Patients presenting with autoimmune hemolytic anemias create inherent challenges to those tasked with providing compatible blood for transfusion therapy. These patients have developed autoantibodies against their own red cell surface antigens. Because these antigens are usually high-incidence, these patients will typically demonstrate panagglutination when their serum is exposed to most commercially procured screening red blood cells. This makes the identification of clinically significant alloantibodies difficult for laboratory personnel. Transfusion history, patient phenotype availability, and previous antibody records all impact the testing methods. The end goal is to identify clinically significant alloantibodies in order to provide antigen negative, compatible red blood cells, which reduces the risk of transfusion related reactions. It is imperative to understand the laboratory results and the techniques available that guide the investigative process.

ABBREVIATIONS: AHG = anti-human globulin; AIHA = autoimmune hemolytic anemia; CAS = cold agglutinin syndrome; DAT = direct antiglobulin test; HDN = hemolytic disease of the newborn; IAT = indirect antiglobulin test; IHA = immune hemolytic anemia; LISS = low ionic strength solution; PAM = prophylactic antigen-matched; PCH = paroxysmal cold hemoglobinuria; PEG = polyethylene glycol; RBC = red blood cell; WAIHA = warm autoimmune hemolytic anemia.

INDEX TERMS: anemia; autoimmune; hemolytic.

In order to effectively manage the technological methodologies employed in the attainment of compatible RBCs for these patients, it is essential for the transfusion service personnel to thoroughly understand the various types of IHAs. Moreover, in order to create an environment which makes the associated project management of these complex work-ups effective, supervisors must understand the background of IHAs, current scientific methods, and decision trigger points. Likewise, it is equally critical to embrace, rather than fear, new and emerging technological methodologies which will decrease processing time and increase efficiency, while simultaneously maintaining testing sensitivity and specificity.

IMMUNE HEMOLYTIC ANEMIAS
Autoimmune hemolytic anemias (AIHA) AIHAs are generally classified as warm, cold, or mixed. Warm reacting autoantibodies are generally an IgG class of antibody that is reacting with all patient RBCs. The reactions are typically stronger at their optimal temperature of 37°C and have a weakened expression at cooler temperatures. They are typically identified by their panreactive characteristics, when a patient’s serum is incubated with commercially procured screening and panel RBCs. The resulting panagglutination is often noted when testing free antibodies found in a patient’s serum and also when testing the antibodies that are eluted from a patient’s RBCs, during in vitro identification techniques.

Immune hemolytic anemia (IHA) results from an immune mediated response to red blood cell (RBC) surface antigens. Based on the class of antibody, predominantly immunoglobulin G (IgG) and immunoglobulin M (IgM), patients may experience varying degrees of hemolysis. The immunological response may result in complement fixation or subsequent RBC destruction by the splenic macrophages. Though various classifications and sub-classifications exist, the AABB has divided IHAs into three main classes: autoimmune hemolytic anemias (AIHA), drug-induced hemolytic anemias, and alloimmune hemolytic anemias. Regardless of the type of anemia, most patients present with diverse, non-specific symptoms that may include dyspnea, pallor, weakness, fatigue, dizziness, abdominal pain, weight loss, and jaundice.2,5

Cold reacting autoantibodies typically consists of IgM classes of antibodies, which are most reactive at 4°C and cause hemolysis, through the fixation of complement. Most of these antibodies are not clinically significant and are only observed because of their interfering characteristics with
room temperature in vitro identification techniques. The most common cold-reactive antibody causes cold agglutinin syndrome (CAS). Though relatively benign, this condition can cause hemolysis in the extremities when exposed to colder temperatures. Moreover, autoantibody agglutination can occur in the vasculature of distal extremities causing a numbing pain and bluish color referred to as acrocyanosis.

A rarer, cold reacting antibody that is actually a biphasic, IgG class is the causative agent of paroxysmal cold hemoglobinuria (PCH). PCH was first identified in the early 1900s in association with syphilis, but has gained more recent notability as a secondary condition associated with viral and bacterial infections in children. In PCH, the antibody attaches to the RBC, fixes complement, and then dissociates as it circulates back to the warmer body core. The unique biphasic nature of this condition can be demonstrated in vitro using the Donath-Landsteiner test. This test allows for the macroscopic visualization of hemolysis in specimens that are cooled and then heated to 37°C, as detailed in the AABB technical manual.

In 1981, mixed type AIHA was proposed, as a new classification, by Sokol and others to categorize patients that appear to have a mixture of autoantibody immunoglobulin classes (IgG and IgM).

These patients typically present with notable autoantibodies that appear reactive at both 37°C and 4°C. The IgM class of the group will also exhibit a larger than normal thermal amplitude, with reactivity from 4°C to > 30°C. In a 1985 study of 144 patients to investigate mixed AIHAs, it was noted that this type of hemolytic anemia constitute only 8.3% of the total number of cases.

Drug-induced hemolytic anemias
Drug-induced hemolytic anemias involve various theoretical mechanisms of activity and account for 12%-18% of AIHA cases. Classifications have involved immune versus non-immune or have centered on the proposed activity thought to cause the ultimate hemolysis. However, the AABB has also presented referenced theories that the antibodies may form against the drug entirely, a combination of the RBC membrane and drug membrane components, or mainly against the RBC membrane. Regardless of the mechanism of activity and the presence of a positive direct antiglobulin test (DAT), hemolytic anemias caused by drug therapies are rare.

Alloimmune hemolytic anemias
In most instances, alloimmune hemolytic anemias occur from alloantibodies that were immunologically derived secondary to a sensitizing event. ABO antibodies, however, are naturally occurring and can provide the most severe acute hemolytic episode, if matched with the wrong donor type. For other antibody types, a sensitizing event is one in which a patient has been exposed to RBC antigens that they lack. This event has a propensity to cause an immunogenic response that may lead to the formation of alloantibodies. Sensitizing events can include blood exposure through transfusion, pregnancy, and intravenous drug use.

However, the sensitizing event does not necessarily cause antibody formation. In a published eight year study of 159,262 patients, it was noted that the mean number of red cell transfusions that elicited the formation of a single specificity antibody was 4.79. Moreover, the formation of multiple antibodies was found to be related to an increase in donor exposures, with all individuals forming >6 antibodies having a mean of 21.73 – 56.00 RBC transfusions. When these alloantibodies form, they must be identified to mitigate the risk of a transfusion reaction from a subsequent antigenic exposure. This is the main reason that the transfusion service must have a mechanism of identifying these alloantibodies, even in patients that have panreactive autoantibodies.

Another form of alloimmune hemolytic anemia is hemolytic disease of the newborn (HDN), caused when the IgG antibodies of the mother cross the placenta. If the baby has antigenic structures corresponding to the mother’s antibodies, these antibodies will react with the baby’s RBCs. This can occur with a mother’s non-ABO alloantibodies or with IgG ABO antibodies for Group O mothers that have group A, B, or AB babies. The severity of the problem can vary from mild to severe. At a facility in San Antonio, Texas, a mother required numerous intrauterine transfusions due to multiple alloantibodies, which were causing fetal hemolysis.

LABORATORY INVESTIGATION
All hemolytic anemias result from an immunological or non-immunological response that decreases the RBC survival rate, but, all patients with IHA will also show varying degrees of reactivity with commercially procured screening RBCs. The degree of reactivity demonstrated will vary based on the IHA classification type, the immunoglobulin type, the antibody titer, the drug history, and the immunological state of the patient.
Indirect antiglobulin testing
Initial transfusion testing typically includes blood typing and performing an indirect antiglobulin test (IAT). The IAT is necessary to identify clinically significant alloantibodies in the patient’s serum. When a patient is showing reactivity to one or more screen cells, the antibody or antibodies that are causing the in vitro agglutination must be identified. Identification involves expanding testing, to include reactions with panel cells of a known phenotypic make-up. Laboratory testing normally identifies the antibody or antibodies present. This facilitates the follow-on investigation to find RBCs that lack the antigen which is eliciting the immunological response, so that crossmatch compatible blood can be provided. However, when initial IAT testing provides only inconclusive, panagglutination with all cells, the investigation continues. Causes of panreactivity include: multiple alloantibodies; antibodies to high-incidence antigens; drug-induced IHA; autoantibodies; or, a combination of these.

Direct antiglobulin testing
One of the key tests in helping to distinguish classes of hemolytic anemia is the direct antiglobulin test (DAT). The DAT is typically performed when the IAT is positive and is used to identify whether or not the patient has RBCs coated with either antibodies or complement. A positive DAT is usually suspect for the presence of alloantibodies. These can be formed by the patient as seen in transfusion reactions or passively acquired as seen in HDN. Unfortunately, most AIHAs will cause both the IAT and DAT to be positive, even if the patient does not have clinically significant alloantibodies.

The DAT is typically performed with a polyspecific, anti-human globulin (AHG), as a screening tool to identify RBCs that are coated, in vivo, from an immunological response. If the test is positive, follow-up testing includes monospecific reagents to distinguish complement fixation from an IgG class of antibody. If patient RBCs are only reacting with anti-C3 AHG, it typically signifies a cold reacting IgM class AIHA, a drug-induced hemolytic anemia, or PCH. However, if the reaction is occurring with only anti-IgG AHG or a combination of both anti-IgG and anti-C3 AHG, then the classification is most likely a warm autoimmune hemolytic anemia (WAHA), mixed AIHA, drug-induced AIHA, a transfusion reaction or HDN as seen in some neonates. DATs that are positive with anti-IgG AHG will have an elution performed to identify the antibody present on the patient’s RBC. Elutions are not performed on DATs that are positive for anti-C3 only, because there is no antibody present to identify. The follow-on testing after a positive IAT is determined by the DAT results (Figure 1).

Either way, the possibility of one or more alloantibodies being present must always be investigated thoroughly. Numerous studies, on the frequency of underlying alloantibodies being present in conjunction with autoantibodies, have provided statistical evidence that the range is between 31%-53%. These alloantibodies can only be identified when the autoantibodies, which are masking them, are removed.

Elution
An elution for antibody identification involves thoroughly washing the RBCs to remove unbound antibodies, prior to treating with an acidic solution. The removal of unbound antibodies is done to ensure the eluate contains only those antibodies which are coating a patient’s RBCs. The bound antibodies are typically dissociated from the RBCs utilizing an acidic solution and then buffered back to a pH of approximately 7.0. The result is an eluate of freed antibodies that can be tested against phenotypically known RBCs. Reactivity that demonstrates
panagglutination is typical of WAIHA. Cold AIHA and drug-induced anemias will usually be non-reactive. If an identifiable pattern is noted, then the thought process should shift to an alloimmune response such as is found in certain transfusion reactions or in babies suffering from passively acquired maternal antibodies.

Caution must be taken, however, when identifying eluted antibodies. Several cases have been presented of warm autoantibodies mimicking certain classes of alloantibodies. In one study, it was found that an apparent 43% of the cases of underlying alloantibodies actually involved mimicking autoantibodies, which reduced the real alloimmunization rate of this patient population to 23%.14 The mimicking specificities typically fall in the Rh system. In a 1977 publication, a case was presented of an autoantibody mimicking the specificity of both anti-E and anti-c alloantibodies.15 In this case, the apparent alloantibodies were adsorbed by RBCs that were antigen negative for both E and c. This is unlikely if the specificities had been actual alloantibodies. In another case, an apparent anti-E was noted in both a patient's plasma and when eluted from the patient's RBCs, following a positive DAT.16 Once again, adsorption was possible with both E-antigen positive cells and E-antigen negative cells.

**Adsorptions and complex testing**

When the IAT and eluate are all demonstrating panreactivity, this is usually conclusive for a WAIHA. But, in order to provide RBCs for transfusion, it is important to identify any alloantibodies present. If the patient has not been recently transfused, then autologous adsorption techniques can be employed to remove the circulating autoantibody from the patient serum. In this technique, the patient cells are treated to remove the bound autoantibodies, which frees up the antigenic binding sites. These cells are then re-exposed to the patient serum to allow for attachment of more autoantibodies. Multiple adsorption techniques may need to be employed depending on the strength of the reactions. When the autoantibodies have been adequately removed from the sample, the remaining serum can be tested against panel cells for the presence of any clinically significant alloantibodies.

Many hospitals will not perform autologous adsorptions if a patient has been transfused within the preceding three months, because RBCs have a normal survival rate of approximately 110-120 days. This survival rate will create a transient, dual RBC population of patient and donor cells. Because these recently transfused patients have a chimera of donor and recipient cells, an autologous adsorption may actually remove a clinically significant alloantibody which may be reacting only with donor cells. It is important to note that the decreased red cell survival in patients with WAIHA affects all cells, donor and recipient, and therefore, some hospitals may perform the technique on recently transfused patients after a 90 day period.

Patients that have been recently transfused will require allogeneic adsorptions. This involves utilizing RBCs of various phenotypic make-ups to perform adsorption techniques. Since all of these cells will remove the autoantibodies, the result is several aliquots of patient serum that may or may not have alloantibodies remaining, depending on the phenotype of the cells utilized. This adsorbed serum is then reacted with screen cells to identify any specificity patterns. The process for allogeneic adsorptions is very complex and time consuming.

**TRANSFUSION TESTING FOR WAIHA**

An option employed by some hospitals involves the initial phenotyping of all patients that present with WAIHA. The phenotype can be obtained utilizing immediate spin or room temperature incubation anti-sera, as long as an auto-control is performed in tandem. For anti-sera that are taken to the AHG phase of testing, the patient RBCs will have to be treated, to remove the autoantibody, before performing antigenic testing. A treated aliquot of phenotypically matched donor RBCs can be used in lieu of patient RBCs, for an autologous adsorption technique.

Taking the concept of initial phenotyping further, the idea of utilizing prophylactic antigen-matched (PAM) donor blood for on-going transfusion therapy of WAIHA patients is definitely worth further investigation. One study supported the effectiveness of PAM blood in meeting the transfusion needs of this population while reducing normal testing time requirements.17 However, this option may not be the best if the rarity of the patient phenotype makes PAM blood more difficult and time-consuming to obtain than normal adsorption procedures. Moreover, the extent of the phenotyping must not be limited to the Rh and Kell specificities. In a recent study of underlying alloantibodies, it was found that 11% of these patients had antibodies found in other blood group systems, including the Duffy and Kidd systems.11

When standard adsorption studies are required, numerous technical procedures are employed nationwide, which include untreated RBCs without antigen-antibody reaction potentiators, untreated RBCs with potentiators such as low ionic strength solutions (LISS) or polyethylene glycol (PEG),
enzymatic treatment of RBCs without potentiators, and enzyme treated RBCs in conjunction with various potentiators. In determining the method of choice for a transfusion service, it is vital to focus on efficiency in reducing the time to provide blood products without sacrificing sensitivity and specificity.

One study, which compared the use of papain-treated RBCs to procedures involving LISS alone or LISS-papain combinations, found that the use of a potentiator in adsorption procedures decreased the mean processing time from 180 minutes to 57.6 and 58 minutes respectively. Moreover, the mean number of adsorptions required per specimen dropped from six to under three without an apparent loss of sensitivity or specificity. Another method that further reduces the total processing time, the number of adsorptions required, and the need for enzyme treating RBCs is the PEG adsorption technique. Two PEG adsorption studies, which detailed procedures involving the use of one part untreated RBCs, one part patient serum (plasma), and one part PEG, reported mean processing times of 22.5 minutes and 28 minutes. In both PEG studies, no dilutional effect was noted and both sensitivity and specificity remained comparable to other methods.

SUMMARY
IHAs present some of the most difficult challenges for transfusion services. In order to provide compatible blood for these patients, it is important for clinical scientists to understand that each case is different and the cumulative, investigative process is step-driven, based on previous findings. Moreover, it is essential that clinicians understand the complexity involved with the testing and the time requirements of both the testing phases and the incubation steps. With close monitoring of patient hematological levels, adequate forecasting of requirements and a thorough understanding of the investigative process, the clinical team of clinicians and scientists will be better prepared to ensure that supportive transfusion therapy requirements are met.

It is also important for laboratories to embrace changes that will improve processes to streamline testing and ensure optimal efficiency. By understanding the various methodologies that are available for performing the required adsorption procedures, for WAIHAs, laboratories can adjust procedures as long as they are validated, maintain acceptable specificities, and do not sacrifice sensitivity.

Clin Lab Sci encourages readers to respond with thoughts, questions, or comments regarding this article. Email responses to ic.ink@mchsi.com. In the subject line, please type “CLIN REFERENCES