CHAPTER

five

The Antiglobulin Test

Ralph E.B. Green, B. App. Sci., FAIMLS, MACE, **and**
Virginia C. Hughes, MS, MT(ASCP)SBB, CLS(NCA)I

OBJECTIVES

On completion of this chapter, the learner should be able to:

1. State the principle of the antiglobulin test.
2. Differentiate monoclonal from polyclonal and monospecific from polyspecific antihuman globulin (AHG) reagents.
3. Describe the preparation of monoclonal and polyclonal AHG reagents.
4. Explain the antibody requirements for AHG reagents.
5. Discuss the use of polyspecific versus monospecific AHG in the indirect antiglobulin test (IAT).
6. Discuss the advantages and disadvantages of anticomplement activity in polyspecific AHG.

7. Compare and contrast the IAT and the direct antiglobulin test (DAT).
8. Include an explanation of (1) principle, (2) applications, and (3) red blood cell sensitization.
9. List the reasons for the procedural steps in the DAT and IAT.
10. Interpret the results of a DAT panel.
11. List the factors that affect the antiglobulin test.
12. List the sources of error associated with the performance of the antiglobulin test.
13. Discuss new techniques for antiglobulin testing.

Introduction

The antiglobulin test (also called Coombs' test) is based on the principle that antihuman globulins (AHGs) obtained from immunized nonhuman species bind to human globulins such as IgG or complement, either free in serum or attached to antigens on red blood cells (RBCs).

There are two major types of blood group antibodies, IgM and IgG. Because of their large pentamer structure, IgM antibodies bind to corresponding antigen and directly agglutinate RBCs suspended in saline. IgG antibodies are termed *nonagglutinating* because their monomer structure is too small to agglutinate sensitized RBCs directly. The addition of AHG containing anti-IgG to RBCs sensitized with IgG antibodies allows for hemagglutination of these sensitized cells. Some blood group antibodies have the ability to bind complement to the RBC membrane. Antiglobulin tests detect IgG and/or complement-sensitized RBCs.

History of the Antiglobulin Test

Before the discovery of the antiglobulin test, only IgM antibodies had been detected. The introduction of the antiglobulin test permitted the detection of nonagglutinating IgG antibodies and led to the discovery and characterization of many new blood group systems.

In 1945, Coombs and associates[1] described the use of the antiglobulin test for the detection of weak and nonagglutinating Rh antibodies in serum. In 1946, Coombs and coworkers[2] described the use of AHG to detect in-vivo sensitization of the RBCs of babies suffering from hemolytic disease of the newborn (HDN). Although the test was initially of great value in the investigation of Rh HDN, its versatility for the detection of other IgG blood group antibodies soon became evident. The first of the Kell blood group system antibodies[3] and the associated antigen were reported only weeks after Coombs had described the test.

Although Coombs and associates[1] were instrumental in introducing the antiglobulin test to blood group serology, the principle of the test had in fact been described by Moreschi[4] in 1908. Moreschi's studies involved the use of rabbit antigoat serum to agglutinate rabbit RBCs that were sensitized with low nonagglutinating doses of goat anti-rabbit RBC serum.

Coombs' procedure involved the injection of human serum into rabbits to produce antihuman serum. After absorption to remove heterospecific antibodies and dilution to avoid prozone, the AHG serum still retained sufficient antibody activity to permit cross-linking of adjacent RBCs sensitized with IgG antibodies. The cross-linking of sensitized RBCs by AHG produced hemagglutination, indicating that the RBCs had been sensitized by an antibody that had reacted with an antigen present on the cell surface.

The antiglobulin test can be used to detect RBCs sensitized with IgG alloantibodies, IgG autoantibodies, and complement components. Sensitization can occur either in vivo or in vitro. The use of AHG to detect in-vitro sensitization of RBCs is a two-stage technique referred to as the indirect antiglobulin test (IAT). In-vivo sensitization is detected by a one-stage procedure, the direct antiglobulin test (DAT). The IAT and DAT still remain the most common procedures performed in blood group serology.

AHG Reagents

Several AHG reagents have been defined by the Food and Drug Administration (FDA) Center for Biologics Evaluation and Research (CBER). These are listed in **Table 5–1** and are

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TABLE 5–1   Antihuman Globulin Reagents

Reagent	Definition
Polyspecific	
1. Rabbit polyclonal	Contains anti-IgG and anti-C3d (may contain other anticomplement and other anti-immunoglobulin antibodies).
2. Rabbit/murine monoclonal blend	Contains a blend of rabbit polyclonal antihuman IgG and murine monoclonal anti-C3b and -C3d.
3. Murine monoclonal	Contains murine monoclonal anti-IgG, anti-C3b, and anti-C3d.
Monospecific Anti-IgG	
1. Rabbit polyclonal	Contains anti-IgG with no anti-complement activity (not necessarily gamma-chain specific).
2. IgG heavy-chain specific	Contains only antibodies reactive against human gamma chains.
3. Monoclonal IgG	Contains murine monoclonal anti-IgG
Anticomplement	
Rabbit polyclonal 1. Anti-C3d and anti-C3b 2. Anti-C3d, anti-C4b, anti-C4d	Contains only antibodies reactive against the designated complement component(s), with no anti-immunoglobulin activity.
Murine Monoclonal 1. Anti-C3d 2. Anti-C3b, anti-C3d	Contains only antibodies reactive against the designated complement component, with no anti-immunoglobulin activity.

Modified from Tyler, V (ed): Technical Manual, ed 12. American Association of Blood Banks, Bethesda, MD, 1996.

discussed in the following paragraphs. Antihuman globulin reagents may be polyspecific or monospecific.

Polyspecific AHG

Polyspecific AHG contains antibody to human IgG and to the C3d component of human complement. Other anticomplement antibodies, such as anti-C3b, anti-C4b, and anti-C4d, may also be present. Commercially prepared polyspecific AHG contains little, if any, activity against IgA and IgM heavy chains. However, the polyspecific mixture may contain antibody activity to kappa and lambda light chains common to all immunoglobulin classes, thus reacting with IgA or IgM molecules.[5]

Monospecific AHG

Monospecific AHG reagents contain only one antibody specificity: either anti-IgG or antibody to specific complement components such as C3b or C3d. Licensed monospecific AHG reagents in common use are anti-IgG and anti-C3b-C3d.[5]

Anti-IgG

Reagents labeled anti-IgG contain no anticomplement activity. Anti-IgG reagents contain antibodies specific for the Fc fragment of the gamma heavy chain of the IgG molecule. If not labeled "gamma heavy chain–specific," anti-IgG may contain anti–light chain specificity and therefore react with cells sensitized with IgM and IgA as well as with IgG.[5]

Anti-Complement

Anti-complement reagents, such as anti-C3b-C3d reagents, are reactive against the designated complement components only and contain no activity against human immunoglobulins.[5]

Preparation of AHG

The classic method of AHG production involves injecting human serum or purified globulin into laboratory animals, such as rabbits. The human globulin behaves as foreign antigen, the rabbit's immune response is triggered, and an antibody to human globulin is produced. For example, human IgG injected into a rabbit results in anti-IgG production; human complement components injected into a rabbit result in anticomplement. This type of response produces a polyclonal antiglobulin serum. Polyclonal antibodies are a mixture of antibodies from different plasma cell clones. The resulting polyclonal antibodies recognize different antigenic determinants (epitopes), or the same portion of the antigen but with different affinities. Hybridoma technology can be used to produce monoclonal antiglobulin serum. Monoclonal antibodies are derived from one clone of plasma cells and recognize a single epitope.

Preparation of Polyspecific AHG

Polyclonal AHG Production

Polyclonal AHG is usually prepared in rabbits, although when large volumes of antibody are required, sheep or goats may be used. In contrast with the early production methods, in which a crude globulin fraction of serum was used as the immunogen, modern production commences with the purification of the immunogen from a large pool of normal sera.

Conventional polyspecific antiglobulin reagents are produced by immunizing one colony of rabbits with human immunoglobulin (IgG) antigen and another colony with human C3 antigen. Because of the heterogeneity of IgG molecules, the use of serum from many donors to prepare the pooled IgG antigen to immunize the rabbits and the pooling of anti-IgG from many immunized rabbits are essential in producing reagents for routine use that are capable of detecting the many different IgG antibodies. This is an advantage of using anti-IgG of polyclonal origin for antiglobulin serum.[6]

Both colonies of animals are hyperimmunized to produce high-titer, high-avidity IgG antibodies. Blood specimens are drawn from the immunized animals, and if the antibody potency and specificity meet predetermined specifications, the animals are bled for a production batch of reagent. Separate blends of the anti-IgG and anticomplement antibodies are made, and each pool is then absorbed with A_1, B, and O cells to remove heterospecific antibodies. The total antibody content of each pool is determined, and the potency of the pools is analyzed to calculate the optimum antibody dilution for use. Block titrations for anti-IgG pools are performed by reacting dilutions of each antibody against cells sensitized with different amounts of IgG. This is a critical step in the manufacturing process because excess antibody, especially with anti-IgG, may lead to prozoning and, hence, false-negative test results.

Because it is not possible to coat cells with measured amounts of complement, the potency of anti-C3 pools is measured using at least two examples each of a C3b- and C3d-coated cell. Both anti-C3b (C3c) and anti-C3d are present in the polyclonal anti-C3 pool. The level of anti-C3d is particularly critical in keeping false-positive tests to a minimum yet detecting clinically significant amounts of RBC-bound C3d. Additionally, if the dilution of the anti-C3 pool is determined on the basis of the amount of anti-C3d present, the level of anti-C3b (C3c) varies. The inability to determine the potency of anti-C3b and anti-C3d individually is one of the difficulties with polyclonal reagents that can be avoided with monoclonal products.[6] Once the required performance characteristics of the trial blend are obtained, a production blend of the separate anti-IgG and anticomplement pools is made.

Monoclonal AHG Production

The monoclonal antibody technique devised by Kohler and Milstein[7] has been used to produce AHG and has proved particularly useful in producing high-titer antibodies with well-defined specificities to IgG and to the fragments of C3.[8–10]

Monoclonal antibody production begins with the immunization of laboratory animals, usually mice, with purified human globulin. After a suitable immune response, mouse spleen cells containing antibody-secreting lymphocytes are fused with myeloma cells. The resulting "hybridomas" are screened for antibodies with the required specificity and affinity. The antibody-secreting clones may then be propagated in tissue culture or by inoculation into mice, in which case the antibody is collected as ascites. Because the clonal line produces a single antibody, there is no need for absorption to remove heterospecific antibodies. All antibody molecules
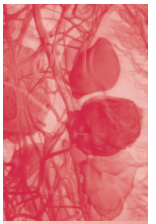
produced by a clone of hybridoma cells are identical in terms of antibody structure and antigen specificity. This has advantages and disadvantages in AHG production. Once an antibody-secreting clone of cells has been established, antibody with the same specificity and reaction characteristics will be available indefinitely. This allows the production of a consistently pure and uncontaminated AHG reagent. The disadvantage is that all antibodies produced by a clone of cells recognize a single epitope present on an antigen. For antigens composed of multiple epitopes such as IgG, several different monoclonal antibodies reacting with different epitopes may need to be blended, or a monoclonal antibody specificity for an epitope on all variants of a particular antigen may need to be selected to ensure that all different expressions of the antigen are detected. Monoclonal antibodies to human complement components anti-C3b and anti-C3d may be blended with polyclonal anti-IgG from rabbits to achieve potent reagents that give fewer false-positive reactions as a result of anticomplement; Gamma Biologicals manufactures AHG reagents from an entirely monoclonal source. The anti-IgG component is produced by exposing mice to RBCs coated with IgG. The resulting monoclonal anti-IgG reacts with the C_H3 region of the gamma chain of IgG subclasses 1, 2, and 3. The antibody does not react with human antibodies of subclass IgG_4, but these are not considered to be clinically significant. Blending the monoclonal anti-IgG with a monoclonal anti-C3b and monoclonal anti-C3d results in a polyspecific AHG reagent. The preparation of polyclonal and monoclonal AHG is dia-

grammed in **Figure 5–1**. Before the AHG is available for purchase, manufacturers must subject their reagents to an evaluation procedure, and the results must be submitted to the United States Food and Drug Administration for approval. Whether produced by the polyclonal or monoclonal technique, the final polyspecific product is one that contains both anti-IgG and anticomplement activity at the correct potency for immediate use. The reagent also contains buffers, stabilizers, and bacteriostatic agents and may be dyed green for identification.
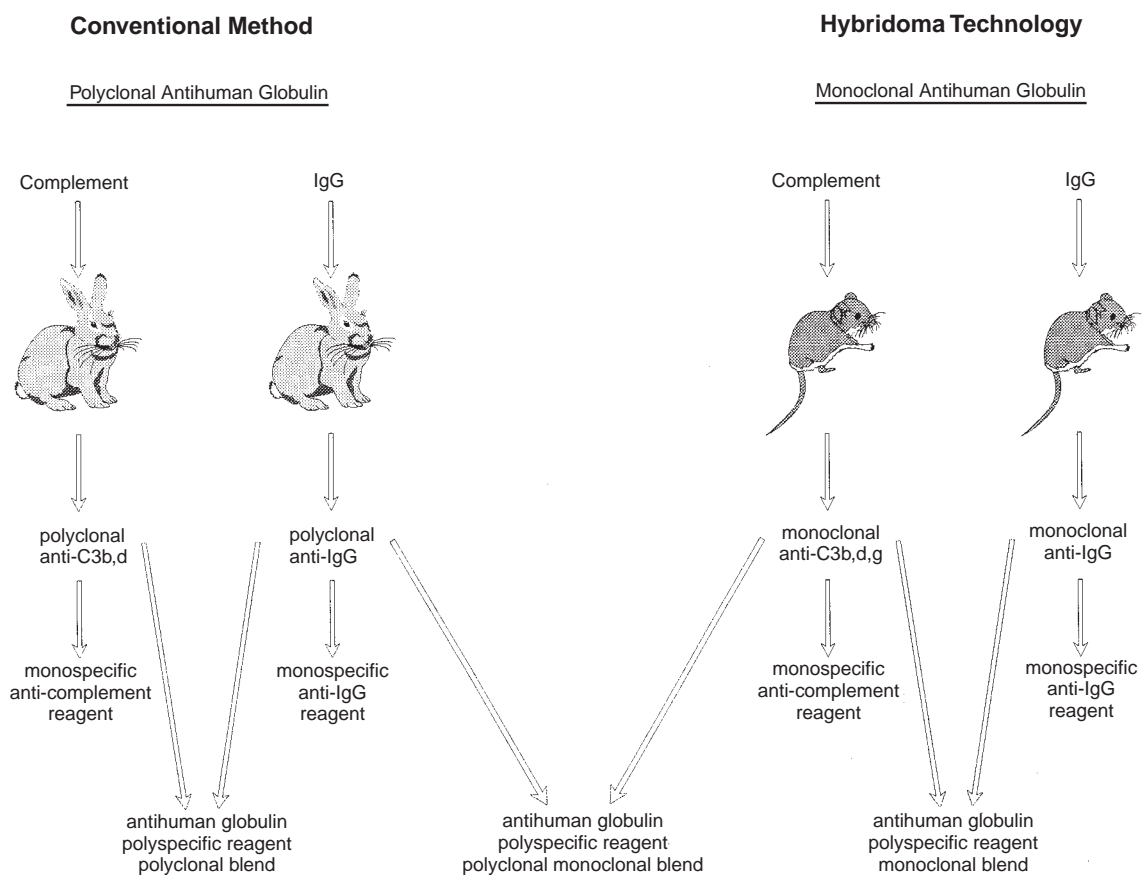
Preparation of Monospecific AHG

Monospecific AHG is prepared by a production process similar to that described for polyspecific AHG; however, it contains only one antibody specificity. Monospecific anti-IgG is usually of polyclonal origin; however, monoclonal anti-IgG has been prepared effectively by hybridoma technology. Monospecific anticomplement reagents are often a blend of monoclonal anti-C3b and monoclonal anti-C3d.

Antibodies Required in AHG

Anti-IgG

AHG must contain antibody activity to nonagglutinating blood group antibodies. The majority of these antibodies are a mixture of IgG_1 and IgG_3 subclass. Rarely, nonagglutinating

■ **FIGURE 5–1**   Preparation of AHG reagents. Polyspecific antihuman globulin may be manufactured by combining polyclonal anti-IgG with either polyclonal or monoclonal anticomplement components. A monoclonal blend may be manufactured by blending monoclonal anti-C3b, monoclonal anti-C3d, and monoclonal anti-IgG. Monospecific antihuman globulin reagents can be manufactured by conventional or hybridoma technology.

IgM antibodies may be found; however, they have always been shown to fix complement and may be detected by anti-complement.[11] IgA antibodies with Rh specificity have been reported; however, IgG antibody activity has always been present as well. The only RBC alloantibodies that have been reported as being solely IgA have been examples of anti-Pr,[12] and those antibodies were agglutinating. IgA autoantibodies have been reported, although very rarely.[13] Therefore, anti-IgG activity must be present in the AHG reagent. Anti-IgM and anti-IgA activity may be present, but neither is essential. The presence of anti–light-chain activity allows detection of all immunoglobulin classes.

Anti-Complement

Some antibodies "fix" complement components to the RBC membrane after complexing of the antibody with its corresponding antigen. These antibodies are listed in **Table 5–2**. These membrane-bound complement components can be detected by the anticomplement activity in AHG.

Early AHG reagents were prepared using a crude globulin fraction as the immunogen. In 1947, Coombs and Mourant demonstrated that the antibody activity that detected Rh antibodies was associated with the anti–gamma globulin fraction in the reagent. The first indication that there might be another antibody activity present that had an influence on the final reaction was presented by Dacie in 1951.[14] He observed that different reaction patterns were obtained when dilutions of AHG were used to test cells sensitized with "warm" as compared with "cold" antibodies. In 1957, Dacie and coworkers[15] published data showing that the reactivity of AHG to cells sensitized with "warm" antibodies resulted from anti–gamma globulin activity, whereas anti–nongamma globulin activity was responsible for the activity of cells sensitized by "cold" antibodies. The nongamma globulin component was shown to be beta globulin and had specificity for complement. Later studies[16,17] revealed that the complement activity was a result of C3 and C4.

During the 1960s many reports were published indicating the need for anticomplement activity in AHG to allow the detection of antibodies by the IAT.[18–21] Many of the specificities mentioned in these reports were ones that are now generally considered to be of little clinical significance (e.g., anti-Le^a, anti-P$_1$, and anti-H). However, one specificity that

TABLE 5–2 Antibodies Capable of Binding Complement		
Most	**Some**	**Rare**
ABO	S	S
Le^a	Xg^a	P$_1$
Le^b	LKE	Lu^a
Jk^a	Lan	Lu^b
Jk^b		Kell
Sc1		Fy^a
Co3		Fy^b
Ge2		Co^b
Ge3		
Ii		
P		
P^k		
Vel		

Note: These antibodies are discussed in detail in Chapters 6 through 9.

was consistently mentioned and that is considered clinically significant was anti-Jk^a. Evidence was also presented showing that the presence of anticomplement activity would enhance the reactions of clinically significant antibodies (e.g., anti-Fy^a and anti-K).[18]

Use of Polyspecific Versus Monospecific AHG in the IAT

As previously stated, polyspecific AHG contains both anti-IgG activity and anti-C3 activity. There is considerable debate among immunotransfusionists over the use of monospecific anti-IgG versus polyspecific AHG for routine antibody detection and pretransfusion testing. Because most clinically significant antibodies detected during antibody screening are IgG, the most important function of polyspecific AHG is the detection of IgG antibodies.

There have been numerous reports of clinically significant RBC alloantibodies that were not detectable with monospecific anti-IgG but were detected with the anticomplement component of AHG.[22] Unfortunately, polyspecific AHG has also been associated with unwanted positive reactions that are not caused by clinically significant antibodies. To investigate these variables, Petz and coworkers[23] examined 39,436 sera comparing monospecific anti-IgG with polyspecific AHG. They also compared the albumin technique with low ionic strength solutions (LISS)-suspended RBCs. Four Jk^a antibodies were detected with polyspecific but not with monospecific anti-IgG using albumin or LISS-suspended RBCs. An additional anti-Jk^a was detected only with polyspecific AHG when using LISS but not with albumin. Also, five antibodies of anti-Kell, anti-Jk^a, and Fy^a specificities were detected when using LISS, but not albumin, with both polyspecific AHG and anti-IgG. Their results concluded that some clinically significant antibodies are detected with the anticomplement component of AHG but not with anti-IgG. This is especially true for anti-Jk^a, a complement-binding IgG antibody often associated with delayed hemolytic transfusion reactions.

Petz and others[22] also determined the number of false-positive reactions obtained when using polyspecific AHG versus anti-IgG with LISS and albumin. False-positive reactions were defined as those caused by antibodies with no definable specificity or by antibodies considered to be clinically insignificant because of optimum reactivity at cold temperatures (anti-I, anti-H, anti-P$_1$, anti-M). Of the unwanted positive reactions, 93 percent were shown to be caused by C3 on the cells. The authors emphasize that, if the first step in evaluating a weakly positive AHG reaction is to repeat using the prewarmed technique, about 60 percent of the false-positive weak reactions become negative.

In a 3-year study, Howard and associates[24] found eight patients whose antibodies were detected primarily or solely by AHG containing anticomplement activity. Seven of these antibodies had anti-Jk^a or anti-Jk^b specificity. Some of them could be detected using homozygous Jk^a or Jk^b cells and an AHG containing only anti-IgG activity. Two of the anti-Jk^a antibodies were associated with delayed hemolytic transfusion reactions. The complement-only Kidd antibodies represented 23 percent of all Kidd antibodies detected during the study. The authors concluded that they would continue to use polyspecific AHG reagent for routine compatibility testing.

In summary, one must balance the advantage of detecting

clinically significant complement-only antibodies with the disadvantages resulting from using antiglobulin serum containing anti-complement activity.[22] A decision on the use of the AHG reagent for indirect tests is the prerogative of the individual blood bank. Many blood banks have adopted the use of monospecific anti-IgG for routine pretransfusion testing, citing cost containment measures necessitated by the high number of repeats versus the rarity of complement-only detected antibodies such as anti-Jk[a]. Milam[25] states rare clinical transfusion intolerance when using monospecific anti-IgG over polyspecific AHG reagents to screen for unexpected antibodies and to test for blood group compatibility offers reliability without interference from common and clinically insignificant IgM-complement fixing antibodies.

AHG Reagents and the DAT

The DAT detects in vivo sensitization of RBCs with IgG and/or complement components. During complement activation, C3 and C4 are split into two components. C3b and C4b bind to the RBC membrane, whereas C3a and C4a pass into the fluid phase. Further degradation of membrane-bound C3b and C4b occurs by removal of C3c and C4c to leave C3d and C4d firmly attached to the RBC membrane.[26–28] Anti-C3c was considered by the ISBT/ICSH Joint Working Party[29] to be the most important anticomplement component, because of its limited capacity to cause nonspecific reactions. However, when RBCs are incubated with serum for longer than 15 minutes, the number of C3c determinants falls rapidly because C3c is split off the C3bi molecule. This finding further supports the use of anti-C3d in international reference reagents by the Joint Working Party. The final degradation step has been shown to occur in vivo[30] and, in fact, is a common occurrence in both warm and cold autoimmune hemolytic anemias. Engelfriet and others[31] have also shown that degradation of C3b to C3d can occur in vitro, providing that the incubation period is greater than 1 hour. In 1976, Garratty and Petz[32] confirmed the need for anti-C3d activity in AHG for use in the DAT. They also confirmed Engelfriet's observation that, given sufficient time, cell-bound C3b could be degraded to C3d in vitro.

The detection of C3d on the RBC membrane is important in the investigation of both warm and cold autoimmune hemolytic anemia (AIHA). Many cases of warm AIHA are associated with both IgG and C3d coating the RBCs. In cold AIHA, C3d may be the only globulin detectable on the RBC. Characterization of AIHA requires the detection of the specific globulin sensitizing the RBCs in vivo, usually IgG or C3d or both. In the investigation of AIHA, a DAT is performed initially with polyspecific AHG. If globulins are detected on the RBC membrane, follow-up testing with monospecific AHG (anti-IgG, anti-C3d) is performed to identify the coating proteins. Although the RBCs of most patients with AIHA are coated with IgG, the cells of some patients will exhibit both IgG and complement coating or complement alone. The presence of complement alone may support the diagnosis of AIHA, rendering the finding significant.[25]

Principles of the Antiglobulin Test

The antiglobulin test is based on the following simple principles:[33]

1. Antibody molecules and complement components are globulins.
2. Injecting an animal with human globulin stimulates the animal to produce antibody to the foreign protein (i.e., AHG). Serologic tests employ a variety of AHG reagents reactive with various human globulins, including anti-IgG, antibody to the C3d component of human complement, and polyspecific reagents that contain both anti-IgG and anti-C3d activity.
3. AHG reacts with human globulin molecules, either bound to RBCs or free in serum.
4. Washed RBCs coated with human globulin are agglutinated by AHG.

The complete procedures for the direct and indirect antihuman globulin tests can be found in the procedural appendix at the end of this chapter. Color Plate 1 summarizes the methodology of both tests. Figure 5–2 illustrates in-vitro sensitization detected in the IAT and in-vivo sensitization detected by the DAT.
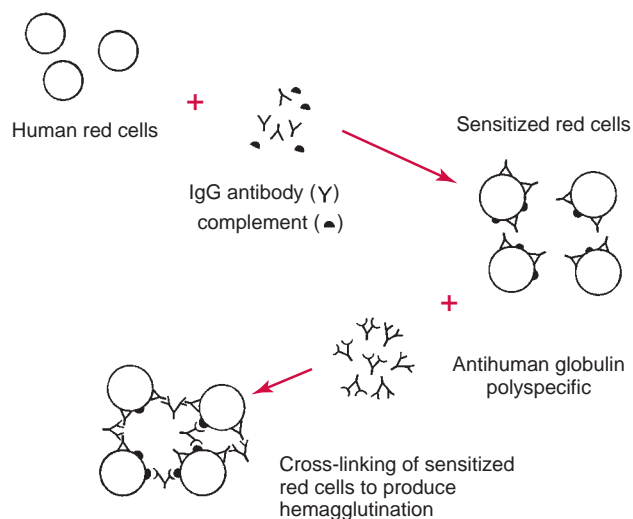
DAT

Principle and Application of the DAT

The DAT detects in-vivo sensitization of RBCs with IgG and/or complement components. Clinical conditions that can result in in-vivo coating of RBCs with antibody and/or complement are:

1. Hemolytic disease of the newborn (HDN)
2. Hemolytic transfusion reaction (HTR)
3. Autoimmune and drug-induced AIHA.

Table 5–3 lists the clinical application and in-vivo sensitization detected for each situation.

The DAT is not a required test in routine pretransfusion protocols. In a study by the College of American Pathologists in 1998,[34] 54 percent of 4299 laboratories surveyed reported

Human red cells

IgG antibody (Y)
complement (▲)

Sensitized red cells

Antihuman globulin
polyspecific

Cross-linking of sensitized
red cells to produce
hemagglutination

■ FIGURE 5–2   AHG test. The IAT is used to determine in-vitro sensitization of RBCs, whereas the DAT is used to detect in-vivo sensitization. Polyspecific AHG contains anti-IgG and anti-complement activity.

TABLE 5–3 Direct Antiglobulin Test	
Principle: Detects In-Vivo RBC Sensitization	
Application	**In-Vivo Sensitization**
HDN	Maternal antibody coating fetal RBCs
HTR	Recipient antibody coating donor RBCs
AIHA	Autoantibody coating individual's RBCs

using the DAT in pretransfusion testing, the primary rationale being early detection of alloimmunization. Eder[35] tested the clinical utility of the DAT at a large tertiary care hospital in Philadelphia. A retrospective study was performed from 1999 to 2002. DATs with anti-IgG were performed on 15,662 pretransfusion patient samples; 15 percent were positive. Subsequent eluate testing revealed nonreactivity in 76 percent; 9 percent panreactive; and 12 percent passively acquired ABO or D antibodies. Only one case demonstrated an RBC antibody in the eluate that was not detected in the serum, concluding that even in a tertiary care setting the routine DAT is inefficient yielding a positive predictive value of 0.16 percent. Judd and coworkers revealed similar findings on 65,049 blood samples in a 29-month period, where only 5.5 percent of samples resulted in a positive DAT.[36]

DAT Panel

Initial DATs include testing one drop of a 3 to 5 percent suspension of washed RBCs with polyspecific (anti-IgG, anti-C3d) reagent. Positive results are monitored by a DAT panel using monospecific anti-IgG and anti-C3d to determine the specific type of protein sensitizing the cell. Some institutions choose to run polyspecific and monospecific reagents at one time as well as a saline control. The saline control serves to detect spontaneous agglutination of cells or reactions occurring without the addition of AHG reagents. In warm AIHA, including drug-induced hemolytic anemia, the RBCs may be coated with IgG or C3d, or both. Patterns of reactivity and the type of protein sensitization in AIHA are summarized in **Table 5–4**. In a transfusion reaction workup, the DAT may demonstrate IgG or C3d, or both, depending on the nature and specificity of the recipient's antibody. In the investigation of HDN, testing for complement proteins is not necessary inasmuch as the

TABLE 5–4 DAT Panel: Patterns of Reactivity in Autoimmune Hemolytic Anemia*		
Anti-IgG	**Anti-C3d**	**Type of AIHA**
+	+	WAIHA (67%)
+	−	WAIHA (20%)
−	+	CHD; PCH, WAIHA (13%)

Modified from Walker, RH (ed): Technical Manual, ed 10. American Association of Blood Banks, Arlington, VA, 1990.

*The DAT with monospecific antiglobulin reagents is helpful in classifying AIHAs. Other procedures and studies are necessary to diagnose and characterize which form of autoimmune disease is present.

WAIHA = warm autoimmune hemolytic anemia; CHD = cold hemagglutinin disease; PCH = paroxysmal cold hemoglobinuria

protein sensitizing the newborn RBCs is presumed to be maternal IgG. Problems can arise in accurate D typing in the case of a newborn with a positive DAT. If the DAT is positive due to IgG and the immediate spin for D typing is negative, a test for weak D cannot be performed. The same is true for a patient with AIHA due to a warm IgG antibody coating the patient cells. The antibody must be removed from the RBCs for accurate phenotyping. Other techniques can be used to remove antibody from the patients RBCs. These include chloroquine diphosphate, EDTA-glycine, and a method using murine monoclonal antibodies.

Evaluation of a Positive DAT

Clinical consideration should dictate the extent to which a positive DAT is evaluated. Interpreting the significance of a positive DAT requires knowledge of the patient's diagnosis, drug therapy, and recent transfusion history. A positive DAT may occur without clinical manifestations of immune-mediated hemolysis. **Table 5–5** describes the in-vivo phenomena that may be associated with a positive DAT.

The American Association of Blood Banks *Technical Manual*[33] states that "results of serological tests are not diagnostic; their significance can only be assessed in relationship to the patient's clinical condition." Answering the following questions before investigating a positive DAT for patients other than neonates will help determine what further testing is appropriate:

1. Is there evidence of in-vivo hemolysis?
2. Has the patient been transfused recently?
3. Does the patient's serum contain unexpected antibodies?
4. Is the patient receiving any drugs?
5. Has the patient received blood products or components containing ABO-incompatible plasma?
6. Is the patient receiving antilymphocyte globulin or antithymocyte globulin?
7. Is the patient receiving IVIG or IV RhIG?

IAT (Indirect Antiglobulin Test)

Principle and Application of the IAT

The IAT is performed to determine in-vitro sensitization of RBCs and is used in the following situations:

1. Detection of incomplete (nonagglutinating) antibodies to potential donor RBCs (compatibility testing) or to screening cells (antibody screen) in serum
2. Determination of RBC phenotype using known antisera (e.g., Kell typing, weak D testing)
3. Titration of incomplete antibodies

Table 5–6 lists the IATs and the in-vitro sensitization detected for each application.

For in-vitro antigen-antibody reactions, the IAT tasks are listed and explained in **Table 5–7**.

The DAT does not require the incubation phase because of the antigen-antibody complexes formed in vivo.

Factors Affecting the Antiglobulin Test

The DAT can detect a level of 100 to 500 IgG molecules per RBC and 400 to 1100 molecules of C3d per RBC.[31]

TABLE 5–5   In-Vivo Phenomena Associated with a Positive DAT

Transfusion	1. Recipient alloantibody and donor antigen	Alloantibodies in the recipient of a recent transfusion that react with antigen on donor RBC.
	2. Donor antibody and recipient antigen	Antibodies present in donor plasma that react with antigen on a transfusion recipient's RBCs.
Drug induced	1. Drug adsorption	Penicillin adsorbed to RBCs in vivo. Antipenicillin reacts with the penicillin bound to the RBCs. Penicillin-coated RBCs become coated with IgG.
	2. Immune complex adsorption mechanism	Drug and specific antibody form complexes that attach nonspecifically to RBCs and initiate complement activation on the RBC surface.
	3. Membrane modification	RBCs coated with cephalothin (Keflin) adsorb albumin, IgA, IgG, IgM, and α and β (i.e., complement) globulins. The DAT is reactive with anti-IgG and anti-C3d.
	4. Autoimmunity	Following α-methyldopa therapy, autoantibodies are formed, which react with intrinsic RBC antigens. DAT is reactive with anti-IgG.

	Type (DAT Reactivity)	
Autoimmune hemolytic anemia	1. WAIHA (IgG and/or C3)	Autoantibody reacts with patient's RBCs in vivo.
	2. CHD (C3)	Cold-reactive IgM autoagglutinin binds to RBCs in peripheral circulation (32°C). IgM binds complement as RBCs return to warmer parts of circulation; IgM dissociates, leaving RBCs coated only with complement.
	3. PCH (IgG)	The IgG autoantibody reacts with RBCs in colder parts of body, causes complement to be bound irreversibly to RBCs, and then elutes at warmer temperature.
Hemolytic disease of newborn	1. Maternal alloantibody crosses placenta (IgG)	Maternal (IgG) alloantibody, specific for fetal antigen, coats fetal RBCs. DAT is reactive with anti-IgG.
Miscellaneous	1. Administration of equine preparations of antilymphocyte globulin and antithymocyte globulin	Heterophile antibodies that are present in ALG or ATG coat recipient's RBCs.
	2. Administration of high-dose IV gamma globulin	Non–antibody-mediated binding of immunoglobulin to RBCs in patients with hypergammaglobulinemia

Modified from Walker, RH (ed): Technical Manual, ed 10. American Association of Blood Banks, Arlington, VA, 1990.
AIHA = autoimmune hemolytic anemia; CHD = cold hemagglutinin disease; PCH = paroxysmal cold hemoglobinuria.

For the IAT there must be between 100 and 200 IgG or C3 molecules on the cell to obtain a positive reaction. The number of IgG molecules that sensitize an RBC and the rate at which sensitization occurs can be influenced by several factors, outlined as follows:

Ratio of serum to cells. Increasing the ratio of serum to cells increases the sensitivity of the test system. Generally, a minimum ratio of 40:1 should be aimed for, and this can be achieved by using 2 drops of serum and 1 drop of a 5 percent volume of solute per volume of solution (v/v) suspension of cells.[35] When using cells suspended in saline, it is often advantageous to increase the ratio of serum to cells in an effort to detect weak antibodies (e.g., 4 drops of serum with 1 drop of a 3 percent [v/v] cell suspension will give a ratio of 133:1).

Reaction medium. *Albumin:* The macromolecules of albumin allow antibody-coated cells to come into closer contact with each other so that aggregation occurs. In 1965, Stroup and MacIlroy[37] reported on the increased sensitivity of the IAT if albumin was incorporated into the reaction medium. Their reaction mixture, consisting of 2 drops of serum, 2 drops of 22 percent (w/v) bovine albumin, and 1 drop of 3 to 5 percent (v/v) cells, was shown to provide the same sensitivity at 30 minutes of incubation as a 60-minute saline test. The use of albumin does not seem to provide any advantage over LISS techniques and adds to the cost of the test.[37] Petz and cowork-

TABLE 5–6   Indirect Antiglobulin Test

Principle: Detects In-Vitro Sensitization		
Application	**Tests**	**In-Vitro Sensitization**
Antibody detection	Compatibility testing	Recipient antibody reacting with donor cells
	Antibody screening	Antibody reacting with screening cells
Antibody identification	Antibody panel	Antibody reacting with panel cells
Antibody titration	Rh antibody titer	Antibody and selected Rh cells
RBC phenotype	RBC antigen detection (ex: weak D, K, Fy)	Specific antisera + RBCs to detect antigen

TABLE 5–7   Tasks and Purposes of the Indirect Antigloblin Test

Task	Purpose
Incubate RBCs with antisera	Allows time for antibody molecule Attachment to RBC antigen
Perform a minimum of three saline washes	Removes free globulin molecules
Add antiglobulin reagent	Forms RBC agglutinates (RBC Ag + Ab + anti-IgG)
Centrifuge	Accelerates agglutination by bringing cells closer together
Examine for agglutination	Interprets test as positive or negative
Grade agglutination reactions	Determines the strength of reaction
Add antibody-coated RBCs to negative reactions	Checks for neutralization of antisera by free globulin molecules (Coombs' control cells are D-positive RBCs coated with anti-D)

ers[23] also showed that an albumin technique may miss a number of clinically significant antibodies.

LISS: These enhance antibody uptake and allow incubation times to be decreased. Some LISS also contain macromolecular substances. The LISS technique introduced by Low and Messeter[38] has critical requirements with respect to the serum-to-cell ratio. Moore and Mollison[39] showed that optimum reaction conditions were obtained using 2 drops of serum and 2 drops of a 3 percent (v/v) suspension of cells in LISS. Increasing the serum-to-cell ratio increased the ionic strength of the reaction mixture, leading to a decrease in sensitivity, thus counteracting the shortened incubation time of the test. A LISS medium may be achieved by either suspending RBCs in LISS or using a LISS additive reagent.

Polyethylene glycol (PEG): PEG is a water-soluble linear polymer and is used as an additive to increase antibody uptake. Its action is to remove water, thereby effectively concentrating antibody. Anti-IgG is the AHG reagent of choice with PEG testing to avoid false-positive reactions.[5] Because PEG may cause aggregation of RBCs, reading for agglutination following 37°C incubation in the IAT is omitted. Several investigators[40] compared the performance of PEG as an enhancement media with that of LISS. Findings indicated that PEG increases the detection of clinically significant antibodies while decreasing detection of clinically insignificant antibodies. Barrett and associates[41] reported that as PEG has been used for pretransfusion antibody screening, 6353 RBC components have been transfused without any reported acute or delayed HTRs.

Temperature. The rate of reaction for the majority of IgG antibodies is optimal at 37°C; therefore, this is the usual incubation temperature for the IAT. This is also the optimum temperature for complement activation.

Incubation time. For cells suspended in saline, incubation times may vary between 30 and 120 minutes. The majority of clinically significant antibodies can be detected after 30 minutes of incubation, and extended incubation times are usually not necessary. If a LISS technique is being used,[38,39] incubation times may be shortened to 10 to 15 minutes. With these shortened times, it is essential that tubes be incubated at a temperature of 37°C. Extended incubation (i.e., up to 40 minutes) in the LISS technique has been shown to cause antibody to elute from the RBCs, causing a decrease in the sensitivity of the test.[42] However, this could not be confirmed by Voak and coworkers.[43]

Washing of RBCs. When both the DAT and IAT are performed, RBCs must be saline-washed a minimum of three times before the addition of AHG reagent. Washing the RBCs removes free unbound serum globulins. Inadequate washing may result in a false-negative reaction because of neutralization of the AHG reagent by residual unbound serum globulins.

Washing should be performed in as short a time as possible to minimize the elution of low-affinity antibodies. The cell pellet should be completely resuspended before adding the next saline wash. All saline should be discarded completely after the final wash because residual saline dilutes the AHG reagent and therefore decreases the sensitivity of the test.

Centrifugation at each wash should be sufficient to provide a firm cell pellet and therefore minimize the possible loss of cells with each discard of saline.

Saline for washing. Ideally, the saline used for washing should be fresh or, alternatively, buffered to a pH of 7.2 to 7.4. Saline stored for long periods in plastic containers has been shown to decrease in pH, which may increase the rate of antibody elution during the washing process.[44] Changes in pH may have important implications when monoclonal AHG is used, inasmuch as monoclonal antibodies have been shown to have narrow pH ranges for optimum reactivity. Significant levels of bacterial contamination in saline have been reported[45]; this situation can contribute to false-positive results.

Addition of AHG. AHG should be added to the cells immediately after washing to minimize the chance of antibody eluting from the cell and subsequently neutralizing the AHG reagent. The volume of AHG added should be as indicated by the manufacturers. However, Voak and associates[46] have shown that adding two volumes of AHG may overcome washing problems when low levels of serum contamination remain. These authors indicated that the neutralization of AHG is a problem only with free IgG left in serum following inadequate saline washings and not with residual serum complement components. The complement fragments free in serum are not the same as the complement fragments bound to RBCs, and therefore residual serum does not contain C3b and C3d to neutralize the anti-C3b and anti-C3d in AHG reagent.

Centrifugation for reading. Centrifugation of the cell pellet for reading of hemagglutination along with the method used for resuspending the cells is a crucial step in the technique. The CBER-recommended method for the evaluation of AHG uses 1000 relative centrifugal forces (RCFs) for 20 seconds, although the technique described in this chapter suggests 500 RCFs for 15 to 20 seconds. The use of higher RCFs yields more sensitive results; however, depending on how the pellet is resuspended, it may give weak false-positive results because of inadequate resuspension or alternatively may give

a negative result if resuspension is too vigorous. The optimum centrifugation conditions should be determined for each centrifuge.

Sources of Error

Some of the more common sources of error associated with the performance of the AHG test have been outlined in the previous section. **Box 5–1** lists reasons for false-negative and false-positive AHG reactions. An anticoagulant such as EDTA should be used to collect blood samples for the DAT in order to avoid the in-vitro complement attachment associated with refrigerated clotted specimens.

   All negative antiglobulin test reactions must be checked by the addition of IgG-sensitized cells. Adding IgG-coated RBCs to negative test reactions should demonstrate hemagglutination of these RBCs with the anti-IgG in the AHG reagent. If no hemagglutination follows the addition of IgG-coated RBCs, the test result is invalid and the test must be repeated. The most common technical errors that result in failure to demonstrate hemagglutination after the addition of IgG-coated RBCs are inadequate washing, nonreactive AHG reagent, and failure to add AHG reagent. While most blood banks do not check monospecific anti-C3d reactivity with the addition of C3d-coated RBCs to negative reactions, these cells are available and may be produced in-house.[47]

BOX 5–1
Sources of Error in the Antihuman Globulin Technique

False-Positive Results

- Improper specimen (refrigerated, clotted) may cause in-vitro complement attachment
- Autoagglutinable cells
- Bacterial contamination of cells or saline used in washing
- Cells with a positive direct AHG test used for the IAT
- Saline contaminated by heavy metals or colloidal silica
- Dirty glassware
- Overcentrifugation and overreading
- Polyagglutinable cells
- Preservative-dependent antibody in LISS reagents (IAT)
- Contaminating antibodies in the AHG reagent
- Centrifugation of test with polyethylene glycol prior to washing

False-Negative Results

- Inadequate or improper washing of cells
- AHG reagent nonreactive because of deterioration or neutralization
- AHG reagent not added
- Serum not added in the indirect test
- Serum nonreactive because of deterioration of complement
- Inadequate incubation conditions in the IAT
- Cell suspension either too weak or too heavy
- Undercentrifuged or overcentrifuged
- Poor reading technique
- Low pH of saline

Rosenfield, RE, et al: Solid phase serology for the study of human erythrocytic antigen-antibody reactions. Proc Fifteenth Congr Int Soc Blood Trans, Paris, 1976, p 27.

Modified and Automated Antiglobulin Test Techniques

Modifications to the antiglobulin test technique (LISS, PEG, and albumin) have been mentioned; however, some other modifications may be used in special circumstances.

Low Ionic Polybrene Technique

In 1980, Lalezari and Jiang[48] reported on the adaptation of the automated low ionic polybrene (LIP) technique for use as a manual procedure. The technique relies on low ionic conditions to rapidly sensitize cells with antibody. Polybrene, a potent rouleaux-forming reagent, is added to allow the sensitized cells to approach each other to permit cross-linking by the attached antibody. A high ionic strength solution is then added to reverse the rouleaux; however, if agglutination is present, it will remain. The test can be carried through to an AHG technique if required. If this is performed, a monospecific anti-IgG reagent must be used because the low ionic conditions cause considerable amounts of C4 and C3 to coat the cells and would give false-positive reactions if a polyspecific reagent were used.

   The antiglobulin test has also been performed using microplates. Crawford and colleagues[49] used microplates for a number of different grouping procedures, including the IAT. Microplate technology is used increasingly in blood group serology, and many techniques are being adapted for it. Redman and associates[50] have adapted the LIP technique for use in microplates. Although their report does not include the use of an AHG phase, this additional step could easily be included.

Enzyme-Linked Antiglobulin Test

In the enzyme-linked antiglobulin test (ELAT), an RBC suspension is added to a microtiter well and washed with saline. AHG, which has been labeled with an enzyme, is added. The enzyme-labeled AHG will bind to IgG-sensitized RBCs. Excess antibody is removed, and enzyme substrate is added. The amount of color produced is measured spectrophotometrically and is proportional to the amount of antibody present. The optical density is usually measured at 405 nm. The number of IgG molecules per RBC can also be determined from this procedure.

Solid Phase

Solid-phase technology may be used for the performance of antiglobulin tests. Several different techniques have been reported using either test tubes[51] or microplates.[52,53] With the availability of microplate readers, this modification lends itself to the introduction of semiautomation. Direct and indirect tests can be performed using solid-phase methodology. In the former, antibody is attached to a microplate well, and RBCs are added. If antibody is specific for antigen on RBCs, the bottom of the well will be covered with suspension; if no such specificity occurs, RBCs will settle to the bottom of the well. In the latter, known RBCs are bound to a well that has been treated with glutaraldehyde or poly L-lysine. Test serum is added to RBC-coated wells, and if antibody in serum is specific for antigen on fixed RBCs, a positive reaction occurs as described above.

Immucor Incorporated manufactures a solid-phase system for the detection and identification of alloantibodies. Group O reagent RBC membranes are bound to the surfaces of polystyrene microtitration strip wells. IgG antibodies from patient or donor sera are bound to the membrane antigens. After incubation, unbound immunoglobulins are rinsed from the wells; then a suspension of anti-IgG–coated indicator RBCs is added to the wells. Centrifugation brings the indicator RBCs in contact with antibodies bound to the reagent RBC membranes. If the test result is negative, a pellet of indicator RBCs forms in the bottom of the wells. A positive test causes adherence of the indicator RBCs, forming anti–IgG-IgG complexes and a second immobilized RBC layer.

The Gel Test

The gel test is a process to detect RBC antigen-antibody reactions by means of using a chamber filled with polyacrylamide gel. The gel acts as a trap; free unagglutinated RBCs form pellets in the bottom of the tube, whereas agglutinated RBCs are trapped in the tube for hours. Therefore, negative reactions appear as pellets in the bottom of the microtube, and positive reactions are fixed in the gel.

There are three different types of gel tests: neutral, specific, and antiglobulin. A neutral gel does not contain any specific reagent and acts only by its property of trapping agglutinates. The main applications of neutral gel tests are antibody screening and identification with enzyme-treated or untreated RBCs and reverse ABO typing. Specific gel tests use a specific reagent incorporated into the gel and are useful for antigen determination. The low ionic antiglobulin test (GLIAT) is a valuable application of the gel test and may be used for the IAT or the DAT. AHG reagent is incorporated into the gel. For example, in an IAT gel, 50 μL of a 0.8 percent RBC suspension is pipetted onto a gel containing AHG, serum is added, and the tube is centrifuged after a period of incubation. At the beginning of centrifugation, the RBCs tend to pass through the gel, but the medium in which they are suspended remains above. This results in separation between the RBCs and the medium without a washing phase. RBCs come in contact with AHG in the upper part of the gel, and the positive and negative reactions are separated. The detection of unexpected antibodies by GLIAT compares favorably with conventional AHG methods and provides a safe, reliable, and easy-to-read AHG test.[54]

For the DAT, 50 μL of a 0.8 percent RBC suspension in LISS solution (ID-Diluent 2) is added to the top of each microtube of the LISS/Coombs ID cards. The cards are centrifuged at 910 rpm for 10 minutes.[55] In the case of a positive reaction, monospecific reagents (anti-IgG, anti-C3d) can be used in the gel test.

Traditional Tube Technique Versus the Gel Test in the DAT

There have been numerous studies comparing the tube and gel test when performing DATs. The main difference in the two techniques is that the former requires washing, and the latter omits a washing stage, resulting in discrepant results between the two methods. Chuansumrit et al[56] compared the conventional tube technique with the gel test in evaluating ABO HDN. Sixty infants with hyperbilirubinemia were tested: 22 cases were ABO-incompatible (A or B infants born to group O mothers), and 38 were ABO-compatible with the mother. Whereas the positive rates of the DAT in the incompatible group were comparable, 54.5 percent (tube) and 50 percent gel test; the second group showed a positive DAT rate of 2.6 percent (tube) and 10.5 percent (gel). The infants were shown to have hyperbilirubinemia, and the antibody coating the cells was found to be IgG only, using monospecific reagents. The authors concluded the DAT via the gel test is beneficial in detecting ABO HDN.

Lai et al[57] described a case of AIHA with a negative DAT using the traditional tube test and a positive result using the gel test. The study found a warm low affinity antibody in the patient's serum, by means of the gel test, that was lost in the tube technique through washing when performing the DAT. The authors concluded that because the gel test does not include washing steps, the elution of low-affinity autoantibody may be avoided but the eluate may yield a negative result. Additionally, the gel test, because of its no-wash nature, might be warranted in the case of a suspected AIHA. Mitek et al[58] compared the gel test to the tube and ELAT techniques. They found the gel test to be more sensitive in the case of hypergammagloblinemic patients, yielding positive results due to IgG in the gel test and negative results in the tube and ELAT. Blood banks should be aware of the differences in the DAT when using the very popular gel test over and tube technique. Additional comparative studies will add to the current body of knowledge.

The changes in blood bank technology, along with the changes in emphasis on the importance of crossmatching versus antibody screening, will probably further modify the role of the antiglobulin test over the coming years. At present, however, it still remains the most important test in the blood bank for the detection of clinically significant antibodies to RBCs and for the detection of immune hemolysis.

CASE STUDIES

Case One

A 32-year old white female gave birth to a 5 lb 3 oz healthy male. The mother was an RhIg candidate in that she typed as O, D-negative. A cord blood was sent down to the blood bank for ABO, Rh, and DAT. The baby forward-typed as A negative, weak D-positive. The DAT was also positive with polyspecific AHG and monospecific anti-IgG. The technologist realized the test for weak D could not be reported in the presence of a positive DAT and reported the type as A unknown.

Questions

1. What further testing is indicated?
2. Why is a weak test for the D antigen not performed in the presence of a positive DAT?

Case Two

A 54-year old white male is admitted for an exploratory laparotomy. A type and antibody screen is ordered prior to his scheduled surgery. ABO and Rh typing reveal the patient is O-positive, and the blood bank technologist performed an antibody screen using the patient serum and a 3-screening cell kit. Reactions were all negative at 37° and AHG. One drop of Coombs' check cells was added to each tube, and the results were nonreactive.

$\boxed{Q}$uestions

1. What is the correct course of action in this case?
2. Give reasons why the addition of Coombs' check cells resulted in nonreactivity.
3. What do Coombs' control cells consist of?

S U M M A R Y C H A R T :

Important Points to Remember (MT/MLT)

➤ The antiglobulin test is used to detect RBCs sensitized by IgG alloantibodies, IgG autoantibodies, and/or complement components.

➤ AHG reagents containing anti-IgG are needed for the detection of IgG antibodies because the IgG monomeric structure is too small to directly agglutinate sensitized RBCs.

➤ Polyspecific AHG sera contain antibodies to human IgG and the C3d component of human complement.

➤ Monospecific AHG sera contain only one antibody specificity: either anti-IgG or antibody to anti–C3b-C3d.

➤ Classic AHG sera (polyclonal) are prepared by injecting human globulins into rabbits, and an immune stimulus triggers production of antibody to human serum.

➤ Hybridoma technology is used to produce monoclonal antiglobulin serum.

➤ The DAT detects in-vivo sensitization of RBCs with IgG and/or complement components. Clinical conditions that can result in a positive DAT include HDN, HTR, and AIHA.

➤ The IAT detects in-vitro sensitization of RBCs and can be applied to compatibility testing, antibody screen, antibody identification, RBC phenotyping, and titration studies.

➤ A positive DAT is followed by a DAT panel using monospecific anti-IgG and anti-C3d to determine the specific type of protein sensitizing the RBC.

➤ EDTA should be used to collect blood samples for the DAT to avoid in-vitro complement attachment associated with refrigerated clotted specimens.

REVIEW QUESTIONS

1. A principle of the antiglobulin test is:
   a. IgG and C3d are required for RBC sensitization
   b. Human globulin is eluted from RBCs during saline washings
   c. Injection of human globulin into an animal engenders passive immunity
   d. AHG reacts with human globulin molecules bound to RBCs or free in serum

2. Polyspecific AHG reagent contains:
   a. Anti-IgG
   b. Anti-IgG and anti-IgM

   c. Anti-IgG and anti-C3d
   d. Anti-C3d

3. Monoclonal anti-C3d is:
   a. Derived from one clone of plasma cells
   b. Derived from multiple clones of plasma cells
   c. Derived from immunization of rabbits
   d. Reactive with C3b and C3d

4. Which of the following is a clinically significant antibody whose detection may be dependent on anticomplement activity in polyspecific AHG?
   a. Anti-Jk^a
   b. Anti-Le^a
   c. Anti-P$_1$
   d. Anti-H

5. After the addition of IgG-coated RBCs to a negative AHG reaction during an antibody screen, a negative result is observed. Which of the following is a correct interpretation?
   a. The antibody screen is negative
   b. The antibody screen needs to be repeated
   c. The saline washings were adequate
   d. Reactive AHG reagent was added

6. RBCs must be washed in saline at least three times before the addition of AHG reagent to:
   a. Wash away any hemolyzed cells
   b. Remove traces of free serum globulins
   c. Neutralize any excess AHG reagent
   d. Increase the antibody binding to antigen

7. An in-vitro phenomenon associated with a positive IAT is:
   a. Maternal antibody coating fetal RBCs
   b. Patient antibody coating patient RBCs
   c. Recipient antibody coating transfused donor RBCs
   d. Identification of alloantibody specificity using panel of reagent RBCs

8. False-positive DAT results are most often associated with:
   a. Use of refrigerated, clotted blood sample in which complement components coat RBCs in vitro
   b. A recipient of a recent transfusion manifesting an immune response to recently transfused RBCs
   c. Presence of heterophile antibodies from administration of globulin
   d. A positive autocontrol caused by polyagglutination

9. Polyethylene glycol enhances antigen-antibody reactions by:
   a. Decreasing zeta potential
   b. Concentrating antibody by removal of water
   c. Increasing antibody affinity for antigen
   d. Increasing antibody specificity for antigen

10. Solid-phase antibody screening is based on:
    a. Adherence
    b. Agglutination
    c. Hemolysis
    d. Precipitation

11. A positive DAT may be found in which of the following situations?
    a. A weak D-positive patient
    b. A patient with anti-K
    c. HDN
    d. An incompatible crossmatch

12. What do Coombs' control cells consist of?
    a. Type A-positive cells coated with anti-D
    b. Type A-negative cells coated with anti-D
    c. Type O-positive cells coated with anti-D
   d. Type O-negative cells coated with anti-D

REFERENCES

1. Coombs, RRA, et al: A new test for the detection of weak and "incomplete" Rh agglutinins. Br J Exp Pathol 26:255, 1945.
2. Coombs, RRA, et al: In vivo isosensisation of red cells in babies with haemolytic disease. Lancet i:264, 1946.
3. Race, RR, and Sanger, R: Blood Groups in Man, ed 6. Blackwell Scientific, Oxford, 1975, p 283.
4. Moreschi, C: Neue Tatsachen über die Blutkorperchen Agglutinationen. Zentralbl Bakteriol 46:49, 1908.
5. Brecher, ME (ed): Technical Manual, ed 14. American Association of Blood Banks, Bethesda, MD, 2002.
6. Issitt, C: Monoclonal Antiglobulin Reagents. Dade International Online, 1997. http://www.dadeinternational.com/hemo/papers/ monoanti.htm.
7. Kohler, G, and Milstein, C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495, 1975.
8. Lachman, PJ, et al: Use of monoclonal antibodies to characterize the fragments of C3 that are found on erythrocytes. Vox Sang 45:367, 1983.
9. Holt, PDJ, et al: NBTS/BRIC 8: A monoclonal anti-C3d antibody. Transfusion 25:267, 1985.
10. Voak, D, et al: Monoclonal antibodies—C3 serology. Biotest Bull 1:339, 1983.
11. Mollison, PL: Blood Transfusion in Clinical Medicine, ed 7. Blackwell Scientific, Oxford, 1983, p 502.
12. Garratty, G, et al: An IgA high titre cold agglutinin with an unusual blood group specificity within the Pr complex. Vox Sang 25:32, 1973.
13. Petz, LD, and Garratty, G: Acquired immune hemolytic anemias. Churchill Livingstone, New York, 1980, p 193.
14. Dacie, JF: Differences in the behaviour of sensitized red cells to agglutination by antiglobulin sera. Lancet ii:954, 1951.
15. Dacie, JV, et al: "Incomplete" cold antibodies: Role of complement in sensitization to antiglobulin serum by potentially haemolytic antibodies. Br J Haematol 3:77, 1957.
16. Harboe, M, et al: Identification of the component of complement participating in the antiglobulin reaction. Immunology 6:412, 1963.
17. Jenkins, GC, et al: Role of C4 in the antiglobulin reaction. Nature 186:482, 1960.
18. Polley, MJ, and Mollison, PL: The role of complement in the detection of blood group antibodies: Special reference to the antiglobulin test. Transfusion 1:9, 1961.
19. Polley, MJ, et al: The role of 19S gamma-globulin blood group antibodies in the antiglobulin reaction. Br J Haematol 8:149, 1962.
20. Stratton, F, et al: The preparation and uses of antiglobulin reagents with special reference to complement fixing blood group antibodies. Transfusion 2:135, 1962.
21. Stratton, F, et al: Value of gel fixation on Sephadex G-200 in the analysis of blood group antibodies. J Clin Pathol 21:708, 1968.
22. Petz, LD, et al: Clinical Practice of Transfusion Medicine, ed 3. Churchill Livingstone, New York, 1996, p 207.
23. Petz, LD, et al: Compatibility testing. Transfusion 21:633, 1981.
24. Howard, JE, et al: Clinical significance of the anti-complement component of antiglobulin antisera. Transfusion 22:269, 1982.
25. Milam JD: Laboratory Medicine Parameter: Utilizing monospecific antihuman globulin to test blood group compatibility. Am J Clin Pathol 104:122, 1995.
26. Lachman, PJ, and Muller-Eberhard, HJ: The demonstration in human serum of "conglutinogen-activating-factor" and its effect on the third component of complement. J Immunol 100:691, 1968.
27. Muller-Eberhard, HJ: Chemistry and reaction mechanisms of complement. Adv Immunol 8:1, 1968.
28. Cooper, NR: Isolation and analysis of mechanisms of action of an inactivator of C4b in normal human serum. J Exp Med 141:890, 1975.
29. Case, J, et al: International reference reagents: Antihuman globulin, an ISBT/ICSH Joint Working Party Report. Vox Sang 77:121, 1999.
30. Brown, DL, et al: The in vivo behaviour of complement-coated red cells: Studies in C6-deficient, Ce-depleted and normal rabbits. Clin Exp Immunol 7:401, 1970.
31. Engelfriet, CP, et al: Autoimmune haemolytic anemias: 111 preparation and examination of specific antisera against complement components and products, and their use in serological studies. Clin Exp Immunol 6:721, 1970.
32. Garratty, G, and Petz, LD: The significance of red cell bound complement components in development of standards and quality assurance for the anti-complement components of antiglobulin sera. Transfusion 16:297, 1976.
33. Brecher, ME (ed): Technical Manual, ed 14. American Association of Blood Banks, Bethesda, MD, 2002.
34. College of American Pathologists: Transfusion medicine survey set J: A final critique, 1998, 1-5.
35. Eder, AF: Evaluation of routine pretransfusion direct antiglobulin test in a pediatric setting. Lab Med 24:680, 2003.
36. Judd, WJ, et al: The evaluation of a positive direct antiglobulin test in pretransfusion testing revisited. Transfusion 26:220, 1986.
37. Stroup, M, and Macilroy, M: Evaluation of the Albumin antiglobulin technique in antibody detection. Transfusion 5:184, 1965.
38. Low, B, and Messeter, L: Antiglobulin test in low-ionic strength salt solution for rapid antibody screening and cross-matching. Vox Sang 26:53, 1974.
39. Moore, HC, and Mollison, PL: Use of a low-ionic strength medium in manual tests for antibody detection. Transfusion 16:291, 1976.
40. Shirley, R, et al: Polyethylene glycol versus low-ionic strength solution in pretransfusion testing: A blinded comparison study. Transfusion 34:5, 1994.
41. Barrett, V, et al: Analysis of the routine use of polyethylene glycol (PEG) as an enhancement medium. Immunohematology 11:1, 1995.
42. Jorgensen, J, et al: The influence of ionic strength, albumin and incubation time on the sensitivity of indirect Coombs' test. Vox Sang 36:186, 1980.
43. Voak, D, et al: Low-ionic strength media for rapid antibody detection: Optimum conditions and quality control. Med Lab Sci 37:107, 1980.
44. Bruce, M, et al: A serious source of error in antiglobulin testing. Transfusion 26:177, 1986.
45. Green, C, et al: Quality assurance of physiological saline used for blood grouping. Med Lab Sci 43:364, 1968.
46. Voak, D, et al: Antihuman globulin reagent specification: The European and ISBT/ICSH view. Biotest Bull 3:7, 1986.
47. Mallory, D, et al: Immunohematology methods and procedures. The American National Red Cross, 1993, pp 40–41.
48. Lalezari, P, and Jiang, RF: The manual polybrene test: A simple and rapid procedure for detection of red cell antibodies. Transfusion 20:206, 1980.
49. Crawford, MN, et al: Microplate system for routine use in blood bank laboratories. Transfusion 10:258, 1970.
50. Redman, M, et al: Typing of red cells on microplates by low-ionic polybrene technique. Med Lab Sci 43:393, 1986.
51. Rosenfield, RE, et al: Solid phase serology for the study of human erythrocytic antigen-antibody reactions. Proc 15th Congr Int Soc Blood Trans, Paris, 1976, p 27.
52. Moore, HH: Automated reading of red cell antibody identification tests by a solid phase antiglobulin technique. Transfusion 24:218, 1984.
53. Plapp, FV, et al: A solid phase antibody screen. Am J Clin Pathol 82:719, 1984.
54. Lapierre, Y, et al: The gel test: A new way to detect red cell antigen-antibody reactions. Transfusion 30:2, 1990.
55. Tissot, JD, et al: The direct antiglobulin test: Still a place for the tube technique? Vox Sang 77:223, 1999.
56. Chuansumrit, A, et al: The benefit of the direct antiglobulin test using gel technique in ABO hemolytic disease of the newborn. Southeast Asian J Trop Med Pub Health 28:428, 1997.
57. Lai, M, et al: Clinically significant autoimmune hemolytic anemia with a negative direct antiglobulin test by routine tube test and positive by column agglutination method. Immunohematology 18:109, 2002.
58. Mitek, JF, et al: The value of the gel test and ELAT in autoimmune haemolytic anaemia. Clin Lab Haem 17:311, 1995.

BIBLIOGRAPHY

Beck, ML, and Marsh, WL: Letter to the editor: Complement and the antiglobulin test. Transfusion 17:529, 1977.
Black, D, and Kay, J: Influence of tube type on the antiglobulin test. Med Lab Sci 43:169, 1986.
Freedman, J, et al: Further observations on the preparation of antiglobulin reagents reacting with C3d and C4d on red cells. Vox Sang 33:21, 1977.
Freedman, J, and Mollison, PL: Preparation of red cells coated with C4 and C3 subcomponents and production of anti-C4d and anti-C3d. Vox Sang 31:241, 1976.
Federal Register 42:41920, 1977.
Federal Register 50:5579, 1985.
Garratty, G, and Petz, LD: An evaluation of commercial antiglobulin sera with particular reference to their anticomplement properties. Transfusion 11:79, 1971.
Giles, C, and Engelfriet, CP: Working party on the standardization of antiglobulin reagents of the expert panel of serology. Vox Sang 38:178, 1980.
Graham, HA, et al: A new approach to prepare cells for the Coombs test. Transfusion 22:408, 1982.
Issitt, PD, et al: Evaluation of commercial antiglobulin sera over a two-year
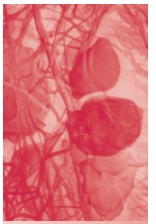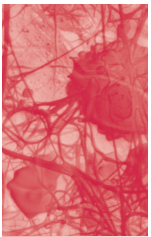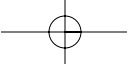
period. Part 1: Anti-beta 1A, anti-alpha 2D, and anti-beta 1E levels. Transfusion 14:93, 1974.

Judd, WJ, et al: Paraben-associated autoanti-Jk^a antibodies: Three examples detected using commercially prepared low-ionic strength saline containing parabens. Transfusion 22:31, 1982.

Petz, LD: Complement in immunohaematology and in neurologic disorders.

International Symposium on the Nature and Significance of Complement Activation. Ortho Research Institute of Medical Science, Raritan, NJ, 1976, p 87.

Plapp, FV, et al: Solid phase red cell adherence tests in blood banking. In Smit Bigings, C Th, Das, PC, and Greenwalt, TJ: Future Development in Blood Banking. Martinus Nijhoff, Boston, 1986, p 177.
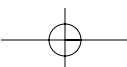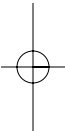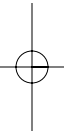
P R O C E D U R A L   A P P E N D I X

Manual Antiglobulin Test Techniques

I. DAT

A. Procedure

1. Label two 10 or 12×75 mm glass test tubes. Test and control, respectively, and add 1 drop of a 3% v/v suspension of test cells to each.
2. Wash the cells a minimum of three times with saline, and *ensure that all saline is completely decanted after the last wash.*
3. To the tube labeled "test," add 1 to 2 drops of AHG as recommended by the manufacturer and mix.
4. To the control tube add 1 to 2 drops of 3% w/v bovine albumin in saline and mix.
5. Centrifuge both tubes at 500 RCF for 15 to 20 seconds.
6. Following centrifugation, completely resuspend the cell pellet by gently tipping and rolling the tube. Read and score agglutination macroscopically with the aid of a background light source and low-power magnification.
7. Incubate the tubes for another 5 minutes at room temperature and repeat steps 5 and 6. Most manufacturers recommend this additional step because it has been shown that some negative or even weak reactions may increase in strength. These reactions have been attributed to the presence of C3d and, to a lesser extent, IgA on the cell surface. Conversely, the reaction with some cells may weaken after the extra incubation; this has been attributed to either detachment of IgG antibody or prozoning when excess anti-IgG has been added.

B. Controls

To all negative tubes add 1 drop of control cells weakly sensitized with IgG, mix the cells, and repeat Steps 5 and 6. A mixed-field weakly positive reaction should now be obtained, indicating that the AHG had been added to the tube and that it was still reactive. All negative results could therefore be considered valid. If a negative result was obtained after addition of the control cells, it would indicate that the AHG had not been added or that, if added, it was nonreactive. This could occur if:

1. The reagent had deteriorated in storage.
2. The reagent had been contaminated by serum and the antibody activity neutralized.

3. The cells had been insufficiently washed and residual serum or plasma had neutralized the AHG reagent when added to the tube.

Control cells weakly sensitized with complement should be used with monospecific anti-C3d reagent to validate negative results.

The control tube containing cells and 3% w/v bovine albumin should give a negative result. If the result is positive, it indicates that the cells are autoagglutinable and that the test cannot be properly interpreted.

For reasons previously outlined, the cells used for DATs should be collected into either EDTA or citrates containing anticoagulant to minimize the possibility of the in-vitro attachment of complement components.

II. IAT

A. Procedure

1. Into a labeled glass 10 or 12×75 mm test tube, place 2 to 4 drops of test serum and 1 drop of a washed 3% v/v suspension of RBCs.
2. Mix the cell suspension, and incubate for 30 minutes in a 37°C water bath.
3. Centrifuge the tube at 500 RCF for 15 to 20 seconds.
4. After centrifugation, completely resuspend the cell pellet by gently tapping and rolling the tube. Read and score agglutination macroscopically with the aid of a background light source and low-power magnification.
5. Wash the cells at least three times with saline, and *ensure that all saline is completely decanted following the final wash.*
6. Add 1 to 3 drops of AHG as recommended by the manufacturer and mix.
7. Repeat Steps 3 and 4.

B. Controls

To all negative tubes, add 1 drop of control cells weakly sensitized with IgG, mix the cells, and repeat Steps 3 and 4. Negative results can be considered valid if a weakly positive mixed-field reaction is obtained after addition of the control cells. If this reaction is not obtained, the test should be repeated.

When phenotyping RBCs using an AHG-reactive typing serum, it is important to follow the antisera manufacturer's recommendations for the use of the reagent.