and in the classification of hematopoietic malignancies (leukemia/lymphomas—further described in Chapter 43). The most commonly

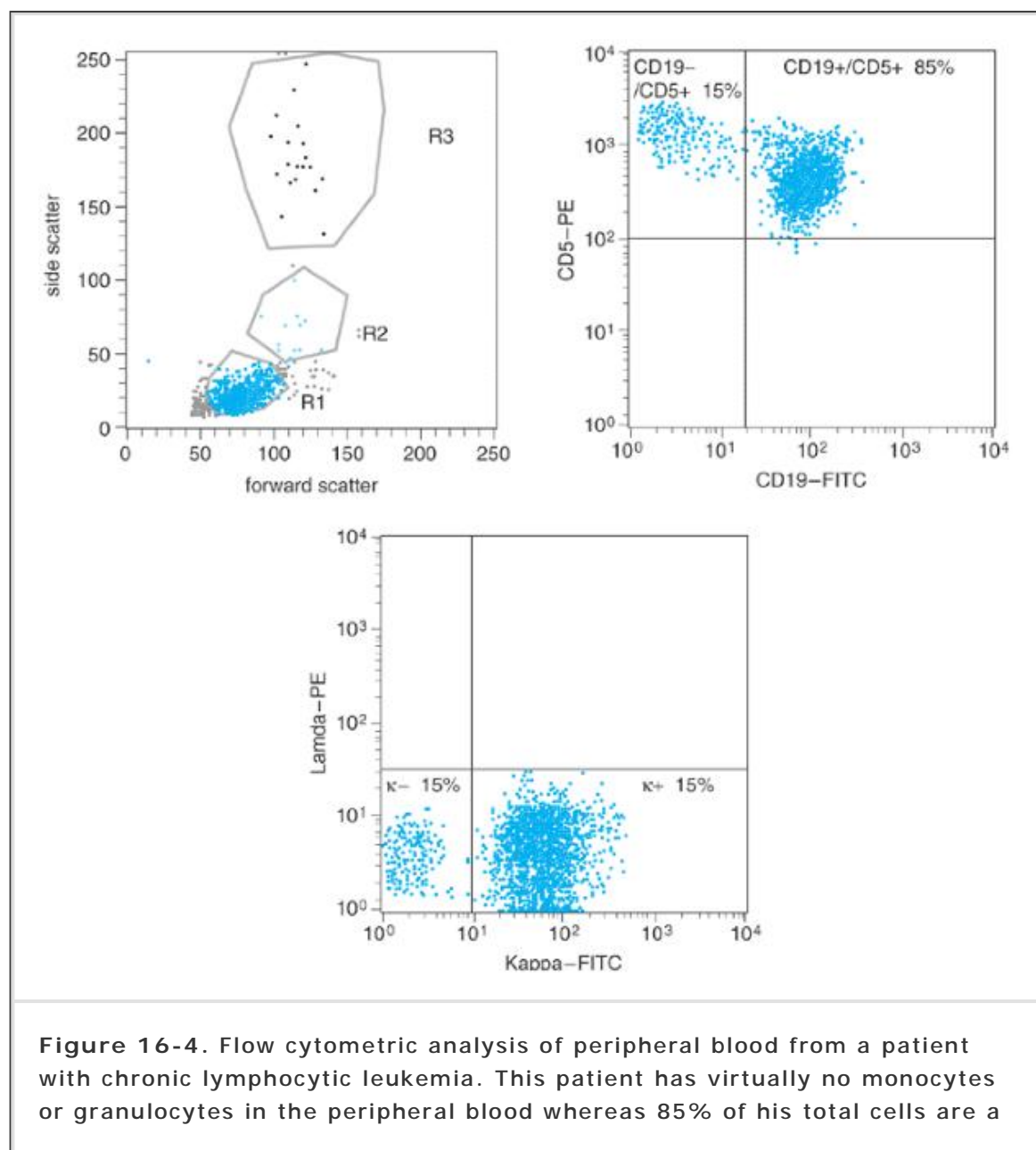
used assays are described as follows.

## T-Cell Antigens

HIV infection causes the progressive loss of CD4 T cells, which heralds the onset of frank immunodeficiency. Flow cytometry is used frequently to enumerate the total number of CD4 cells in these patients. This is often done by multiplying

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the percentage of CD4 lymphocytes times the total lymphocyte count provided by the hematology laboratory. Commercially available methods of determination of absolute CD4 cell counts by flow cytometry have been recently developed using a known number of fluorescent beads added to the sample to provide a standard against which to compare the cell percentage.



monospecific type of B lymphocyte that marks aberrantly with the CD5 antigen. The lack of monocytes and granulocytes indicate that this patient is at great risk for infection.

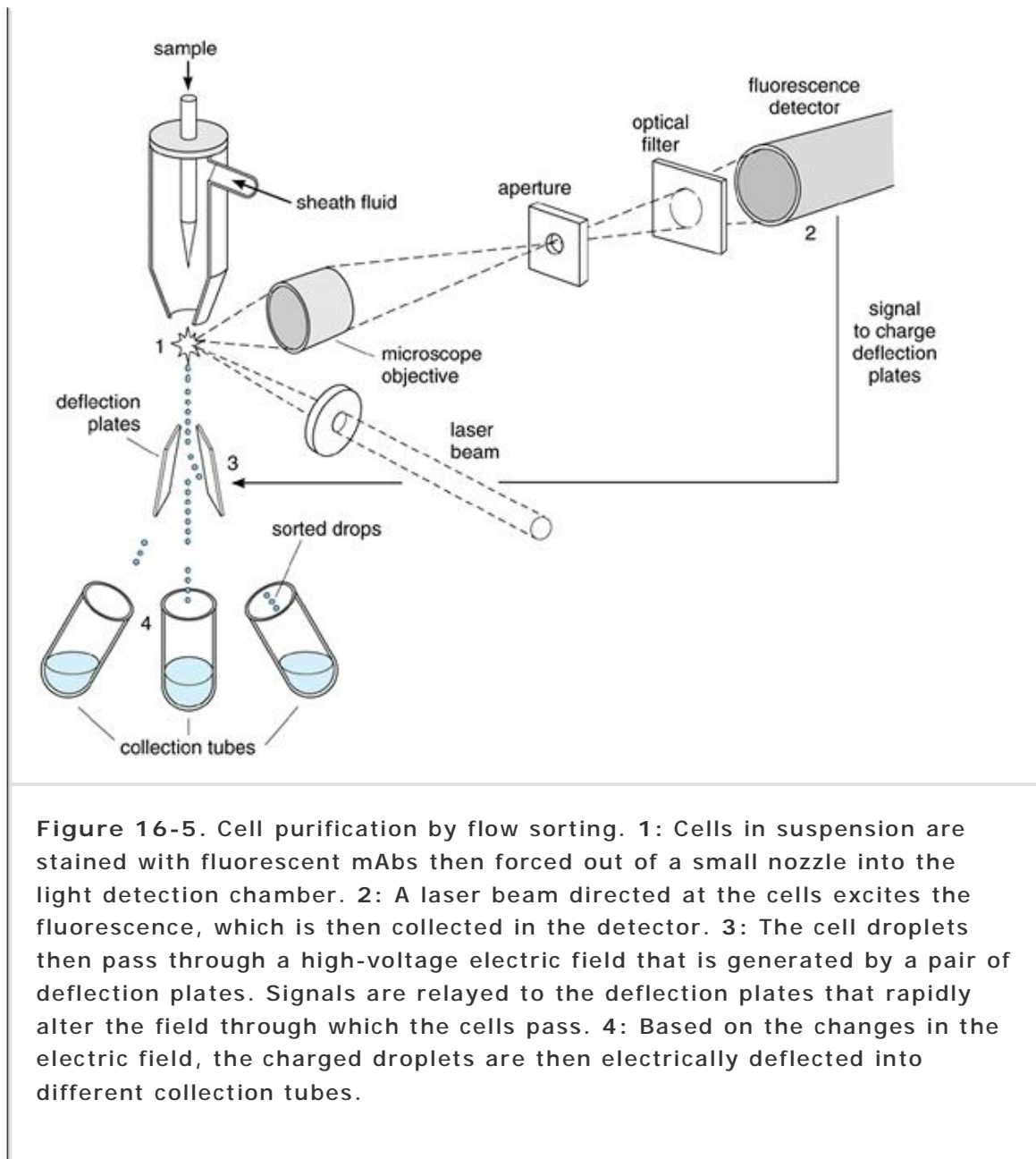
Routine monitoring of T lymphocytes is also performed in the setting of induced immunosuppression in transplantation patients, such as during the treatment of transplant rejection by the administration of T-cell-depleting mAbs. Likewise, recovery of T cells during bone marrow transplantation is routinely followed by flow cytometry. Classification of primary forms of immunodeficiency that result from defects in T-lymphopoiesis depends on leukocyte phenotyping. Primary T-cell immunodeficiencies include **Wiskottâ€Aldrich syndrome**, **DiGeorge syndrome**, and a variety of **severe combined immunodeficiency states (SCID)**, all of which result in loss of T-cell subsets. Alterations in the ratio of CD4 to CD8 T cells can also occur in many other diseases, including autoimmune disorders and infectious disease. The diagnosis of T-cell malignancies depends critically on flow cytometry.

## B-Cell Antigens

Characterization of B-cell phenotypes has its primary use in diagnosis of hematopoietic malignancy. Different antigenic markers appear at different stages of B-cell differentiation. These different markers can determine the stage of B-cell development at which the malignant transformation

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occurred and can be used to classify the different subtypes of B-cell leukemias and lymphomas. Knowledge of the surface antigen phenotype of these malignancies is important for prognostic and therapeutic decision making.



**Table 16-1. Clinical applications of flow cytometry.**

**Leukocyte phenotyping**

Diagnosis of congenital immunodeficiency diseases

Assessment of prognosis of HIV-positive patients

Monitoring of immunotherapy or chemotherapy in immunodeficiency



diseases

Monitoring of immune reconstitution in bone marrow transplant recipients

#### **Tumor cell phenotyping**

Diagnosis and classification of leukemias and lymphomas

Determination of clonality of immunoglobulin-bearing cells from lymphomas and leukemias

Differentiation of hematopoietic from nonhematopoietic tumors or cells

Assessment of prognosis of cancers

#### **DNA analysis**

Determination of aneuploidy

Determination of cell cycle kinetics

#### **Neutrophil function analysis**

#### **Other applications**

Reticulocyte counting

Platelet-associated immunoglobulin detection

Leukocyte crossmatching in transplant recipients

Cytogenetics

Enumeration of B cells is also important in evaluating primary immunodeficiency. The most common form of immunodeficiency, **common variable immunodeficiency** can present with reduced immunoglobulin levels and low numbers of B cells. Likewise, the X-linked **Bruton agammaglobulinemia** disorder results from a defect in B-cell development revealed by a profound defect in their numbers. In the **hyper-IgM syndrome** class switching does not occur, and patients fail to express IgG or other isotypes on their cell surface.

## **Myeloid Antigens**

Phenotypic analysis of myeloid antigens is used extensively to characterize **myelodysplastic syndromes** and **myeloid leukemias**.

**Hematopoietic stem**—**progenitor cells** express CD34. Detection of these cells by anti-CD34 staining

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has become a critical component of bone marrow transplantation procedures. This assay allows one to count the number of progenitor cells in a bone marrow sample and thus determine the effective dosage of stem cells being delivered to the recipient during transplantation. In many centers, stem cell transplantation is now performed by treating donors with cytokine cocktails (eg, granulocyte colony-stimulating factor [G-CSF]) that result in the production and release of large numbers of stem—progenitor cells directly into the peripheral blood. These cells can be easily harvested from the blood, concentrated, and enumerated by anti-CD34 staining. Specific doses of CD34 cells can then be

delivered to the recipients (whose own hematopoietic systems are usually destroyed following intensive chemotherapy for malignancy) to ensure rapid and complete engraftment by donor cells. Extra donor cells are often stored in liquid nitrogen and can be delivered to the patient at a later date as “salvage” therapy if needed.

A technical problem with staining myeloid cells (and to a lesser extent, B cells) is the nonspecific binding of labeled mAbs to Fc receptors on the cell surface. Myeloid cells express abundant levels of the receptors that recognize the Fc region of certain immunoglobulin isotypes (eg, Fc $\gamma$  receptors bind the Fc portion of IgG). Monocytes—macrophages and granulocytes use these receptor systems to facilitate the uptake and killing of Ig-bound microorganisms (and other foreign particles). However, these receptors can also bind fluorescently labeled mAbs that are used as staining reagents and are readily detected by flow cytometry, giving the false impression that specific binding has occurred. To control for this phenomenon, the staining of the specific mAb is compared with the staining of an **isotype-matched mAb** that is raised against an unrelated antigen, such as a hapten or nonmammalian protein. Alternatively, many laboratories routinely block Fc $\gamma$ Rs on cell samples by preincubation with large amounts unlabeled rabbit polyclonal antisera. This preincubation step blocks all the Fc receptors and reduces nonspecific staining.

## Intracellular Antigens

Flow cytometry can also be used to detect a variety of intracellular antigens. In these circumstances cells must be first treated with agents that make the cells permeable (ie, result in holes in the membrane) without completely breaking them apart. At the same time, the cells are treated with mild fixatives that will lightly cross-link cytoplasmic proteins in place and prevent them from leaking outside the cell. The newly permeable cells are then incubated with mAbs that recognize the intracellular antigens, the mAbs enter the cells and bind directly, after which the cells are analyzed by flow cytometry. This procedure tends to have more technical problems than traditional cell surface staining and is influenced both by the antigen to be studied and the particular mAb used (not all mAbs work). Nevertheless, detection of **intracellular terminal deoxynucleotidyl transferase (TdT)** and **myeloperoxidase** are routinely performed in clinical laboratories to help define early B-cell malignancies and myeloid leukemias, respectively. Tdt, an enzyme expressed during immunoglobulin gene rearrangement, is responsible for addition of random oligonucleotides at the junctions of Ig segments. Hence, its expression in a malignant B-cell population is indicative of a very immature B-cell phenotype. Likewise, myeloperoxidase is a major constituent of primary granules in neutrophils; expression of this protein develops midway through granulocyte maturation and hence is used to classify various subtypes of myeloid leukemias.

## Other Uses of Flow Cytometry: DNA-Ploidy, Functional Assays, and Apoptosis.

The DNA content of cells is a direct reflection of their position in the cell cycle

(Chapter 1). In populations of rapidly dividing cells, a large number are in S, G<sub>2</sub>, and M phases of the cell cycle and hence have more than  $2n$  chromosomes. Similarly in tumors with a large number of genetic rearrangements, chromosomal duplications, and amplifications (**aneuploidy**), a large percentage of cells have an aberrant amount of DNA. Flow cytometry is routinely used to assay the amount of genetic material in a cell to determine the **S-phase fraction** or amount of aneuploidy in a population of cells. This technique is often applied to solid tumors (breast, colon, prostate cancers) because in many circumstances the number of highly replicative cells and the degree of genetic rearrangement correlate with prognosis. DNA analysis is performed by incubation of permeabilized cells with fluorescent dyes (eg, propidium iodide) that will intercalate into the DNA and fluoresce when bound; the amount of fluorescence is directly proportional to DNA content.

A number of leukocyte functional assays use flow cytometric detection methods. The production of O<sub>2</sub><sup>-</sup> (superoxide) as an assay for chronic granulomatous disease is routinely performed in clinical laboratories (see under Neutrophil Function Assays). Other assays tend to be restricted to the research laboratory, but include methods for measuring phagocytic uptake of fluorescent particles, degranulation assays, determination of intracellular Ca<sup>2+</sup> mobilization (during leukocyte activation), and changes in cytoskeletal structure.

Cell viability is routinely determined in the clinical laboratory by flow cytometry. In the simplest method, cells are incubated directly with propidium iodide, and the proportion of fluorescent (dead) cells is directly counted. Because the dye will only enter cells that already have broken membranes and a disrupted nuclear structure, only dead cells fluoresce. Because the fluorescence of propidium iodide is in the far red portion of the spectrum, this method is often combined with regular mAbs staining in mixed cell populations. The dead cells can be identified in the population, and their staining properties can be

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ignored during data analysis. A number of other fluorescent markers have been developed to detect cells undergoing **apoptosis** (the process of active cell death), including binding proteins (eg, annexin V) that recognize abnormal lipids on apoptotic cell membranes. The binding of these proteins is usually detected by flow cytometry.

The number of different methodologies that use flow cytometry continues to grow. The preceding is only a partial list—other assays include bacterial determination, viral assays, and nucleic acid sequence detection. Basically, any assay that has a fluorescent readout and that can be performed on single-cell suspension is amenable to detection by flow cytometry. Flow cytometry is one of the most heavily used and rapidly growing methodologies (perhaps second only to molecular nucleic acid based assays) in the clinical laboratory.

## DELAYED-TYPE HYPERSENSITIVITY SKIN TESTING

Delayed-type hypersensitivity (DTH) skin testing is used clinically for two primary reasons: (1) to assess immune competence and (2) to determine whether a patient has memory T cells that recognize a particular pathogen (ie,

evidence of prior infection). Testing is performed by intradermal (not subcutaneous) injections of sterilely prepared antigens into the forearm or other easily accessible skin site. The degree of induration (swelling due to inflammation) is measured 48 hours after injection. The DTH skin response requires antigen-specific memory T cells and produces inflammation that peaks approximately 48 hours after the injection. Inflammation results from the production of local cytokines and chemokines at the injection site, which results in the recruitment of large numbers of neutrophils and mononuclear cells. The DTH response is a true measure of the cellular-dependent arm of the immune response.

The timing and induration help to distinguish DTH reactions from two other inflammatory responses to the intradermal injection of antigen. IgE-mediated (mast cell-dependent) hypersensitivity responses produce immediate wheal and flare at the site of injection and, occasionally, late-phase responses after several hours. Inflammatory reactions that result from the formation of immune complexes at the skin site—the so-called Arthus reaction—develop 12–24 hours after injection. This response is indicative of high levels of preexisting IgG to the test antigen.

Skin testing with a battery of antigens against common fungal agents (some pathogenic, others not) can be used to validate the general competence of the cellular immune system. Common test antigens (also known as recall antigens) are listed in Table 16-2. In some cases, competence testing can be done using antigens to which most patients in the United States have been immunized as children and hence should mount vigorous responses. Patients who fail to respond to common antigens are referred to as **anergic** and have some defect in cell-mediated immune responses. Clinical situations that may result in anergy are outlined in Table 16-3.

Table 16-2. Examples of common antigen preparations used for DTH testing.	
Antigen	Comments
<i>Candida albicans</i>	Common, nonpathogenic organism against which normal patients should respond
Trichophyton (dermatophyton)	As with <i>C. albicans</i> , used as control
Coccidioidin	Antigen of coccidioidomycosis

Histoplasmin	Antigen of histoplasmosis
Tuberculin purified protein derivative (PPD)	Antigen of <i>Mycobacterium tuberculosis</i>
Mumps	Used to validate prior vaccination
Tetanus toxoid	As with mumps testing

In the United States, the DTH test most commonly used to determine prior infection with a particular pathogen is the response to **purified protein derivative**

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(PPD), which assesses past infection with tuberculosis. The PPD test permits the identification and treatment of latently infected individuals prior to the onset of clinical disease. Obviously, anergic patients who fail to respond to recall test antigens may have false-negative results with PPD (or any other DTH test).

### Table 16-3. Clinical conditions associated with anergy.

- I. Pharmacologic Treatments
  - Corticosteroids
  - Immunosuppressive drugs (cyclosporine, anti-T-cell Ab treatment)
  - Chemotherapeutics for malignancy
  - Some nonsteroidal therapies (in some patients)
- II. Immunologic Deficiencies
  - Congenital immunodeficiency (SCID, Wiskottâ€Aldrich, DiGeorge)
  - Ataxiaâ€telangiectasia
  - AIDS
- III. Coexistent Diseases
  - Carcinoma
  - Chronic lymphocytic leukemia
  - Lymphomas (Hodgkins and non-Hodgkins)
  - Sarcoidosis
  - Uremia
  - Liver disease (cirrhosis)

- Autoimmune disorders—rheumatoid arthritis
- IV. Coexistent infectious diseases
  - Influenza, measles, mumps
  - Miliary or active tuberculosis
  - Disseminated mycotic infections
  - Lepromatous leprosy
- V. Technical errors in skin testing
  - Improper Ag concentrations (too dilute)
  - Injection too deep into skin
  - Improper interpretation of test results

Repeated PPD testing may produce a so-called booster effect. This phenomenon is seen in individuals, particularly the elderly, who have had prior infection with tuberculosis but whose cell-mediated immune response to tuberculosis has waned over the years such that it does not produce induration when first challenged with PPD. The initial test, however, “boosts” the patient's immune response to PPD antigens, and, as a result, a positive skin test occurs on subsequent challenge with PPD. Unless one is aware of the booster effect, the combination of an initial negative PPD test and a subsequent positive test can lead to the incorrect conclusion that the patient has been infected with tuberculosis in the interim. To avoid being misled by the booster effect, many nursing homes employ a two-stage test in which PPD is administered twice, one month apart.

DTH skin testing is of relatively little value in assessing cellular immunity during the first year of life. Infants may have limited exposure to the various recall antigens used, so control results are difficult to interpret. Consequently, leukocyte phenotyping and in vitro assays for T-cell function are much more useful in the diagnosis of congenital immunodeficiency.

A variant form of DTH testing is used in the diagnosis and evaluation of dermatitis—so-called **contact hypersensitivity** testing. Contact hypersensitivity develops as a result of cutaneous exposure to a sensitizing antigen. Upon reexposure to the antigen, inflammation develops at the skin contact site 48–72 hours later. A classic form of contact hypersensitivity is the skin inflammatory responses to the antigens of plants such as poison oak or poison ivy. **Patch testing** is commonly employed by allergists and dermatologists to detect cutaneous hypersensitivity to various substances thought to be responsible for contact dermatitis. The test substance is applied in a low concentration and the area covered with an occlusive dressing. After 48–96 hours, the dressing is removed and the site is examined for the presence of inflammatory reactions. Patch testing is commonly done with panels of known and common antigens, which can often reveal the presence of unsuspected clinical sensitivity to various agents. False-positive reactions can

result from too high a concentration of the test substance, irritation rather than allergy, and allergy to the adhesive used in the dressing. False-negative tests usually result from too low a concentration of the test substance and inadequate skin penetration. The results of patch testing must be weighed with clinical history—the combination of a positive test in the setting of a strong history of exposure is best for reliable diagnosis.

Occasionally, patients who are highly sensitive to various antigens have marked local reactions to skin testing. These severe reactions include marked induration and even skin necrosis at the challenge site. This can occur in patients with robust immune response to PPD antigens. Injection of corticosteroids into the site of severe induration will significantly abort the immune response. If unusual sensitivity is suspected (ie, if there is a high probability that the patient may have been exposed to the test antigen or may have had a strong positive result in the past), preliminary testing with dilute solutions of antigen are indicated. Similarly, painful blistering and inflammation can sometimes occur following surface application of contact sensitizers during patch testing; this is also adequately treated by topical steroid application.

## LYMPHOCYTE ACTIVATION ASSAYS

The two primary general types of assay of lymphocyte activation are (1) determination of changes in cell surface phenotype (ie, acquisition of “activation” markers on the cell surface) and (2) the ability of lymphocytes to proliferate following stimulation. In most cases, lymphocyte activation assays evaluate mainly T-cell responses, but under certain circumstances evaluation of B-cell activation may also be important. Related to these methods are assays that measure the products of activated lymphocytes (T-cell cytokines or B-cell-produced immunoglobulins) or the acquisition of effector function by cytolytic T cells. Most of these assays are performed by in vitro culture of lymphocytes isolated from various body sites. Determination of the ability of lymphocytes to become activated is used principally to characterize immunodeficiency states. Lymphocyte activation measures the *functional* capability of lymphocytes to respond to antigenic or mitogenic stimulation and is therefore a more direct test of immunocompetence than simple enumeration of lymphocyte numbers. Most of these assays are not directly amenable to standardization between different laboratories, and, although they appear to be quantitative, they should be viewed more as qualitative assessments of immune response. Furthermore, activation type assays demonstrate the greatest biologic variation between individuals.

### **Activation Markers**

Activated T cells undergo a series of morphologic and phenotypic changes. This includes an expansion in size, the manifestation of open chromatin by histologic staining, and the expression of surface proteins not found on small resting cells. The expression of activation markers by T cells can be determined in fresh cells directly isolated from inflammatory sites or in cultured cells that have been stimulated in vitro. In both circumstances, determination is made by flow

cytometry. The activation

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of T cells in vitro can be accomplished either with specific antigens (plus antigen presenting cells) or through use of general lymphocyte mitogens. The low frequency of T cells specific for a particular antigen can render these cells difficult to detect in many samples. The number of activated T cells isolated from an inflamed site, however, may be much greater than the number of such cells circulating in the peripheral blood. For example, activated T cells can be isolated by bronchoalveolar lavage from sarcoidosis patients or from the cerebrospinal fluid of patients with active multiple sclerosis.

### ***Lymphocyte Proliferation***

Lymphocyte proliferation is usually determined using polyclonal activators of lymphocytes or lymphocyte mitogens. The most commonly used T-cell stimuli are lectins, such as phytohemagglutinin (PHA) and concanavalin A (Con A); bacterial toxins that act as superantigens; chemical compounds, such as phorbol myristate acetate (PMA) and calcium ionophores; cytokines; and mAbs to surface receptors (especially CD3). B-cell proliferation is often induced with pokeweed mitogen (PWM—although this will also activate T cells), superantigens, lipopolysaccharide (LPS), or mAbs that cross-link the surface immunoglobulin (B-cell receptor). The mixed lymphocyte reaction, which assesses responses to histocompatibility antigens, is reviewed in Chapter 19.

Proliferation responses are measured using purified lymphocytes cultured in vitro in small 96-well microtiter plates. Cells are stimulated for defined periods (usually 48 hours), after which DNA synthesis is measured by pulse labeling the cultures with tritiated thymidine ( $^3\text{H}$ -Tdr). The incorporation of  $^3\text{H}$ -Tdr into chromosomal DNA reflects the rate of cell proliferation. One can also use nonradioactive assays to determine cell proliferation, such as using fluorescent dyes that incorporate into DNA or dyes that measure oxidative respiration such as MTT (a tetrazolium substrate that is converted to an insoluble product and is measured in the spectrophotometer). Bromodeoxyuridine (BrdU) is also often used to assay proliferation—like  $^3\text{H}$ -Tdr it will be incorporated into DNA in highly replicative cells—and it can be detected in DNA using mAb staining (similar to the methods used for other intracellular antigens) followed by flow cytometry. The advantage of BrdU staining is that it can be delivered to patients in vivo, hence one can directly follow the proliferative fraction of a lymphocyte subset during a natural immune response. BrdU staining has also found a clinical use in grading the proliferative fraction of tumors in vivo. Patients are given an injection of BrdU after which tumor tissue is removed and the proliferative fraction is determined by flow cytometry.

Both the culture time and the dose response can affect the interpretation of lymphocyte proliferation assays. Since clinically important defects in cellular responses are rarely absolute, quantitative relationships between normal control samples and patient samples need to be established as much as possible. Using both microtiter culture systems and semiautomated cell harvesters, one can attempt to determine both the dose and time response kinetics of either



mitogen-activated or antigen-stimulated T cells. Altered lymphocyte function can result in shifts in either time or dose response curves. Such comparison may allow one to tease out subtle or partial defects in lymphocyte responsiveness that may occur in different disease states. Considerable controversy exists in the literature concerning the form in which data are presented. Many laboratories report simply the counts per minute of incorporated  $^3\text{H}$ -Tdr. It is also common, however, to use a "stimulation index" — the ratio of the incorporated  $^3\text{H}$ -Tdr in stimulated versus resting cultures. Neither method is entirely satisfactory. Because the stimulation index is a ratio, marked differences between patient and control samples can result simply from changes in the low levels of  $^3\text{H}$ -Tdr incorporated by resting cells.

Antigen-dependent proliferative responses can be assessed using antigens to which the patient mounts a vigorous DTH response. In general, normal subjects show agreement between the results of skin tests and antigen-induced lymphocyte activation. In certain conditions, however, the *in vitro* technique may be a more sensitive index of cell-mediated immunity to a specific antigen. As in the case of mitogen-induced activation, time and dose response kinetics are crucial in generating reliable data. Compared with mitogen-induced lymphocyte activation, antigen stimulation results in lower total DNA synthesis because only a fraction of the T cells respond, and the time to maximal response is usually delayed.

### ***Cytolytic T-Cell Responses***

Assays for cytolytic T lymphocytes (CTL) in patients can be performed as a variant of a mixed lymphocyte culture using allogeneic cells (see Chapter 19) or can be done using autologous target cells that express the antigen of interest and are loaded with  $^{51}\text{Cr}$ . Cytotoxicity is measured as the percentage of  $^{51}\text{Cr}$  released from specific target cells compared with the percentage released from control (nonspecific) targets. Because CTL responses are restricted by MHC class I molecules, CTL assays require the generation of custom target cells for each patient and thus are almost exclusively limited to research applications.

### ***Cytokine Production***

Activated T cells produce a large repertoire of cytokine products. One can assay cytokine production to assess the type of immune response occurring within a given site in the body, for example, to distinguish  $\text{T}_\text{H}1$  versus  $\text{T}_\text{H}2$  type responses. In the simplest system, T cells isolated from inflammatory sites are cultured *in vitro*, and the spectrum of cytokines they release into the media is determined by enzyme-linked immunosorbent (ELISA)-type assays (see Chapter 15). Alternatively, resting T cells can be activated in culture and

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cytokine production determined. These methods measure the total amount of any given cytokine produced by a T-cell population, but do not allow one to judge the percentage of T cells within the population that are producing a given cytokine. To determine this, the enzyme-linked immunospot (ELISPOT) assay can be used. Activated lymphocytes are incubated in a semisolid agar that limits diffusion of

the cytokine products to the immediate area of the cell. The agar is then dried and probed with labeled anticytokine mAbs, producing spots that represent T cells producing the cytokine of interest. Finally, one can determine cytokine production cell by cell by mAb staining for intracellular cytokines followed by flow cytometric analysis. Currently, assays of cytokine responses remain primarily a research tool.

## MONOCYTE-MACROPHAGE ASSAYS

Monocytes and macrophages, which are considered part of the innate immune system, coordinate adaptive immune responses through cytokine production, act as effectors to remove specific types of pathogens, and play a central role in clearing apoptotic cells during tissue remodeling and development. One can readily identify these cell types in tissue samples or cell suspensions using mAb staining. As shown in Figure 16-3, identification of monocytes in the peripheral blood by flow cytometry is most reliably performed using CD14 mAb staining combined with appropriate forward and side scatter gating. Mononuclear cells also express a host of other markers (CD11b, CD11c, CD16, CD32, CD64, and scavenger receptors of various types) that facilitate their identification. Histochemical staining for nonspecific esterase ( $\bar{I}\pm$ -naphthol esterase) is commonly performed on leukemic samples to define monocyte-derived disease. Remember that the great heterogeneity in the types of tissue macrophages (Langerhans' cells, Kupffer cells, osteoclasts, alveolar macrophages, bone marrow macrophages, lymph node dendritic cells) is also reflected in the variety of surface markers expressed by these cells.

## NEUTROPHIL FUNCTION ASSAYS

Polymorphonuclear neutrophils (PMNs) are the primary effector cells of the innate immune system. These cells have a finite life span (a relatively short 24 hours) and are constantly being produced in the bone marrow. PMNs are the first cells to enter an inflammatory site, where, following activation by a variety of stimuli, they undergo respiratory burst to release superoxide, degranulate to release antimicrobial peptides and proteins, and produce limited numbers of proinflammatory cytokines. PMN deficiency results in susceptibility to bacterial (or other pathogen) infection. Such deficiencies are due either to reduced numbers of PMNs (as occurs with bone marrow suppression following chemotherapy) or to defects intrinsic to the PMN. This discussion will focus on the latter and review methods to evaluate intrinsic defects that are clinically significant. These include assays to evaluate adhesion, chemotaxis, phagocytosis, production of superoxide, and bacterial killing. Many of the methods to evaluate PMN function use nonstandardized procedures; hence, different methods are often used by different laboratories.

### *Neutrophil Adhesion*

The ability of PMNs to adhere to endothelial surfaces and migrate into inflammatory sites is critical for their ability to control bacterial infections. PMNs use a host of cell surface receptors, including selectins and integrins, to carry

out this function. The major leukocyte-associated selectin, L-selectin, acts in concert with endothelial selectins to allow PMNs to roll along the endothelial surface. In the presence of inflammatory mediators (chemokines,  $\text{TNF}\alpha$ , or bacterial products such as LPS), the PMNs become activated and firmly attach to the endothelial surface using their integrin receptors. Deficiency of either selectins or integrins results in a specific deficiency of leukocyte adhesion (so-called **leukocyte adhesion deficiency** or LAD). In such patients, PMNs fail to enter into inflammatory sites, and bacterial infections rapidly spread. Clinically these patients are recognized by their inability to make pus at the site of an infection. Lack of these surface molecules can be readily determined by mAb staining and flow cytometry: LAD I patients lack the  $\alpha_x$ -subunit (CD18) of the major leukocyte integrins, and LAD II patients lack a fucosyl transferase enzyme involved in the expression of selectin ligands on the leukocyte surface. The inability of these PMNs to adhere to appropriate surfaces can be evaluated using in vitro adhesion assays. PMNs are activated with various agents and allowed to bind to ligand-coated surfaces, and the strength of binding is determined by resistance to removal with washing. Neutrophils also use a variety of cell surface receptors to bind opsonized pathogens. These include  $\text{Fc}\gamma\text{Rs}$  and various sugar-binding proteins (scavenger receptors). One can determine the expression of these receptors by mAb staining or by determining the binding of fluorescently labeled particles, coated with the appropriate ligands, to the surface of the PMNs. Clinically recognized immunodeficiency resulting from the lack of these receptor systems has yet to be defined. The ability of PMNs to respond to inflammatory stimuli, however, is manifested by up-regulation of these receptors on the cell surface. Hence, increased expression of many of these adhesion receptors can be used as activation markers for PMNs.

## ***Chemotaxis***

Directional motility of PMNs to inflammatory sites is mediated by a host of chemotactic molecules (bacterial products such as formylated peptides or host-derived chemokines). The inability

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of PMNs to respond to these stimuli results in defective migratory responses. This can be quantitated in the laboratory by use of the modified Boyden chamber assay. Cells to be tested are placed in the upper chamber and are separated from the lower chamber containing a chemotactic substance by a filter membrane with small pore size. Cells enter the filter and are either trapped in it or migrate all the way through. The extent of migration is determined by counting cells in filter and lower chamber (by flow cytometry or using labeled cells). A more rigorous method of determining PMN chemotaxis involves the use of semisolid agarose media formed in a small Petri dish, into which a hole is cut that contains the cells, the chemotactic stimulus, or control nonchemotactic proteins. The dish is incubated for several hours, and the migration of the PMNs under the agarose toward the well containing chemotactic factors is determined microscopically. Migration toward the nonchemokine-containing wells represents random motion of PMNs (so called **chemokinesis**). Defects in chemotactic responses are used to evaluate idiopathic immunodeficiencies.

## ***Phagocytosis***

Ingestion of microorganisms by neutrophils is an active process that requires energy production by the phagocytic cell. Internalization of antibody-coated and complement-coated microorganisms occurs rapidly following their surface contact with PMNs and macrophages. Because subsequent intracellular events, such as superoxide production and degranulation into the phagocytic vesicle, depend on successful ingestion, assays that evaluate phagocytosis allow one to determine the step at which potential functional defects may occur. The term *phagocytosis* is usually limited to evaluation of this initial step of bacterial killing. Assays of phagocytosis are very simple—“as easy as incubating cells directly with opsonized (IgG or complement-bound) particles and microscopically observing the cells for uptake of the particles. One must have a method to distinguish between surface-bound but not internalized particles. This can be accomplished using chemical means to remove surface-bound particles (acid treatment) or by staining with dyes that obscure the surface-bound but not the intracellular particle. More quantitative assays have been developed that use fluorescently labeled or radioactive particles that permit direct counting of phagocytosis in larger cultures of PMNs or macrophages. One can test the effect of various cellular activators (or the ability of different opsonins) to stimulate phagocytosis. A variety of particles can be used, including yeast, bacteria, and various types of red blood cells.

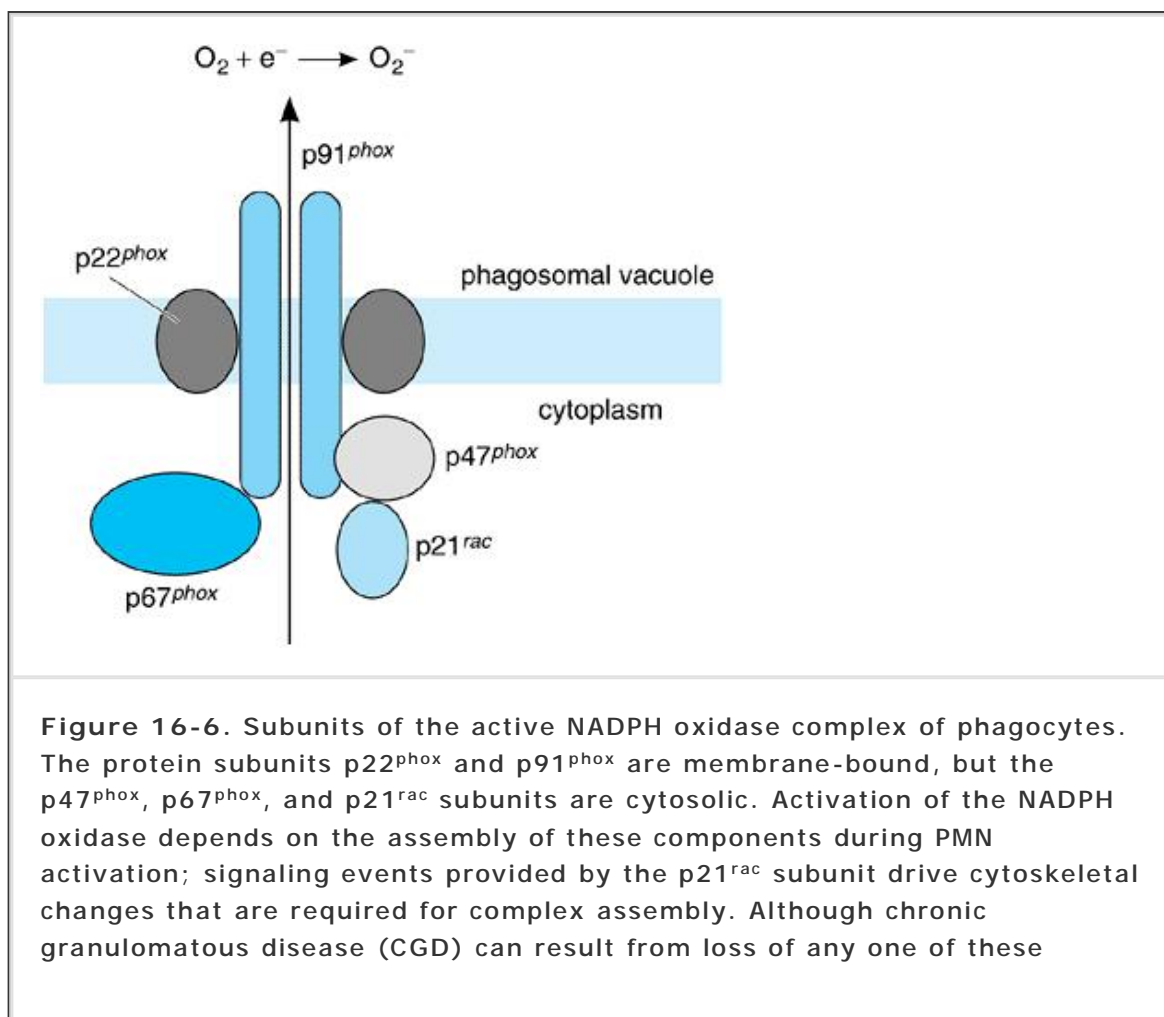
## ***Determination of Respiratory Burst and Degranulation***

Perhaps the most frequently applied test of PMN function is the determination of the ability of these cells to produce superoxide ( $O_2^-$ ). This test is used as a functional assay to screen for **chronic granulomatous disease (CGD)**, a well-recognized disorder of phagocytes caused by inherited deficiency of one of several subunits of the oxidase that acts on the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), most commonly the p91<sup>phox</sup> and p47<sup>phox</sup> proteins (Figure 16-6). Two assays are used to determine the ability of activated PMNs to produce superoxide: (1) the slide **nitroblue tetrazolium (NBT)** test and (2) the flow cytometric 2',7'-**dichlorofluorescein (DCF)** test. NBT is a clear, yellow, water-soluble compound that forms formazan, a deep blue dye, on reduction. Incubation of activated PMNs (achieved by treatment with PMA, exposure to LPS, or incubation with opsonized particles to stimulate phagocytosis) with NBT results in production of  $O_2^-$  and reduction of the dye. The activated PMNs then appear blue when visualized under simple light microscopy. This simple screening test can be performed on as little as a single drop of blood. This qualitative assay can be quantitated by extraction of the blue precipitate from the PMNs and measurement by spectroscopy. Children with complete deficiency of NADPH oxidase activity have striking defects in the slide NBT test, but mutations that result in only partial loss of function are better detected using the quantitative assay. The flow cytometric DCF assay provides a simpler and more reproducible method for CGD screening. This assay actually measures the formation of  $H_2O_2$ , which is

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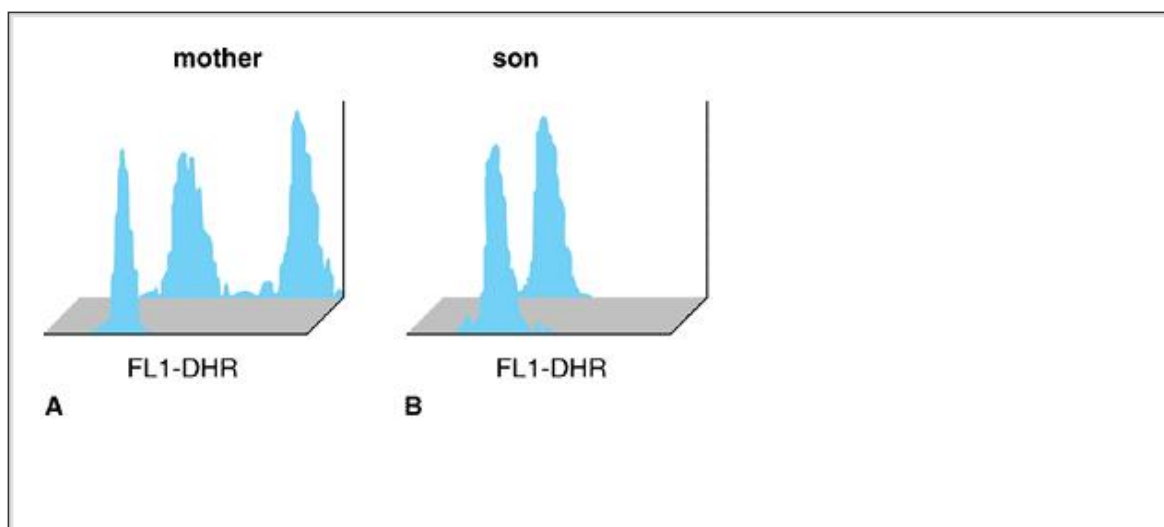
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derived from  $O_2^-$  by the enzyme superoxide dismutase.  $H_2O_2$  can be measured by the oxidation of the nonfluorescent compound 2,7-dichlorodihydrofluorescein (DCFH) to the fluorescent compound DCF, which is easily detected by flow cytometry. PMNs are incubated with DCFH, which readily enters the cells, and are then activated by incubation with PMA (or other agents). Soon thereafter, the cells are analyzed by flow cytometry, and the increase in mean fluorescence of the PMN population is determined. Because the DCF assay is based on flow cytometry, it has the tremendous advantage of enabling the visualization of individual cellular differences, thereby allowing for detection of partial defects in NADPH oxidase function or for screening for heterozygous carriers of the X-linked forms of the disease. Because about two thirds of all cases of CGD are due to mutations in the X-linked  $p91^{phox}$ , women who are heterozygous for the disease will have a population of PMNs that have randomly inactivated the normal X chromosome (by the process of Lyonization) during their differentiation from stem cells. These cells will fail to undergo respiratory burst, while other cells within the carrier will behave normally because they have inactivated the mutant allele. Hence, carriers will demonstrate two populations of reactive PMNs using the DCF or similar assays (Figure 16-7).



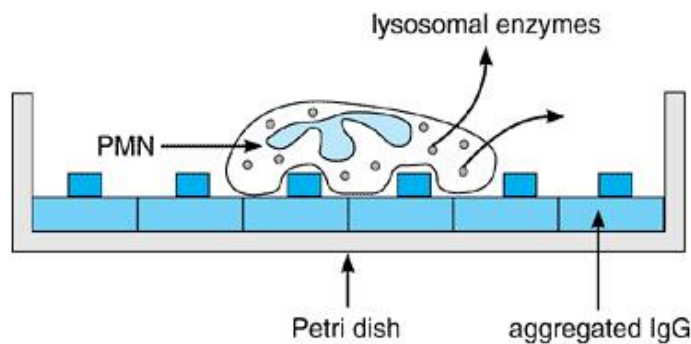
subunits, more than 90% of all cases can be accounted for by mutations in  $p47^{\text{phox}}$  (autosomal-recessive) or  $p91^{\text{phox}}$  (X-linked).

**Degranulation** assays are usually used for the diagnosis of immunodeficiency due to lack of granule constituent proteins. Degranulation is the process of fusion of lysosomes and phagosomes, with the subsequent discharge of intralysosomal contents into the phagolysosome. Degranulation is an active process and requires energy expenditure by the cell. Thus, impairment of normal metabolic pathways of the neutrophil—especially oxygen consumption and the metabolism of glucose through the hexose mono-phosphate shunt—interferes with degranulation and subsequent intracellular bacterial killing. Degranulation of PMNs can be induced in suspended cells by treatment with various activating agents and compounds that affect the actin cytoskeleton of the cell (eg, cytochalasin B). The cells will release mainly contents of secondary and tertiary granules, and these components can be measured directly by ELISA assay. The most common marker protein of secondary granules is lactoferrin, and albumin is used as a marker for tertiary granule release. Assays for release of primary granules are best done by looking for release of these granules into enclosed spaces. The “frustrated-phagocytosis system” (Figure 16-8) provides such an assay. Heat-aggregated IgG or immune complexes are fixed to a tissue culture dish, and PMNs are then incubated on the dish. The cells bind the IgG complexes through  $\text{Fc}\gamma\text{R}$  and attempt to phagocytose the particles. At the same time, primary granules fuse with the phagosomes, which remain at the cell surface, resulting in the release of primary granule constituents into media. The rate of release of primary granule proteins, such as myeloperoxidase (MPO) and  $\beta$ -glucuronidase, is used to estimate degranulation. These assays are used to search for functional defects in granule release. Deficiency in either secondary or primary granule proteins can also be diagnosed by intracellular mAb staining and flow cytometry. Surprisingly, although absence of secondary granules produces severe immunocompromise, lack of MPO (a common disorder found in 1 in 2000 people) has relatively mild effects on immune function.

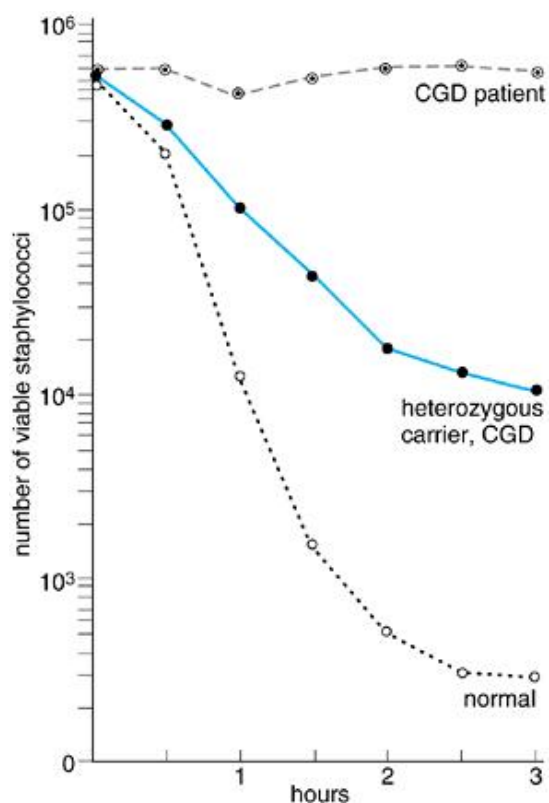


**Figure 16-7.** Three-dimensional flow-cytometric display of dihydrorhodamine (DHR) fluorescence in a patient with chronic granulomatous disease and his mother, a carrier. DHR reduction occurs by the same mechanism as DCF, although most laboratories prefer to use DCF.

**A:** Fluorescent intensity of DHR (horizontal axis) of neutrophils from the mother. The peak in the foreground shows the histogram of unstimulated cells. After stimulation with phorbol myristate acetate (PMA), two peaks are shown (background of panel A). The population to the left are neutrophils that did not reduce the DHR and thus show no increase in fluorescence. The peak to the right demonstrates an increase in fluorescence of PMNs that have reduced the DHR. In panel B the patient's PMNs do not reduce the DHR and thus there is no difference in fluorescence intensity of unstimulated (peak in foreground) versus PMA-stimulated cells (peak in background).



**Figure 16-8.** Assay of granulocyte degranulation by the "frustrated phagocytosis" method. The neutrophil is attached to aggregated IgG fixed to the bottom of a Petri dish. Lysosomal enzymes are discharged into the supernatant as the cell attempts to phagocytose the IgG but is "frustrated." (Courtesy of S Barrett.)



**Figure 16-9.** Bactericidal assay of granulocytes. Curves represent the number of viable intracellular organisms that survive after being ingested by granulocytes. Note the marked decline in bacterial survival in normal cells compared with reduced to absent killing by cells from patients and relatives with chronic granulomatous disease (CGD).

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### ***Bacterial Killing***

The microbicidal assay has long been considered the best functional assay for the evaluation of potential PMN disorders. Because efficient killing of bacteria requires all the steps described earlier, a defect in microbicidal activity can result from a defect in any of these systems. It would therefore be logical to assume that this assay is performed first in the evaluation of patients with defects in innate immune responses. This is not the case, however, because microbicidal assays are difficult to perform and labor-intensive. Microbicidal assays are usually used only when other simpler assays of PMN function (eg, respiratory burst and immunophenotyping) have failed to reveal a diagnosis.

**Table 16-4. Disorders of neutrophil function.**



### Leukocyte adherence deficiency

Chronic granulomatous disease (X-linked or autosomal-recessive)

Job's syndrome

Chediak-Higashi syndrome

Myeloperoxidase deficiency

Glucose-6-phosphate dehydrogenase deficiency

Acute leukemia

Down syndrome

Premature infants

Transient neutrophil dysfunction

Acute infections

Ataxia-telangiectasia

Many strains of bacteria and fungi are effectively engulfed and killed by human neutrophils in vitro. The bactericidal capacity of PMNs for the test strain 502A of *Staphylococcus aureus* is commonly performed. Bacteria growing in log phase are incubated with human serum (to provide a source of complement proteins and IgG as opsonins) and freshly isolated PMNs at a ratio of roughly five to ten bacteria per PMN. After a short time (30 minutes), the extracellular bacteria are killed by addition of gentamicin. Because the antibiotic does not enter the cells, intracellular organisms survive. Aliquots of PMNs are sampled following addition of antibiotic and at 30-minute intervals thereafter. Intracellular bacteria are liberated by lysis of neutrophils with sterile water, and the number of viable organisms is determined by plating serial dilutions of the lysates on blood agar bacterial plates. Results are plotted as in Figure 16-9. Normal PMNs show a two-log reduction in viable intracellular *S. aureus* after 1 hour incubation, but killing is virtually absent in PMNs derived from CGD patients. Carriers can show an intermediate phenotype. By varying the test organism or the source of opsonin, this assay can be used to measure a wide range of microbial killing activities. Because failure to ingest bacteria results in complete loss of all viable organisms following addition of the antibiotics, the laboratory must be aware of how to interpret what may appear as extremely rapid killing.

Examples of the commonly recognized neutrophil functional disorders are shown in Table 16-4.

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## 17

# Blood Banking & Immunohematology

Maurene Viele MD

Elizabeth Donegan MD

The ability to successfully transfuse whole blood, or more specific blood components, has saved countless lives and supported the advance of modern surgery and cancer chemotherapy. The first lifesaving transfusion was performed almost 200 years ago by James Blundell in 1818. Today, more than 20 million blood components, prepared from approximately 12.6 million blood donations, are transfused in the United States annually. The safety of blood transfusion has steadily improved since the first US blood bank was founded in the 1940s. Tests were developed and implemented to detect the infectious diseases recognized as transmitted in blood products. New molecular diagnostic techniques are now being investigated to improve the sensitivity of the tests used for donor blood analysis.

Nevertheless, transfusion continues to require the removal of blood from one human being for infusion into another. This “living transplant” carries with it the complexities of its human source and thereby brings with it the potential of undesirable side effects in the recipient. Some risks of transfusion are now known, and others have yet to be described. Consequently, the need for transfusion must be judged carefully in light of these risks.

## BLOOD GROUPS

The first blood group system was described at the turn of the 20th century by Karl Landsteiner. He observed that erythrocytes from some individuals clumped when mixed with the serum of others but not with their own. Using this agglutination technique, he classified an individual's erythrocytes into four types: A, B, AB, and O. It is now recognized that A and B represent carbohydrate antigens on the erythrocyte. Group O individuals have neither of these antigens on their erythrocytes, whereas erythrocytes from AB individuals have both A and B antigens. The ABO system is the most important blood group system for transfusion purposes.

Knowledge about blood groups has expanded to include a diverse and numerous array of antigenic determinants on erythrocytes. Approximately 600 erythrocyte

antigens are known, of which 207 belong to 23 recognized blood group systems. Each blood group system has members, each of which may be composed of one or more different antigens. Each antigen is controlled by one gene. The antigenic determinants of a blood group are produced either directly (for proteins) or indirectly (for carbohydrates) by alleles at a single gene locus or at other gene loci so closely linked that crossing over is extremely rare. For any antigen of a blood group, a single allele is present at that locus and other alleles are therefore excluded. A specific antigen on the erythrocyte surface is usually detected in the blood bank laboratory by reacting erythrocytes with sera known to contain antibodies reactive with that antigen. This test defines a phenotype.

## ERYTHROCYTE ANTIGENS

### *H & ABO*

Antigenic determinants of the H and ABO systems are carbohydrate moieties whose specificity resides in the terminal sugars of an oligosaccharide. On erythrocyte and endothelial surfaces, most of the antigens are bound to glycosphingolipids. Genetic control is via the production of transferase enzymes that conjugate terminal sugars to a stem carbohydrate. The H and ABO systems have separate gene loci and are independent of each another (Figure 17-1).

The H gene codes for a fucosyl transferase enzyme that adds fucose to precursor chains and completes the stem chain. The H gene is rarely absent; this phenotype (*hh*) is called *O<sub>h</sub>*, or Bombay, type. In the absence of a complete stem chain, additional sugars cannot be added despite the presence of A or B transferase, and high-titer anti-H is produced.

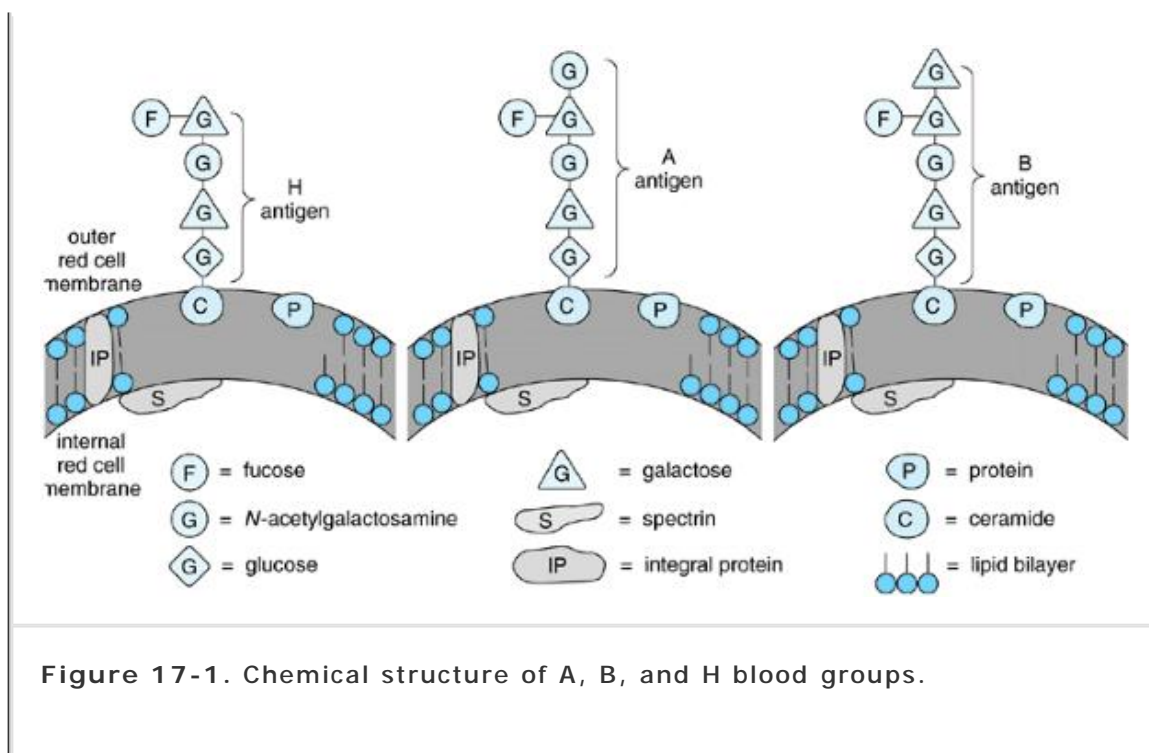
The ABO blood groups are determined by allelic genes A, B, and O (Table 17-1). The A-group transferase adds *N*-acetylglucosamine to the completed stem chain. The B-group transferase adds a terminal D-galactose. The O gene produces no transferase to modify the blood group substance (see Figure 17-1).

Both groups A and B can be divided into subgroups. Many subgroups of A have been described,

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but most are rare. The most important are A<sub>1</sub> and A<sub>2</sub>. Differences between subtypes of group A appear to be quantitative, that is, in the number of antigenic sites per erythrocyte surface. AB blood can also be divided into A<sub>1</sub>B and A<sub>2</sub>B types. Although less frequently detected, subgroups of group B can also be distinguished. Subgroups of group B, like those of group A, demonstrate a continuum in the number of antigenic sites per erythrocyte.





The naturally occurring antibodies to groups A and B are thought to be stimulated by very common substances. Intestinal bacteria are known to have substances chemically similar to and therefore antigenically cross-reactive with A and B. Antibodies to A or B antigens (or both) are first detected in children at 3â€"6 months of age, peaking at 5â€"10 years of age and falling with age and in some immunodeficiency states.

Two other systems directly interact with the ABO and H systems: Lewis and secretor. Secretion of ABH substances in body fluids (saliva, sweat, milk, etc) is controlled by the allelic genes *Se* and *se*. These genes are independent of ABO and are inherited in a mendelian dominant manner. Eighty percent of people are *Se*; they secrete Lewis antigens in addition to ABH substances. Typing of body fluids for these antigens has been useful in forensic investigations.

**Table 17-1. Routine ABO groupings.**

Blood Group	Erythrocyte Antigens	Serum Antibody	Frequency (%) in US Population			
			White	Black	Native American	Asian
O	H	Anti-A, Anti-B	45	49	79	40

A	A	Anti-B	40	27	16	28
B	B	Anti-A	11	20	4	27
AB	A and B	â€”	4	4	<1	5

### ***Rh (Rhesus)***

The Rhesus blood group system is second in importance only to the ABO system. Anti-Rh antibodies are the leading cause of hemolytic disease of the newborn (HDN) and may also cause delayed hemolytic transfusion reactions.

Recent investigations have elucidated the genetic basis of the primary Rh antigens: D, C, c, E, e. The Rh locus on chromosome 1 consists of two adjacent structural genes designated *RHD* and *RHCE*. The *RHD* gene encodes the D polypeptide present on the erythrocyte in Rh-positive individuals. The *RHD* gene is completely absent in the genome of Rh- negative individuals, which explains why no D antigen counterpart (d) has ever been found in Rh-negative people. The *RHCE* gene encodes for both C/c and E/e proteins via alternative splicing events.

Previous theories explaining the genetic basis of the Rh system gave rise to different nomenclatures.

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In the Wiener nomenclature, multiple Rh alleles were designated as either *R* or *r* with one of many superscripts. *R* alleles produced the antigen Rh<sub>0</sub>(D) in a particular phenotype in addition to two other antigens; *r* alleles denote the absence of Rh<sub>0</sub>. In the Fisher and Race system (Table 17-2), three allelic gene pairs were thought to commonly produce five antigens (the remaining antigens are rare variants). Each antigen (D, C, c, E, and e) has a corresponding designation in the Wiener system (ie, D = Rh<sub>0</sub>, C = rhâ€², etc). *C* and *c*, as well as *E* and *e*, function as alleles. No d antigen was known, so d describes the absence of D. The Rh antigens were believed to be inherited as two sets of three, one from each parent.

Clinically, Rh-positive (Rh+) means the presence of D (Rh<sub>0</sub>) and Rh-negative (Rhâ€”) indicates the absence of D (Rh<sub>0</sub>). D is the most immunogenic of the Rh antigens. Slightly less than half of Rh+ people are homozygous for D. Because there are no antisera to detect the absence of D, determination of zygosity depends on family studies or gene amplification techniques. Roughly 15% of



whites are Rh<sup>+</sup>. Rh-negativity is less common in other races. Erythrocytes with less than the normal number of D antigen sites are described and designated weak D (previously termed D<sup>u</sup>). A weak D can appear as D negative (Rh<sup>-</sup>) in testing if blood is typed only with routine anti-D antisera but is detected if the indirect antiglobulin test is used. Blood-banking standards require all donor blood to be tested using methods that detect weak D antigen. If weak D is detected, the blood unit is labeled Rh-positive.

### Other Erythrocyte Antigens

Many of the remaining 20 blood group systems are rarely implicated in transfusion reactions. Antibodies to the Kidd, Duffy, Kell, and MNS systems, however, are known for their ability to cause hemolysis if antigen-positive blood is transfused into a sensitized recipient. In general, hemolytic antibodies are IgG and react at 37°C (body temperature). IgM antibodies rarely cause hemolysis.

Antibodies to Kidd antigens are a frequent cause of delayed hemolytic transfusion reaction and can cause HDN. These antibodies are often difficult to identify in test systems because of poor reactivity. Four antigenic phenotypes have been described: Jk(a+ b-), Jk(a- b+), Jk(a+ b+), and Jk(a- b-).

**Table 17-2. Rh blood group terminology.**

Fisher-Race	Wiener	Common Genotypes
<b>Rh-Positive</b>		<b>White</b>
<i>DCE</i>	<i>R<sub>1</sub></i>	<i>R<sub>1</sub></i>
<i>DcE</i>	<i>R<sub>2</sub></i>	<i>R<sub>2</sub></i>
<i>Dce</i>	<i>R<sub>0</sub></i>	<i>r</i>
<i>DCE</i>	<i>R<sub>z</sub></i>	<b>Black</b>
<b>Rh-Negative</b>	<i>r</i>	<i>R<sub>0</sub></i>
<i>dce</i>	<i>râ€²</i>	<i>R<sub>1</sub></i>
<i>dCe</i>	<i>râ€³</i>	<i>r</i>
<i>dcE</i>	<i>r<sup>y</sup></i>	<b>Asian</b>
<i>dCE</i>		<i>R<sub>1</sub></i>
		<i>R<sub>2</sub></i>

The antigens of the Duffy system (Fy<sup>a</sup> and Fy<sup>b</sup>) are controlled by codominant alleles. Antibodies to Fy<sup>a</sup> are more commonly associated with delayed hemolytic transfusion reactions than are those to Fy<sup>b</sup>. Many blacks have a third allele, which produces the Fy(a- b-) phenotype. Duffy antigens on erythrocytes serve as receptors for the entry of *Plasmodium vivax* into the erythrocytes. Fy(a- b-) individuals who lack Duffy antigens are resistant to *P vivax* infection.

The Kell system, as first described, included the allelic pair *K* and *k*, *k* antigen

being the more frequent. The system now includes two additional allelic pairs and several variants. The K antigen is highly immunogenic, with one of 20 individuals transfused with K+ cells developing antibody. Antibodies to Kell antigen cause HDN, hemolytic transfusion reactions, and, occasionally, autoimmune hemolytic anemia. Individuals of the McLeod phenotype lack Kx antigen, which is a precursor in the synthesis of Kell antigens. These individuals have erythrocyte and neuromuscular system abnormalities. The McLeod phenotype is also associated with some cases of chronic granulomatous disease (see Chapter 24).

## METHODS FOR DETECTION OF ANTIGEN & ANTIBODIES TO ERYTHROCYTES

### *Antiglobulin Tests*

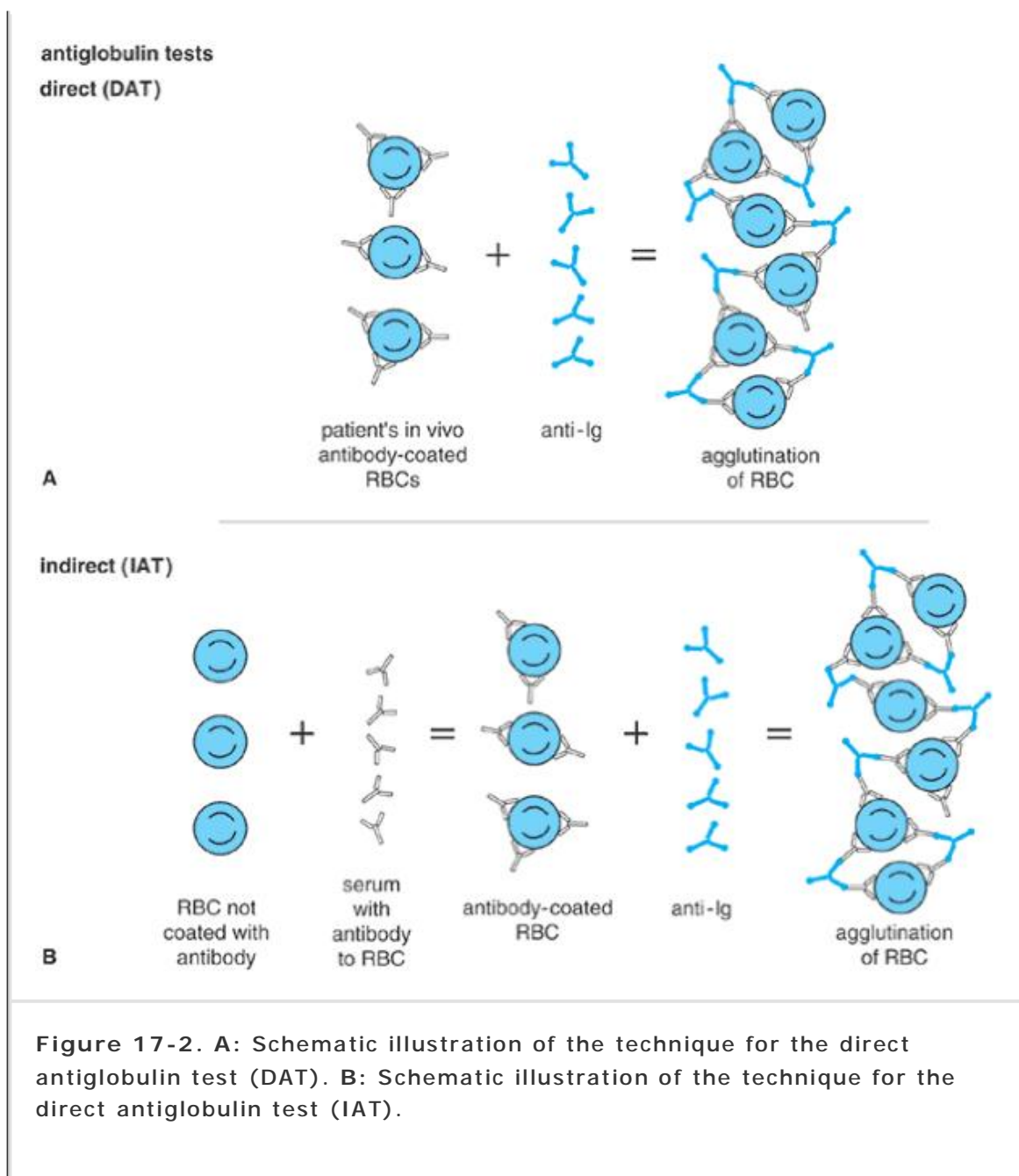
Antibody or complement adsorbed onto erythrocytes is detected by using antibodies to human serum globulins (AHG). AHG reagents are produced either in animals or in tissue culture by using monoclonal antibody techniques (see Chapter 15). These reagents may be polyspecific (a mixture of antibodies to IgG, complement, and heavy and light chains) or monospecific (antibodies to specific immunoglobulin or components of complement). The direct antiglobulin test (DAT) detects antibody or complement coating the surface of erythrocytes, whereas the indirect antiglobulin test (IAT) identifies antibody in serum or plasma.

To perform the DAT (Figure 17-2) erythrocytes are washed with saline to remove unbound antibody or complement and then AHG is added. If antibody is present on the erythrocytes, the Fab portion of AHG attaches to the Fc portion of the erythrocyte-bound antibody. Bridging of AHG Fab molecules between erythrocytes results in visually detectable agglutination. The DAT is used in the investigation of autoimmune or drug-induced hemolytic anemia, HDN, and suspected hemolytic transfusion reactions.

The IAT detects serum or plasma antibodies, which can attach in vitro to erythrocytes (see Figure 17-2).

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This test differs from the DAT in that before an IAT is performed, the serum or plasma to be tested is incubated with erythrocytes so that antibody, if present, binds to erythrocyte antigen. The erythrocytes are then washed to remove any unbound globulin, and AHG is added. If agglutination is observed, antibodies to erythrocyte antigens are present. The IAT is used by blood banks in three ways. First, to identify the presence and specificity of recipient plasma antibody, plasma is tested using panels of reagent erythrocytes with known antigens on their surface. Second, to select donor blood that is free of specific erythrocyte antigens, commercial reagents, containing known erythrocyte antibodies, are used to test donor blood for the absence of the antigen. Third, to confirm the absence of an antigen-antibody reaction, recipient plasma is tested against donor blood cells (crossmatch).



## Pretransfusion Testing

Blood is tested prior to transfusion to prevent clinically significant destruction of the transfused erythrocytes. Clinically significant antibodies are those known to have caused unacceptably shortened erythrocyte survival in vivo or frank hemolysis. Generally, these antibodies react at 37°C (body temperature) and in the indirect antiglobulin test. Prior to transfusion, the recipient's erythrocytes and plasma are tested for ABO and Rh<sub>0</sub> (D) types and for antibodies to erythrocyte antigens, often called the *œtype and screen*. Additionally, the recipient's plasma is tested for compatibility with the erythrocytes from the

intended donor (crossmatch).

### **Type & Screen**

ABO and Rh<sub>o</sub> (D) types are determined by mixing the recipient's erythrocytes with anti-A, anti-B, and

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anti-D antisera. The ABO group is then confirmed by testing the recipient's plasma against commercial reagent A and B cells to detect isoagglutinins.

The recipient's plasma is screened for alloantibodies that may not be demonstrated in the crossmatch. In antibody screens, suspensions of reagent O erythrocytes that contain known erythrocyte antigens on their surface are incubated at 37°C with the recipient's plasma. If antigen-antibody complexes are formed, hemolysis or agglutination of erythrocytes is observed. The screen is completed by the IAT and again observed for agglutination.

In the crossmatch, compatibility between donor and recipient is determined. Donor cells are combined with recipient plasma, centrifuged, and observed for hemolysis or agglutination (called the "immediate spin" crossmatch). If the recipient either has a history of previous erythrocyte antibody or has had antibody detected during the antibody-screening procedure, the IAT using recipient plasma and donor red cells must be performed before a crossmatch may be considered compatible.

A variety of methods to increase the sensitivity of the IAT has been developed. These methods add albumin, low-ionic-strength solution (LISS), polybrene, or polyethylene glycol (PEG) to the test system. Reagent erythrocytes can also be treated with proteolytic enzymes to enhance the reactivity of some erythrocyte antigens (Rh and Kidd) and to abolish the reactivity of others (M, N, Fy<sup>a</sup>, and Fy<sup>b</sup>).

## **TRANSFUSION REACTIONS**

Blood transfusion has become increasingly safe, but a variety of adverse reactions, only some of which are preventable, continues to occur (Table 17-3). Patients who are transfused must be monitored during infusion for immediate reactions and over time to detect delayed reactions.

**Table 17-3. Transfusion reactions.**

Cause	Incidence	Manifestations	Treatment
Erythrocyte antibodies Hemolytic	<0.02%	Fever, chills, hypotension. Pain in back or	Stop transfusion; blood/urine to blood bank

(acute)  Hemolytic (delayed)		infusion site. Hemoglobin in blood and urine.	Hydrate Monitor hematocrit, liver, and renal function.
		Lowered hematocrit, increased bilirubin; elevated LDH days to weeks posttransfusion.	Monitor hematocrit, also liver and renal function if severe.
Cytokines, WBC	<2%	Temperature raised $\pm 1^{\circ}\text{C}$ , chills.	Stop transfusion: rule out hemolytic reaction blood/urine to blood bank; premedicate with antipyretics; give leukocyte-reduced products if available.
Donor WBC antibodies	<0.2%	Noncardiac pulmonary edema, bronchospasm	Stop transfusion, treat symptoms.
Plasma proteins	2-3%	Itching, urticaria, rarely asthma, bronchospasm, anaphylaxis.	Stop transfusion; give antihistamines for urticaria; treat symptoms.
<i>Abbreviations:</i> LDH = lactate dehydrogenase; WBC = white blood cells.			

## ***Hemolytic Reactions***

The transfusion of incompatible blood may cause immediate hemolysis.

Immediate hemolytic transfusion reactions, which are fatal in approximately 10–40% of cases, generally occur when ABO-incompatible blood is transfused.

The cause is most often managerial or clerical error, such as transfusing patients

with units intended for other recipients. Two thirds of these errors occur in areas other than the hospital blood bank. Incompatible transfusions involving other blood groups are usually less severe, but deaths have been reported. The most common presentation of a hemolytic transfusion reaction is fever or fever with chills. Other signs or symptoms are chest pain, hypotension, nausea, flushing, dyspnea, and hemoglobinuria. The hemolytic transfusion reaction may progress to shock, disseminated intravascular coagulation (DIC), and renal failure.

Delayed hemolytic transfusion reactions occur 3–10 days after transfusion and may be clinically undetected. This reaction occurs from an anamnestic immune response to transfused erythrocytes in a previously sensitized person with undetectable antibody in pretransfusion testing. Presenting symptoms are fever, anemia, and jaundice. The patient's transfused erythrocytes are coated with antibody demonstrated by a positive DAT. The antibody specificity is identified by removing it from the surface of the coated transfused erythrocytes by a procedure called elution. The eluted antibody is then tested against a panel of reagent erythrocytes by the IAT. The frequency of delayed hemolytic transfusion reactions is 1 per 4000

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units of blood transfused. Mortality from delayed hemolytic transfusion reactions is uncommon.

### ***Febrile Reactions***

In the past, febrile nonhemolytic transfusion reactions (FNHTR) were thought to be caused by cytotoxic or agglutinating antibodies in the recipient, directed against donor leukocyte antigens. Leukocyte reduction filters used at the time of red cell or platelet transfusion decreased the amount of leukocytes transfused and should have eradicated FNHTRs. When this anticipated effect was not observed, researchers looked for other causes to explain the fever, chills, and rare rigors that describe a FNHTR. It was observed that during storage, cytokines (IL-1 $\beta$ , IL-6, TNF $\alpha$ ) are released from leukocytes present in red cell and platelet components. These cytokines are known to have pyrogenic activity and thus may be the cause of this adverse reaction. FNHTR must be distinguished from fever associated with hemolytic transfusion reactions and from the high fever ( $>40^{\circ}\text{C}$ ) and rigors associated with bacterial contamination of blood components. Only one in eight patients with a febrile reaction has another reaction on subsequent transfusion. Recurrent febrile reactions are often controlled with antipyretics, leukocyte-reduced components, or recently collected components.

### ***Transfusion-Related Acute Lung Injury***

High-titer leukocyte antibodies in donor plasma can cause pulmonary edema (see Chapter 40). Donor antibodies bound to recipient granulocytes (or infrequently, recipient antibodies bound to donor granulocytes) activate complement. Complement activation leads to the sequestration of antibody–granulocyte complexes in the lung microvasculature. The presence of activated complement fragments and leukocyte enzymes or free radicals are thought to cause lung

injury with resultant pulmonary edema. The sequelae are fever, dyspnea, and marked hypoxemia. The acute respiratory distress occurs within 1–6 hours of a transfusion and often requires aggressive respiratory support. Although some deaths have been reported, most patients with transfusion-related acute lung injury (TRALI) improve within 48–96 hours if promptly treated. The risk of TRALI is approximately 1 per 5000 units transfused.

## ***Allergic Reactions***

Allergic reactions to transfusion are characterized by itching, hives, and local erythema. Rarely are they accompanied by cardiopulmonary instability. They are thought to be caused by infused plasma proteins and occur in 1–2% of transfusions. Patients with a history of allergy more frequently have allergic reactions to blood. Mild reactions can be treated with antihistamines and the transfusion continued. Pretreatment with antihistamines often prevents recurrent allergic reactions. If the allergic reaction is severe, washed erythrocytes may be indicated. After transfusion of as little as 10–15 mL of a blood component, some IgA-deficient recipients with anti-IgA experience anaphylactic reactions. Fortunately, these reactions are rare. The reaction is due to the IgA present in transfused plasma and is prevented by transfusing plasma-free or IgA-deficient components.

Other transfusion reactions include those caused by bacterial contamination of blood components, congestive heart failure due to intravascular volume overload, and donor erythrocyte destruction prior to infusion. Erythrocytes may be destroyed by inadvertent overheating, improper freezing technique, or mixing with nonisotonic solutions.

## ***Transfusion-Transmitted Infection***

Transfusion may be complicated by a variety of infectious microorganisms, only some of which can be detected by current donor-screening methods (Table 17-4). The most frequently reported posttransfusion infections in developed countries are various bacterial contaminants, hepatitis, cytomegalovirus (CMV), human immunodeficiency virus-1 (HIV-1), and human T-cell lymphotropic virus I/II (HTLV-I/II). Elimination of potentially infected blood depends on successful donor screening by medical history, aseptic blood collection, and adequate laboratory testing of the donated blood. The presence of hepatitis B surface antigen (HBsAg), antibody to hepatitis B core antigen (anti-HBc), antibody to hepatitis C virus (anti-HCV), anti-HIV 1/2, HIV-1 antigen (p24), anti-HTLV I/II, and syphilis (STS) is currently tested in all US blood donors.

The prevalence of posttransfusion hepatitis (PTH) is estimated to be <1%. PTH is caused by hepatitis B virus in 5% of cases and by hepatitis C virus in 95% of cases. Of transfusion recipients who develop posttransfusion hepatitis, 50% develop chronic hepatitis; 10% of these develop cirrhosis. All blood components can potentially transmit hepatitis, except those that can be pasteurized, such as albumin and other plasma proteins.

CMV is transmitted to CMV-seronegative transfusion recipients by leukocytes

contaminating erythrocyte and platelet components. Roughly 50% of blood donors are infected with CMV, which limits availability

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of CMV-negative blood. CMV disease causes significant morbidity and mortality in severely immunocompromised patients. When possible, CMV-seronegative blood should be given to low-birth-weight infants (<1250 g), CMV-seronegative pregnant women, and CMV-seronegative recipients of CMV-seronegative bone marrow or organ transplants.

**Table 17-4. Transfusion-transmitted infection.<sup>a</sup>**

Infection	Risk/Unit Transfused
Hepatitis C	1:103,000
Hepatitis B	1:63,000
HTLV-I/II	1:640,000
HIV-1 infection	1:675,000
<i>Abbreviations:</i> HTLV-I/II = human T-cell lymphotropic virus-I/II; HIV-1 = human immunodeficiency virus-1.	
<sup>a</sup> Rare infections include syphilis, malaria, Epstein-Barr virus infection, delta hepatitis, brucellosis, Chagas' disease, babesiosis, and leishmaniasis.	

HIV-1 infection due to transfusion is rare since implementation of donor HIV-1 antibody testing (March 1985). HIV-1 can be transmitted by erythrocytes, platelets, cryoprecipitate, fresh-frozen plasma, and possibly other blood components. The risk of infection by transfusion is now estimated to be about 1 in 675,000 per unit transfused. The virus can be transmitted by blood collected from donors who have been recently infected but don't yet have detectable levels of HIV antigen or antibodies (called the "window period"). Even though HIV-2 infection is rare in the United States, isolated cases are reported in parts of Europe and West Africa. Consequently, all US blood donations are screened for antibodies to both HIV-1 and HIV-2 as well as to HIV p24 antigen. To date 3 US blood donors were found to have been infected with HIV-2 since HIV-2 testing was implemented in 1992.



Human T-lymphotrophic viruses type I and type II (HTLV-I and HTLV-II) are also retroviruses known to be transmitted by transfused blood products. Donor screening histories and serologic testing for evidence of HTLV-I/II infection has reduced the risk of transfusion-transmitted HTLV-I/II infection to 1:641,000. Both viruses are associated with a slowly progressive spinal cord disorder known as tropical spastic paraparesis/HTLV-associated myelopathy (TSP-HAM). Blood donors found to be infected with HTLV-I or HTLV-II by serologic testing have been shown to have an increased incidence of infections (bladder–kidney infections with HTLV-I and bladder–kidney infections, bronchitis, and oral herpes with HTLV-II) when compared with seronegative donor controls. In addition, HTLV-I can cause adult T-cell leukemia (See Chapter 43).

### ***Other Diseases Transmitted by Transfusion***

Bacterial contamination of blood products is an important cause of morbidity and mortality. The source of blood product contamination is either silent bacteremia in the donor or skin contaminants at the venipuncture site. Storage of products at standard refrigerator temperatures (4°C) retards the growth of most bacteria so the risk of transfusion-transmitted bacteria in red blood cells is about 1:500,000 units transfused. In stark contrast, platelet products carry a much higher bacterial contamination risk of 1:12,000 units transfused due to their storage at room temperature. Gram-negative organisms are more often found in refrigerated products, whereas in platelets stored at ambient temperature the organisms are gram-positive. The mortality rate from transfused bacterially contaminated blood products has been estimated to be as high as 25%.

Epstein–Barr virus (EBV) may be transmitted by transfusion. In most cases it results in asymptomatic seroconversion, but it can cause a mononucleosis syndrome.

Posttransfusion syphilis is now rare. There is a low prevalence of syphilitic infection in blood donors, and all donors are screened for antibody. Since the organism does not survive cold storage for more than 96 hours, it can be transmitted only by fresh blood or platelets.

Malaria remains a disease of major worldwide importance. The parasite can be present in erythrocytes of carriers for years after infection. No available laboratory tests are simple and sensitive enough to screen the blood donor population; therefore, blood banks in the United States rely on histories taken at the time of donation. Donors who have traveled to areas where malaria is endemic are deferred for 12 months.

Other parasites are transmitted by transfusion. In the United States, *Babesia microti*, the causative agent of babesiosis, is the second most common parasitical infection transmitted by blood products. *Trypanosoma cruzi*, which causes Chagas' disease, is a very rare cause of transfusion-transmitted parasitic infection in the United States; however, in the endemic countries of Central and South America, blood transfusion is a common source for this infection. Microfilariasis is a transfusion risk in the tropical areas of the world where *Wuchereria bancrofti*, *Loa loa*, and other filarial parasites are found. Transfusion-

transmitted leishmaniasis is also reported.

### ***Immunologic Mechanisms of Transfusion Reactions***

Hemolytic transfusion reactions are caused by antigen-antibody complexes on the erythrocyte membrane. These complexes activate Hageman factor (factor XIIa) and complement and induce the production of several cytokines. Hageman factor activates the kinin system (see Chapter 12). Bradykinins thus generated increase capillary permeability and dilate arterioles, causing hypotension. Complement is activated and leads to intravascular hemolysis as well as to histamine release from mast cells. Hageman factor and free incompatible erythrocyte stroma activate the intrinsic clotting cascade, with consequent DIC. Systemic hypotension with renal vasoconstriction and the formation of intravascular thrombi lead to renal failure. When complement activation is incomplete, the reaction is less severe. Erythrocytes coated with C3b are cleared from the circulation by phagocytes, resulting in extravascular hemolysis.

The mechanism of graft-versus-host disease (GVHD) depends on the engraftment and clonal expansion

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of donor lymphocytes in the recipient. Donor lymphocytes recognize recipient tissue antigens as "foreign" and cause a clinical syndrome characterized by fever, skin rash, hepatitis, and diarrhea. In transfusion-associated GVHD (TA-GVHD), bone marrow is also a target of donor lymphocytes, and a significant aplasia results. Most cases of TA-GVHD are poorly responsive to treatment and result in death. Gamma irradiation of lymphocyte-containing blood components to preclude lymphocyte activation and expansion prevents TA-GVHD. Patients at risk for TA-GVHD are fetuses receiving intrauterine transfusions, patients transfused with HLA-matched platelets, newborns undergoing exchange transfusion, patients with T-cell immunodeficiencies, and patients severely immunosuppressed by intensive irradiation and chemotherapy (see Chapter 53). There are rare reports of graft-versus-host disease following transfusion of blood from a haploidentical donor into an immunocompetent recipient. Consequently, designated blood donations collected from blood relatives are now irradiated before transfusion.

### **RH ISOIMMUNIZATION**

The D antigen is a common, strongly immunogenic antigen, 50 times more immunogenic than the other Rh antigens. The prevalence of antibody formation to Rh+ blood depends on the dose of Rh+ cells: 1 mL of cells sensitizes 15% of individuals exposed; 250 mL sensitizes 60-70%. After the initial exposure to Rh+ cells, weak IgM antibody can be detected as early as 4 weeks. This is followed by a rapid conversion to IgG antibody. A second exposure to as little as 0.03 mL of Rh+ erythrocytes may result in the rapid formation of IgG antibodies.

The majority of potential transfusion reactions to Rh can be prevented by transfusing Rh+ individuals with Rh- blood. Immunization and antibody formation to D antigen still occur owing to occasional Rh sensitization during pregnancy or to transfusion errors, particularly during emergencies.

Immunization to other Rh antigens may occur because donor blood is typed routinely for D but not for other Rh antigens.

Hemolytic disease of the newborn occurs with the passage of Rh<sup>+</sup> cells from the fetus to the circulation of the Rh<sup>-</sup> mother. Once anti-D antibody is formed in the mother, IgG but not IgM anti-D antibodies cross the placenta, causing hemolysis of fetal erythrocytes. Rh<sup>-</sup> mothers become sensitized during pregnancy or at the time of delivery as a result of transplacental fetal hemorrhage. Following delivery, 75% of women will have had transplacental fetal hemorrhage. Some obstetric complications increase the risk of transplacental fetal hemorrhage: antepartum hemorrhage, toxemia of pregnancy, cesarean section, external version, and manual removal of the placenta. Transplacental fetal hemorrhage can also occur following spontaneous or therapeutic abortion, amniocentesis, chorionic villus sampling (CVS), or percutaneous umbilical cord sampling (PUBS). Overall Rh immunization occurs in 8% of Rh<sup>-</sup> women following the delivery of the first Rh<sup>+</sup> ABO-compatible baby and in 1.5%–2.0% of Rh<sup>-</sup> women who deliver Rh<sup>+</sup> ABO-incompatible babies.

### ***Rh Prophylaxis***

Rh immunization can now be suppressed almost entirely in antepartum or postpartum Rh<sup>-</sup> women if high-titer anti-Rh immunoglobulin (RhIg) is administered within 72 hours after the potentially sensitizing dose of Rh<sup>+</sup> cells.

The protective mechanism of RhIG administration is not clear. RhIG does not effectively block Rh antigen from immunosuppressive cells by competitive inhibition, since effective doses of RhIG do not cover all D antigen sites. Intravascular hemolysis and rapid clearance of RhIg-coated erythrocytes is also unlikely. Although this mechanism appears to explain the 90% protective effect of ABO incompatibility between mother and fetus, RhIG-induced erythrocyte hemolysis is extravascular. Rh<sup>+</sup> fetal cells are removed primarily by highly phagocytic cells in the spleen and liver. The most likely mechanism is a negative modulation of the primary immune response, which thereby depresses antibody formation. Antigen-antibody complexes are bound to cells bearing Fc receptors in the lymph nodes and spleen. These cells presumably stimulate suppressor T-cell responses, which prevent antigen-induced B-cell proliferation and antibody formation.

A prophylactic dose of 300 µg of RhIG intramuscularly prevents Rh immunization following exposure to up to 15 mL of Rh<sup>+</sup> erythrocytes, which corresponds to 30 mL of fetal whole blood. Initial recommendations were that 300 µg of RhIG be given to nonimmunized Rh<sup>-</sup> mothers within 72 hours after delivery of an Rh<sup>+</sup> infant. The postpartum dose of RhIG decreased the incidence of anti-D development to 1% in Rh<sup>-</sup> women giving birth to Rh<sup>+</sup> infants. To further decrease the chances of developing anti-D in this population of women, antepartum RhIG is also now administered at 28 weeks' gestation. A dose of RhIG is also indicated for an Rh<sup>-</sup> woman after any terminated pregnancy, amniocentesis, CVS, PUBS, and fetal surgery or manipulation. Additional doses may have to be given in cases of massive transplacental fetal hemorrhage.

Large doses of RhIG can effectively suppress immunization following inadvertent transfusion of Rh+ blood into Rh- patients if given within 72 hours of transfusion. Once Rh immunization is demonstrated by the IAT, administration of RhIG is ineffective.

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## BLOOD COMPONENT THERAPY

Improvements in the medical care of previously fatal illnesses has placed increasing demands on the blood supply. As the need for blood products has expanded, the pool of eligible blood donors has decreased due to more intensive screening and testing. The separation of a whole-blood donation into its component parts (fresh-frozen plasma, platelets, and erythrocytes) has helped stretch a limited blood supply.

### *Erythrocytes*

During acute blood loss, 1 hour or more is required for equilibration of intravascular and extravascular fluids and an accurate assessment of the fall in the hemoglobin level. Generally, a loss of 20% of blood volume can be corrected with crystalloid (electrolyte) solution alone, which can then be supplemented with colloid (protein) solution. Whole blood is indicated if blood loss exceeds one third of blood volume. Operative blood loss of 1000–1200 mL rarely requires transfusion in an otherwise healthy adult. If increased oxygen-carrying capacity is required, erythrocyte transfusion is indicated (Table 17-5).

A decreased hemoglobin level is tolerated better in a patient with chronic anemia than in a patient with acute blood loss. Patients with a slow decline in their hemoglobin level compensate for the decreased oxygen-carrying capacity by increasing their cardiac output. 2,3-Diphosphoglycerate is also increased in patients with chronic anemia, shifting the oxyhemoglobin dissociation curve to the right. This rightward shift enhances oxygen release to the tissues.

**Table 17-5. Guidelines for component therapy.**

Component	Indications for Use
Red blood cells	<p>Use to increase O<sub>2</sub>-carrying capacity; 1 unit increases hemoglobin 1 g/dL in a 70-kg patient. Consider the degree of anemia, intravascular volume, and presence of coexisting cardiac, pulmonary, or vascular conditions.</p> <ol style="list-style-type: none"> <li>1. If hemoglobin &gt;10 g/dL, transfusion is rarely indicated.</li> <li>2. If hemoglobin &lt;7 g/dL, transfusion is usually</li> </ol>

	<p>indicated.</p> <p>3. If hemoglobin is 7-10 g/dL, assess clinical status, mixed venous pO<sub>2</sub>, and O<sub>2</sub> extraction ratio.</p>
Platelets	<p>Use to control or prevent bleeding due to low platelet count or abnormal platelet function; one concentrate increases platelet count by approximately 5000 platelets/<math>\mu</math>L.</p> <ol style="list-style-type: none"> <li>1. Generally, patients with platelet counts &lt;50,000-10,000 should receive platelets to prevent bleeding.</li> <li>2. Actively bleeding patients with platelet counts &lt;50,000 may benefit from platelets.</li> </ol>
FFP	<p>Used to increase clotting factors in patients with documented deficiencies (PT &gt;1.5 <math>\times</math> normal, 1 unit increases the level of any factor 2-3%.</p> <ol style="list-style-type: none"> <li>1. FFP should not be used as a volume expander or nutritional source.</li> <li>2. FFP is useful for treatment of factor II, V, VII, X, XI, or XIII deficiencies when specific concentrates are not available.</li> <li>3. FFP is useful for patients with warfarin overdose who have life-threatening bleeding or who require emergency surgery.</li> <li>4. FFP may be useful in massive blood transfusion (&gt;1 blood volume within a few hours).</li> <li>5. FFP is useful as a source of C1-esterase inhibitor in deficient patients with life-threatening angioedema and in patients with thrombotic thrombocytopenic purpura.</li> </ol>
<p><i>Source:</i> Data used with permission, from the following sources: Fresh frozen plasma—indication and risks, <i>JAMA</i> 1985;253:551; Platelet transfusion therapy, <i>JAMA</i> 1987;257:1777; and Perioperative red blood cell transfusion, <i>JAMA</i> 1988;260:2700.</p> <p><i>Abbreviations:</i> PT= prothrombin time; FFP = fresh-frozen plasma.</p>	

All erythrocyte components should be administered through blood filters. Medications, especially solutions containing calcium or glucose, should not be infused with blood components.

## ***Platelets***

Platelets function to control bleeding by acting as hemostatic plugs on vascular endothelium. Platelet abnormalities that require platelet transfusion may be either quantitative or qualitative. The vast majority of platelet transfusions are given to supplement decreased numbers of circulating platelets due to suppressed production, pooling, or dilution.

Platelets are available as either platelet concentrates (recovered from a whole-blood donation) or as plateletpheresis (collected by using a cytopheresis instrument). The transfusion of one platelet concentrate is expected to increase the platelet count of a 70-kg adult by  $5000 \times 10,000/\mu\text{L}$ . A plateletpheresis is equivalent to four to eight platelet concentrates because both have the same number of platelets. The survival of transfused platelets decreases in patients who are actively bleeding; who have splenomegaly, fever, infection, or DIC; or who are sensitized to platelet antigens. The transfusion of ABO-incompatible platelets may be associated with slightly decreased platelet survival.

Much discussion ensues whenever the subject of indications for the appropriate use of platelet transfusions arises. Little good clinical evidence addresses the indications for platelet therapy. General guidelines suggest that stable, afebrile thrombocytopenic

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adults and older children are not at high risk of serious bleeding unless their platelet counts fall below  $5,000 \times 10,000/\mu\text{L}$ . Indications for transfusion of unstable patients are more problematic. Bleeding patients should be more aggressively transfused, and many experts suggest transfusion when platelet counts fall below  $30,000 \times 50,000/\mu\text{L}$ . Thrombocytopenic patients undergoing invasive procedures do not generally experience increased complications unless their platelet counts are  $<50,000/\mu\text{L}$ ; however, the patient's clinical situation and the site of the procedure or surgery should influence the decision to transfuse. Patients undergoing surgery on the eye, brain, spinal cord, or airway are at higher risk of serious sequelae due to bleeding and may require higher platelet counts for safety.

## ***Plasma Products***

Fresh-frozen plasma (FFP), stored plasma, and cryoprecipitate are valuable sources of coagulation factors. Stored plasma and FFP may often be used interchangeably. Levels of factors V and VIII in stored plasma are half those in FFP, but levels of other factors are equivalent. Cryoprecipitate was initially produced to provide therapeutic doses of factor VIII and von Willebrand's factor. This use has been greatly supplanted by the development of recombinant or treated factor VIII, which have lower infectious risks to recipients. Cryoprecipitate is now most often used to treat bleeding in patients with fibrinogen less than 100 mg/dL.

FFP is used for treating isolated congenital factor deficiencies, for which a safer factor concentrate product is not available. It is also used to correct warfarin overdoses in patients with significant bleeding. FFP is also used to treat

thrombotic thrombocytopenic purpura and C1 esterase inhibitor deficiency. Massively transfused patients with a prothrombin time or partial thromboplastin time greater than 1.5 times normal and platelet counts above 50,000/ $\mu$ L may benefit from FFP treatment. FFP or plasma should never be used for volume expansion because colloid solutions without infectious risk are available (ie, albumin).

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## 18

# Molecular Genetic Techniques for Clinical Analysis of the Immune System

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Genetic information in humans and most other organisms is encoded in the linear sequence of four nucleotide bases (abbreviated A, T, G, and C) along the strands of a DNA molecule. The sequence of the human genome is more than 3 billion DNA bases long, is divided among 23 chromosomes, and is present twice in each diploid nucleus. The human genome contains an estimated 100,000 genes, each comprising, on average, no more than a few thousand bases of coding sequence that specify a particular protein or structural RNA. The coding information of a typical human gene is rarely contained in a single, uninterrupted stretch of DNA but, instead, is divided into shorter coding segments called **exons**, which are separated by noncoding regions called **introns**. Individual genes are also widely separated from one another along the DNA, with noncoding sequences in between. Altogether, coding sequences are thought to make up only 5%–10% of the human genome, and the function of the remaining sequences is, for the most part, unknown. The complete sequences of many human genes have been determined (by using techniques that lie outside the scope of this chapter), and the sequence of the entire human genome is likely to become known within a few years.

During the past two decades, advances in nucleic acid chemistry and recombinant DNA technology have made it practical to analyze individual genes rapidly and precisely. The techniques involved are now commonplace in research and are gradually being adapted for use in clinical laboratories as well. DNA offers numerous advantages as a substrate for clinical analysis: It is a remarkably sturdy biomolecule that is fairly easy to handle; it can be obtained from either fresh or fixed tissue or blood specimens; and it can be manipulated and dissected in ways that are not possible with proteins. Most importantly, access to the information contained in DNA enables us to diagnose and investigate many disease processes at the most fundamental level. This chapter summarizes the basic concepts and practical techniques for analyzing DNA from clinical specimens, along with some specialized applications to the immune system. At the end of the chapter, related techniques for studying cellular RNA

are briefly discussed.

## NUCLEIC ACID PROBES

Underlying the complexity of DNA is a simple but profound symmetry. Each DNA molecule is composed of two linear strands of bases, which are bound to each other side by side and coiled to form a double helix (Figure 18-1). The two strands are held together by hydrogen bonding between adjacent bases: A on one strand always binds to T on the other, and similar binding occurs between G and C. In normal DNA, the two strands are said to be **complementary** in that every base is appropriately paired to the corresponding position on the opposite strand. Bases within a strand are held together by strong covalent bonds, but the base-pairing bonds between strands are relatively weak, so that the two strands can easily be separated (*“denatured”* or *“melted apart”*) by heat or alkaline pH. When slowly returned to physiologic conditions, the strands reanneal spontaneously and in perfect alignment to re-form the original double-stranded helix.

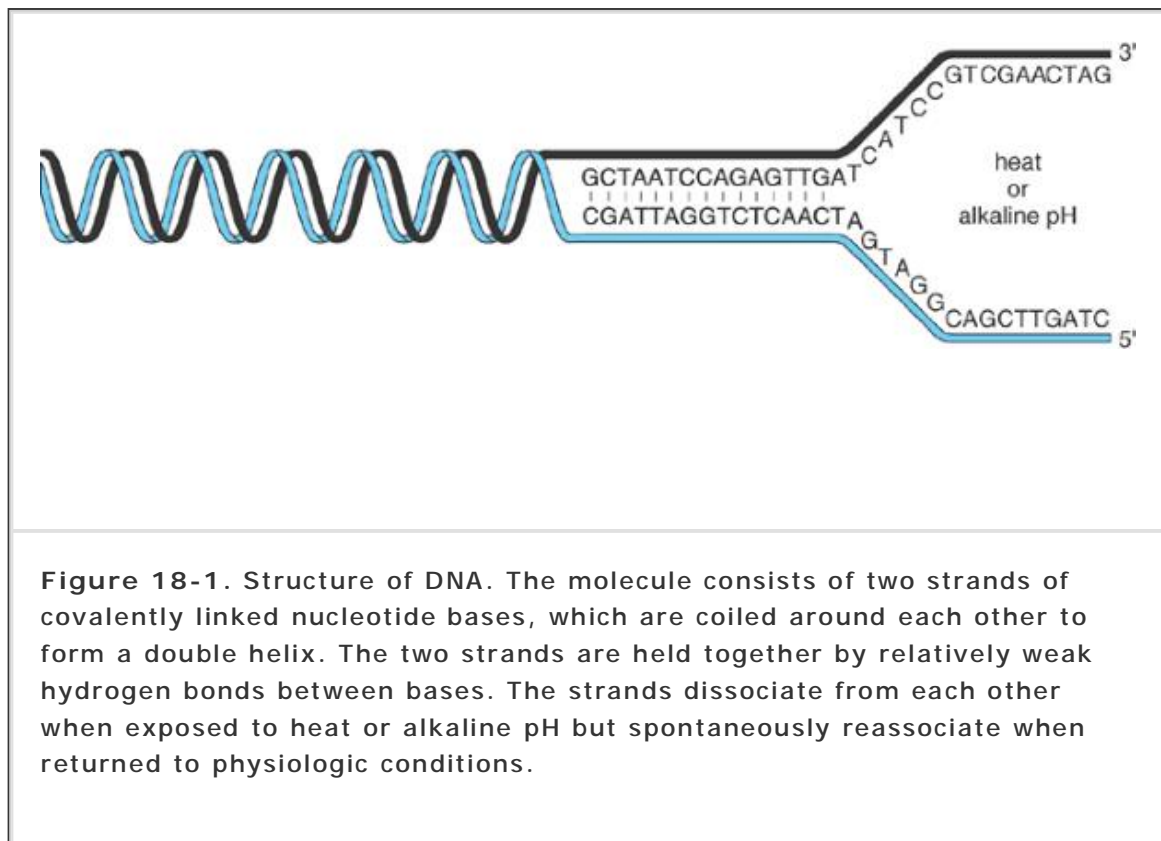
This spontaneous pairing between complementary strands provides the basis for many of the techniques that are used to detect and characterize genes. These techniques employ short strands of known sequence as **probes** to detect strands with the complementary sequence. Probes of any desired sequence can readily be obtained in abundant quantities and at very high purity: Single DNA strands up to about 100 bases long are easily prepared by using automated chemical synthesizers, whereas larger DNA sequences are generally introduced (*“cloned”*) into bacteria to be replicated biologically. It is also possible to use probes made of RNA—a molecule that, for the purposes of this chapter, can be considered equivalent to single-stranded DNA—since these also anneal specifically to a complementary DNA strand. RNA probes are most often prepared enzymatically by cloning the corresponding DNA sequence and using this as a template for in vitro transcription, that is, producing a complementary RNA strand from the template DNA.

Cellular DNA can be isolated by chemical extraction from a blood or tissue specimen followed by enzymatic treatment to remove traces of contaminating

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RNA or protein. Unless special precautions are taken, the extremely long strands of chromosomal DNA are usually sheared by mechanical forces into random fragments of roughly 50,000–100,000 bp during the purification process. To use a nucleic acid probe, this target DNA is first heated or exposed to alkali in order to separate the strands and then mixed with the labeled probe and returned to normal temperature and pH. As the molecules reassociate, some of the target strands anneal (*“hybridize”*) to the probe rather than to the unlabeled complementary strand, forming labeled duplexes. To maximize the likelihood that a target strand will anneal to the probe rather than to its original partner, the hybridization reaction is usually carried out with a great molar excess of probe. The stability of the complex formed by a probe and its target is influenced by many factors, the most important of which are temperature, salt concentration, the length and base composition of the probe, and the presence of

any mismatched bases. Under the conditions used in most assays, two strands must share at least 16–20 consecutive bases of perfect complementarity to form a stable hybrid. The probability of such a match occurring by chance is less than one in a billion ( $10^{-9}$ ). Thus, nucleic acid probes possess an extraordinary degree of specificity: A typical probe is capable of recognizing and binding selectively to a single copy of its complementary sequence among the 3 billion bp in the human genome. DNA or RNA probes can easily be tagged with radioisotopes, fluorochromes, or enzymatic markers prior to use (Figure 18-2) and can then act as “molecular stains” that recognize and bind only to the exact complementary sequence.



## HYBRIDIZATION ASSAYS

Several different methods can be used to test whether a DNA specimen contains sequences complementary to a particular probe. One common approach takes advantage of the fact that, under certain conditions (eg, when exposed to ultraviolet light or when heated in a high concentration of salt), DNA strands can be made to bind tightly onto nylon or nitrocellulose membranes. In a procedure called **dot blot hybridization** (Figure 18-3A), a solution of target DNA is denatured, spotted onto the surface of such a membrane, and then treated so that the separated DNA strands adhere irreversibly to the membrane. When immobilized in this manner, the target strands remain accessible on the membrane surface but are prevented from reannealing with one another. The membrane is then incubated with labeled probes under conditions in which the

probe does not adhere to the membrane but may hybridize with the target strands. Afterward, the filter is washed extensively to remove unhybridized probe. Any probe that has hybridized to the bound DNA can then be detected by autoradiography or enzymatic assay, depending on the particular label that it carries.

In an alternative approach, called a **nuclease protection assay**, target and probe DNAs are denatured, allowed to anneal together in solution, and then treated with an enzyme that specifically cleaves single-stranded but not double-stranded DNA. A probe survives this enzymatic digestion only if it has become stably hybridized to the target DNA (Figure 18-3B).

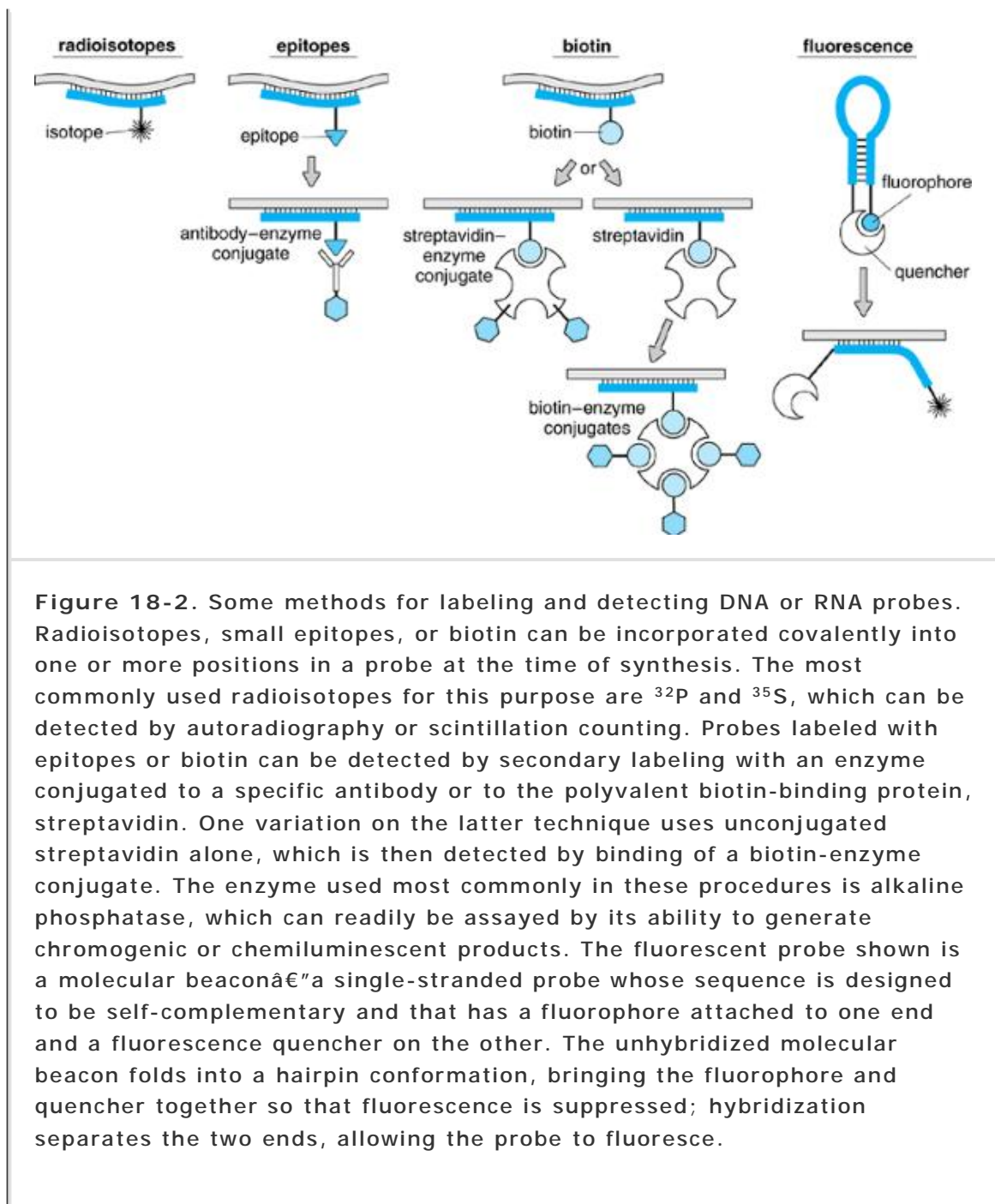
The interaction between probe and target occurs with one-to-one stoichiometry, and this tends to limit the sensitivity of hybridization assays. One way of maximizing the signal obtained is to incorporate multiple labels into a single probe, such as by radioactively labeling many bases in the probe (Figure 18-4). It may also be appropriate to use multiple probes that each recognize adjacent regions of a longer target sequence or to attach secondary probes onto a long, unhybridized "tail" on the primary probe (see Figure 18-4). A recent innovation is to attach

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short DNA sidechains onto the primary probe by means of synthetic chemistry, creating an artificial **branched DNA** molecule that can interact with many copies of a secondary probe. Still another approach is to use probes that form polyvalent complexes with an enzyme or fluorochrome marker, similar to those used in immunohistochemistry (see Chapter 15). For example, hybrids containing a probe that has been labeled with biotin can first be incubated with the polyvalent biotin-binding protein streptavidin and then secondarily tagged with many copies of a biotinylated marker enzyme (see Figure 18-2). The use of enzymatic detection systems that produce colored or chemiluminescent products can greatly amplify the signal obtained, as can the use of molecular beacons—probes that become fluorescent only after they hybridize (see Figure 18-2). Even when such measures are taken, however, about  $10^4$ - $10^5$  copies of a target sequence must usually be present in a sample to be detectable by routine hybridization.

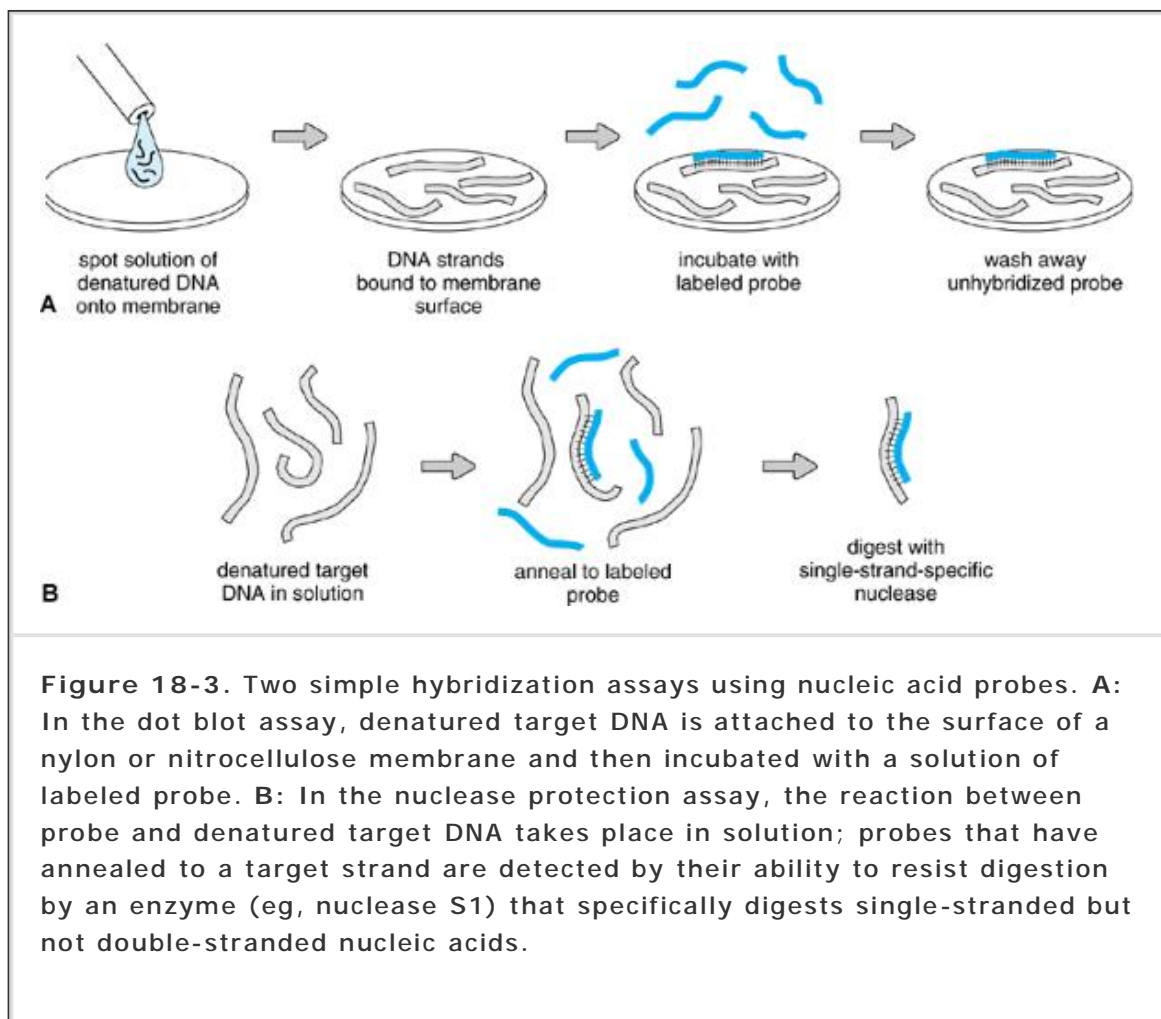




## SOUTHERN BLOT

The simplest hybridization assays, such as the dot blot assay, indicate whether a particular sequence is present in the target DNA and may also give an estimate of its abundance. These assays are rarely used clinically, because easier and more sensitive tests can provide the same information (see the section, Target Amplification Techniques). Nucleic acid probes, however, offer special advantages when they are used in conjunction with restriction enzymes, a

class of bacterial enzymes that cut both strands of a linear DNA molecule at specific short recognition sequences, usually 4–6 bp long. For example, the enzyme *EcoRI* cuts only within the sequence GAATTC, whereas the enzyme *BamHI* cleaves only GGATCC. Each restriction enzyme therefore cleaves long target DNA molecules into specific smaller segments called **restriction fragments**, whose number and length are determined by the sequence of the substrate DNA.



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Because of the enormous size and complexity of the human genome, cleaving human DNA with a restriction enzyme yields millions of unique restriction fragments ranging up to tens of thousands of bases long. Nevertheless, the fragment that carries any particular gene can readily be identified, provided that a DNA probe complementary to the gene is available. The technique used for this purpose (Figure 18-5A) is called the **Southern blot**, after its inventor, E. M. Southern. DNA extracted from a tissue or blood specimen is first cleaved with one or more restriction enzymes, and the resulting DNA fragments are then subjected to electrophoresis through an agarose gel, which separates them according to length. Afterward, the gel is immersed in alkali solution to melt

apart the complementary strands of each fragment. A sheet of nylon or nitrocellulose is then pressed firmly against the gel; the denatured DNA fragments bind tightly to this sheet and are drawn out of the gel. When the sheet is peeled away, it retains on its surface the immobilized DNA fragments, still arranged according to length as they had been in the gel but now exposed and accessible to further analysis. The sheet is then incubated with the labeled probe, which binds only to the fragment bearing its complementary sequence. Unbound probe is washed away, and the location of the remaining hybridized probe is determined by virtue of the label it carries. The size of the bound target fragment can then be deduced from its location on the membrane because this corresponds to the distance it migrated in the agarose gel.

The Southern blot reveals not only the presence of a particular sequence but also the size of the restriction fragment on which it lies. This size, in turn, is determined by the distribution of nearby restriction sites and so reflects the local DNA sequence.

## GENE REARRANGEMENT ASSAY FOR LYMPHOCYTE CLONALITY

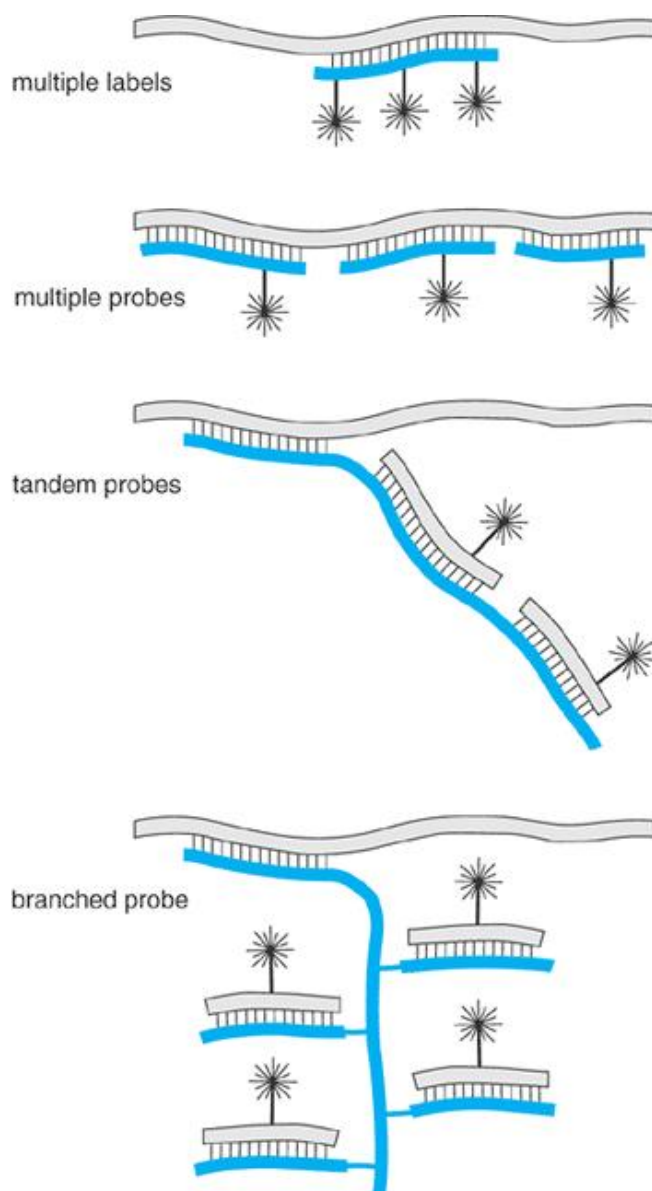
If all the cells in a population contain identical DNA, the restriction fragment carrying any given gene will have the same length in every cell, and all of these fragments will appear together as a single band on a Southern blot. This is the case for most cellular genes, including the immunoglobulin (Ig) and T-cell receptor (TCR) genes of nonlymphoid cells. In lymphocytes, however, the Ig and TCR genes undergo specific rearrangements (see Chapter 7), which markedly alter the DNA sequences in and around these loci. Such rearrangements can be detected on the Southern blot by a shift in the size of the restriction fragment that carries an Ig or TCR gene. Moreover, because the size of the shifted fragment depends on the exact rearrangement that has

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occurred, it represents a unique and characteristic property of each lymphocyte clone—a molecular fingerprint that can be used to distinguish one lymphoid clone from another.





**Figure 18-4.** Some approaches for increasing the sensitivity of nucleic acid hybridization assays. These can be used singly or in combination.

This provides a powerful means of estimating the clonal composition of lymphocyte populations (Figure 18-5B). In normal polyclonal lymphocyte populations, each of the innumerable clones contributes its own distinctively sized Ig or TCR fragment, but none of these is abundant enough to be detectable. Only when large numbers of clonally related cells are present do the rearranged genes appear in sufficient quantity to produce a detectable band. The presence of abnormally sized Ig or TCR bands on the Southern blot thus suggests the presence of a predominant clone of lymphoid cells, and this, in an



appropriate setting, can be taken as evidence of lymphoid malignancy.

By using the Southern blot assay, clonal rearrangements of the Ig heavy-chain genes can be found in the neoplastic B cells in essentially all cases of B-cell lymphoma regardless of histologic type. The clinical utility of this approach is somewhat limited, however, because B-cell clonality can often be assessed more easily and cheaply by comparing the ratio of kappa ( $\bar{\kappa}$ ) and lambda ( $\bar{\lambda}$ ) light-chain proteins, using immunohistochemical stains (see Chapter 15).

Nevertheless, the rearrangement assay is invaluable for demonstrating clonality in cases when malignant B cells either fail to express Ig protein or are heavily contaminated with polyclonal lymphocytes. Ig heavy-chain gene rearrangements are usually demonstrable in the lymphoid blast crisis of chronic myelogenous leukemia, in hairy cell leukemia, in  $\bar{\alpha}$ non-T, non-B acute lymphoblastic leukemia, and in most null large-cell lymphomas (see Chapter 43).

The analysis of TCR rearrangements has even greater potential usefulness because no other practical method is available for assessing T-cell clonality. For technical reasons, most clinical assays focus on the TCR  $\bar{\alpha}$ -chain genes, which have been found to be clonally rearranged in nearly all cases of T-cell leukemia and lymphoma, including plaque- or tumor-stage mycosis fungoides, SÅ©zary syndrome, and adult T-cell leukemia-lymphoma. The assay is especially useful for distinguishing reactive lymphadenopathy from T-cell lymphoma (see Chapter 43).

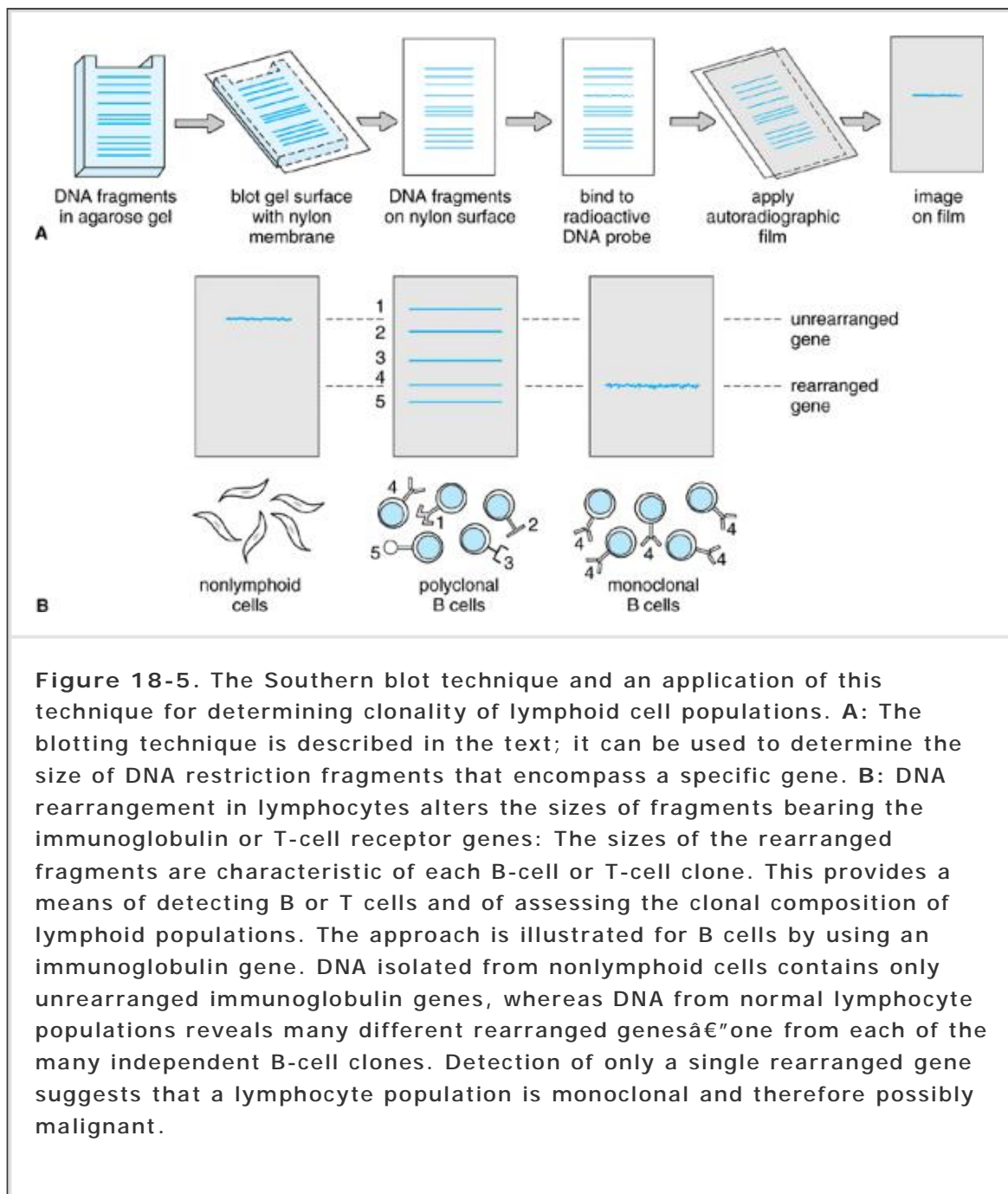
Although Ig and TCR gene rearrangements are generally confined to the B- and T-cell lineages, respectively, the correlation is not absolute. Roughly 15% of poorly differentiated lymphoid malignancies harbor rearrangements of both TCR  $\bar{\alpha}$ - and Ig heavy-chain genesâ€”an example of lineage infidelity. Ig light-chain rearrangements, which occur later in normal ontogeny than heavy-chain rearrangements (see Chapter 7), are more specific for the B lineage but less sensitive for detecting clonality. Absence of any rearrangements argues strongly that a tumor is not of lymphoid origin. Specimens from separate lymphomatous lesions in a single patient usually show identical rearrangements. Because the rearrangements in recurrent cancers are identical to those seen prior to treatment, the Southern blot technique offers special advantages in monitoring remission and recurrence, since it may reveal persistence of a malignant clone that is not yet detectable morphologically.

The Southern assay for lymphocyte clonality has several limitations. Although it is potentially more sensitive than histologic examination (a clonal subpopulation can be detected even when diluted 100-fold with polyclonal cells), this degree of sensitivity requires prior knowledge of the position of an abnormal band on the gel. Faint bands seen in a case being analyzed de novo must be interpreted with great care because they may represent technical artifacts.

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To minimize degradation of the DNA, fresh or frozen tissue must be used, and processing must begin promptly. The method has been applied successfully to specimens obtained during fine-needle biopsies of lymph nodes, but obtaining sufficient DNA for a complete analysis generally requires a blood or tissue

specimen that contains at least 25 million leukocytes.

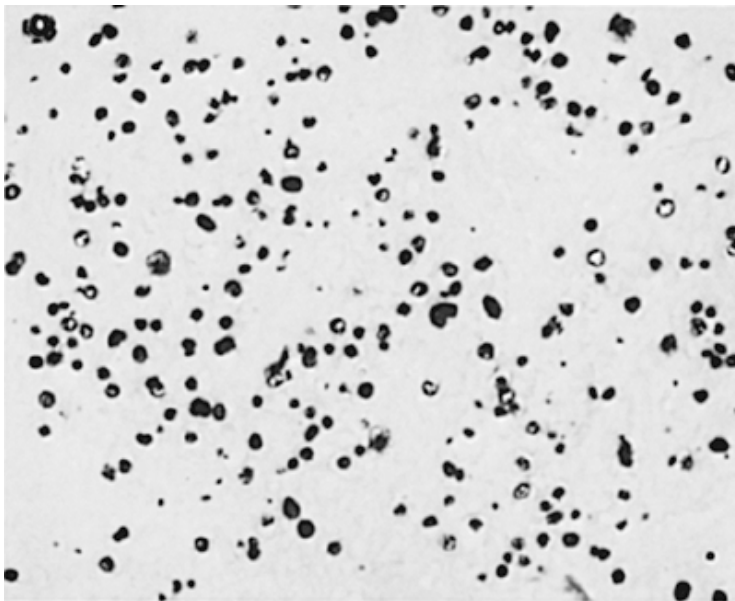


It is also important to recognize that the TCR  $\hat{I}^2$  locus includes far fewer V-gene segments than are found in the Ig loci. This greatly increases the probability that unrelated T-cell clones will coincidentally have the same rearrangement. Moreover, benign inflammatory responses to some antigens have been shown to use a particular TCR  $\hat{I}^2$  V region preferentially and so might appear monoclonal by this assay. These facts may ultimately limit the validity of TCR rearrangements for diagnosing T-cell neoplasia. More fundamentally, it is unclear that monoclonality signifies malignancy in every case. Therefore, as with any

other single test, results from gene rearrangement analyses must always be interpreted in the context of all other available clinical and laboratory data.

## IN SITU HYBRIDIZATION

Another specialized hybridization technique, called **in situ hybridization**, is based on the ability of labeled probes to bind target DNA in thin tissue sections or cytologic smears (Figure 18-6). This technique reveals not only the presence of a specific sequence but also its spatial distribution within tissues or individual cells. In brief, cells or tissues attached to the surface of a glass microscope slide are fixed, incubated with a labeled probe, and then washed to remove unbound probe. The specimen is then coated with a thin layer of photographic emulsion or chromogenic substrate that reveals the location of any bound radiolabeled or enzymatically labeled probe. The assay is technically arduous and not very sensitive. It is very useful for detecting abundant RNA species or viral DNA, which may be present in large amounts in a single infected cell. It can also be used cytogenetically to map the chromosomal locations of individual genes or to identify large-scale chromosomal anomalies.



**Figure 18-6.** Detection of viral DNA in human cells by in situ hybridization. A lymph node biopsy specimen from a patient with Hodgkin's disease was fixed onto the surface of a glass slide and then hybridized with a biotinylated nucleic acid probe specific for sequences from Epstein-Barr virus. Hybridized probe was detected with streptavidin-conjugated alkaline phosphatase. The nuclei of cells that harbor the viral DNA stain darkly. (Courtesy of Lawrence M Weiss.)

## TARGET AMPLIFICATION TECHNIQUES: POLYMERASE CHAIN REACTION

In the past, a major drawback of hybridization assays was their need for relatively large amounts of sample DNA to compensate for their low sensitivity. This problem has been surmounted in recent years by the development of powerful enzymatic techniques that can exponentially replicate specific DNA sequences in the test tube. With these techniques, it is now possible to analyze vanishingly small samples that initially contain fewer than ten copies of the sequence of interest. The new methods take advantage of the chemical properties of nucleic acids and of highly specialized enzymes that can repair and replicate DNA in vitro.

Every single-stranded DNA molecule has two ends, called the 5' and 3' ends, whose chemical and biologic properties differ. In double-stranded DNA, the two strands are always antiparallel (ie, their 3' and 5' ends are in opposite orientation to each other). Cellular enzymes known as **DNA polymerases**, which elongate these strands during DNA replication, can do so only by adding new nucleotide bases sequentially onto the 3' end of a preexisting strand, which serves as a **primer**. Moreover, most DNA polymerases function only when the primer is annealed to a longer second strand, which serves as a **template** for DNA synthesis; the enzyme adds nucleotides in a sequence complementary to that of the template, producing a base-paired double helix.

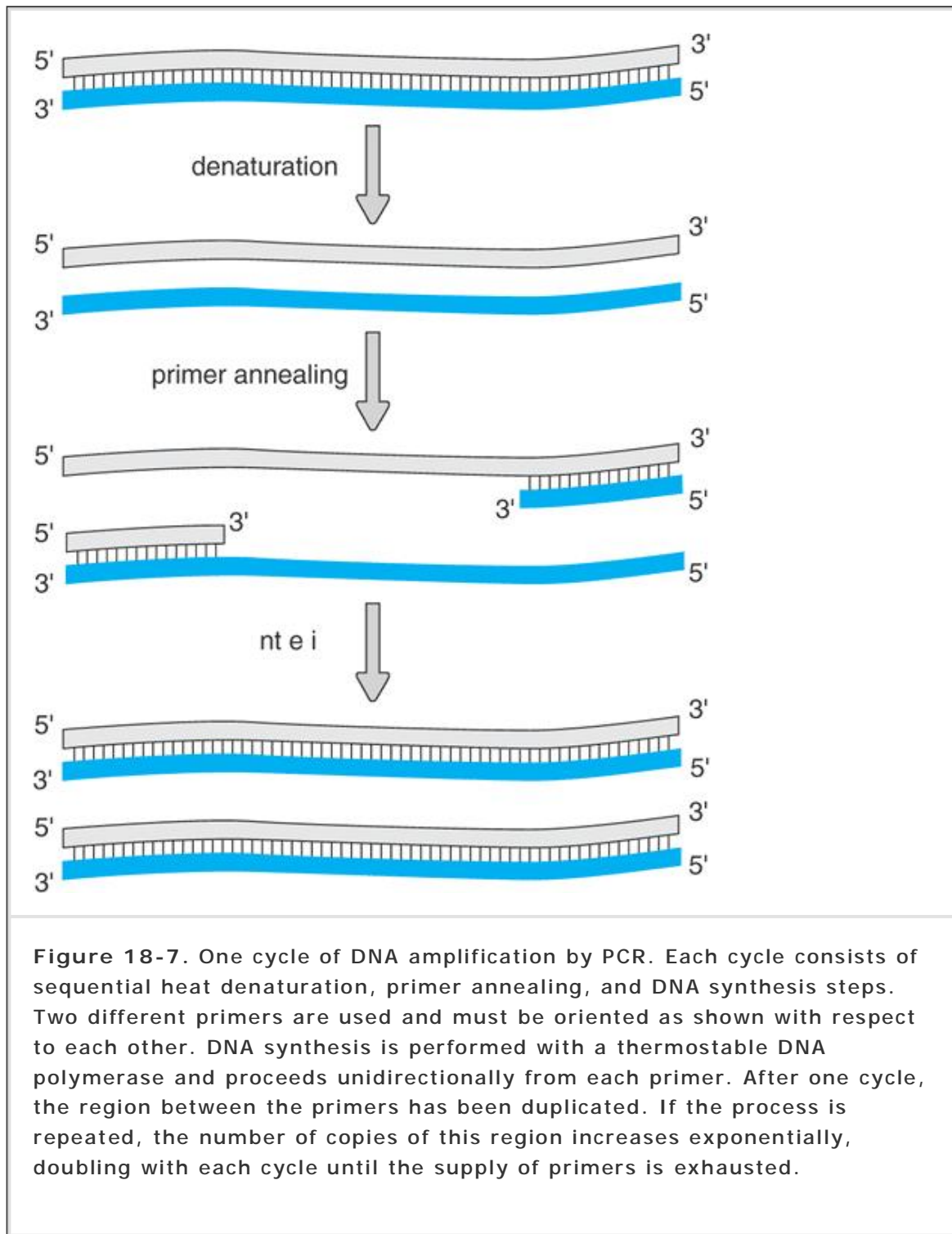
These properties of DNA polymerases are exploited in a technique called the **polymerase chain reaction (PCR)**, which can be used to replicate a particular region of target DNA selectively in vitro (Figure 18-7). Beginning with sample DNA from a very small number of cells, PCR can be used to synthesize multiple copies of a particular gene or gene segment that is present in those cells. PCR works best for copying regions less than about 2000 bp, and the DNA sequences flanking the region of interest must be known in advance. To use PCR, two short DNA primers (usually at least 16–20 bases long) are synthesized whose sequences are complementary to those of the flanking regions but on opposite strands; the two primers must be chosen so that their 3' ends are directed toward each other (see Figure 18-7). A vast molar excess of these primers is added to the sample DNA, which is then denatured by heating and

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allowed to anneal with the primers. A bacterial DNA polymerase is then added, which initiates synthesis at the 3' end of each annealed primer and produces a new strand complementary to a portion of the adjacent template strand. Synthesis is continued for long enough that the newly synthesized strands extend through the entire region of interest. When the mixture is then denatured and reannealed again, each newly synthesized strand provides a new template for synthesis from the opposite primer. By repeated cycles of denaturation annealing, and synthesis, the region between the two primers is amplified exponentially, with the number of double-stranded copies of this region doubling at each cycle. The thermal cycling is performed in automated, programmable instruments that can accommodate many samples, and using thermostable DNA polymerases, isolated from thermophilic bacteria because these can better

withstand exposure to high temperatures. Under ideal conditions, 220,000 copies should theoretically be produced from a single original DNA molecule after only 20 cycles of PCR. This is enough copies to allow detection by routine hybridization techniques.



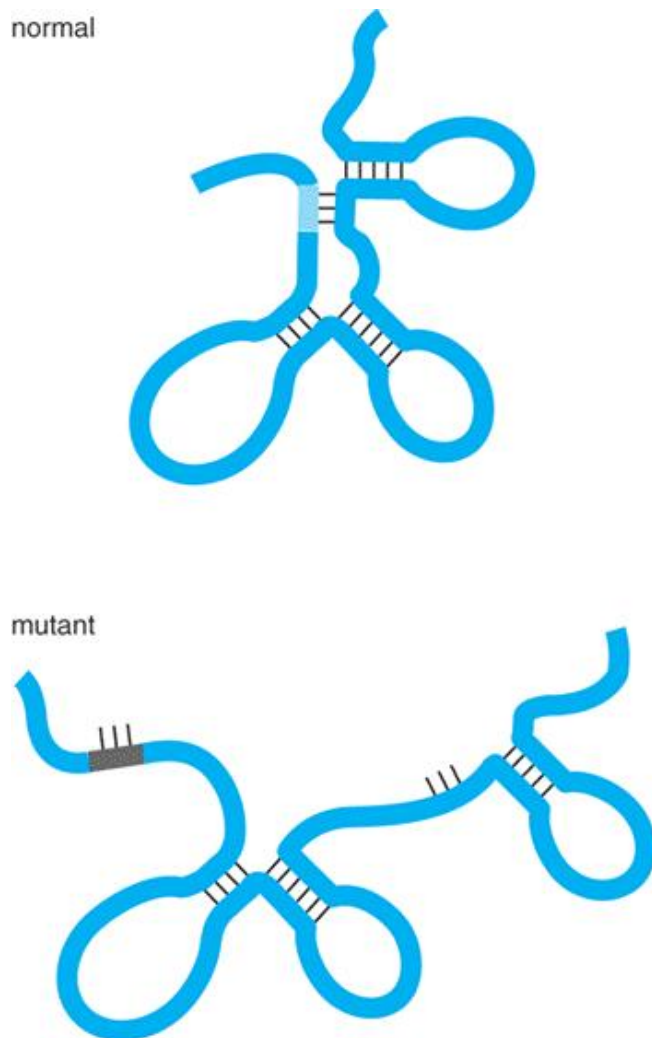
Perhaps the most common problem encountered when using PCR is cross-

contamination: Because the method is so sensitive, extreme care must be taken to avoid transferring even a trace of target DNA from one specimen to another. Another limitation of this technique arises from the fact that the bacterial polymerases frequently make errors when synthesizing new strands and so can introduce mutations that are not present in the original sample.

The basic technique of PCR amplification has been adapted in a great many ways to serve particular purposes. For example, it is widely used to facilitate detection of minute amounts of viral or bacterial DNA in clinical specimens because it can often identify these microorganisms much more rapidly than conventional culture techniques. A similar approach can be used to monitor lymphoid cancers; if primers are chosen that selectively amplify only a uniquely rearranged Ig or TCR V/(D)/J gene segment in the malignant clone, this can be used as an extremely sensitive assay for detecting persistence or regrowth of that clone in blood or tissues after cancer therapy. The PCR reaction can be monitored quantitatively by using primers designed as molecular beacons (see earlier section); each beacon that is used to prime DNA synthesis unfolds and becomes fluorescent, so that the rate at which fluorescence increases over successive PCR cycles reflects the original concentration of template DNA in the sample. Methods have also been developed that allow *in situ* PCR on tissue sections so that cells harboring a distinctive DNA sequence, such as a viral genome, can be identified morphologically. The amplifying power of PCR is so great that it allows analysis of genes from individual cells, which can be excised from frozen tissue sections using laser-capture microdissection. This has made it possible, for example, to detect clonal Ig gene rearrangements in the rare Reedâ€”Sternberg cells of Hodgkin's disease, confirming their B-cell origin.

It is also possible to search for single-point mutations within a target sequence by testing PCR-amplified DNA for **single-strand conformational polymorphisms (SSCP)**. For this purpose, the amplified product is treated with alkali to separate the DNA strands and is then quickly applied to an electrophoretic gel under nondenaturing conditions (Figure 18-8). Because the individual strands are not given the opportunity to reanneal with other strands, they tend instead to fold up on themselves by forming base pairs at short regions of intrastrand complementarity. Even subtle mutations can greatly affect the folding pattern and, hence, the three-dimensional shape of the folded strand, causing it to migrate anomalously on the gel relative to the normal sequence. One advantage of this technique is that it can detect many alternative mutations within an amplified region, even if their identities are not known in advance.





**Figure 18-8.** Single-strand conformational polymorphism. Under suitable conditions, a single strand of DNA often anneals with itself to form a complex folded structure as a result of regions of internal complementarity. Mutations may alter the folding pattern, and so change the electrophoretic mobility of the strand on a gel.

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## METHODS OF ANALYZING RNA

DNA and RNA probes can also be used to analyze the RNAs from a clinical specimen, and this can be highly advantageous for some purposes. Whereas DNA analysis can reveal the presence and structure of a particular gene sequence, RNA analysis indicates whether, and how strongly, it is being expressed. Another important advantage is sensitivity: A cell that expresses a particular gene often contains hundreds of copies of the RNA derived from it, and this RNA may be

readily detectable even though the gene itself is not. Techniques such as in situ or dot blot hybridization can easily be adapted to search for specific sequences in cellular RNA. In a modified form of the Southern blot, called the **Northern blot**, a mixture of cellular RNAs can be separated according to length by agarose gel electrophoresis, transferred to the surface of a nylon or nitrocellulose membrane, and then hybridized to a labeled nucleic acid probe to determine the size and abundance of any particular RNA species.

RNA analysis has some inherent limitations, however. Because expression of a given RNA varies widely depending on the lineage and physiologic state of a cell, it is critical to sample the right tissues at the right time. RNA is less durable than DNA (eg, it degrades rapidly and irreversibly at alkaline pH) and must be handled with correspondingly greater care. In addition, the number and types of enzymes that are available to manipulate RNA sequences are very limited. For some applications, it is necessary to begin by making a DNA copy of the target RNA, which then serves as the substrate for further analysis. For example, PCR amplification of RNA is carried out by a two-stage procedure known as **reverse transcriptase PCR (RT-PCR)**. The first stage employs an enzyme called reverse transcriptase, which synthesizes a DNA strand complementary to the RNA of interest by using one of the PCR primers as its primer. This complementary DNA (**cDNA**) is then used, in the second stage, as the starting material for PCR amplification by a conventional thermostable DNA polymerase.

## GENOMIC ARRAYS

As an increasing proportion of all human genes are discovered and sequenced, techniques are being developed that allow vast numbers of these genes to be examined simultaneously in a single tissue specimen. These techniques are variations on the dot-blot hybridization assay described earlier, but involve the use of a **genomic array**—a large collection of different DNA probes individually displayed in a pattern of tiny spots on a glass microscope slide or other two-dimensional surface. One common type of probe (called an **expressed sequence tag**, or **EST**) is a short sequence complementary to part of the coding region from a particular gene whose function may or may not be known. Genomic arrays can be prepared either by painting a minute amount of each probe onto a slide robotically or by synthesizing each probe directly on the surface using microlithographic technology similar to that used in manufacturing computer chips. Either approach yields a high-density array of as many as hundreds of thousands of short, defined DNA probes immobilized on a small surface. The array is then incubated with fluorescently labeled DNA prepared from a tissue specimen, under conditions that allow each tissue DNA fragment to hybridize only to a perfectly complementary probe. Afterward, the amount of tissue DNA bound to each spot in the array is measured fluorometrically. Depending on the probes used, a single array can scan for a multitude of human or pathogen genes, or can screen individual genes to detect specific sequence variations or mutations in the chromosomes. Alternatively, RNA extracted from a tissue sample can be used to prepare labeled cDNA, which can then be screened against an array of EST probes to determine which genes are active in the tissue and at



what levels they are expressed.

Genomic arrays have already proven their ability to provide enormous amounts of information, but the challenge of interpreting and applying that information remains. Nevertheless, one can envision a future in which, for example, routine evaluation of biopsies using such chips could detect, classify, and reveal the etiology of a lymphoid cancer and simultaneously provide information on metabolic phenotype and drug sensitivities to guide individualized therapy.

## OVERVIEW & PROSPECTS

Tests based on nucleic acid technology are a relatively new addition to the armamentarium of the clinical immunology laboratory, and it is not yet clear to what extent they will supplement or replace conventional assays. They are particularly well suited to the detection of viruses and other microorganisms in tissue specimens because such organisms can often be recognized and positively identified by their unique RNA or DNA sequences much more quickly and inexpensively than by culture. Hence, PCR-based tests have already assumed an important role in microbiologic diagnosis, and it seems likely that this role will increase in the future. Immunologically important organisms that are currently assayed in this manner include Epstein-Barr virus, human immunodeficiency viruses, and human T-cell leukemia viruses (see Chapters 43 and 46).

DNA-based assays are also very useful for detecting large-scale chromosomal deletions or rearrangements that occur at fairly constant locations in the genome and that characterize several types of hematologic

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malignancies. Examples include the Philadelphia chromosome of chronic myelogenous leukemia, the t(14,18) of follicular lymphoma, and the t(8,14) and related anomalies of Burkitt's lymphoma (see Chapters 7 and 43). Detection of these rearrangements can be a useful adjunct in diagnosis and also provides a simple means to monitor disease progression or to search for minimal residual disease after therapy. The Southern blot assay for lymphocyte clonality has similar potential utility and may be especially useful for evaluating poorly differentiated malignancies; however, it is currently too labor-intensive and technically demanding to be adopted by many clinical laboratories. PCR, LCR, and related techniques can detect extremely subtle DNA anomalies, including single-base point mutations, and are likely to be used increasingly for the diagnosis of congenital immunodeficiencies and of hereditary predispositions to cancer or other disorders. The emerging technologies for genomic analysis will certainly help identify genes that have diagnostic and prognostic value for individual patients and may also yield new and unexpected insights into disease biology.

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## 19

# Histocompatibility Testing

Lee Ann Baxter-Lowe PhD

Beth W. Colombe PhD

Today most histocompatibility laboratories provide human leukocyte antigen (HLA) typing and perform a variety of tests to support allogeneic transplantation, including assays to detect humoral and cellular responses to alloantigens. HLA typing plays an important role in contemporary medicine because HLA molecules are (1) the primary targets of immune responses to allogeneic transplants, (2) critical for responses to antigenic stimuli, and (3) implicated in genetic susceptibility to autoimmune disease. During the last decade histocompatibility laboratories have developed many new techniques that have supplemented, and sometimes supplanted, conventional methods for HLA typing and other tests for compatibility.

HLA molecules were first recognized as the “major transplantation antigens” that determine compatibility of allogeneic grafts. Although these molecules were first recognized for their antigenic qualities, it is now known that HLA molecules play a pivotal role in intercellular communication with T and natural killer (NK) cells (see Chapters 4, 6, and 9).

One of the hallmarks of HLA molecules is their diversity in the human population. HLA typing detects and classifies this diversity. HLA typing emanated from observations made by scientists using an agglutination technique to study leukocyte antigens in patients who had received multiple blood transfusions. The first HLA specificities (types) were defined when statistical methods were applied to complex patterns of agglutination reactions with sera containing alloantibodies. Over the ensuing years many technical advances occurred in HLA typing, including complement-dependent lymphocytotoxicity, microlymphocytotoxicity, and molecular typing. Application of these methods has resulted in continuous discovery of novel HLA molecules, which, by 1999, surpassed 1000 officially recognized HLA alleles.

Many patients who are candidates for allogeneic transplantation have developed alloantibodies in response to foreign HLA molecules encountered from pregnancy, transfusion, or prior allografts. These antibodies can cause rejection of allogeneic organ and tissue grafts. Crossmatching was developed to detect donor-specific alloantibodies in the serum of patients who are candidates for allogeneic transplants. Use of sensitive crossmatch techniques to assess compatibility of patients and kidney donors has virtually eliminated hyperacute rejection of renal grafts and made a major contribution to improved graft and patient survival.

Current practices in histocompatibility testing for allogeneic transplantation are influenced by the organ or tissue to be transplanted as well as the transplant protocol. Although the routine tests performed by histocompatibility laboratories are fundamentally similar, the tests are often customized for each transplant program (eg, variation in the sensitivity of lymphocytotoxicity tests, the use of flow cytometric methods for detection of antibodies, and the differences in the level of resolution for HLA typing).

During the last decade, high-resolution HLA typing methods have become available that can define HLA polymorphism at an allele level. High-resolution HLA typing methods are now routinely used for selection of unrelated donors for hematopoietic stem cell transplantation and sometimes for disease association. These methods are rarely used for solid organ transplantation, in which donor limitations preclude the ability to search for well-matched

unrelated donors.

This chapter describes the histocompatibility tests most frequently used today and new methods that offer potential for improved histocompatibility testing in the future. Internet sites that provide useful information about HLA polymorphism, histocompatibility, and transplantation are listed in Table 19-1.

## HISTOCOMPATIBILITY TESTING FOR TRANSPLANTATION

The primary goal of pretransplant histocompatibility testing is to optimize compatibility between organ or tissue donors and recipients in order to prevent rejection of the graft and to prevent graft-versus-host disease (GvHD) for grafts that potentially transfer sufficient numbers of lymphoid cells (eg, bone marrow, liver) to cause GvHD. HLA matching between donor and recipient is desirable for allogeneic transplantation.

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In practice, however, the extensive polymorphism of HLA makes it difficult to locate HLA-matched donors for transplants unless an HLA-matched sibling donor exists. Based on Mendelian inheritance, 25% of offspring will inherit the same HLA type. Thus, family size influences the likelihood of having an HLA-identical sibling. In the United States approximately 30% of patients have an HLA-identical sibling donor. In contrast, in Japan approximately 12% of patients have an HLA-identical sibling donor. The majority of patients therefore require transplants from donors who are not HLA-identical (partially HLA-matched related donors or unrelated donors). Routine tests used to assess histocompatibility for allogeneic transplants are summarized in Table 19-2.

**Table 19-1. Useful Websites for histocompatibility testing and transplantation.**

Anthony Nolan HLA Informatics	<a href="http://www.ashi-hla.org">http://www.ashi-hla.org</a>	IMGT/HLA database; HLA sequence data; official HLA nomenclature
American Society for Histocompatibility and Immunogenetics	<a href="http://www.ashi-hla.org">http://www.ashi-hla.org</a>	Histocompatibility information for general readers and another for patients; news updates; useful links; laboratory standards
British Society for Histocompatibility and Immunogenetics	<a href="http://www.umds.ac.uk/tissue/bshil.html">http://www.umds.ac.uk/tissue/bshil.html</a>	Introduction to tissue typing and blood groups; laboratory standards
National Marrow Donor Program	<a href="http://www.marrow.org">http://www.marrow.org</a>	Sections for patients, donors;

Home		news; Page information for HLA typing labs and transplant coordinators
United Network for Organ Sharing Home Page	<a href="http://www.unos.org">http://www.unos.org</a>	Information for transplant patients; transplant statistics
Immunogenetics (IMGT) database	<a href="http://www.ebi.ac.uk/imgt/hla/">http://www.ebi.ac.uk/imgt/hla/</a>	HLA sequence database information, documentation, nomenclature, query tools, and submission forms for novel alleles
Sanger Centre Home Page	<a href="http://www.sanger.ac.uk/">http://www.sanger.ac.uk/</a>	Human genome project and related databases; chromosome 6 maps
MHCPEP	<a href="http://wehih.wehi.edu.au/mhcpep/">http://wehih.wehi.edu.au/mhcpep/</a>	Database of peptides that bind to HLA molecules
<i>Abbreviations:</i> HLA = human leukocyte antigen; IMGT = immunogenetics.		

**Table 19-2. Selection of histocompatibility tests.**

Typing Method	Low-resolution	Low-resolution	High-resolution	High-resolution				
Transplant	AB	DR/DQ	AB	DR/DQ	C	DP	ABO	XM
Blood or bone marrow, sibling or related donor	U	U	R	R	R	V	V	V

Blood or bone marrow, unrelated donor	U	U	I	U	V	V	V	V
Kidney	U	U	R	R	V	R	U	U
Pancreas	U	U	R	R	V	R	U	U
Heart/lung	U	U	R	R	V	R	U	U
Liver	V	V	R	R	V	R	V	V
Cornea	V	R	R	R	V	R	R	R
<i>Abbreviations:</i> U = usually; V = variable, depends on transplant center practice; R = rarely; I = increasing use.								

The method and resolution of HLA typing or tissue typing that is optimal for donor selection depends on several factors, including the extent of HLA matching required for transplant (eg, organ, transplant protocol), relationship to the recipient (ie, relative or unrelated), time constraints, and specimen characteristics (eg, cell source, quality, quantity). For example, for solid organ transplants from cadaver donors in which HLA disparity usually occurs between donor and recipient, low-resolution typing is adequate and turnaround time must be minimal. HLA matching is most important for transplantation of

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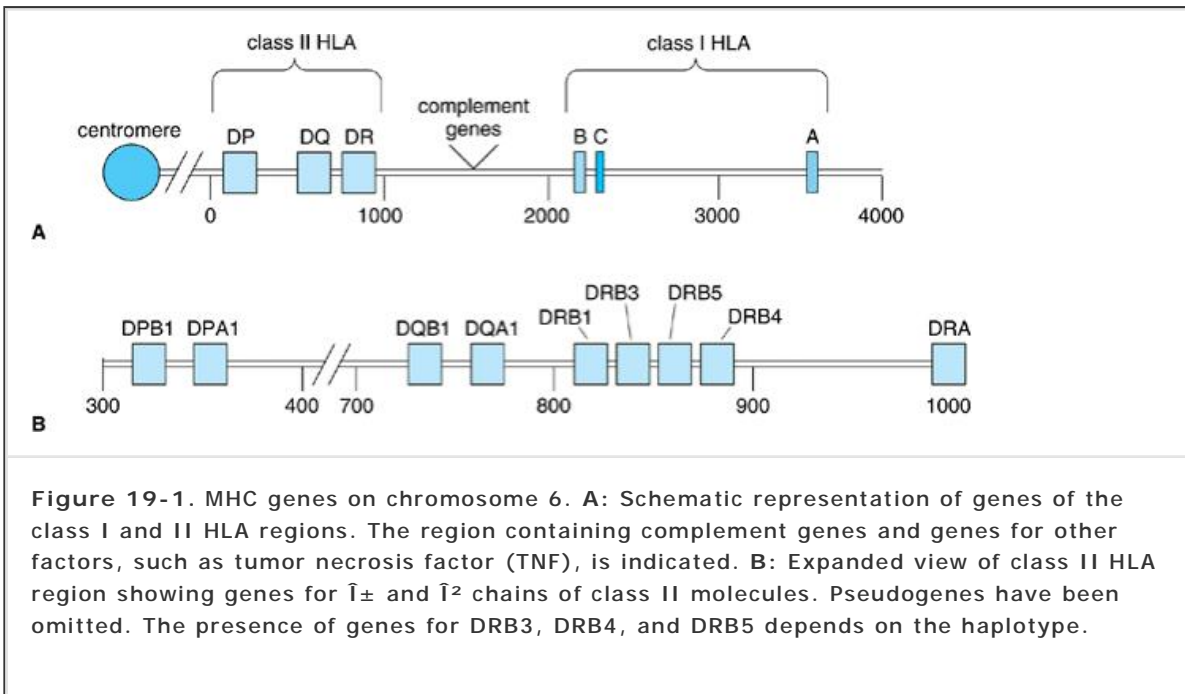
lymphohematopoietic cells. Large registries of volunteer donors are now available to locate well HLA-matched unrelated donors for patients who lack a suitably matched family donor. High-resolution molecular HLA typing is often used for evaluation of these donors. Typical HLA typing requirements are listed in Table 19-2.

Some patients have existing antibodies directed toward HLA mismatches of potential donors. These antibodies are formed after contact with foreign HLA molecules (eg, pregnancy, transfusion, transplantation). The formation of specific antibodies to alloantigens is referred to as **sensitization**. Preformed antibodies directed against mismatched antigens of the donor can cause hyperacute rejection of certain solid organ grafts and have been associated with increased rates of rejection following blood and marrow transplants. A **crossmatch** is used to test for the presence of donor-specific preformed antibodies in the patient's serum. Crossmatches can be performed using lymphocytotoxicity sometimes supplemented with more sensitive flow cytometry assays. A positive crossmatch attributable to specific antibodies toward donor HLA mismatches is considered to be a contraindication for most transplants.

Antibody screening is used to detect alloantibodies and attempt to define the specificity of alloantibodies in patients who are transplant candidates who are at risk for preformed alloantibodies. Lymphocytotoxicity is the traditional method for antibody screening, and a number of alternative methods for detecting alloantibodies have been used during recent years (eg, flow cytometry, enzyme-linked immunosorbent assay [ELISA]).

Functional assays that detect T-cell responses to alloantigens are sometimes used to supplement

HLA typing. These include the mixed lymphocyte culture (MLC) and the cell-mediated lympholysis test (CML). Assays that measure the precursor frequency of helper and cytolytic T cells are available but are primarily used in research settings.



## HLA POLYMORPHISM & TYPING

### HLA Genetics

The genetics of the HLA system is described in detail Chapter 6. Briefly, the gene products detected by HLA typing are encoded by a cluster of related genes located in the major histocompatibility complex (MHC) on the short arm of chromosome 6 (Figure 19-1). Historically the MHC has been considered to encompass about 4 megabases that are subdivided into three regions: The class I region of approximately 2000 kilobases includes the polymorphic HLA-A, B, and C loci, the class II region of approximately 1000 kilobases includes the HLA-DR, -DQ, and -DP loci, and the class III region of approximately 1000 kilobases encodes genes with diverse functions. Recent discovery of genes that are homologous to HLA genes (eg, HFE) or that play a role in HLA function (eg, tapasin) has defined an extended MHC that spans 8 megabases. These genes are not currently included in HLA typing, but it is conceivable that certain HLA-like genes may be included in the future.

In addition to the expressed HLA genes, numerous class I and class II pseudogenes are located in the MHC. These pseudogenes provided challenges in the development of molecular typing methods because it is necessary to type the expressed genes without detecting the closely related pseudogenes. Rarely, the

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presence of a pseudogene can confound molecular HLA typing.

### HLA Polymorphism & Nomenclature

The extensive polymorphism of HLA was initially shown by the large number of serologic specificities (types) defined by alloantibodies (Table 19-3). Functional assays suggested that a cells with the same HLA specificity could have differences that are recognized by T cells. The molecular basis for these observations was revealed by sequencing the genes encoding these antigens. This investigation demonstrated that cells with the same serologic specificity may have



different amino acid sequences. By 1999, the number of known HLA alleles exceeded 1000, and the number continues to increase. The number of known alleles corresponding to particular specificity ranges from 1 to more than 50. This is demonstrated Figure 19-2, which shows the number of alleles for each HLA-A specificity that were known in October 1999.

Establishing the nomenclature for HLA alleles has been challenging because HLA is extremely polymorphic. The first sequenced alleles were assigned names with two digits that corresponded to their serologic specificity followed by two digits that indicated the chronologic order of naming by the World Health Organization (WHO) Nomenclature Committee for Factors of the HLA System. For example, the first allele sequenced from an HLA-A2 gene was named HLA-A\*0201, and the second was named HLA-A\*0202. Discovery of polymorphism that does not alter the protein sequence was addressed by adding from one to three numbers and a letter to names to identify synonymous (silent) mutations and polymorphism located in noncoding regions of the genes (Figure 19-3). By October 31, 1999, 40 alleles had been named in the HLA-A2 group, including 5 distinguished by silent mutations in the coding regions and 2 null alleles (no protein expressed).

Today, HLA typing may be performed by serologic typing methods that report specificities listed in Table 19-3 or molecular types that are derived from the allele names (Figure 19-3). The genetic diversity of HLA, HLA nomenclature, and use of a variety of HLA typing methods has reached such complexity that the subject is rarely understood by individuals outside the histocompatibility field.

The relationship between serologic specificities and molecular types is not always straightforward. For example, many recently discovered alleles have not been typed by serologic methods and, therefore, have no defined serologic specificity. These alleles were assigned names using homology for certain key sequences located in previously named alleles. Thus, the first two digits of a name currently considered to belong to an "allele group" rather than a particular serologic specificity. The relationship between alleles and serologic specificities is sometimes complicated

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by the presence of epitopes associated with two or more serologic specificities in a single allele. Some alleles lack epitopes corresponding to the known specificities and cannot be assigned a specificity using conventional typing sera. These are called blanks. These circumstances and others confound the relationship between certain serologic specificities and molecular types. For example HLA-B\*1522 is consistently typed as HLA-B35 by serologic methods. A major effort is underway to establish a correlation between known alleles and defined serologic specificities; updates of these correlations are routinely published. In practice, these relationships can be important because it is often necessary to compare molecular types for one individual with serologic specificities determined for another (eg, typing data from both methods in unrelated donor registries).

**Table 19-3. HLA antigen specificities.<sup>a</sup>**

A	B	C	DR	DQ	DP
A1	B5	Cw1	DR1	DQ1	DPw1
A2	B7	Cw2	DR103	DR2	DPw2
A203	B703	Cw3	DR2	DR3	DPw4
A210	B8	Cw4	DR3	DR4	DPw5
A3	B12	Cw5	DR4	DR5(1)	DPw6
A9	B13	Cw6	DR5	DR6(1)	

A10	B14	Cw7	DR6	DR7(3)
A11	B15	Cw8	DR7	DR8(3)
A19	B16	Cw9(w3)	DR8	DR9(3)
A23(9)	B17	Cw10(w3)	DR9	
A24(9)	B18		DR10	
A2403	B21		DR11(5)	
A25(10)	B22		DR12(5)	
A26(10)	B27		DR13(6)	
A28	B35		DR14(6)	
A29(19)	B37		DR1403	
A30(19)	B38(16)		DR1404	
A31(19)	B39(16)		DR15(2)	
A32(19)	B3901		DR16(2)	
A33(19)	B3902		DR17(3)	
A34(10)	B40		DR18(3)	
A36	B4005		DR51	
A43	B41		DR52	
A66(10)	B42		DR53	
A68(28)	B44(12)			
A69(28)	B45(12)			
A74(19)	B46			
A80	B47			
	B48			
	B49(21)			
	B50(21)			
	B51(5)			
	B5102			
	B5103			
	B52(5)			
	B53			
	B54(22)			
	B55(22)			
	B56(22)			
	B57(17)			
	B58(17)			
	B59			
	B60(40)			
	B61(40)			
	B62(15)			
	B63(15)			
	B64(14)			
	B65(14)			
	B67			
	B70			
	B71(70)			
	B72(70)			
	B73			
	B75(15)			
	B76(15)			
	B77(15)			
	B7801			
	B8101			

B8201  
Bw4  
Bw6

<sup>a</sup>Antigens as recognized by the World Health Organization. Antigens listed in parentheses are the broad antigens; antigens followed by broad antigens in parentheses are the antigen splits. Antigens of the Dw series are omitted.

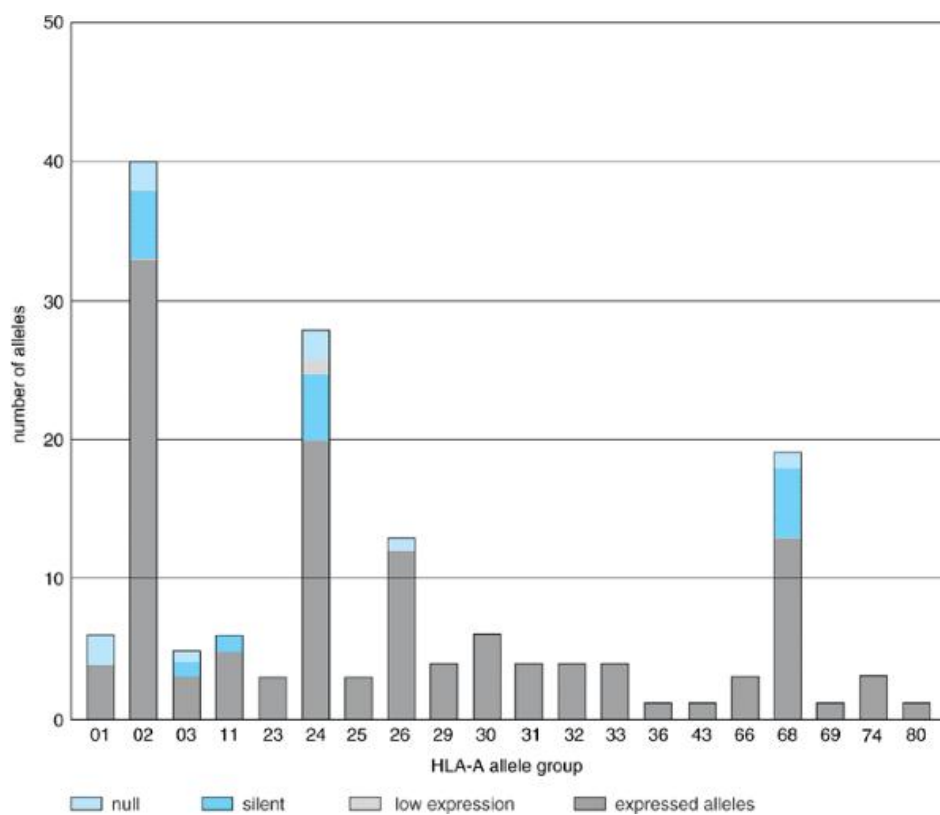
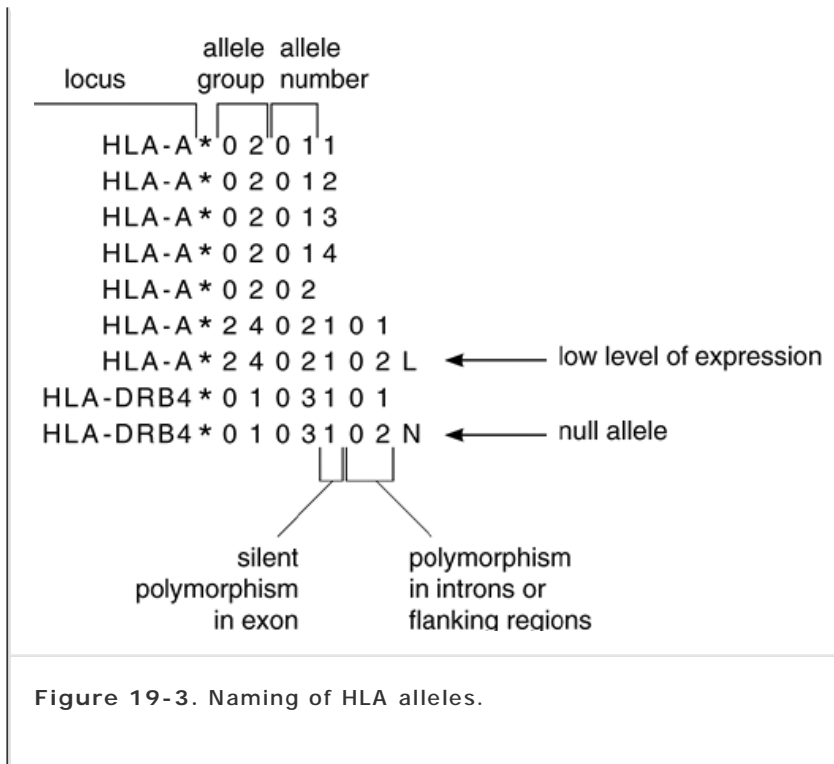


Figure 19-2. Number of HLA alleles known in 1999.



In contrast to serologic typing methods that depend on detection of epitopes that are recognized by alloantibodies, molecular HLA typing methods can detect any polymorphic nucleotide sequence. This provides the opportunity to perform typing at different levels of resolution ranging from groups of alleles to individual alleles. Typing that defines groups of alleles, usually approximating serologic specificities, is referred to as **low-resolution**, or **generic**, typing (eg, HLA-DRB1-04). Typing methods that resolve all known alleles are often referred to as **high-resolution** HLA typing (eg, HLA-DRB1\*0401). Typing that resolves HLA types beyond serologic specificities but that does not achieve allele level is described as intermediate-resolution typing (eg, HLA-DRB1-0401/09/13/16/21/26/33). The National Marrow Donor Program has created codes to facilitate management of these complex intermediate resolution data. In this system, the name for HLA-DRB1-0401/09/13/16/21/26/33 is DRB1-04EJV.

Further complexity is caused by differences in molecular HLA typing methods and the continual need to update interpretation of molecular typing data as the number of known HLA alleles increases. Several molecular methods detect key polymorphic sequences and use these data to predict the allele. The interpretation of these data is influenced by the library of HLA sequences used for interpreting these data. For example, in 1987 five sequences were known for the HLA-DRB1-04 group. At that time, a probe for a polymorphic sequence at codon 71 was used to assign DRB1-0401 because this polymorphism was believed to be unique to this allele. By 1999, six additional HLA-DRB1\*04 alleles had been discovered that shared this polymorphic sequence. Thus data that would have been typed as DRB1\*0401 in 1987 may be interpreted as DRB1\*0401, 0409, 0413, 0416, 0421, 0426, or 0433 in 1999.

There are more complicated examples in which the assignment of HLA types based on detection of key polymorphic sequences is affected by knowledge of HLA polymorphism. For example, certain reagents used to detect HLA-A-03 in 1998 would also detect HLA-A\*3204, which was discovered in 1999. Thus a sample containing HLA-A\*3204 might be typed as HLA-A\*03 before 1998 and with appropriate adjustments in reagents, typed as HLA-A\*32 after the discovery in HLA-A\*3204 in 1999. Situations such as this have stimulated interest in use of automated nucleotide sequencing for molecular HLA typing because this approach minimizes the likelihood that the presence of an unknown allele will produce an HLA typing assignment.

The continual discovery of additional HLA alleles also complicates use of National Marrow Donor Program (NMDP) codes. The same molecular typing data may have different code depending on when the sample was typed because the sequence libraries used for data interpretation differed. In practice this discrepancy can be important because a potential donor that is not matched to the patient may actually be matched if a patient's allele is not included in the type because the allele was unknown at the time of the typing. One potential solution currently under investigation is to store typing data in registries as the polymorphic sequences rather than the HLA types.

### ***Inheritance***

One of the consequences of the clustering of HLA genes on chromosome 6 is that most individuals inherit a set of nonrecombined HLA alleles from each parent. These genes are codominantly expressed. Thus, if the HLA types of family members are determined, segregation of HLA types within the family can be used to construct the HLA types from each chromosome. The set of HLA alleles found on one chromosome is called a **haplotype**. Determination of haplotypes is important for identification of HLA-identical siblings because sharing of antigens from different haplotypes is common. Determining the inheritance of haplotypes within the family permits identification of HLA-identical siblings with a high degree of confidence. If haplotypes are unknown, some antigens may appear to be matched using low-resolution HLA typing but differ at high-resolution typing. This circumstance is particularly important for certain types of transplants that are strongly influenced by the extent of HLA matching (eg, bone marrow transplant).

## **SEROLOGIC METHODS IN HISTOCOMPATIBILITY TESTING**

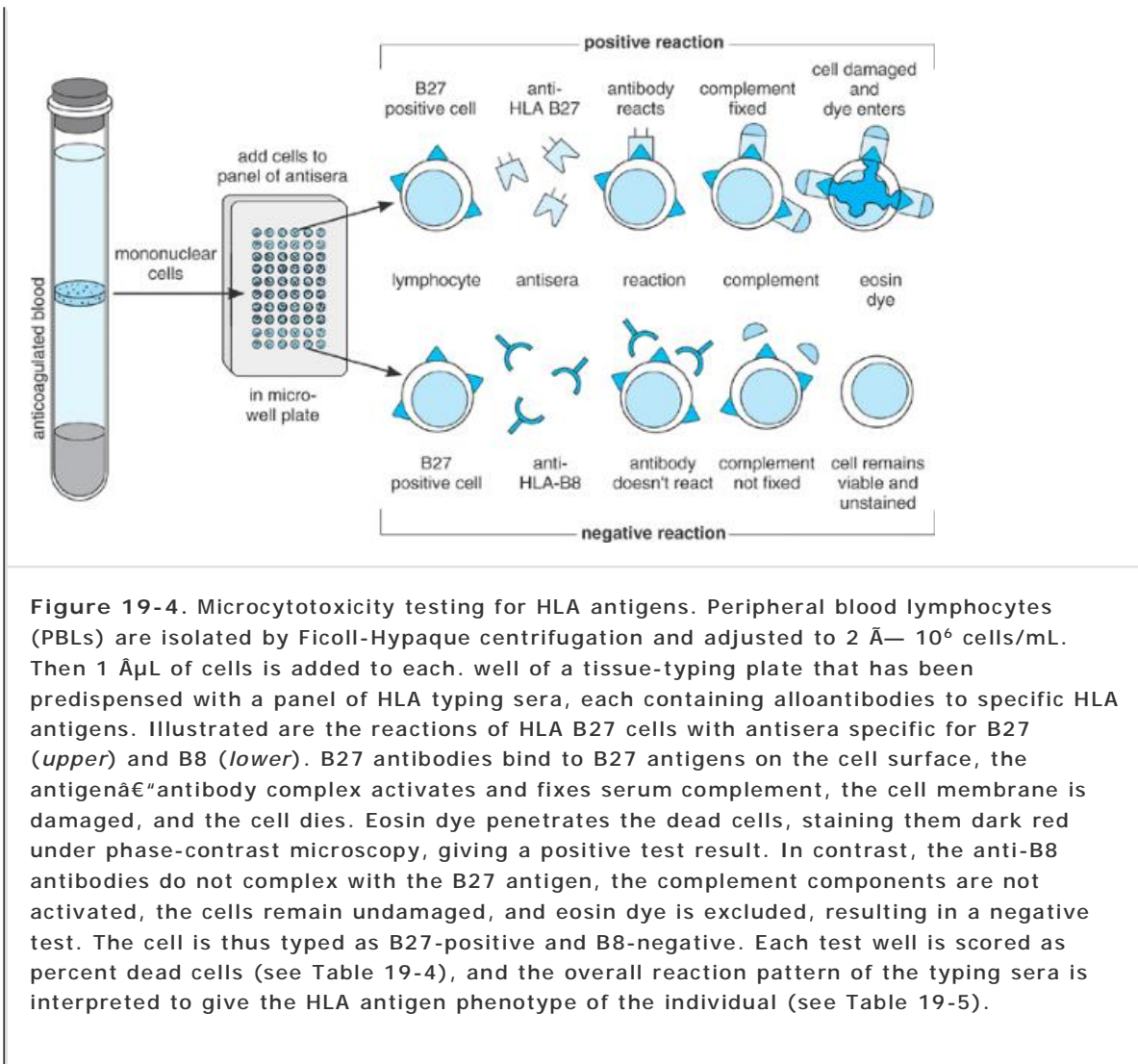
Serologic techniques provide one of the simplest and fastest methods for histocompatibility testing. These methods use serum that contains antibodies to HLA antigens. Anti-HLA antibodies are highly specific for the individual structural determinants that characterize the different antigens of the HLA system.

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Thus, when sera containing HLA antibodies are mixed with lymphocytes, the antibodies bind only to their specific target antigens (Figure 19-4).

When the antigen-antibody complex is formed on the cell surface in the presence of complement, complement activation leads to cell lysis. Thus, cell death is an indicator of the shared specificity of antigen and antibody and is a "positive" test result. Detection of this specificity between antibody and antigen provides the answer to several fundamental questions in histocompatibility testing: (1) What are the HLA antigens of a particular cell? When the antibody specificity is known, as for the HLA typing reagents, and the cell is of unknown phenotype, the positive test results with specific antisera identify the antigens of the cell. (2) Are there anti-HLA antibodies in a particular serum? When the serum is being tested for the presence of HLA antibodies, a positive test indicates antilymphocyte activity in the serum. From the pattern of positive and negative reactions with a panel of HLA-typed cells, the specificity of the antibodies may be inferred. If the patient's serum reacts with the donor cell, the two individuals are incompatible.





Thus, through an iterative process of testing serum and typing cells, HLA antigens are defined, panels of typed lymphocytes are generated, and collections of HLA typing sera of known specificity are created.

## TISSUE TYPING BY THE LYMPHOCYTOTOXICITY TEST

Serologic HLA typing is accomplished by exposing the unknown cell to a battery of antisera of known HLA specificity (see Figure 19-4). The typing sera are selected to give unequivocally strong positive scores to ensure reproducibility. If the cells are killed in the presence of antiserum and complement, the cell is presumed to have the same HLA antigen as the specificity of the antibody.

### Cell Isolation

Lymphocytes are the preferred cell type for serologic HLA typing, antibody screening, and crossmatching. Traditionally lymphocytes were isolated from whole peripheral blood by buoyant density gradient

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separation, from buffy coat, or, in cadaveric testing, from lymph nodes and spleen. Care must be taken to prepare a cell suspension of excellent viability as well as one that is free of erythrocyte and platelet contamination. Although other cell types that bear HLA molecules, such

as platelets, amniocytes, and fibroblasts, can be used for HLA typing in special circumstances, lymphocytes are the most responsive and reproducible target for the standard cytotoxicity assay. HLA typing for class II HLA antigens, HLA-DR and -DQ, is performed on lymphocyte preparations enriched for B lymphocytes. Special isolation procedures are required because approximately 80% of normal peripheral blood lymphocytes (PBL) are resting T cells that lack class II HLA antigens on their surface.

Isolation of T and B lymphocytes by magnetic beads is now the most common method for HLA typing and crossmatching. The antibody-coated beads offer versatility, speed of cell recovery, and relative purity of the final cell preparation. Beads with anti-CD2 or anti-CD8 are used to isolate T cells and anti-CD19 for B cells. The beads can be used to obtain adequate numbers of cells from whole blood, buffy coats, or PBL preparations. Cells with bound beads also become more sensitive as targets, possibly due to disturbance of their cell membranes, and thus tests require less incubation time than the standard cytotoxicity assays.

### ***The Complement-Dependent Lymphocytotoxicity Test: NIH Standard Method***

Individual HLA antisera are predispensed in 1- $\mu$ L quantities into the microtest wells of specifically designed plastic trays composed of 60 or 72 wells of 15- $\mu$ L capacity. An array of anti-HLA sera is chosen with specificities covering the full range of HLA types. Usually each type is represented by at least two antisera. Today most laboratories obtain frozen typing trays from commercial sources. Some laboratories continue to prepare batches of typing trays that are stored for use over several months or years.

For HLA typing, the sera in a test tray are thawed, approximately 2000 lymphocytes are dispensed per well, and the tray is incubated for 30 minutes at room temperature to allow anti-HLA antibodies to bind to their specific target HLA antigens. Complement (5  $\mu$ L) is added, usually as rabbit serum, and the tray is incubated for another 60 minutes. The complement-dependent cytotoxicity assay for HLA-DR and -DQ typing is performed with appropriate class II typing sera but is modified as follows for B cells isolated over nylon wool: Initial incubation of cells and serum is performed at 37 $^{\circ}$ C or 22 $^{\circ}$ C for 60 minutes; after addition of complement, the mixture is incubated for 120 minutes at room temperature. The extended incubation times are used to promote binding of antibodies and complement. The temperature increase is used to avoid the false-positive reactions that can result from the binding of cold-reactive nonspecific antibodies. When immunoabsorbent beads are used for class II typing, the incubation times are generally decreased by approximately half, possibly because of the weakening of the cell membrane by attachment to the beads.

To visualize the dead and live cells under phase-contrast microscopy, a vital dye, eosin Y, is added, followed by formalin to fix the reaction. Live cells exclude the dye and appear bright and refractile, but dead cells take up the dye and are swollen and dark (see Figure 19-4). In an alternative method known as fluorochromasia, the cells are prelabeled with a fluorochrome such as fluorescein diacetate (green) prior to plating. When the cells are killed in the positive test, the fluorescein leaks out, and the cells disappear. Positive results are compared with a negative well where all the cells are visible. A second fluorochrome of contrasting color such as ethidium bromide (red) may be added to visualize the dead cells.

Each test well is scored individually by inspection, with the percentage of dead cells per well being noted. The test is unequivocally positive when at least half of the cells are killed (Table 19-4).

An HLA type is assigned by interpreting the patterns of reactivity of the individual sera and their specificities. The typing sera that reacted positively with the test cells should have antibody specificities in common. For an HLA type to be assigned, the majority of sera of that specificity must be unequivocally positive. In phenotyping an individual for class I HLA, one expects to find patterns with two antigens each from the A, B, and C loci. When only a single antigen is identified at a locus, the individual may be homozygous for that type, or the laboratory has failed to identify the second antigen, because the sera may not contain antibodies for the

epitopes present on the target cells or there is low expression of the HLA molecules. Table 19-5 shows a representation of a subset of HLA-typing test results.

### ***Serologic Typing Reagents***

An ideal HLA-typing serum should be monospecific, that is, would have specificity for a single HLA antigen; however, in reality, the observed alloantibodies

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in a single serum are usually polyspecific. An alloantiserum can contain antibodies to multiple determinants on the immunizing antigen(s). Some determinants are the classic HLA **private** specificities that characterize each HLA type (see Table 19-3). Others are **public**, that is, are shared by several antigens that collectively constitute a cross-reacting antigen group (CREG group). Some complex sera can be rendered monospecific by dilution, whereas others lose all activity for all specificities simultaneously.

**Table 19-4. Scoring the lymphocytotoxicity test for HLA typing.**

% Dead Lymphocytes in Test Well	Score	Interpretation
0â€"10	1	Negative
11â€"20	2	Doubtful positive
21â€"50	4	Weak positive
51â€"80	6	Positive
81â€"100	8	Strong positive

**Table 19-5. An example of HLA typing test results.<sup>a</sup>**

Serum Name	Specificities	Score
A-001	A1	1
A-002	A1, A36	1
A-003	A1, A11	1
A-004	A2	1
A-005	A2, A28	6



A-006	A2, A28, B7	8
A-007	A3	8
A-008	A3	6
A-009	A3, A10, A11, A19	8
A-010	A11	1
A-011	A10, A11	1
A-012	A11, A1, A3 (weak)	4
B-001	B51, B52, B35	1
B-002	B51, B52	1
B-003	B51, B52, B53	1
B-004	B7, B42	8
B-005	B7, B27	8
B-006	B7, B55	8
B-007	B8	1
B-008	B8, B59	2
B-009	B44, B45, B21	6
B-010	B44, B45	8
B-011	B44	8
B-012	B45	1
<sup>a</sup> Interpretation: HLA phenotype is: A28, A3, B7, B44.		

To determine the specificities of HLA antibodies, sera are tested against panels of cells of known

HLA phenotype, a process termed "screening." The cell panel, usually from 40 to 60 cells, is preselected to provide a minimum of two to three representations of the most frequent HLA antigens. The antigens must be distributed among the cells so that the reaction pattern for one antigen is not entirely included within the pattern for a second antigen; for example, all of the HLA-A1 cells must not also be the only HLA-B8 cells. If they were, the reaction patterns for both antibodies would be identical and the determination of A1 or B8, or both, could not be made with certainty. When the sera react with a subset of the panel, the specificity of the antibody is deduced by inspecting the HLA phenotypes of the positive cells.

The majority of HLA antisera are complex sera obtained from multiparous women. Maternal exposure to the mismatched paternal HLA antigens in the fetus gives rise to a polyclonal antibody response that frequently results in sera with multiple specificities. Consequently, several different antisera are used to type for a specific antigen. It is not unusual for a laboratory to use typing trays composed of more than 200 different sera to type a single individual for HLA-A, -B, -C, and -DR, -DQ antigens.

Placental fluid, a mixture of serum and tissue fluids, has proved to be a valuable second source of alloantisera for tissue typing. Antibodies of high titer can be recovered from the fluid shed from fresh placentae that have been refrigerated for 24 hours after delivery.

HLA antibodies are also found in the sera of patients exposed to HLA antigens through transfusions of blood and organ grafts. Generally, patients are not used as sources of HLA-typing sera, since the quantities obtainable would be limited by their medical conditions.

Attempts to immunize other animals, such as rabbits, for production of HLA antisera have largely failed. The xenoantisera reacted primarily with common human antigens such as the HLA-DR constant region. Efforts in numerous laboratories have produced monoclonal antibodies to certain HLA specificities. It was hoped that hybridoma cell lines (see Chapter 15) would produce inexhaustible quantities of monospecific antibodies to HLA antigens. In reality, many murine monoclonal antibodies have been directed against human monomorphic framework determinants or to common epitopes rather than to the private polymorphic determinants of the individual HLA antigens. Unfortunately, most murine monoclonal antibodies do not fix complement. Noncomplement-fixing antibodies can be used in assays that require only antigen binding, such as ELISA (Chapter 15) and flow cytometry (Chapter 16). Many monoclonal antibodies are reactive with more than one HLA specificity, consistent with the existence of common antigenic determinants on HLA molecules.

As use of molecular HLA typing has increased, serum screening efforts have diminished. A small number of laboratories currently maintain serum screening programs. New methods for producing monoclonal antibodies may be used for preparation of serologic typing reagents if serologic typing is not supplanted by molecular typing methods. The majority of histocompatibility laboratories currently use molecular methods for HLA-DR and -DQ typing, and the use of molecular methods for class I typing is increasing each year. The ease, speed, and technical similarity to crossmatch tests are factors that favor continuation of serologic HLA typing. Another strength of serologic typing methods is that expression of the HLA molecules is determined.

### ***Variability in Serologic Typing Results***

HLA-typing sera are not uniform because the complexity of HLA polymorphism and scarcity of the defining antisera make it impossible to create a standard reagent for each specificity. Each tissue-typing laboratory has the responsibility of obtaining the appropriate

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antisera and monitoring their performance (including commercial products). Results with the laboratory's routine typing trays may be compared with those of other collections of sera for confirmation of an HLA phenotype. All tissue-typing laboratories are required by the standards set by the American Society for Histocompatibility and Immunogenetics (ASHI) to control the quality of serologic and cellular reagents used for clinical testing (see Table 19-1). International and national quality control programs are available for typing, crossmatching, and serum

analysis, and satisfactory performance is mandatory for ASHI accreditation of the laboratory.

A second variable in tissue typing is the serum complement, a reagent commercially available as the pooled serum from several hundred rabbits. Rabbit serum contains heterophile antibodies with antihuman lymphocyte activity that enhances its effectiveness in the cytotoxicity assay. Overabundance of these antihuman antibodies render the complement innately cytotoxic and therefore produces false-positive results. As with typing sera, each laboratory must screen its source of complement to find one that promotes strong cytotoxic reactions without causing nonspecific toxicity. Because there is no standard complement source, the same serum tested in different laboratories has the potential to yield disparate results.

Other variables are the method used to visualize the live and dead cells, incubation times, and the definition of the end of the test period. Table 19-6 illustrates the type of reaction patterns obtained on reagent or patient serum screens.

## ANTIBODY SCREENING

Antibody screening is used to detect the presence of HLA antibodies in patients who are candidates for transplant. Screening has traditionally been performed using complement-dependent lymphocytotoxicity methods. Most laboratories perform monthly screens for patients on waiting lists for solid organ donors. Sera from each patient are tested for the presence of antibodies against a panel of cells that contain the most frequent HLA types in the donor population present in several different HLA-A and -B combinations (or DR and DQ for class II) to allow determination of the specificities of alloantibodies. The format may be designed as a batch test for all patients or a test for a single patient.

**Table 19-6. An example of results of serum screening for class I HLA antibodies.<sup>a</sup>**

Panel Cell HLA Antigens	Cytotoxicity Test Score for Patient Serum Number				
	1	2	3	4	5
A, A, B, B (locus)					
1, 2, 7, 60	1	1	8	1	8
2, 3, 8, 51	1	8	8	8	8
3, 29, 35, 44	1	8	1	6	8
30, 33, 55, 60	1	1	1	1	8
1, 30, 13, 51	1	1	1	8	8
24, 28, 35, 55	1	1	4	6	4
3, 28, 7, 44	1	8	4	1	6

2, 28, 35, 38	1	1	8	8	8
<sup>a</sup> PRA (panel-reactive antibody) was 0, 38, 50, 63, and 100%, for patient sera 1 through 5, respectively. PRA is calculated as (number of positive tests/number of cells tested) $\bar{A}$ — 100. Analyzed antibodies were as follows (patient sera 1 through 5, respectively): None; A3; A2, weak A28; B51, B35; and Unknown (autoantibody?).					

The percent panel reactive antibody (PRA) is calculated as the ratio of the number of positive cells to the number of total panel cells multiplied by 100. PRA is indicative of the extent of sensitization of the patient to HLA. Note that the antibody specificities of sera with high PRA cannot be determined. Special procedures must be used, such as dilution or treatment of the sera, or both, to determine antibody specificities.

### ***HLA Antibody Screening by the ELISA Method***

A rapid and highly sensitive solid-phase method for the detection of antibodies to HLA has been commercially developed using the ELISA technique. Purified preparations of HLA antigens affixed to plastic plates are used to capture anti-HLA antibodies in patient sera, thereby avoiding the use of whole cells with their complex array of surface markers. False-positive reactions due to antibody binding to non-HLA antigens is avoided with the use of this technology. Affinity-purified HLA antigens are isolated from platelets or from lymphoblastoid cell lines, pooled, and fixed to the bottom of test wells in a plastic test plate. Antigens derived from platelets are HLA class I only, whereas antigens from cell lines may be class I, class II, or a combination of both types of antigens. Advantages of the ELISA assay over conventional lymphocyte panel testing by cytotoxicity include the ease of rapid batch processing via automated plate readers and the elimination of the need for viable lymphocytes and complement for the assay.

An aliquot of the patient's serum is incubated in the test well where any HLA-reactive antibody of appropriate avidity binds to the fixed antigen. After removal of unbound antibody, an enzyme-linked antibody that is directed against human IgG is added. Addition of enzyme substrate develops positive tests. The readout of the basic ELISA antibody test is either positive (antibody present) or negative (antibody absent). No PRA can be calculated, nor can specificity be assigned to the antibody. Further testing with standard lymphocyte panels (or with newly developed flow cytometry methods, see section on HLA Antibody Detection by Flow Cytometry) is necessary to obtain these parameters. The ELISA test is formulated to detect only IgG, the HLA antibody isotype

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that is considered to be the most deleterious to solid organ transplant outcome. Consequently, standard ELISA cannot detect cytotoxic IgM and non-HLA antibodies and detects noncomplement-fixing IgG antibodies that cytotoxicity assays miss. ELISA may fail to detect an antibody with specificity for a rare HLA antigen that may be absent or at low levels in the antigen pool. Recently, ELISA products have been developed to provide antibody identification and PRA capability, and an IgM-specific second antibody is available.

### ***Antibody Detection (PRA) by Flow Cytometry***

Antibodies binding to non-HLA cell surface antigens can generate false-positive results in both the standard complement-dependent lymphocytotoxicity assay and the flow cytometry crossmatch test, both of which are routinely used to detect antibodies to HLA antigens. A recent

product for general HLA antibody detection (measurement of PRA) takes advantage of the additional sensitivity afforded by the flow cytometer. Microbeads are coated with HLA antigens extracted from a cell panel of HLA-typed cell lines. The beads are available with either purified HLA class I or class II HLA antigens or as a mixture of both. The class II particles are distinguishable from the class I particles by fluorescence, making it possible to detect both types of antibody in a single flow cytometer run. The microparticles are incubated with the patient's serum and then stained with an anti-human IgG-fluorescent conjugate that binds to those IgG-anti-HLA antibodies that formed a complex with the beads. The percentage of the beads that stain above background provides a measure of the patient's PRA. This assay can measure noncomplement-fixing HLA antibody but is limited to antibodies of the IgG isotype. Other isotype-specific anti-human Ig conjugates can be used if detection of, for example, IgM HLA antibodies is desired. An additional product of coated microbeads offers several separate bead panels of HLA class I and class II antigens that allow characterization of the specificity of the antibody detected. In addition to the measurement of PRA, the microspheres can be used as a diagnostic test to confirm that the antibody detected by the cell-based assays is indeed anti-HLA. This information can be critical to the decision to transplant in the event of an equivocal positive crossmatch.

## CROSSMATCHING

The purpose of the crossmatch test is to detect the presence of antibodies in the patient's serum that are directed against the HLA antigens of the potential donor. If present, the antibodies signal that the immune system of the recipient has been sensitized to donor antigen(s) and is primed to vigorously reject any graft bearing the antigen(s). In the transplanted kidney, the main target of these antibodies is probably the HLA antigens on vascular endothelium of capillaries and arterioles. HLA antigen-antibody complexes on endothelium activate complement and lead to cell damage. Platelets then aggregate, eventually producing fibrin clots, which clog the vessels and cause ischemic necrosis. Even weak, low-titer antibodies, particularly those directed against class I antigens, can contribute to graft rejection. The ultimate goal of the crossmatch is a test of both great sensitivity and specificity for HLA antigens.

### *PBL Lymphocytotoxicity Crossmatching*

A simple crossmatch by the standard cytotoxicity method (see earlier discussion) may be performed with donor PBL as targets. PBL crossmatches are usually included in the preliminary evaluation of potential living related donors for renal graft recipients. PBL are normally about 80% T cells, which carry class I HLA antigens only, and 20% B cells and monocytes, which bear both class I and class II antigens. A strongly positive crossmatch by cytotoxicity (50% or more cell death per well) clearly indicates the presence of antibodies to class I antigens. However, 10%–20% cell killing could result from an antibody specific for class II or could be due to a weak anticlass I antibody. To resolve the specificity of the antibody, crossmatching is then performed on cell preparations enriched for either T or B lymphocytes.

### *T-Cell Lymphocytotoxicity Crossmatching*

T-cell crossmatches depicted in Figure 19-5 are performed at room temperature and also at 37°C in some laboratories to avoid the binding of cold-reactive antibodies, presumed to be autoreactive. A positive T-cell crossmatch by any method contraindicates transplantation, no matter how weak the reaction level; that is, a reaction of 4+ (20%–50% dead cells per well above background) is considered just as positive a result as 6+ or 8+ (51% dead cells or greater). Some laboratories even consider a reaction of 2 (10%–20% dead over background) as a positive crossmatch result.

Several methods to improve the sensitivity of T-cell crossmatches by complement-dependent cytotoxicity have been developed. These include

#### 1. Extended incubation

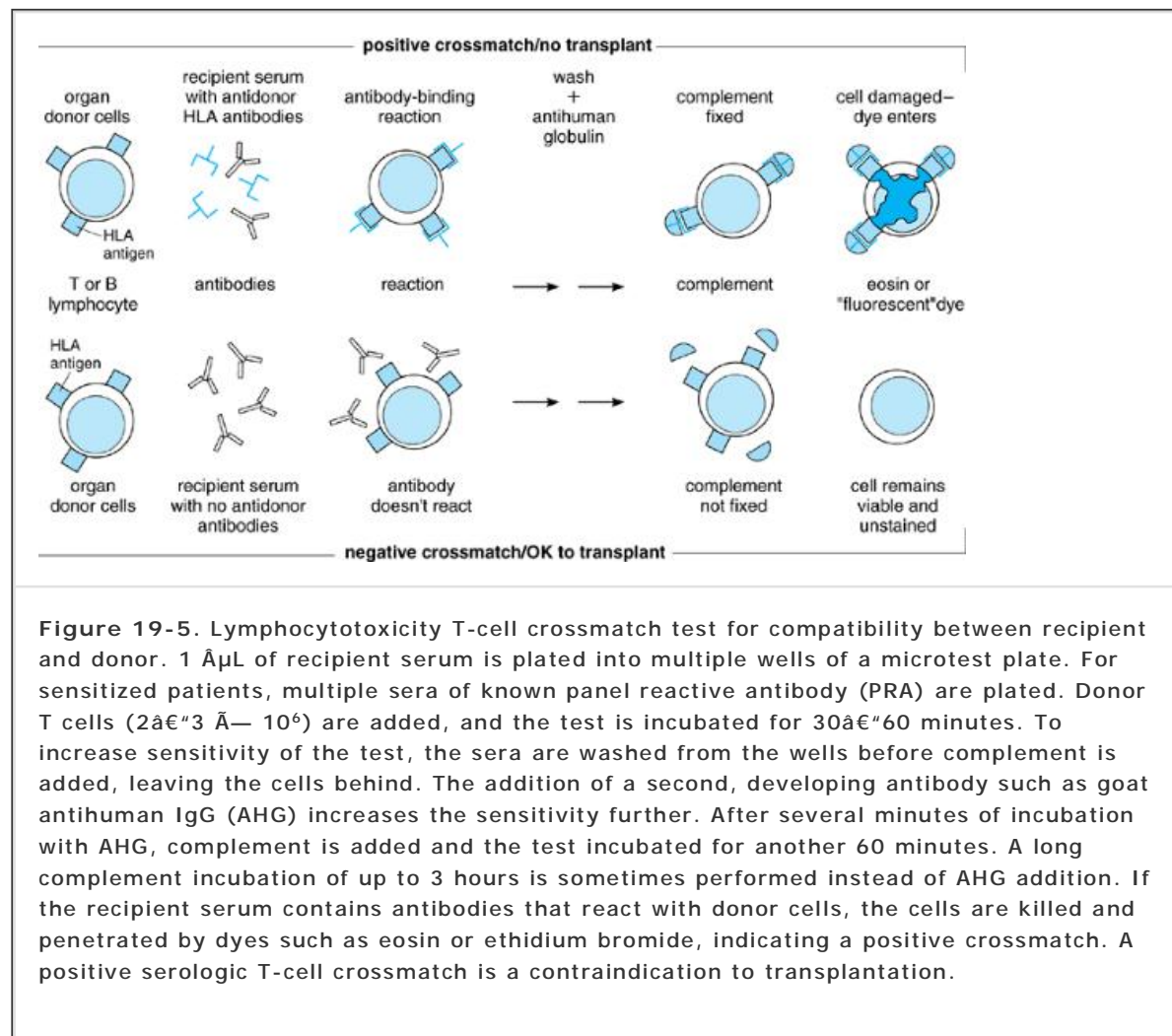
The simplest modification in the cytotoxicity assay to increase sensitivity is to extend the incubation time of cells, serum, and complement.

## 2. The Amos wash step

This method interjects a wash step after the incubation of cells and serum and prior to the addition of complement to remove anticomplementary factors in the serum.

## 3. Antihuman globulin

The cytotoxicity of some antibodies may be enhanced by the addition of a second-step antibody, usually a polyclonal antihuman immunoglobulin (AHG) reagent.



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## B-Cell Lymphocytotoxicity Crossmatching

Crossmatching for antibodies to class II HLA antigens requires the use of B lymphocytes as targets and the same extended incubation times as HLA-DR and -DQ serologic typing for nylon-wool isolated B cells. A positive B-cell crossmatch may result from antibodies binding to class I or class II HLA antigens. Moreover, B cells are a more sensitive indicator for weak class I antibodies because they carry class I molecules in greater density than T cells. Crossmatching by flow cytometry can readily distinguish between  $\alpha$  antibodies and weak class I antibodies of a positive B-cell cytotoxicity crossmatch. The significance for transplant outcome

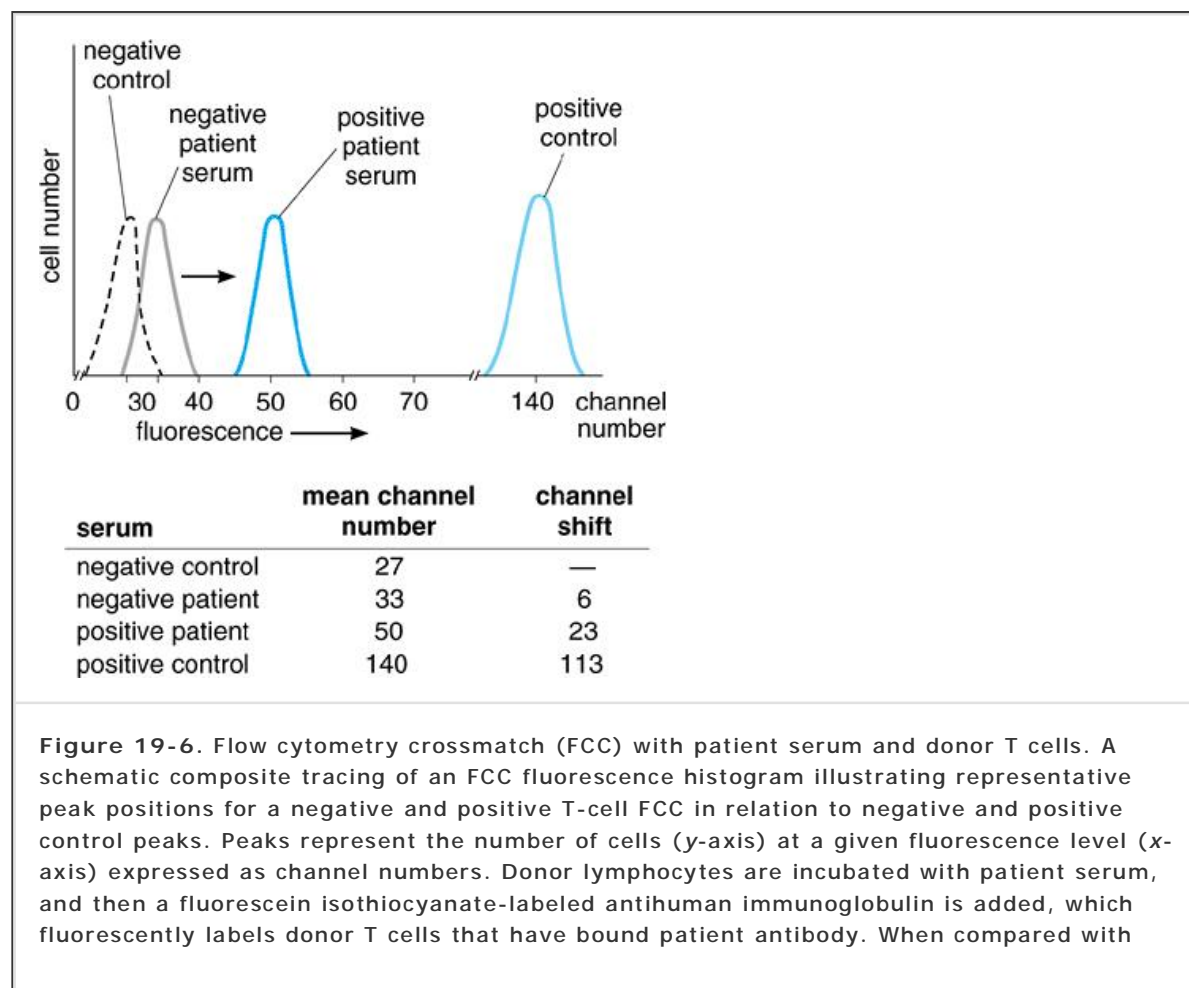
of preformed antibodies to class II antigens is not yet clear. Successful transplantation into patients with low-titer anticlass II antibodies (titer of 1:1 or 1:2) has been reported, as has the acute rejection of grafts transplanted in the face of high-titer (1:8) antibodies. It is possible that the loss of grafts transplanted in the face of T-negative, B-positive crossmatches is due to the anticlass I component of these alloreactive sera.

### Flow-Cytometry Crossmatching

Cross-matching by flow cytometry (FCC) (see Chapter 16) has been shown to be 30–250 times more sensitive than visual serologic methods for the detection of IgG HLA antibodies on lymphocytes (Figure 19-6). In this crossmatching application, T cells can be separated from B cells electronically through the use of a fluoresceinated antibody to a T-cell surface antigen such as the CD3 T-cell receptor complex. An electronic "gate" can be created so that only the fluorescently labeled T cells are selected. Donor lymphocytes are incubated with patient's serum to allow binding of any antidonor antibodies. Antibody bound to the selected T-cell population is detected by addition of an antihuman IgG anti-Fc-specific F(ab')<sub>2</sub> antibody labeled with a fluorochrome of a different color from the T-cell marking antibody (eg, green, if the anti-CD3 was red). The flow cytometer counts the number of labeled T cells and creates a histogram displaying the number of cells versus fluorescence intensity (see Figure 19-6). Unlabeled cells lie near the origin, and labeled cells lie

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to the right of the origin on the x-axis. A shift to the right of the T-cell peak in the experimental test compared with the negative control indicates that anticlass I HLA antibody from the patient serum has bound to the donor T cells. FCCs can also be performed on gated B cells labeled with a B-cell-specific antibody.



the peak of T cells having no antibody bound, the fluorescent T-cell peak is brighter and shifted to the right on the x-axis. When the mean channel fluorescence of the T-cell peak shifts to the right by more than 10 channels on a 256-channel scale, the crossmatch is considered to be positive.

FCC is generally performed with the most recent serum and a selection of historically reactive sera for all patients who are on the waiting list for cadaver donors and who have increased risk of rejection (eg, rejected prior transplants, high PRA, living related donors with a negative T-cell and positive B-cell serologic crossmatch).

Occasionally, positive FCC crossmatches occur when both the serologic T- and B-cell crossmatches are negative. The indications for transplant remain controversial. Two possible explanations for these observations are the presence of HLA antibodies that are not cytotoxic or the presence of blocking antibodies that interfere with testing. One alternative for further testing is use of HLA-bound beads (described earlier) to clarify the presence of HLA-specific antibodies. Clinical decisions concerning transplantation of the patient can then be made including this finding.

### ***Crossmatching for Autoantibodies***

In crossmatching patient serum for donor compatibility, it is most important to distinguish nonspecific antilymphocyte antibodies, referred to as autoantibodies, from the specific antidonor antibodies. The presence of autoantibodies is detected by the autocrossmatch, in which the patient's own serum and cells are combined in the standard cytotoxicity test. Autoantibodies can give a false-positive result in a donor crossmatch, leading to the erroneous disqualification of that donor. Alternatively, preexisting autoantibodies can mask the presence of specific antidonor antibodies. Autoantibody crossmatches are routinely performed in conjunction with all living-donor crossmatches for each serum that is tested. Auto FCC crossmatches are also recommended, particularly in the case of a negative serologic crossmatch coupled with an unexpectedly positive FCC. False-positive FCCs can occur when the patient has autoantibodies of undetermined specificity. Such autoantibody-positive FCCs are not considered to be a contraindication to transplantation.

## **HISTOCOMPATIBILITY TESTING BY MOLECULAR-BIOLOGIC METHODS**

### ***Introduction***

The serologic specificities have been identified through the binding patterns of antibodies in serologic tests and from the activation of lymphocytes by disparate MHC antigens in the mixed lymphocyte culture (MLC) test. With the advent of gene cloning and DNA sequencing, HLA antigen specificities are now known to derive from sequence differences localized to several hypervariable regions in the MHC molecules. Most of the differences have arisen from intragene and intergene conversion events that have resulted in the complex polymorphism that characterizes the HLA system. In the HLA class II genes, the variable regions are found mainly in exon 2 and in class I genes; the polymorphisms are concentrated in exons 2 and 3. These variations in sequence are localized in the antigen-binding cleft and to the alpha-helical regions facing the T-cell receptor, locations strategic to the ability of the immune system to recognize and respond to pathogenic (and possibly autoimmunogenic) endogenous and exogenous antigens.

HLA alleles share many of the same sequence motifs but in different combinations. Consequently, HLA class I and class II antigens can be thought of as a patchwork of combinations of these various sequence



polymorphisms occurring on a background of shared (consensus) nucleotide base sequences. The long-observed phenomenon of crossreactivity of antisera and, more recently, the cross-hybridization of oligonucleotide probes, can be explained by the fact that several antigens can share the same sequence motifs. For example, the concept of the "public" (ie, held in common) HLA antigen specificity, such as antigens Bw4 and Bw6 that are associated with all HLA B locus specificities, is confirmed by finding a polymorphic amino acid sequence located at residues 77-83 in all B locus antigen sequences. An even greater degree of sharing of specific polymorphic sequences takes place among the class I alleles, even occurring across the class I loci, making typing for class I alleles more technically demanding than for class II alleles. An important consequence of these shared sequence motifs is that certain heterozygous combinations of alleles cannot always be distinguished from a second, different combination. All DNA typing methods must take this into consideration when proposing schema for allele identification. Clearly, the most definitive HLA typing method would be to carry out a complete sequence analysis of the DNA of the HLA genes of each individual. Automated nucleotide sequencing is currently regarded as the gold standard for HLA typing, but this technology is currently limited to laboratories that support hematopoietic stem cell transplant programs that frequently use unrelated donors.

Table 19-7 compares major features of molecular and serologic typing methods. Molecular typing methods offer several advantages over serologic methods, including improved accuracy, higher resolution, and specimen flexibility. Molecular typing methods can be particularly advantageous for typing samples containing HLA molecules that are in the same cross-reactive group. Several publications document the clinical benefits of molecular typing that have been attributed to improved accuracy or higher resolution typing. The sequence data provided by molecular methods along with knowledge of the structure and function of HLA are currently being used to better understand the molecular basis for allorecognition and HLA-associated diseases.

**Table 19-7. Comparison of HLA typing methods: DNA-based and serologic.**

Method	Serologic	DNA:SSP/SSOP	DNA:SBT
Number of identifiable types	21 43	21-151 43-301	151 301
HLA-A	10	10-83	83
HLA-B	18	18-282	282
HLA-C	9	9-43	43
HLA-DR	-	6-87	87
HLA-DQ			
HLA-DP			
Sample material	2-3 million live lymphocytes	Minute amount of DNA	Minute amount of DNA
Reagents	Alloantisera (supply exhaustible) some monoclonals	Synthetic oligonucleotide primers/probes (supply unlimited)	Synthetic digonudeo tide primers (supply unlimited)
Power to	Very limited:	Limited: based on	Unlimited: new

identify new alleles	depends on availability and specificity of sera	knowledge of sequences and on novel reaction patterns	alleles identified by their sequences
Level of resolving power for known alleles	Generic level	Generic to allele level	Allele level
Important factors	Expression of HLA on cell surface Viability of test cells	Quality and quantity of genomic DNA and amplification factors Stringency of test conditions	Quality and quantity of genomic DNA and amplification factors
<p><i>Abbreviations:</i> SSP/SSOP = sequence-specific priming/sequence-specific oligonucleotide probing; SBT = sequence-based typing; HLA = human leukocyte antigen; PCR = polymerase chain reaction.</p>			

### ***Molecular Typing Methods***

The first molecular typing method detected restriction fragment length polymorphism (RFLP) in genomic DNA and was primarily used for class II HLA typing. A few reports also described the use of sequence-specific oligonucleotide probe (SSOP) hybridization to RNA templates. After the discovery of the polymerase chain reaction (PCR), several easier and more powerful molecular typing methods were rapidly developed. The first used SSOP hybridization to amplify templates. This was followed by typing by sequence-specific priming (SSP), which relies on the specificity of the amplification to determine HLA types. The most recent development has been sequence-based typing (SBT), which takes advantage of automated nucleotide sequencing. These methods are summarized in Table 19-8.

**Table 19-8. Molecular histocompatibility testing techniques.**

Name	Characteristic Reagents	Characteristic Processes	Polymorphisms Detected
RFLP	Bacterial restriction endonucleases	Southern blotting	Restriction fragment length
SSP	Sequence-specific PCR primers	PCR/gel electrophoresis	Generic to allele-level
SSOP	Sequence-specific	PCR/hybridization of	Generic to

	oligonucleotide probes	probes to PCR product	allele-level
SBT	Labeled primers/labeled sequence terminators	PCR/nucleotide sequencing of PCR product	Allele-level
Heteroduplex analysis RSCA	Denatured, single-strand DNA/Artificial universal heteroduplex generator (UHG)	Reannealing of strands of DNA/electrophoresis of recombined DNA	DNA complexes characteristic of alleles
<p><i>Abbreviations:</i> RFLP = restriction fragment-length polymorphism; SSP = sequence-specific priming; SSOP = sequence-specific oligonucleotide probing; SBT = sequence-based typing; PCR = polymerase chain reaction; HLA = human leukocyte antigen; RSCA = Reference Strand Conformational Analysis.</p>			

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## Gene Amplification

The majority of molecular HLA typing methods use PCR to selectively amplify the segments of HLA genes that are required for typing. The specificity of the amplification can be **locus-specific** (eg, HLA-A, HLA-B, HLA-DRB1), **group-specific** (eg, DRB1-01, DRB1-02), or **allele-specific** (eg, DRB1-0401, DRB1\*0402). For PCR, the specificity is determined by the sequence of the primers and amplification conditions (eg, thermal cycling,  $[Mg^{2+}]$ ). Most typing schemes require conditions that avoid coamplification of pseudogenes.

## SSP Typing

One of the most frequently used molecular typing methods takes advantage of sequence-specific priming (SSP), which is depicted in Figure 19-7. Primer pairs are designed to specifically amplify each polymorphic sequence that must be detected to provide the desired level of typing resolution. A primer pair that amplifies a different segment of DNA is usually included in the same tube as a positive control. Target DNA is added to a tube containing the primers and the other reaction components (eg, DNA polymerase, buffer,  $Mg^{2+}$ ), and thermal cycling is performed. PCR products are usually detected by separating the amplified DNA on an agarose gel. After electrophoresis, the DNA is stained (eg, ethidium bromide), and the reaction is scored for the presence of the product of the internal control primers and the presence or absence of the HLA-specific product.

The combination of positive and negative HLA reactions is used to assign the HLA type. If no HLA-specific products and no internal control products are present, the reaction is scored as a failure and must be repeated. The number of primers included in the test varies according to the locus and level of resolution required. For a low-resolution HLA-DRB typing, typically about 20–30 reactions are needed; for an ABC typing usually about 100–200 reactions are required. Sets of typing primers are currently provided by several commercial vendors. As the number of known HLA alleles increases, the minimum number of reactions required for SSP typing also increases. Variations on this method include use of multiplex PCR and different techniques for detecting positive reactions (eg, hybridization with labeled probes).

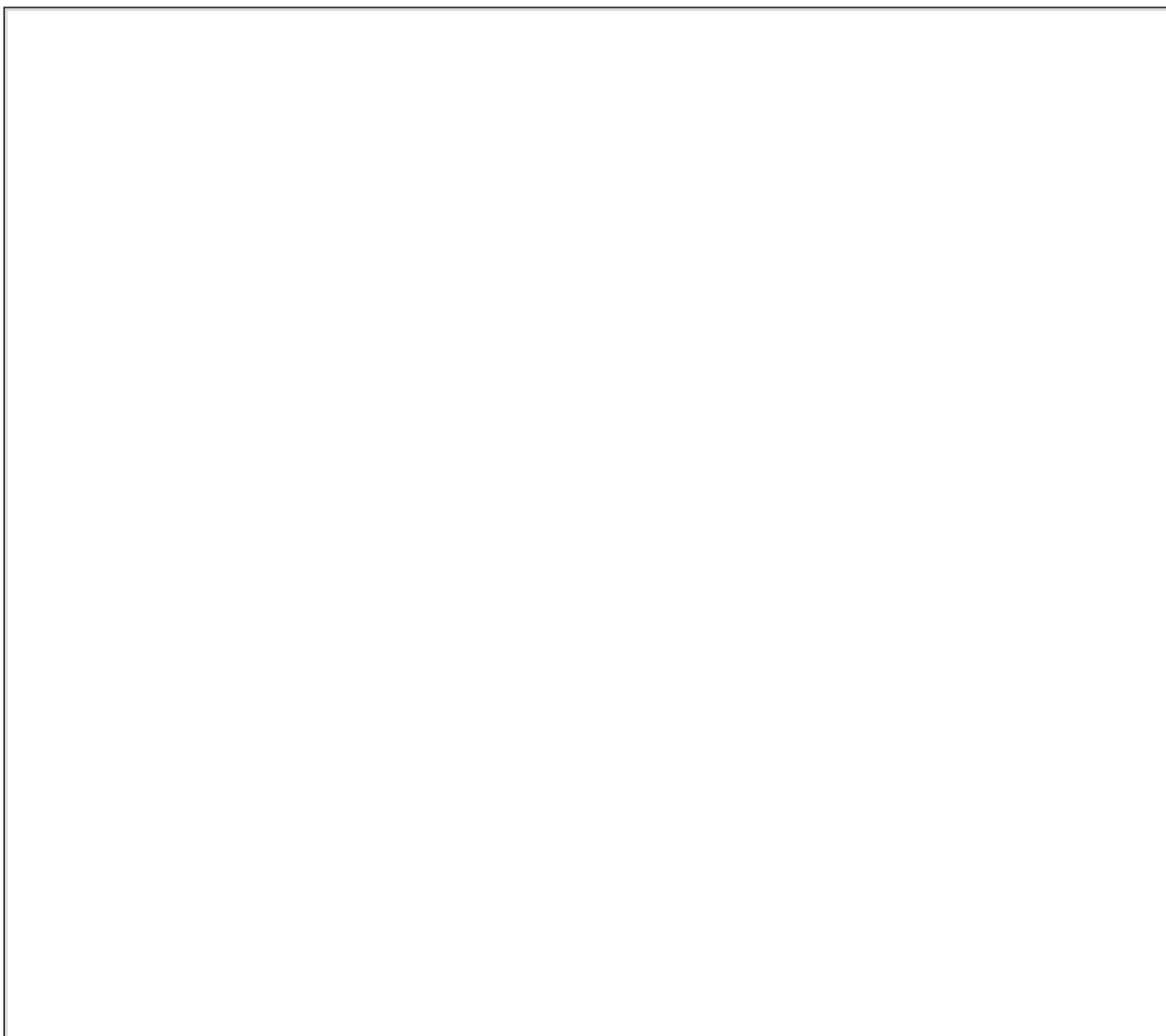
The major advantages of this method are that equipment requirements are minimal, the time required for testing is short, and it is fairly easy to learn. Major disadvantages are requirements for a large number of reactions and for high-purity DNA to ensure that reaction specificity is not compromised. One weakness in the formats that are currently in use is that the HLA typing primers are not internally controlled. Thus, a false-negative result can be obtained if the internal control (a different primer pair) is positive, but the HLA-specific primers are dysfunctional. Although it is theoretically possible to construct more appropriate internal controls, to date, this has not been accomplished.

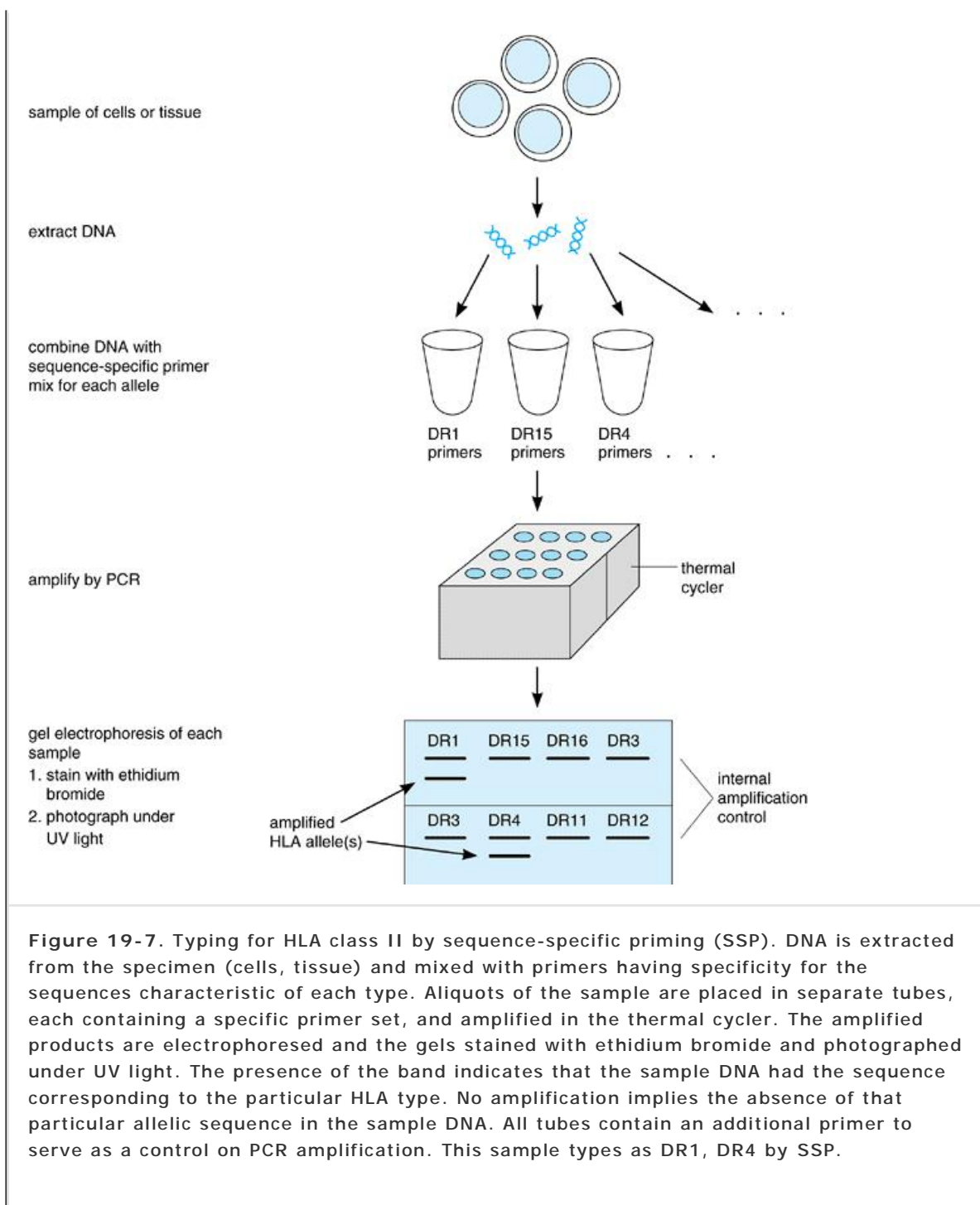
### ***SSOP Typing***

Sequence-specific oligonucleotide probe hybridization was the first PCR-based HLA typing method. This method involves the selective amplification of the HLA target followed by hybridization to a panel of oligonucleotide probes. The two most frequently used formats are depicted in Figure 19-8: (1) dot or slot blots with amplified DNA bound to a solid support (eg, membrane) and hybridized to probes in solution and (2) reverse dot or slot blots with probes bound to a solid support hybridized to amplified DNA in solution. The dot blot format is favorable for typing large numbers of samples. Some high-volume laboratories use this approach for typing batches of 96 or 384 samples. The reverse dot blot is favorable for testing small numbers of samples. In general, if the number of probes exceeds the number of samples,

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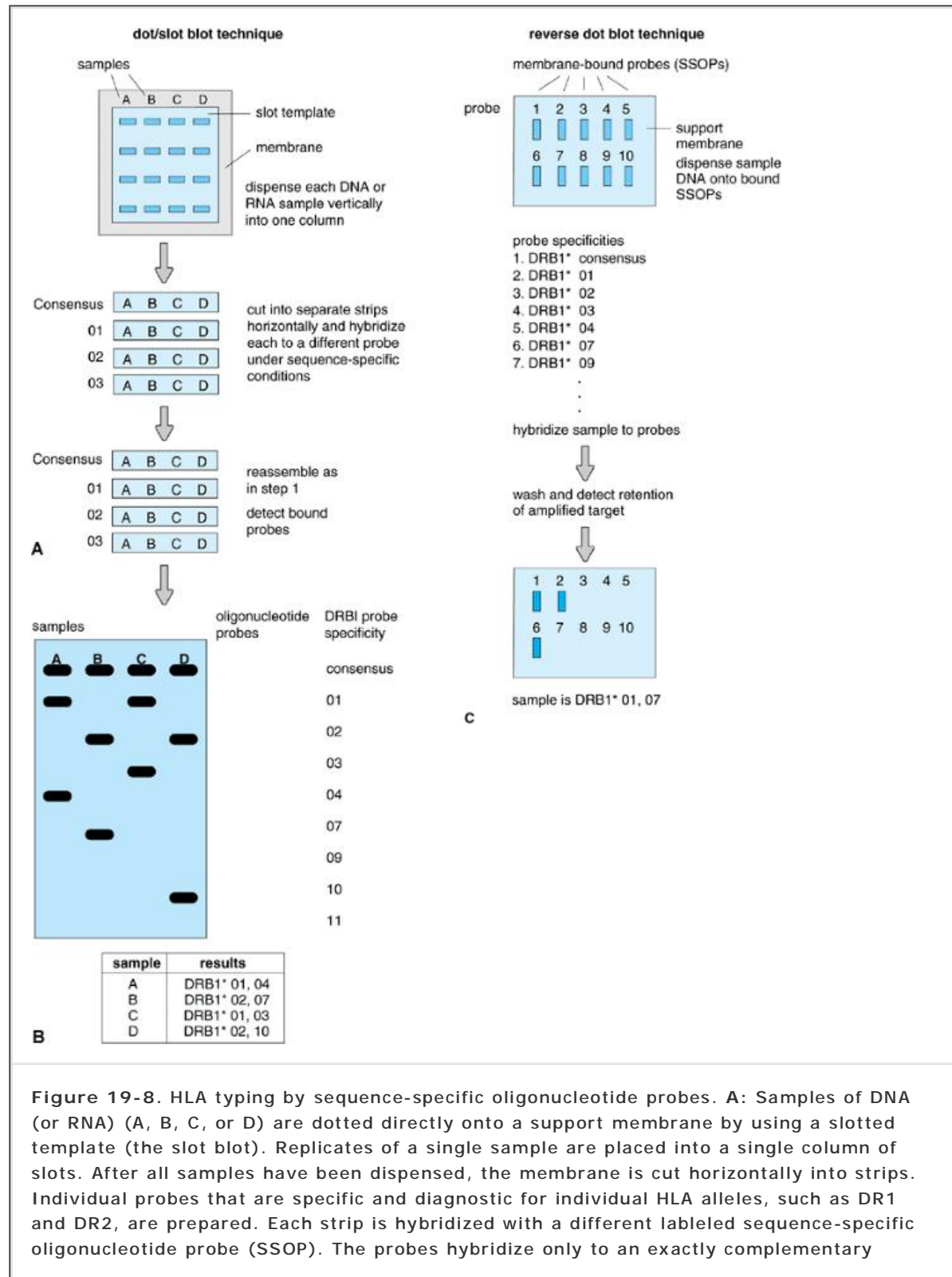
the reverse dot blot is most efficient, and if the number of samples exceeds the number of probes, the reverse dot blot is most efficient.





Reagents for dot/slot blots are commercially available or made by individual laboratories. Typically 1–5 µL of amplified DNA from each sample is applied to nylon membranes using a manifold with dots or slots. Certain high-volume procedures transfer less than 2 µL of amplified DNA directly to the membrane without use of a manifold. Probes can be synthesized by standard methods or purchased in HLA typing kits that are available from several vendors. In 1999, the approximate minimum number of probes required for low-resolution typing was 30 for HLA-A, 60 for HLA-B, and 30 for DRB1. After hybridization with probes, the membranes are washed to remove

nonspecifically bound probes. Labels attached to the probe are detected (eg, chemiluminescence, enzymatic methods with colorimetric detection, radioactivity), and the patterns of positive and negative hybridization are used to assign HLA types. One major advantage of this format is that positive and negative controls can be included for each probe.



**Figure 19-8.** HLA typing by sequence-specific oligonucleotide probes. **A:** Samples of DNA (or RNA) (A, B, C, or D) are dotted directly onto a support membrane by using a slotted template (the slot blot). Replicates of a single sample are placed into a single column of slots. After all samples have been dispensed, the membrane is cut horizontally into strips. Individual probes that are specific and diagnostic for individual HLA alleles, such as DR1 and DR2, are prepared. Each strip is hybridized with a different labeled sequence-specific oligonucleotide probe (SSOP). The probes hybridize only to an exactly complementary

nucleotide sequence in the sample. **B:** The membrane is reassembled and probes are detected. A band indicates the presence in the sample of the sequence of the corresponding HLA type. **C:** In the reverse dot blot, the sequence-specific probes are bound to the membrane support, and the sample DNA is hybridized with amplified DNA. The membrane is washed, and the hybridized DNA is detected by colorimetric methods.

Reverse dot blots require a substantial investment to develop conditions that maintain sequence specificity for a large number of probes under the same hybridization and wash conditions. This format is therefore generally limited to use of commercial products. Initially the probes were applied as dots, and more recently some companies apply the probes in lines. In this format, reading the type is analogous to reading a bar code.

Several variations of the SSOP format have been reported. One method uses probes bound to the bottom of 96-well trays, and hybridization of amplified DNA to the probes is detected using ELISA methods. This variation of the reverse dot blot provides an opportunity to include positive and negative controls for each probe and takes advantage of automated systems for ELISA methods.

### **SBT**

Sequence-based HLA typing involves determining the nucleotide sequence of an amplified segment of an HLA gene. This is usually accomplished using an automated nucleotide sequencer. Briefly, the HLA gene segment is amplified, the excess nucleotides and primers are removed, the amplified DNA is used as a template for a sequencing reaction, and the products of the sequencing reactions are purified and applied to a sequencing gel.

Sequencing reactions contain a mixture of normal and modified nucleotides (dideoxynucleotides) that terminate polymerization when they are incorporated into the replicating strand of DNA. A primer is used to initiate DNA synthesis using DNA polymerase. When a dideoxynucleotide is incorporated into the new DNA molecule, the polymerization is terminated. Thus, primer extension products are generated that are terminated at every position of the DNA molecule. Fluorescent labels are used to distinguish chains terminated by each base (A, C, G, or T). The labels are incorporated using a dye-labeled primer (dye primer chemistry) or dye-labeled dideoxynucleotide terminators (dye terminator chemistry).

Custom dye primers can be purchased in kits or obtained by custom synthesis (expensive). Another alternative is to use PCR primers that contain a tail that can be hybridized to a labeled primer. Drawbacks of dye primer chemistry include detection of premature termination products, which can cause substantial problems during interpretation of the sequencing data, and cumbersome set up (four reactions per sequence). These problems are eliminated by using dye-labeled dideoxyterminators that are insensitive to premature termination products because these are unlabeled and therefore not detected by the sequencer. Dye-terminator reactions are performed in a single tube (Figure 19-9). One disadvantage of the dye-terminator method is that the signal from the dye-labeled products can be significantly decreased by the presence of primers and nucleotide remaining from the PCR reactions (present in unpurified template). Early dye-labeled primer chemistries suffered from variable peak heights caused by enzymatic differences in nucleotide incorporation. This is now minimized with new enzymes that reduce discrimination against dideoxynucleotides (eg, AmpliTaq, FS which has a point mutation in the active site).

The primer extension products are separated on a gel that resolves single base differences in DNA (or sequencing gels). The gel is run in an automated sequencer that usually contains a laser that excites the dye molecules and a detector that records the emissions from each dye. Software converts the primary data into a chromatogram format and automates nucleotide assignments for each position. The data are manually edited, and the sequence is compared with

a library containing all known sequences to assign the allele(s) in the sample. One advantage of this method is that the sequence of each of the DNA strands can be determined to confirm the types. This is recommended because technical artifacts can cause the occasional loss of a particular nucleotide, which can cause incorrect interpretation of the data from heterozygotes.

### ***Limitations of Molecular Typing Methods***

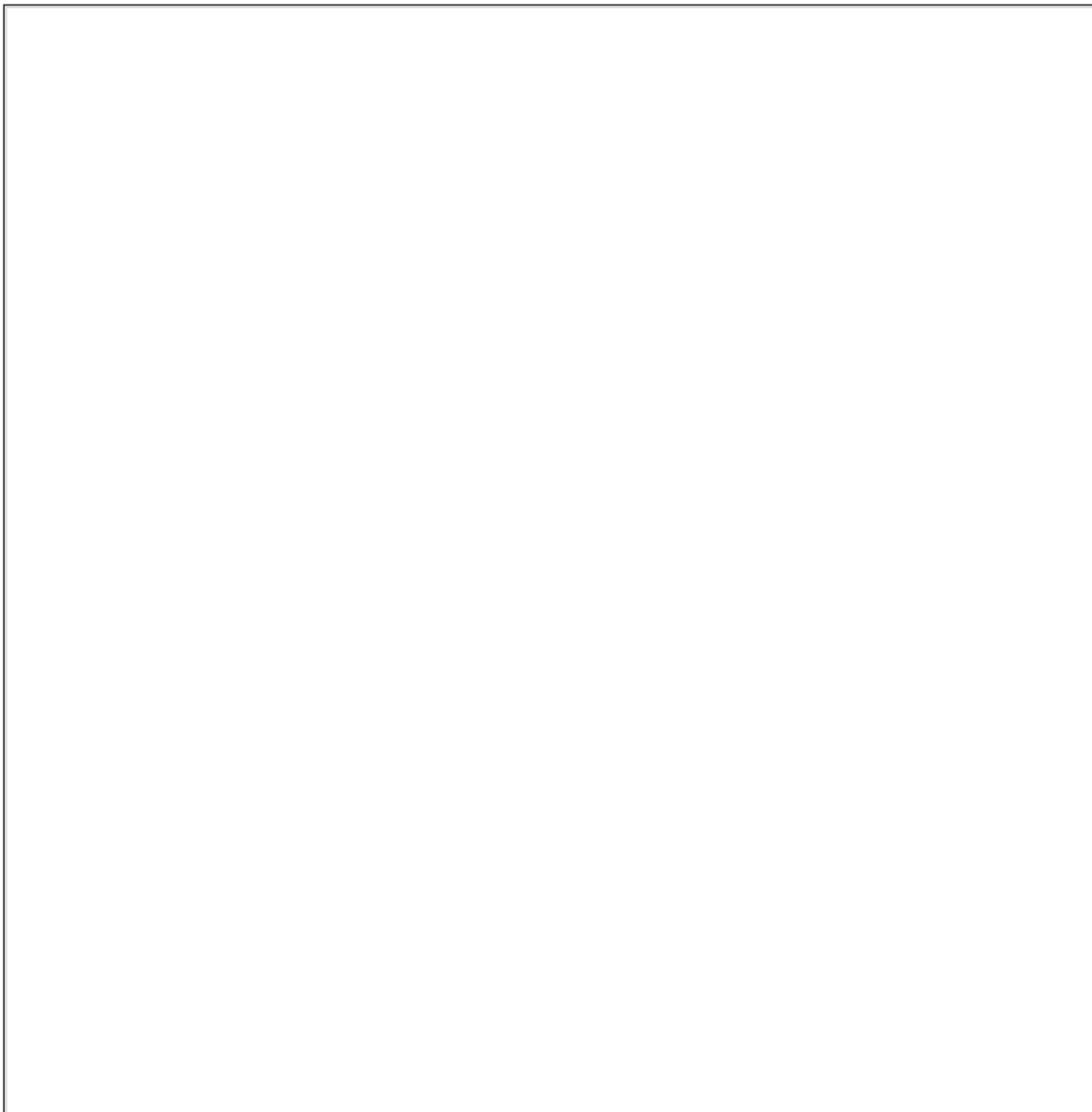
Methods that detect a few key polymorphic sequences to deduce an HLA type can sometimes assign an incorrect type if an unknown allele is present. These methods cannot detect novel alleles that are distinguished by sequences that are not present at the polymorphic sites tested. Furthermore problems arise with different interpretations of the data depending on the list of HLA sequences used for assignment of HLA types (detailed earlier). This circumstance makes it difficult to compare typings

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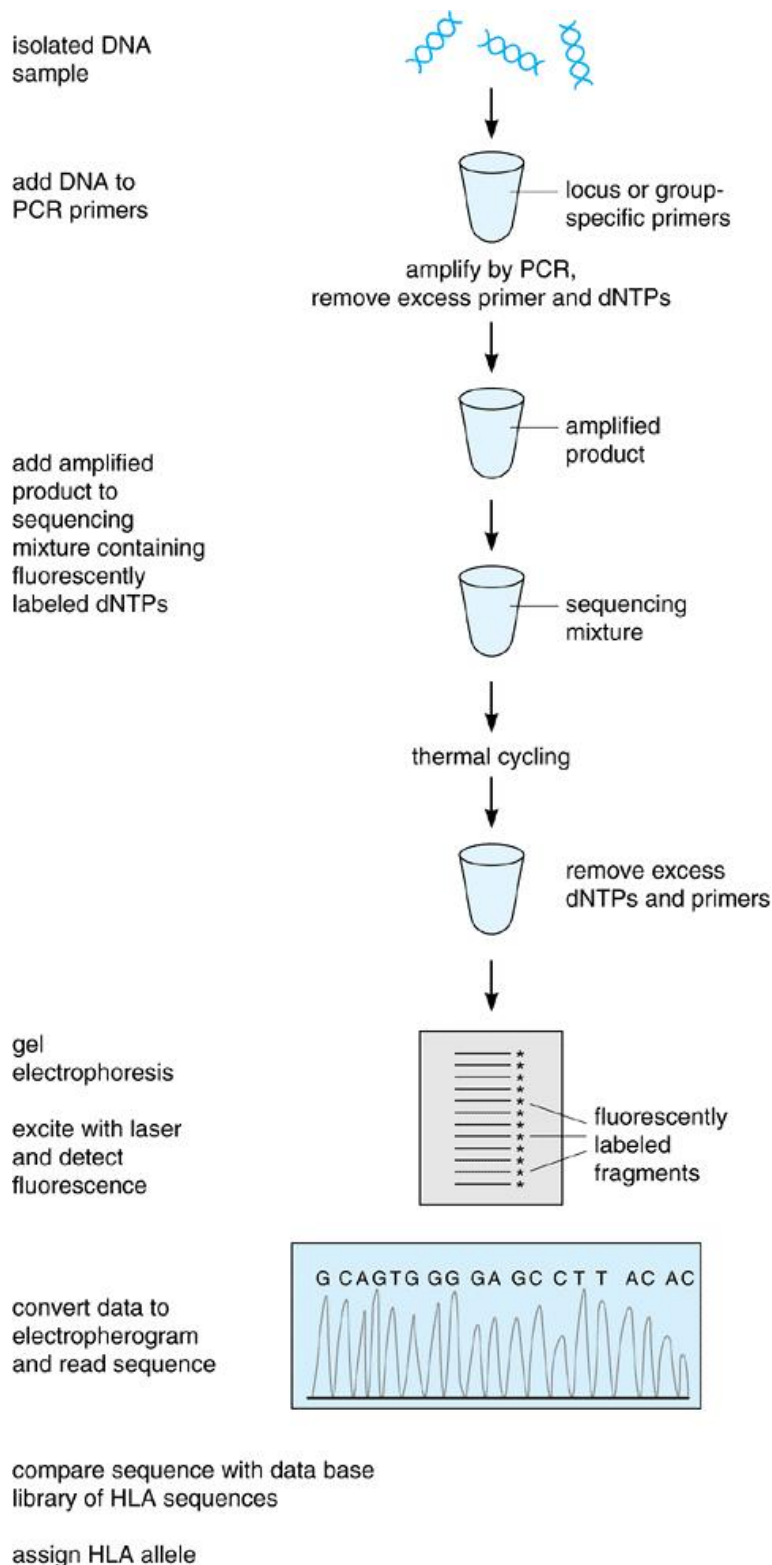
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performed at different dates. In addition, the extrapolation from limited sequence data to a type can sometimes cause assignment of different types depending on the method and reagents used for the typing.







**Figure 19-9.** Automated DNA sequence-based HLA typing using dye-terminator chemistry. DNA is isolated from a cell or tissue sample and amplified by PCR, using locus-, group-, or allele-specific primers. The amplified product is distributed into four tubes, each

one a polymerase, dNTPs, and sequencing mixtures. Each of the four sequencing mixtures is labeled with a fluorescently tagged sequence terminator: tube 1 with labeled cytosine (C\*), tube 2 with labeled guanine (G\*), tube 3 with labeled thymine (T\*), and tube 4 with labeled adenine (A\*). The product is amplified by PCR to incorporate the labeled terminators. The four sample tubes are pooled and electrophoresed to separate primer extension products according to size. The sequencer detects the fluorescence in each band and software converts the data into nucleotide sequences. Software compares the sequence to the library of HLA sequences and assigns the HLA type.

A major limitation that affects typing of heterozygous amplicons is that multiple interpretations of the data are possible for certain combinations of alleles. This situation can be resolved by performing the typing on selectively amplified alleles. Sometimes ambiguities can be resolved by using a combination of data from two methods (eg, SSOP and SSP or SBT and SSP).

Sometimes novel alleles that are distinguished by different patterns of SSOP or SSP data can be missed if the data are consistent with the presence of known alleles. A major limitation is that interpretation of the data is influenced by the library of sequences used to interpret the data.

SBT, which typically determines the sequence of a segment representing substantial portions of the HLA gene, is less susceptible to these problems. If the sequence is determined for a selectively amplified allele, the sequence is precise. Because most laboratories do not determine the sequence for the entire HLA gene, polymorphism outside the sequenced region is not detected. Many laboratories perform SBT using heterozygous templates (eg, two HLA-A alleles that are coamplified). These data are typically interpreted by comparing the determined sequence with the sequences of all possible combinations of known alleles. Sometimes these results are ambiguous (ie, multiple interpretations of the sequence data of the heterozygous data). If the library of HLA sequences is increased in number, the number of ambiguities or alternative interpretations of the data increases.

### ***Other Molecular Testing Methods***

Molecular HLA typing per se may not be required when only a preliminary assessment of HLA identity is desired, (eg, multiple donors are available for unrelated bone marrow transplantation).

### **Heteroduplex Methods**

Heteroduplex formation is a rapid method that uses the property that denatured DNA strands reanneal into heteroduplexes (ie, to form a double helix in alternative combinations that are less than perfectly matched at all base pairs). If the individuals are genetically disparate for HLA alleles, mismatching at the polymorphic bases modifies the bending or increases the superhelical diameter of the DNA and causes a retardation in its electrophoretic mobility (see Chapter 15). When denatured DNA from two genetically disparate individuals is mixed, novel heteroduplexes form, generating new band patterns in electrophoresis. Thus, the genetic identity between two individuals can be quickly assessed by mixing PCR DNA from their HLA genes. Alternatively, a reference DNA for a single allele or a synthetic universal heteroduplex generator (UHG) molecule can be added to a PCR sample. This forms heteroduplex complexes that are unique for each allele, thus generating unique diagnostic bands and, that can be used to assign an HLA type.

### **Chimerism Testing**

Posttransplant monitoring of hematopoietic stem cell transplants includes testing to monitor the engraftment process. Increasing use of nonmyeloablative conditioning regimens has stimulated use of chimerism testing to evaluate the relative proportions of donor cells after transplant. Historically, engraftment was detected using serologic methods to detect donor-specific HLA

molecules. Today, chimerism testing is usually accomplished using molecular methods. If HLA disparities exist between donor and recipient, a test can be designed to quantify the relative amounts of HLA genes that are unique to donor and recipient (ie, the HLA mismatches). If the donor and recipient have different gender, Y chromosome-specific sequences can be detected using in situ hybridization (Chapter 18) or amplification-based methods. Because these approaches are applicable to only a subset of patients, most centers use tests that detect other polymorphic loci that can be used for all donor-recipient pairs (including HLA and gender-identical pairs). These tests discriminate between donor and recipient cells using highly polymorphic loci that have polymorphism in the number of tandemly repeated sequences. One type of target, termed variable number tandem repeats (VNTR), typically detects repeats of 30–100 bp. Another type, termed short tandem repeats (STR), detects repeats of 2–6 bp.

Usually a large number of STR or VNTR loci are examined for each donor–recipient pair, and the most informative (donor and recipient-specific alleles that are easily resolved) loci are selected for testing. The relative amount of donor-and-patient-specific alleles are determined to monitor engraftment. The methods used for this analysis vary substantially in sensitivity, which typically is between 1 and 10%, but can be as low as 50% for certain methods. Some methods are quantitative and others are qualitative.

## CELLULAR ASSAYS FOR HISTOCOMPATIBILITY

In vivo, recognition of nonself antigens and destruction of cells bearing such markers is accomplished by cells of the immune system. Some of the clinically relevant HLA molecules that can trigger the immune response are not readily detected by the serologic methods discussed previously. Instead, lymphocytes are used as discriminatory reagents to detect histoincompatibility between donor and recipient.

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Cellular tests include the MLC (described in Chapter 16), the CML (described in Chapter 16), primed lymphocyte typing (PLT), and measurement of cytotoxic T cell precursors (CTLp) and helper T cell precursors (HTLp). In general, these tests are cumbersome and expensive, most laboratories have replaced cellular tests with high-resolution molecular typing.

For many years the MLC was considered to be an in vitro model for detecting histoincompatibility that is not revealed by serologic typing methods. Several studies involving bone marrow transplantation have failed to show a significant association between MLC reactivity and rejection or GvHD. For blood or marrow transplants, most centers have replaced MLC tests with high-resolution molecular HLA typing. The MLC continues to be used by some centers to monitor induction of tolerance following hematopoietic stem cell and solid organ transplants. The PLT test involves use of homozygous typing cells in an MLC to determine the type of the responder cell. This test is rarely used today because it has been supplanted by molecular class II HLA typing.

Limiting dilution analysis is used to measure CTLp (measurement of cytotoxicity or interferon secretion) and HTLp (measurement of IL-2 secretion). The clinical value of CTLp and HTLp tests, which have been investigated in the bone marrow transplant setting, remains controversial. It has been suggested that this test may be most useful to T-cell-depleted transplants.

**Table 19-9. Serologic and cellular methods used in histocompatibility testing.**

Test	Test Type and Components	Time	Application

<b>Tissue typing</b> Complement-dependent lymphocytotoxicity	Serologic (HLA, antisera; complement; test cells)	3 h	Identification of class I and II HLA antigens.
<b>Crossmatching</b> PBL crossmatch TB-cell crossmatch MLC test CML test FCC Autocrossmatch	Serologic (recipient serum; donor cells; complement; AHG optional) Serologic (purified donor T or B cells; recipient serum; AHG optional with T cells) Cellular (donor and recipient cells combined in tissue culture) Cellular (patient cells from primary MLC; fresh donor stimulators as targets) Serologic (patient serum; donor cells; fluorescent antihuman immunoglobulin) Serologic (patient PBL, T and B cells, and serum) and FCC.	3 h 3-6 h 6 d 4 h 3-4 h 3-4 h	Detection of preformed antidonor antibodies in patient serum. T cells: detection of antidonor class I HLA antibodies; B cells: detection of antibodies to class I and II HLA. Class II HLA antigen compatibility. Detection of anti-donor CTL. Detection of very weak and non-cytotoxic antidonor antibodies. Detection of nonspecific antilymphocyte antibodies (autoantibodies).
<b>Screening</b> Screening for class I HLA antibodies Screening for class II HLA antibodies	Serologic (patient serum; panel of HLA-typed T cells or PBL) Serologic (patient serum absorbed; B-cell panel typed for HLA-DR, DQ)	3 h $\bar{A}$ — 60 cells 4 h $\bar{A}$ — no. of cells plus absorption time	Detection of class I HLA antibodies; identification of antibody specificity. Detection of class II HLA antibodies; identification of antibody specificity.
<p><b>Abbreviations:</b> PBL = peripheral blood lymphocyte; MLC = mixed leukocyte culture; CML = cell-mediated lympholysis test; FCC = flow cytometry crossmatch; HLA = human leukocyte antigen; AHG = antigen to human serum globulins; CTL = cytotoxic T lymphocyte.</p>			

A summary of the serologic and cellular methods for histocompatibility testing is presented in

Table 19-9. These methods are in current use and are accepted as appropriate (in some instances mandatory) procedures for clinical histocompatibility testing.

### ***HLA Typing for Allogenic Transplantation***

The routine selection of tests for histocompatibility testing is summarized in Table 19-2. HLA typing currently plays an important role in selection of compatible donors for hematopoietic stem cell transplants. In general, HLA matching is associated with decreased rates of rejection and GvHD and increased survival. Large studies reported in 1998 and 1999 suggest that there are advantages for matching HLA-A, -B, -C, -DR, -DQ, and -DP at a high-resolution level. Nevertheless, issues related to the relative importance of mismatching for each locus as well as permissible HLA disparities are unresolved. Other histocompatibility tests (eg, cellular assays and crossmatching) are sometimes used.

In general, the association between HLA matching and solid organ transplants is influenced by the organ(s) transplanted and the transplant protocol. In renal

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transplantation numerous reports show that HLA matching (low resolution) is associated with increased graft and patient survival. Evidence for an anti-class I antibody determined using positive T-cell lymphocytotoxicity crossmatch is a contraindication for transplant. Other more sensitive crossmatch tests (flow cytometry) or B-cell crossmatches are subject to practices at individual transplant centers. At the other extreme, there is little support for histocompatibility in liver transplantation, and testing is usually minimal.

A positive effect of HLA matching on kidney graft outcome has been clearly documented in reports from the two largest studies of renal transplant data: the UCLA Transplant Registry, Los Angeles, California, which has collected data on 106,000 transplants, and the Collaborative Transplant Study (CTS), Heidelberg, Germany, with data on 107,500 renal transplants. Both of these studies agree that the main factor that improves long-term (up to 10-year) renal allograft survival is donor-recipient matching for HLA antigens (Table 19-10). Table 19-10 indicates the decreasing graft survival rates obtained in primary renal transplants when the donor is HLA-identical (sibling), half identical (parent), and completely mismatched and unrelated (cadaver).

Beneficial effects of HLA matching on short-term graft survival (1 year) are no longer apparent in the results from many individual transplant centers. Immunosuppression with cyclosporine has improved first-transplant graft survival of cadaver and living related transplants to near that of HLA-identical transplants: approximately 80-85% after 1 year.

Matching for the splits (subtypes) of HLA broad antigens may be even more significant for graft outcome than simple matching of the "generic" HLA antigens (eg, matching for B51 or B52 rather than for the broad B5 antigen). As shown in Table 19-11, from a CTS review of 33,000 transplants, matching for A and B locus antigen splits in conjunction with HLA-DR shows a striking correlation with graft survival. Table 19-11 shows percent graft survival for patients with 0, 3, and 6 mismatches for HLA-A, -B, and -DR antigens and the estimated half-life survival time for those grafts. Well-matched grafts (zero mismatches) survive approximately 60% longer than do completely mismatched (six mismatches) grafts (half-life of 12.3 and 7.5 years, respectively). Corroborative data is provided by the UCLA Transplant Registry showing graft half-lives of 20.3, 8.4, and 7.7 years for zero, three to four, and five to six antigen mismatches, respectively.

**Table 19-10. Effect of HLA matching on long-term renal allograft survival.**

Organ Donor	Number of Haplotypes Matched <sup>b</sup>	% Graft Survival (10 year)	Transplant Half-Life (years)

HLA-identical sibling	2	74	24
Parent	1	54	12
Cadaver <sup>a</sup>	0	40	9

Source: Data from Terasaki PI (editor): *Clinical Transplants 1992*. UCLA Tissue Typing Laboratory, 1993, p. 501.

<sup>a</sup>Recipient treated with cyclosporine.

<sup>b</sup>N = 40,765 transplants.

**Table 19-11. Effect of HLA-A, -B, and -DR mismatches on primary renal graft survival.<sup>a</sup>**

Number of Mismatches	Estimated 10-year Graft Survival(%)		Half-Life of Graft (years)	
	Study 1	Study 2	Study 1	Study 2
0	53	65	12.3	20.3
1&"2	"	47	"	10.4
3&"4	42	38	9.4	8.4
5&"6	32	32	7.5	7.7

Sources: Study 1 data from G. Opelz: *Collaborative Transplant Study*, Newsletter personal communication, May 1992; Study 2 data from Zhou & Cecka: in *Clinical Transplants*, 1993 (see References).

<sup>a</sup>Matching for split HLA-A and -B locus antigens.

Despite the dramatic improvement in 1-year survival, however, the ensuing rate of graft loss due to chronic rejection remains essentially unchanged; that is, half of cadaver grafts are still lost by 9 years, compared with 7.3 years in 1978. Thus, the use of cyclosporine has not established an operational state of long-term organ tolerance. It is estimated that if all kidneys were shared nationally, 25% of all waiting patients could be transplanted with kidneys with no HLA-A, -B, or -DR mismatches. These statistics argue in favor of sharing organs on a regional and national scale to promote the most beneficial usage of scarce organ resources. To achieve this end, the National Organ Transplant Act of 1987 established the United Network for Organ Sharing (UNOS). UNOS links local and regional transplant procurement centers with a national

registry of waiting recipients and establishes mandatory criteria for selection of recipients based on a point system for the following attributes: quality of HLA matching, degree of sensitization (panel-reactive antibody [PRA]), time on the waiting list, medical emergency status, and geographic factors.

In heart transplantation, distribution of hearts based on HLA matching is impractical because of the lack of availability of the organ. Retrospective analysis indicates that HLA matching offers a benefit to graft survival, although there are few well-matched grafts to evaluate. For those prospective heart recipients who are sensitized to HLA antigens, a pretransplant crossmatch is routinely performed using preorgan-harvest donor blood where possible to minimize ischemia time of the heart.

For liver transplantation, better HLA-matched livers are associated with fewer rejection episodes, but, paradoxically, liver graft survival results show no advantage from HLA matching and, possibly, a detrimental

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effect. Those patients who suffer recurrence of an autoimmune-type disease in the new, well-matched liver grafts presumably express the autoantigen better with shared rather than with disparate HLA antigens, thereby encouraging renewed disease. Some liver patients are transplanted despite positive crossmatches, whereas others receive no pretransplant testing. No consensus has been reached regarding the utility of crossmatching liver patients prior to transplant.

Data on over 2100 pancreas plus kidney transplants from the UNOS transplant registry indicates the beneficial effect of matching for HLA antigens. Patients with technically successful transplants who were mismatched for zero or one HLA antigens had significantly better ( $p < 0.05$ ) 5-year graft survival than did those mismatched for from two to six HLA antigens. Preliminary data for cornea transplantation show that patients with previously rejected transplants benefit from a well-matched transplant.

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## 20

# Laboratory Evaluation of Immune Competence

Clifford Lowell MD, PhD

The integrity of the human immune system depends on a complex interplay of cells (lymphocytes, monocytes, neutrophils), secreted factors (immunoglobulins, cytokines, chemokines), and serum proteins (complement, acute phase reactants). Defects in the production or function of any of these components can result in impairments that range from catastrophic immunodeficiency to subtle increases in the frequency of infections with specific classes of organisms.

The indications for testing immune competence are listed in Table 20-1. Of these the most important is suspected immunodeficiency. Clinical clues to the presence of an immunodeficiency syndrome include (1) increased frequency of infections, (2) failure to clear infections rapidly despite adequate therapy, (3) dissemination of local infections to distant sites, (4) occurrence of opportunistic infections, and (5) development of certain types of cancer (eg, Kaposi's sarcoma in AIDS patients).

The clinical presentation or initial test results may strongly suggest a certain syndrome or defect, allowing the physician to focus on a particular aspect of the immune system. In other cases, the evaluation proceeds by examining the four major components of the immune system (1) B cells (humoral immunity) (2) T cells (cellular immunity) (3) phagocyte function, and (4) complement components.

<b>Table 20-1. Indications for laboratory testing for immune competence.</b>

**Clinical diagnosis, therapeutic monitoring, or prognosis of<sup>a</sup>:**  
 Congenital and acquired immunodeficiency diseases (see Chapters 20,21,22,23,24,25,46)

Immune reconstitution following bone marrow or other lymphoid tissue grafts (see Chapter 52)

Immunosuppression induced by drugs, radiation, or other means (see Chapter 53) for transplant rejection, cancer treatment, or autoimmune diseases

Autoimmune disorders as a possible adjunct to diagnosis (rarely useful) or to monitor therapy

Immunization to monitor efficacy or immune status

Clinical or basic research

<sup>a</sup>Tests must be interpreted in the clinical context, particularly in conjunction with a thorough history and physical examination.

## VARIABLES & LIMITATIONS IN IMMUNOLOGIC TESTING

The results of immunologic tests should always be interpreted within the context of the clinical history and presentation. A number of limitations warrant emphasis. Results of tests of cellular immunity can vary between different laboratories for technical reasons. There also can be biologic causes for variability. Genetic polymorphisms, gender, age, and environmental influences affect normal immune function, sometimes rendering it difficult to assess the clinical significance of differences in test results between individuals. Intercurrent infections, exacerbation of autoimmunity, and medications can affect the evaluation of immune competence. For example, nitroblue tetrazolium (NBT) slide or 2,7-dichlorofluorescein (DCF) flow cytometric testing for **chronic granulomatous disease (GCD)** (see Chapter 16) should not be performed in patients who have severe ongoing infections because many of the polymorphonuclear neutrophils (PMNs) in these patients are already fully active or have degranulated. These PMNs will fail to respond to further stimulation in the tests, giving the misleading impression that the patient has a primary defect in PMN function. Nearly all the simple assays of immune function use cells or serum isolated from the peripheral blood, even though the blood is not the most common site of immunologic activity. Immune responses ongoing in lymph nodes, the spleen, or even the bone marrow may not be reflected by changes in the blood. Finally,

bear in mind that the presence of normal numbers of cells or normal levels of immunoglobulins does not exclude the possibility of immune dysfunction. A normal serum IgG level does not necessarily mean that the patient's antibody repertoire is adequate to recognize all pathogens. The same is true for T-cell responses. Functional assays can help to address this problem, but our inability to

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fully evaluate the immunologic repertoire of individual patients is perhaps our greatest limitation in the assessment of the immune system.

## ASSESSMENT OF IMMUNOLOGIC COMPETENCE

A brief summary of one approach to evaluating the basic elements of the immune system is described in the following steps. Most of this discussion focuses on the evaluation and diagnosis of an immunodeficiency, but some of the individual tests described play a role in diagnostic work-ups in other diseases. More complete descriptions of testing procedures and methods are provided in Chapters 15 and 16.

### *Initial Evaluation*

The patient's age, general clinical history, history of infections, and findings on physical examination help guide the evaluation of immune competence. Identification of the specific types of pathogens to which a patient is susceptible can provide significant insight into the type of immunodeficiency involved. For example, patients with deficiencies in the late-acting components of the complement cascade are uniquely susceptible to *Neisseria* infections. Likewise, defects in T-cell function often manifest as susceptibility to viruses and intracellular pathogens.

The initial evaluation of childhood immunodeficiency should include chest radiographs to rule out thymic agenesis (especially if lymphopenia is present). Simple analyses, such as a complete blood count and morphologic examination, often are adequate to recognize major deficiencies in hematopoietic lineages or leukemia. Normal values for lymphocyte, monocyte, and neutrophil levels are very different in children versus adults; results must therefore be viewed in the context of age. True lymphopenia is seen in severe combined immunodeficiency (SCID), in common variable immunodeficiency (CVID), in major histocompatibility (MHC) class II deficiency (bare lymphocyte syndrome), and in X-linked agammaglobulinemia (XLA). In contrast, lymphocytosis may suggest X-linked lymphoproliferative syndrome (Duncan's syndrome) or malignancy. Leukocytosis (both lymphocytes and granulocytes) is a feature of the leukocyte adhesion deficiency (LAD) syndromes as well as hyperresponsive immune states. Abnormal appearing neutrophils are often seen in patients with chronic

granulomatous disease (CGD) and are a hallmark of Chédiak-Higashi syndrome. Abnormal platelet morphology is seen in Wiskott-Aldrich syndrome.

## ***Evaluation of Humoral Immunity***

Evaluation of virtually any abnormality in the immune system requires determination of immunoglobulin levels and subtypes. Indeed, the most common form of primary immunodeficiency, **selective IgA deficiency**, is readily recognized by low levels of this immunoglobulin, especially in mucosal secretions. XLA patients usually have IgG levels of less than 100 mg/dL unless studies are performed in the first few months of life when maternal IgG persists. Patients with **hyper-IgM syndrome** (congenital deficiency of CD40L) are recognized by the combination of extremely high levels of IgM and virtually absence of other immunoglobulin types. High IgE levels are seen in many allergic and hypersensitivity syndromes. **Paraproteins** (monoclonal immunoglobulins) can be indicative of malignancy, such as lymphoma or multiple myeloma. Consideration of the patient's age is critical in interpreting immunoglobulin levels because normal levels differ with age.

Specific testing for IgG subclasses (IgG1, IgG2, IgG3, or IgG4) may also be useful in some patients. Deficiency of certain subtypes, such as IgG2, may be associated with recurrent infections and an inability to respond to polysaccharide antigens. Clinical features of selective deficiency of specific IgG subclasses is reviewed in Chapter 21.

Immunophenotyping by flow cytometry is a key part of the evaluation of immune function. Surface staining for all leukocyte subsets is performed simultaneously. Alterations or defects in B-cells are revealed by staining for a host of B-cell-surface markers (Chapter 16).

Determining the levels of antibodies specific for particular antigens can provide an estimate of the ability of the individual to make a humoral immune response. One approach is to examine serum for the presence of antibodies directed against antigens to which the individual should have been exposed. For example, antibodies against tetanus and diphtheria toxoids should be present in individuals who have received the appropriate vaccinations. Alternatively, development of a specific humoral immune response can be examined directly by deliberate immunization with agents such as pneumococcal polysaccharide, capsular antigens of *Haemophilus influenzae*, and keyhole limpet hemocyanin (KLH). Serum is collected at 2- to 3-week intervals and specific antibody titers measured by ELISA assay.

Antibody function can also be assessed by measurement of naturally occurring isohemagglutinins. These IgM antibodies are directed against microbial polysaccharides but cross-react with the human A and B

antigens present on red blood cells. Although isohemagglutinin titers usually rise with age, testing is still useful in infants, because newborns can make IgM, and maternally derived IgG does not interfere with measurement of isohemagglutinins.

Additional assessments of humoral immunity include in vitro proliferation and immunoglobulin production by B cells in response to mitogenic stimulation. A variety of agents can be used, most of which nonspecifically activate B cells (eg, **bacterial lipo-polysaccharide [LPS]**

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or infection with Epstein-Barr virus). Often, patients with CVID manifest defects in Ig production in these in vitro assays.

## ***Evaluation of Cellular Immune Function***

The simplest evaluation of cellular (T-cell) immunity is enumeration of T-cell numbers and subtypes by mAb staining and flow cytometry. A large number of mAbs that recognize and distinguish immature, mature, resting, and activated T cells have been developed. Using this methodology, T-cell deficiency is easily recognized in diseases such as SCID and AIDS. Indeed, the quantitation of CD4 T cells is widely used to monitor disease progression in AIDS and the response to therapy.

The major in vivo functional assay for evaluation of T-cell responses is **delayed-type hypersensitivity (DTH) testing**. The ability to mount DTH responses to intradermally injected antigens depends on the ability of antigen-specific, memory T cells to secrete the appropriate cytokines and chemokines to initiate mononuclear infiltration (see Chapter 16).

Responses are assessed to test antigens to which the patient either has been immunized or should have been exposed (see Table 16-2). Lack of response to a wide range of antigens suggests a T-cell defect. The reliability of the test is influenced by several factors (Chapter 16), including the antigen used, injection technique, medication, and the age of the patient. DTH reactions are often minimal in young infants because of lack of exposure to the test antigen.

The simplest in vitro analysis of T-cell function is the measurement of proliferation induced with nonspecific **mitogenic lectins**, such as phytohemagglutinin (PHA) or concanavalin A (Con A). Proliferative responses are measured by incorporation of radiolabeled nucleotides into DNA or by other means (Chapter 16). Patients with defects in signaling molecules (eg, forms of SCID lacking the tyrosine kinase ZAP-70) have reduced numbers of T cells that fail to proliferate in these assays.

Additional evaluation of lymphocyte responses in vitro may be indicated, such as tests for antigen-specific responses, cytolytic function, or cytokine responses.

## ***Evaluation of Phagocyte Function***

Assessment of phagocyte function is indicated in patients with chronic bacterial infections, repeated pneumonias, or abnormal blood counts. Like all assays of immune function, the first step is enumeration and marker analysis by staining for known surface antigens and flow cytometry. Certain markers are immediately informative for phagocytic defects, such as the absence of CD11b/CD18 ( $\alpha$ -M $\beta$ 2-integrin) in **leukocyte adhesion deficiency 1 (LAD1)**. In contrast to lymphocyte disorders, careful morphologic examination of myeloid cells is important—various **myelodysplastic syndromes**, **granule deficiencies** or disorders such as **Chédiak-Higashi syndrome** can be recognized by alterations in neutrophil morphology. Similarly, bone marrow biopsy and examination of myeloid precursors (as well as other hematopoietic elements) play a more central role in evaluation of phagocyte disorders than lymphocyte defects. In particular, defects in myeloid cell production, presenting as neutropenia, are recognized in this fashion. Defects in cytokine production or receptors (**granulocyte colony-stimulating factor [G-CSF] receptor**) are known causes of congenital neutropenia (**Kostmann's syndrome**). These patients have varying levels of peripheral blood neutropenia and an accumulation of immature myeloid forms in the marrow that are recognized morphologically and by flow cytometry. Histochemical staining is helpful in the recognition of granule disorders, such as **myeloperoxidase deficiency**. Of course, neutropenia due to malignancy (from either infiltration of the marrow by nonhematopoietic cells or as a result of leukemia) is also diagnosed by bone marrow biopsy and analysis.

A number of functional tests of myeloid cells are helpful in characterizing phagocyte defects (see Chapter 16). Of these, tests for superoxide production (to rule out CGD) and microbicidal function are central. CGD is caused by congenital deficiency of one of the subunits of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. As a result, myeloid cells (both neutrophils and macrophages) fail to undergo respiratory burst to produce  $O_2^-$  following activation. This respiratory burst is assayed by reduction of NBT (a simple screening test) or, more quantitatively, by the flow DCF assay. Bactericidal function is tested by quantitative assessment of killing of *Staphylococcus aureus* that has been opsonized with serum proteins (complement). Specific defects in fungicidal responses can also be evaluated. These functional methods result in abnormal tests if any one of the steps of bacterial phagocytosis and killing are defective. Usually these assays are performed on neutrophils, but in research protocols, monocyte-macrophage function can be separately determined.



## ***Evaluation of Complement Deficiencies***

Assays for complement function often are performed prior to extensive evaluation of lymphocyte and phagocyte function. Hereditary deficiencies leading to the complete absence of individual components of the classical pathway can be associated with a breakdown in host resistance to certain bacteria and with autoimmune disease (systemic lupus erythematosus and glomerulonephritis; Chapter 25). The CH50 (Chapter 15) is an excellent screening test for the hereditary deficiencies of the classical pathway, because detectable CH50 activity requires the presence of at least some of each component of the pathway. If there is detectable CH50 activity, then a homozygous deficiency in the classical pathway is excluded. When the clinical picture suggests a homozygous complement deficiency, a CH50 of zero should prompt evaluation of individual complement components. Note that a reduction in the CH50 to

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zero is not specific for homozygous deficiencies and can result from disease activity in lupus and other immune complex-mediated disorders.

## ***Subsequent Evaluation***

Results of initial testing guide the laboratory immunologist in deciding about subsequent testing. Research protocols tailored to the individual patient may be needed to define the defect leading to immunodeficiency. Alternatively, initial results may lead to testing for known mutations that result in immunodeficiency, such as mutation in the *BTK* gene (leading to XLA), the *WASP* gene (leading to Wiskott-Aldrich syndrome), or various kinase genes associated with SCID syndromes (*ZAP-70* or *JAK-3*). Indeed, as the molecular understanding of immunodeficiency improves, genetic test-ing may become a routine part of screening.

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