# Review: evaluation of patients with immune hemolysis

LAWRENCE D. PETZ

The presence of hemolysis is usually easily confirmed by simple tests such as reticulocyte count, review of the peripheral blood film, serum bilirubin (direct and indirect), LDH, and haptoglobin.[1] When it has been established that the patient has a hemolytic anemia, the cause of the hemolysis should be sought next. The DAT should be performed on the RBCs of every patient in whom the presence of hemolysis has been established, and a positive DAT in a patient with hemolytic anemia does, of course, indicate that the most likely diagnosis is one of the immune hemolytic anemias (IHAs).[2] However, there are numerous causes of IHA and the clinical manifestations, prognosis, and therapy vary so that defining the precise diagnosis is of considerable importance.

## Classification of IHAs

In evaluating a patient with IHA, one should first consider a differential diagnosis as outlined in Table 1. Although a definitive diagnosis rests with serologic studies, some clinical and routine laboratory procedures are sufficiently distinctive as to strongly suggest the type of IHA that is present.

**Table 1.** Differential diagnosis of immune hemolytic anemias

**Autoimmune hemolytic anemias (AIHA)**

- Warm-antibody AIHA
  - Idiopathic
  - Secondary (e.g., chronic lymphocytic leukemia, lymphomas, systemic lupus erythematosus)
- Cold agglutinin syndrome
  - Idiopathic
  - Secondary
    - Nonmalignant disorders (e.g., mycoplasma pneumoniae infection, infectious mononucleosis, other virus infections)
    - Malignant disorders (e.g., lymphoproliferative disorders)
- Combined cold and warm AIHA
- Paroxysmal cold hemoglobinuria
  - Idiopathic
  - Secondary
    - Viral syndromes
    - Syphilis
- Atypical AIHA
  - AIHA with a negative direct antiglobulin test
  - Warm-antibody AIHA caused by IgM or IgA autoantibodies
- Drug-induced immune hemolytic anemia
  - Drug-related antibody identifiable
  - Drug-induced AIHA
- Alloantibody-induced immune hemolytic anemia
  - Hemolytic transfusion reactions
  - Hemolytic disease of the fetus and newborn

Note: Tables 1, 2, 4, 5, and 6 were taken from Petz LD, Garraty G. Immune hemolytic anemias. 2nd ed. Philadelphia: Churchhill Livingstone, 2004.[1]

## Distinctive Clinical Signs and Routine Laboratory Procedures

*Association with exposure to cold*

A history of acrocyanosis and/or hemoglobinuria on exposure to cold in an elderly patient with an acquired hemolytic anemia strongly suggests a diagnosis of cold agglutinin syndrome (CAS). However, these manifestations are absent in a majority of patients with CAS even though they were often emphasized in the early medical literature.

Although one might assume that paroxysmal cold hemoglobinuria (PCH) is commonly precipitated by exposure to cold, this is only occasionally true. Indeed Wolach et al.[3] pointed out that the most common form of PCH is the transient type, secondary to infection (e.g., in childhood); is rarely paroxysmal; is only occasionally clearly precipitated by cold; and is not invariably expressed as hemoglobinuria, although the latter finding is very common.[4]

*Autoagglutination*

Autoagglutination is a finding that may be noted by technologists in all sections of the laboratory, not just those in the blood transfusion or immunohematology laboratories. Indeed, cold autoagglutinins that react strongly at room temperature cause such striking findings that they are difficult to ignore. Autoagglutination visible to the naked eye occurring at room temperature is characteristic of the CAS, but may also be noted in about one-third of patients with warm autoimmune hemolytic anemia (WAIHA).[1] Although

the autoagglutination caused by cold agglutinins is often 2+ to 4+, it almost always completely disperses after a few minutes of incubation at 37°C, whereas that caused by warm autoantibodies is usually much weaker and will not disperse at 37°C. If the blood sample has been obtained from a patient known to have hemolytic anemia, such simple observations offer an important clue to the correct diagnosis.

However, a common error is the over-interpretation of cold agglutination. Many cold antibodies are reactive at room temperature but are clinically benign, albeit somewhat a nuisance in the laboratory. The serologic criteria for distinguishing clinically benign cold agglutinins from pathologic cold agglutinins capable of causing a CAS are discussed later.

*Drug ingestion*

A temporal history of drug ingestion may suggest the etiology of the patient's IHA. A critical aspect of evaluation of a patient with IHA is the elicitation of a history of drug ingestion which, in some instances, may have occurred a week or more prior to the onset of hemolysis or be a single dose given for surgery (e.g., cefotetan). Knowledge of the drugs that have been implicated as a cause of drug-induced IHA is essential.

Many drug-induced IHAs can be distinguished from autoimmune hemolytic anemia (AIHA) by laboratory findings, i.e., the demonstration of a drug-dependent RBC antibody. However, the administration of some drugs causes hemolytic anemia that is serologically indistinguishable from cases of idiopathic WAIHA. Such cases are appropriately termed drug-induced AIHA, whereas those cases wherein a drug-dependent antibody can be identified are termed drug-induced IHA.

*Alloantibody-induced IHA*

Alloantibody-induced hemolytic anemias include HDN and hemolytic transfusion reactions. The clinical setting usually strongly suggests these diagnoses. Although autoantibody-induced HDN can occur as a result of transplacental passage of a mother's IgG warm autoantibody, this is very unlikely unless the mother has obvious and quite severe WAIHA.

Also, when hemolysis occurs in the immediate aftermath of a RBC transfusion, the diagnosis of an acute hemolytic transfusion reaction is quite evident. However, distinguishing a delayed hemolytic transfusion reaction from AIHA is, on occasion, difficult.[5–7]

*Hemoglobinemia and hemoglobinuria*

Hemoglobinuria (Hb in the urine) is far less common than hematuria (RBCs in the urine), and a common clinical error is the assumption that a patient's red urine is caused by hematuria. It should be remembered that hemoglobinuria, associated with hemolytic anemia, cannot occur without hemoglobinemia. If red urine is present without hemoglobinemia, it should be suspected that the cause is hematuria and not hemolysis. The presence of hemoglobinemia and hemoglobinuria should alert the clinician to a specific group of diagnoses and, when considered in association with the clinical setting, often makes the specific diagnosis evident (Table 2). Probably the most common associated diagnoses are hemolytic transfusion reactions and severe acute WAIHA, although hemoglobinuria may occur in patients with CAS, especially after exposure to cold. Also, drug-induced IHAs caused by cefotetan and ceftriaxone are commonly associated with hemoglobinemia and hemoglobinuria.

**Table 2.** Causes of hemoglobinuria

| |
|---|
| **Acute Hemoglobinuria** |
| Incompatible blood transfusion |
| Transfusion of damaged blood (overheating or freezing, bacterial contamination, pump-oxygenation) |
| Drugs and chemical agents (immune or nonimmune mechanisms) |
| Paroxysmal cold hemoglobinuria |
| Acute severe warm-antibody AIHA |
| *Clostridium perfringens* infection |
| Malaria (blackwater fever) |
| Bartonellosis, babesiosis, leptospirosis, toxoplasmosis |
| Peritoneal hemorrhage |
| Severe hypophosphatemia |
| Snake and spider bites |
| Cold agglutinin syndrome* |
| March hemoglobinuria |
| Microangiopathic hemolytic anemia |
| Hypotonic bladder irrigation during prostatic surgery |
| Mistaken intravenous administration of water |
| **Chronic Hemoglobinuria** |
| Paroxysmal nocturnal hemoglobinuria** |
| Prosthetic cardiovascular materials |

*Chronic low grade intravascular hemolysis is common, with acute hemoglobinuria resulting from exposure to cold
**Characteristically associated with intermittent episodes of grossly evident hemoglobinuria

Hemoglobinuria and hemoglobinemia are much more common in children, and both warm and cold AIHA can be the cause. In a child, PCH should be strongly suspected and a Donath-Landsteiner (DL) test should be performed whenever an acute severe hemolytic anemia occurs with hemoglobinemia and hemoglobinuria. Indeed, hemoglobinuria is a common presenting manifestation of PCH[4] and therefore becomes an important diagnostic clue.

*RBC morphology and erythrophagocytosis*

One easily performed and very valuable test for determining the specific diagnosis in a patient with hemolytic anemia is examination of the peripheral blood film. RBC morphology often strongly suggests a specific diagnosis or a limited number of diagnostic possibilities. Spherocytosis may be a prominent feature in IHAs, especially WAIHA, ABO HDN, hemolytic transfusion reactions, and some instances of drug-induced hemolysis.

Less well appreciated is the fact that RBC adherence and erythrophagocytosis by neutrophils is a prominent finding in PCH, but is seen rarely in other forms of IHA. Erythrophagocytosis is rarely observed in the peripheral blood film of WAIHA and when it is observed, monocytes, not neutrophils, are more often involved.

*Association with* Mycoplasma pneumoniae

If a patient with *Mycoplasma pneumoniae* infection develops AIHA, the diagnosis of a CAS must be strongly suspected, since AIHA in this setting is almost always caused by cold agglutinins.

*Association with underlying disorders (secondary IHAs)*

Patients should be evaluated for certain disease entities, since approximately half of AIHAs are associated with an underlying disorder (Table 3). AIHAs are classified as secondary for any of several reasons. One reason is the association of AIHA with an underlying disease with a frequency greater than can be explained by chance alone, as in chronic lymphocytic leukemia or systemic lupus erythematosus.

Another criterion for categorizing a given case of AIHA as secondary is the reversal of the hemolytic anemia simultaneously with the correction of the associated disease. Ovarian tumors are a good example; well-documented cases of cure of the AIHA after surgical removal of the tumor have been reported.[8–11] Similarly, AIHA in association with ulcerative colitis almost invariably goes into remission after colectomy, even when hemolysis is refractory to other therapeutic approaches.[12–14]

Still another reason for suspecting a relationship between the occurrence of AIHA and an associated disease consists of evidence of immunologic aberration as part of the underlying disorder, especially if the associated disease is thought to have an autoimmune pathogenesis. In general, the evidence for a relationship between immunologic disorders, including immune deficiency states, and AIHA is strong.[15–19] Although the pathogenetic basis for the association between cytopenias and congenital immune deficiency is unclear, defects in T-cell regulation, cytokine defects, abnormal apoptosis, and abnormal production of immunoglobulins with autoimmune features are potential mechanisms.[15]

**Table 3.** Secondary autoimmune hemolytic anemias

| |
|---|
| Ovarian tumors |
| Ulcerative colitis |
| Chronic lymphocytic leukemia |
| Hodgkins disease |
| Non-Hodgkins lymphoma |
| Other lymphoproliferative disorders |
| Systemic lupus erythematosus (SLE) |
| Antiphospholipid syndrome |
| Collagen disorders other than SLE |
| Thymoma |
| Carcinomas |
| Primary immunodeficiency diseases |
| Autoimmune lymphoproliferative syndrome (ALPS) |
| Miscellaneous disorders |
| **AIHA associated with infectious agents** |
| *Mycoplasma pneumoniae* |
| Infectious mononucleosis (Epstein-Barr Virus) |
| Cytomegalovirus (CMV) |
| Human immunodeficiency virus (HIV) |
| Varicella (chickenpox) |
| Rubella |
| Parvovirus B19 |
| Hepatitis |
| Malaria and other blood parasites |
| Bacterial infections |

## Laboratory Diagnosis of IHAs

Even in the presence of valuable clinical clues that may suggest a specific diagnosis, the confirmation of the precise diagnosis of the type of IHA present depends on the laboratory. The serologic tests to be performed determine whether the patient's RBCs are coated with IgG, complement components, or both. The performance of the DAT supplies such information. Further tests must be performed to determine the characteristics of the antibodies in the patient's serum and in a RBC eluate.

*Significance of the DAT in the diagnosis of IHAs*

The DAT, using polyspecific and monospecific antiglobulin reagents, provides useful information in the evaluation of a patient with IHA. However, the results must always be interpreted in conjunction with clinical and other laboratory data to avoid erroneous conclusions. It must be remembered that a positive DAT occurs in situations other than IHAs. A positive DAT does not necessarily indicate the presence of

autoantibody; furthermore, even if autoantibody is present, the patient may or may not have a hemolytic anemia. Thus, an independent assessment must be made to determine the presence or absence of hemolytic anemia, and the role of the DAT is to aid in the evaluation of the etiology of hemolysis when present. The results of DATs in patients with various types of IHAs are indicated in Table 4.

**Table 4.** Typical DAT results in patients with immune hemolytic anemias

| Causes of hemolytic anemia | Anti-IgG | Anti-C3* |
|---|---|---|
| Warm-antibody AIHA | | |
| 67% | + | + |
| 20% | + | 0 |
| 13% | 0 | + |
| Warm-antibody AIHA associated with systemic lupus erythematosus | + | + |
| Cold agglutinin syndrome (100%)* | 0 | + |
| Paroxysmal cold hemoglobinuria (100%) | 0 | + |
| Drug-induced immune hemolytic anemias | | |
| *Drug-dependent antibodies* | | |
| Penicillin and first-generation cephalosporins | + | (+)† |
| Second- and third-generation cephalosporins | + | + |
| Associated with "Immune Complex Mechanism" | + | + |
| *Drug-independent antibodies* | + | (+) |
| *Drug-induced nonimmunologic adsorption of proteins** | + | (+) |
| Hemolytic disease of fetus/newborn | + | 0 |
| Hemolytic transfusion reactions | + | (+) |

*IgG +C3 were both detected on the RBCs of one patient with CAS because the patient was on methyldopa therapy and made IgG autoantibodies
†(+) = sometimes positive

AIHA associated with warm IgM autoantibodies without the presence of IgG occurs on rare occasions. However, even with potent antisera, RBC-bound IgM is difficult to detect with the antiglobulin test.[1,20,21] Fortunately, IgM antibodies that cause IHA characteristically fix complement, which is much more readily detected. IgA antibodies only infrequently play a role in RBC sensitization, and in such cases other immune globulins and/or complement components are almost always, although not invariably, found on the cell surface as well. The clinical and hematologic features of WAIHA associated only with IgA autoantibodies are very similar to AIHA associated with warm IgG autoantibodies.

### *The AIHA serum screen test*

The "AIHA screen" is an elaboration of the routine antibody screen performed for pretransfusion testing. In summary, the patient's serum is tested against a pool of untreated and enzyme (e.g., ficin)-treated allogeneic group O RBCs and autologous RBCs at 20°C (room temperature can be used) and 37°C (all stages strictly at 37°C). A duplicate set of tubes is used with added fresh complement (pooled normal sera) at optimal pH (6.5 to 6.8) for lysis. After incubation, the tubes are inspected for hemolysis, agglutination, and sensitization (antiglobulin test using polyspecific antiglobulin serum).

The results of this screen usually indicate whether one is dealing with a cold autoagglutinin, a "warm" autoantibody, or possibly a combination of both. Other points of interest are whether there is a hemolysin present and whether it is a "cold" or "warm" hemolysin. If agglutination occurs at 20°C, a cold agglutinin titer and thermal amplitude should be performed. Results of the AIHA serum screen in WAIHA and in CAS are indicated in Tables 5 and 6, respectively.

With the results of the DAT, AIHA screen, and possibly a cold agglutinin titer/thermal amplitude, one generally has a good idea of whether the diagnosis is WAIHA or CAS. Sometimes the patient's history and results of the DAT/AIHA screen will lead to further tests, such as the DL test for PCH, or detection of drug-dependent antibodies.

### *Characteristic serology of WAIHA*

Autoantibodies causing WAIHA are usually IgG but can be IgM or IgA. These proteins can be present together; it is rare for only IgM or IgA to be the cause of AIHA. Autoantibodies are usually only found in the serum when all autoantigens are saturated, apparently because the patient's RBCs are adsorbing warm autoantibodies continuously. Only about 60 percent of patients' sera will react with saline-suspended RBCs,

**Table 5.** Results of serum screening in 244 patients with warm-antibody AIHA

| Results | Untreated serum | Acidified serum plus acidified complement |
|---|---|---|
| Serologic reactions | (% Positive reactions) | (% Positive reactions) |
| **20°C Untreated RBCs** | | |
| Lysis | 0.4 | 0.8 |
| Agglutination | 34.8 | 34.8 |
| **20°C Enzyme-treated RBCs** | | |
| Lysis | 1.6 | 2.5 |
| Agglutination | 78.6 | 78.6 |
| **37°C Untreated RBCs** | | |
| Lysis | 0.4 | 0.4 |
| Agglutination | 4.9 | 4.9 |
| Indirect anti-globulin test | 57.4 | 57.4 |
| **37°C Enzyme-treated RBCs** | | |
| Lysis | 8.6 | 12.7 |
| Agglutination | 88.9 | 88.9 |

**Table 6.** Results of serum screening in 57 patients with CAS

| Results | Untreated serum | Acidified serum plus acidified complement |
|---|---|---|
| Serologic reactions | (% Positive reactions) | (% Positive reactions) |
| **20°C Untreated RBCs** | | |
| Lysis | 2.0 | 14.3 |
| Agglutination | 98 | 98 |
| **20°C Enzyme-treated RBCs** | | |
| Lysis | 24.5 | 93.8 |
| Agglutination | 100 | 100 |
| **37°C Untreated RBCs** | | |
| Lysis | 0 | 0 |
| Agglutination | 10.7 | 10.7 |
| Indirect anti-globulin test | 5.4 | 5.4 |
| **37°C Enzyme-treated RBCs** | | |
| Lysis | 12.2 | 22.5 |
| Agglutination | 28.6 | 28.6 |

but a higher percentage will react in the presence of potentiators (e.g., PEG) or enzyme-treated RBCs.

Some sera contain warm hemolysins, which react optimally at 37°C but may react at 20°C. The hemolysis is enhanced by adding fresh complement at pH 6.5 to 6.8 to the patients' sera. von dem Borne et al.[22] showed that such hemolysins were usually IgM autoantibodies; they were maximally reactive at a pH of 6.5 and 7 of 11 (64%) reacted optimally at 30°C; 4 of 11 reacted optimally at 37°C. About one-third of sera from patients with WAIHA contain IgM cold autoagglutinins that can react quite strongly at 20°C (or room temperature), but have a normal cold agglutinin titer at 4°C and do not react at 30°C. These may be naturally occurring cold antibodies that become boosted (e.g., raised thermal amplitude) during the pathogenic autoimmune response.

A diagnosis can usually be reached on the basis of the serologic tests described thus far. In spite of the seemingly complicated nature of the foregoing, the usual or "typical" essential diagnostic tests that lead to a reasonably confident diagnosis of WAIHA may be very simply summarized as follows: (a) the presence of an acquired hemolytic anemia, (b) a positive DAT, and (c) an unexpected antibody in the serum and eluate that reacts optimally at 37°C. The antibody usually reacts with all normal erythrocytes but, in some cases, it can readily be shown to react preferentially with antigens on the patient's own RBCs.

*Characterization of antibodies in CAS*

The mere presence of cold autoagglutinins is not diagnostic of CAS, and when they are encountered, the task is to determine whether the patient has clinically insignificant, albeit abnormal, cold agglutinins; has WAIHA with an associated but probably insignificant elevation of cold agglutinin titer and/or thermal amplitude; or has CAS. A rather common diagnostic error is over-diagnosis of CAS in patients who have benign cold antibodies and pathogenic warm autoantibodies. Also, in rare patients, characteristic findings of both WAIHA and CAS occur simultaneously.

A diagnosis of CAS must be considered in all patients with acquired hemolytic anemia who have a positive DAT using anti-C3 and a negative DAT using anti-IgG. A practical initial serum screening procedure is to test the ability of the patient's serum to agglutinate saline-suspended normal RBCs at 20°C (or room temperature) after incubation for 30 to 60 minutes. If this screening test is negative, a CAS is extremely unlikely; if positive, further studies are necessary to determine the thermal amplitude of the antibody.

When CAS appears to be a possible diagnosis on the basis of the preceding evaluation, studies of the thermal range of reactivity of the antibody in saline and albumin are indicated. It is also convenient to simultaneously determine possible Ii blood group specificity of the antibody. The titer of the cold agglutinin in CAS is invariably highest at 4°C and progressively decreases at higher temperatures. Of particular note are the reactions at 30°C and 37°C. If the reactions at 30°C are positive in saline or albumin, the antibody may well be of pathogenic significance, i.e., it may be causing short RBC survival in vivo. If the reactions at 37°C are also positive in the presence of albumin (as is true in about 68% of patients) or even when albumin is not present (only about 7% of patients), the antibody will cause problems in compatibility testing.

Using clinical information, the results of the DAT, and the preceding screening tests, a reasonably confident assessment of the presence or absence of CAS may be made. CAS may be diagnosed if the following are present: (1) clinical evidence of acquired hemolytic anemia, (2) a positive DAT caused by sensitization with C3, (3) a negative DAT using anti-IgG, (4) the presence of a cold autoagglutinin with reactivity up to at least 30°C in saline or albumin, and (5) a cold agglutinin titer (at 4°C) $\geq$ 256, except in exceptional cases. An alternative diagnosis must be sought for patients who do not satisfy all these criteria.

*Patients who have warm and cold autoantibodies*

Approximately one-third of WAIHA patients have cold agglutinins that can react quite strongly at room

temperature but have a normal titer (at 4°C) and do not react at 30°C and 37°C. Such antibodies are probably not pathogenic. However, some patients with WAIHA have cold antibodies that react up to 30°C or above and therefore may be pathogenic. Some of these patients have IgG and C3 on their RBCs, and their sera contain IgG 37°C-reactive antibodies together with high-titer, high-thermal amplitude cold autoagglutinins (i.e., the combined serology of classical WAIHA and CAS).[1,23–28] Other patients have IgG and/or C3 on their RBCs, and their sera contain IgG 37°C-reactive antibodies together with cold autoagglutinins of normal titer that react at 37°C and/or 30°C. There are only three published reports that relate to this group, and the report by Sokol et al.[28] does not give any cold agglutinin titers, so some of the patients may belong to the first group. Shulman et al.[27] believe that up to 8 percent of WAIHAs may belong to this group.

## PCH

The diagnosis of PCH or the exclusion of that diagnosis in the laboratory is usually considerably easier than that of either WAIHA or CAS. The essential laboratory test is the DL test. A negative test excludes the diagnosis of PCH and a positive test is, with rare exceptions (described below), diagnostic of the disorder.

The autoantibody associated with PCH is termed a biphasic hemolysin, that is to say, it sensitizes RBCs in the cold but only hemolyzes them when the RBCs reach 37°C. The diagnostic test is the DL test, wherein RBCs are incubated with the patient's serum at 0°C (e.g., melting ice) and then moved to 37°C for a further incubation. No lysis occurs following the incubation at 0°C, and no lysis occurs if the incubation is carried out only at 37°C. The thermal amplitude of this antibody is usually less than 20°C, that is to say, the antibody will give a positive DL test only when the initial incubation is < 20°C; stronger results will occur as the temperature of the initial incubation is lowered. Rare patients have been described, in whom the DL test is positive when the first incubation phase is as high as 32°C, or their DL antibody sensitized RBCs up to 37°C, as detected by the IAT.[29–31]

The autoantibody may sometimes agglutinate RBCs in addition to giving a positive DL test.[1] The agglutination is usually of low titer (< 64) at 4°C and of low thermal amplitude (< 20°C). The DL antibody is IgG but is usually only detectable by IAT if, following incubation of the patient's serum and RBCs at 0°C, the RBCs are washed with ice-cold saline and ice-cold antiglobulin serum is used. Indeed, Dacie found that the IAT was a more sensitive way of demonstrating antibody activity than looking for lysis.[32] Such agglutination tests must be carefully controlled because many sera give positive results under these circumstances if a broad-spectrum antiglobulin serum is used, due to the presence in human sera of normal incomplete cold antibody. It is important, therefore, to be sure that monospecific anti-IgG antiglobulin serum is used.

Since PCH is quite rare, one may justifiably question the advisability of performing a DL test routinely in patients with acquired hemolytic anemia. However, one should be liberal with the indications for performance of the test, since it is simple to perform and its inclusion avoids diagnostic errors. The performance of the test is indicated in any child, any patient with hemoglobinuria, patients with a history of hemolysis exacerbated by cold, and in all cases with "atypical" serologic findings. If positive results are obtained in the DL test, determination of the specificity of the autoantibody is indicated. In almost all cases of PCH the antibody has anti-P specificity. Sokol et al.[33] found anti-P specificity in 27 of 30 (90%) patients with PCH; specificity was not clearly defined in the other three cases. Nevertheless, rare reports of other specificities said to be associated with PCH have been reported. RBCs necessary for determining anti-P specificity are rare but, with the assistance of reference laboratories, specificity testing can be carried out. It should be noted that the DL test must be used to determine specificity.

### *Cautions regarding the interpretation of the DL test*

The DL test is essentially diagnostic for PCH, but one must be cautious when using serum from patients with CAS. This is true because about 15 percent of sera from patients with CAS contain monophasic cold hemolysins that will hemolyze untreated RBCs at around 20°C. Up to 95 percent of such sera will cause direct lysis of enzyme-treated RBCs at 20°C. During the performance of the DL test, there is a brief period of time when cells and serum are at room temperature after being moved from the ice bath to a 37°C water bath. In about 15 percent of patients with CAS, falsely positive (weak) DL tests will occur on this basis. If enzyme-treated RBCs are used for the DL test, as suggested by some investigators,[4] the falsely positive rate is likely to be higher. Therefore, if enzyme-treated

RBCs are used for the DL test, a control for monophasic lysis set up in parallel should be mandatory.

*Performing the DL test in patients with hemoglobinemia*

It may be impossible to determine if in vitro hemolysis has occurred in the DL test if the patient's serum is red because of marked hemoglobinemia. In this case, a simple procedure is to perform the cold phase of the DL test using the patient's serum, but after incubation at 4°C, carefully replace the patient's serum with fresh normal serum before moving to the 37°C phase of the test. As a control, a similar tube can be kept at 4°C, or a similar test can be performed with P-negative RBCs, if available, and if the specificity of the DL antibody is anti-P. Another simple technical aid is to compare the size of the RBC buttons following centrifugation. If these approaches fail, then a "cold IAT" can be performed.

## Differentiating Delayed Hemolytic Transfusion Reactions From Autoimmune Hemolytic Anemia

Delayed hemolytic transfusion reactions (DHTRs) are a recognized risk of blood transfusion. The reaction is caused by the reappearance of an antibody, presumably first stimulated by pregnancy or a previous transfusion. Unlike immediate transfusion reactions, which are usually caused by human error,[34–38] delayed reactions are usually not avoidable. Since hemolysis is delayed in onset (typically 3 to 14 days after transfusion), the relationship of hemolytic anemia to prior transfusion may not be suspected, and a diagnosis of AIHA may seem more appropriate. Further, laboratory tests are likely to reveal the presence of a positive DAT and IAT, spherocytosis, and reticulocytosis. If multiple alloantibodies or an alloantibody against a high-frequency antigen is formed, the findings may be difficult to differentiate from AIHA. The diagnostic problem is compounded by the fact that, in some cases, AIHA may actually develop as a consequence of blood transfusion.[1] Indeed, therapy with corticosteroids for suspected AIHA has been instituted[5,39] or contemplated[40,41] in some reported cases of DHTRs before the correct diagnosis was made.

## Diagnostic Aids

If a patient has been transfused within recent weeks, careful evaluation is needed to distinguish between a DHTR and AIHA. The following measures will afford important clues.

*Comparison of DAT and IAT*

A simple observation that may yield valuable information is the comparison of the strength of the DAT and the IAT. In WAIHA, the DAT is almost always stronger than the IAT. It appears that autoantibody is largely adsorbed onto the patient's RBCs, and only when the RBCs are heavily coated does one find a large amount of antibody in the serum. In contrast, strongly reactive alloantibodies may be present in a patient's serum, but this finding cannot result in a strongly positive DAT unless large numbers of transfused RBCs of appropriate antigenic type are present. Thus, the presence of a weakly positive DAT in association with a strongly positive IAT is presumptive evidence for the presence of an alloantibody. These findings are therefore highly suggestive of a DHTR. Further, even if the DAT is strongly positive at the time of initial evaluation, it may soon become weaker in subsequent tests as a result of the destruction of the transfused RBCs. This is true even though the DAT may remain weakly positive for several months following a DHTR. In contrast, a rapid diminution in the strength of the DAT would not be expected in AIHA except as a result of treatment, as with corticosteroids, immunosuppressive drugs, or splenectomy.

*Antibody specificity*

An important means of differentiating AIHA from a DHTR relates to the specificity of the antibody(ies) present in the serum and in a RBC eluate. Some antibodies that commonly cause DHTRs have not been found or have been reported only rarely as autoantibodies in AIHA. Examples are anti-K and anti-Fy$^a$, which are frequently encountered in published cases of DHTRs.[6,42–45] Many autoantibodies in warm antibody AIHA demonstrate specificity within the Rh system but, even here, a distinction between autoantibodies and alloantibodies with Rh specificity is often possible. Whereas alloantibodies demonstrate truly specific reactions and give clearly negative reactions with cells lacking the appropriate antigen, autoantibodies commonly demonstrate "relative specificity." That is, autoantibodies that are described as having specificity within the Rh system react more strongly or to a higher titer against RBCs bearing a particular Rh antigen, but they will nevertheless react with RBCs lacking the antigen. Thus, a truly specific Rh

antibody strongly suggests that it is an alloantibody, whereas an antibody demonstrating "relative specificity" is characteristic of autoantibody.

A further clue to the differentiation of autoantibodies and alloantibodies having Rh specificity is the specificity itself. That is, anti-e is a rare cause of a DHTR,[6] but it is the most frequently described autoantibody specificity. In contrast, anti-E is the most common Rh alloantibody responsible for DHTRs,[6,44] but it is a relatively unusual autoantibody. Again, if pretransfusion RBCs are still available, it will be possible to use them to distinguish alloantibody from autoantibody with certainty.

If the antibody shows a defined specificity, the patient's RBCs should be tested for the relevant antigen. If it is an autoantibody, the patient's RBCs should possess the antigen. It is not always easy to determine the patient's phenotype, as DAT+ RBCs are difficult to phenotype and transfused RBCs may be present. If transfused RBCs are present, several methods are available for determining the phenotype of only the patient's RBCs:

1) One method depends on separating out the younger RBCs (e.g., reticulocytes are presumed to be the patient's own RBCs).[46–50]
2) Another method, which may be more reliable than the reticulocyte method, utilizes flow cytometry.[51–53]
3) A recent approach is to use DNA typing.[54–56]

*Additional approaches*

If the recipient's DAT was known to be negative prior to transfusion, or if the recipient's RBCs are still available for the performance of the DAT and it is demonstrated to be negative, this can be valuable information. An abrupt change in the DAT from negative to positive is strong evidence that the patient has a HTR rather than AIHA.

If it is possible to test the donor units of blood, this procedure may be of significance if an antibody having specificity that could be that of either an alloantibody or an autoantibody is detected, such as, for example, anti-E. If by chance, none of the donor units contained the E antigen, AIHA or an alloantibody-induced HTR caused by an undetected antibody must be considered.

Thus, with careful serologic testing, it is possible to distinguish a DHTR from AIHA in almost all cases. However, if pretransfusion RBCs are not available (as, unfortunately, is usually the case), and if the patient has an alloantibody or a mixture of alloantibodies with a broad range of reactivity, the distinction may be difficult.

A final note of caution is that the IHA may be neither a DHTR nor AIHA, but instead may be drug-induced IHA. In particular, cephalosporin drugs are frequently used in association with surgical procedures that may require transfusion. In this setting, abrupt onset of a positive DAT and hemolysis may be misinterpreted as a DHTR or the sudden onset of AIHA.

## References


1. Petz LD, Garratty G. Immune hemolytic anemias. 2nd ed. Philadelphia: Churchill Livingstone, 2004.
2. Kaplan HS, Garratty G. Predictive value of direct antiglobulin test results. Diagn Med 1985;8:29-33.
3. Wolach B, Heddle N, Barr RD, Zipursky A, Pai KR, Blajchman MA. Transient Donath-Landsteiner haemolytic anaemia. Br J Haematol 1981;48:425-34.
4. Heddle NM. Acute paroxysmal cold hemoglobinuria. Transfus Med Rev 1989;3:219-29.
5. Croucher BEE, Crookston MC, Crookston JH. Delayed haemolytic transfusion reactions simulating auto-immune haemolytic anaemia. Vox Sang 1967;12:32.
6. Croucher BEE. Differential diagnosis of delayed hemolytic transfusion reaction. In: Bell CA, editor. A Seminar on Laboratory Management of Hemolysis. Washington, DC: American Association of Blood Banks, 1979:151-60.
7. Worlledge SM. The interpretation of a positive direct antiglobulin test. Br J Haematol 1978;39: 157-62.
8. Barry KG, Crosby WH. Auto-immune hemolytic anemia arrested by removal of an ovarian teratoma: review of the literature and report of a case. Ann Intern Med 1957;47:1002-7.
9. Dawson MA, Talbert W, Yarbro JW. Hemolytic anemia associated with an ovarian tumor. Am J Med 1971;50:552-6.
10. De Bruyere M, Sokal G, Devoitille JM, Fauchet-Dutrieux MC, De Spa V. Autoimmune haemolytic anaemia and ovarian tumour. Br J Haematol 1971; 20:83-94.
11. Payne D, Muss HB, Homesley HD, Jobson VW, Baird FG. Autoimmune hemolytic anemia and ovarian dermoid cysts: case report and review of the literature. Cancer 1981;48:721-4.
12. Shashaty GG, Rath CE, Britt EJ. Autoimmune hemolytic anemia associated with ulcerative colitis. Am J Hematol 1977;3:199-208.

13. Ramakrishna R, Manoharan A. Auto-immune haemolytic anaemia in ulcerative colitis. Acta Haematol 1994;91:99-102.
14. Arner O, Brohult J, Engstedt L, Karlson R, Sallstrom T. Autoimmune hemolytic anemia in ulcerative colitis, cured by colectomy. Report of a case. Acta Med Scand 1971;189:275-7.
15. Cunningham-Rundles C. Hematologic complications of primary immune deficiencies. Blood Rev 2002;16:61-4.
16. Cunningham-Rundles C, Bodian C. Common variable immunodeficiency: clinical and immunological features of 248 patients. Clin Immunol 1999;92:34-48.
17. Oyefara BI, Kim HC, Danziger RN, Carroll M, Greene JM, Douglas SD. Autoimmune hemolytic anemia in chronic mucocutaneous candidiasis. Clin Diagn Lab Immunol 1994;1:38-43.
18. Cummins L, Spearer W, Stevenson S, et al. Wiskott-Aldrich syndrome: clinical, immunologic, and pathologic observations. Am J Dis Child 1959;98:579.
19. Pinchas-Hamiel O, Mandel M, Engelberg S, Passwell JH. Immune hemolytic anemia, thrombocytopenia and liver disease in a patient with DiGeorge syndrome. Isr J Med Sci 1994;30:530-2.
20. Hsu TC, Rosenfield RE, Burkart P, Wong KY, Kochwa S. Instrumented PVP-augmented antiglobulin tests. II. Evaluation of acquired hemolytic anemia. Vox Sang 1974;26:305-25.
21. Garratty G, Petz LD. An evaluation of commercial antiglobulin sera with particular reference to their anticomplement properties. Transfusion 1971;11: 79-88.
22. Von dem Borne AE, Engelfriet CP, Beckers D, Van Der Kort-Henkes G, Van Der Giessen M, Van Loghem JJ. Autoimmune haemolytic anaemias. II. Warm haemolysins—serological and immunochemical investigations and $^{51}$Cr studies. Clin Exp Immunol 1969;4:333-43.
23. Sokol RJ, Hewitt S, Stamps BK. Autoimmune haemolysis: an 18-year study of 865 cases referred to a regional transfusion centre. Br Med J (Clin Res Ed) 1981;282:2023-7.
24. Crookston JH. Hemolytic anemia with IgG and IgM autoantibodies and alloantibodies. Arch Intern Med 1975;135:1314.
25. Freedman J, Lim FC, Musclow E, Fernandes B, Rother I. Autoimmune hemolytic anemia with concurrence of warm and cold red cell autoantibodies and a warm hemolysin. Transfusion 1985; 25:368-72.
26. Kajii E, Miura Y, Ikemoto S. Characterization of autoantibodies in mixed-type autoimmune hemolytic anemia. Vox Sang 1991;60:45-52.
27. Shulman IA, Branch DR, Nelson JM, Thompson JC, Saxena S, Petz LD. Autoimmune hemolytic anemia with both cold and warm autoantibodies. JAMA 1985;253:1746-8.
28. Sokol RJ, Hewitt S, Stamps BK. Autoimmune hemolysis: mixed warm and cold antibody type. Acta Haematologica 1983;69:266-74.
29. Ries CA, Garratty G, Petz LD, Fudenberg HH. Paroxysmal cold hemoglobinuria: report of a case with an exceptionally high thermal range Donath-Landsteiner antibody. Blood 1971;38:491-9.
30. Lindgren S, Zimmerman S, Gibbs F, Garratty G. An unusual Donath-Landsteiner antibody detectable at 37°C by the antiglobulin test. Transfusion 1985; 25:142-4.
31. Nordhagen R. Two cases of paroxysmal cold hemo-globinuria with a Donath-Landsteiner antibody reactive by the indirect antiglobulin test using anti-IgG. Transfusion 1991;31:190-1.
32. Dacie JV. The haemolytic anaemias. Part II. Autoimmune haemolytic anaemias. 2nd ed. London: J. & A Churchill Ltd., 1962.
33. Sokol RJ, Booker DJ, Stamps R. Erythropoiesis: paroxysmal cold haemoglobinuria: A clinicopathological study of patients with a positive Donath-Landsteiner test. Hematol 1999;4:137-64.
34. Myhre BA. Fatalities from blood transfusion. JAMA 1980;244:1333-5.
35. Honig CL, Bove JR. Transfusion-associated fatalities: review of Bureau of Biologics reports 1976–1978. Transfusion 1980;20:653-61.
36. Sazama K. Reports of 355 transfusion-associated deaths: 1976 through 1985. Transfusion 1990;30: 583-90.
37. Linden JV, Kaplan HS. Transfusion errors: causes and effects. Transfus Med Rev 1994;8:169-83.
38. Williamson L, Cohen H, Love E, Jones H, Todd A, Soldan K. The Serious Hazards of Transfusion (SHOT) initiative: the UK approach to haemovigilance. Vox Sang 2000(78 suppl);2:291-5.
39. Thomson S, Johnstone M. Delayed haemolytic transfusion reaction due to anti-Jk$^a$ and anti-M. Can J Med Technol 1972;34:159-61.
40. Holland PV, Wallerstein RO. Delayed hemolytic transfusion reaction with acute renal failure. JAMA 1968;204:1007-8.

41. Rothman IK, Alter HJ, Strewler GJ. Delayed overt hemolytic transfusion reaction due to anti-U antibody. Transfusion 1976;16:357-60.
42. Pineda AA, Brzica SM Jr., Taswell HF. Hemolytic transfusion reaction. Recent experience in a large blood bank. Mayo Clin Proc 1978;53:378-90.
43. Mollison PL, Engelfriet CP, Contreras M, eds. Blood transfusion in clinical medicine. 10th ed., Oxford: Blackwell Science Ltd., 1997.
44. Pineda AA, Taswell HF, Brzica SM Jr. Delayed hemolytic transfusion reaction. An immunologic hazard of blood transfusion. Transfusion 1978;18:1-7.
45. Redman M, Regan F, Contreras M. A prospective study of the incidence of red cell alloimmunisation following transfusion. Vox Sang 1996;71:216-20.
46. Branch DR, Hian AL, Carlson F, Maslow WC, Petz LD. Erythrocyte age-fractionation using a Percoll-Renografin density gradient: application to autologous red cell antigen determinations in recently transfused patients. Am J Clin Pathol 1983;80:453-8.
47. Brecher M, ed. Technical manual. 14th ed. Bethesda, MD: American Association of Blood Banks, 2002.
48. Reid ME, Toy PT. Simplified method for recovery of autologous red blood cells from transfused patients. Am J Clin Pathol 1983;79:364-6.
49. Renton PH, Hancock JA. A simple method of separating erythrocytes of different ages. Vox Sang 1964;9:183.
50. Vengelen-Tyler V, Gonzales B. Reticulocyte rich RBCs will give weak reactions with many blood typing antisera (abstract). Transfusion 1985;25: 476.
51. Garratty G, Arndt PA. Applications of flow cytofluorometry to red blood cell immunology. Cytometry 1999;38:259-67.
52. Griffin GD, Lippert LE, Dow NS, Berger TA, Hickman MR, Salata KF. A flow cytometric method for phenotyping recipient red cells following transfusion. Transfusion 1994;34:233-7.
53. Wagner F. Identification of recipient Rh phenotype in a chronically transfused child by two-colour immunofluorescence. Transfus Med 1994; 4:205-8.
54. Wenk RE, Chiafari PA. DNA typing of recipient blood after massive transfusion. Transfusion 1997; 37:1108-10.
55. Reid ME, Rios M, Powell VI, Charles-Pierre D, Malavade V. DNA from blood samples can be used to genotype patients who have recently received a transfusion. Transfusion 2000;40:48-53.
56. Castilho L, Rios M, Pellegrino J, Rodrigues A, Costa FF. Blood group genotyping for the management of patients with "warm" antibody-induced hemolytic anemia (WAIHA) (abstract). Blood 2001;98:62a.

---

*Lawrence D. Petz, MD, StemCyte, 400 Rolyn Place, Arcadia, CA 91007; lpetz@mednet.ucla.edu.*