Review

A 13-Question Approach to Resolving Serological Discrepancies in the Transfusion Medicine Laboratory

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ABSTRACT

Laboratory professionals, consultants, and treating physicians may encounter discrepancies in serological testing results for numerous reasons; identifying the reason(s) for the presence of an unexpected antibody or antigen can be challenging. A question-based approach can be useful in identifying the underlying cause of the discrepancy. We describe a new approach to serological problems in a transfusion-service laboratory. The approach we outline herein is targeted towards a general transfusion medicine service, rather than a center that offers complex antibody investigations using specialized techniques. This question-based problem-solving approach considers patient factors including diagnosis, transfusion history, previous pregnancies, and medication history, along with serological test results: ABO and Rh groups, direct and indirect antiglobulin tests, reacting temperature of the antibody, effect of enzyme treatment of cells, strength of reactivity, and antibody reactivity with umbilical cord cells. We also demonstrate the usefulness of this approach through a case scenario.

Keywords: serological testing, antibody discrepancy, antigen discrepancy, transfusion

Discrepancies in serological testing may occur for a variety of reasons; these data have often been categorized according to a lack of expected antigens or antibodies or by the presence of unexpected antigens or antibodies. Resolving them, however, requires a systematic approach for gathering relevant information that will assist the laboratory professional, consultant, and/or physician to understand the significance of the findings and to identify additional tests that may resolve discrepancies.

Abbreviations

TM, transfusion medicine; IgG, immunoglobulin G; DAT, direct antiglobulin test; IgM, immunoglobulin M; EBV, Epstein-Barr virus; PCH, paroxysmal cold hemoglobinuria; RBC, red blood cell; IVIG, intravenous immunoglobulin; DSTR, delayed serological transfusion reaction; RHIG, Rh immunoglobulin; ITP, idiopathic thrombocytopenic purpura; AIHA, autoimmune hemolytic anemia; IV, intravenous; PVP, polyvinylpyrrolidone; HTLA, high titer low avidity; NSAIDs, nonsteroidal anti-inflammatory drugs; NA, not applicable

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Unresolved serological antibody discrepancies can delay the identification of compatible units for transfusion, the availability of compatible blood, or identification of an underlying abnormality.

A question-based approach was developed for serological problems in which the response to each question provides a piece of the puzzle that will eventually resolve the clinical picture. This approach has been taught to many physician trainees and can be applied to serological discrepancies encountered in transfusion services; it is designed for physicians who serve as directors of transfusion laboratories, trainees who consult for the transfusion medicine (TM) laboratory as part of their training, and laboratory technologists. It is meant to be a tool to aid in the investigation and resolution of serological discrepancies in the routine hospital setting. The approach presented herein is targeted towards a general TM service rather than toward centers that undertake complex antibody investigations using specialized techniques.

In this article, we identify and discuss 13 essential questions that laboratory professionals, consultants, and treating physicians should ask (**Figure 1**). The order in which the questions should be asked varies according to the serological problem encountered and the answers

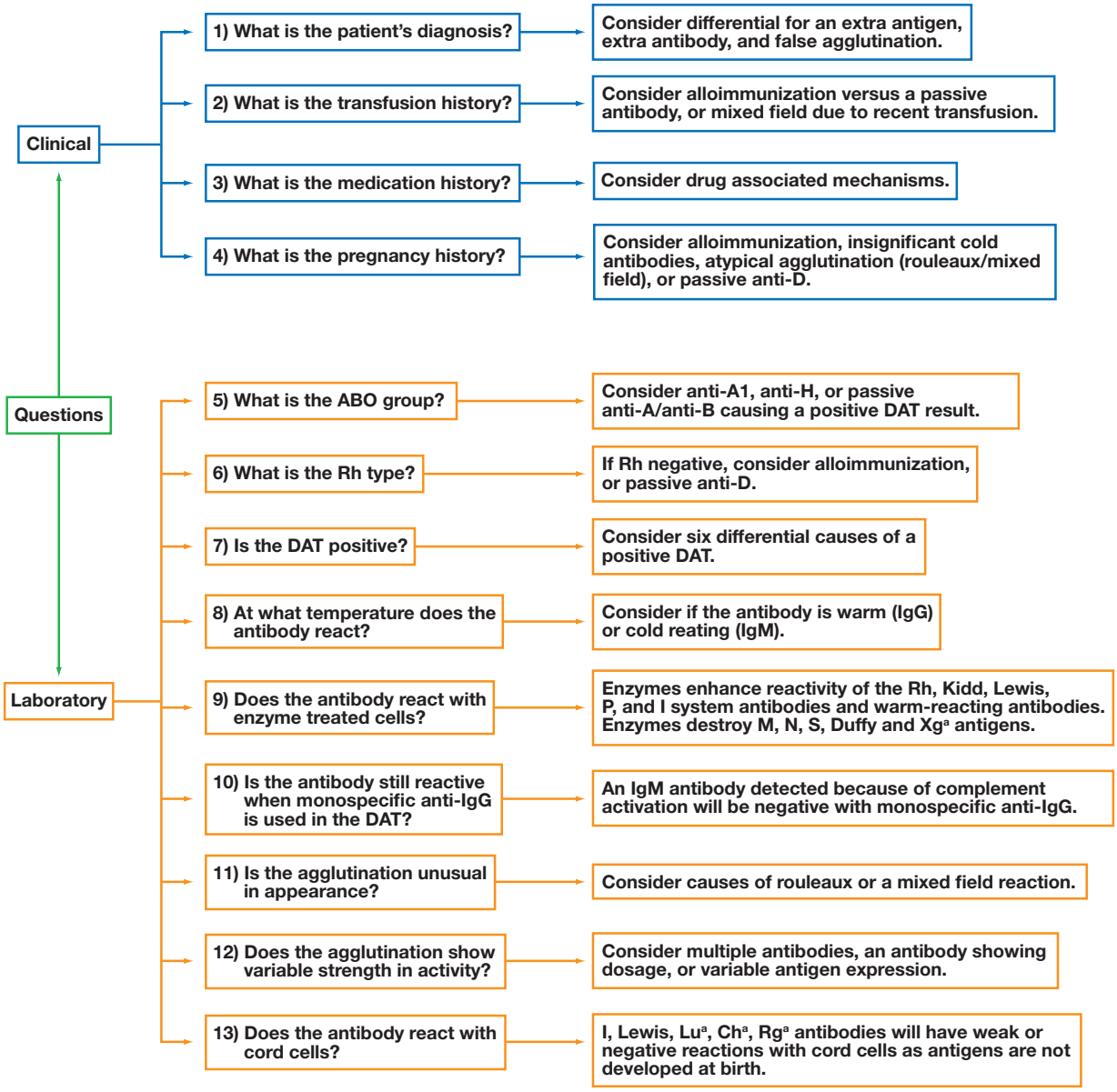

Figure 1

Thirteen questions (clinical- and laboratory-based) that laboratory profesionals, consultants, and treating physicians can ask themselves to guide their investigations of the serological discrepancies and atypical findings they encounter in the transfusion medicine laboratory. *DAT indicates direct antiglobulin test; IgG, immunoglobulin G; IgM, immunoglobulin M; RBCs, red blood cells.*

revealed through the process. In some situations, the problem may be resolved without addressing all 13 questions. It is important not only to ask the questions but also to understand the relevance and potential implications of the answers because this may reveal which question is most relevant to ask next.

Question #1: What is the diagnosis for the patient?

It is helpful to know the diagnosis for the patient because certain serological problems are more common with particular diseases. Diseases associated with warm or

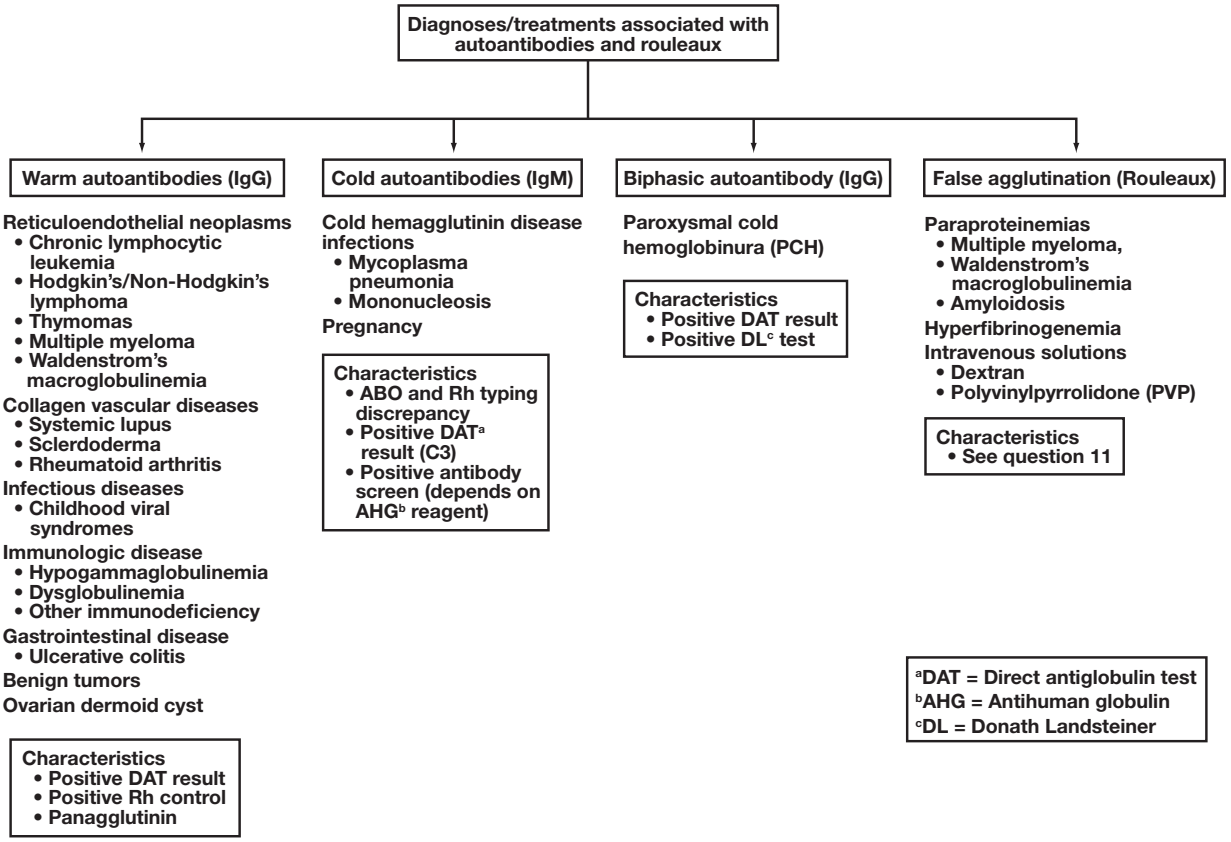

Figure 2

Summary of conditions that may be associated with autoantibody formation and conditions in which pseudoagglutination (rouleaux) can be present. *IgG indicates immunoglobulin G; IgM, immunoglobulin M; DAT, direct antiglobulin test; DL, Donath-Landsteiner.*

cold autoantibody formation are summarized in **Figure 2**. Warm autoantibodies are typically immunoglobulin G (IgG) antibodies that may be primary or can be secondary to underlying disease states, such as lymphoproliferative diseases or in collagen vascular diseases.[1,2] Typical features of warm autoantibodies include a positive direct antiglobulin test (DAT) result; a positive Rh control (depending on the reagent being used); and, in most cases, a positive antibody screening (panagglutinin) result. Warm autoantibodies do not typically cause an ABO-grouping discrepancy. Cold autoantibodies, which usually are immunoglobulin M (IgM) type, are produced in primary or secondary cold hemagglutinin disease.[1,3] *Mycoplasma pneumoniae* infection is associated with production of anti-I; Epstein-Barr virus (EBV) is associated with production of anti-i.[3]

Cold autoantibodies typically cause discrepancies in ABO grouping and Rh typing, a positive DAT (usually C3), and positive results in the antibody screen and cross-match if the antibody has high thermal activity and/or if a polyspecific antiglobulin reagent is being used.[4] The biphasic IgG antibody found in paroxysmal cold hemoglobinuria (PCH), which occurs primarily in children after a viral infection,[5] is unlikely to be detected in routine serological testing because biphasic testing would be required as part of the investigation for an IgG that is associated with PCH. In some cases, however, the DAT result may be positive with complement present on the red cell surface.

Some diagnoses are associated with abnormal plasma proteins that can cause artifactual agglutination, or rouleaux. Rouleaux is also observed in the laboratory when blood specimens are collected after infusion of certain intravenous fluids (eg, high-molecular-weight dextran) and in medical situations in which plasma protein levels are elevated, such as multiple myeloma, inflammatory

Table 1. Medical Conditions and Mechanisms of Polyagglutination That Can Cause Serological Discrepancies

Extra Antigen	Diagnosis/Comorbidities	Mechanism
T activation[7]	Clostridia Corynebacteria *Escherichia coli* Streptococcus Staphylococcus *Vibrio cholera* Influenza virus Primary and metastatic carcinoma (breast, bladder, lung and pancreas)	Unmasking of the cryptic T antigen by bacterial sialidases, which cleaves sialic acid residues from the red cell membrane glycoproteins and glycolipids leaving a terminal D-galactose.
Tn activation[7]	Leukemia Breast carcinoma	The Tn antigen is similar to the T antigen but lacks the terminal galactose due to a somatic mutation.
Tk activation[7]	*Bacteroides fragilis* *Serratia marcescens* *Candida albacans* Clostridia Pneumococci	The Tk receptor is associated with normal cellular sialic acid content, but is formed with microbial endo- or exo-B-galactosidases act on the red cell surface.
Acquired B[8]	*Escherichia coli* *Clostridium tertium* Gastric or colonic malignancy Bowel disorders	The acquired B phenomenon refers to the deacetylation of group A receptors (N-acetyl-D-galactosamine), via microbial activity leaving a terminal D-galactosamine that minimics the terminal D-galactose of a normal B antigen.

and connective tissue disorders, cancers, and pregnancy (**Figure 2**). Rouleaux is discussed further under question 11: Is the agglutination unusual in appearance?"

Some diseases are associated with production of an extra antigen. An extra antigen can be suspected when a discrepancy between the forward and reverse ABO grouping occurs; our experience has been that the discrepant result occurs within the forward grouping. These discrepancies are often categorized under the term polyagglutination (red blood cells [RBCs]) that agglutinate in the presence of almost all adult human sera but not with autologous serum or the sera of neonates[6]). Polyagglutination is a rare event in which cryptic antigens become unmasked (T antigen) or antigen structures on the RBC membrane become modified (to become Tn, Tk, or acquired B antigens). Diagnoses and comorbidities, as well as the mechanisms by which these extra antigens appear, are summarized in **Table 1**. Most of these extra antigens are rarely encountered; in some cases, they would not be detected in a modern laboratory because of the switch from human to monoclonal ABO typing reagents.[7-9]

Knowing the diagnosis of the patient can also be useful in cases of a suppressed antigen or missing antibodies. In acute leukemia, the A antigen may decrease in strength or disappear, and the group A RBCs of the patient may show a weak or negative reaction with anti-A. The suspected mechanism for a weak or absent A antigen is a decrease in the production of *N*-acetyl-D-galactosaminyltransferase, the enzyme responsible for adding the terminal *N*-acetyl-D-galactosamine to the H subunit to form the A antigen. Cases of missing antibodies may be observed in neonates, elderly individuals (greater than 70 years of age), patients who have just undergone bone marrow transplantation, or in association with hypo- or agammaglobulinemia.[10] Neonates do not produce anti-A or anti-B antibodies until they reach the age of 3 to 6 months; elderly patients may have reduced levels of anti-A or anti-B along with generally reduced levels of immunoglobulins.[11] It is useful to know the common diagnoses that can be associated with serological anomalies because this information may provide clues to possible reasons for the results.

Question 2: What is the transfusion history?

Has the patient ever received a transfusion? Has he or she received a transfusion within the previous 3 months? If so, which blood products were administered?

A history of previous transfusion may reveal a patient that has been alloimmunized to RBC antigens with formation of a potentially clinically relevant IgG antibody. Clinically significant alloantibodies occur most commonly after RBC transfusion; however, active alloimmunization can be induced by RBC contamination of platelet products; also, in less common situations, the presence of passive antibody can be detected in the patient's blood after the transfusion of large volumes of plasma. Hence, it is important to ask not only about RBC transfusions but also about transfusions with other blood products. The frequency of RBC alloimmunization varies according to the antigen distribution among patients and donors in a given geographic area, with estimates of 1% to 2% in the general hospital population[12] and 15% or greater in patients with transfusion-dependent diseases such as sickle cell disease or hemoglobinopathies.[13-15] If a patient has never received a transfusion or been pregnant, IgG RBC alloantibodies should not be present; however, a patient of this type can possess IgM alloantibodies to RBCs because these can be environmentally stimulated. Environmentally stimulated IgM antibodies are not usually clinically relevant; however, this will depend on the thermal range of the antibody and the ability of the antibody to activate complement. Other than ABO antibodies, the typical IgM alloantibodies detected in the transfusion laboratory are directed against the following antigens: M, N, S (some are of IgM and some of IgG type), Le^a, Le^b, P_1, Lu^a, and anti-A_1.

If the patient has received a transfusion, it is important to know whether the transfusion occurred within the past 3 months and, if so, which blood product(s) was (were) administered. When a patient has recently received a transfusion, certain factors should be considered. If the recent transfusion consisted of RBCs, the patient could be having a delayed serological or delayed hemolytic transfusion reaction. If the recent transfusion was a plasma product or intravenous immunoglobulin (IVIG), passive antibody might be causing the problem. If phenotyping of the RBCs is required, transfused donor cells may still be present in the circulatory system of the patient and cause erroneous results. If a patient has received a transfusion or has been pregnant within the past 3 months, the laboratory should perform phenotyping using a pretransfusion specimen, reticulocyte separation, or RBC genotyping. Packed RBCs, plasma, platelets, and cryoprecipitate may contain immunoglobulin contaminants; hence, the laboratory must consider the possibility of passive antibody transfer. Immunoglobulin concentrates, such as IVIG, are a source of additional antibodies because they always contain ABO antibodies and sometimes also contain antibodies against other blood group antigens.

If the patient has received a transfusion recently, the laboratory should consider the possibility of passive antibodies; however, if the transfusion history is remote, alloimmunization should be considered. Formation of an RBC alloantibody can occur in the days or weeks after transfusion. Typically, alloimmunization by this mechanism is clinically silent, and the alloantibody will only be detected during subsequent pretransfusion testing. If antibody testing is performed within the first few weeks after transfusion and a new antibody is identified, this might be accompanied by a positive DAT result in which antibody is bound to the transfused antigen-positive RBCs. This phenomenon is called a delayed serological transfusion reaction (DSTR) and occurs when there are no additional laboratory signs and/or clinical symptoms of hemolysis.

If the patient has no history of recent or remote transfusion and no history of pregnancy, it is rare for this individual to possess IgG RBC alloantibodies. If an antibody is detected in the serum of such an individual, it is likely that this entity is of the IgM (allo- or autoantibody) or IgG autoantibody type.

Question 3: What is the pregnancy history of the patient?

In the serum of a female patient with a history of previous pregnancy, IgG RBC antibodies can be detected due to alloimmunization caused by fetal-maternal bleeds. If the patient is currently pregnant, a number of serological problems can occur and should be considered, including passive anti-D, clinically insignificant cold agglutinins, and atypical agglutination (as discussed in question 11).

Passive Anti-D

Identification of anti-D in the serum of a pregnant patient may occur because of active production of the antibody due to alloimmunization or, more commonly, due to passive antibody transfer following injection of Rh immunoglobulin (RHIG). RHIG is typically administered,

Table 2. Drugs Associated With Immune Hemolytic Anemia or a Positive Direct Antiglobulin Test[20, 21]

Drug Category	Drugs Implicated		
Analgesic and NSAID			
	Acetaminophen[a]	Glafenine[a]	Naproxen[a]
	Azapropazone[a]	Ibuprofen[a]	Phenacetin[a]
	Diclofenac[a]	Methadone[a]	Sulindac[a]
	Dipyrone[a]	Mefenamic acid[b]	Tolmetin[a]
Antiarrhythmic	Procainamide[b]		
Antidepressant	Nomifensine[a]		
Antidiabetic	Chlorpropamide[a]	Insulin[a]	Tolbutamide[a]
Antidiarrheal	Catechin[a]		
Antiemetic	Chlorpromazine[a]		
Antihistamine	Antazoline[a]		
Antihypertensive	Captopril[b]	Methyldopa[b]	
Antimicrobial and β-lactamase inhibitor			
	Amphotericin B[a]	Chloramphenicol[a]	Quinine[a]
	Amoxicillin[a]	Cloxacillin[a]	Quinidine[a]
	Cefalothin[c]	Clavulinic acid[c]	Rifampin[a]
	Cefazolin[a]	Erythromycin[a]	Stibophen[a]
	Cefotaxime[a]	Isoniazid[a]	Sulbactam[c]
	Cefotetan[a,c]	Levofloxacin[a]	Streptomycin[a]
	Cefoxitin[a]	Mefloquine hydrochloride[a]	Tazobactam[c]
	Ceftazidime[a]	Nafcillin[a]	Temafloxacin[a]
	Ceftizoxime[a]	Nalidixic acid[b]	Tetracycline[a]
	Ceftriaxone[a]	P-aminosalicylic acid[a]	Ticarcillin[a]
	Cefuroxime[a]	Penicillin G[a]	Trimethoprim-sulfamethoxazole[a]
	Cephalexin[a]	Piperacillin[a]	
	Cephalothin[a]	Pyrimethamine[a]	
Antineoplastic	Carboplatin[a]	Elliptinium acetate[a]	Interleukin-2[b]
	Cisplatin[a]	Fludarabine phosphate[b]	Methotrexate[a]
	Cisplatin[c]	Fluorouracil[a]	Oxaliplatin[a,c]
	Cladribine[b]	Imatinib mesylate[a]	
	Diglycoaldehyde[c]	Interferon[b]	
Antiparkinsonian	Levodopa[b]		
Antithyroid	Carbimazole[a]		
Diuretic	Furosemide[a]	Hydrochlorothiazide/Triamterene[a]	
Estrogen	Diethylstillbestrol[a]		
H2 blocker	Ranitidine Hydrocloride[a]	Cimetidine[b]	
Immunomodulators	Cyclosporine[a]	Tacrolimus[b]	
	Sulfasalazine[a]		
Uricosuric	Probenecid[a]		

[a] *Drug dependent mechanisms–Drug Adsorption and Immune-Complex Mechanisms*
[b] *Drug Independent–Autoimmune Mechanism*
[c] *Drug Independent–Nonimmunologic Protein Adsorption*

at 28 weeks' gestational age, to nonalloimmunized obstetrical patients with Rh negativity to prevent sensitization to fetal D antigen, which may enter the circulation of the mother during pregnancy.[16] It is also routinely administered after delivery if the patient with Rh negativity has an infant with Rh positivity. RHIG should be administered in other situations that involve procedures or trauma during pregnancy. RHIG has a half-life of approximately 3 weeks; using a sensitive antiglobulin test, the passive antibody can be detected in the serum at least 8 to 10 weeks after injection.

Clinically Insignificant IgM Antibodies

Cold agglutinins that are not clinically significant (anti-I, anti-H, anti-HI, and Lewis antibodies) may be detected in the serum of a pregnant patient, depending on the screening method used. These antibodies are not clinically significant; however, when detected, they generate additional investigative work for the laboratory.

Atypical Agglutination

An increase in fibrinogen is a normal physiologic change during pregnancy[17] and can result in rouleaux.[18] Mixed-field

Table 3. Typical Serological Findings Associated With a Drug-Induced Positive DAT Result[20,21]

Mechanism	Mechanistic Details	Monospecific DAT Results (IgG, C3, or Both)	IAT	Eluate
Drug Dependent				
Drug adsorption	IgG antibodies bind to drugs that coat the RBCs; antibodies interact with Fc receptors of macrophages to cause extravascular hemolysis	IgG	Negative[a]	Nonreactive with normal RBCs but positive with drug-coated RBCs
Immune complex	Antibody reacts with the drug and the RBC membrane; complement is activated, causing predominately intravascular hemolysis	C3	Typically negative[b]	Usually nonreactive
Drug Independent				
Nonimmunologic adsorption of proteins	Drug causes nonimmunologic binding of proteins to the RBC membrane (IgG, complement, albumin, etc). Typically, hemolysis has not been observed; however, more recently, some drugs have been reported to cause hemolysis via this mechanism	IgG or C3	Negative	Nonreactive
Autoimmune	The drug causes dysregulation of the immune system, resulting in autoantibody production	IgG	Positive or negative[c]	Positive[c]

DAT, direct antiglobulin test; IgG, immunoglobulin G; IAT, indirect antiglobulin test; RBCs, red blood cells.
[a]IAT results are negative unless RBCs are coated with the drug.
[b]Negative unless drug and fresh normal serum (as a source of complement) are added to the test.
[c]Antibody typically reacts as a panagglutinin.

reactions have also been reported[19] after a fetomaternal hemorrhage of a large volume of blood[19] (see question 11).

Question 4: What is the medication history of the patient?

A detailed drug history is helpful to determine whether serological and clinical findings are consistent with drug-induced hemolytic anemia. Although it is not a complete list, **Table 2** indicates many of the drugs that have caused a positive direct antiglobulin test result and/or hemolysis. Use of several other medications by patients with drug-induced hemolytic anemia has been reported in the literature; a more comprehensive list by Garratty and Arndt[20] and another by Garratty[21] includes drugs implicated in single instances of this condition. Contrast material and dyes have also been implicated. Three categories of medications are responsible for most positive serological-test results: antimicrobials, 42%; anti-inflammatory drugs, 15%; and antineoplastic agents, 11%.[20]

There are 2 types of drug-induced antibodies: drug dependent and drug independent. Drug-dependent antibodies require that a certain drug be present to detect the antibody; the drug is bound covalently to the RBC membrane or exists freely in the plasma. Drug-independent antibodies do not require the presence of any drug to be detectable in the serum (ie, the antibody reacts similar to an autoantibody observed in patients with warm autoimmune hemolytic anemia). Drug-dependent antibodies are more common than drug-independent antibodies.[21] **Table 3** shows typical serological findings for different mechanisms of drug-induced hemolytic anemias. Laboratory professionals should consider the possibility of drug-induced hemolytic anemia if the patient has hemolysis and/or a positive DAT result, with or without a positive antibody screening result.[21] A comprehensive drug history (current and within the past 6 months) of the patient is essential when investigating serological problems.

Question 5: What is the ABO group for the patient's blood?

Some antibodies that cause serological puzzles are directed against antigens in the ABO blood groups. If

Table 4. Summary of Serological Reactions to Differentiate Cold Autoantibodies (Anti-A_1 and Antibodies in the I and H Systems)

Antibody	ABO Group of Individuals Who Can Form Antibody	Reactions with RBCs by Group				
		OI (adult)	A_1I (adult)	A_2I (adult)	Oi (cord)	A_1 (cord)
Anti-HI	A_1, A_1B	++++	+	++++	+/–	–
Anti-H	A_1, A_1B	++++	+/–	++	++++	++++
Anti-I	All groups	++++	++++	++++	+/–	–
Anti-i	All groups	+/–	+/–	+/–	++++	++++
Anti-A_1	A_2, A_2B	–	++ or +++	–	–	+/–

RBCs, red blood cells.
Grading system for agglutination typically used in immunohematology: ++++, One solid clump of cells; +++, Several large clumps of cells; ++, moderate size clumps of cells; +, very small clumps of cells; +/–, very weak with red cell clumping only visible using a microscope; –, Negative.

the patient is blood group A or AB, the possibility that anti-A_1 or anti-H is present should be considered. Anti-A_1 is an IgM-type, environmentally stimulated antibody that typically reacts at colder temperatures; its presence is usually not clinically significant. It is found in the plasma of 1% to 3% of individuals with A_2 and may be present in the plasma of approximately 25% of individuals who are the A_2B phenotype.[22] The ABO serotyping result can sometimes reveal that the patient may be an A_2B phenotype because the anti-A typing result may not give a typical 4+ reaction (it often gives a 2+ or 3+ result). Anti-A_1 typically appears as a discrepancy between the results of ABO forward and reverse testing: the RBCs of the patient appear as type as A or AB, but the serum reacts with the reagent A cells. Unexpected reactions in the reverse grouping may also be caused by the presence of other IgM alloantibodies (anti-M, N, S Le^a, Le^b, P_1, or Lu^a); however, when the blood of the patient is of the group A or AB designation, the laboratorian should also consider the possible presence of anti-A_1.

Antibodies against the H antigen can be detected in the plasma of individuals who red cells group as A_1 and A_1B.[22] These are autoantibodies (A_1 and A_1B RBCs contain small amounts of H antigen in their surface); however, the autocontrol typically tests negative. Anti-H is usually not detected in routine serological testing because it typically reacts only at cold temperatures; however, sometimes the thermal range of this reaction extends to 22°C or higher, which causes interference with serological testing. Anti-H may be detected via the antibody screen if a polyspecific antiglobulin reagent is being used. The strongest reactions occur with the group O screening cells, which strongly express H-antigen, whereas the cross-matched A_1 or A_1B units yield weak or negative results since H antigen is weakly expressed on A_1 and A_1B RBCs. Anti-H antibodies may be detected when using an immediate spin cross-match if the antibody has activity at room temperature and if A_2 donor units are selected for cross-match (A_2 RBCs have greater H-antigen expression). There is also an antibody that reacts with RBCs that express the H and I antigens. **Table 4** summarizes the typical reactivity of anti-A_1, anti-H, and anti-HI, and compares this reactivity to IgM autoantibodies in the I–blood-group system.[22]

Question 6: What is the Rh type of the patient's blood?

In Rh-negative patients, anti-D is always a possibility. With routine prophylactic use of RHIG in the obstetrical population, the frequency of anti-D alloimmunization has decreased dramatically; however, anti-D formation can still occur. More commonly, passive anti-D may be detected in the plasma of Rh-negative pregnant women because they have been injected with RHIG at 28 weeks' gestation or have received RHIG after delivery or for other indications throughout their pregnancy. If a female patient is Rh negative with a positive antibody screening result when tested during pregnancy or after delivery, it should be verified whether the patient has recently received RHIG.

Question 7: Is the DAT result positive? If yes, what were the results of eluate testing?

A positive DAT result reveals that circulating RBCs of the patient are coated with IgG and/or C3.[22] However, a positive DAT result may or may not be associated with hemolysis and should be correlated with clinical and laboratory findings. Likewise, a negative DAT result does not exclude the possibility of immune-mediated hemolysis. The differential diagnosis for a positive DAT result includes a delayed serological or delayed hemolytic transfusion reaction; warm autoimmune hemolytic anemia; cold autoimmune hemolytic anemia; immune hemolysis due to the presence of a certain drug or drugs in the plasma; hemolytic disease of the newborn; and hypergammaglobulinemia.[23] The plasma of as many as 15% of hospitalized patients may yield a positive DAT result.[24] When a patient has a positive DAT result, it is important to consider the information received from the previous questions asked. For example, if a patient has idiopathic thrombocytopenic purpura (ITP) and received IVIG a week ago, the most likely causes for the positive DAT result is a delayed serological and/or hemolytic transfusion reaction due to passive antibody in the IVIG, or treatment-induced hypergammaglobulinemia. A positive DAT result associated with hypergammaglobulinemia occurs in patients whose disease process or treatment results in elevated immunoglobulin levels. IgG binds nonspecifically to the RBC surface; however, typically, hemolysis does not occur.[25] The use of monospecific antiglobulin reagents (Anti-IgG and Anti-C3d) and elution studies, combined with a comprehensive clinical history, usually helps to identify the cause of the positive DAT result.

There are a variety of techniques to elute IgG from the RBC surface: namely, chemical methods that disrupt the RBC membrane, causing antibodies bound to the RBC surface to be released, and the use of heat (56°C) to dissociate antibodies from the membrane—this latter approach works well when ABO antibodies are on the RBC surface. A negative eluate result when IgG is detected on the RBC surface is consistent with a positive DAT result due to the presence of certain drugs in the plasma, hypergammaglobulinemia, or passive anti-A and anti-B if elution studies are only carried out using group-O screening or panel cells. Whenever IgG is present on RBCs, it is useful to perform elution studies; however, creating an eluate when only C3 is detected by the DAT can also be informative in suspected cases of warm autoimmune hemolytic anemia (AIHA) because it may be possible to elute IgG from the RBCs.

Question 8: At what temperature does the antibody react?

RBC antibodies fall into 2 categories, namely, cold reacting (optimal temperature around 4°C) and warm reacting (37°C). Cold-reacting antibodies are usually IgM and can be alloantibodies or autoantibodies. The most common IgM alloantibodies are directed against antigens M, N, S, Le^a, Le^b, P_1, and A_1. Antibodies to Lu^a are also of IgM type but are far less common.[26,27] IgM autoantibodies usually are specific to the I antigens (I or i) but may be directed against the HI or Pr antigens.[28]. IgM antibodies do not cross the placenta and therefore do not cause hemolytic disease of the newborn; however, they can occasionally cause RBC hemolysis after transfusion if the antibody has thermal activity above 30°C and can activate complement. In most situations, the presence of these antibodies is not clinically relevant; however, these antibodies may cause serological discrepancies in tests that are performed at room temperature, such as the ABO-grouping or Rh-typing tests, or in the antiglobulin phase if a polyspecific antiglobulin reagent is used.

IgG antibodies react optimally at 37°C and frequently display specificity for Rh, Kell, Duffy, Kidd, and Ss antigens. Antibodies that are specific for Lu^b and Xg^a are rare; these antibodies typically are detectable by the antiglobulin test. Because these antibodies react at the normal body temperature of 37°C and can cross the placenta, their presence is usually clinically significant with regard to hemolytic transfusion reactions and can cause hemolytic disease in newborns.

Question 9: Does the antibody react with enzyme-treated RBCs?

Proteolytic enzymes (eg, papain, ficin, trypsin, and bromelin) have been used in serological testing for many years. Papain and ficin are used most frequently. Proteolytic

enzymes cleave amino acids at specific sites, removing protein fragments on the RBC surface. If blood group antigens are located on the cleaved protein, antibodies directed against these antigens will no longer react. Antigens that are destroyed by proteolytic enzymes include Fy^a, Fy^b, M, N, S, Xg^a, Ch^a, Rg^a, and JMH.[29] Proteolysis can also make other antigen sites more accessible to antibodies, resulting in enhanced reactivity of antibodies toward Rh, Kidd, Lewis, P, and I antigens, including enhancement of warm-reacting autoantibodies. Enzyme treatment is not a routine procedure but it can be useful as an additional test, especially when multiple antibodies are present in the plasma and 1 or more of the antibodies are directed against an antigen that is destroyed by enzymes.

Question 10: Is the antibody still reactive when monospecific Anti-IgG is used in the DAT?

Many laboratories routinely use Anti-IgG to detect the presence of clinically significant antibodies; however, in laboratories that use a polyspecific antiglobulin reagent, it is important to consider whether the reactivity occurs due to IgG or C3 on the RBC surface. When a room temperature–reacting IgM antibody binds to RBCs, complement may be activated and bind to the RBC membrane when the serological test is set up at room temperature. Although the IgM antibody may elute from the RBC when the test is incubated at 37°C, complement (C3b) may remain on the RBC surface and be detectable via the polyspecific antiglobulin reagent. Hence, if complement activation by an IgM antibody is suspected, it may be helpful to repeat the test with monospecific Anti-IgG reagent or using a prewarming technique.

Question 11: Is the agglutination unusual in appearance?

Agglutination typically results in clumps of RBCs that appear somewhat homogeneous; however, 2 unusual patterns of RBC clumping can occur in serological testing. Rouleaux formation can mimic agglutination; however, on microscopic assessment, the cells have the appearance of a stack of coins. Rouleaux formation typically occurs when patients have an altered albumin:globulin ratio. Conditions in which this phenomenon occurs include multiple myeloma, cryoglobulinemia, macroglobulinemia, cirrhosis, and hyperfibrinogenemia (the latter occurs in patients with acute infections and during pregnancy).[17,18,30] Rouleaux has also been observed following infusion of intravenous (IV) solutions containing high-molecular-weight dextran or polyvinylpyrrolidone (PVP). When rouleaux is suspected, phenotyping may be done with washed RBCs and a saline-replacement technique can be used for tests involving direct agglutination.[31] Rouleaux is not observable in antiglobulin test results because the washing phase that is performed before adding the antiglobulin reagent typically removes the plasma proteins that cause the phenomenon.

The second unusual pattern that may be observed is a mixed-field reaction in which some of the RBCs are agglutinated, whereas others are not. This pattern may be observed macroscopically if the test is performed in tubes: visible agglutination is present, whereas the background appears pink because of the nonagglutinated RBCs. Mixed-field reactions are easier to detect microscopically or when using column-agglutination methods (2 RBC bands are visible). The differential diagnosis for mixed-field reactions depends on whether the pattern is observed when phenotyping or when performing antibody-screening procedures. When a mixed-field reaction is observed with phenotyping, the following possibilities should be considered: recent transfusion including intrauterine and exchange transfusions; recent allogeneic hematopoietic stem-cell transplantation; the A3 phenotype; extensive fetomaternal or maternal-fetal bleeds; in utero exchange of hematopoietic stem-cell tissue in twins (ie, the chimera phenomenon); and polyagglutination.[10] Antibodies that typically cause mixed-field reactions include anti-Lu^a, anti-Sd^a (the appearance of agglutinates is spherical and refractory); and, some antibodies that exhibit high titer and low avidity (HTLA) characteristics.[32]

Question 12: Does the agglutination show variable reactivity?

When panel cells, screening cells, or cross-matched units show variable reactivity, there are 3 possibilities

Table 5. Useful Reference Lists for Solving Serological Problems

Variable	Antibodies/Antigens
Common IgM alloantibodies (react at room temperature or lower)	Have specificity for: M N S (rarely—usually IgG) Lewis (Le^a, Le^b) P_1 Lu^a A_1
IgM autoantibodies (typically react at room temperature or lower but may have higher thermal activity in autoimmune hemolytic anemia).	Have specificity for: I I H HI Pr (uncommon)
Antigens destroyed by proteolytic enzymes	M N S Duffy (Fy^a Fy^b) Xg^a
Antibodies enhanced by RBC enzyme treatment	Antibodies of the following types: Rh Kidd Lewis P_1 I-system
Antibodies that can show dosage	Usually have specificity for: Rh antigens MNS antigens
Antigens not developed or weakly developed in umbilical-cord RBCs	Lewis (Le^a, Le^b) P_1 I Lu^a
Causes of variable reaction strength	Dosage Multiple antibodies Variable antigen expression unrelated to dosage (eg, P_1, I, Lewis antigens)

IgM, immunoglobulin M; RBCs, red blood cells.

to consider: 1) multiple antibodies, 2) stronger reactions with double-dose (homozygous) versus single-dose (heterozygous) cells, suggesting an antibody that shows dosage, and 3) variable antigen expression unrelated to dosage (such as P_1, Lewis, and I-system antigens).

Question 13: Does the antibody react with umbilical cord cells?

Antibody panel manufacturers do not supply umbilical cord cells on their panels (hereafter, cord cells); however, when certain types of antibodies are suspected it may be useful

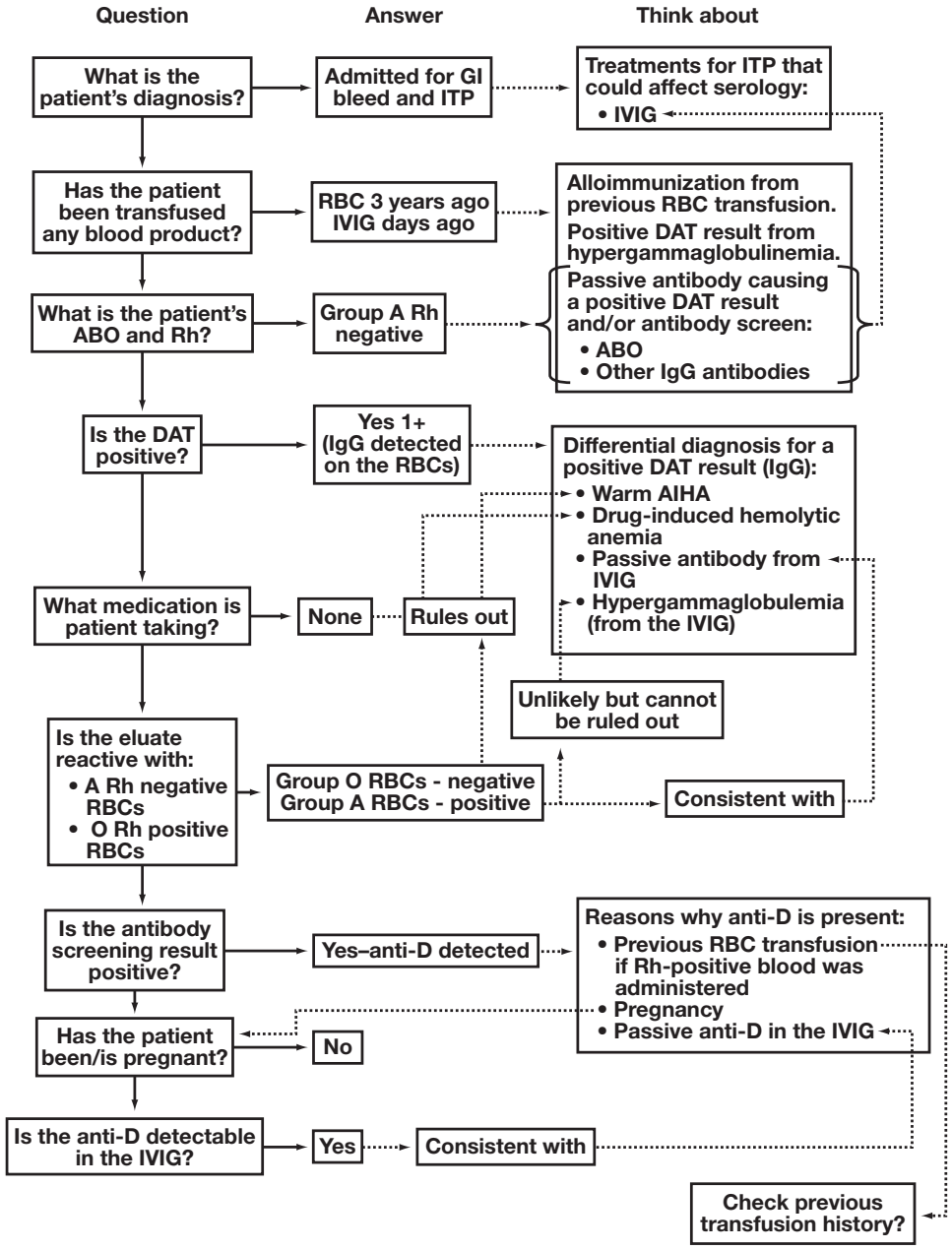

Figure 3

Data from a case scenario, examined using the question-based approach. A 40-year-old woman is admitted to the hospital for gastrointestinal bleeding, and the transfusion laboratory is called on to provide compatible units. A positive direct antiglobulin test (DAT) result is identified, and the laboratorian is asked to consider the most likely cause of these findings. Depending on the case, it may not be necessary to ask every question; the order in which the questions are asked may vary depending on the clinical and laboratory findings. *GI indicates gastrointestinal; ITP, idiopathic thrombocytopenic purpura; IVIG, intravenous immunoglobulin; DAT, direct antiglobulin test; IgG, immunoglobulin G; RBCs, red blood cells; AIHA, autoimmune hemolytic anemia.*

to select and/or pool several group-O cord cells for use in serological testing. Some antibodies do not react with cord cells (or yield weaker reactions than with other cell types) because they are reacting with an antigen that is not well developed in neonate RBCs, namely, anti-I, Lewis antibodies; anti-Lu^a, anti-Ch^a, and anti-Rg^a.[33]

Useful Lists

Table 5 provides a useful reference list of the various antibodies and antigens that present challenging diagnostic scenarios in transfusion medicine.

Conclusion

These 13 questions and the logical approach outlined herein are targeted towards a general transfusion medicine service, rather than a blood center that undertakes complex antibody investigations using specialized techniques. Nevertheless, when complex serological problems occur, these 13 key questions can be helpful to identify the cause of the discrepancy and resolve the diagnostic mystery. An example of the application of this question-based approach is presented in **Figure 3**. This example illustrates several important points: not all problems may require every question to be asked; not all questions will provide helpful information to solve the serological discrepancy; and the order of the questions may vary because the answer to one question may dictate the next most relevant question to ask. The case also illustrates that understanding the information obtained from each question relates to other questions that will help resolve the discrepancy by putting together the pieces of the diagnostic puzzle. The information gained through these pertinent questions will help to resolve the serological data and provide useful information to clinicians. LM

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