REPORT

How we investigate drug-induced immune hemolytic anemia

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Drugs are a rare cause of immune hemolytic anemia, but an investigation for a drug antibody may be warranted if a patient has definitive evidence of immune hemolysis, other more common causes of hemolysis have been excluded, and there is a good temporal relationship between the administration of a drug and the hemolytic event. Drug antibodies are either drug-dependent (require drug to be in the test system) or drug-independent (reactive without drug present in the test). Drug-dependent antibodies are investigated by testing drug-treated red blood cells (RBCs) or by testing RBCs in the presence of a solution of drug. Drug-independent antibodies are serologically indistinct from idiopathic warm autoantibodies and cannot be defined or excluded by serologic testing. Nonimmunologic protein adsorption, caused by some drugs, is independent of antibody production but may also cause immune hemolytic anemia. Serologic methods for testing for drug antibodies are presented, and observations from more than 30 years of this laboratory's experience are discussed. ***Immunohematology* 2014;30:85–94.**

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Dr. Garratty's Immunohematology Research Laboratory at the American Red Cross in Southern California has investigated drug-induced immune hemolytic anemia (DIIHA) for 35 years. When contacted to investigate a possible DIIHA, we ask a series of questions:

- Does the patient have hemolytic anemia (e.g., decreased hemoglobin or hematocrit, increased reticulocytes, increased indirect bilirubin and lactate dehydrogenase, decreased haptoglobin)?
- Does the patient have a positive direct antiglobulin test (DAT)? Is there IgG or C3 or both present on the patient's red blood cells (RBCs)? The DAT is used to establish an immune etiology for a hemolytic anemia. For DIIHA the DAT should be positive for IgG or C3 (or both) close to the time of the observed hemolysis. If not tested until weeks later, the DAT may be weak or even negative, especially if the patient has been transfused with multiple RBC units.[1]
- Was an eluate tested and what were the results? The typical prompt for pursuing DIIHA in a patient with hemolytic anemia is a positive DAT and nonreactive eluate. Some recent findings, however, show that an eluate may be reactive while the patient is still receiving the drug (e.g., piperacillin, cefotetan).
- What drug(s) is (are) the patient taking now or recently? Have any been implicated in causing DIIHA? It is important to obtain a complete medication history with dates of administration. This includes antibiotics the patient may have received for a surgery in the previous 2 to 3 weeks; such medications might only appear on the surgical record and not on a listing of current medications.

And most importantly:

- Is there a temporal relationship between the drug administration and the hemolytic anemia? If the hemolysis began before the drug was administered, then there is no temporal relationship. In some situations, there may not be any prior administration of the drug in question (e.g., cefotetan), while in others, the patient may have received the drug previously without any obvious adverse effects (ceftriaxone, piperacillin).

In summary, because DIIHA is rare, the time and effort of an investigation should only be undertaken when the patient has definite evidence of a hemolytic anemia and a good temporal relationship to treatment with a particular drug exists. When this has been established, the clinician should consider stopping the drug, and specimens should be collected for an investigation.

The approach and methods described in this article have been used in our laboratory for several decades.[2] Additional information gleaned from our experience is added here.

Types of Drug-Induced Antibodies

There are two types of drug-induced antibodies: drug-dependent and drug-independent. Serologically, we can only determine the presence of drug-dependent antibodies. Drug-dependent antibodies are subdivided into those that react with drug-treated RBCs (e.g., antibodies to penicillin and some cephalosporins) and those that react with untreated RBCs in the presence of a solution of the drug (e.g., antibodies to

ceftriaxone and quinine). Some drug-dependent antibodies react both with drug-treated RBCs and in the presence of a soluble drug; others react by only one of these methods. Drug-independent antibodies (e.g., autoantibodies induced by methyldopa and fludarabine) have serologic reactivity without the drug being present and are indistinguishable from idiopathic IgG warm autoantibodies.

Nonimmunologic Protein Adsorption

Some drugs modify the RBC membrane and cause nonimmunologic adsorption of protein (NIPA), resulting in positive direct and indirect antiglobulin tests.[3] NIPA is independent of antibody production, but may be a cause of hemolytic anemia.[4] Drugs that cause NIPA include some cephalosporins, platinum-based chemotherapies (cisplatin, carboplatin, and oxaliplatin), and β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam). NIPA should be suspected when a patient's plasma or serum and most normal plasma or sera react in an indirect antiglobulin test with drug-treated RBCs, but the eluate from the patient's RBCs is nonreactive with the drug-treated cells. When testing RBCs treated with a drug known to cause NIPA, the patient's serum and the controls (negative and positive) are tested both undiluted (for detection of a hemolysin or a directly agglutinating drug antibody) and at a dilution of 1 in 20 (to avoid NIPA in the antiglobulin test). Normal sera diluted 1 in 20 generally do not contain enough protein for NIPA to be detected.

To verify that NIPA has occurred, we test the patient's RBCs (DAT) or the drug-treated RBCs after incubation with plasma (indirect antiglobulin test) using antihuman albumin (Sigma-Aldrich, St. Louis, MO).[5] The antihuman albumin is not licensed for RBC indirect antiglobulin testing, so it must be standardized for this purpose. To determine the appropriate dilution of antihuman albumin, doubling dilutions prepared in phosphate-buffered saline (PBS) are tested against untreated and cephalothin-treated RBCs after incubation at 37°C with normal plasma.[5] The amount of protein that adsorbs to the RBCs increases with time (e.g., 2 hours vs. 30 minutes). When new dilutions of antihuman albumin are prepared, expected reactivity with cephalothin-treated and -untreated RBCs (after incubation with normal plasma) is verified.

Sample From Patient

We request at a minimum 5 mL of EDTA whole blood and one or two 10-mL clot tubes (serum is required to detect hemolysis or complement activation, e.g., for testing in the presence of a soluble drug). It is preferable to have a sample from when in vivo hemolysis was first observed. If the patient's serum or plasma appears to have an autoantibody, it is also beneficial to have a sample collected 2 to 3 days after the drug was stopped. This second sample helps to determine whether the observed autoantibody reactivity is truly autoantibody or caused by a drug antibody reacting with a circulating drug present in the sample obtained while the patient is still receiving the drug. This apparent autoantibody is observed quite often with antibodies to piperacillin. Once the drug has cleared from the patient's circulation (e.g., about 1–2 days after the drug is stopped), this apparent autoantibody reactivity disappears, whereas true autoantibody would persist.

Initial immunohematology testing should be completed before beginning drug studies. The presence or absence of alloantibodies and autoantibodies and eluate results will guide the selection of RBCs used for the drug studies and the methods to be used. Autoantibodies will need to be removed by adsorption before performing tests for drug antibodies for the same reason they are removed by adsorption to detect the presence of other alloantibodies.

We prepare eluates from the patient's EDTA RBCs using a rapid acid elution kit (e.g., Gamma Elu-Kit II, Immucor, Norcross, GA). To wash the RBCs in preparation for the elution, we substitute cold (4°C) low-ionic strength saline for the kit wash solution. In 1995, we showed that washing RBCs with the low-ionic kit wash solution can result in false-positive eluates when high-titer antibodies are present.[6] Cold PBS can also be substituted but may be less efficient for lower affinity antibodies. The original study on false-positive eluates began with detection of anti-D in the eluate from D– RBCs after adsorption with a polyclonal anti-D. Coincidental to that research, anti-cefotetan was detected in acid eluates prepared from maternal and cord RBCs that were collected many weeks after the mother received cefotetan. In retrospect, we realized that these eluates (prepared using the low-ionic kit wash solution) probably gave false-positive results because of nonspecific adsorption of high-titer anti-cefotetan to the RBCs during the washing for the eluate preparation. This was later confirmed with in vitro studies when the mother's anti-cefotetan was adsorbed and eluted from untreated RBCs when the kit wash solution was used but not when cold PBS was used.[6]

Sample of Drug

We also request a sample of the drug under investigation unless we already have it on hand (e.g., powder [1-g vial],

tablets, or capsules [2–4]; the drug should not be dissolved before sending). We have not been successful using drugs in a pediatric oral suspension formula because they hemolyze RBCs.[5,7]

Although it would seem important to use the particular drug that a patient received, it is optimal to use pure drug rather than a commercial tablet, capsule, or suspension product. These commercial formulations contain inert ingredients that can make it difficult to dissolve the drug and test with RBCs. It is also possible that antibodies detected in drug studies are directed against one of these inert ingredients rather than the drug itself.[8] Initial testing can be performed with the particular drug the patient received, but positive results should be confirmed using pure drug to ensure correct interpretation. Drugs administered by intravenous injection are free of excipients. Some pure drugs are available from chemical companies (e.g., Sigma-Aldrich).

Some drugs are combinations of two drugs (e.g., Zosyn is a combination of piperacillin plus tazobactam). Tazobactam causes NIPA, which can create a problem with the interpretation of results, so it is preferable to test with the separate components.

Test Methods

If the suspected drug has previously been reported as a cause for DIIHA (see tables in Garratty and Arndt[9]), it is best to follow the techniques used in the relevant report. If the drug has never been reported or sufficient serologic details are not provided, testing the patient's serum and an eluate from the patient's RBCs against drug-treated RBCs and testing the patient's serum against untreated and enzyme-treated RBCs in the presence of the soluble drug is recommended. Many more drug antibodies are detected by testing in the presence of soluble drug than by testing drug-treated RBCs (see Table 1 in Garratty and Arndt[9]).

We perform all our DIIHA investigations in tubes. Salama et al.[10] described using the gel test for detecting drug antibodies. We have done limited testing in the presence of soluble drug by the gel test (ID-Micro Typing System, Pompano Beach, FL) using buffered gel and anti-IgG, -C3d gel cards and comparing the results with tube tests. Anti-ceftriaxone could be identified by the gel test for 9 of 11 examples tested; the presence of anti-ceftriaxone could not be discerned for two examples. Some agglutination and hemolysis detected by tube test was not detected by the gel test, and some antiglobulin tests with the PBS substituted for the drug were stronger by gel test (e.g., 2+ by gel vs. negative or ½+ by tube test).[11]

Table 1. Preparation of drug-treated RBCs

Variable	Penicillin-treated RBCs	Cephalosporin-/other-treated RBCs
RBCs, group O	Fresh, <7 days old	Fresh, <7 days old
Amount of drug	600 mg	400 mg
Buffer (pH)	0.1 M sodium barbital (pH 9.6–9.8)	Phosphate-buffered saline (pH 7.1–7.4)
Volume buffer	15 mL	10 mL
Volume packed RBCs	1 mL	1 mL
Temperature of incubation	Room temperature	37°C
Time of incubation	1 hour	1 hour

RBCs = red blood cells.

Preparing Drug Solutions

The solution of drug must be isotonic for use with RBCs. Dissolving drugs in PBS, pH 7.1 to 7.4, is the preferred method. Some drugs are readily soluble in PBS, others are somewhat soluble, and still others are insoluble. The Merck Index is an excellent source for looking up the solubility of a drug.[12] For drugs that are somewhat soluble, we incubate the solution at 37°C for about 10 to 15 minutes and mix vigorously on a vortex mixer; the solution is then centrifuged, and the supernatant is transferred to a clean tube. Even if the drug is not completely soluble, sufficient drug may be present for an antibody to be detected. Tablets can be crushed using a mortar and pestle, taking care to tease apart and remove as much of the outer coating as possible. Drug or inert ingredients that are not dissolved can damage the RBCs used in the test, so efforts to remove particulate matter or find a better medium for solution are needed. Drugs that are insoluble in PBS are problematic. A lower pH or solvent (e.g., alcohol) may be helpful, but the solution would need to be diluted in PBS for an appropriate pH and isotonicity to be used with RBCs. Drugs that are supplied as an aqueous solution may also need to be diluted in PBS before adding to RBCs. Dissolving drugs in an albumin solution has been suggested,[13] but we have found that 6 percent albumin decreases the binding of some drugs (e.g., cefotetan, cephalothin) to RBCs.[14] Thus, albumin should only be used if the drug is insoluble in water and PBS; perhaps 1 percent albumin in PBS, as used in drug-induced thrombocytopenia investigations, would be a better alternative than 6 percent albumin.[15]

Some drugs are not stable in solution. We have found that anti-cefotetan titers were dramatically reduced when the solution of cefotetan was just 2 hours old compared with tests

with a fresh solution for both 40 and 1 mg/mL preparations. Unless the stability of a particular drug in solution has been verified by reproducible titers over time, these solutions need to be used as soon as possible after preparation both for treating RBCs and for testing in the presence of the drug. We have found 1 mg/mL solutions of ceftriaxone in PBS to be stable up to 5 days[11] and piperacillin to be stable up to 1 week (unpublished observations) when stored at 4°C.

Preparing Drug-Treated RBCs

For preparing most drug-treated RBCs, we start with a drug concentration of 40 mg/mL in PBS. This concentration originated from early studies for optimally preparing penicillin- and cephalothin-treated RBCs.[16] It is interesting to note one cannot make penicillin-coated RBCs with the concentration of drug found in vivo (Garratty, unpublished data).

Optimal binding of penicillin G to RBCs occurs with 600 mg of penicillin G in 15 mL of high-pH buffer (40 mg/mL), such as barbital, 0.1 M (pH 9.6–9.8) incubated with 1 mL of RBCs at room temperature for 1 hour.[16,17] After treating, the RBCs are washed three to four times with PBS or until no hemolysis is visible. Increased hemolysis can occur if treating is extended beyond 1 hour.

Cephalosporin-treated RBCs are prepared slightly differently: 400 mg of the drug is dissolved in 10 mL of PBS, pH 7.3 (for 40 mg/mL), and incubated with 1 mL of RBCs at 37°C for 1 hour.[2] So 40 mg/mL of drug is used but at a 10:1 ratio of drug solution to RBCs (vs. 15:1 for penicillin). Also, binding of cephalosporins is greater at 37°C than at room temperature (the opposite of penicillin).[16] Originally, pH 10.0 buffer was used to prepare cephalosporin-treated RBCs, but a high pH is not required for optimal coating.[16] In fact, a lower pH (e.g., pH 6–7) decreases NIPA that occurs when some cephalosporin- and other drug-treated RBCs are incubated with plasma or serum (i.e., during testing).[18,19] When testing a drug that we have not previously tested or for which there is no previous report, we typically use the cephalosporin treatment method, unless the drug is in the penicillin family. The preparation of penicillin-treated and cephalosporin-treated RBCs is compared in Table 1.

Some drugs require a different buffer (e.g., borate for nafcillin[20,21]) or incubation at 4°C (e.g., for erythromycin[7,22]). Occasionally, a drug is only available in aqueous solution at a lower concentration or contains an ingredient that damages RBCs (e.g., hemolysis) during treatment. This takes a trial and error approach, testing different concentrations, temperatures, and time of treatment incubation. Chemotherapeutic drugs in aqueous solution, such as oxaliplatin and carboplatin, have been successfully used at 1 or 5 mg/mL in PBS, respectively, to treat RBCs. Cimetidine, which was supplied as an aqueous solution containing alcohol, hemolyzed RBCs when prepared at 40 mg/mL at both 37°C and room temperature, but was used successfully at 15 mg/mL in PBS with a room temperature treatment incubation.[23]

For whatever method is used, another aliquot of RBCs is prepared under the same conditions in the appropriate buffer or PBS (without the drug) to serve as the control untreated RBCs. These untreated RBCs are tested in parallel with the drug-treated RBCs. The volume of drug-treated and untreated RBCs prepared can be scaled down as long as the appropriate ratio is kept constant (e.g., dissolving 100 mg of cephalosporin in 2.5 mL of PBS and adding 0.25 mL of packed RBCs maintains the 40 mg/mL concentration with a 10:1 ratio).

Some drug-treated (e.g., penicillin, cephalothin, cefotetan) and untreated control RBCs can be stored for a short time (a few days) at 4°C, with Alsever's solution added; however, reactivity of treated RBCs will weaken over time. These RBCs can also be stored frozen in liquid nitrogen. Other drug-treated RBCs might not store well. For example, cimetidine-treated RBCs were nonreactive after storage overnight at 4°C.[23]

Testing Drug-Treated RBCs

Drug-treated RBCs are tested in parallel with the control untreated RBCs by tube test. Two sets of tubes are labeled for each sample to be tested. For the patient, serum (or plasma) and an eluate and last wash are tested. Negative controls include normal sera or plasma (pooled or several individual sera) and PBS. A positive control is tested whenever available. If the drug under investigation is known to cause NIPA, the patient's serum and the normal sera or plasma are also tested at a dilution of 1 in 20, and the positive control is tested at a dilution of 1 in 20 or greater.

Two drops of each sample and control are tested with one drop of the drug-treated or control untreated RBCs (3–5% suspension in PBS). The tubes are incubated at 37°C for 1 hour and then centrifuged and examined for hemolysis (if serum was tested) and agglutination. The RBCs are washed four times with PBS, two drops of antihuman globulin are added, and the tubes are centrifuged and examined for agglutination. If the patient's plasma is tested, testing with anti-IgG is sufficient; if patient's serum is tested, we use polyspecific antihuman globulin to also detect complement activation.

Interpretation of Results

Test results with drug-treated RBCs are often straightforward. Results definitive for a drug antibody are (1) reactivity of the patient's serum and eluate with the drug-treated RBCs and no reactivity with the untreated RBCs, (2) no reactivity of the normal sera or plasma and PBS controls, and (3) reactivity of the positive control, if available, with only the drug-treated RBCs (see expected results for a drug antibody that reacts with drug-treated RBCs in Table 2). Reactivity can be hemolysis, direct agglutination, or a positive indirect antiglobulin test, or a combination of these results. In cases of DIIHA caused by high-dose intravenous penicillin, the anti-penicillin is detectable in an eluate prepared from the patient's RBCs. Theoretically, this should be true for other drug antibodies reactive with drug-treated RBCs, but eluates in these cases have not always been reactive.

For some drugs (e.g., cefotetan, oxaliplatin), the results can be confusing and potentially misleading unless the appropriate controls described previously are included. Table 3 shows the interpretation of various results with drug-treated RBCs.

No reactivity of the patient's serum and eluate together with a positive result for the positive control indicates the absence of a drug antibody. No reactivity of the patient's serum and eluate without a positive control, however, does not exclude a drug antibody and can only be interpreted that the drug antibody was not detected; without a positive control, it is unknown whether the drug was truly bound to the test RBCs. The report must state that a positive control was not tested. No reactivity of the patient's serum and eluate and no reactivity of the positive control is an invalid test; there may be no drug bound to those RBCs or the positive control may have deteriorated.

Reactivity of the patient's serum and eluate with both the drug-treated and untreated RBCs indicates the presence of alloantibody, autoantibody, or circulating drug or drug–anti-drug complexes. Additional work is required. If an alloantibody is present, new drug-treated RBCs that are negative for the appropriate antigen(s) need to be tested. If autoantibody is

Table 2. Expected results when drug antibody reacts with drug-treated RBCs

	Drug-treated RBCs		Untreated RBCs	
Sample tested	60 min at 37°C	AHG	60 min at 37°C	AHG
Patient's serum	+/0	+	0	0
Patient's serum diluted 1 in 20*	+/0	+	0	0
Eluate	+/0	+	0	0
Last wash	0	0	0	0
Normal sera (pooled or 4–6 individuals)	0	0/+*	0	0
Normal sera diluted 1 in 20*	0	0	0	0
Positive control	+/0	+	0	0
PBS	0	0	0	0

*If drug causes nonimmunologic protein adsorption, normal sera will react at the antiglobulin test; a 1 in 20 dilution should not react.

AHG = antihuman globulin; PBS = phosphate-buffered saline; RBCs = red blood cells.

Table 3. Interpretation of tests with drug-treated RBCs

Drug-treated RBCs								Untreated RBCs	
Patient's serum	Patient's serum 1/20	Eluate	Last wash	Normal sera/plasma	Normal sera/ plasma 1/20	PBS	Positive control	Patient's serum	Interpretation
+	NT	+	0	0	NT	0	+	0	Drug antibody
+	+	+	0	+	0	0	+	0	Drug antibody
+	NT	+	0	0	NT	0	NA	0	Drug antibody
0	NT	0	0	0	NT	0	+	0	No drug antibody detected
0	NT	0	0	0	NT	0	NA	0	No drug antibody detected; ?drug bound to RBCs; no positive control
+ (AGT only)	0	0	0	+ (AGT only)	0	0	+ at ≥1/20*	0	NIPA but no drug antibody detected
+ direct aggl, + or 0 AGT	0	0	0	with some sera, + direct aggl, + or 0 AGT	0	0	+	0	Not DIIHA; environmental exposure?
+	NT	+ /0	0	0	NT	0	+	+	Alloantibody? Autoantibody? Drug–anti-drug? needs additional testing

*When NIPA occurs, the positive control should be shown to react at dilution ≥1 in 20.

RBCs = red blood cells; PBS = phosphate-buffered saline; NT = not tested; NA = not available; AGT = antiglobulin test; NIPA = nonimmunologic protein adsorption; aggl = agglutination; DIIHA = drug-induced immune hemolytic anemia.

present, the serum can be adsorbed and retested. Circulating drug or drug–anti-drug complexes will also be removed by adsorption. Alternatively, the patient's serum can be dialyzed to remove circulating drug. If the reactivity is caused by circulating drug or drug–anti-drug complexes, the reactivity should disappear after the patient is no longer receiving the drug and the drug has cleared from the circulation.

If the normal sera or plasma reacts with the drug-treated RBCs but does not react with the untreated RBCs, there are two likely explanations. First, if the reactivity is at the antiglobulin phase only, the reactivity is probably attributable to NIPA. Several individual sera should be retested both undiluted and diluted 1 in 20; the 1 in 20 dilution should be nonreactive (the protein content should be too low to be a problem). For drugs that cause NIPA, most or all normal sera will react with the drug-treated RBCs; however, the strength can vary from weak to strong among sera.

If the normal sera or plasma causes direct agglutination of the drug-treated RBCs, but not the untreated RBCs, a low-titer antibody to the drug could be present. Antibodies to several drugs (e.g., penicillin, cephalothin, cefotetan, piperacillin, meropenem, oxaliplatin) have been detected in plasma from blood donors and patients who do not have hemolytic anemia.[24] Widespread use of antibiotics in agriculture and environmental contamination have been suggested as the source of exposure to many drugs.[25,26] Most of these antibodies are IgM and low titer, but occasionally these antibodies react in sera diluted greater than 1 in 20, especially anti-cefotetan. This agglutination can be anywhere from 1+ to 4+ in strength. The presence of these antibodies in the plasma of healthy individuals underscores the importance of testing normal sera or plasma against drug-treated RBCs. It is imperative that the presence of a drug antibody should not be determined on the basis of a single test of undiluted patient's plasma without sufficient normal sera or plasma (either pooled or several individual sera) tested in parallel. Anti-cefotetan found in normal sera has reacted up to a dilution of 1 in 100, so in tests against cefotetan-treated RBCs, the patient's plasma should be strongly reactive, at least at a dilution of 1/100 for identification of anti-cefotetan as a cause of DIIHA (unpublished observations). Titration of the patient's plasma against drug-treated RBCs, in parallel with normal sera, may help to distinguish a low-titer antibody in normal sera from a high-titer antibody that is causing hemolysis (see subsequent discussion).

Even though piperacillin is a semisynthetic penicillin, we do not recommend testing piperacillin-treated RBCs because plasma from a high percentage of blood donors (91%) and patients (49%) directly agglutinated piperacillin-coated RBCs.[25] In contrast to cefotetan antibodies, piperacillin antibodies in patients with DIIHA do not react to high titers with piperacillin-treated RBCs (unpublished observations). Thus, testing in the presence of a soluble drug is more reliable for detecting clinically significant antibodies to piperacillin.

Hapten Inhibition to Prove Specificity

If positive results for a drug antibody are obtained when testing drug-treated RBCs without a positive control or no previous report, we would attempt hapten inhibition to prove the specificity of the antibody. Inhibition of reactivity occurs when the antibody has combined with the hapten of the same specificity as the subsequently added antigen. Dilutions of the patient's serum incubated with different concentrations of the drug are tested against the drug-treated RBCs. Alternatively, a dilution of the patient's serum that reacts 2+ can be selected for testing with different concentrations of drug. For example, two drops of plasma are incubated with two drops of drug solution (10 mg/mL, 1 mg/mL, and 0.1 mg/mL) for 60 minutes at 37°C for the inhibition phase. The control against which the inhibition tests are compared is two drops of plasma plus two drops of PBS (substituted for the drug). One drop of 3 to 5 percent drug-treated RBCs is added after the inhibition phase and incubated for 60 minutes at 37°C. Inhibition by the soluble drug is shown by a weaker reaction (partial inhibition) or no reaction (complete inhibition) with the drug-treated RBCs. As shown in Table 4, a solution of oxaliplatin added to the plasma inhibited direct agglutination of oxaliplatin-treated RBCs, thus proving the specificity of the antibody. In this example, the antibody reactivity of the 1 in 2 dilution of plasma was too strong (3½+ with the PBS control) to be inhibited until

Table 4. Inhibition test: reactivity of plasma (diluted) plus solution of oxaliplatin or PBS versus oxaliplatin-treated red blood cells

Solution added	Plasma #1 1 in 2 dilution	Plasma #1 1 in 5 dilution
Oxaliplatin (mg/mL)		
1	3+s	1+
2.5	3+	±
5	2+	0
PBS	3+s	2+
Interpretation	Partial inhibition with 5 mg/mL of drug; proof of specificity of anti-oxaliplatin would have been missed if only tested with 1 mg/mL of drug	Complete inhibition with 5 mg/mL of drug and partial inhibition with 2.5 and 1 mg/mL of drug

PBS = phosphate-buffered saline; s = strong.

the higher concentration of drug (5 mg/mL) was added for the inhibition; when the serum was diluted further so that the PBS control yielded a 2+ reaction, the inhibition was clear even with the lower 1 mg/mL concentration. If the serum plus drug reacts more strongly than the respective dilution of serum plus PBS control, then the drug antibody works preferentially when testing untreated RBCs in the presence of drug. Drug antibodies that react only by testing in the presence of a solution of drug (e.g., ceftriaxone) should not be inhibited. IgG antibodies are more difficult to inhibit than IgM antibodies of the same titer.[27] Drugs with a common chemical core might demonstrate cross-reactivity in hapten inhibition tests (e.g., some anti-piperacillin can be inhibited by penicillin).[25]

Testing in the Presence of Soluble Drug

Antibodies to many drugs are detected by testing untreated RBCs in the presence of a solution of the drug. Piperacillin and some of the second- and third-generation cephalosporins react by this method; anti-ceftriaxone has been detected only by testing in the presence of drug.

For testing a patient's sample in the presence of soluble drug, we prepare a 1 mg/mL solution of the drug in PBS. For example, 10 mg of powder, capsule contents, or crushed tablet are dissolved in 10 mL of PBS. As described above, if the drug is not completely dissolved, the solution is centrifuged and the supernate is transferred to a clean tube. The drug solution should be approximately pH 7 to be compatible with RBCs (i.e., adjusted to pH 6–8, if necessary). The 1 mg/mL concentration used for testing in the presence of soluble drug has no correlation to the therapeutic in vivo concentration of the drug. However, this concentration has been successfully used for decades to detect most drug antibodies.

Patient's serum is incubated in the presence of the soluble drug, with and without the addition of fresh normal serum as a source of complement, and tested with untreated and enzyme-treated RBCs (see details in Table 5). Serum, rather than plasma, is the preferred specimen for this testing for the observation of hemolysis; this also allows for the addition of fresh normal serum as a source of complement. Testing with enzyme-treated RBCs and the addition of a complement source may increase the sensitivity of the test, especially for hemolysis. Pooled RBCs (e.g., R_1R_1 and R_2R_2 RBCs) are prepared at a heavier concentration (6 to 10 percent); an aliquot of these RBCs are enzyme-treated. The heavier RBC suspension enhances detection of hemolysis. Because of the heavier RBC suspension, more thorough washing for the antiglobulin test may be accomplished if performed manually rather than using an automated cell washer. Antigen-negative RBCs are selected if alloantibodies are present. If autoantibodies are present, the serum is adsorbed with allogeneic RBCs before testing (any alloantibodies can also be adsorbed out).

Table 5. Testing in the presence of a drug solution

1. Label two sets of tubes for untreated and enzyme-treated RBCs:
   - Serum + drug
   - Serum + PBS
   - Serum + complement + drug
   - Serum + complement + PBS
   - Complement + drug
   - Complement + PBS
   - Positive control (if available) + drug*
   - Positive control (if available) + PBS
2. Place 2 drops of serum, complement source, drug, and PBS in the appropriate tubes
3. Add 1 drop of untreated or enzyme-treated RBCs (6–10% suspension)
4. Mix and incubate at 37°C for 1 hour
5. Centrifuge and examine for hemolysis and agglutination
6. Wash RBCs four times and add polyspecific antihuman globulin; examine for agglutination
7. Add IgG-coated RBCs to nonreactive tests

*Positive control need not be tested with both untreated and enzyme-treated RBCs.

RBCs = red blood cells; PBS = phosphate-buffered saline.

The complement source is serum from two or more individuals, screened for reactivity with enzyme-treated RBCs, pooled, aliquoted, and frozen at –55°C or less on the same day that it was collected.[28] To show that the complement source has hemolytic ability, the complement source is added to serum containing a hemolytic antibody (e.g., anti-I) that has been inactivated by incubation at 56°C for 30 minutes (if necessary) and tested with antigen-positive RBCs. Complement source prepared in this manner can be stored at –55°C or less for 1 year.

Interpretation of Results

Hemolysis, agglutination, or positive indirect antiglobulin tests may occur together or separately. The expected results for a positive test for drug antibody are reactivity in the tests with patient's serum plus drug and no reactivity in the respective control tests of patient's serum plus PBS or the tests with complement source but no patient's serum. The tests with serum plus PBS serve as controls for the respective tests with serum plus drug solution (see Table 6). No reactivity in any of the tests indicates no drug antibody is present.

There are times when results are not so straightforward (see Table 7). The PBS control test(s) may be reactive. This can be because of autoantibody, alloantibody, or circulating drug or drug–anti-drug complexes. A positive result in the test with drug and a significantly weaker result in the corresponding

Table 6. Expected results when drug antibody reacts in the presence of a soluble drug

	Untreated RBCs		Enzyme-treated RBCs	
Sample tested	60 min at 37°C	AHG	60 min at 37°C	AHG
Patient's serum + drug	+/0	+/0	+	+
Patient's serum + PBS	0	0	0	0
Patient's serum + C + drug	+/0	+/0	+/H	+
Patient's serum + C + PBS	0	0	0	0
Positive control + drug	+/0	+/0	+	+
Positive control + PBS	0	0	0	0
C + drug	0	0	0	0
C + PBS	0	0	0	0

RBCs = red blood cells; AHG = antihuman globulin; PBS = phosphate-buffered saline; C = complement source; H = hemolysis.

test with PBS indicate drug antibody is present. Equivalent reactivity of patient's serum in both the drug and PBS tests may be caused by alloantibody or autoantibody. Adsorbing the serum to remove alloantibody or autoantibody and drug or drug–anti-drug complexes, or dialyzing the serum to remove circulating drug, may resolve the problem. If the reactivity is caused by circulating drug or drug–anti-drug complexes, the reactivity should disappear after the patient is no longer receiving the drug and the drug has cleared from the circulation. For piperacillin, ceftriaxone, and cefotetan, the reactivity should disappear about 1 to 2 days after the drug is stopped; other drugs may have a longer elimination half-life.

The addition of fresh normal serum (complement) can sometimes enhance agglutination of a drug antibody tested against enzyme-treated RBCs. This phenomenon is not completely understood. For one patient who probably had a circulating drug that resulted in positive antiglobulin tests in the PBS controls, direct agglutination of enzyme-treated RBCs in the presence of ceftriaxone plus fresh normal serum (complement source) provided the distinguishing reactivity for a ceftriaxone antibody.[11] The controls of complement plus drug and complement plus PBS should be negative; the complement plus drug test ensures the complement source by itself does not cause complement activation in the presence of the drug or react with the test RBCs (with or without drug). If the complement plus drug test is positive at the antiglobulin test and the complement plus PBS test is negative, NIPA may be

Table 7. Interpretation of various example tests in the presence of a soluble drug

	Untreated RBCs		Enzyme-treated RBCs		
Test	37°C	AHG	37°C	AHG	Interpretation
Patient's serum + drug	0	0	3+	1+	Positive (drug antibody)
Patient's serum + PBS	0	0	0	0	
Patient's serum + C + drug	0	0	4+	1+	
Patient's serum + C + PBS	0	0	0	0	
Patient's serum + drug	2+H/3+	3+	4+H	—	Positive (drug antibody)
Patient's serum + PBS	0	0	0	0	
Patient's serum + C + drug	2+H/3+	3+	4+H	—	
Patient's serum + C + PBS	0	0	0	0	
Patient's serum + drug	0	1+s	4+	3+	Positive (drug antibody) and circulating drug or drug–anti-drug complexes
Patient's serum + PBS	0	m+	±	1+	
Patient's serum + C + drug	0	1+	4+	2+	
Patient's serum + C + PBS	0	0	1+	±	
Patient's serum + drug	3+s	2+	2+H/4+	4+	Positive (drug antibody) and circulating drug or drug–anti-drug complexes
Patient's serum + PBS	0	0	3+	1+	
Patient's serum + C + drug	2+s	2+	2+H/4+	4+	
Patient's serum + C + PBS	0	0	3+	1+	
Patient's serum + drug	0	0	4+	4+	Not interpretable as a result of autoantibody or alloantibody; adsorb and retest
Patient's serum + PBS	0	0	4+	4+	
Patient's serum + C + drug	0	1+	4+	4+	
Patient's serum + C + PBS	0	1+	4+	4+	

RBCs = red blood cells; AHG = antihuman globulin; PBS = phosphate-buffered saline; C = complement; H = hemolysis; — = no cells left; m+ = micro.

occurring. If both the complement plus drug and complement plus PBS tests are positive and the patient's serum plus drug or PBS (without complement added) was not informative, the test should be repeated with a new source of complement.

If negative results are obtained using this method but the patient's history warrants further evaluation, use of a different concentration of drug (e.g., saturated solution[18]), wash for the antiglobulin test using a solution of drug (e.g., 1 mg/mL[29]), or testing with drug metabolites substituted for the drug solution (see later discussion) should be considered.

Titration of Drug Antibodies

Antibody titration against drug-treated RBCs or in the presence of drug is of interest when investigating a new drug antibody. Antibody titration against drug-treated RBCs should be performed if normal serum also reacted with the drug-treated RBCs for a correct interpretation. For agglutination and indirect antiglobulin test titers, dilute the serum in PBS. For a hemolysin titer, dilute the serum in fresh normal serum (complement source) and read the test only for hemolysis. The titer is the reciprocal of the last dilution that reacts 1+ (e.g., a serum that reacts 2+ at the dilution 1 in 128, 1+ at 1 in 256, and ± at 1 in 512 has a titer of 256).

Test Metabolites of Drug

If there is convincing history incriminating a particular drug and the DAT is positive but the drug workup is not informative, testing the patient's serum and eluate against metabolites of the drug should be considered. Salama et al.[30] reported detection of antibodies to buthiazide and nomifensine only when ex vivo antigen (i.e., serum from volunteers after ingestion of the drugs) was tested.[30] Antibodies to some nonsteroidal anti-inflammatory drugs also have required testing in the presence of a urine metabolite for detection.[31] Uncharacterized metabolites can be obtained from serum or urine of a patient or volunteer after taking the drug. The serum or urine is collected before the drug is taken and at various times after ingestion and is substituted for the drug solution in testing in the presence of a soluble drug. Previous reports may be helpful to determine the appropriate time(s) of collection. For diclofenac, the morning void after an evening dose of diclofenac (150 mg) was successful.[32] The urine is centrifuged, and the supernatant is adjusted to pH 6.0 to 8.0, if necessary. Purified metabolites are sometimes available from drug manufacturers.

Dialysis to Remove Drug From Patient's Sample

When it is suspected that drug is present in a patient's sample collected when the patient was still receiving the drug or shortly thereafter, the blood sample can be dialyzed to remove the drug. The sample is placed in dialysis tubing (e.g., with a 12,000 to 14,000 molecular weight cutoff [MWCO]) and dialyzed in PBS overnight at 4°C.[31] Alternatively, Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific, Rockford, IL), 2,000 MWCO, 0.5- to 3.0-mL capacity, work well. A large volume of PBS dialysate and two to three changes of the PBS in 24 hours will drive the diffusion of drug out of the sample. The dialyzed sample is tested with and without drug added. No reactivity in the test without drug added (e.g., in the PBS control) and reactivity in the test with soluble drug added confirms the presence of a drug-dependent antibody.

Unexpected Observations

It should be obvious from the preceding discussion that interpreting results of tests for drug antibodies is not always easy. We have encountered three serologic problems when investigating drug antibodies: (1) normal plasma (e.g., from blood donors) directly agglutinates some drug-treated RBCs (e.g., cefotetan,[19] piperacillin,[25] oxaliplatin[26]); (2) some drugs cause nonimmunologic adsorption of protein, resulting in positive indirect and direct antiglobulin tests[3]; and (3) drug that is still circulating in a patient's plasma when the sample is collected can result in positive tests without adding drug in vitro (e.g., when testing the serum plus PBS control in the presence of drug).[11] This latter problem has been evident in many cases of antibodies to piperacillin; this is most disconcerting because serologists are misled to interpret the reactivity as warm autoantibody, especially if the eluate is also reactive.[33,34] Unfortunately, some of these patients with antibodies to piperacillin are treated for warm autoimmune hemolytic anemia and not immediately taken off the drug that is causing the hemolysis.

Another unexpected result is reactivity of the last wash control for eluates when testing cefotetan-treated RBCs. Increasing the number of washes (e.g., 8 to 12) before preparing the eluate does not eliminate the problem.[19]

In summary, the serologic methods described here have been successful in identifying most drug antibodies. Including the appropriate controls will help to ensure correct interpretation of results. As new drugs come into use, we will continue to learn.

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