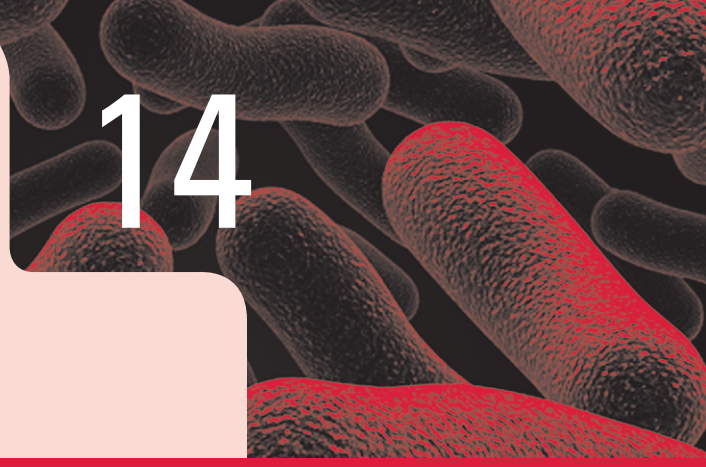

14 Antigen–Antibody Reactions

Introduction

The interactions between antigens and antibodies are known as ***antigen–antibody reactions***. The reactions are highly specific, and an antigen reacts only with antibodies produced by itself or with closely related antigens. Since these reactions are essentially specific, they have been used in many diagnostic tests for the detection of either the antigen or the antibody *in vitro*. The antigen and antibody reactions also form the basis of immunity against microbial diseases *in vivo*. In the host, it may cause tissue injury in hypersensitivity reactions and in autoimmune diseases.

General Features of Antigen–Antibody Reactions

Antigen and antibody bind through noncovalent bonds in a manner similar to that in which proteins bind to their cellular receptors, or enzymes bind to their substrates. But antigen–antibody reactions differ from the latter as there is no irreversible chemical alteration in either of the participants, i.e., antigen or the antibody. The antigen and antibody binding is reversible and can be prevented or dissociated by high ionic strength or extreme pH. Following are some of the general features of these interactions:

Physicochemical Properties

Electrostatic bonds, hydrogen bonding, van der Waals bonds, and hydrophobic interactions are the intermolecular forces involved in antigen–antibody reactions. All these types of intermolecular forces depend on the close proximity of the antigen and antibody molecules. For that reason, the "good fit" between an antigenic determinant and an antibody-combining site determines the stability of the antigen–antibody reaction. Multiple bonding between the antigen and the antibody ensures that the antigen will be bound tightly to the antibodies.

Affinity

Affinity denotes the intensity of attraction between antigen and antibody.

- Low-affinity antibodies bind antigen weakly and tend to dissociate readily, whereas high-affinity antibodies bind antigen more tightly and remain bound longer.
- High-affinity binding is believed to result from a very close fit between the antigen-binding sites and the corresponding antigenic determinants that facilitates development of strong noncovalent interactions between antigen and antibody.

Avidity

Avidity is a measure of the overall strength of binding of an antigen with many antigenic determinants and multivalent antibodies. Avidity is a better indicator of the strength of interactions in real biological systems than affinity. Therefore, the avidity of an antigen–antibody reaction is dependent on the valencies of both antigens and antibodies and is greater than the sum total of individual affinities.

Specificity

The term specificity refers to the ability of an individual antibody-combining site to react with only one antigenic determinant or the ability of a population of antibody molecules to react with only one antigen. Antigen–antibody reactions usually show a high degree of specificity.

Key Points

Antibodies can specifically recognize differences in:

- primary structure of an antigen,
- isomeric forms of an antigen, and
- secondary and tertiary structure of an antigen.

Despite this, cross-reactions between antigens and antibodies, however, do occur and are sometimes responsible for causing diseases in hosts and for causing false results in diagnostic tests.

Cross-Reactivity

Although antigen–antibody 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. Antisera containing polyclonal antibodies can often be found to cross-react with immunogens partially related to those used for immunization, due to the existence of common epitopes or of epitopes with similar configurations.

Stages of Antigen–Antibody Reactions

The antigen–antibody reaction occurs in two stages: primary and secondary.

Primary Stage

Primary stage is the initial interaction between antigen and antibody. It is rapid and reversible, but without any visible effects. The ionic bonds, hydrogen bonds, van der Waals forces, and hydrophobic interactions are the weaker intermolecular forces that bind antigen and antibodies together in this primary stage.

Covalent binding, which is a stronger intermolecular force between antigen and antibody, however, does not occur in this stage.

Secondary Stage

Secondary stage is an irreversible interaction between antigen and antibody, with visible effects, such as agglutination, precipitation, neutralization, complement fixation, and immobilization of motile organisms. The binding between antigen and antibody during this stage occurs by covalent binding.

A single antibody is capable of causing different types of antigen–antibody reactions, and a single antigen is capable of inducing production of different classes of immunoglobulins, which differ in their biological properties.

The results of agglutination, precipitation, neutralization, and other tests are usually expressed as a titer. ***Titer*** is defined as the highest dilution of serum that gives a positive reaction in test. Higher titer means greater level of antibodies in serum. For example, a serum with a titer of 1/128 contains more antibodies than a serum with a titer of 1/8.

Types of Antigen–Antibody Reactions

Serological tests are widely used for detection of either serum antibodies or antigens for diagnosis of a wide variety of infectious diseases (Table 14-1). These serological tests are also used for diagnosis of autoimmune diseases and in typing of blood and tissues before transplantation. The following are the examples of antigen–antibody reactions: (*a*) precipitation, (*b*) agglutination, (*c*) complement-dependent serological tests, (*d*) neutralization test, (*e*) opsonization, (*f*) immunofluorescence, (*g*) enzyme immunoassay, (*h*) radioimmunoassay, (*i*) western blotting, (*j*) chemiluminescence assay, and (*k*) immunoelectronmicroscopic tests.

Precipitation

Precipitation test shows the following features:

- It is a type of antigen–antibody reaction, in which the antigen occurs in a soluble form.
- It is a test in which antibody interacts with the soluble antigen in the presence of electrolyte at a specified pH and temperature to produce a precipitate. A lattice is formed between the antigens and antibodies; in certain cases, it is visible as an insoluble precipitate.
- Antibodies that aggregate soluble antigens are called precipitins.

TABLE 14-1 Commonly used tests in clinical microbiology

Test	Uses
Flocculation test	Detection of reaginic antibodies in syphilis by VDRL test
Radial immunodiffusion	Detection of fungal antigen and antibodies
Counter-current immunoelectrophoresis	Detection of both antigen and antibodies in bacterial, viral, fungal, and parasitic diseases
Slide agglutination test	Identification of bacterial isolates, such as *Salmonella*, *Shigella*, *Vibrio*, etc.
Tube agglutination test	Detection of antibodies in bacterial infections, e.g., Widal test for enteric fever
Latex agglutination test	Quantitation and detection of antigen and antibodies
Hemagglutination test	Detection of both antigens and antibodies in viral and parasitic infections
Coagglutination test	Detection of microbial antigens
Complement fixation test	Quantitation and detection of antibodies
Direct immunofluorescence test	Detection and localization of antigen in a cell or tissue
Indirect immunofluorescence test	Detection of specific antibodies in the serum
Sandwich ELISA	Detection of antigens and antibodies
Indirect ELISA	Quantitation and detection of antibodies
Radioimmunoassay	Quantitation of hormones, drugs, etc.
Western blot	Detection of antigen-specific antibody

- When instead of sedimenting, the precipitate remains suspended as floccules, the reaction is known as ***flocculation***.
- Formation of an antigen–antibody 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.

Prozone phenomenon

Antigen and antibody reaction occurs optimally only when the proportion of the antigen and antibody in the reaction mixture is equivalent ***(zone of equivalence)*** (Fig. 14-1). On either side of the equivalence zone, precipitation is actually prevented because of an excess of either antigen or antibody. The zone of antibody excess is known as the ***prozone phenomenon*** and the zone of antigen excess is known as ***postzone phenomenon***.

Marrack in 1934 proposed the *lattice hypothesis* to explain the prozone phenomenon. Marrack's hypothesis is based on the assumptions that each antibody molecule must have at least two binding sites, and antigen must be multivalent. In the zone of equivalence where optimum precipitation occurs, the number of multivalent sites of antigen and antibody are approximately equal. In this zone, precipitation occurs as a result of random, reversible reactions whereby each antibody binds to more than one antigen and vice versa, forming a stable network or lattice. As they combine, it results in a multimolecular lattice that increases in size until it precipitates out of solution.

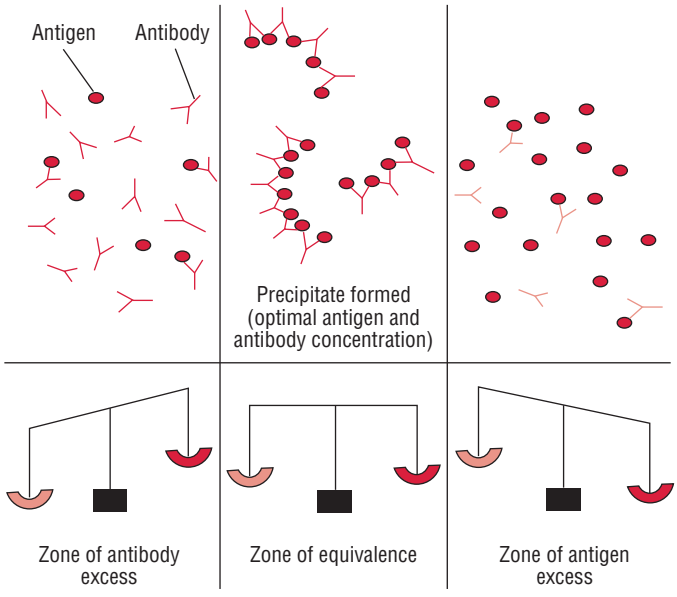

FIG. 14-1. Prozone phenomenon.

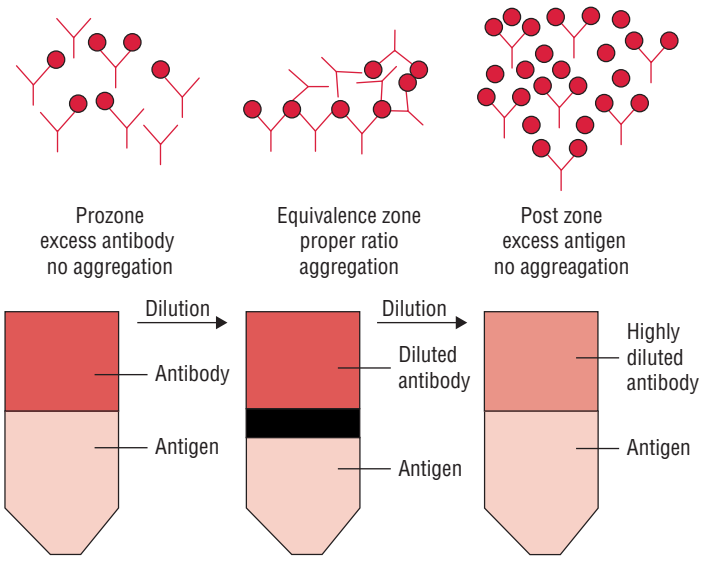

FIG. 14-2. Marrack's lattice hypothesis.

Key Points

In the prozone phenomenon, there is too much antibody for efficient lattice formation. This is because antigen combines with only few antibodies and no cross-linkage is formed.

In postzone phenomenon, small aggregates are surrounded by excess antigen and again no lattice network is formed.

Thus, for precipitation reactions to be detectable, they must be run in the zone of equivalence (Fig. 14-2).

The prozone and postzone phenomena are taken into consideration in the interpretation of serological tests, because false negative reactions can occur in either of these conditions. A false negative reaction suspected to be due to prozone phenomenon can be rectified by diluting out the antibody and performing the test. In the postzone phenomenon, excess antigen may obscure the presence of small amount of antibodies. Typically, such a test is repeated with an additional patient specimen taken about a week later. This would give time for the further production of antibodies. If the test is negative on this occasion, it is unlikely that the patient has that particular antibody.

Types of precipitation reactions

Precipitation reactions can be broadly of three types:

1. Precipitation in solution
2. Precipitation in agar
3. Precipitation in agar with an electric field

Precipitation in solution

Ring test and flocculation test are examples of precipitation in solution.

- **Ring test:** In this test, antigen solution is layered over antiserum in a test tube. Precipitation between antigen and antibodies in antiserum solution is marked by the appearance of a ring of precipitation at the junction of two liquid layers. C-reactive protein (CRP) and streptococcal grouping by the Lancefield methods are the examples of the ring test.
- **Flocculation test:** Flocculation test may be performed in a slide or tube. VDRL test for detection of reaginic antibodies in syphilis is an example of a *slide flocculation test*. In this test, a drop of VDRL antigen suspension is added to a drop of patients' serum on a cavity slide, and the result is recorded after shaking the slide on a VDRL shaker. In a positive test, the floccules appear, which can be demonstrated well under a microscope. Kahn test for syphilis is an example of *tube flocculation test*. The tube flocculation test for standardization of toxins and toxoids is another example.

Precipitation in agar

The precipitation test in agar gel is termed as ***immunodiffusion*** test. In this test, reactants are added to the gel and antigen–antibody combination occurs by means of diffusion. The rate of diffusion is affected by the size of the particles, temperature, gel viscosity, amount of hydration, and interactions between the matrix and reactants.

An agar concentration of 0.3–1.5% allows for diffusion of most reactants. Agarose is often preferred to agar because agar has a strong negative charge, while agarose has almost none, so that interactions between the gel and reactants are minimized.

Key Points

Immunodiffusion reactions have the following advantages:

- In this test, the line of precipitation is visible as a band, which can also be stained for preservation.
- The test can be used to detect identity, cross-reaction, and nonidentity between different antigens in a reacting mixture.

Types of immunodiffusion reactions: Immunodiffusion reactions are classified based on the (*a*) number of reactants diffusing and (*b*) direction of diffusion, as follows:

- ***Single diffusion in one dimension*:** Single diffusion in one dimension, as the name suggests, is the single diffusion of antigen in agar in one dimension. It is otherwise called *Oudin procedure* because this technique was pioneered by Oudin who for the first time used gels for precipitation reactions. In this method, antibody is incorporated into agar gel in a test tube and the antigen solution is poured over it. During the course of time, the antigen diffuses downward toward the antibody in agar gel and a line of precipitation is formed. The number of precipitate bands shows the number of different antigens present in the antigen solution.
- ***Single diffusion in two dimensions*:** Single diffusion in two dimensions is also called *radial immunodiffusion*. In this method, antiserum solution containing antibody is incorporated in agar gel on a slide or Petri dish. The wells are cut on the surface of gel. The antigen is then applied to a well cut into the gel. When antibody already present in the gel reacts with the antigen, which diffuses out of the well, a ring of precipitation is formed around the wells. The diameter of the ring is directly proportional to th=e concentration of antigen. The greater the amount of antigen in the well, the farther the ring will be from the well (Fig. 14-3, Color Photo 6).

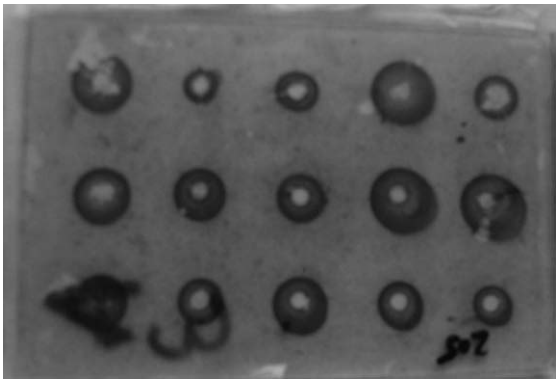

FIG. 14-3. Radial immunodiffusion.

Key Points

Radial immunodiffusion has been used for the quantitative estimation of antibodies and antigens in the serum. It is used to measure:

- IgG, IgM, IgA, and complement components in the serum,
- antibodies to influenza virus in sera, and
- serum transferrin and α-fetoprotein.

However, the test has recently been replaced by more sensitive and automated methods, such as nephelometry and enzyme-linked immunosorbent assays (ELISAs).

- ***Double diffusion in one dimension*:** This method is also called *Oakley–Fulthrope procedure*. In this method, the antibody is incorporated in agar gel in a test tube, above which a layer of plain agar is placed. The antigen is then layered on top of this plain agar. During the course of time, the antigen and antibody move toward each other through the intervening layer of plain agar. In this zone of plain agar, both antigen and antibody react with each other to form a band of precipitation at their optimum concentration.
- ***Double diffusion in two dimensions*:** This method is also called the *Ouchterlony procedure*. In this method, both the antigen and antibody diffuse independently through agar gel in two dimensions, horizontally and vertically. The test is performed by cutting wells in the agar gel poured on a glass slide or in a Petri dish. The antiserum consisting of antibodies is placed in the central well, and different antigens are added to the wells surrounding the center well. After an incubation period of 12–48 hours in a moist chamber, the lines of precipitins are formed at the sites of combination of antigens and antibodies (Color Photo 7). Three types of reactions can be demonstrated as follows (Fig. 14.4):

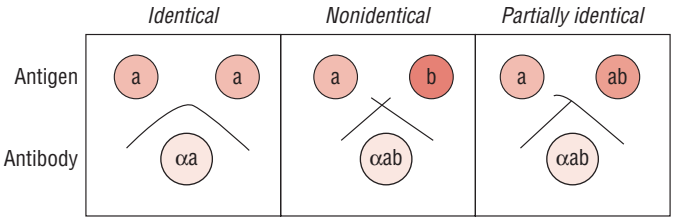

FIG. 14-4. Ouchterlony procedure.

1. Line of precipitation at their junction forming an arc represents serologic identity or the presence of a common epitope in antigens.
2. A pattern of crossed lines demonstrates two separate reactions and indicates that the compared antigens are unrelated and share no common epitopes.
3. Fusion of two lines with a spur indicates cross-reaction or partial identity. In this last case, the two antigens share a common epitope, but some antibody molecules are not captured by the antigen and traverse through the initial precipitin line to combine with additional epitopes found in the more complex antigen.

Key Points

Double diffusion in two dimension has been used for:

- demonstration of antibodies in serodiagnosis of small pox,
- identification of fungal antigens, and
- detection of antibodies to extractable nuclear antigens.

Elek's precipitation test in gel is a special test used for demonstration of toxigenicity of *Corynebacterium diphtheriae*.

Precipitation in agar with an electric field

Immunoelectrophoresis: Immunoelectrophoresis is a process of combination of immunodiffusion and electrophoresis. It is a method in which different antigens in serum are separated according to their charge under an electric field. In this method, a drop of antigen is placed into a well in agar on a glass slide. An electric current is then passed through the agar. During electrophoresis, antigens move in the electric field according to their charge and size. Following electrophoresis, a trough is cut into the agar and is filled with the antibody and diffusion is allowed to occur. As the antigen and antibody diffuse toward each other, they form a series of lines of precipitation (Fig. 14-5). The main advantage of immunoelectrophoresis is that a number of antigens can be identified in serum. The method is used to detect normal as well as abnormal proteins, such as myeloma proteins in human serum.

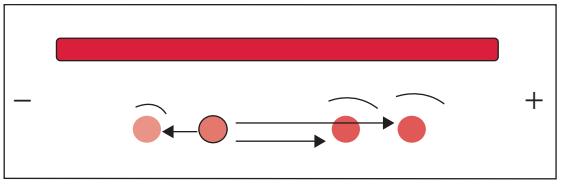

FIG. 14-5. Immunoelectrophoresis.

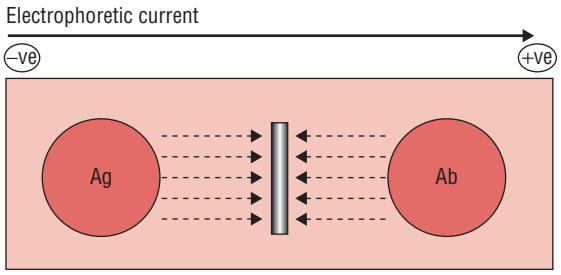

FIG. 14-6. Counter-current immunoelectrophoresis.

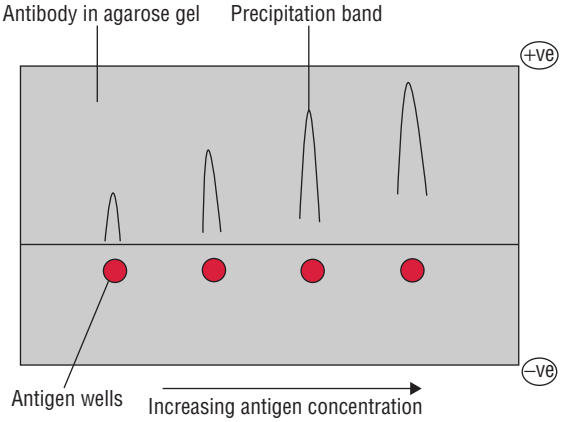

FIG. 14-7. Rocket electrophoresis.

Counter-current immunoelectrophoresis: Counter-current immunoelectrophoresis depends on movement of antigen towards the anode and of antibody towards the cathode through the agar under electric field. The test is performed on a glass slide with agarose in which a pair of wells is punched out. One well is filled with antigen and the other with antibody. Electric current is then passed through the gel. The migration of antigen and antibody is greatly facilitated under electric field, and the line of precipitation is made visible in 30–60 minutes (Fig. 14-6).

Key Points

The counter-current immunoelectrophoresis has many uses:

- It is a rapid and a highly specific method for detection of both antigen and antibodies in the serum, cerebrospinal fluid, and other body fluids in diagnosis of many infectious diseases including bacterial, viral, fungal, and parasitic.
- It is commonly used for Hepatitis B surface antigen (HBsAg), α-fetoprotein, hydatid and amoebic antigens in the serum, and cryptococcal antigen in the CSF.

Rocket electrophoresis: This technique is an adaptation of radial immunodiffusion developed by Laurell. It is called so due to the appearance of the precipitin bands in the shape of cone-like structures (rocket appearance) at the end of the reaction (Fig. 14-7, Color Photo 8). In this method, antibody is incorporated in the gel and antigen is placed in

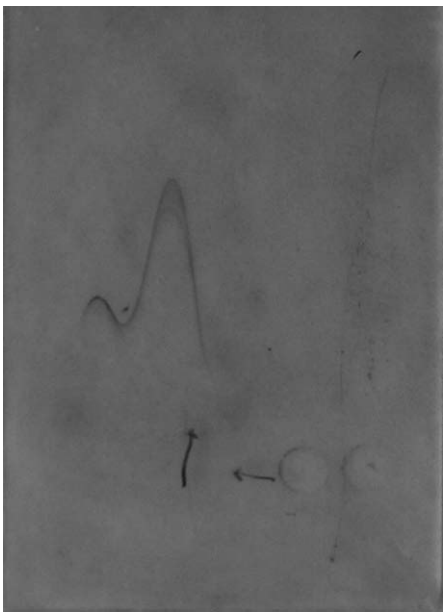

FIG. 14-8. Photograph of rocket electrophoresis.

wells cut in the gel. Electric current is then passed through the gel, which facilitates the migration of antigen into the agar. This results in formation of a precipitin line that is conical in shape, resembling a rocket. The height of the rocket, measured from the well to the apex, is directly in proportion to the amount of antigen in the sample (Fig. 14-8). Rocket electrophoresis is used mainly for quantitative estimation of antigen in the serum.

Two-dimensional immunoelectrophoresis: Two-dimensional immunoelectrophoresis is a variant of rocket electrophoresis. It is a double diffusion technique used for qualitative as well as quantitative analysis of sera for a wide range of antigens. This test is a two-stage procedure. In the first stage, antigens in solution are separated by electrophoresis. In the second stage, electrophoresis is carried out again, but perpendicular to that of first stage to obtain rocket-like precipitation.

In this test, first, a small trough is cut in agar gel on a glass plate and is filled with the antigen solution. Electric current is then passed through the gel, and the antigens migrate into the gel at a rate proportional to their net electric charge. In the second stage, after electrophoresis, the gel piece containing the separated antigens is placed on a second glass plate and the agar containing antibody is poured around the gel piece. A second electric potential is applied at right angles to the first direction of migration. The preseparated antigens then migrate into the gel containing antibodies at a rate proportional to their net charge and precipitate with antibodies in the gel, forming precipitates.

This method is both qualitative, in that it identifies different antigens that are present in the serum solution, and quantitative, in that it detects the amount of different antigens present in the solution.

Turbidimetry and nephelometry: Turbidimetry and nephelometry are the two methods used to detect and quantitate precipitation reactions in serum and are based on the phenomenon of light scattering by precipitates in a solution. ***Turbidimetry*** is a measurement of turbidity or cloudiness of precipitate in a solution. In this method, a detection device is placed in direct line with the incident light that collects the light after it has passed through the solution. It thus measures the reduction in the intensity of light due to reflection, absorption, or scatter. Scattering of light occurs in proportion to the size, shape, and concentration of precipitates present in solution.

Nephelometry is an improvement on this technique in that it measures the light that is scattered at a particular angle from the incident beam as it passes through a suspension containing the antigen–antibody precipitate. The amount of light scattered is an index of the concentration of the solution. Beginning with a constant amount of antibody, an increasing amount of antigen would result in an increase in antigen–antibody complexes. Thus the relationship between antigen concentrations, as indicated by the antigen–antibody complex formation, and light scattering approaches linearity. By using a computer, the exact values of the antigen or antibody in the serum can be estimated through this system. To improve the sensitivity of this system, laser beams have been used as the source of incident light.

Key Points

Nephelometry is now becoming the method of choice for use in various laboratories for the measurement of plasma proteins including IgG, IgM, and IgA, complement components, RA (rheumatoid arthritis) factor, ASLO (anti-streptolysin O), etc.

Agglutination

Agglutination is an antigen–antibody reaction in which a particulate antigen combines with its antibody in the presence of electrolytes at a specified temperature and pH resulting in formation of visible clumping of particles. Agglutination occurs optimally when antigens and antibodies react in equivalent proportions.

- Agglutination reactions are mostly similar to precipitation reactions in their fundamentals and share similar features. This reaction is analogous to the precipitation reaction in that antibodies act as a bridge to form a lattice network of antibodies and the cells that carry the antigen on their surface. Because cells are so much larger than a soluble antigen, the result is more visible when the cells aggregate into clumps.
- Agglutination differs from precipitation reaction in that since the former reaction takes place at the surface of the particle involved, the antigen must be exposed and be able to bind with the antibody to produce visible clumps.

In agglutination reactions, serial dilutions of the antibody solution are made and a constant amount of particulate antigen is added to serially diluted antibody solutions. After several hours of incubation at 37°C, clumping is recorded by visual inspection. The titer of the antiserum is recorded

as the reciprocal of the highest dilution that causes clumping. Since the cells have many antigenic determinants on their surface, the phenomenon of antibody excess is rarely encountered.

Occasionally, antibodies are formed that react with the antigenic determinants of a cell but does not cause any agglutination. They inhibit the agglutination by the complete antibodies added subsequently. Such antibodies are called ***blocking antibodies***. Anti-Rh antibodies and anti-brucella antibodies are few examples of such blocking antibodies.

Agglutination reactions have a wide variety of applications in the detection of both antigens and antibodies in serum and other body fluids. They are very sensitive and the result of the test can be read visually with ease.

Types of agglutination reactions

Agglutination reactions where the antigens are found naturally on a particle are known as direct agglutination. This is different from passive agglutination, which employs particles that are coated with antigens not normally found on their surfaces.

Direct agglutination

Direct agglutination reactions can broadly be of the following types: (*a*) slide agglutination, (*b*) tube agglutination, (*c*) heterophile agglutination, and (*d*) antiglobulin (Coombs') test.

Slide agglutination test: It is a basic type of agglutination reaction that is performed on a slide. Identification of bacterial types represents a classic example of a direct slide agglutination that is still used today. In this test, a suspension of bacteria is prepared and is added to a drop of standardized antiserum. A positive reaction is indicated by clumping of bacteria and clearing of the background solution. Clumping occurs instantly or within seconds in a positive test. A control consisting of antigen suspension in saline without adding antiserum is included on the same slide. It is used to validate the results and also to detect possible false positives due to autoagglutination of the antigen.

Key Points

Slide agglutination is used:

- As a routine procedure to identify bacterial strains, such as *Salmonella*, *Shigella*, *Vibrio*, etc., isolated from clinical specimens.
- For blood grouping and cross-matching.

Tube agglutination test: Tube agglutination test, as the name suggests, is performed in glass tubes. Typically, in these tests, patient's serum is diluted in a series of tubes and bacterial antigens specific for the suspected disease are added to it. Antigen and antibody reactions are demonstrated by demonstration of visible clumps of agglutination. It is a standard method used for quantitative estimation of antibodies in the serum. Tube agglutination tests are routinely used for demonstration of antibodies in the serum for serodiagnosis of enteric fever and brucellosis, as follows:

Key Points

- Widal test is used to diagnose enteric fever and uses different *Salmonella* antigens (T_O, T_H, A_H, and B_H) to detect the presence of antibodies to *Salmonella typhi*, *S. paratyphi* A, and *S. paratyphi* B in patient's serum.
- The standard agglutination test is a commonly used test for serodiagnosis of brucellosis. The tube agglutination test for brucellosis, however, is complicated by the prozone phenomenon. This is due to high concentration of brucella antibodies in patient's serum, resulting in false negative reactions. This problem is obviated by use of several dilutions of serum to prevent false positive reactions. The presence of blocking or incomplete antibodies in the serum is another problem. This is avoided by using antiglobulin (Coombs' test) to detect these antibodies.

Heterophile agglutination test: This test depends on demonstration of heterophilic antibodies in serum present in certain bacterial infections:

Key Points

- Weil–Felix test is an example of heterophile agglutination reaction for serodiagnosis of rickettsial infections. In this test, the cross-reacting antibodies produced against rickettsial pathogen are detected by using cross-reacting related antigens (e.g., *Proteus* strains OXK, OX19, and OX2). Although the antibodies are produced against rickettsial organisms, they cross-react with antigens of *Proteus* strains OXK, OX19, and OX2.
- Paul–Bunnell test is another heterophile agglutination test, which is used to detect antibodies in infectious mononucleosis by using sheep erythrocytes as antigens.
- *Streptococcus* MG agglutination test is a similar test used for detection of antibodies to *Mycoplasma pneumoniae* causing primary atypical pneumonia.

Antiglobulin (Coombs') test: Coombs' test was devised originally by Coombs', Mourant, and Race for detection of incomplete anti-Rh antibodies that do not agglutinate Rh+ erythrocytes in saline. When serum containing incomplete anti-Rh antibodies is mixed with Rh+ erythrocytes in saline, incomplete antibody antiglobulin coats the surface of erythrocytes but does not cause any agglutination. When such erythrocytes are treated with antiglobulin or Coombs' serum

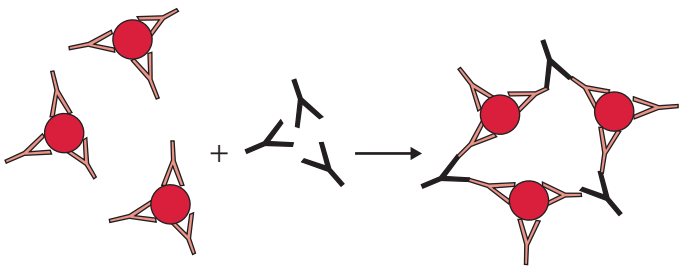

FIG. 14-9. Principle of the direct Coombs' test.

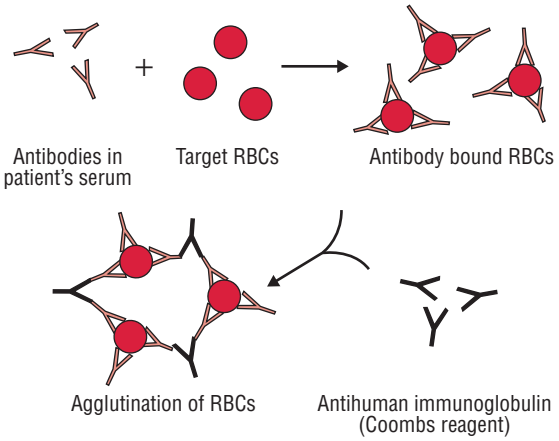

FIG. 14-10. Principle of the indirect Coombs' test.

(rabbit antiserum against human γ globulin), then the cells are agglutinated. Coombs' test is of two types: (*a*) direct Coombs' test and (*b*) indirect Coombs' test.

- **Direct Coombs' test**: In this test, the sensitization of red blood cells (RBCs) with incomplete antibodies takes place *in vivo*. The cell-bound antibodies can be detected by this test in which antiserum against human immunoglobulin is used to agglutinate patient's red cells (Fig. 14-9).
- **Indirect Coombs' test**: In this test, the sensitization of RBCs with incomplete antibodies takes place *in vitro*. In this test, the patient's serum is mixed with normal red cells and antiserum to human immunoglobulin is added. Agglutination occurs if antibodies are present in the patient's serum (Fig. 14-10).

Coombs' tests are used for detection of (*a*) anti-Rh antibodies and (*b*) incomplete antibodies in brucellosis and other diseases.

Passive agglutination

Passive agglutination employs carrier particles that are coated with soluble antigens. This is usually done to convert precipitation reactions into agglutination reactions, since the latter are easier to perform and interpret and are more sensitive than precipitation reactions for detection of antibodies. When the antibody instead of antigens is adsorbed on the carrier particle for detection of antigens, it is called ***reverse passive agglutination.***

Until the 1970s, erythrocytes were the major carrier particles used for coating of antigens. Recently, however, a variety of other particles including polystyrene latex, bentonite, and charcoal are used for this purpose. Particle size vary from 7 μ for RBCs to 0.05 μ for very fine latex particles. The use of synthetic beads or particles provides the advantage of consistency, uniformity, and stability. Reactions are also easy to read visually. Passive agglutination reaction, depending on the carrier particles used, can be of the following types: (*i*) latex agglutination test, (*ii*) hemagglutination test, and (*iii*) coagglutination test.

Latex agglutination test: It is a test that employs latex particles as carrier of antigen or antibodies. In 1955, Singer and Plotz accidentally found that IgG was naturally adsorbed to the surface of polystyrene latex particles.

Latex particles are inexpensive, relatively stable and are not subject to cross-reactivity with other antibodies. These particles can be coated with antibodies to detect antigen in the serum and other body fluids. Use of monoclonal antibodies has reduced the cross-reactions resulting in reduction of false positive reactions.

Additionally, the large particle size of the latex facilitates better visualization of antigen–antibody reactions by the naked eye observation. The tests are usually performed on cardboard cards or glass slides and positive reactions are graded on a scale of 1+ to 4+.

Key Points

The latex agglutination tests have following uses:

- The tests are used for rapid identification of antigens of group B *Streptococcus*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Cryptococcus neoformans*, etc.
- The tests have also been found to be useful for detection of soluble microbial antigens in urine, spinal fluid, and serum for diagnosis of a variety of infectious diseases.
- These tests are being used to detect RA factor, ASLO, CRP, etc., in serum specimens.

Hemagglutination test: RBCs are used as carrier particles in hemagglutination tests. RBCs of sheep, human, chick, etc. are commonly used in the test. When RBCs are coated with antigen to detect antibodies in the serum, the test is called ***indirect hemagglutination (IHA) test***. The IHA is a most commonly used test for serodiagnosis of many parasitic diseases including amoebiasis, hydatid disease, and toxoplasmosis.

When antibodies are attached to the RBCs to detect microbial antigen, it is known as ***reverse passive hemagglutination (RPHA)***. The RPHA has been used extensively in the past to detect viral antigens, such as in HBsAg in the serum for diagnosis of hepatitis B infection. The test has also been used for detection of antigens in many other viral and parasitic infections.

Viral hemagglutination: Many viruses including influenza, mumps, and measles have the ability to agglutinate RBCs without antigen–antibody reactions. This process is called viral hemagglutination. This hemagglutination can be inhibited by antibody specifically directed against the virus, and this phenomenon is called ***hemagglutination inhibition***. This forms the basis of the viral hemagglutination inhibition test, which is used to detect antibodies in patient's sera that neutralize the agglutinating viruses. To perform this test, patient's serum is first incubated with a viral preparation. Then RBCs that the virus is known to agglutinate are added to the mixture. If antibody is present, this will combine with viral particles and prevent

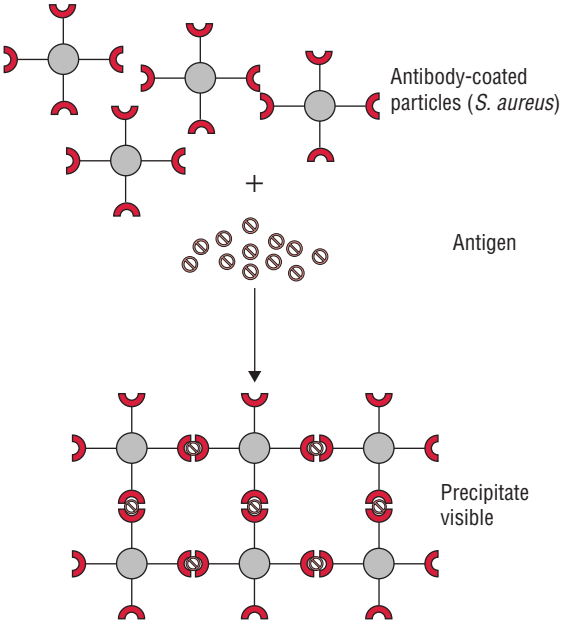

FIG. 14-11. Principle of the coagglutination.

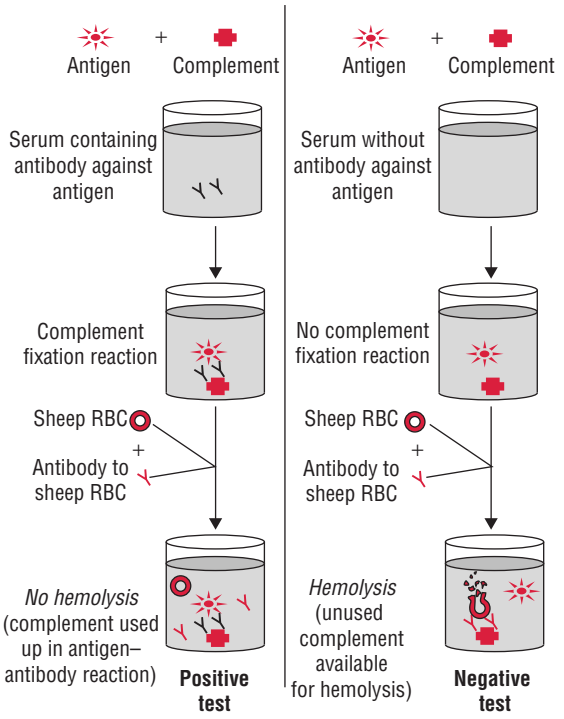

FIG. 14-12. Complement fixation test.

agglutination, and a lack of or reduction in agglutination indicates presence of antibody in patient's serum.

Coagglutination test: Coagglutination is a type of agglutination reaction in which Cowan I strain of *S. aureus* is used as carrier particle to coat antibodies. Cowan I strain of *S. aureus* contains protein A, an anti-antibody, that combines with the Fc portion of immunoglobulin, IgG, leaving the Fab region free to react with the antigen present in the specimens (Fig. 14-11). In a positive test, protein A bearing *S. aureus* coated with antibodies will be agglutinated if mixed with specific antigen. The advantage of the test is that these particles show greater stability than latex particles and are more refractory to changes in ionic strength.

Key Points

Coagglutination test has been used for:

- Detection of cryptococcal antigen in the CSF for diagnosis of cryptococcal meningitis;
- Detection of amoebic and hydatid antigens in the serum for diagnosis of amoebiasis and cystic echinococcosis, respectively; and
- Grouping of streptococci and mycobacteria and for typing of *Neisseria gonorrhoeae.*

Complement-Dependent Serological Tests

The complement system is a group of serum proteins that is present in normal serum. The system consists of 20 or more serum proteins that interact with one another and with cell membrane. It is a biochemical cascade that helps to clear pathogens from the body. It aids the antibodies in lysing bacteria, promoting phagocytosis, and in immune adherence. The complement-dependent serological tests may be of the following types:

1. Complement fixation test
2. Immune adherence test
3. Immobilization test
4. Cytolytic or cytocidal reactions

Complement fixation test

The principle of the complement fixation test is that when antigen and antibodies of the IgM or the IgG classes are mixed, complement is "fixed" to the antigen–antibody complex. If this occurs on the surface of RBCs, the complement cascade will be activated and hemolysis will occur. The complement fixation test consists of two antigen–antibody complement systems: (*a*) an indicator system and (*b*) a test system.

Indicator system: It consists of RBCs that have been preincubated with a specific anti-RBC antibody, in concentrations that do not cause agglutination, and no hemolysis of RBCs occurs in the absence of complement. Such RBCs are designated as "sensitized" red cells.

Test system: In the test system, patient's serum is first heated to 56°C to inactivate the native complement. Then the inactivated serum is adsorbed with washed sheep RBC to eliminate broadly cross-reactive anti-RBC antibodies (also known as Forssman-type antibodies), which could interfere with the assay. The serum is then mixed with purified antigen and with a dilution of fresh guinea pig serum, used as source of

complement. The mixture is incubated for 30 minutes at 37°C to allow antibody in the patient's serum to form complexes with the antigen and to fix complement (Fig. 14-12).

In complement fixation test, "sensitized" red cells are then added to the mixture. If the red cells are lysed, it indicates that there were no antibodies specific to the antigen in the serum of the patient. The complement therefore was not consumed in the test system and was available to be used by the anti-RBC antibodies, resulting in hemolysis. This reaction is considered negative. The test is considered positive if the red cells are not lysed. Nonlysis of the cells indicates that patient's serum had antibodies specific to the antigen, which have "fixed" complement. Hence, no complement was available to be activated by the indicator system.

Key Points

The complement fixation reactions were used earlier for diagnosis of many infections, such as:

- Wassermann test for syphilis and
- Tests for demonstration of antibodies to *M. pneumoniae*, *Bordetella pertussis*, many different viruses, and to fungi (such as *Cryptococcus* spp., *Histoplasma*, and *Coccidioides immitis*).

Since this test is technically very cumbersome, and often difficult, it is no longer used now-a-days.

Indirect complement fixation test: Indirect complement fixation test is carried out to test the sera that cannot fix guinea pig complement. These include avian sera (e.g., parrot, duck) and mammalian sera (e.g., cat, horse). The test is carried out in duplicate and after the first test, the standard antiserum known to fix the complement is added to one set. Hemolysis indicates a positive test. In a positive test, if the serum contains antibody, the antigen would have been used up in the first test, standard antiserum added subsequently would fail to fix the complement, therefore causing hemolysis.

Conglutinating complement adsorption test: It is an alternative method for systems that do not fix guinea pig complement. Sheep erythrocytes sensitized with bovine serum are used as the indicator system. The bovine serum contains conglutinin, a β globulin that acts as antibody to the complement. Therefore, conglutinin causes agglutination of sensitized sheep erythrocytes if these are combined with complement, which is known as ***conglutination***. If the horse complement had been used up by the antigen–antibody reaction in the first step, the agglutination of the sensitized cells does not occur.

Immune adherence test

Immune adherence test is a test in which certain pathogens (e.g., *Vibrio cholerae*, *Treponema pallidum*, etc.) react with specific antibodies in the presence of complement and adhere to erythrocytes or platelets. The adherence of cells to bacteria is known as immune adherence, which facilitates phagocytosis of the bacteria.

Immobilization test

Immobilization test is a complement-dependent test in which certain live bacteria, such as *T. pallidum,* are immobilized when mixed with patient's serum in the presence of complement. This forms the basis of *T. pallidum* immobilization test. A positive test shows serum to contain treponemal antibodies.

Cytolytic or cytocidal reactions

When a live bacterium, such as *V. cholerae,* is mixed with its specific antibody in the presence of complement, the bacterium is killed and lysed. This forms the basis of test used to measure anti-cholera antibodies in the serum.

Neutralization Tests

Neutralization is an antigen–antibody reaction in which the biological effects of viruses and toxins are neutralized by homologous antibodies known as neutralizing antibodies. These tests are broadly of two types: (*a*) virus neutralization tests and (*b*) toxin neutralization tests.

Virus neutralization tests

Neutralization of viruses by their specific antibodies are called virus neutralization tests. Inoculation of viruses in cell cultures, eggs, and animals results in the replication and growth of viruses. When virus-specific neutralizing antibodies are injected into these systems, replication and growth of viruses is inhibited. This forms the basis of virus neutralization test.

Viral hemagglutination inhibition test is an example of virus neutralization test frequently used in the diagnosis of viral infections, such as influenza, mumps, and measles. If patient's serum contains antibodies against certain viruses that have the property of agglutinating the red blood cells, these antibodies react with the viruses and inhibit the agglutination of the red blood cells.

Toxin neutralization tests

Toxin neutralization tests are based on the principle that biological action of toxin is neutralized on reacting with specific neutralizing antibodies called antitoxins. Examples of neutralization tests include:

- *In vivo*—(*a*) Schick test to demonstrate immunity against diphtheria and (*b*) *Clostridium welchii* toxin neutralization test in guinea pig or mice.
- *In vitro*—(*a*) antistreptolysin O test and (*b*) Nagler reaction used for rapid detection of *C. welchii*.

Opsonization

Opsonization is a process by which a particulate antigen becomes more susceptible to phagocytosis when it combines with *opsonin*. The opsonin is a heat-labile substance present in fresh normal sera. Unlike opsonin, *bacteriotropin* is heat-stable substance present in the serum but with similar activities.

The term "opsonic index" is defined as the ratio of the phagocytic activity of patient's blood for a particular bacterium to the phagocytic activity of blood from a normal individual. It is used to study the progress of resistance during the course of disease. It is measured by incubating fresh citrated blood with the suspension of bacteria at 37°C for 15 minutes and estimating the average number of phagocytic bacteria from the stained blood films.

Immunofluorescence

The property of certain dyes absorbing light rays at one particular wavelength (ultraviolet light) and emitting them at a different wavelength (visible light) is known as ***fluorescence***. Fluorescent dyes, such as fluorescein isothiocyanate and lissamine rhodamine, can be tagged with antibody molecules. They emit blue-green and orange-red fluorescence, respectively under ultraviolet (UV) rays in the fluorescence microscope. This forms the basis of the immunological test. Immunofluorescence tests have wide applications in research and diagnostics. These tests are broadly of two types:

1. Direct immunofluorescence test
2. Indirect immunofluorescence test

Direct immunofluorescence test

Direct immunofluorescence test is used to detect unknown antigen in a cell or tissue by employing a known labeled antibody that interacts directly with unknown antigen. If antigen is present, it reacts with labeled antibody and the antibody-coated antigen is observed under UV light of the fluorescence microscope (Fig. 14-13). Direct immunofluorescence test is widely used for detection of bacteria, parasites, viruses, fungi, or other antigens in CSF, blood, stool, urine, tissues, and other specimens. Few examples include:

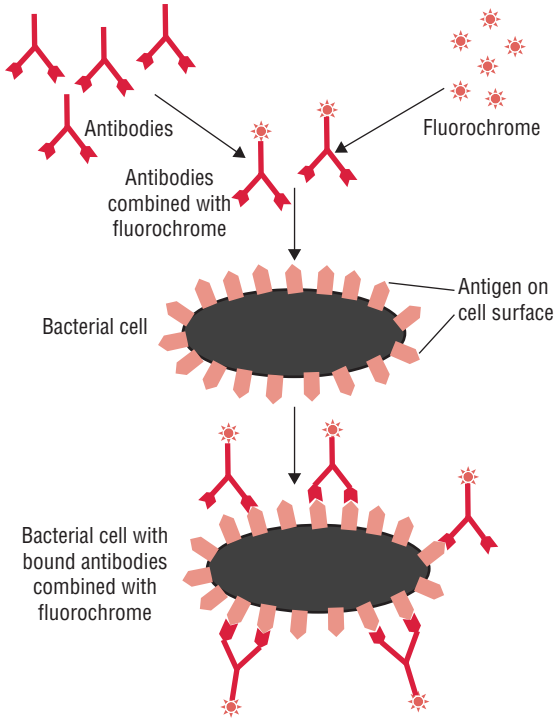

FIG. 14-13. Direct fluorescent antibody test.

Key Points

- Direct immunofluorescence test for antemortem diagnosis of rabies: The test is used for detection of rabies virus antigen in the skin smear collected from the nape of the neck in humans and in the saliva of dogs.
- Also used for detection of *N. gonorrhoeae*, *C. diphtheriae*, *T. pallidum*, etc. directly in appropriate clinical specimens.

The need for preparation of separate labeled antibody for each pathogen is the major disadvantage of the direct immunofluorescence test.

Indirect immunofluorescence test

The indirect immunofluorescence test is used for detection of specific antibodies in the serum and other body fluids for serodiagnosis of many infectious diseases.

Indirect immunofluorescence is a two-stage process. In the first stage, a known antigen is fixed on a slide. Then the patient's serum to be tested is applied to the slide, followed by careful washing. If the patient's serum contains antibody against the antigen, it will combine with antigen on the slide. In the second stage, the combination of antibody with antigen can be detected by addition of a fluorescent dye-labeled antibody to human IgG, which is examined by a fluorescence microscope.

The first step in the indirect immunofluorescence test is the incubation of a fixed antigen (e.g., in a cell or tissue) with unlabeled antibody, which becomes associated with the antigen. Next, after careful washing, a fluorescent antibody (e.g., fluorescent labeled anti-IgG) is added to the smear. This second antibody will become associated to the first, and the antigen–antibody complex can be visualized on the fluorescence microscope.

The indirect method has the advantage of using a single labeled antiglobulin (antibody to IgG) as a "universal reagent" to detect many different specific antigen–antibody reactions. The test is often more sensitive than the direct immunofluorescence test.

Key Points

Indirect immunofluorescence test is used widely to:

- Detect specific antibodies for serodiagnosis of syphilis, leptospirosis, amoebiasis, toxoplasmosis, and many other infectious diseases;
- Identify the class of a given antibody by using fluorescent antibodies specific for different immunoglobulin isotypes;
- Identify and enumerate lymphocyte subpopulations by employing monoclonal antibodies and cytofluorographs; and
- Detect autoantibodies, such as antinuclear antibodies in autoimmune diseases.

The major limitation of immunofluorescence is that the technique requires (*a*) expensive fluorescence microscope and reagents, (*b*) trained personnel, and (*c*) have a factor of subjectivity that may result in erroneous results.

Enzyme Immunoassays

Enzyme immunoassays (EIAs) can be used for detection of either antigens or antibodies in serum and other body fluids of the patient. In EIA techniques, antigen or antibody labeled with enzymes are used. Alkaline phosphatase, horseradish peroxidase, and galactosidase are the enzymes used in the EIA tests.

The commonly used EIAs are enzyme-linked immunosorbent assays (ELISAs). The ELISA technique was first conceptualized and developed by Peter Perlmann and Eva Engvall at Stockholm University, Sweden.

These assays involve the use of an immunosorbent specific to either the antigen or antibody. Following the antigen–antibody reaction, chromogenic substrate specific to the enzyme (o-phenyldiamine dihydrochloride for peroxidase, p-nitrophenyl phosphate for alkaline phosphatase, etc.) is added. The reaction is detected by reading the optical density. Usually, a standard curve based on known concentrations of antigen or antibody is prepared from which the unknown quantities are calculated. There are different types of ELISAs available for the detection and quantitation of either the antigen or antibodies in serum and other body fluids. These include: (*a*) indirect ELISA, (*b*) sandwich ELISA, (*c*) competitive ELISA, and (*d*) ELISPOT assay.

Indirect ELISA

The indirect ELISA is used for the quantitative estimation of antibodies in the serum and other body fluids. In this method, specimens are added to microtiter plate wells coated with antigen to which specific antibodies are to be detected. After a period of incubation, the wells are washed. If antibody was present in the sample, antigen–antibody complex would have been formed and will not get washed away. On the other hand, if the specific antibody was not present in the specimen, there would not be any complex formation. Next, an anti-isotype antibody conjugated with an enzyme is added and incubated. After another washing step, a substrate for the enzyme is added. If there was complex formation in the initial step, the secondary anti-isotype antibody would have bound to the primary antibody, and there would be a chromogenic reaction between the enzyme and substrate. By measuring the optical density values of the wells, after a stop solution has been added to arrest the chromogenic reaction, one can determine the amount of antigen–antibody complex formed in the first step (Fig. 14-14).

Key Points

The test is extensively used for determination of serum antibodies for diagnosis of human immunodeficiency virus (HIV) infection, Japanese encephalitis, dengue, and many other viral infections.

Sandwich ELISA

The sandwich ELISA is used for the detection of antigen. In this test, the known antibody is coated and immobilized onto the wells of microtiter plates. The test sample containing the suspected antigen is added to the wells and is allowed to react with the antibodies in the wells. After the step of washing the well, a second enzyme-conjugated antibody specific for a different epitope of the antigen is added and allowed to incubate. After removing any free secondary antibody by rewashing, the specific substrate is added, and the ensuing chromogenic reaction is measured. The chromogenic reaction is then compared with a standard curve to determine the exact amount of the antigen present in the test sample. In a positive test, an enzyme acts on the substrate to produce a color, and its intensity can be measured by spectrophotometer or ELISA reader. The change of color can also be observed by the naked eye (Fig. 14-15).

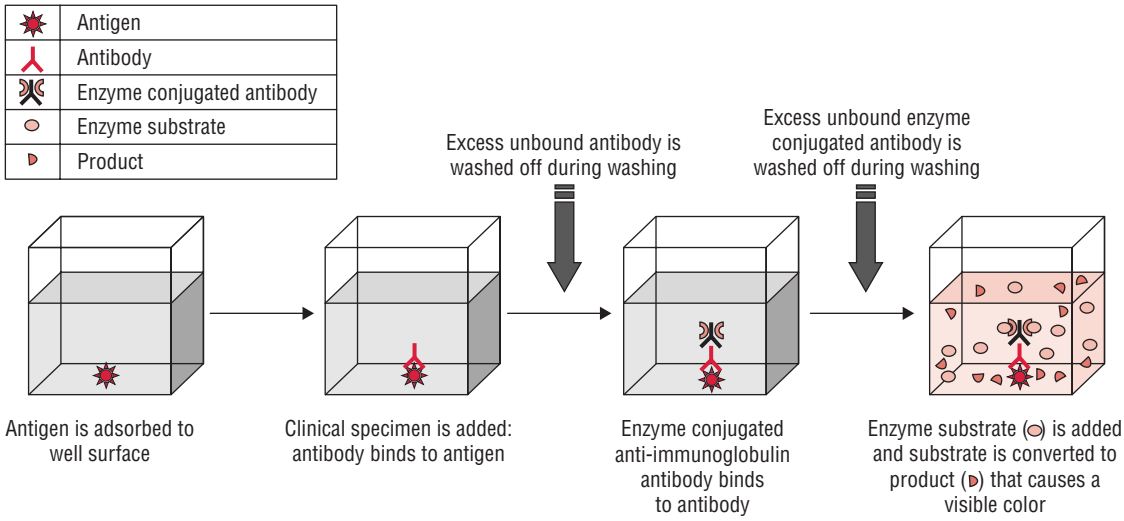

FIG. 14-14. Indirect ELISA test.

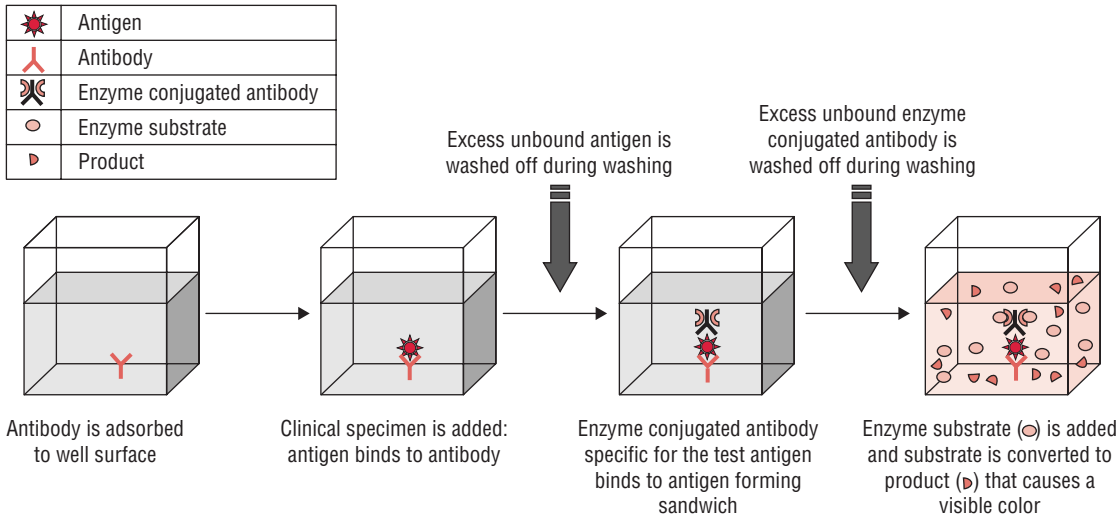

FIG. 14-15. Sandwich ELISA test.

Key Points

The sandwich ELISA is used to detect rotavirus and enterotoxin of *Escherichia coli* in feces.

Competitive ELISA

Competitive ELISA is another technique used for the estimation of antibodies present in a specimen, such as serum. Principle of the test is that two specific antibodies, one conjugated with enzyme and the other present in test serum (if serum is positive for antibodies), are used. Competition occurs between the two antibodies for the same antigen. Appearance of color indicates a negative test (absence of antibodies), while the absence of color indicates a positive test (presence of antibodies).

In this test, the microtiter wells are coated with HIV antigen. The sera to be tested are added to these wells and incubated at 37°C and then washed. If antibodies are present in the test serum, antigen–antibody reaction occurs. The antigen–antibody reaction is detected by adding enzyme-labeled-specific HIV antibodies. In a positive test, no antigen is left for these antibodies to act. Hence, the antibodies remain free and are washed away during the process of washing. When substrate is added, no enzyme is available to act on it. Therefore, positive result indicates no color reaction. In a negative test, in which no antibodies are present in the serum, antigen in the coated wells is available to combine with enzyme-conjugated antibodies and the enzyme acts on the substrate to produce color.

Key Points

Competitive ELISA is the most commonly used test for detection of HIV antibodies in serum in patients with HIV.

ELISPOT Assay

ELISPOT assay is a modification of ELISA. It allows the quantitative determination of 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. These tests have found application widely in the measurement of cytokines.

Radioimmunoassay

When radioisotopes instead of enzymes are used as labels to be conjugated with antigens or antibodies, the technique of detection of the antigen–antibody complex is called as radioimmunoassay (RIA). RIA was first described in 1960 for measurement of endogenous plasma insulin by Solomon Berson and Rosalyn Yalow of the Veterans Administration Hospital in New York. Yalow was awarded the 1977 Nobel Prize for Medicine for the development of the RIA for peptide hormones, but because of his untimely death in 1972, Berson could not share the award.

The classical RIA methods are based on the principle of competitive binding. In this method, unlabeled antigen competes with radiolabeled antigen for binding to antibody with the appropriate specificity. Thus, when mixtures of radiolabeled and unlabeled antigen are incubated with the corresponding antibody, the amount of free (not bound to antibody) radiolabeled antigen is directly proportional to the quantity of unlabeled antigen in the mixture.

In the test, mixtures of known variable amounts of cold antigen and fixed amounts of labeled antigen and mixtures of samples with unknown concentrations of antigen with identical amounts of labeled antigen are prepared in the first step. Identical amounts of antibody are added to the mixtures. Antigen–antibody complexes are precipitated either by cross-linking with a second antibody or by means of the addition of reagents that promote the precipitation of antigen–antibody complexes. Counting radioactivity in the precipitates allows

the determination of the amount of radiolabeled antigen precipitated with the antibody. A standard curve is constructed by plotting the percentage of antibody-bound radiolabeled antigen against known concentrations of a standardized unlabeled antigen, and the concentrations of antigen in patient samples are extrapolated from that curve. The extremely high sensitivity of RIA is its major advantage:

Key Points

Uses of RIA:

- The test can be used to determine very small quantities (e.g., nanogram) of antigens and antibodies in the serum.
- The test is used for quantitation of hormones, drugs, HBsAg, and other viral antigens.

The main drawbacks of the RIA include: (*a*) the cost of equipment and reagents, (*b*) short shelf-life of radiolabeled compounds, and (*c*) the problems associated with the disposal of radioactive waste.

Western Blotting

Western blotting is called so because the procedure is similar to Southern blotting, which was developed by Edwin Southern for the detection of DNA. While Southern blotting is done to detect DNA, Western blotting is done for the detection of proteins.

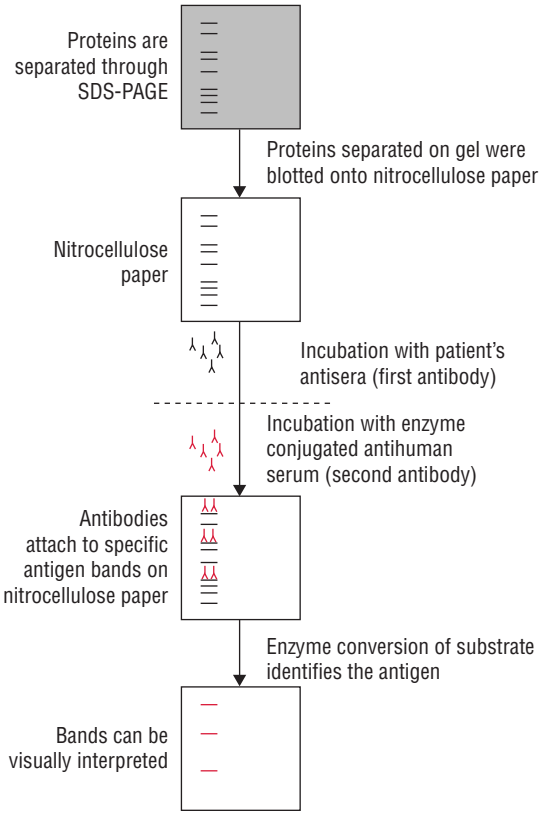

FIG. 14-16. Western blot test.

Western blotting is usually done on a tissue homogenate or extract. It uses SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), a type of gel electrophoresis to first separate various proteins in a mixture on the basis of their shape and size. The protein bands thus obtained are transferred onto a nitrocellulose or nylon membrane where they are "probed" with antibodies specific to the protein to be detected. The antigen–antibody 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 (Fig. 14-16).

Key Points

Western blot technique has many uses as follows:

- It is used for identification of a specific protein in a complex mixture of proteins. In this method, 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 specifi c 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.
- It is also used for estimation of the size of the protein as well as the amount of protein present in the mixture.
- The Western blot test is most widely used as a confirmatory test for diagnosis of HIV, where this procedure is used to determine whether the patient has antibodies that react with one or more viral proteins or not.
- The Western blotting is also used for demonstration of specific antibodies in the serum for diagnosis of neurocysticercosis and tubercular meningitis.

Chemiluminescence Assay

The chemiluminescence assay uses chemiluminescent compounds that emit energy in the form of light during the antigen–antibody reactions. The emitted lights are measured and the concentration of the analyze is calculated. The assay is a fully automated method, which is used commonly for drug sensitivity testing of *Mycobacterium tuberculosis*.

Immunoelectronmicroscopic Tests

These are the types of antigen–antibody reactions that are visualized directly by electron microscope. These are of the following types:

Immunoelectronmicroscopy

This is a test used to detect rotavirus and hepatitis A virus directly in feces. In this test, viral particles are mixed with specific antisera and are demonstrated as clumps of virion particles under the electron microscope.

Immunoenzyme test

This test is used to detect antigen directly in tissue specimens, in which tissue sections are treated with peroxidase-labeled antisera to detect corresponding antigen. The peroxidase bound to the antigen is visualized under the electron microscope.

Immunoferritin test

Electron-dense substances, such as ferritin are conjugated with antibody and such labeled antibodies reacting with antigen can be visualized under the electron microscope.