New Developments in the Understanding and Treatment of Autoimmune Hemolytic Anemia: Traditional and Novel Tests

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KEYWORDS

• Autoimmune hemolytic anemia • AIHA • DAT-Positive

KEY POINTS

- Correlation of clinical history, standard laboratory, and transfusion laboratory testing is critical to properly diagnosing autoimmune hemolytic anemia (AIHA).
- The standard ABO type, antibody screen, and direct antiglobulin test (DAT) is a key starting point in the evaluation.
- Dialogue between the clinical provider and the laboratorian can help with test selection and interpretation of findings.

BACKGROUND

Autoimmune hemolytic anemias (AIHAs) are heterogeneous disorders characterized by antibody-mediated red blood cell destruction. The destruction of red blood cells may take place in the intravascular space if the immune response results in the activation of the classical complement cascade or, more commonly, in the extravascular space when macrophages in the spleen and liver phagocytose opsonized red cells. The risk for intravascular or extravascular hemolysis is determined by the presence or absence of complement and/or immunoglobulin on the red blood cells. This risk can be assessed by serologic investigations carried out in the transfusion medicine laboratory, which guides a more precise diagnostic workup essential to providing appropriate therapeutic management.

The monospecific direct antiglobulin test (DAT) is a standard laboratory test used to determine if a patient's red blood cells have been coated in vivo with immunoglobulin,

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complement, or both. However, the DAT result alone does not define AIHA. A positive DAT may or may not be associated with immune-mediated hemolysis, and as shown in **Box 1**, there are multiple causes for a positive DAT result that are unrelated to immune-mediated hemolysis. Therefore, clinical findings and additional laboratory data, such as hemoglobin or hematocrit values, bilirubin, haptoglobin and lactate dehydrogenase (LDH) levels, reticulocyte count, and red blood cell morphology must be considered in addition to the DAT result. Likewise, standard transfusion tests such as a type and screen, the DAT and eluate as well as more specialized testing found in reference laboratories such as adsorptions, the Donath–Landsteiner test, and the modified monocyte assay (MMA) may be beneficial in the diagnostic workup of AIHA when used and interpreted appropriately. Understanding the value and limitations of the available tests is critical to appropriate test utilization and interpretation.

DISCUSSION

Overview of Autoimmune Hemolytic Anemia Classification

At the most basic level, the AIHAs are classified as "warm," "cold," and "mixed" forms. Warm forms of AIHA are typically polyclonal IgG autoantibodies and optimally reactive with red blood cells at 37°C; however, IgM or IgA autoantibodies can also present. The DAT results in warm forms may be variable; whereas, some older data indicate that the DAT results are most commonly positive for both IgG and complement more recent data show that more warm autoimmune hemolytic anemia (WAIHA) cases are positive for IgG only (43%) than those positive for both IgG and complement (17%).[1,2] Cold forms of AIHA are due to IgM, which is typically reactive at 22°C or below and has an optimal temperature of reaction at 4°C. These are often directed against the I/i system. IgM fixes complement more efficiently than other antibody isotypes and thus is more prone to cause intravascular hemolysis. The amount of red blood cell destruction by intravascular hemolysis is understood to be 200 mL of RBC in 1 hour; whereas, the destruction by extravascular hemolysis is 10-fold less.[3]

Recent recommendations from the First International Consensus Meeting provided standardization to the definitions of AIHA, and the appropriate definitions are provided in **Table 1** for diseases discussed in this article. Regardless of the specific criteria, it is often the isotype and optimal reactivity temperature of the antibody that categorize the AIHA into either WAIHA, cold agglutinin syndrome (CAS) or cold agglutinin disease

Box 1
Causes of a positive dat[5]

Auto- or alloantibodies to red blood cell antigens

Drug-induced antibodies to red blood cell antigens

Passively acquired antibodies (eg, from IVIG, antilymphocyte or antithymocyte globulin, or donor platelets/plasma)

Nonspecific adsorbed proteins (eg, hypergammaglobulinemia)

Complement activation due to bacterial infections

Sickle cell disease, beta-thalassemia

Autoimmune disorders

Modified from: Borge PD, Mansfield PM. The Positive Direct Antiglobulin Test and Immune-Mediated Hemolysis. In: Cohn CS, Delaney M, Johnson ST, Katz LM, eds. Technical Manual. 20th ed. Bethesda, MD: AABB; 2020:429-478.

Table 1
Disease definitions in AIHA[6]

Autoimmune Hemolytic Anemia (AIHA)	Hemolytic Anemia Caused by Antibody-Mediated Destruction of RBCs.
Warm AIHA (WAIHA)	WAIHA characteristically has a DAT positive for IgG, IgA (rarely), or C3d ± IgG and a clinically significant cold reactive antibody has been excluded.
Cold agglutinin disease (CAD)	CAD typically has a DAT positive for complement (and negative or weakly positive with IgG) and a cold agglutinin (CA) titer of 64 or greater at 4°C. Patients may have a B-cell clonal lymphoproliferative disorder without clinical or radiological evidence of malignancy.
Cold agglutinin syndrome (CAS)	CAS often has a DAT positive for complement (and negative or weakly positive with IgG) and a CA titer of 64 or greater at 4°C. Patients have an associated condition such as autoimmune disorder, infection, B-cell lymphoma, or other malignancy.
Mixed AIHA	Mixed AIHA is diagnosed in patients with a DAT positive for both complement and IgG. There is a cold antibody with a thermal amplitude ≥30°C and evidence of a warm IgG antibody.
Paroxysmal cold hemoglobinuria (PCH)	PCH is diagnosed in patients with hemolysis and a positive Donath–Landsteiner test.

Modified from: Jager U, Barcellini W, Broome CM, et al. Diagnosis and treatment of autoimmune hemolytic anemia in adults: Recommendations from the First International Consensus Meeting. *Blood Rev.* 2020;41:100648.

(CAD), mixed-type AIHA, or paroxysmal cold hemoglobinuria (PCH). WAIHA is classically defined by DAT-positive for IgG with or without complement. CAS and CAD are both DAT-positive for the complement, typically have anti-I immunoglobulin specificity and have a cold agglutinin titer greater than 64. Differentiation of these 2 entities will be discussed later in discussion. Mixed-type AIHA has elements of both warm and cold forms; it is DAT-positive for complement and IgG and has evidence of both a cold antibody with a thermal amplitude at or greater than 30°C and a warm IgG antibody. PCH is a rare finding of a biphasic anti-P immunoglobulin that fixes complement at cold temperatures and then causes hemolysis at 37°C. PCH is diagnosed with the Donath–Landsteiner test, which is often only available at reference laboratories. The details of the Donath–Landsteiner test are discussed later in discussion. **Table 2** shows the typical serologic characteristics of the AIHAs.

STANDARD TESTING

Type and Screen

The start of any work up in transfusion medicine should begin with the blood group type and antibody screen. This provides valuable information including a basic forward and reverse typing, which defines the ABO type of the patient and demonstrates the presence of anti-A and anti-B in their plasma. The forward and reverse typing should correspond, or discrepancies investigated further. The antibody screen can hint at pan-reactive antibodies versus alloantibodies reactive with just one or more

Table 2
Types of AIHA[5,6]

	WAIHA	CAS/CAD	MIXED-TYPE AIHA	PCH
DAT (Routine)	IgG ± Complement	Complement only	IgG + Complement	Complement only
Ig Type	IgG	IgM	IgG, IgM	IgG
Eluate	IgG	Nonreactive	IgG	Nonreactive
Specificity	Pan-reactive	Usually anti-I/i	Usually unclear	Anti-P
In Vivo Hemolysis	Extravascular	Intravascular	Extravascular and Intravascular	Intravascular

Modified from: Borge PD, Mansfield PM. The Positive Direct Antiglobulin Test and Immune-Mediated Hemolysis. In: Cohn CS, Delaney M, Johnson ST, Katz LM, eds. *Technical Manual*. 20th ed. Bethesda, MD: AABB; 2020:429-478. Jager U, Barcellini W, Broome CM, et al. Diagnosis and treatment of autoimmune hemolytic anemia in adults: Recommendations from the First International Consensus Meeting. *Blood Rev.* 2020;41:100648.

screening cells. A follow-up antibody panel for any positive screening cell can confirm the presence of a pan-reactive antibody versus an alloantibody. In addition, reactivity at room temperature immediate spin hints at an IgM antibody, versus the 37°C-reactivity with antihuman globulin that is more typical of IgG. The use of an autocontrol then helps to distinguish an antibody to a high incidence antigen from an autoantibody. If the autocontrol is negative, it suggests the antibody in the plasma targets an antigen that is absent from the patient's red blood cells; thus, it is most likely an alloantibody being detected. Conversely, if the autocontrol is positive, it suggests there is an antibody in the plasma that reacts with the patient's own cells. In the presence of a positive autocontrol a reflex DAT is performed. With these basic tests, one can quickly identify a warm autoantibody, a cold autoantibody, and the need for further testing. As discussed previously, an autoantibody alone is not pathognomonic of an AIHA, as more typically they are found in nonhemolytic states. Clinical-pathologic correlation is essential.

The Direct Antiglobulin Test

The DAT helps distinguish immune from nonimmune hemolytic anemia. While the DAT should be performed to identify AIHA in patients with hemolytic anemia, it should not be used as a screening test for hemolytic anemia if the presence of hemolysis has not been established. The predictive value of a positive DAT is 83% in a patient with hemolytic anemia; however, this drops to 1.4% in a patient without hemolytic anemia.[4] In addition, positive DATs have been reported in 1% to 15% of hospitalized patients and fewer than 0.1% of healthy blood donors.[2]

Samples submitted for DAT testing should be collected in lavender topped EDTA tubes. The EDTA present in the tube chelates calcium in the sample and prevents in vitro fixation of complement.[5] The DAT is performed by first washing red blood cells to remove plasma containing free globulins (immunoglobulin and complement). Then antihuman globulin (AHG) reagent is added. In most laboratories, a polyspecific AHG is used initially, which detects both IgG and complement. The IgG is detected by an antibody targeting the IgG heavy chains (Fc portion), and complement is detected by an antibody to the C3d fragment of complement. If agglutination is observed following the addition of polyspecific AHG, then there is either IgG and/or complement present on the red cells. This agglutination can be assessed and scored by the strength of reactivity, which is usually proportional to the amount of bound protein.[5] If the results are positive with polyspecific AHG, tests with monospecific anti-IgG and anticomplement antisera are performed to appropriately distinguish the immune process involved.[5] Some laboratories do not use a polyspecific reagent and choose to test initially with monospecific anti-IgG and anticomplement antisera. Monospecific anticomplement often contains anti-C3b in addition to the anti-C3d used in polyspecific AHG. Although polyspecific AHG does not contain anti-IgM, complement deposition remains an indirect sign of prior IgM binding because IgM is a potent activator of complement.[6] It is important to remember that IgG1 and IgG3 are able to activate complement; however, most of the complement detected by the DAT is caused by IgM.[7] While some manufactures offer additional monospecific anticomplement antisera (anti-C3, -C3c, -C3d, -C4, -C4c), anti-C3d provides optimal detection of sensitization and is the only anticomplement antisera required by US FDA regulations.[8] With a positive result for the monospecific IgG reagent, an eluate is often performed. In the case of a WAIHA, the eluate will show pan-reactivity at 37°C with an AHG reagent, thus confirming that the patient's positive DAT is caused by an IgG antibody reacting against red cells at 37°C (see later in discussion for further discussion on eluates).

When no agglutination is observed, the DAT result is negative. A negative DAT result does not necessarily mean that the red cells have no attached globulin molecules. Known diagnostic pitfalls include IgG bound at a quantitative level below the threshold of sensitivity, low-affinity IgG, warm IgM, and IgA autoantibodies in addition to a myriad of technical issues. In manual tube testing, polyspecific and monospecific reagents detect 100 to 500 molecules of IgG per cell and 400 to 1100 molecules of complement per cell, but patients may still experience autoimmune hemolytic anemia when the coating molecules are below these levels of detection.[9] Testing with microcolumn and solid-phase methods is able to detect approximately 200 to 300 molecules of IgG per cell.[10] Novel tests, capable of detecting smaller amounts of antibodies, are discussed briefly later in discussion and in more detail in a subsequent article in this issue. Alternatively, the DAT result can be negative if low-affinity autoantibodies were present but then removed in the initial wash step. False-negative DAT results due to these low-affinity autoantibodies may be overcome by testing with low ionic strength saline (LISS) or performing the initial wash step using cold saline.[3] IgM autoantibodies that react with a thermal range close to 37°C (warm IgM) produce a rare but severe form of AIHA that also may be DAT-negative.[11] Testing with a dual DAT (DDAT), which is discussed in more detail later in discussion, aids in the identification of warm IgM autoantibodies that are typically missed by testing with a standard DAT only.[12] In addition, an IgA antibody will not be detected by a standard DAT; therefore, falsely negative-DATs occur in rare cases when the patient's hemolysis is due to IgA-mediated red blood cell destruction.[13,14] In 800 patients with DAT-negative hemolytic anemia, IgA alone was found on 2% of samples.[15]

As mentioned previously, a positive DAT result alone is not diagnostic of hemolytic anemia. The significance of a positive DAT result can only be interpreted in the context of a patient's medical history, including medication, pregnancy, transfusion, and hematopoietic transplantation history. It is also important to consider the specific testing method used when interpreting the results. In a comparative study of various DAT methods, Barcellini and colleagues found that the manual tube DAT method was the most specific but least sensitive test (0.87 and 0.43, respectively); whereas, other traditional DAT methods of microcolumn and solid phase showed reduced specificity but increased sensitivity (0.70 and 0.65, respectively).[16] Despite these limitations, additional serologic investigations, such as an eluate, can be helpful in the evaluation of an AIHA.

The Eluate

Elution frees antibody from sensitized red blood cells and recovers the antibody in a useable/testable/analyzable form, enabling the laboratory to determine the antibody's specificity. Like the antibody screen, this is accomplished by testing the freed antibodies present in the eluate against panel cells. An elution is typically only performed if the DAT is positive with monospecific anti-IgG antisera; however, one of the most common causes for AIHA associated with a negative DAT is RBC-bound IgG below the sensitivity threshold of the DAT, and it has been suggested that an eluate can be useful when there is a high clinical suspicion for AIHA and the DAT is negative.[15] In such a case, the eluate may be useful as it effectively concentrates the antibody present and therefore can gain sensitivity.

If the DAT was only positive for complement the eluate is likely to be nonreactive. If the DAT demonstrates the presence of IgG and the eluate reacts with all cells tested, including an autocontrol cell previously treated to remove bound antibody that on retesting demonstrates rebinding of the antibody, then an autoantibody is the most likely explanation. In this case, an adsorption should be performed to aid in identifying

whether there are any alloantibodies present in addition to the pan-reactive autoantibodies (see later in discussion).

The eluate may also demonstrate the presence of alloantibodies that may have sensitized red blood cells in the context of a delayed hemolytic transfusion reaction or hemolytic disease of the fetus and newborn. Working closely with the transfusion medicine laboratory to determine transfusion history and with the obstetrics teams to determine pertinent pregnancy history often provides the clinical correlation necessary to appropriately interpret the significance of alloantibodies present in the eluate.

Adsorptions

It was mentioned above that pan-reactivity in the antibody screen and eluate is characteristic of warm AIHA. The presence of a pan-reactive autoantibody in the plasma increases the complexity of the transfusion laboratory's testing and results in significant time requirements to complete required pretransfusion testing, which must always assess whether there are alloantibodies present in addition to any autoantibodies identified. The root of the problem is that the pan-reactive antibody can mask an underlying alloantibody. Additional testing using adsorptions is required to unmask such alloantibodies.

Adsorption is a method that removes autoantibody from the sample while preserving any alloantibodies that may exist. Fundamentally, a patient will only have an alloantibody to an antigen that they lack; therefore, testing will use phenotypically similar red cells (ie, cells negative for the same antigens the patient lacks) to absorb autoantibody while leaving behind any alloantibody. Depending on the patient's medical history, the absorbing cell can be an autologous red cell or an allogenic red cell. If there is no history that might result in an unclear red cell phenotype, such as recent transfusion, pregnancy, or stem cell transplantation, then autologous adsorption may be performed. If such a history does exist, allogenic red cell adsorption must be performed to prevent absorbing an alloantibody onto an unexpected antigen. Performing adsorptions to identify any underlying alloantibodies can increase testing time significantly, sometimes up to 8 to 12 hours.

Alternative to performing adsorption is to transfuse phenotypically matched blood. This approach is often utilized at institutions that do not have a reference laboratory in house; however, it is dependent on having an accurate phenotype. If the patient has not been transfused, serologic methods can identify the phenotype of the patient; however, in the context of recent transfusion, DNA-based methods are necessary to provide an accurate phenotype.

Warm Autoimmune Hemolytic Anemia

Most of the AIHA is caused by polyclonal warm reactive autoantibodies that react optimally at 37°C. In making the diagnosis of WAIHA, a clinically significant cold reactive antibody should be excluded. WAIHA is classically defined by reactivity at 37° C and a DAT that is positive for IgG with or without complement. However, a high thermal amplitude IgM or IgA can also result in WAIHA.[3,11,15] In IgG-positive WAIHA, the elution is typically pan-reactive with all cells tested, including the autocontrol.

In approximately one-third of patients with WAIHA, agglutination is also detected at room temperature due to nonpathologic IgM antibodies; however, these cold-reacting agglutinins (CA) have titers less than 64 at 4°C and are nonreactive at warmer temperatures of 30 to 37°C. Because the CA titer is <64, they are not considered clinically significant and the patient does not meet defined criteria for CAS or CAD in addition to WAIHA.[2,6] This is important to distinguish from the more severe and rare form of mixed-type AIHA given the difference in prognosis and treatment approach.

IgM-mediated WAIHA is associated with IgM autoantibodies that react at 37°C and may portend a poorer prognosis with rapid progression of fatal hemolysis noted.[2,11] These autoantibodies tend to cause spontaneous agglutination in the DAT, making identification a challenge. Traditional testing methods can overcome the interference caused by a warm IgM by treatment with dithiothreitol (DTT) or 2-mercaptoethanol (2-ME), which disrupts the disulfide bonds of IgM molecules and enables controls to be appropriately evaluated. It is important to distinguish warm IgM from clinically significant cold agglutinins, which can be easily done by titration studies. Warm IgM often has low titers of less than 64 at 4°C, which makes the distinction from typical CAS and CAD.[5] Of note some authors would still consider this to be "low titer high thermal amplitude" and a part of the spectrum of CAS/CAD. Below in the novel testing section, we discuss the dual DAT, which is another method to identify warm IgM.

Cold Agglutinin Syndrome and Cold Agglutinin Disease

Clinically significant cold agglutinins are usually IgM-mediated with complement as the only protein detected on the DAT. As in vitro testing demonstrates that IgM binding is optimal at colder temperatures, it is assumed that in vivo IgM binding occurs in the peripheral circulation and causes complement activation. As the cells are circulated to warmer central regions of the vasculature, the IgM dissociates, and the bound complement remains.

CAS and CAD both have a DAT positive for complement (and negative for IgG) with a cold agglutinin titer greater than 64 when tested at 4°C. Of note, while the accepted threshold for diagnosis is 64, many experts consider a range between 64 and 512 to be equivocal and a titer more than 512 to be clinically significant.[17] The titer is determined by testing serial dilutions of patient serum and each dilution's ability to agglutinate red blood cells. The titer is the inverse of the highest dilution at which agglutination occurs. As such, the titer reflects the concentration and avidity of the antibody. Usually, the titer needs to be ordered separately and results are reported separately from the DAT. Depending on how the test has been validated, some institutions may report the IgM titer based on critical thresholds, such as less than 64, 64 to 512, or greater than 512. Therefore, a low titer would be reported as less than 64.

Patients with CAS have a known associated condition, for example, autoimmune disorders, infection, clinical or radiologic evidence of B-cell lymphoma, or another malignancy. On the other hand, patients with CAD lack an associated condition; however, they may have a B-cell clonal lymphoproliferative disorder detectable in blood or marrow, but no clinical or radiological evidence of malignancy.[6]

Cold agglutinins that are clinically significant for causing hemolysis are usually present at high titers (greater than 512) when tested at 4°C and they often react at 30°C; however, it has been noted that some clinically significant cold agglutinins may have a lower titer. Thermal amplitude testing identifies the highest temperature at which the antibody will bind to an antigen and whether the cold IgM autoantibody will be clinically significant at relevant temperatures. The thermal amplitude test is performed at 4°C, 22°C, 30°C, and 37°C to determine the temperatures at which the cold autoantibody is reactive. Cold antibodies that are reactive at temperatures less than 30°C are not considered to be clinically significant in most situations. When the IgM antibody reactions at greater than 30°C, it may be causing hemolysis; therefore, it has the potential to be clinically significant even at a low titer. As such, thermal amplitude testing is best used when a hemolytic patient has a DAT positive for complement only and cold agglutinins are present at low titers because thermal amplitude testing identifies the "low titer high thermal amplitude" cold agglutinins that might be missed when using greater than 64 or greater than 512 as a cut off for clinically significant cold agglutinins. It is important to remember

that to determine the true thermal amplitude or titer of a suspected cold agglutinin, the specimen must be collected and maintained at 37°C until the serum and red cells are separated to avoid in vitro autoadsorption of the IgM autoantibody.[5]

Mixed-Type Autoimmune Hemolytic Anemia

While there is a set of patients that have both WAIHA and nonpathogenic cold antibodies (discussed in the WAIHA section above), there is a smaller group of patients that have both WAIHA and a pathogenic cold component. This latter group is often referred to as "mixed-type" AIHA. Typically, mixed-type AIHA will have a DAT positive for both IgG and complement. In addition, there is evidence of both a warm IgG antibody and a cold antibody with a thermal amplitude $\geq$30°C.[6]

Usually both IgG and complement are detected in the DAT of mixed forms, but it's been reported that IgG, complement, or IgA alone were detected in mixed-type AIHA.[2] As with other IgG-mediated AIHAs, the eluate is usually pan-reactive. Unique to this group of patients, however, is that the adsorption testing necessary to detect the presence of underlying alloantibodies must be performed at both 4°C and 37°C.[5]

Paroxysmal Cold Hemoglobinuria

PCH is the rarest form of DAT-positive AIHA. It often presents secondary to a viral infection in young children. As such, the classic biphasic (also termed bithermic) hemolysin may only be transiently detectable. This hemolysin associated with PCH is a cold-reactive IgG antibody capable of binding complement. As with CAS/CAD, the antibody binds in the colder periphery, and complement is fixed before movement to warmer body parts. Activation of the classical complement pathway occurs after warming to 37°C in the central circulation at which time the IgG dissociates.[18] Therefore, routine DAT results will often only be positive for the complement. These patients typically do not have IgG positivity on their red blood cell surface and no evidence of autoantibody activity in the serum or the eluate made from the DAT-positive cells.

Testing for PCH with a Donath–Landsteiner (DL) test is suggested for patients with a positive DAT result due to complement alone with demonstrable hemoglobinemia or hemoglobinuria. A DL tests the patient's serum at different temperatures to mimic the presumed in vivo activation of the biphasic antibody: constantly cold, a bithermic stage that begins cold and finishes warm, and constantly warm. In each temperature set, there are 3 tubes: a tube with patient plasma only, a tube with patient plasma and additional donor plasma, and a negative control without patient plasma. The donor plasma supplements complement levels that may be low due to consumption in a patient with active hemolysis. If the DL test demonstrates hemolysis in the biphasic set of tests alone, it is diagnostic of PCH, as seen in **Fig. 1**. This is a cumbersome test with a stringent requirement for the patient sample to remain at 37° C; therefore, it is typically only performed at in-house reference laboratories.

Additional Testing

Autoantibodies most often have broad specificities leading to the classic pan-reactive testing described above; whereas, in other cases, specificity to single antigen types can be seen. Regardless of the specificities of the antibodies, distinguishing an alloantibody versus an autoantibody remains essential. This is typically done by using commercial antigen typing reagents (polyclonal and monoclonal antibodies) to determine if the patient possesses the antigen to which the antibody is specific. This is analyzed in combination with the autocontrol as in some cases patients express only partial antigen molecules and can make true alloantibodies against the nonexpressed portions. Additional genetic testing using single nucleotide polymorphism

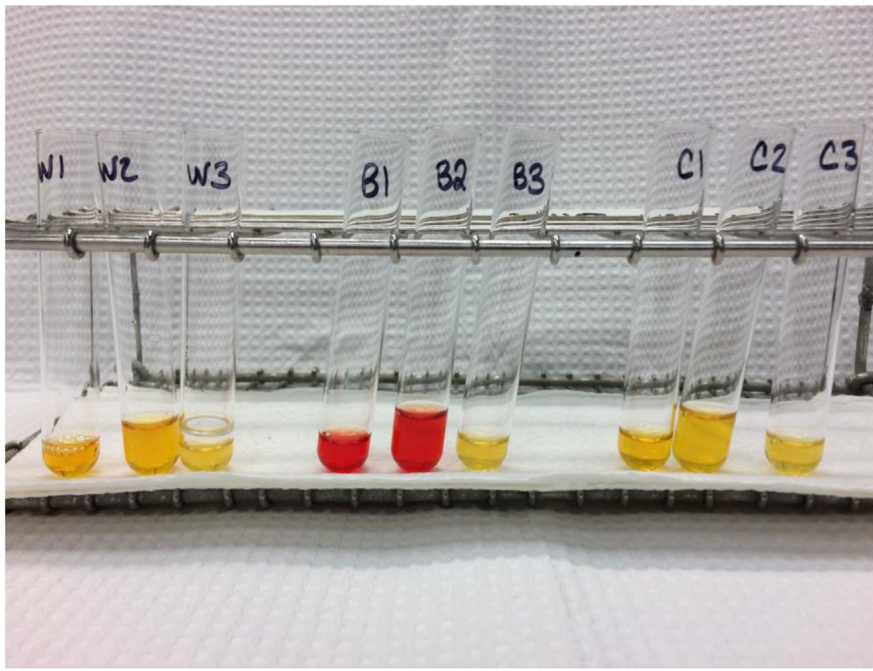

Fig. 1. Positive Donath–Landsteiner Test. W1–W3 are tested at warm temperatures; B1–3 are the biphasic tubes, and C1–3 are the cold tubes. For each set, tube 1 contains patient plasma, tube 2 contains patient plasma and additional donor plasma in case the patient's complement levels are low, and tube 3 is a negative control without patient plasma. A positive test demonstrates hemolysis that occurs only in the biphasic tubes (B1 and B2) and negative in the negative control (B3). (*Courtesy of* Mayo Clinic Laboratories, Mayo Clinic, Rochester, MN.)

(SNP) analysis with commercial arrays or gene sequencing at reference laboratories can also be helpful to confirm such findings.

Monocyte Monolayer Assay

Occasionally, an allo- or autoantibody is present that is of unclear clinical significance. The MMA is designed to assess the clinical relevance of antibodies. This is done by mixing the patient's plasma with red blood cells expressing the antigen to which the antibodies have specificity. Third party donor monocytes are added and both opsonization and phagocytosis of red blood cells are assessed microscopically. If more than 5% of the red blood cells are either tethered to a monocyte or phagocytosed, then the antibody is considered clinically significant. Although not always done, it is possible to use the patient's own cells rather than donor red blood cells.

Drug-Induced Hemolytic Anemia

Drug-induced hemolytic anemia (DIHA) is covered elsewhere in this issue and will not be discussed at length. It is important to be aware, however, that the findings can mimic a standard warm autoantibody typical of AIHA or show no reactivity. This is due to the neo-epitopes target and the immune mechanisms that generate the antibodies in this underrecognized entity. It is necessary to obtain a detailed clinical history with a clear timeline in relation to the start of hemolysis and the start and stop time

of new drugs in the weeks and months preceding hemolysis. Specialized testing methodologies can help confirm suspicions of DIHA.

Novel Direct Antiglobulin Test Testing

Additional variations of DAT are discussed elsewhere in this addition of the journal but are briefly mentioned here. Novel tests, typically only performed in reference laboratories, are capable of detecting smaller amounts of antibodies bound to the patient's red blood cells compared with the standard manual tube DAT testing; however, positivity in these novel tests must be interpreted with caution given their higher sensitivities and lower specificities.

Flow cytometry provides a simple and low-cost method to detect immunoglobulin and complement bound to red blood cells; however, positive results have limited stand-alone value. While a standard tube DAT identifies IgG on red blood cells in 1 in 1400 healthy donors, flow cytometry is so sensitive that it can be positive for IgG-bound red blood cells in most, if not all, individuals.[2,19,20] Recall that a manual tube DAT will result as positive if a minimum of 100 to 500 molecules of IgG are present per red blood cell. Flow cytometry is so sensitive that it will detect as few as 30 to 40 molecules of IgG per red blood cell.[20] Therefore, the use of flow cytometry may be useful in the DAT-negative AIHA patient in which there is an extremely high level of clinical suspicion. Specifically, the flow DAT can aid in the detection of IgM AIHA and can subclass IgG molecules to assess the risk of complement-mediated intravascular hemolysis.[21] The extreme sensitivity of the test necessitates careful clinical correlation with any positive results.

Mitogen stimulated DAT (MS-DAT) is a functional and quantitative method that detects red blood cell antibodies that may be too few for standard DAT methods to detect. A culture of a patient's whole blood is performed in the presence of IL-6 and mitogens, which results in an amplification of antibody production and significantly increases the IgG bound to autologous red blood cells compared with unstimulated cultures.[22] Compared with standard manual tube DAT and microcolumn/solid-phase DATs, the MS-DAT is the least specific but the most sensitive test (0.59 and 0.88, respectively).[16] Although highly sensitive, the utility of MS-DAT is limited to patients not on steroid therapy, as steroids affect the in vitro lymphocyte capability to respond to mitogen stimulation and may result in false-negative results.[23]

There are numerous other novel methods to identify the presence of antiglobulin and/or complement on red blood cells. These include the immunoradiometric assay (IRMA), the complement fixation antibody consumption test, the enzyme-linked immunoassay test (ELAT), as well as the enzyme-linked immunosorbent assay (ELISA).[24–27] While these tests each may identify red blood cell-bound antibody, the quantity of bound antibody remains only one of the many factors influencing the degree of erythrocyte destruction, and as such, the manual tube DAT remains the gold standard to be interpreted in the patient with hemolysis.[19,20] However, as echoed by Barcellini, Petz, and Garratty, no single test is optimal in the diagnosis of AIHA.

The dual DAT (DDAT) method was developed because warm IgM AIHA is a serious disease, yet laboratory testing was inadequate at identifying IgM autoantibodies. The presence of IgM is often implicated when complement is detected in the DAT result; however, direct identification of IgM in the DAT has been limited by multiple factors. The use of anti-human IgM is limited due to spontaneous red blood cell agglutination, which results in invalid test controls, and as such, there remains a lack of standardized anti-IgM reagents. The DDAT bypasses this limitation by performing 2 stages of sensitization with IgG rabbit anti-human IgM and then IgG goat anti-rabbit IgG. First, the DAT cells are incubated with a rabbit IgG antibody that is specific to human IgM. The cells are washed, and any unbound antibody is removed. A second incubation

step occurs following the addition of a goat IgG with anti-rabbit IgG specificity. The presence of agglutination following the dual incubations indicates that the original DAT cells had human IgM bound to them.[12] By controlling the temperatures of the incubation and wash steps, the dual DAT method enables one to identify IgM bound in vivo to the red blood cells without invalidating testing.

SUMMARY

AIHA is a series of clinical entities diagnosed by standard and specialized transfusion laboratory testing used in the appropriate clinical setting. In patients with confirmed hemolysis, an ABO type and screen with a DAT can often diagnose the standard WAIHA. Additional specialized testing such as cold agglutinin titers and the Donath–Landsteiner test for biphasic hemagglutinins can further define the presence of more rare entities such as CAS/CAD or PCH. Other specialized testing such as enhanced DATs and testing for drug-induced hemolytic anemias can also be useful in the appropriate setting. An open dialogue and consultation between the clinician and laboratorian can often lend efficiency and specificity to the workup while correlating the clinical history with the laboratory findings.

CLINICS CARE POINTS

- Standard tests such as ABO typing and antibody screening along with a DAT are the intial starting point for testing for AIHA.
- Specialized testing such as sensitive DAT testing should only be performed with a known history of AIHA.
- All testing should be interpreted within the clinical context.

DISCLOSURE

The authors have nothing to disclose.

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