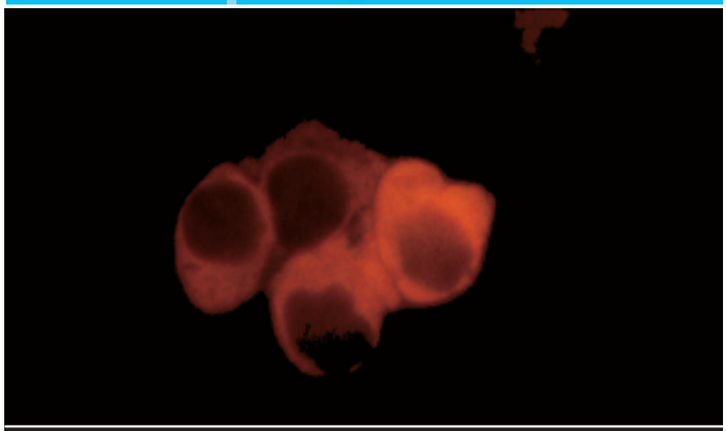
chapter 6

Antigen-Antibody Interactions:

Principles and Applications



Fluorescent Antibody Staining Reveals Intracellular Immunoglobin

THE ANTIGEN-ANTIBODY INTERACTION IS A BIMOlecular association similar to an enzyme-substrate interaction, with an important distinction: it does not lead to an irreversible chemical alteration in either the antibody or the antigen. The association between an antibody and an antigen involves various noncovalent interactions between the antigenic determinant, or epitope, of the antigen and the variable-region (V_H/V_L) domain of the antibody molecule, particularly the hypervariable regions, or complementarity-determining regions (CDRs). The exquisite specificity of antigen-antibody interactions has led to the development of a variety of immunologic assays, which can be used to detect the presence of either antibody or antigen. Immunoassays have played vital roles in diagnosing diseases, monitoring the level of the humoral immune response, and identifying molecules of biological or medical interest. These assays differ in their speed and sensitivity; some are strictly qualitative, others are quantitative. This chapter examines the nature of the antigen-antibody interaction, and it describes various immunologic assays that measure or exploit this interaction.

Strength of Antigen-Antibody Interactions

The noncovalent interactions that form the basis of antigen-antibody (Ag-Ab) binding include hydrogen bonds, ionic bonds, hydrophobic interactions, and van der Waals interactions (Figure 6-1). Because these interactions are individually weak (compared with a covalent bond), a large number of such interactions are required to form a strong Ag-Ab interaction. Furthermore, each of these noncovalent interactions operates over a very short distance, generally about 1×10^{-7} mm (1 angstrom, Å); consequently, a strong Ag-Ab interaction depends on a very close fit between the antigen and antibody. Such fits require a high degree of complementarity between antigen and antibody, a requirement that underlies the exquisite specificity that characterizes antigen-antibody interactions.

Antibody Affinity Is a Quantitative Measure of Binding Strength

The combined strength of the noncovalent interactions between a *single* antigen-binding site on an antibody and a *single* epitope is the **affinity** of the antibody for that epitope. Low-affinity antibodies bind antigen weakly and tend to dissociate readily, whereas high-affinity antibodies bind antigen more tightly and remain bound longer. The association between a binding site on an antibody (Ab) with a monovalent antigen (Ag) can be described by the equation

$$\text{Ag} + \text{Ab} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Ag-Ab}$$

VISUALIZING CONCEPTS

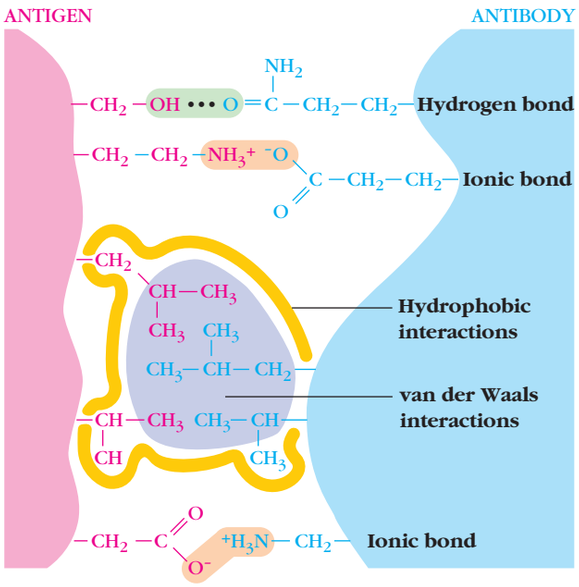


FIGURE 6-1 The interaction between an antibody and an antigen depends on four types of noncovalent forces: (1) hydrogen bonds, in which a hydrogen atom is shared between two electronegative atoms; (2) ionic bonds between oppositely charged residues; (3) hydrophobic interactions, in which water forces hydrophobic groups together; and (4) van der Waals interactions between the outer electron clouds of two or more atoms. In an aqueous environment, noncovalent interactions are extremely weak and depend upon close complementarity of the shapes of antibody and antigen.

where k_1 is the forward (association) rate constant and k_{-1} is the reverse (dissociation) rate constant. The ratio k_1/k_{-1} is the association constant K_a (i.e., $k_1/k_{-1} = K_a$), a measure of affinity. Because K_a is the equilibrium constant for the above reaction, it can be calculated from the ratio of the molar concentration of bound Ag-Ab complex to the molar concentrations of unbound antigen and antibody at equilibrium as follows:

$$K_a = \frac{[\text{Ag-Ab}]}{[\text{Ab}][\text{Ag}]}$$

The value of K_a varies for different Ag-Ab complexes and depends upon both k_1, which is expressed in units of liters/mole/second (L/mol/s), and k_{-1}, which is expressed in units of 1/second. For small haptens, the forward rate constant can be extremely high; in some cases, k_1 can be as high as 4×10^8 L/mol/s, approaching the theoretical upper limit of diffusion-limited reactions (10^9 L/mol/s). For larger protein antigens, however, k_1 is smaller, with values in the range of 10^5 L/mol/s.

The rate at which bound antigen leaves an antibody's binding site (i.e., the dissociation rate constant, k_{-1}) plays a major role in determining the antibody's affinity for an antigen. Table 6-1 illustrates the role of k_{-1} in determining the association constant K_a for several Ag-Ab interactions. For example, the k_1 for the DNP-L-lysine system is about one fifth that for the fluorescein system, but its k_{-1} is 200 times greater; consequently, the affinity of the antifluorescein antibody K_a for the fluorescein system is about 1000-fold higher than that of anti-DNP antibody. Low-affinity Ag-Ab complexes have K_a values between 10^4 and 10^5 L/mol; high-affinity complexes can have K_a values as high as 10^{11} L/mol.

For some purposes, the dissociation of the antigen-antibody complex is of interest:

$$\text{Ag-Ab} \rightleftharpoons \text{Ab} + \text{Ag}$$

The equilibrium constant for that reaction is K_d, the reciprocal of K_a

$$K_d = [\text{Ab}][\text{Ag}]/[\text{Ab-Ag}] = 1/K_a$$

and is a quantitative indicator of the stability of an Ag-Ab complex; very stable complexes have very low values of K_d, and less stable ones have higher values.

The affinity constant, K_a, can be determined by **equilibrium dialysis** or by various newer methods. Because equilibrium dialysis remains for many the standard against which

TABLE 6-1 Forward and reverse rate constants (k_1 and k_{-1}) and association and dissociation constants (K_a and K_d) for three ligand-antibody interactions

Antibody	Ligand	k_1	k_{-1}	K_a	K_d
Anti-DNP	ϵ-DNP-L-lysine	8×10^7	1	1×10^8	1×10^{-8}
Anti-fluorescein	Fluorescein	4×10^8	5×10^{-3}	1×10^{11}	1×10^{-11}
Anti-bovine serum albumin (BSA)	Dansyl-BSA	3×10^5	2×10^{-3}	1.7×10^8	5.9×10^{-9}

SOURCE: Adapted from H. N. Eisen, 1990, *Immunology,* 3rd ed., Harper & Row Publishers.

other methods are evaluated, it is described here. This procedure uses a dialysis chamber containing two equal compartments separated by a semipermeable membrane. Antibody is placed in one compartment, and a radioactively labeled ligand that is small enough to pass through the semipermeable membrane is placed in the other compartment (Figure 6-2). Suitable ligands include haptens, oligosaccharides, and oligopeptides. In the absence of antibody, ligand added to compartment B will equilibrate on both sides of the membrane (Figure 6-2a). In the presence of antibody, however, part of the labeled ligand will be bound to the antibody at equilibrium, trapping the ligand on the antibody side of the vessel, whereas unbound ligand will be equally distributed in both compartments. Thus the total concentration of ligand will be greater in the compartment containing antibody (Figure 6-2b). The difference in the ligand concentration in the two compartments represents the concentration of ligand bound to the antibody (i.e., the concentration of Ag-Ab complex). The higher the affinity of the antibody, the more ligand is bound.

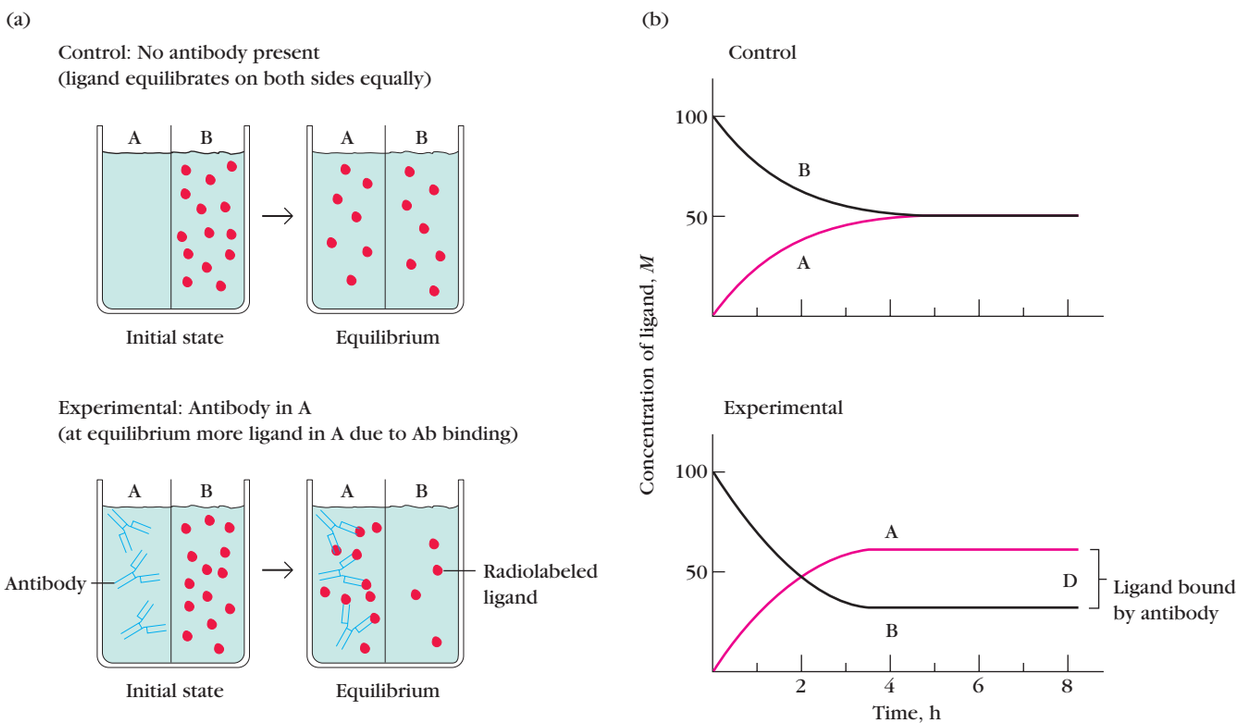


FIGURE 6-2 Determination of antibody affinity by equilibrium dialysis. (a) The dialysis chamber contains two compartments (A and B) separated by a semipermeable membrane. Antibody is added to one compartment and a radiolabeled ligand to another. At equilibrium, the concentration of radioactivity in both compartments is measured. (b) Plot of concentration of ligand in each compartment with time. At equilibrium, the difference in the concentration of radioactive ligand in the two compartments represents the amount of ligand bound to antibody.

Since the total concentration of antibody in the equilibrium dialysis chamber is known, the equilibrium equation can be rewritten as:

$$K_a = [\text{Ab-Ag}]/[\text{Ab}][\text{Ag}] = \frac{r}{c(n - r)}$$

where r equals the ratio of the concentration of bound ligand to total antibody concentration, c is the concentration of free ligand, and n is the number of binding sites per antibody molecule. This expression can be rearranged to give the **Scatchard equation:**

$$\frac{r}{c} = K_a n - K_a r$$

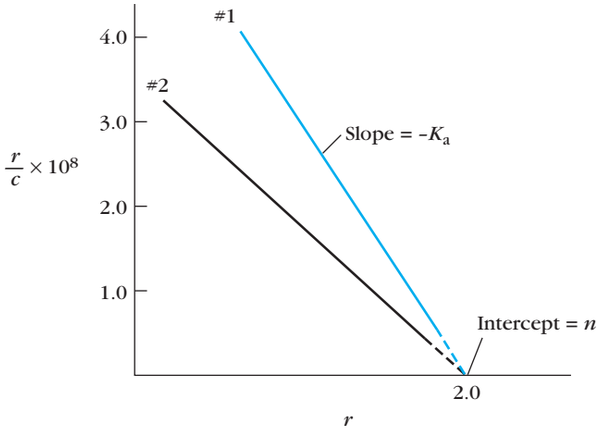
Values for r and c can be obtained by repeating the equilibrium dialysis with the same concentration of antibody but with different concentrations of ligand. If K_a is a constant, that is, if all the antibodies within the dialysis chamber have the same affinity for the ligand, then a Scatchard plot of r/c versus r will yield a straight line with a slope of $-K_a$ (Figure 6-3a). As the concentration of unbound ligand c increases, r/c approaches 0, and r approaches n, the **valency,** equal to the number of binding sites per antibody molecule.

Most antibody preparations are polyclonal, and K_a is therefore not a constant because a heterogeneous mixture of antibodies with a range of affinities is present. A Scatchard plot of heterogeneous antibody yields a curved line whose slope is constantly changing, reflecting this antibody heterogeneity (Figure 6-3b). With this type of Scatchard plot, it is possible to determine the average affinity constant, K_0, by determining the value of K_a when half of the antigen-binding sites are filled. This is conveniently done by determining the slope of the curve at the point where half of the antigen binding sites are filled.

Antibody Avidity Incorporates Affinity of Multiple Binding Sites

The affinity at one binding site does not always reflect the true strength of the antibody-antigen interaction. When complex antigens containing multiple, repeating antigenic determinants are mixed with antibodies containing multiple binding sites, the interaction of an antibody molecule with an antigen molecule at one site will increase the probability of reaction between those two molecules at a second site. The strength of such multiple interactions between a multivalent antibody and antigen is called the **avidity.** The avidity of an antibody is a better measure of its binding capacity within biological systems (e.g., the reaction of an antibody with antigenic determinants on a virus or bacterial cell) than the affinity of its individual binding sites. High avidity can compensate for low affinity. For example, secreted pentameric

(a) Homogeneous antibody



(b) Heterogeneous antibody

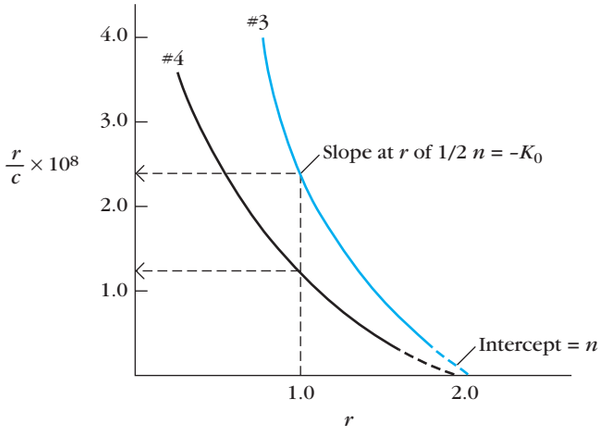


FIGURE 6-3 Scatchard plots are based on repeated equilibrium dialyses with a constant concentration of antibody and varying concentration of ligand. In these plots, r equals moles of bound ligand/mole antibody and c is the concentration of free ligand. From a Scatchard plot, both the equilibrium constant (K_a) and the number of binding sites per antibody molecule (n), or its valency, can be obtained. (a) If all antibodies have the same affinity, then a Scatchard plot yields a straight line with a slope of $-K_a$. The x intercept is n, the valency of the antibody, which is 2 for IgG and other divalent Igs. For IgM, which is pentameric, $n = 10$, and for dimeric IgA, $n = 4$. In this graph, antibody #1 has a higher affinity than antibody #2. (b) If the antibody preparation is polyclonal and has a range of affinities, a Scatchard plot yields a curved line whose slope is constantly changing. The average affinity constant K_0 can be calculated by determining the value of K_a when half of the binding sites are occupied (i.e., when $r = 1$ in this example). In this graph, antiserum #3 has a higher affinity ($K_0 = 2.4 \times 10^8$) than antiserum #4 ($K_0 = 1.25 \times 10^8$). Note that the curves shown in (a) and (b) are for divalent antibodies such as IgG.

IgM often has a lower affinity than IgG, but the high avidity of IgM, resulting from its higher valence, enables it to bind antigen effectively.

Cross-Reactivity

Although Ag-Ab reactions are highly specific, in some cases antibody elicited by one antigen can cross-react with an unrelated antigen. Such **cross-reactivity** occurs if two different antigens share an identical or very similar epitope. In the latter case, the antibody's affinity for the cross-reacting epitope is usually less than that for the original epitope.

Cross-reactivity is often observed among polysaccharide antigens that contain similar oligosaccharide residues. The **ABO blood-group antigens,** for example, are glycoproteins expressed on red blood cells. Subtle differences in the terminal residues of the sugars attached to these surface proteins distinguish the A and B blood-group antigens. An individual lacking one or both of these antigens will have serum antibodies to the missing antigen(s). The antibodies are induced not by exposure to red blood cell antigens but by exposure to cross-reacting microbial antigens present on common intestinal bacteria. These microbial antigens induce the formation of antibodies in individuals lacking the similar blood-group antigens on their red blood cells. (In individuals possessing these antigens, complementary antibodies would be eliminated during the developmental stage in which antibodies that recognize self epitopes are weeded out.) The blood-group antibodies, although elicited by microbial antigens, will cross-react with similar oligosaccharides on foreign red blood cells, providing the basis for blood typing tests and accounting for the necessity of compatible blood types during blood transfusions. A type A individual has anti-B antibodies; a type B individual has anti-A; and a type O individual thus has anti-A and anti-B (Table 6-2).

A number of viruses and bacteria have antigenic determinants identical or similar to normal host-cell components. In some cases, these microbial antigens have been shown to elicit antibody that cross-reacts with the host-cell components, resulting in a tissue-damaging autoimmune reaction. The bacterium *Streptococcus pyogenes,* for example, expresses cell-wall proteins called M antigens. Antibodies produced to streptococcal M antigens have been shown to cross-react with several myocardial and skeletal muscle proteins and have been implicated in heart and kidney damage following streptococcal infections. The role of other cross-reacting antigens in the development of autoimmune diseases is discussed in Chapter 20.

Some vaccines also exhibit cross-reactivity. For instance, vaccinia virus, which causes cowpox, expresses cross-reacting epitopes with variola virus, the causative agent of smallpox. This cross-reactivity was the basis of Jenner's method of using vaccinia virus to induce immunity to smallpox, as mentioned in Chapter 1.

TABLE 6-2 ABO blood types

Blood type	Antigens on RBCs	Serum antibodies
A	A	Anti-B
B	B	Anti-A
AB	A and B	Neither
O	Neither	Anti-A and anti-B

Precipitation Reactions

Antibody and soluble antigen interacting in aqueous solution form a lattice that eventually develops into a visible precipitate. Antibodies that aggregate soluble antigens are called **precipitins.** Although formation of the soluble Ag-Ab complex occurs within minutes, formation of the visible precipitate occurs more slowly and often takes a day or two to reach completion.

Formation of an Ag-Ab lattice depends on the valency of both the antibody and antigen:

- The antibody must be bivalent; a precipitate will not form with monovalent Fab fragments.
- The antigen must be either bivalent or polyvalent; that is, it must have at least two copies of the same epitope, or have different epitopes that react with different antibodies present in polyclonal antisera.

Experiments with myoglobin illustrate the requirement that protein antigens be bivalent or polyvalent for a precipitin reaction to occur. Myoglobin precipitates well with specific polyclonal antisera but fails to precipitate with a specific monoclonal antibody because it contains multiple, distinct epitopes but only a single copy of each epitope (Figure 6-4a). Myoglobin thus can form a crosslinked lattice structure with polyclonal antisera but not with monoclonal antisera. The principles that underlie precipitation reactions are presented because they are essential for an understanding of commonly used immunological assays. Although various modifications of the precipitation reaction were at one time the major types of assay used in immunology, they have been largely replaced by methods that are faster and, because they are far more sensitive, require only very small quantities of antigen or antibody. Also, these modern assay methods are not limited to antigen-antibody reactions that produce a precipitate. Table 6-3 presents a comparison of the *sensitivity,* or minimum amount of antibody detectable, by a number of immunoassays.

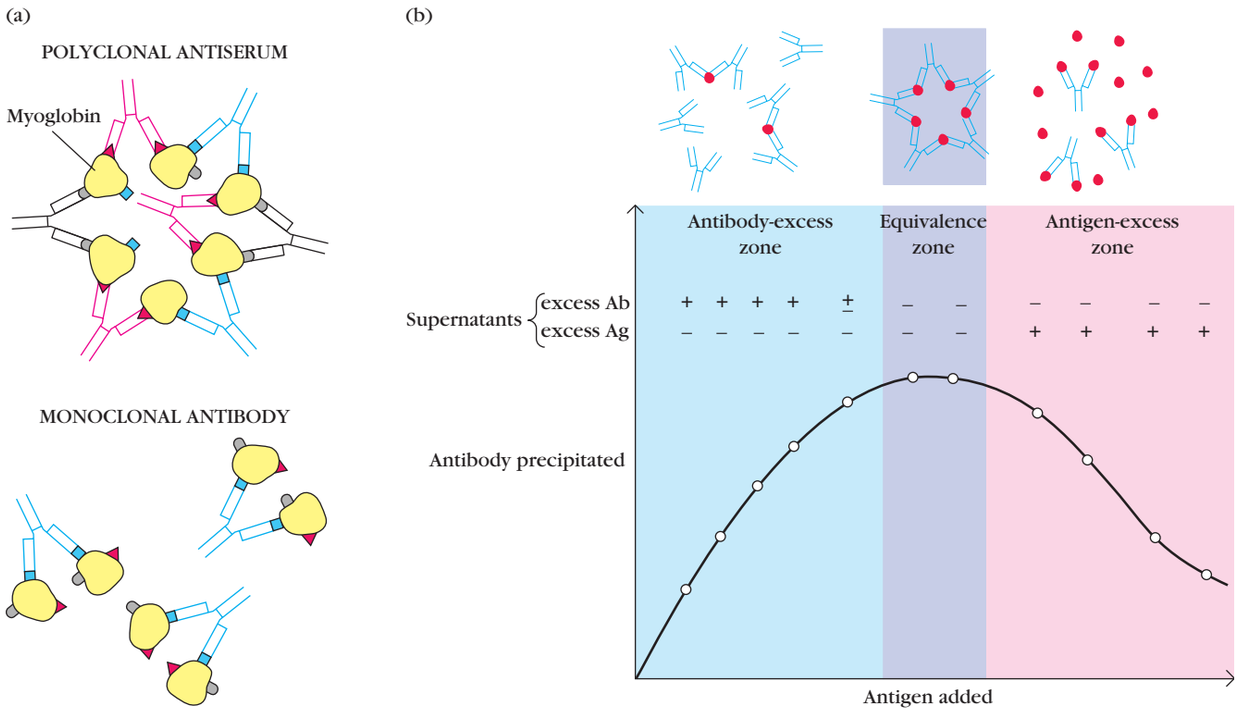


FIGURE 6-4 Precipitation reactions. (a) Polyclonal antibodies can form lattices, or large aggregates, that precipitate out of solution. However, if each antigen molecule contains only a single epitope recognized by a given monoclonal antibody, the antibody can link only two molecules of antigen and no precipitate is formed. (b) A precipitation curve for a system of one antigen and its antibodies. This plot of the amount of antibody precipitated versus increasing antigen concentrations (at constant total antibody) reveals three zones: a zone of antibody excess, in which precipitation is inhibited and antibody not bound to antigen can be detected in the supernatant; an equivalence zone of maximal precipitation in which antibody and antigen form large insoluble complexes and neither antibody nor antigen can be detected in the supernatant; and a zone of antigen excess in which precipitation is inhibited and antigen not bound to antibody can be detected in the supernatant.

Precipitation Reactions in Fluids Yield a Precipitin Curve

A quantitative precipitation reaction can be performed by placing a constant amount of antibody in a series of tubes and adding increasing amounts of antigen to the tubes. At one time this method was used to measure the amount of antigen or antibody present in a sample of interest. After the precipitate forms, each tube is centrifuged to pellet the precipitate, the supernatant is poured off, and the amount of precipitate is measured. Plotting the amount of precipitate against increasing antigen concentrations yields a precipitin curve. As Figure 6-4b shows, excess of either antibody or antigen interferes with maximal precipitation, which occurs in the so-called **equivalence zone,** within which the ratio of antibody to antigen is optimal. As a large multimolecular lattice is formed at equivalence, the complex increases in size and precipitates out of solution. As shown in Figure 6-4, under conditions of *antibody excess* or *antigen excess,* extensive lattices do not form and precipitation is inhibited. Although the quantitative precipitation reaction is seldom used experimentally today, the principles of antigen excess, antibody excess, and equivalence apply to many Ag-Ab reactions.

Precipitation Reactions in Gels Yield Visible Precipitin Lines

Immune precipitates can form not only in solution but also in an agar matrix. When antigen and antibody diffuse toward one another in agar, or when antibody is incorporated into the agar and antigen diffuses into the antibody-containing matrix, a visible line of precipitation will form. As in a precipitation reaction in fluid, visible precipitation occurs in the region of equivalence, whereas no visible precipitate forms in regions of antibody or antigen excess. Two types of *immunodiffusion reactions* can be used to determine relative concentrations of antibodies or antigens, to compare antigens, or to determine the relative purity of an antigen preparation. They are **radial immunodiffusion** (the Mancini method) and **double immunodiffusion** (the Ouchterlony method); both are carried out in a semisolid medium such as agar. In radial immunodiffusion, an antigen sample is placed in a well and allowed to diffuse into

TABLE 6-3 Sensitivity of various immunoassays

Assay	Sensitivity* (μg antibody/ml)
Precipitation reaction in fluids	20–200
Precipitation reactions in gels	
Mancini radial immunodiffusion	10–50
Ouchterlony double immunodiffusion	20–200
Immunoelectrophoresis	20–200
Rocket electrophoresis	2
Agglutination reactions	
Direct	0.3
Passive agglutination	0.006–0.06
Agglutination inhibition	0.006–0.06
Radioimmunoassay	0.0006–0.006
Enzyme-linked immunosorbent assay (ELISA)	<0.0001–0.01
ELISA using chemiluminescence	<0.0001–0.01†
Immunofluorescence	1.0
Flow cytometry	0.06–0.006

*The sensitivity depends upon the affinity of the antibody as well as the epitope density and distribution.

†Note that the sensitivity of chemiluminescence-based ELISA assays can be made to match that of RIA.

SOURCE: Adapted from N. R. Rose et al., eds., 1997, *Manual of Clinical Laboratory Immunology,* 5th ed., American Society for Microbiology, Washington, D.C.

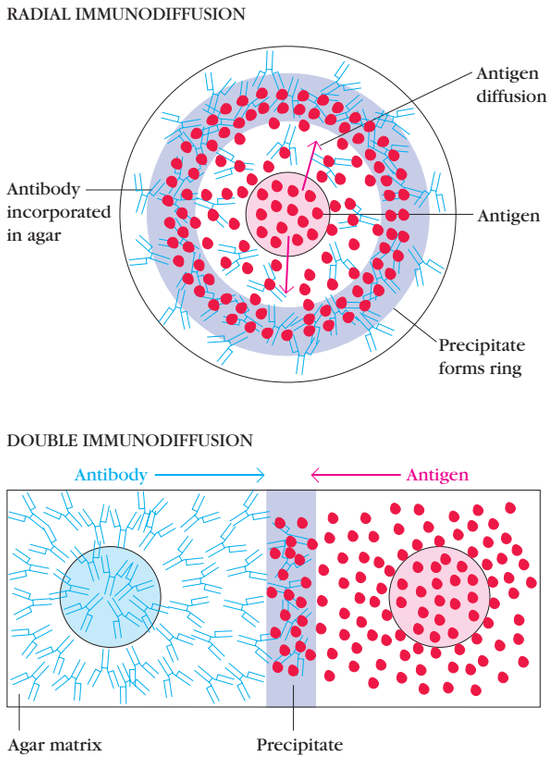


FIGURE 6-5 Diagrammatic representation of radial immunodiffusion (Mancini method) and double immunodiffusion (Ouchterlony method) in a gel. In both cases, large insoluble complexes form in the agar in the zone of equivalence, visible as lines of precipitation (purple regions). Only the antigen (red) diffuses in radial immunodiffusion, whereas both the antibody (blue) and antigen (red) diffuse in double immunodiffusion.

agar containing a suitable dilution of an antiserum. As the antigen diffuses into the agar, the region of equivalence is established and a ring of precipitation, a precipitin ring, forms around the well (Figure 6-5, upper panel). The area of the precipitin ring is proportional to the concentration of antigen. By comparing the area of the precipitin ring with a standard curve (obtained by measuring the precipitin areas of known concentrations of the antigen), the concentration of the antigen sample can be determined. In the Ouchterlony method, both antigen and antibody diffuse radially from wells toward each other, thereby establishing a concentration gradient. As equivalence is reached, a visible line of precipitation, a precipitin line, forms (Figure 6-5, lower panel).

Immunoelectrophoresis Combines Electrophoresis and Double Immunodiffusion

In **immunoelectrophoresis,** the antigen mixture is first electrophoresed to separate its components by charge. Troughs are then cut into the agar gel parallel to the direction of the electric field, and antiserum is added to the troughs. Antibody and antigen then diffuse toward each other and produce lines of precipitation where they meet in appropriate proportions (Figure 6-6a). Immunoelectrophoresis is used in clinical laboratories to detect the presence or absence of proteins in the serum. A sample of serum is electrophoresed, and the individual serum components are identified with antisera specific for a given protein or immunoglobulin class (Figure 6-6b). This technique is useful in determining whether a patient produces abnormally low amounts of one or more isotypes, characteristic of certain immunodeficiency diseases. It can also show whether a patient overproduces some serum protein, such as albumin, immunoglobulin, or transferrin. The immunoelectrophoretic pattern of serum from patients with multiple myeloma, for example, shows a heavy distorted arc caused by the large amount of myeloma protein, which is monoclonal Ig and therefore uniformly charged (Figure 6-6b). Because immunoelectrophoresis is a strictly *qualitative* technique that only detects relatively high antibody concentrations (greater than several hundred μg/ml), it utility is limited to the detection

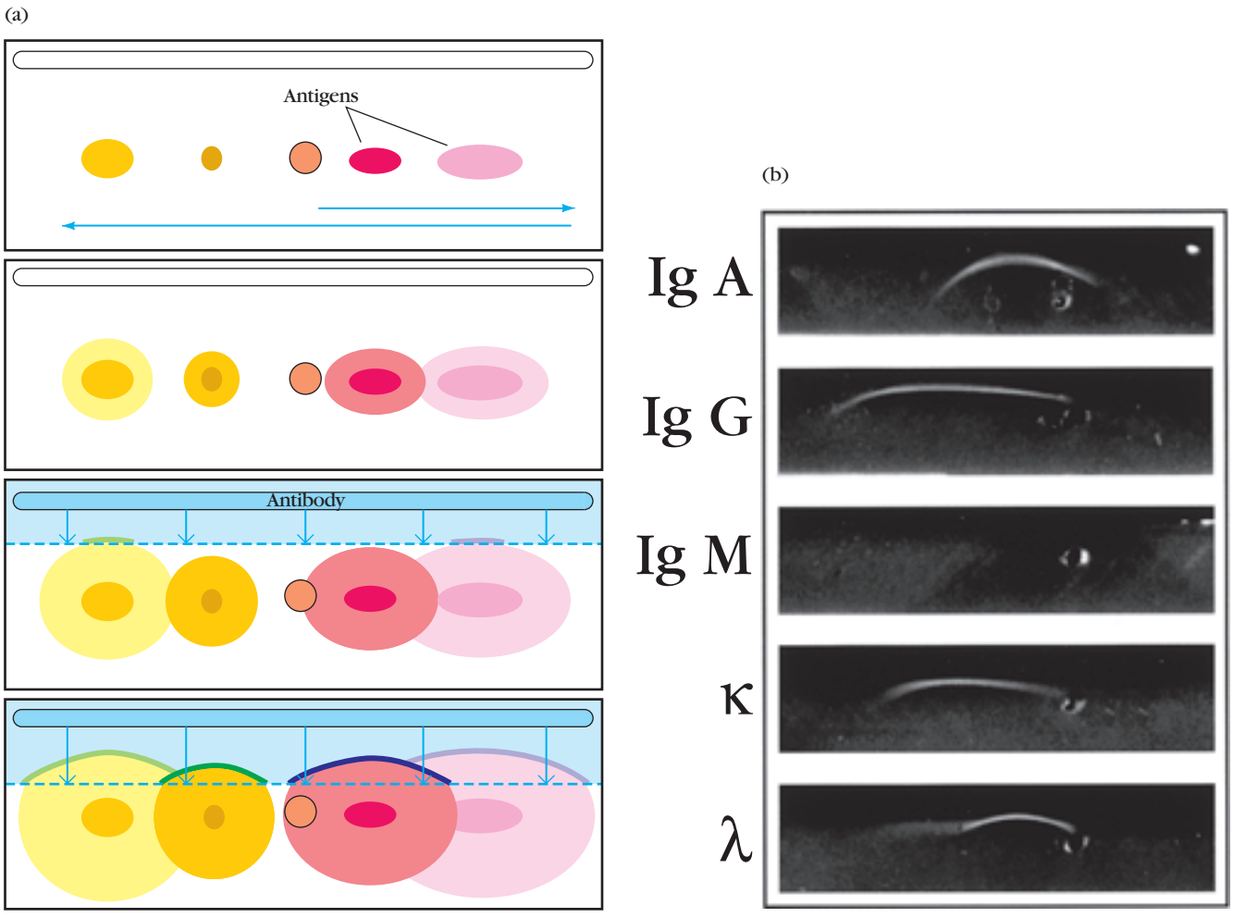


FIGURE 6-6 Immunoelectrophoresis of an antigen mixture. (a) An antigen preparation (orange) is first electrophoresed, which separates the component antigens on the basis of charge. Antiserum (blue) is then added to troughs on one or both sides of the separated antigens and allowed to diffuse; in time, lines of precipitation (colored arcs) form where specific antibody and antigen interact. (b) Immunoelectrophoretic patterns of human serum from a patient with myeloma. The patient produces a large amount of a monoclonal IgG (λ-light-chain-bearing) antibody. A sample of serum from the patient was placed in the well of the slide and electrophoresed. Then antiserum specific for the indicated antibody class or light chain type was placed in the top trough of each slide. At the concentrations of patient's serum used, only anti-IgG and anti-λ antibodies produced lines of precipitation. *[Part (b),* Robert A. Kyle and Terry A. Katzman, *Manual of Clinical Immunology, 1997, N. Rose, ed., ASM Press, Washington, D.C., p. 164.]*

of quantitative abnormalities only when the departure from normal is striking, as in immunodeficiency states and immunoproliferative disorders.

A related *quantitative* technique, **rocket electrophoresis,** does permit measurement of antigen levels. In rocket electrophoresis**,** a negatively charged antigen is electrophoresed in a gel containing antibody. The precipitate formed between antigen and antibody has the shape of a rocket, the height of which is proportional to the concentration of antigen in the well. One limitation of rocket electrophoresis is the need for the antigen to be negatively charged for electrophoretic movement within the agar matrix. Some proteins, immunoglobulins for example, are not sufficiently charged to be quantitatively analyzed by rocket electrophoresis; nor is it possible to measure the amounts of several antigens in a mixture at the same time.

Agglutination Reactions

The interaction between antibody and a particulate antigen results in visible clumping called **agglutination.** Antibodies that produce such reactions are called **agglutinins.** Agglutination reactions are similar in principle to precipitation reactions; they depend on the crosslinking of polyvalent antigens. Just as

an excess of antibody inhibits precipitation reactions, such excess can also inhibit agglutination reactions; this inhibition is called the **prozone effect.** Because prozone effects can be encountered in many types of immunoassays, understanding the basis of this phenomenon is of general importance.

Several mechanisms can cause the prozone effect. First, at high antibody concentrations, the number of antibody binding sites may greatly exceed the number of epitopes. As a result, most antibodies bind antigen only univalently instead of multivalently. Antibodies that bind univalently cannot crosslink one antigen to another. Prozone effects are readily diagnosed by performing the assay at a variety of antibody (or antigen) concentrations. As one dilutes to an optimum antibody concentration, one sees higher levels of agglutination or whatever parameter is measured in the assay being used. When one is using polyclonal antibodies, the prozone effect can also occur for another reason. The antiserum may contain high concentrations of antibodies that bind to the antigen but do not induce agglutination; these antibodies, called **incomplete antibodies,** are often of the IgG class. At high concentrations of IgG, incomplete antibodies may occupy most of the antigenic sites, thus blocking access by IgM, which is a good agglutinin. This effect is not seen with agglutinating monoclonal antibodies. The lack of agglutinating activity of an incomplete antibody may be due to restricted flexibility in the hinge region, making it difficult for the antibody to assume the required angle for optimal cross-linking of epitopes on two or more particulate antigens. Alternatively, the density of epitope distribution or the location of some epitopes in deep pockets of a particulate antigen may make it difficult for the antibodies specific for these epitopes to agglutinate certain particulate antigens. When feasible, the solution to both of these problems is to try different antibodies that may react with other epitopes of the antigen that do not present these limitations.

Hemagglutination Is Used in Blood Typing

Agglutination reactions (Figure 6-7) are routinely performed to type red blood cells (RBCs). In typing for the ABO antigens, RBCs are mixed on a slide with antisera to the A or B blood-group antigens. If the antigen is present on the cells, they agglutinate, forming a visible clump on the slide. Determination of which antigens are present on donor and recipient RBCs is the basis for matching blood types for transfusions.

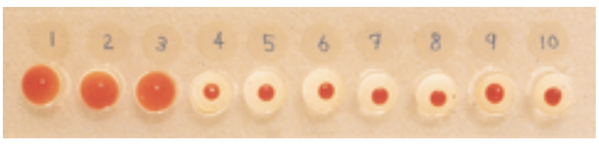


FIGURE 6-7 Demonstration of hemagglutination using antibodies against sheep red blood cells (SRBCs). The control tube (10) contains only SRBCs, which settle into a solid "button." The experimental tubes 1–9 contain a constant number of SRBCs plus serial two-fold dilutions of anti-SRBC serum. The spread pattern in the experimental series indicates positive hemagglutination through tube 3. *[Louisiana State University Medical Center/MIP. Courtesy of Harriet C. W. Thompson.]*

Bacterial Agglutination Is Used To Diagnose Infection

A bacterial infection often elicits the production of serum antibodies specific for surface antigens on the bacterial cells. The presence of such antibodies can be detected by bacterial agglutination reactions. Serum from a patient thought to be infected with a given bacterium is serially diluted in an array of tubes to which the bacteria is added. The last tube showing visible agglutination will reflect the serum antibody **titer** of the patient. The agglutinin titer is defined as the reciprocal of the greatest serum dilution that elicits a positive agglutination reaction. For example, if serial twofold dilutions of serum are prepared and if the dilution of 1/640 shows agglutination but the dilution of 1/1280 does not, then the agglutination titer of the patient's serum is 640. In some cases serum can be diluted up to 1/50,000 and still show agglutination of bacteria.

The agglutinin titer of an antiserum can be used to diagnose a bacterial infection. Patients with typhoid fever, for example, show a significant rise in the agglutination titer to *Salmonella typhi.* Agglutination reactions also provide a way to type bacteria. For instance, different species of the bacterium *Salmonella* can be distinguished by agglutination reactions with a panel of typing antisera.

Passive Agglutination Is Useful with Soluble Antigens

The sensitivity and simplicity of agglutination reactions can be extended to soluble antigens by the technique of passive hemagglutination. In this technique, antigen-coated red blood cells are prepared by mixing a soluble antigen with red blood cells that have been treated with tannic acid or chromium chloride, both of which promote adsorption of the antigen to the surface of the cells. Serum containing antibody is serially diluted into microtiter plate wells, and the antigen-coated red blood cells are then added to each well; agglutination is assessed by the size of the characteristic spread pattern of agglutinated red blood cells on the bottom of the well, like the pattern seen in agglutination reactions (see Figure 6-7).

Over the past several years, there has been a shift away from red blood cells to synthetic particles, such as latex beads, as matrices for agglutination reactions. Once the antigen has been coupled to the latex beads, the preparation can either be used immediately or stored for later use. The use of synthetic beads offers the advantages of consistency,

uniformity, and stability. Furthermore, agglutination reactions employing synthetic beads can be read rapidly, often within 3 to 5 minutes of mixing the beads with the test sample. Whether based on red blood cells or the more convenient and versatile synthetic beads, agglutination reactions are simple to perform, do not require expensive equipment, and can detect small amounts of antibody (concentrations as low as nanograms per milliliter).

In Agglutination Inhibition, Absence of Agglutination Is Diagnostic of Antigen

A modification of the agglutination reaction, called **agglutination inhibition,** provides a highly sensitive assay for small quantities of an antigen. For example, one of the early types of home pregnancy test kits included latex particles coated with human chorionic gonadotropin (HCG) and antibody to HCG (Figure 6-8). The addition of urine from a pregnant woman, which contained HCG, inhibited agglutination of the latex particles when the anti-HCG antibody was added; thus the absence of agglutination indicated pregnancy.

Agglutination inhibition assays can also be used to determine whether an individual is using certain types of illegal drugs, such as cocaine or heroin. A urine or blood sample is first incubated with antibody specific for the suspected drug. Then red blood cells (or other particles) coated with the drug are added. If the red blood cells are not agglutinated by the antibody, it indicates the sample contained an antigen recognized by the antibody, suggesting that the individual was

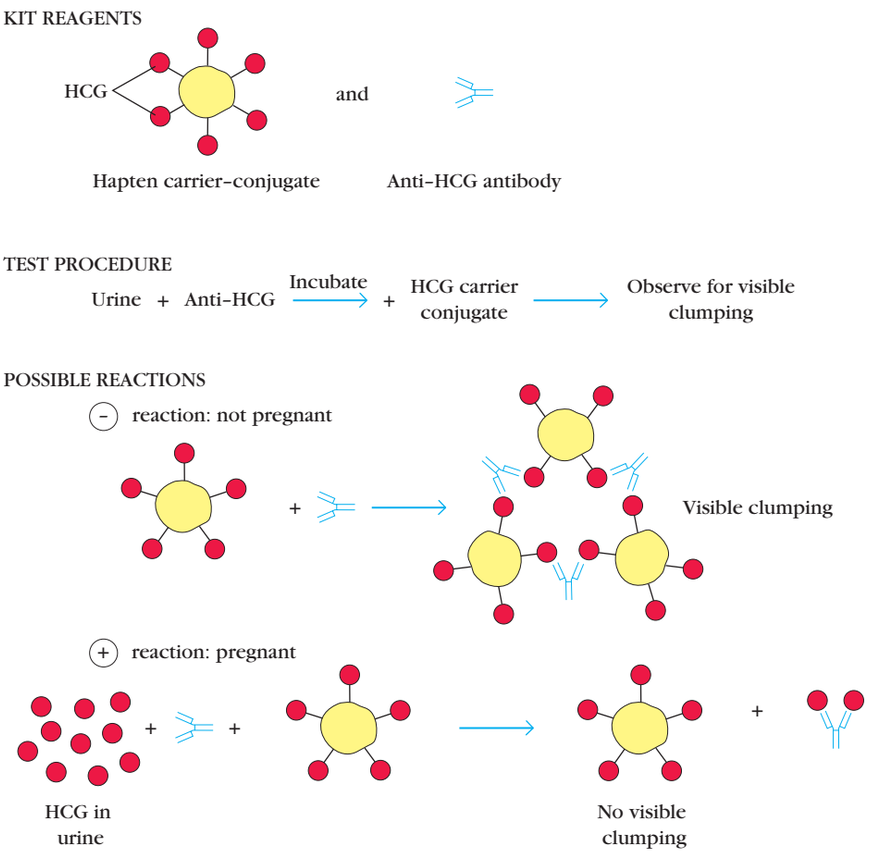


FIGURE 6-8 The original home pregnancy test kit employed hapten inhibition to determine the presence or absence of human chorionic gonadotropin (HCG). The original test kits used the presence or absence of visible clumping to determine whether HCG was present. If a woman was not pregnant, her urine would not contain HCG; in this case, the anti-HCG antibodies and HCG-carrier conjugate in the kit would react, producing visible clumping. If a woman was pregnant, the HCG in her urine would bind to the anti-HCG antibodies, thus inhibiting the subsequent binding of the antibody to the HCG-carrier conjugate. Because of this inhibition, no visible clumping occurred if a woman was pregnant. The kits currently on the market use ELISA-based assays (see Figure 6-10).

using the illicit drug. One problem with these tests is that some legal drugs have chemical structures similar to those of illicit drugs, and these legal drugs may cross-react with the antibody, giving a false-positive reaction. For this reason a positive reaction must be confirmed by a nonimmunologic method.

Agglutination inhibition assays are widely used in clinical laboratories to determine whether an individual has been exposed to certain types of viruses that cause agglutination of red blood cells. If an individual's serum contains specific antiviral antibodies, then the antibodies will bind to the virus and interfere with hemagglutination by the virus. This technique is commonly used in premarital testing to determine the immune status of women with respect to rubella virus. The reciprocal of the last serum dilution to show inhibition of rubella hemagglutination is the titer of the serum. A titer greater than 10 (1:10 dilution) indicates that a woman is immune to rubella, whereas a titer of less than 10 is indicative of a lack of immunity and the need for immunization with the rubella vaccine.

Radioimmunoassay

One of the most sensitive techniques for detecting antigen or antibody is **radioimmunoassay (RIA).** The technique was first developed in 1960 by two endocrinologists, S. A. Berson and Rosalyn Yalow, to determine levels of insulin–anti-insulin complexes in diabetics. Although their technique encountered some skepticism, it soon proved its value for measuring hormones, serum proteins, drugs, and vitamins at concentrations of 0.001 *micrograms* per milliliter or less. In 1977, some years after Berson's death, the significance of the technique was acknowledged by the award of a Nobel Prize to Yalow.

The principle of RIA involves competitive binding of radiolabeled antigen and unlabeled antigen to a high-affinity antibody. The labeled antigen is mixed with antibody at a concentration that saturates the antigen-binding sites of the antibody. Then test samples of unlabeled antigen of unknown concentration are added in progressively larger amounts. The antibody does not distinguish labeled from unlabeled antigen, so the two kinds of antigen compete for available binding sites on the antibody. As the concentration of unlabeled antigen increases, more labeled antigen will be displaced from the binding sites. The decrease in the amount of radiolabeled antigen bound to specific antibody in the presence of the test sample is measured in order to determine the amount of antigen present in the test sample.

The antigen is generally labeled with a gamma-emitting isotope such as ^{125}I, but beta-emitting isotopes such as tritium (^{3}H) are also routinely used as labels. The radiolabeled antigen is part of the assay mixture; the test sample may be a complex mixture, such as serum or other body fluids, that contains the unlabeled antigen. The first step in setting up an RIA is to determine the amount of antibody needed to bind 50%–70% of a fixed quantity of radioactive antigen (Ag^*) in the assay mixture. This ratio of antibody to Ag^* is chosen to ensure that the number of epitopes presented by the labeled antigen always exceeds the total number of antibody binding sites. Consequently, unlabeled antigen added to the sample mixture will compete with radiolabeled antigen for the limited supply of antibody. Even a small amount of unlabeled antigen added to the assay mixture of labeled antigen and antibody will cause a decrease in the amount of radioactive antigen bound, and this decrease will be proportional to the amount of unlabeled antigen added. To determine the amount of labeled antigen bound, the Ag-Ab complex is precipitated to separate it from free antigen (antigen not bound to Ab), and the radioactivity in the precipitate is measured. A standard curve can be generated using unlabeled antigen samples of known concentration (in place of the test sample), and from this plot the amount of antigen in the test mixture may be precisely determined.

Several methods have been developed for separating the bound antigen from the free antigen in RIA. One method involves precipitating the Ag-Ab complex with a secondary anti-isotype antiserum. For example, if the Ag-Ab complex contains rabbit IgG antibody, then goat anti-rabbit IgG will bind to the rabbit IgG and precipitate the complex. Another method makes use of the fact that protein A of *Staphylococcus aureus* has high affinity for IgG. If the Ag-Ab complex contains an IgG antibody, the complex can be precipitated by mixing with formalin-killed *S. aureus.* After removal of the complex by either of these methods, the amount of free labeled antigen remaining in the supernatant can be measured in a radiation counter; subtracting this value from the total amount of labeled antigen added yields the amount of labeled antigen bound.

Various solid-phase RIAs have been developed that make it easier to separate the Ag-Ab complex from the unbound antigen. In some cases, the antibody is covalently cross-linked to Sepharose beads. The amount of radiolabeled antigen bound to the beads can be measured after the beads have been centrifuged and washed. Alternatively, the antibody can be immobilized on polystyrene or polyvinylchloride wells and the amount of free labeled antigen in the supernatant can be determined in a radiation counter. In another approach, the antibody is immobilized on the walls of microtiter wells and the amount of bound antigen determined. Because the procedure requires only small amounts of sample and can be conducted in small 96-well microtiter plates (slightly larger than a 3×5 card), this procedure is well suited for determining the concentration of a particular antigen in large numbers of samples. For example, a microtiter RIA has been widely used to screen for the presence of the hepatitis B virus (Figure 6-9). RIA screening of donor blood has sharply reduced the incidence of hepatitis B infections in recipients of blood transfusions.

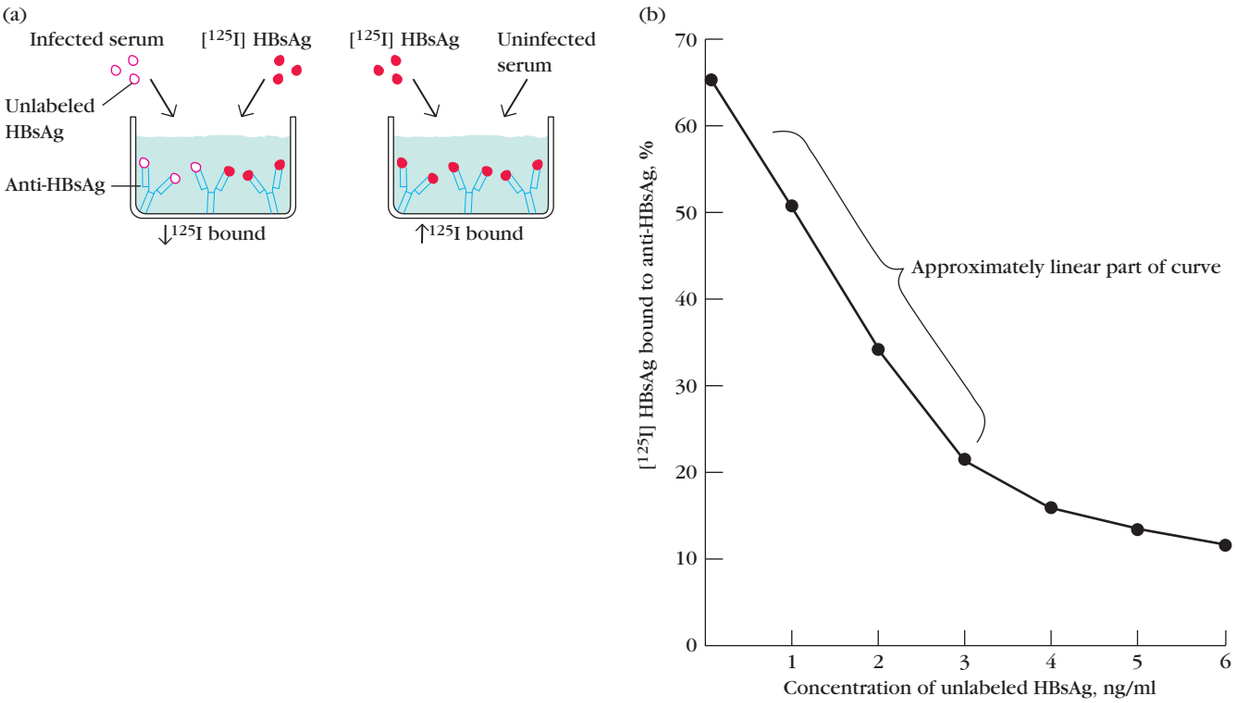


FIGURE 6-9 A solid-phase radioimmunoassay (RIA) to detect hepatitis B virus in blood samples. (a) Microtiter wells are coated with a constant amount of antibody specific for HBsAg, the surface antigen on hepatitis B virions. A serum sample and [^{125}I]HBsAg are then added. After incubation, the supernatant is removed and the radioactivity of the antigen-antibody complexes is measured. If the sample is infected, the amount of label bound will be less than in controls with uninfected serum. (b) A standard curve is obtained by adding increasing concentrations of unlabeled HBsAg to a fixed quantity of [^{125}I]HBsAg and specific antibody. From the plot of the percentage of labeled antigen bound versus the concentration of unlabeled antigen, the concentration of HBsAg in unknown serum samples can be determined by using the linear part of the curve.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay, commonly known as **ELISA** (or EIA), is similar in principle to RIA but depends on an enzyme rather than a radioactive label. An enzyme conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product. Such a substrate is called **a chromogenic substrate.** A number of enzymes have been employed for ELISA, including alkaline phosphatase, horseradish peroxidase, and β-galactosidase. These assays approach the sensitivity of RIAs and have the advantage of being safer and less costly.

There Are Numerous Variants of ELISA

A number of variations of ELISA have been developed, allowing qualitative detection or quantitative measurement of either antigen or antibody. Each type of ELISA can be used qualitatively to detect the presence of antibody or antigen. Alternatively, a standard curve based on known concentrations of antibody or antigen is prepared, from which the unknown concentration of a sample can be determined.

INDIRECT ELISA

Antibody can be detected or quantitatively determined with an indirect ELISA (Figure 6-10a). Serum or some other sample containing primary antibody (Ab_1) is added to an antigen-coated microtiter well and allowed to react with the antigen attached to the well. After any free Ab_1 is washed away, the presence of antibody bound to the antigen is detected by adding an enzyme-conjugated secondary anti-isotype antibody (Ab_2), which binds to the primary antibody. Any free Ab_2 then is washed away, and a substrate for the enzyme is added. The amount of colored reaction product that forms is measured by specialized spectrophotometric plate readers, which can measure the absorbance of all of the wells of a 96-well plate in seconds.

Indirect ELISA is the method of choice to detect the presence of serum antibodies against human immunodeficiency virus (HIV), the causative agent of AIDS. In this assay, recombinant envelope and core proteins of HIV are adsorbed

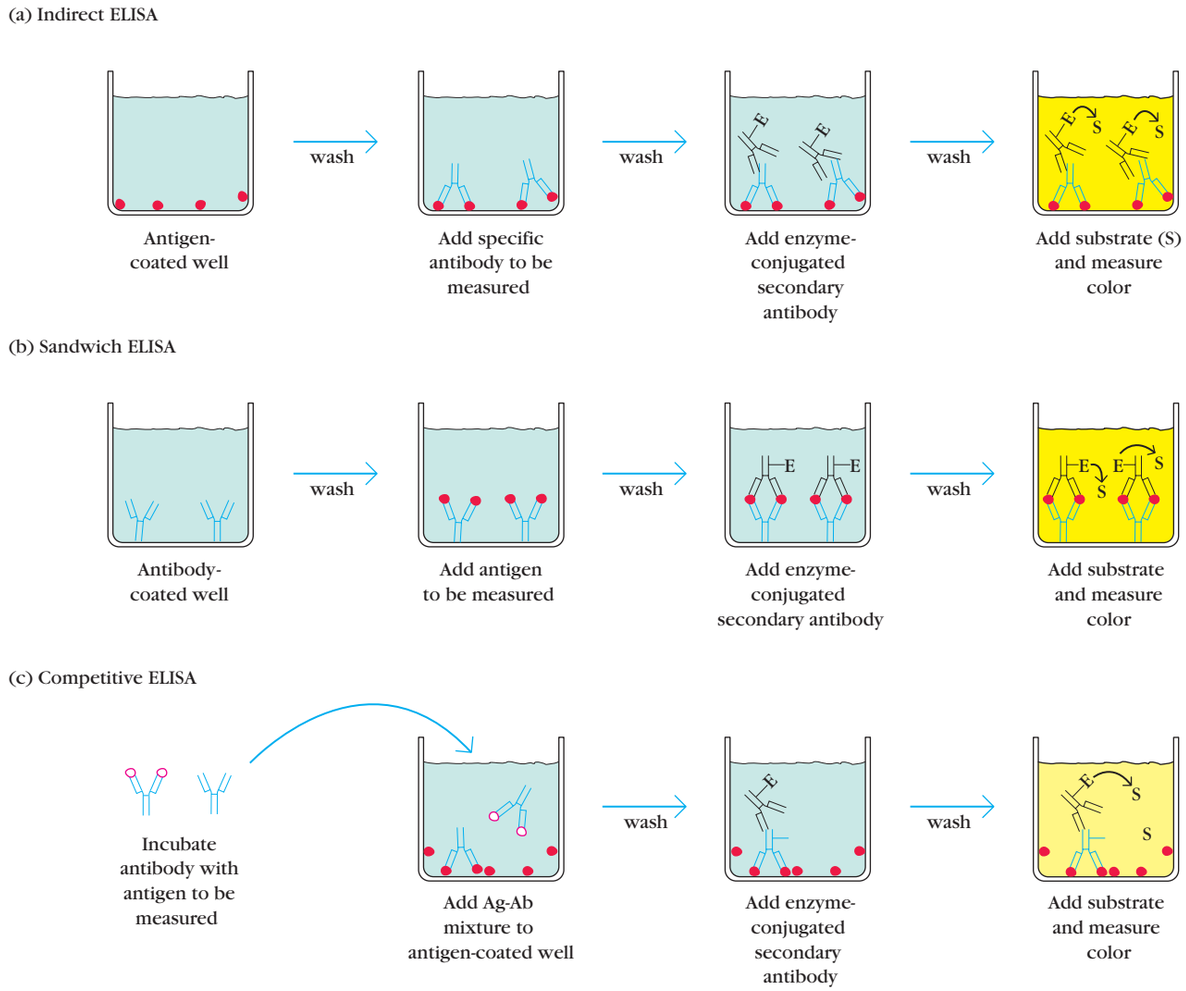


FIGURE 6-10 Variations in the enzyme-linked immunosorbent assay (ELISA) technique allow determination of antibody or antigen. Each assay can be used qualitatively, or quantitatively by comparison with standard curves prepared with known concentrations of antibody or antigen. Antibody can be determined with an indirect ELISA (a), whereas antigen can be determined with a sandwich ELISA (b) or competitive ELISA (c). In the competitive ELISA, which is an inhibition-type assay, the concentration of antigen is inversely proportional to the color produced.

as solid-phase antigens to microtiter wells. Individuals infected with HIV will produce serum antibodies to epitopes on these viral proteins. Generally, serum antibodies to HIV can be detected by indirect ELISA within 6 weeks of infection.

SANDWICH ELISA

Antigen can be detected or measured by a sandwich ELISA (Figure 6-10b). In this technique, the antibody (rather than the antigen) is immobilized on a microtiter well. A sample containing antigen is added and allowed to react with the immobilized antibody. After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen. After any free second antibody is removed by washing, substrate is added, and the colored reaction product is measured.

COMPETITIVE ELISA

Another variation for measuring amounts of antigen is competitive ELISA (Figure 6-10c). In this technique, antibody is first incubated in solution with a sample containing antigen. The antigen-antibody mixture is then added to an antigen-coated microtiter well. The more antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well. Addition of an enzyme-conjugated secondary antibody (Ab_2) specific for the isotype of the primary antibody can be used to determine the amount of primary antibody bound to the well as in an indirect ELISA. In the

competitive assay, however, the higher the concentration of antigen in the original sample, the lower the absorbance.

CHEMILUMINESCENCE

Measurement of light produced by chemiluminescence during certain chemical reactions provides a convenient and highly sensitive alternative to absorbance measurements in ELISA assays. In versions of the ELISA using chemiluminescence, a luxogenic (light-generating) substrate takes the place of the chromogenic substrate in conventional ELISA reactions. For example, oxidation of the compound luminol by H_2O_2 and the enzyme horseradish peroxidase (HRP) produces light:

$$\text{Ab-HRP} + \text{Ag} \longrightarrow \text{Ab-HRP-Ag} \xrightarrow{\text{luminol} + H_2O_2} \text{light}$$

The advantage of chemiluminescence assays over chromogenic ones is enhanced sensitivity. In general, the detection limit can be increased at least ten-fold by switching from a chromogenic to a luxogenic substrate, and with the addition of enhancing agents, more than 200-fold. In fact, under ideal conditions, as little as 5×10^{-18} moles (5 attomoles) of target antigen have been detected.

ELISPOT ASSAY

A modification of the ELISA assay called the ELISPOT assay allows the quantitative determination of the number of cells in a population that are producing antibodies specific for a given antigen or an antigen for which one has a specific antibody (Figure 6-11). In this approach, the plates are coated with the antigen (capture antigen) recognized by the antibody of interest or with the antibody (capture antibody) specific for the antigen whose production is being assayed. A suspension of the cell population under investigation is then added to the coated plates and incubated. The cells settle onto the surface of the plate, and secreted molecules reactive with the capture molecules are bound by the capture molecules in the vicinity of the secreting cells, producing a ring of antigen-antibody complexes around each cell that is producing the molecule of interest. The plate is then washed and an enzyme-linked antibody specific for the secreted antigen or specific for the species (e.g., goat anti-rabbit) of the secreted antibody is added and allowed to bind. Subsequent development of the assay by addition of a suitable chromogenic or chemiluminescence-producing substrate reveals the position of each antibody- or antigen-producing cell as a point of color or light.

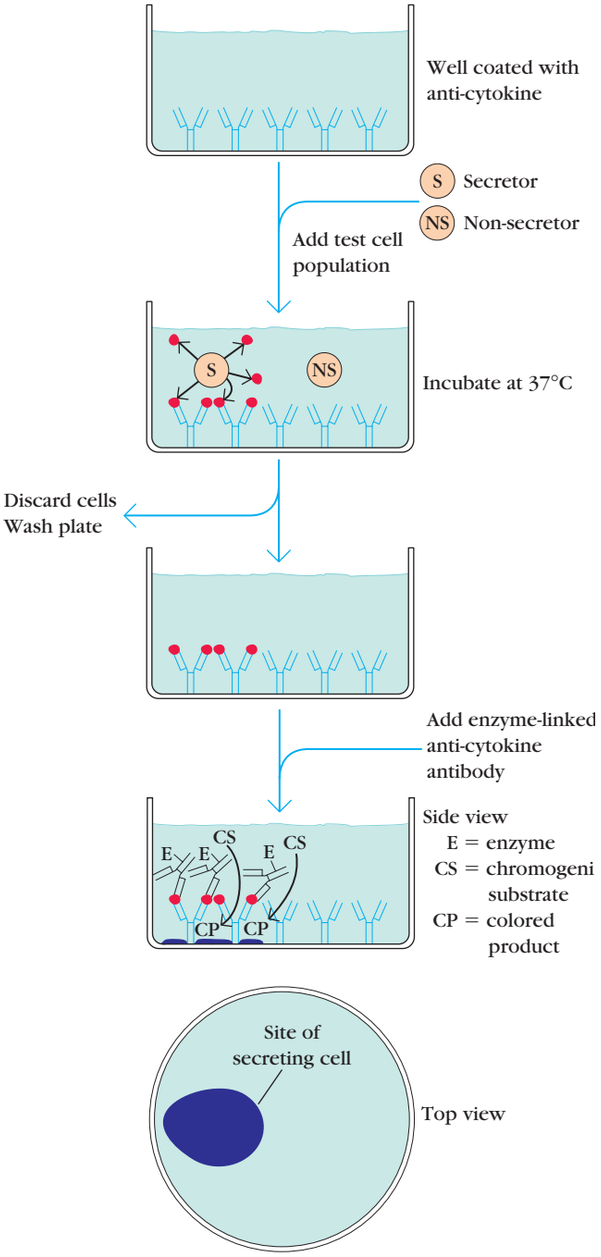


FIGURE 6-11 In the ELISPOT assay, a well is coated with antibody against the antigen of interest, a cytokine in this example, and then a suspension of a cell population thought to contain some members synthesizing and secreting the cytokine are layered onto the bottom of the well and incubated. Most of the cytokine molecules secreted by a particular cell react with nearby well-bound antibodies. After the incubation period, the well is washed and an enzyme-labeled anti-cytokine antibody is added. After washing away unbound antibody, a chromogenic substrate that forms an insoluble colored product is added. The colored product (purple) precipitates and forms a spot only on the areas of the well where cytokine-secreting cells had been deposited. By counting the number of colored spots, it is possible to determine how many cytokine-secreting cells were present in the added cell suspension.

Western Blotting

Identification of a specific protein in a complex mixture of proteins can be accomplished by a technique known as **Western blotting,** named for its similarity to Southern blotting,

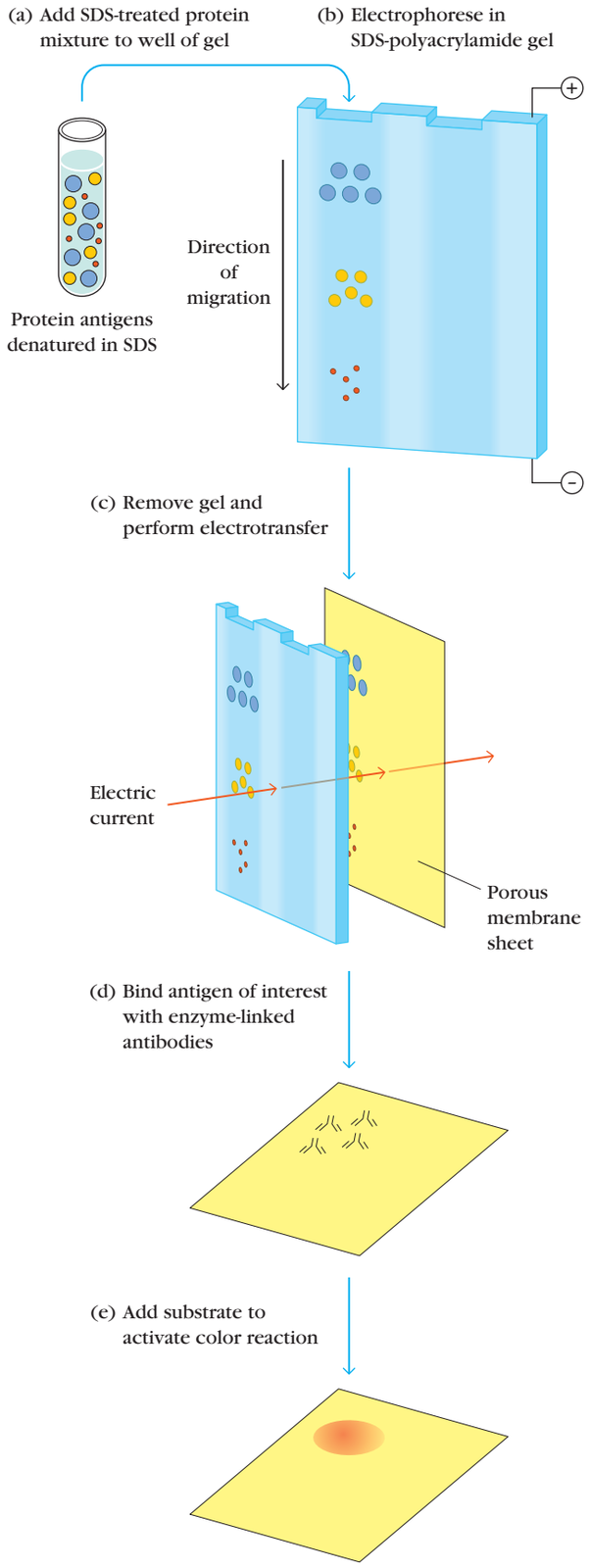


FIGURE 6-12 In Western blotting, a protein mixture is (a) treated with SDS, a strong denaturing detergent, (b) then separated by electrophoresis in an SDS polyacrylamide gel (SDS-PAGE) which separates the components according to their molecular weight; lower molecular weight components migrate farther than higher molecular weight ones. (c) The gel is removed from the apparatus and applied to a protein-binding sheet of nitrocellulose or nylon and the proteins in the gel are transferred to the sheet by the passage of an electric current. (d) Addition of enzyme-linked antibodies detects the antigen of interest, and (e) the position of the antibodies is visualized by means of an ELISA reaction that generates a highly colored insoluble product that is deposited at the site of the reaction. Alternatively, a chemiluminescent ELISA can be used to generate light that is readily detected by exposure of the blot to a piece of photographic film.

which detects DNA fragments, and Northern blotting, which detects mRNAs. In Western blotting, a protein mixture is electrophoretically separated on an **SDS-polyacrylamide gel (SDS-PAGE),** a slab gel infused with sodium dodecyl sulfate (SDS), a dissociating agent (Figure 6-12). The protein bands are transferred to a nylon membrane by electrophoresis and the individual protein bands are identified by flooding the nitrocellulose membrane with radiolabeled or enzyme-linked polyclonal or monoclonal antibody specific for the protein of interest. The Ag-Ab complexes that form on the band containing the protein recognized by the antibody can be visualized in a variety of ways. If the protein of interest was bound by a radioactive antibody, its position on the blot can be determined by exposing the membrane to a sheet of x-ray film, a procedure called autoradiography. However, the most generally used detection procedures employ enzyme-linked antibodies against the protein. After binding of the enzyme-antibody conjugate, addition of a chromogenic substrate that produces a highly colored and insoluble product causes the appearance of a colored band at the site of the target antigen. The site of the protein of interest can be determined with much higher sensitivity if a chemiluminescent compound along with suitable enhancing agents is used to produce light at the antigen site.

Western blotting can also identify a specific antibody in a mixture. In this case, known antigens of well-defined molecular weight are separated by SDS-PAGE and blotted onto nitrocellulose. The separated bands of known antigens are then probed with the sample suspected of containing antibody specific for one or more of these antigens. Reaction of an antibody with a band is detected by using either radiolabeled or enzyme-linked secondary antibody that is specific for the species of the antibodies in the test sample. The most widely used application of this procedure is in confirmatory testing for HIV, where Western blotting is used to determine whether the patient has antibodies that react with one or more viral proteins.

Immunoprecipitation

The immunoprecipitation technique has the advantage of allowing the isolation of the antigen of interest for further analysis. It also provides a sensitive assay for the presence of a particular antigen in a given cell or tissue type. An extract produced by disruption of cells or tissues is mixed with an antibody against the antigen of interest in order to form an antigen-antibody complex that will precipitate. However, if the antigen concentration is low (often the case in cell and tissue extracts), the assembly of antigen-antibody complexes into precipitates can take hours, even days, and it is difficult to isolate the small amount of immunoprecipitate that forms.

Fortunately, there are a number of ways to avoid these limitations. One is to attach the antibody to a solid support, such as a synthetic bead, which allows the antigen-antibody complex to be collected by centrifugation. Another is to add a secondary antibody specific for the primary antibody to bind the antigen-antibody complexes. If the secondary antibody is attached to a bead, the immune complexes can be collected by centrifugation. A particularly ingenious version of this procedure involves the coupling of the secondary antibody to magnetic beads. After the secondary antibody binds to the primary antibody, immunoprecipitates are collected by placing a magnet against the side of the tube (Figure 6-13).

When used in conjunction with biosynthetic radioisotope labeling, immunoprecipitation can also be used to determine whether a particular antigen is actually synthesized by a cell or tissue. Radiolabeling of proteins synthesized by cells of interest can be done by growing the cells in cell-culture medium containing one or more radiolabeled amino acids. Generally, the amino acids used for this application are those most resistant to metabolic modification, such as leucine, cysteine, or methionine. After growth in the radioactive medium, the cells are lysed and subjected to a primary antibody specific for the antigen of interest. The Ag-Ab complex is collected by immunoprecipitation, washed free of unincorporated radiolabeled amino acid and other impurities, and then analyzed. The complex can be counted in a scintillation counter to obtain a quantitative determination of the amount of the protein synthesized. Further analysis often involves disruption of the complex, usually by use of SDS and heat, so that the identity of the immunoprecipitated antigen can be confirmed by checking that its molecular weight is that expected for the antigen of interest. This is done by separation of the disrupted complex by SDS-PAGE and subsequent autoradiography to determine the position of the radiolabeled antigen on the gel.

Immunofluorescence

In 1944, Albert Coons showed that antibodies could be labeled with molecules that have the property of fluorescence. Fluorescent molecules absorb light of one wavelength

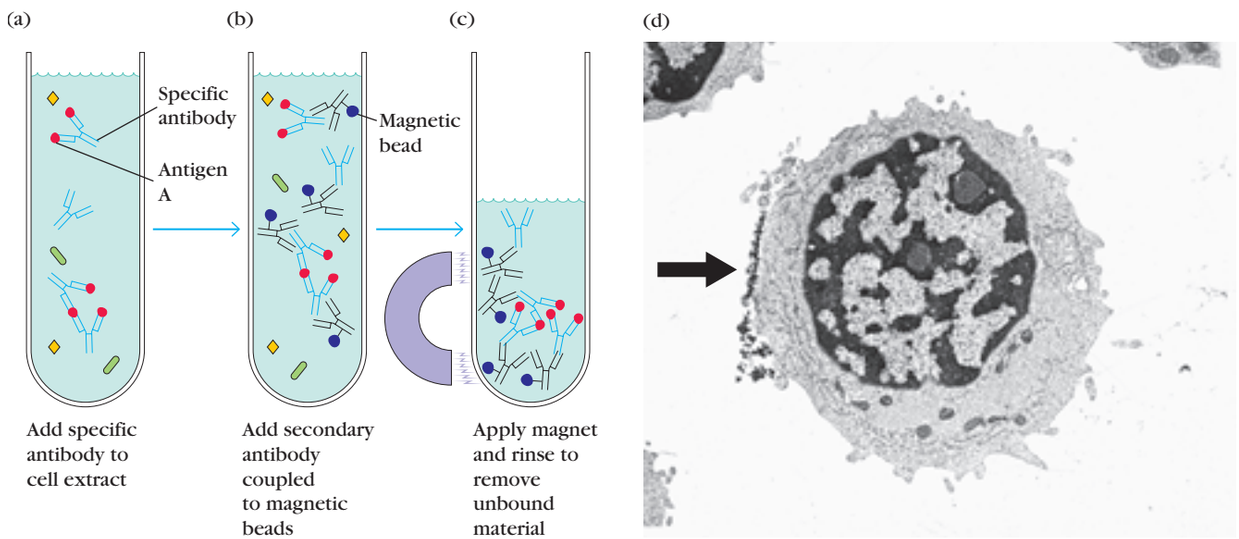
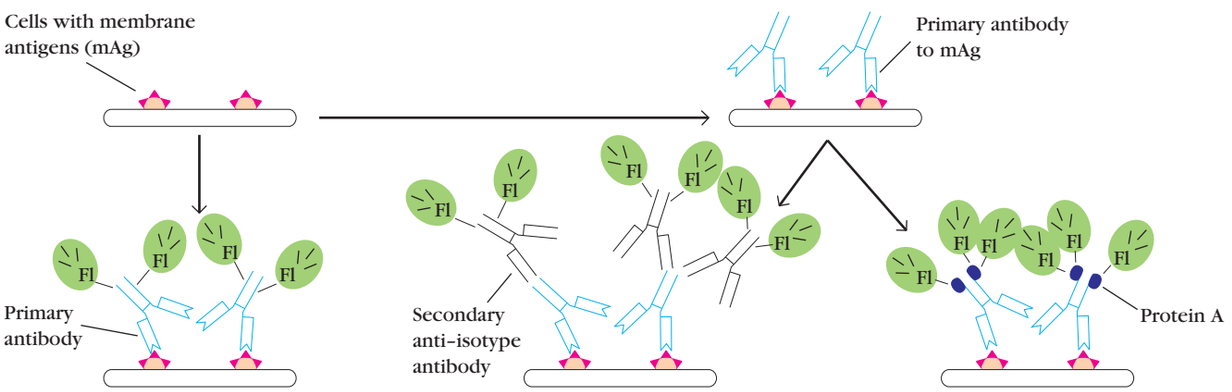


FIGURE 6-13 Immunoprecipitates can be collected using magnetic beads coupled to a secondary antibody. (a) Treatment of a cell extract containing antigen A (red) with a mouse anti-A antibody (blue) results in the formation of antigen-antibody complexes. (b) Addition of magnetic beads to which a rabbit anti-mouse antibody is linked binds the antigen-antibody complexes (and any unreacted mouse Ig). (c) Placing a magnet against the side of the tube allows the rapid collection of the antigen-antibody complexes. After rinsing to remove any unbound material, the antigen-antibody complexes can be dissociated and the antigen studied. (d) An electron micrograph showing a cell with magnetic beads attached to its surface via antibodies. *[Part (d), P. Groscurth, Institute of Anatomy, University of Zurich-Irchel.]*

(excitation) and emit light of another wavelength (emission). If antibody molecules are tagged with a fluorescent dye, or **fluorochrome,** immune complexes containing these fluorescently labeled antibodies (FA) can be detected by colored light emission when excited by light of the appropriate wavelength. Antibody molecules bound to antigens in cells or tissue sections can similarly be visualized. The emitted light can be viewed with a fluorescence microscope, which is equipped with a UV light source. In this technique, known as **immunofluorescence,** fluorescent compounds such as fluorescein and rhodamine are in common use, but other highly fluorescent substances are also routinely used, such as phycoerythrin, an intensely colored and highly fluorescent pigment obtained from algae. These molecules can be conjugated to the Fc region of an antibody molecule without affecting the specificity of the antibody. Each of the fluorochromes below absorbs light at one wavelength and emits light at a longer wavelength:

- **Fluorescein,** an organic dye that is the most widely used label for immunofluorescence procedures, absorbs blue light (490 nm) and emits an intense yellow-green fluorescence (517 nm).
- **Rhodamine,** another organic dye, absorbs in the yellow-green range (515 nm) and emits a deep red fluorescence (546 nm). Because it emits fluorescence at a longer wavelength than fluorescein, it can be used in two-color immunofluorescence assays. An antibody specific to one determinant is labeled with fluorescein, and an antibody recognizing a different antigen is labeled with rhodamine. The location of the fluorescein-tagged antibody will be visible by its yellow-green color, easy to distinguish from the red color emitted where the rhodamine-tagged antibody has bound. By conjugating fluorescein to one antibody and rhodamine to another antibody, one can, for example, visualize simultaneously two different cell-membrane antigens on the same cell.
- **Phycoerythrin** is an efficient absorber of light (~30-fold greater than fluorescein) and a brilliant emitter of red fluorescence, stimulating its wide use as a label for immunofluorescence.

Fluorescent-antibody staining of cell membrane molecules or tissue sections can be direct or indirect (Figure 6-14). In **direct staining,** the specific antibody (the primary antibody) is directly conjugated with fluorescein; in **indirect staining,** the primary antibody is unlabeled and is detected with an additional fluorochrome-labeled reagent. A number of reagents have been developed for indirect staining.



(d)

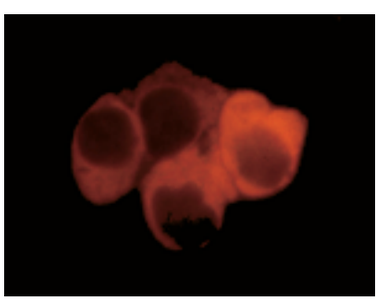


FIGURE 6-14 Direct and indirect immunofluorescence staining of membrane antigen (mAg). Cells are affixed to a microscope slide. In the direct method (a), cells are stained with anti-mAg antibody that is labeled with a fluorochrome (Fl). In the indirect methods (b and c), cells are first incubated with unlabeled anti-mAg antibody and then stained with a fluorochrome-labeled secondary reagent that binds to the primary antibody. Cells are viewed under a fluorescence microscope to see if they have been stained. (d) In this micrograph, antibody molecules bearing μ heavy chains are detected by indirect staining of cells with rhodamine-conjugated second antibody. *[Part(d), H. A. Schreuder et al.,* 1997, Nature ***386:****196, courtesy H. Schreuder, Hoechst Marion Roussel.]*

The most common is a fluorochrome-labeled secondary antibody raised in one species against antibodies of another species, such as fluorescein-labeled goat anti-mouse immunoglobulin.

Indirect immunofluorescence staining has two advantages over direct staining. First, the primary antibody does not need to be conjugated with a fluorochrome. Because the supply of primary antibody is often a limiting factor, indirect methods avoid the loss of antibody that usually occurs during the conjugation reaction. Second, indirect methods increase the sensitivity of staining because multiple molecules of the fluorochrome reagent bind to each primary antibody molecule, increasing the amount of light emitted at the location of each primary antibody molecule.

Immunofluorescence has been applied to identify a number of subpopulations of lymphocytes, notably the $CD4^+$ and $CD8^+$ T-cell subpopulations. The technique is also suitable for identifying bacterial species, detecting Ag-Ab complexes in autoimmune disease, detecting complement components in tissues, and localizing hormones and other cellular products stained in situ. Indeed, a major application of the fluorescent-antibody technique is the localization of antigens in tissue sections or in subcellular compartments. Because it can be used to map the actual location of target antigens, fluorescence microscopy is a powerful tool for relating the molecular architecture of tissues and organs to their overall gross anatomy.

Flow Cytometry and Fluorescence

The fluorescent antibody techniques described are extremely valuable qualitative tools, but they do not give quantitative data. This shortcoming was remedied by development of the flow cytometer, which was designed to automate the analysis and separation of cells stained with fluorescent antibody. The flow cytometer uses a laser beam and light detector to count single intact cells in suspension (Figure 6-15). Every time a cell passes the laser beam, light is deflected from the detector, and this interruption of the laser signal is recorded. Those cells having a fluorescently tagged antibody bound to their cell surface antigens are excited by the laser and emit light that is recorded by a second detector system located at a right angle to the laser beam. The simplest form of the instrument counts each cell as it passes the laser beam and records the level of fluorescence the cell emits; an attached computer generates plots of the number of cells as the ordinate and their fluorescence intensity as the abscissa. More sophisticated versions of the instrument are capable of sorting populations of cells into different containers according to their fluorescence profile. Use of the instrument to determine which and how many members of a cell population bind fluorescently labeled antibodies is called analysis; use of the instrument to place cells having different patterns of reactivity into different containers is called cell sorting.

The flow cytometer has multiple applications to clinical and research problems. A common clinical use is to determine the kind and number of white blood cells in blood samples. By treating appropriately processed blood samples with a fluorescently labeled antibody and performing flow cytometric analysis, one can obtain the following information:

- How many cells express the target antigen as an absolute number and also as a percentage of cells passing the beam. For example, if one uses a fluorescent antibody specific for an antigen present on all T cells, it would be possible to determine the percentage of T cells in the total white blood cell population. Then, using the cell-sorting capabilities of the flow cytometer, it would be possible to isolate the T-cell fraction of the leukocyte population.
- The distribution of cells in a sample population according to antigen densities as determined by fluorescence intensity. It is thus possible to obtain a measure of the distribution of antigen density within the population of cells that possess the antigen. This is a powerful feature of the instrument, since the same type of cell may express different levels of antigen depending upon its developmental or physiological state.
- The size of cells. This information is derived from analysis of the light-scattering properties of members of the cell population under examination.

Flow cytometry also makes it possible to analyze cell populations that have been labeled with two or even three different fluorescent antibodies. For example, if a blood sample is reacted with a fluorescein-tagged antibody specific for T cells, and also with a phycoerythrin-tagged antibody specific for B cells, the percentages of B and T cells may be determined simultaneously with a single analysis. Numerous variations of such "two-color" analyses are routinely carried out, and "three-color" experiments are common. Aided by appropriate software, highly sophisticated versions of the flow cytometer can even perform "five-color" analyses.

Flow cytometry now occupies a key position in immunology and cell biology, and it has become an indispensable clinical tool as well. In many medical centers, the flow cytometer is one of the essential tools for the detection and classification of leukemias (see the Clinical Focus). The choice of treatment for leukemia depends heavily on the cell types involved, making precise identification of the neoplastic cells an essential part of clinical practice. Likewise, the rapid measurement of T-cell subpopulations, an important prognostic indicator in AIDS, is routinely done by flow-cytometric analysis. In this procedure, labeled monoclonal antibodies against the major T-cell subtypes bearing the CD4 and CD8 antigens are used to determine their ratios in the patient's blood. When the number of CD4 T cells falls below a certain level, the patient is at high risk for opportunistic infections.

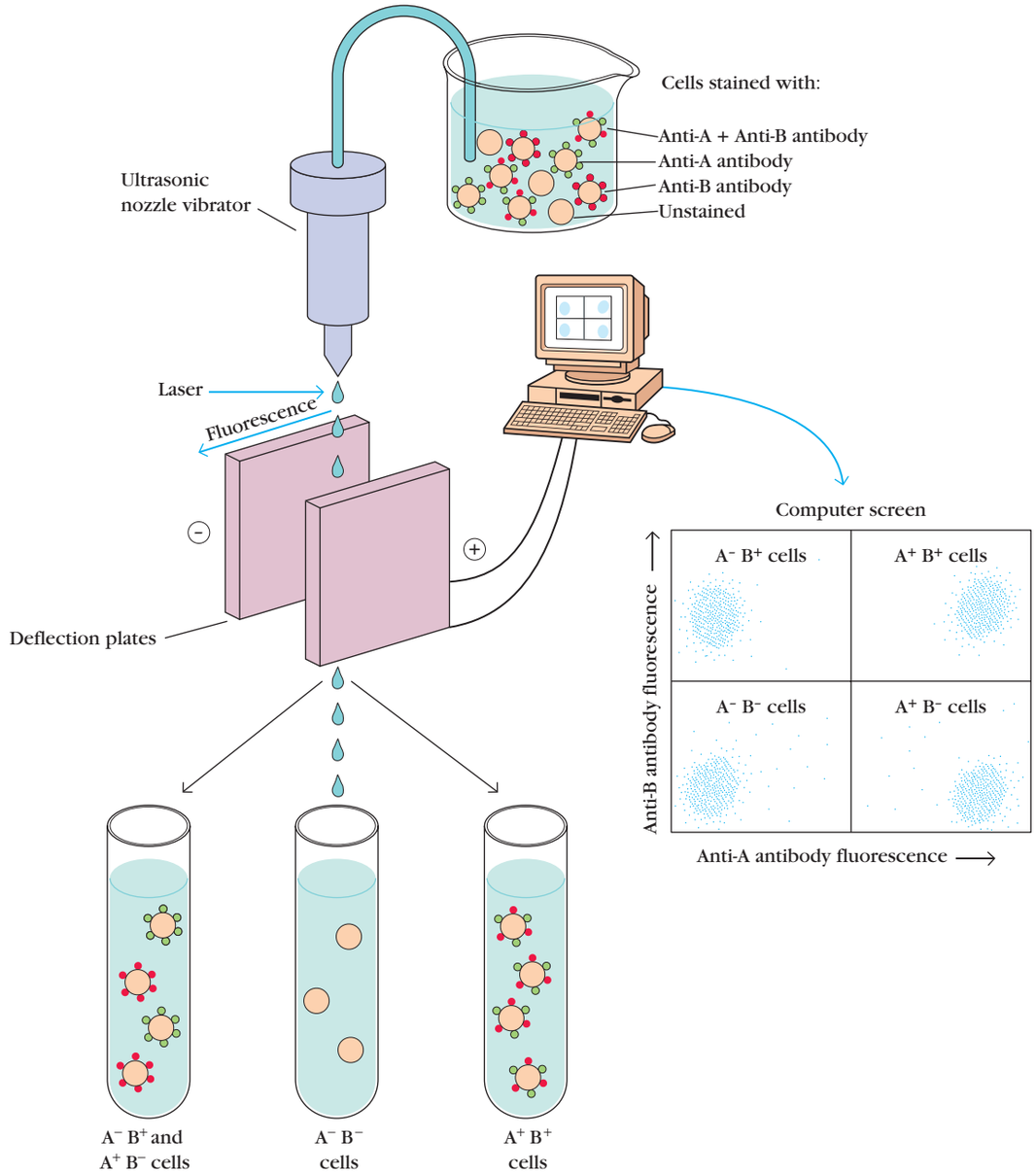


FIGURE 6-15 Separation of fluorochrome-labeled cells with the flow cytometer. In the example shown, a mixed cell population is stained with two antibodies, one specific for surface antigen A and the other specific for surface antigen B. The anti-A antibodies are labeled with fluorescein (green) and the anti-B antibodies with rhodamine (red). The stained cells are loaded into the sample chamber of the cytometer. The cells are expelled, one at a time, from a small vibrating nozzle that generates microdroplets, each containing no more than a single cell. As it leaves the nozzle, each droplet receives a small electrical charge, and the computer that controls the flow cytometer can detect exactly when a drop generated by the nozzle passes through the beam of laser light that excites the fluorochrome. The intensity of the fluorescence emitted by each droplet that contains a cell is monitored by a detector and displayed on a computer screen. Because the computer tracks the position of each droplet, it is possible to determine when a particular droplet will arrive between the deflection plates. By applying a momentary charge to the deflection plates when a droplet is passing between them, it is possible to deflect the path of a particular droplet into one or another collecting vessel. This allows the sorting of a population of cells into subpopulations having different profiles of surface markers.

In the computer display, each dot represents a cell. Cells that fall into the lower left-hand panel have background levels of fluorescence and are judged not to have reacted with either antibody anti-A or anti-B. Those that appear in the upper left panel reacted with anti-B but not anti-A, and those in the lower right panel reacted with anti-A but not anti-B. The upper right panel contains cells that react with both anti-A and anti-B. In the example shown here, the A^-B^-—and the A^+B^+—subpopulations have each been sorted into a separate tube. Staining with anti-A and anti-B fluorescent antibodies allows four subpopulations to be distinguished: A^-B^-, A^+B^+, A^-B^+, and A^+B^-.

CLINICAL FOCUS

Flow Cytometry and Leukemia Typing

Leukemia is the unchecked proliferation of an abnormal clone of hematopoietic cells. Typically, leukemic cells respond poorly or inappropriately to regulatory signals, display aberrant patterns of differentiation, or even fail to differentiate. Furthermore, they sometimes suppress the growth of normal lymphoid and myeloid cells. Leukemia can arise at any maturational stage of any one of the hematopoietic lineages. Lymphocytic leukemias display many characteristics of cells of the lymphoid lineage; another broad group, myelogenous leukemias, have attributes of members of the myeloid lineage. Aside from lineage, many leukemias can be classified as acute or chronic. Some examples are acute lymphocytic leukemia (ALL), the most common childhood leukemia; acute myelogenous leukemia (AML), found more often in adults than in children; and chronic lymphocytic leukemia (CLL), which is rarely seen in children but is the most common form of adult leukemia in the Western world. A fourth type, chronic myelogenous leukemia (CML), occurs much more often in older adults than in children.

The diagnosis of leukemia is made on the basis of two findings. One is the detection of abnormal cells in the bloodstream, and the other is observation of abnormal cells in the bone marrow. Clinical experience has shown that designing the most appropriate therapy for the patient requires knowing which type of leukemia is present. In this regard, two of the important questions are: (1) What is the lineage of the abnormal cells and (2) What is their maturational stage? A variety of approaches, including cytologic examination of cell morphology and staining characteristics, immunophenotyping, and, in some cases, an analysis of gene rearrangements, are useful in answering these questions. One of the most powerful of these approaches is immunophenotyping, the determination of the profile of selected cell-surface markers displayed by the leukemic cell. Although leukemia-specific antigens have not yet been found, profiles of expressed surface antigens often can establish cell lineage, and they are frequently helpful in determining the maturational stages present in leukemic cell populations. For example, an abnormal cell that displays surface immunoglobulin would be assigned to the B-cell lineage and its maturational stage would be that of a mature B cell. On the other hand, a cell that had cytoplasmic μ heavy chains, but no surface immuno-globulin, would be a B-lineage leukemic cell but at the maturational stage of a pre-B cell. The most efficient and precise technology for immunophenotyping uses flow cytometry and monoclonal antibodies. The availability of monoclonal antibodies specific for each of the scores of antigens found on various types and subtypes of hematopoietic cells has made it possible to identify patterns of antigen

Alternatives to Antigen-Antibody Reactions

As a defense against host antibodies, some bacteria have evolved the ability to make proteins that bind to the Fc region of IgG molecules with high affinity ($K_a \sim 10^8$). One such molecule, known as **protein A,** is found in the cell walls of some strains of *Staphylococcus aureus*, and another, **protein G,** appears in the walls of group C and G Streptococcus. By cloning the genes for protein A and protein G and generating a hybrid of both, one can make a recombinant protein, known as **protein A/G,** that combines some of the best features of both. These molecules are useful because they bind IgG from many different species. Thus they can be labeled with flourochromes, radioactivity, or biotin and used to detect IgG molecules in the antigen-antibody complexes formed during ELISA, RIA, or such fluorescence-based assays as flow cytometry or fluorescence microscopy. These bacterial IgG-binding proteins can also be used to make affinity columns for the isolation of IgG.

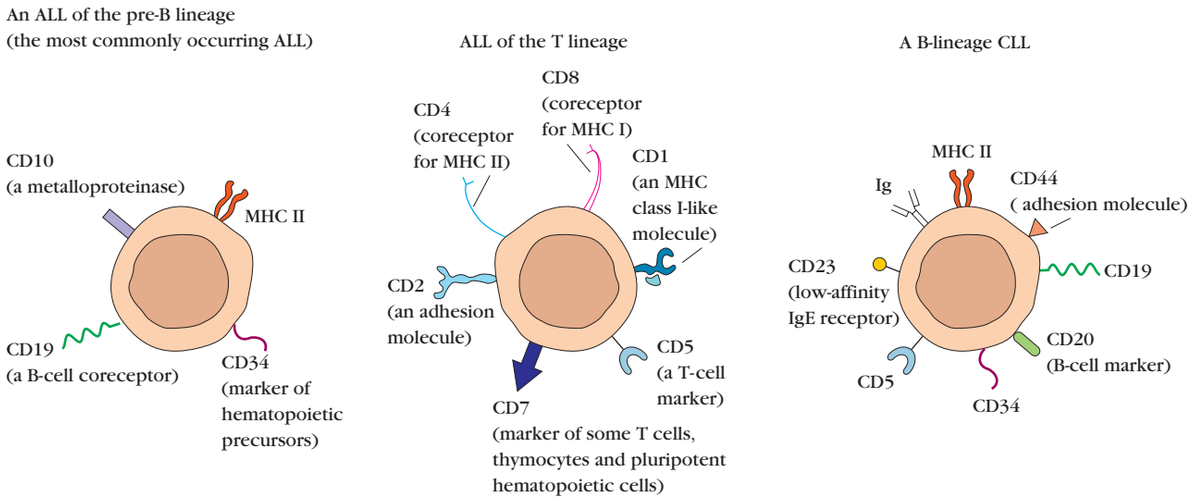
Egg whites contain a protein called *avidin* that binds biotin, a vitamin that is essential for fat synthesis. Avidin is believed to have evolved as a defense against marauding rodents that rob nests and eat the stolen eggs. The binding between avidin and biotin is extremely specific and of much higher affinity ($K_a \sim 10^{15}$) than any known antigen-antibody reaction. A bacterial protein called **streptavidin,** made by *streptomyces avidinii,* has similarly high affinity and specificity. The extraordinary affinity and exquisite specificity of the interaction of these proteins with biotin is widely used in many immunological procedures. The primary or secondary antibody is labeled with biotin and allowed to react with the target antigen, and the unbound antibody is then washed away. Subsequently, streptavidin or avidin conjugated with an enzyme, flourochrome, or radioactive label is used to detect the bound antibody.

Immunoelectron Microscopy

The fine specificity of antibodies has made them powerful tools for visualizing specific intracellular tissue components

expression that are typical of cell lineages, maturational stages, and a number of different types of leukemia. Most cancer centers are equipped with flow cytometers that are capable of performing and interpreting the multiparameter analyses necessary to provide useful profiles of surface markers on tumor cell populations. Flow cytometric determination of immuno-phenotypes allows:

- Confirmation of diagnosis
- Diagnosis when no clear judgment can be made based on morphology or patterns of cytochemical staining
- Identification of aberrant antigen profiles that can help identify the return of leukemia during remission
- Improved prediction of the course of the disease



Distribution of selected markers on some leukemic cell types. Shown are typical surface antigen profiles found on many, but not all, ALLs and CLLs.

by **immunoelectron microscopy.** In this technique, an electron-dense label is either conjugated to the Fc portion of a specific antibody for direct staining or conjugated to an anti-immunoglobulin reagent for indirect staining. A number of electron-dense labels have been employed, including *ferritin* and *colloidal gold.* Because the electron-dense label absorbs electrons, it can be visualized with the electron microscope as small black dots. In the case of immunogold labeling, different antibodies can be conjugated with gold particles of different sizes, allowing identification of several antigens within a cell by the different sizes of the electron-dense gold particles attached to the antibodies (Figure 6-16).

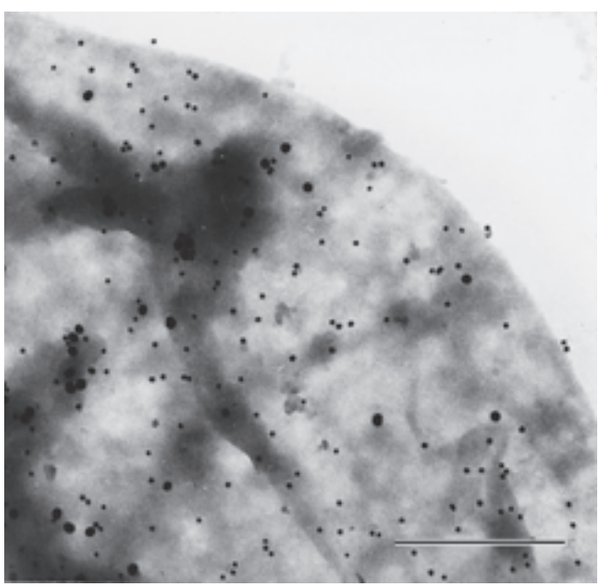


FIGURE 6-16 An immunoelectronmicrograph of the surface of a B-cell lymphoma was stained with two antibodies: one against class II MHC molecules labeled with 30-nm gold particles, and another against MHC class I molecules labeled with 15-nm gold particles. The density of class I molecules exceeds that of class II on this cell. Bar = 500 nm. *[From A. Jenei et al., 1997,* PNAS ***94:****7269–7274; courtesy of A. Jenei and S. Damjanovich, University Medical School of Debrecen, Hungary.]*

SUMMARY

- Antigen-antibody interactions depend on four types of noncovalent interactions: hydrogen bonds, ionic bonds, hydrophobic interactions, and van der Waals interactions.
- The affinity constant, which can be determined by Scatchard analysis, provides a quantitative measure of the strength of the interaction between an epitope of the antigen and a single binding site of an antibody. The avidity reflects the overall strength of the interactions between a multivalent antibody molecule and a multivalent antigen molecule at multiple sites.
- The interaction of a soluble antigen and precipitating antibody in a liquid or gel medium forms an Ag-Ab precipitate. Electrophoresis can be combined with precipitation in gels in a technique called immunoelectrophoresis.
- The interaction between a particulate antigen and agglutinating antibody (agglutinin) produces visible clumping, or agglutination that forms the basis of simple, rapid, and sensitive immunoassays.
- Radioimmunoassay (RIA) is a highly sensitive and quantitative procedure that utilizes radioactively labeled antigen or antibody.
- The enzyme-linked immunosorbent assay (ELISA) depends on an enzyme-substrate reaction that generates a colored reaction product. ELISA assays that employ chemiluminescence instead of a chromogenic reaction are the most sensitive immunoassays available.
- In Western blotting, a protein mixture is separated by electrophoresis; then the protein bands are electrophoretically transferred onto nitrocellulose and identified with labeled antibody or labeled antigen.
- Fluorescence microscopy using antibodies labeled with fluorescent molecules can be used to visualize antigen on or within cells.
- Flow cytometry provides an unusually powerful technology for the quantitative analysis and sorting of cell populations labeled with one or more fluorescent antibodies.

References

Berzofsky, J. A., I. J. Berkower, and S. L. Epstein. 1991. Antigen-antibody interactions and monoclonal antibodies. In *Fundamental Immunology,* 3rd ed., W. E. Paul, ed. Raven Press, New York.

Coligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober. 1997. *Current Protocols in Immunology.* Wiley, New York.

Harlow, E., and D. Lane. 1999. *Using Antibodies: A laboratory manual.* Cold Spring Harbor Laboratory Press.

Herzenberg, L. A., ed. 1996. *Weir's Handbook of Experimental Immunology,* 5th ed. Oxford, Blackwell Scientific Publications.

Rose, N. R., E. C. de Macario, J. D. Folds, C. H. Lane, and R. M. Nakamura. 1997. *Manual of Clinical Laboratory Immunology.* American Society of Microbiology, Washington, D.C.

Stites, D. P., C. Rodgers, J. D. Folds, and J. Schmitz. 1997. Clinical laboratory detection of antigens and antibodies. In *Medical Immunology,* 9th ed., D. P. Stites, A. I. Terr, and T. G. Parslow, eds., Appelton and Lange, Stamford, CT.

Wild, D., ed. 2001. *The Immunoassay Handbook.* Nature Publishing Group, NY.

USEFUL WEB SITES

http://pathlabsofark.com/flowcyttests.html

Explore the Pathology Laboratories of Arkansas to see what kinds of samples are taken from patients and what markers are used to evaluate lymphocyte populations by flow cytometry.

http://jcsmr.anu.edu.au/facslab/AFCG/standards.html

At the highly informative Australian Flow Cytometry Group Web site, one can find a carefully detailed and illustrated guide to the interpretation of flow cytometric analyses of clinical samples.

http://www.kpl.com

The Kirkegaard & Perry Laboratories Web site contains a subsite, http://www.kpl.com/support/immun/pds/50datasht/54-12-10.html, which allows one to follow a step-by-step procedure for using a chemiluminescent substrate in a sensitive immunoassay.

Study Questions

CLINICAL FOCUS QUESTION Flow-cytometric analysis for the detection and measurement of subpopulations of leukocytes, including those of leukemia, is usually performed using monoclonal antibodies. Why is this the case?

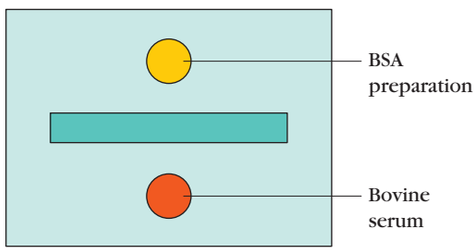
1. Indicate whether each of the following statements is true or false. If you think a statement is false, explain why.

   a. Indirect immunofluorescence is a more sensitive technique than direct immunofluorescence.
   b. Most antigens induce a polyclonal response.
   c. A papain digest of anti-SRBC antibodies can agglutinate sheep red blood cells (SRBCs).
   d. A pepsin digest of anti-SRBC antibodies can agglutinate SRBCs.
   e. Indirect immunofluorescence can be performed using a Fab fragment as the primary, nonlabeled antibody.
   f. For precipitation to occur, both antigen and antibody must be multivalent.
   g. Analysis of a cell population by flow cytometry can simultaneously provide information on both the size distribution and antigen profile of cell populations containing several different cell types.

h. ELISA tests using chemiluminescence are more sensitive than chromogenic ones and precipitation tests are more sensitive than agglutination tests.
i. Western blotting and immunoprecipitation assays are useful quantitative assays for measuring the levels of proteins in cells or tissues.
j. Assume antibody A and antibody B both react with an epitope C. Furthermore, assume that antibody A has a K_a 5 times greater than that of antibody B. The strength of the monovalent reaction of antibody A with epitope C will always be greater than the avidity of antibody B for an antigen with multiple copies of epitope C.

2. You have obtained a preparation of purified bovine serum albumin (BSA) from normal bovine serum. To determine whether any other serum proteins remain in this preparation of BSA, you decide to use immunoelectrophoresis.

a. What antigen would you use to prepare the antiserum needed to detect impurities in the BSA preparation?
b. Assuming that the BSA preparation is pure, draw the immunoelectrophoretic pattern you would expect if the assay was performed with bovine serum in a well above a trough containing the antiserum you prepared in (a) and the BSA sample in a well below the trough as shown below:



3. The labels from four bottles (A, B, C, and D) of hapten-carrier conjugates were accidentally removed. However, it was known that each bottle contained either 1) hapten 1–carrier 1 (H1-C1), 2) hapten 1–carrier 2 (H1-C2), 3) hapten 2–carrier 1 (H2-C1), or 4) hapten 2–carrier 2 (H2-C2). Carrier 1 has a molecular weight of 60,000 daltons and carrier 2 has a molecular weight of over 120,000 daltons. Assume you have an anti-H1 antibody and an anti-H-2 antibody and a molecular-weight marker that is 100,000 daltons. Use Western blotting to determine the contents of each bottle and show the Western blots you would expect from 1, 2, 3, and 4. Your answer should also tell which antibody or combination of antibodies was used to obtain each blot.

4. The concentration of a small amount (250 nanograms/ml) of hapten can be determined by which of the following assays: (a) ELISA (chromogenic), (b) Ouchterlony method, (c) RIA, (d) fluorescence microscopy, (e) flow cytometry, (f) immunoprecipitation, (g) immunoelectron microscopy, (h) ELISPOT assay, (i) chemiluminescent ELISA.

5. You have a myeloma protein, X, whose isotype is unknown and several other myeloma proteins of all known isotypes (e.g., IgG, IgM, IgA, and IgE).

a. How could you produce isotype-specific antibodies that could be used to determine the isotype of myeloma protein, X?
b. How could you use this anti-isotype antibody to measure the level of myeloma protein X in normal serum?

6. For each antigen or antibody listed below, indicate an appropriate assay method and the necessary test reagents. Keep in mind the sensitivity of the assay and the expected concentration of each protein.

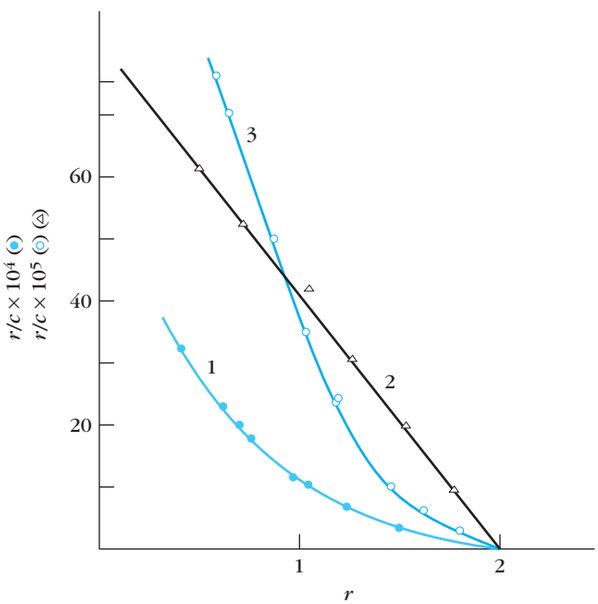
a. IgG in serum
b. Insulin in serum
c. IgE in serum
d. Complement component C3 on glomerular basement membrane
e. Anti-A antibodies to blood-group antigen A in serum
f. Horsemeat contamination of hamburger
g. Syphilis spirochete in a smear from a chancre

7. Which of the following does *not* participate in the formation of antigen-antibody complexes?

a. Hydrophobic bonds
b. Covalent bonds
c. Electrostatic interactions
d. Hydrogen bonds
e. Van der Waals forces

8. Explain the difference between antibody affinity and antibody avidity. Which of these properties of an antibody better reflects its ability to contribute to the humoral immune response to invading bacteria?

9. You want to develop a sensitive immunoassay for a hormone that occurs in the blood at concentrations near 10^{-7} M. You are offered a choice of three different antisera whose affinities for the hormone have been determined by equilibrium dialysis. The results are shown in the Scatchard plots.



a. What is the value of K_0 for each antiserum?
b. What is the valence of each of the antibodies?
c. Which of the antisera might be a monoclonal antibody?
d. Which of the antisera would you use for your assay? Why?

10. In preparing a demonstration for her immunology class, an instructor purified IgG antibodies to sheep red blood cells (SRBCs) and digested some of the antibodies into Fab, Fc, and $F(ab.)_2$ fragments. She placed each preparation in a separate tube, labeled the tubes with a water-soluble marker, and left them in an ice bucket. When the instructor returned for her class period, she discovered that the labels had smeared and were unreadable. Determined to salvage the demonstration, she relabeled the tubes 1, 2, 3, and 4 and proceeded. Based on the test results described below, indicate which preparation was contained in each tube and explain how you identified the contents.

a. The preparation in tube 1 agglutinated SRBCs but did not lyse them in the presence of complement.
b. The preparation in tube 2 did not agglutinate SRBCs or lyse them in the presence of complement. However, when this preparation was added to SRBCs before the addition of whole anti-SRBC, it prevented agglutination of the cells by the whole anti-SRBC antiserum.
c. The preparation in tube 3 agglutinated SRBCs and also lysed the cells in the presence of complement.
d. The preparation in tube 4 did not agglutinate or lyse SRBCs and did not inhibit agglutination of SRBCs by whole anti-SRBC antiserum.

11. You are given two solutions, one containing protein X and the other containing antibody to protein X. When you add 1 ml of anti-X to 1 ml of protein X, a precipitate forms. But when you dilute the antibody solution 100-fold and then mix 1 ml of the diluted anti-X with 1 ml of protein X, no precipitate forms.

a. Explain why no precipitate formed with the diluted antibody.
b. Which species (protein X or anti-X) would likely be present in the supernatant of the antibody-antigen mixture in each case?

12. Consider equation 1 and derive the form of the Scatchard equation that appears in equation 2.

1. $S + L = SL$

2. $B/F = K_a([S]_t - B)$

Where: S = antibody binding sites; [S] = molar concentration of antibody binding sites; L = ligand (monovalent antigen); [L] = molar concentration of ligand; SL = site-ligand complex; [SL] = molar concentration of site ligand complex; B is substituted for [SL] and F for [L]. Hint: It will be helpful to begin by writing the law of mass action for the reaction shown in equation 1.