This chapter describes the three major types of antigen-antibody reactions, the factors that affect agglutination reactions, the antiglobulin test and its effect on the second stage of agglutination, and the methods and reagents used for laboratory detection of antigen-antibody reactions.

Major Types of Antigen-Antibody Reactions

Agglutination

Agglutination is the antibody-mediated clumping of particles that express antigen on their surface. Clumping occurs because antibody molecules unite with antigenic determinants on adjacent red cells, bringing them together into a visible aggregate. Agglutination is the endpoint for most tests involving red cells and blood group antibodies. In some tests, antibody directly bridges the gap between adjacent cells; in others, antibody molecules attach to, but do not aggregate, the antigen-bearing particles, and an additional antibody is needed to induce visible agglutination.

Precipitation

Precipitation is the formation of an insoluble, usually visible, complex when soluble antibody reacts with soluble antigen. Such complexes are seen in test tubes as a sediment or ring and in agar gels as a white line. Precipitation is the endpoint of procedures such as immunodiffusion and immunoelectrophoresis.

Precipitation may not occur, even though soluble antigen and its specific antibody are present. Precipitation of the antigen-antibody complex requires that antigen and antibody be present in optimal proportions. If antibody is present in excess, too few antigen sites exist to crosslink with the molecules and the lattice structure is not formed. Antigen-antibody complexes do form, but do not accumulate sufficiently to form a visible lattice. This is called a prozone phenomenon.
Hemolysis

Hemolysis is the rupture of red blood cells with release of intracellular hemoglobin. Antibody-mediated hemolysis, in vitro, depends on activity of the membrane attack unit of complement and does not occur if the antigen and antibody interact in serum that lacks complement or in plasma if the anticoagulant has chelated cations (calcium and magnesium) necessary for complement activation. In tests for antibodies to red cell antigens, hemolysis is considered a positive result because the union of antibody with antigen activates the complement cascade. Pink or red supernatant fluid in a test system of antibody and red cells is an important observation; antibodies that are lytic in vitro are likely to cause intravascular hemolysis in a transfusion recipient.

Factors Affecting Agglutination

Agglutination is thought to occur in two stages: 1) sensitization, the attachment of antibody to antigen on the red cell membrane; and 2) formation of bridges between the sensitized red cells to form the lattice that constitutes agglutination. A number of variables affect each stage.

The First Stage

Affinity Constant of the Antibody

The association of antigen with antibody is reversible. The amount of antibody attached to antigen at equilibrium depends in part on an intrinsic property of the antibody molecule, its equilibrium constant or affinity constant. Generally, the higher the equilibrium constant, the more association occurs during the first stage of agglutination. Association is also affected by the concentrations of antigen and antibody and by physical conditions such as pH, ionic strength of the suspending medium, and temperature at which the reaction occurs. In laboratory tests that use agglutination as an endpoint, altering the physical conditions of the system can increase or decrease test sensitivity.

Temperature

Most blood group antibodies are reactive within a restricted temperature range. In general, IgM antibodies are more reactive at lower temperatures (4-27 C), whereas IgG antibodies react better at 37 C. Antibody detection procedures can be performed at various temperatures, usually in the 22-37 C or 30-37 C ranges. Antibodies that react in vitro only at temperatures significantly below 37 C rarely cause destruction of transfused, antigen-positive red cells, and are not considered to be clinically significant. A notable exception is the anti-P found in the serum of some patients with paroxysmal cold hemoglobinuria or PCH

Some cold-reactive IgM antibodies activate complement at temperatures above 30 C but rarely affect survival of transfused red cells that express the corresponding antigen. Identification of these antibodies is discussed in Chapter 18. A clinically significant antibody is one that shortens in-vivo red cell survival. This is usually detected by in-vitro reactivity at 37 C. Testing is not performed at temperatures above 37 C, because of possible damage to red cell antigens or to antibody molecules.

pH

For most clinically significant blood group antibodies, optimal pH has not been determined, but is assumed to approximate the physiologic pH range. Occasional antibodies, notably many exam-
amples of anti-M, react best at a lowered pH. For most routine testing, a pH around 7.0 should be used. Stored saline often exhibits pH of 5.0-6.0, causing some workers to use buffered saline in serologic testing.

**Incubation Time**

The time needed to reach equilibrium differs for different blood group antibodies. Significant variables include immunoglobulin class and specific interactions between antigen configuration and the Fab site of the antibody. The addition of enhancement agents to the system can increase the amount of antibody that attaches to antigen in the first 15 minutes and, therefore, decrease the incubation time needed to reach equilibrium.

For saline or albumin systems in which antiglobulin serum is used to demonstrate antibody attachment, 30 minutes of incubation at 37°C is adequate to detect most clinically significant antibodies. With some weakly reactive antibodies, however, association may not reach equilibrium at 30 minutes and extending the incubation time may increase test system sensitivity. Prolonging the incubation time beyond 30 minutes has few disadvantages except for the delay before results are available.

**Ionic Strength**

In normal saline, Na\(^+\) and Cl\(^-\) ions cluster around and partially neutralize opposite charges on antigen and antibody molecules. This hinders the association of antibody with antigen, but can be reduced by lowering the ionic strength of the reaction medium. Reducing the salt concentration of the serum-cell system tends to increase the speed of antibody attachment and possibly the amount of antibody bound. The use of low ionic strength saline (LISS) decreases the time required for incubation in routine antibody detection procedures.

**Antigen-Antibody Proportions**

The number of antibody molecules in the system and the number of antigen sites per red cell affect the speed with which antigen and antibody associate. Increasing the quantity of antibody present can increase test sensitivity. Raising the serum-to-cell ratio provides more antibody molecules to combine with the fixed number of available antigen sites. Very rarely, significant antibody excess may inhibit agglutination, producing a prozone phenomenon comparable to what occurs with precipitation reactions. Usually, however, increasing antibody concentration enhances the sensitivity of agglutination tests. A commonly used ratio in red cell serology is 2 drops of serum to 1 drop of a 2-5% red cell suspension. Reducing the concentration of red cells from 5% to 2-3% could double the serum-to-cell ratio, as could adding 4 drops of serum to the standard cell suspension. The use of an increased serum-to-cell ratio may detect weakly-reactive antibodies not demonstrable under standard test conditions. Sometimes it is useful to increase the volume of serum to 10 or even 20 drops, particularly during investigation of a hemolytic transfusion reaction in which routine testing reveals no antibody. Alterations in the volume of serum significantly affect the ionic strength of test systems in which LISS has reduced the dielectric constant, so procedures must be modified accordingly. Chapter 17 gives more details about antibody detection and pretransfusion testing.

**The Second Stage**

Once antibody molecules attach to antigens on the red cell surface, the sensitized cells must be linked into a lattice.
The size and physical properties of the antibody molecules, the concentration of antigen sites on each cell, and the distance between cells all have an effect on the development of agglutinates.

**Distance Between Cells**

Red cells suspended in saline have a net negative charge at their surface. Negatively charged molecules on the membrane attract positively charged cations, which reduce but do not neutralize the charge at the surface of shear between the surrounding medium and the cloud of ions attracted to each cell. The net charge is reflected in the zeta potential; since like charges repel, the distance between red cells in an ionic medium is proportional to the zeta potential.

Another physical property that maintains distance between saline-suspended red cells is the water of hydration. Water molecules tightly bound to cell surfaces are thought to act as insulating bubbles, preventing close association between cells.

When large, multivalent IgM molecules associate with surface antigens of saline-suspended cells, agglutination readily occurs. IgG molecules characteristically fail to bridge the distance between red cells and cause sensitization without lattice formation. Reducing the distance between cells enhances the likelihood that visible agglutinates will form. Strategies to achieve this goal include: reducing the negative charge of surface molecules; modifying the ionic composition of the suspending medium; reducing the ionic strength of the suspending medium by introducing macromolecules; or reducing the hydration layer around the cells.

**Positively Charged Molecules**

In the presence of positively charged polymers, such as Polybrene®, normal red cells exhibit spontaneous aggregation, which can be dispersed by sodium citrate. This may be due to neutralization of the significant negative charge contributed by the numerous sialic acid residues characteristic of the red cell membrane, a theory supported by the observation that Polybrene® does not affect cells lacking sialic acid (from genetic causes or after treatment with various enzymes). Another explanation for this polycation-induced aggregation is that association of the macromolecules with charged molecules on the cell membrane extrudes the water molecules that form the hydration shell.

**Other Factors**

Properties of the membrane itself affect agglutination. Mobility and clustering of antigen-bearing molecules exert an incompletely understood effect. Antibody molecules will attach to cells in which the membrane has been rendered nondeformable without denaturation of antigens, but agglutination will not occur. Treating red cells with certain proteolytic enzymes cleaves polypeptides from the membrane, thereby altering the steric configuration and modifying intercellular interactions. By removing many molecules that carry sialic acid residues, these enzymes also affect surface charge.

**The Antiglobulin Test**

In 1945, Coombs, Mourant, and Race described procedures for detecting attachment of antibodies that did not produce agglutination. This was first used to demonstrate antibody in serum, but later the same principle was used to demonstrate in-vivo coating of red cells with antibody or complement components. This test uses antibody to human globulins and is known as the anti-
man globulin (AHG) test. As used in immunohematology, AHG testing generates visible agglutination of sensitized red cells. The direct antiglobulin test (DAT) is used to demonstrate in-vivo sensitization of red cells, and is a one-step procedure. It is used in investigating autoimmune hemolytic anemia (AIHA), drug-induced hemolysis, hemolytic disease of the newborn (HDN), and alloimmune reactions to recently transfused red cells. Two-step procedures, sometimes called indirect antiglobulin testing (IAT), are used to demonstrate in-vitro reactions between red cells and antibodies that sensitize but do not agglutinate cells that express the corresponding antigen. This is useful for detection and identification of antibodies, blood grouping, and compatibility testing.

Principles of the AHG Test

Antiglobulin testing depends on the following principles:

1. All antibody molecules are globulins.
2. Animals injected with human globulins produce antibody to the foreign protein. After the animal serum is adsorbed to remove unwanted agglutinins, it will react specifically with human globulins, and can be called antihuman globulin serum. Depending on the material used for immunization and the procedures used to separate the resulting immune products, AHG sera with varying specificities can be produced, notably anti-IgG and antibodies to several complement components. (Hybridoma techniques, now used for most AHG manufacture, are described in Chapter 10.)
3. The antiglobulin antibody combines with the Fc portion of the sensitizing antibody molecules, not with any epitopes native to the red cell

(see Fig 11-1). The two Fab sites of the AHG molecule form a bridge between adjacent antibody-coated cells to produce visible agglutination. Cells that have no attached globulin will not be agglutinated. The strength of the observed agglutination is usually proportional to the amount of bound globulin.

4. AHG will react with human globulin molecules that are bound to red cells or are present, free in serum. Unbound globulins react preferentially with AHG, and may prevent the indicator serum from combining with membrane-bound globulin molecules. Unless the red cells are washed free of unbound proteins before addition of AHG serum, the unbound globulins may neutralize AHG and cause a false-negative result.

Direct Antiglobulin Testing

The DAT is used to demonstrate in-vivo coating of red cells with globulins, in particular IgG and C3dg. Washed red cells...
cells from a patient or donor are tested directly with AHG reagents. (See Method 3.6.)

Two-Stage (Indirect) Antiglobulin Testing

In indirect AHG procedures, serum is incubated with red cells, which are then washed to remove unbound globulins. Agglutination that occurs when AHG is added indicates a reaction between antibody in the serum and antigen present on the red cells. The antibody may be known and the antigen unknown, as in blood grouping tests with AHG-reactive reagents such as anti-Fy\textsuperscript{a}; the antigenic composition of the red cells may be known and the presence or specificity of antibody unknown, as in antibody detection and identification tests. Both serum and cells are unknown in the AHG cross-match, and the procedure is used to determine whether any sort of antigen-antibody interaction has occurred. Anything that affects the speed or the intensity of antibody attachment (first stage of agglutination) will influence the sensitivity of indirect antiglobulin testing.

It may be possible to eliminate the need for washing the serum-cell system by processing the antiglobulin-requiring reagent antibody. The one technique involves binding the specific antibody onto antigen-positive cells, and then dissociating the antibody, usually by preparing an eluate. Reagent sera processed in this way are not contaminated with unwanted human globulin, so the test system need not be washed before the addition of the AHG reagent. Another technique is the gel test described later in this chapter. The gel test uses a micro-column filled with mixtures of either glass beads or gel, buffer, and sometimes reagents, and can be used for direct or indirect antiglobulin procedures.

Sources of Error

False-Negative Results

The following considerations apply to both direct and indirect tests.

1. Failure to wash red cells adequately is a major cause of false-negative AHG tests, because globulins not bound to red cells react preferentially with AHG sera. One volume of human serum diluted as much as 1:4000 in saline can neutralize an equal volume of AHG serum. Three or four manual washes or automated washing will adequately remove unbound globulins, provided that saline fills the test tubes at least three-quarters full and the red cell button is thoroughly resuspended with each saline addition. The supernatant saline should be decanted thoroughly at each wash phase. Covering the tube with finger, thumb, or palm during resuspension is forbidden; it not only endangers the worker’s health but can also introduce globulins into the wash solution and partially or completely inactivate the AHG reagent.

2. False-negative results can occur if testing is interrupted or delayed. If AHG reagent is not added immediately after washing, previously bound globulins may dissociate from the red cells, and either leave too little IgG on the cells to detect or partially neutralize the AHG reagent, or both. Once AHG has been added, tests should be centrifuged and read immediately because agglutination of IgG-coated cells often weakens with time.

3. Improper technique may cause weakly reactive tests to be misinterpreted as negative. In a study of potential causes of error, testing personnel had great difficulty reading, grading, and interpreting weakly positive tests. Between 5 and 60%
of tests that should have been interpreted as positive were incorrectly interpreted as negative; no positive relationship was observed between results obtained and testing personnel’s experience.

4. AHG reagents can lose activity because of improper storage, bacterial contamination or contamination with human serum. Freezing AHG sera may impair antibody reactivity. Contamination with reagents that contain human serum may completely or partially neutralize AHG reagents. This may not be apparent visually and will only be detected when agglutination of IgG-coated control red cells is diminished. Partial neutralization may not be detected at all, particularly if the control cells are heavily coated with IgG.

5. If AHG reagent has not been added to the system, globulin coating will go undetected. Colored AHG reagents protect against this, but give no indication about preservation of activity.

6. Improper centrifugation influences the sensitivity of AHG tests. Under-centrifugation provides suboptimal conditions for agglutination; over-centrifugation packs red cells so tightly that the agitation required for resuspension may break up fragile agglutinates.

7. Red cell concentration influences reactivity. Weak reactions occur if too many cells are present, but with too few red cells it is difficult to observe agglutination accurately.

8. High concentrations of IgG paraprotein in a patient’s serum may inhibit anti-IgG even after numerous wash phases. Inhibition by cold-precipitating paraprotein can be averted if 1) all phases of collection, storage, separation, and testing are performed at 37 C; or 2) the specimen is incubated overnight at 4 C, followed by cold centrifugation and removal of the supernatant serum or plasma from the cryoprecipitated paraprotein before testing.

9. Low pH of the saline wash solutions can decrease sensitivity of the AHG test. Commercially available saline often has a pH around 6.0; the optimal saline wash solution is phosphate-buffered to a pH between 7.0 and 7.2.

Note: The addition of IgG-coated cells to negative AHG tests, as required by AABB Standards for antibody detection and crossmatching procedures, does not detect all false-negative results. The conditions described in items 3, 6, and 7 will not be detected by a control system. For the other items, the use of cells too heavily coated with IgG significantly reduces the likelihood of detecting diminished AHG reactivity.

False-Negative DAT Results. Additional causes of false-negative direct antiglobulin results are listed below.

1. Cells coated only with complement may not be agglutinated if the test is read immediately. For maximal detection of bound complement, manufacturers recommend incubation at room temperature for approximately 5 minutes and subsequent centrifugation and examination. Such incubation can convert a negative DAT into a positive DAT when red cells are coated with complement. IgG-coated red cells, however, may give weaker reactions after incubation than at immediate reading. Reading after incubation should never replace an immediate reading. Although a tube may be read immediately after centrifugation and then incubated and read again, optimal sensitivity is achieved with two tubes—one examined after immediate centrifugation and the other after incubation.
2. Cells that have very few bound globulin molecules may give negative results on standard DATs. Polyspecific and anti-IgG antiglobulin reagents have a detection threshold of 200-500 IgG molecules per cell. 

**False-Negative Results in Indirect Procedures.** Additional causes of false-negative results in indirect antiglobulin procedures are listed below.

1. Red cells and serum lose reactivity if improperly stored. Exposure to excessive heat or repeated freezing and thawing impairs antibody reactivity. Red cells undergo hemolysis after freezing or exposure to excessive heat, but some antigens undergo subtle changes at temperatures that do not visibly damage the cells.

2. Rare examples of anti-Jk\(^a\) and anti-Jk\(^b\) may be detected only when polyspecific AHG is used and active complement is present. Most anticoagulants chelate calcium and magnesium ions, essential for complement binding. The use of plasma instead of serum may cause these rare antibodies to go undetected. Serum specimens that are old or have been improperly stored will have impaired complement activity.

**False-Positive Results**

The following considerations apply to both direct and indirect tests:

1. Red cells may already be agglutinated before washing and addition of the AHG serum. Agglutination observed after the addition of AHG may be incorrectly interpreted as indicating IgG or complement attachment. In specimens containing potent cold-reactive autoantibodies, red cells may agglutinate at or below room temperature. This should be detected by observation of the appearance of the washed red cell suspension before addition of immunologically active material. Some antibodies cause direct agglutination of red cells without the addition of AHG. This is, indeed, an antigen-antibody reaction, but the presence of agglutinated cells after addition of AHG should not be interpreted as indicating the presence of IgG or complement components on the red cells.

2. Particles or contaminants in dirty glassware may cause clumping of red cells. If all tests on all blood specimens are weakly reactive, it is worthwhile to use test tubes from another source.

3. Overcentrifugation may pack red cells so tightly that clumping cannot be completely dispersed and may be mistaken for agglutination.

**False-Positive DAT Results.** Additional causes of false-positive direct antiglobulin results are listed below.

1. Complement components, primarily C4, may bind to red cells from blood clots or donor segments in CPDA-1 kept at 4 C, and occasionally when these specimens are kept at room temperature. This reflects the activity of naturally occurring, cold-reactive, complement-activating autoagglutinins often present in human serum. The cells are, in fact, coated with complement, but attachment occurred during storage and not in vivo. DATs should be performed on red cells from specimens anticoagulated with EDTA, ACD, or CPD.

2. Red cells from specimens collected into tubes containing silicone gel may have spurious complement attachment. Geisland and Milam found 13% of blood specimens collected into such tubes gave inappropriately positive results on direct antiglobulin testing.

3. Complement may attach to cells in specimens collected from infusion...
lines used to administer dextrose-containing solutions. The strongest reactions are observed when large-bore needles are used or when the sample volume obtained is less than 0.5 mL.

**False-Positive Results on Indirect Testing.** Red cell specimens with a positive DAT will give positive results in any and every indirect antiglobulin procedure. IgG-coated red cells generally cannot be accurately tested with antiglobulin-reactive reagents. Procedures for removing IgG from DAT-positive red cells are given in Methods 2.11, 2.13, and 2.14. With these procedures, it is often possible to remove sufficient IgG to allow testing with antisera that require addition of AHG, but such tests must be rigorously controlled. The use of a solution containing proteolytic enzyme and a thiol reagent (ZZAP) denatures LW, γ, Fy, S, s, Yt, Ch, Rg, Pr, and Tn antigens, and all the antigens of the Kell blood group system. This reagent should only be used when other techniques for IgG removal, such as gentle heat treatment or chloroquine, have failed and in tests for antigens other than those listed above. Any manipulation for IgG removal may alter red cell antigens and distort tests with blood grouping reagents. This is particularly true for the Kell blood group system. It is essential, therefore, that control cells and test cells be treated simultaneously and tests be performed in parallel.

**Reagents for Antiglobulin Testing**

The Center for Biologics Evaluation and Research of the Food and Drug Administration (FDA) has established definitions for a variety of AHG reagents as shown in Table 11-1. The properties and applications of these reagents are discussed below.

**Polyspecific AHG**

Polyspecific AHG reagents are used for direct antiglobulin testing and, in many laboratories, for routine compatibility tests and antibody detection. These reagents contain antibody to human IgG and to the C3d component of human complement. Other anticomplement antibodies may be present, including anti-C3b, -C4b, and -C4d. Commercially prepared polyspecific antiglobulin sera contain little, if any, activity against IgA and IgM heavy chains. However, they may react with IgA or IgM molecules, because the polyspecific mixture may react with lambda and kappa light chains, which are present in immunoglobulins of all classes.

Because most clinically significant antibodies are IgG, the most important function of polyspecific AHG, in most procedures, is detection of IgG. AHG reagents are prepared and standardized to detect a wide variety of IgG antibodies. In addition, the FDA requires that reagents marketed as polyspecific AHG contain anti-C3d activity at a level that equals or exceeds the FDA's anti-C3d reference serum. Anticomplement activity has limited usefulness in crossmatching and in antibody detection because antibodies detectable only by their ability to bind complement are quite rare. Anti-C3d activity is important, however, for the DAT, especially in the investigation of AIHA. In some patients with AIHA, C3dg may be the only globulin detectable on their red cells.

**Monospecific AHG Reagents**

Monospecific antibodies to human globulins can be prepared by injecting animals with purified IgG, IgA, IgM, C3, or C4. Such sera generally require ad-
sorption to remove unwanted antibodies from the monospecific AHG reagent. Monospecific reagents can effectively be prepared from hybridomas. Hybridoma-derived antibodies can be combined into reagent preparations containing any desired combination of specificities, or a combination of different clonal products all recognizing the same specificity.

Licensed monospecific AHG reagents in common use are anti-IgG and anti-C3b,-C3d. The FDA has established labeling requirements for other anticomplement reagents, including anti-C3b, anti-C4b, and anti-C4d, but these products are not generally available. After direct antiglobulin testing reveals globulins on a red cell specimen, monospecific AHG reagents are used to characterize the coating proteins. Anti-IgG and anti-C3d are also used in indirect antiglobulin testing to distinguish patterns of reactivity in a single serum that contains both complement-binding and non-complement-binding antibodies, eg, a mixture of anti-Le\(^a\) and anti-E.

### Anti-IgG
Reagents labeled “anti-IgG” contain no anticomplement activity. The major component of anti-IgG is antibody to human gamma chains, but unless labeled as “heavy chain specific,” these reagents may exhibit some reactivity with light chains, which are common to all immunoglobulin classes. An anti-IgG reagent not designated “heavy chain spe-

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**Table 11-1. Antihuman Globulin Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyspecific (Rabbit polyclonal; rabbit/murine monoclonal blend; and murine monoclonal)</td>
<td>Rabbit polyclonal contains anti-IgG and anti-C3d (may contain other anticomplement and other anti-immunoglobulin antibodies); rabbit/murine monoclonal blend contains a blend of rabbit polyclonal antihuman IgG and murine monoclonal anti-C3b and -C3d; murine monoclonal contains murine monoclonal anti-IgG, -C3b, and -C3d.</td>
</tr>
<tr>
<td>Anti-IgG (rabbit polyclonal; IgG heavy chains; monoclonal IgG)</td>
<td>Rabbit polyclonal contains anti-IgG with no anticomplement activity (not necessarily gamma chain specific); IgG heavy chains contain only antibodies reactive against human gamma chains; monoclonal IgG contains murine monoclonal anti-IgG.</td>
</tr>
<tr>
<td>Anti-C3d and anti-C3b, (rabbit polyclonal) and anti-C3d, -C4b, -C4d (rabbit polyclonal)</td>
<td>Contain only antibodies reactive against the designated complement component(s), with no anti-immunoglobulin activity.</td>
</tr>
<tr>
<td>Anti-C3d (murine monoclonal) and anti-C3b, -C3d (murine monoclonal)</td>
<td>Contains only antibodies reactive against the designated complement component, with no anti-immunoglobulin activity.</td>
</tr>
</tbody>
</table>

* As defined by the FDA.
specific” must be considered capable of reacting with light chains of IgA or IgM. A positive DAT with such an anti-IgG does not prove the presence of IgG, although it is rare to have in-vivo coating with IgA or IgM, in the absence of IgG. Many workers prefer anti-IgG over polyspecific AHG in antibody detection and compatibility tests because anti-IgG AHG does not react with complement bound to red cells by cold-reactive antibodies that are not clinically significant.

**Anti-C3b,-C3d**

Anti-C3b,-C3d reagents prepared by animal immunization contain no activity against human immunoglobulins and are used in situations described for anti-C3d. This type of anti-C3d characteristically reacts with C3b and possibly other epitopes present on C3-coated red cells. Murine monoclonal anti-C3b,-C3d reagent is a blend of hybridoma-derived antibodies. Monoclonal anti-C3d may be less likely to react with C3b than anti-C3d obtained by animal immunization.

**Role of Complement in Antiglobulin Reactions**

Complement components may attach to red cells in vivo or in vitro by one of two mechanisms:

1. Complement-binding antibody specific for a red cell antigen may cause attachment of complement to the cell surface as a consequence of specific alloimmune recognition.
2. Immune complexes, of various specificities unrelated to red cell antigens, may be present in plasma and activate complement components that adsorb onto red cells in a non-specific manner. Attachment of complement to the membrane of cells not involved in the specific antigen-antibody reaction is often described as “innocent bystander” complement coating.

Red cells coated with elements of the complement cascade may or may not undergo hemolysis. If the cascade does not go to completion, the presence of bound early components of the cascade can be detected by anticomplement reagents. The component most readily detected is C3, because several hundred C3 molecules may be bound to the red cell by the attachment of only a few antibody molecules. C4 coating also can be detected, but C3 coating has more clinical significance.

**Complement as the Only Coating Globulin**

Complement alone, without immunoglobulin, may be present on washed red cells in certain situations.

1. IgM antibodies reacting in vitro occasionally attach to red cell antigens without agglutinating the cells. IgM coating is difficult to demonstrate in AHG tests, partly because IgM molecules tend to dissociate during the washing process and partly because polyspecific AHG contains little if any anti-IgM activity. IgM antibodies characteristically activate complement, so the reaction of antibody with antigen can best be demonstrated by identifying the several hundred C3 molecules bound to the cell membrane near the site of antibody attachment.
2. About 10-20% of patients with warm AIHA have red cells with a positive DAT due to C3 coating alone. No IgG, IgA, or IgM coating is demonstrable with routine procedures, although some specimens may be coated with IgG at levels below the detection threshold for the standard DAT.
3. In cold hemagglutinin disease, the cold-reactive autoantibody can react with red cell antigens at tempera-
tures up to 32 C, although it does not cause agglutination. Red cells passing through vessels in the skin at this temperature become coated with autoantibody, which activates complement. If the cells escape hemolysis, they return to the central circulation where the temperature is 37 C, and the autoantibody dissociates from the cells, leaving complement components firmly bound to the red cell membrane. The component usually detected by AHG reagents is C3dg.

4. Immune complexes that form in the plasma and bind weakly and nonspecifically to red cells may cause complement coating. The activated complement remains on the red cell surface after the immune complexes dissociate. C3 remains as the only detectable surface globulin.

**Nontraditional Methods to Detect Antigen-Antibody Reactions**

The methods that follow are not an exhaustive listing. They represent some commonly discussed methods and alternatives to traditional tube testing and use of antiglobulin serum.

**Inhibition of Agglutination**

In agglutination inhibition tests, the presence of either antigen or antibody is detected by its ability to inhibit agglutination in a system with known reactants. For example, the saliva from a secretor contains soluble blood group antigens that combine with anti-A, -B, or -H. The indicator system is a standardized dilution of antibody that agglutinates the corresponding cells to a known degree. If the saliva contains blood group substance, incubating saliva with antibody will wholly or partially abolish agglutination of cells added to the incubated mixture. Absence of expected agglutination indicates the presence of soluble antigen in the material under test. Agglutination of the indicator cells is a negative result.

**Immunofluorescence**

Immunofluorescence testing allows identification and localization of antigens inside or on the surface of cells. A fluorochrome such as fluorescein or phycoerythrin can be attached to an antibody molecule, without altering its specificity or its ability to bind antigen. Attachment of fluorescent-labeled antibody to cellular antigen makes the antibody-coated cells appear as brightly visible yellow-green or red (depending on the fluorochrome).

Immunofluorescent antibodies can be used in direct or indirect procedures, with the fluorescence endpoint analogous to agglutination as an endpoint. In a direct test, the fluorescent-labeled antibody is specific for a single antigen of interest. In an indirect test, fluorescent-labeled antiglobulin serum is added to cells that have been incubated with an unlabeled antibody of known specificity. Immunofluorescent techniques were initially used to detect antigens in or on lymphocytes or in tissue sections. More recently, immunofluorescent antibodies have been used in flow cytometry where, among their many applications, they have been used to quantify fetomaternal hemorrhage, to identify transfused cells and follow their survival in recipients, to measure low levels of cell-bound IgG, and to distinguish homozygous from heterozygous expression of blood group antigens.

**Radioimmunoassay**

Radioimmunoassay procedures use a suitable radionuclide as a marker for either
antigen or antibody, to be used in either direct or indirect tests. Radiolabeling does not affect antibody specificity, and the quantity of antibody bound can be accurately measured. In one indirect technique, the presence and quantity of antigen can be demonstrated by allowing the test material to interact with an unlabeled, known antigen bound to a solid phase. If present, antigen combines with and is immobilized by the solid-phase antibody. Radiolabeled antibody of the same specificity can then attach to the immobilized antigen in a quantitative manner, and be precisely measured with a gamma counter. Radiolabeling is also used in competitive binding procedures in which labeled and unlabeled antibody of the same specificity react with the corresponding antigen. Calculating the proportion of a known dose of labeled material bound in a test system permits calculation of the amount of unlabeled material that must have been present.

**Enzyme-Linked Immunosorbent Assay**

Enzyme-linked immunosorbent assays (ELISAs) are used to measure either antigen or antibody. Enzymes such as alkaline phosphatase can be bound to antibody molecules without destroying either the antibody specificity or the enzyme activity. The enzyme acts as a quantifiable label, similar to radiolabeling, but enzymes are more stable; are safer, cheaper, and simpler to measure; and, in many cases, provide results that are comparably sensitive. Many tests to detect antibodies to transfusion-transmitted viruses use this principle. ELISA has also been used to detect and measure cell-bound IgG and to demonstrate fetomaternal hemorrhage. When red cells are examined, the test often is called an enzyme-linked antiglobulin test (ELAT).

**Solid-Phase Red Cell Adherence Tests**

Solid-phase microplate techniques long used in immunologic assays (eg, for HBsAg) are now being applied to identification of red cell antigens or antibodies. In a direct test, antibody is fixed to the microplate well and red cells are added. If the cells express the corresponding antigen, they will adhere to the sides of the well; if no antigen-antibody reaction occurs, the red cells settle to the bottom of the well. An indirect test uses red cells of known antigenic composition bound to a well pretreated with a compound such as glutaraldehyde and poly L-lysine or with a potent serum-specific antibody. The test serum is added to the red cell-coated wells and allowed to react with the cells, after which the plates are washed free of unbound serum proteins. The indicator for attached antibody is a suspension of IgG-coated red cells. The reaction is positive if the indicator cells adhere to the sides of the well. If they settle to the bottom, it demonstrates that no antigen-antibody reaction has occurred.

**Gel Test**

In 1986, Lapierre patented a technology that uses gel particles. The gel test uses six microtubes instead of test tubes, contained in what is called a gel card. The microtube card format allows simultaneous centrifugation of six different antigen-antibody systems. The gel particles function as filters that trap red cell agglutinates when the cards are centrifuged. Larger agglutinates are trapped at the top of the microtubes; smaller agglutinates are filtered out near the lower portion of the microtubes; and unagglutinated cells are forced through the microtubes and settle at the tapered tips. With suitably selected reagents, the procedure can be used to detect and identify
cell-surface antigens or serum antibodies, and to perform crossmatching.

Additives

Enzymes
Proteolytic enzymes are used in serologic tests primarily to reduce the red cell surface charge by cleaving sialic acid molecules from polysaccharide chains. Sialic acid is a major contributor to the net negative charge at the red cell surface, which keeps red cells separated from each other in an ionic suspending medium. Any mechanism that reduces the net charge should enhance red cell agglutination, and red cells pretreated with proteolytic enzymes often show enhanced agglutination by IgG molecules. However, red cells pretreated with neuraminidase demonstrate no comparable increase in agglutinability. This may reflect differences among enzymes in the effect they have on the water of hydration. The proteolytic enzymes used most often in immunohematology laboratories are bromelin, ficin, papain, and trypsin. While enhancing agglutination by some antibodies, the enzymes destroy certain red cell antigens, notably M, N, S, Fy^a, and Fy^b.

Polybrene®
Hexadimethrine bromide (Polybrene®) can be used to increase test sensitivity in certain systems. Polybrene® generally is added to red cells that have been incubated with antibody at low ionic strength and low pH. Polybrene® causes the cells to aggregate very closely, but the aggregation can be dissociated by the addition of a salt solution such as sodium citrate. If antibody had attached to the red cells at the time Polybrene® was added, the addition of citrate would not disperse the antibody-linked agglutinates. If AHG is used in a Polybrene® system, care must be taken to avoid false-positive reactions due to the detection of bound complement components. Problems have been encountered in Polybrene® procedures with antibodies in the Kell system.

Polyethylene Glycol
Polyethylene glycol (PEG) is used as an additive to increase antibody uptake. Its action is to remove water, thereby effectively concentrating antibody, promoting antibody uptake, and, in many cases, enhancing reaction strength. Anti-IgG is the AHG reagent of choice with PEG testing, to avoid false-positive reactions. IgM antibodies, especially those of the ABO and Lewis systems, have diminished reactivity or nonreactivity in PEG procedures. If too high a concentration of PEG is added to a test mixture, proteins may precipitate.

Albumin Additives
Bovine serum albumin (BSA) is available as solutions of 22% or 30% concentration, as a polymerized solution, or as solutions with low ionic strength buffers. BSA, unless used under low ionic conditions, does little to promote antibody uptake; it influences the second stage of agglutination by reducing the net negative charge of the red cells or by affecting the water of hydration.

LISS Additives
Most commercially available LISS additives contain macromolecules in addition to ionic salts and buffers. LISS solutions increase the rate of antibody association when volume proportions are correct. (See Methods 3.2.2 and 3.2.3.) Increasing the volume of serum used in a test will increase the ionic strength of a LISS-additive system. When LISS is used as an additive re-
agent, the manufacturer’s instructions must be followed. Some antibodies in the Kell blood group system give weaker-than-expected reactions in LISS-additive systems.

References
