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on our cover

George Garratty was my boss, my mentor, and, later, my friend. He instilled in each of his staff the requirement to associate clinical findings with laboratory and serologic findings and that these aspects contributed to optimal care of patients with immune hemolytic anemias. In his preface to Immunobiology of Transfusion Medicine, published in 1994, he said, “Avoiding the immune destruction of circulating cells such as erythrocytes, leukocytes, and platelets is one of the major goals of transfusion medicine. The in vivo and in vitro reactions involved in these immune reactions provide easily studied human models for complement- and macrophage-mediated cell destruction, autoimmunity, and drug-induced immune destruction of cells. Although complement-mediated cell destruction is understood quite well, many aspects of the more common extravascular destruction of blood cells are still not understood. Over the last four decades, we have learned that important factors such as immunoglobulin class and subclass, complement-activating ability of the antibody, quantity of cell-bound antibody and complement components, affinity of the antibody, and activity of the mononuclear phagocyte system all play a role, but there are still many anomalies between the observed in vivo destruction and our in vitro results. We need to reconcile these differences before in vitro assays can be improved and to forecast accurately the survival of transfused cellular components.” We miss his presence among us.

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Introduction to *Immunohematology* Special Edition on Drug-Induced Immune Cytopenias

This issue of *Immunohematology* on drug-induced immune cytopenias (hemolytic anemia, thrombocytopenia, and neutropenia) was organized and guest edited by the late Dr. George Garratty. Dr. Garratty started writing regular reviews for *Immunohematology* on drug-induced immune hemolytic anemia (DIIHA) and positive direct antiglobulin tests in 1985. His first review covered “some controversies, unusual aspects, or subjects that have not been thoroughly discussed before” and included a table with 45 drugs. The second review 4 years later concentrated on mechanisms and ended with the statement “[i]t seems to me that in 1989 we still have more questions than answers.” In his 1994 review, Dr. Garratty updated information on current theories about DIIHA and included a table with 76 drugs. In 2004, about 100 drugs had been associated with DIIHA, but a large number of DIIHAs were associated with cephalosporins so they were the emphasis of the review that year. With regard to mechanisms of DIIHA, Dr. Garratty stated that “personally I do not think that, in 2004, we have any better explanations than we did in 1994!” The most recent review in 2007 was by Dr. Garratty and Pat Arndt and included three tables: the first table contained information on 108 drugs associated with drug-dependent antibodies, the second table listed 17 drugs possibly or probably associated with drug-independent antibodies (autoantibodies), and the third table showed 9 drugs that had been shown to cause nonimmunologic protein adsorption.

This issue contains the final installment of this series of reviews by Dr. Garratty. The review by Dr. Garratty and Pat Arndt updates the three previous tables and summarizes new information on drugs currently associated with DIIHA. In addition, in this issue, there are three others articles on DIIHA. The article by Pat Arndt is a written version of her Sally Frank Award Lecture given at the 2012 AABB meeting; it covers some of the changes seen with DIIHA during the last 30 years. The article by Gina Leger, Pat Arndt, and Dr. Garratty describes in detail how Dr. Garratty’s laboratory investigates DIIHA; there are differences in how laboratories perform testing for DIIHA (e.g., gel vs. tube; untreated vs. enzyme-treated red blood cells), which could affect the results. The article by Dr. Abdulgabar Salama and Dr. Beate Mayer covers some of the pitfalls and errors they have observed that occur both clinically and in the laboratory that can result in misdiagnosis or lack of serologic evaluation for DIIHA. Rounding out the drug-induced cytopenias are two thorough reviews by Dr. Brian Curtis on drug-induced immune thrombocytopenia (DIIT) and drug-induced immune neutropenia (DIIN). Some of the same drugs are found in association with DIIHA, DIIT, and DIIN, but usually not in the same patients. Other similarities can be seen among these three different types of drug-induced immune cytopenias.

Unfortunately, Dr. Garratty passed away during the final preparation of this issue. His reviews and other publications on DIIHA have been a valuable source of information for clinicians and laboratory scientists for almost 40 years. It is fitting that this issue of *Immunohematology* is published as a tribute to him.

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Drug-induced immune hemolytic anemia (DIIHA) is a rare condition that occurs primarily as a result of drug-induced antibodies, either drug-dependent or drug-independent. Drug-dependent antibodies can be detected by testing drug-treated red blood cells (RBCs) or untreated RBCs in the presence of a solution of drug. Drug-independent antibodies react with untreated RBCs (no drug added) and cannot be distinguished from warm autoantibodies. Many changes have occurred during the last 30 years, such as which drugs most commonly cause DIIHA, the optimal testing methods for identifying them, and the theories behind the mechanisms by which they react. This article reviews the major changes in DIIHA since the early 1980s involving the immune complex mechanism, cephalosporins, nonimmunologic protein adsorption, and penicillins. Because serologic results associated with DIIHA can mimic those expected with autoimmune hemolytic anemia or hemolytic transfusion reactions, DIIHA may go undetected in some cases. Immunohematology 2014;30:44–54.

Key Words: immune hemolytic anemia, drugs, antibodies, red blood cells, IgG, complement

It was a case of being at the right place at the right time. Shortly after my graduation in 1983 from the Specialist in Blood Banking (SBB) program at the American Red Cross in Los Angeles, there was an opening for a research associate in Dr. George Garratty’s research laboratory, and I was hired. Dr. Garratty’s main research focus was the immune hemolytic anemias. My coworkers in the research laboratory in the 1980s were Nina Postoway and Sandy Nance. Sandy taught me how to perform flow cytometric analyses on red blood cells (RBCs) and the monocyte monolayer assay (MMA). Nina taught me how to perform the enzyme-linked antiglobulin test for direct antiglobulin test (DAT)-negative autoimmune hemolytic anemia (AIHA) workups and also how to do drug-induced immune hemolytic anemia (DIIHA) workups. Nina said to expect that most, if not all, DIIHA workups would be negative. She was right...at first...and then things started to change. I worked in Dr. Garratty’s research laboratory at the Red Cross in Southern California for a little over 30 years and during that time saw many changes relating to DIIHA. This presentation will review some of those changes.

Background

The incidence of DIIHA is unknown, but Dr. Garratty suggested that it may be about 1 in a million. Causes of DIIHA can be classified into two broad categories: drug-induced antibodies and nonimmunologic protein adsorption (NIPA). Drug-induced antibodies can be further subdivided into drug-dependent antibodies and drug-independent antibodies. The first reported drug-dependent antibody was an antibody to stibophen (an anthelmintic drug) in 1956, and the first described drug-independent antibodies were autoantibodies induced by methyldopa (an antihypertensive drug) in 1966. The first drug noted to cause NIPA was cephalothin (an antibiotic); positive DATs were first reported in 1967, and positive indirect antiglobulin tests (IATs), in 1971.

The next few paragraphs summarize knowledge with regard to DIIHA in the early 1980s, using the 1980 textbook Acquired Immune Hemolytic Anemias by Petz and Garratty as a reference. At that time, 32 drugs had been reported to cause immune hemolytic anemia (IHA) or positive DATs. The Petz and Garratty book described four mechanisms: (1) immune complex, (2) drug adsorption, (3) NIPA, and (4) AIHA.

Immune Complex

This mechanism was said to occur when drugs and drug antibodies (IgG or IgM) combined to form immune complexes, which were then nonspecifically adsorbed onto RBCs and activated complement. Most drugs that caused DIIHA reacted by this mechanism: prototype drugs were quinidine (an antiarrhythmic drug) and phenacetin (a nonsteroidal anti-inflammatory drug [NSAID]). Typical characteristics of this mechanism were (1) a previously sensitized patient needed to take only a small amount of the drug; (2) as a result of complement activation, patients had acute intravascular hemolysis (hemoglobinemia and hemoglobinuria) and often...
renal failure; (3) the DAT was positive, often with C3 only; and (4) in vitro reactions (agglutination, hemolysis, and sensitization) could only be demonstrated by incubating a mixture of the patient’s serum, the drug, and test RBCs (the “immune complex” method).

Drug Adsorption

This mechanism was used to describe the few drugs that bound firmly to RBC membranes; antibodies to these drugs reacted with the RBC-bound drug. Penicillin (an antibiotic) was the prototype drug for this mechanism. Typical characteristics of penicillin-induced DIIHA were (1) the patients had received large doses of penicillin intravenously, e.g., 10 million units daily for one or more weeks; (2) the extravascular hemolysis developed over the course of 7–10 days, and hemolysis of decreasing severity could persist for several weeks after the penicillin was stopped; (3) the DAT was strongly positive for IgG, and sometimes C3 was also present; and (4) in vitro tests of the patient’s serum and eluate with drug-treated RBCs were positive (serum IgG antibody titer usually ≥1000).

Nonimmunologic Protein Adsorption

This mechanism occurred when drugs modified the RBC membrane so that plasma proteins were adsorbed nonimmunologically. Cephalothin (trade name Keflin) was the prototype drug for this mechanism. Characteristics of NIPA included the following: (1) patients taking cephalothin had a positive DAT (using antisera directed to albumin, complement, fibrinogen, immunoglobulins, etc.) but no hemolytic anemia, and (2) in vitro, cephalothin-treated RBCs incubated in normal plasma were coated with numerous proteins (the IAT was positive using various antisera as above), but those same RBCs incubated with an eluate were typically nonreactive because protein levels in the eluate were too low.

Autoimmune Hemolytic Anemia

This mechanism occurred when the drug induced an autoantibody that reacted with normal RBCs (no drug added), similar to reactivity seen with autoantibodies found in idiopathic IgG warm AIHA. The prototype drug for this mechanism was methyldopa (trade name Aldomet). The characteristics of methyldopa-induced autoantibodies were (1) 10 to 36 percent of patients developed a strongly positive DAT (IgG ± C3) after taking methyldopa for 3 to 6 months (this appeared to be dose-dependent), and the DAT could take up to 2 years to become negative after methyldopa was stopped; (2) about 0.8 percent of patients gradually developed extravascular hemolytic anemia, which resolved after cessation of methyldopa; and (3) the patient’s serum and eluate both contained an autoantibody serologically indistinguishable from other IgG warm autoantibodies. One proposed mechanism was that methyldopa caused an alteration of the immune system.⁹ Proof that methyldopa caused AIHA came only from clinical observations.

Changes Since the Early 1980s

The Research Laboratory

In 1990, Nina and Sandy left the research laboratory to pursue other transfusion medicine careers, and in 1993, Gina Leger joined our laboratory from the Immunohematology Reference Laboratory. It was my turn to teach her most of the standard tests, and by then, DIIHA workups had become very interesting. So she, too, was in the right place at the right time.

The Immune Complex Mechanism

The immune complex mechanism was proposed in the 1950s to 1960s to explain drug-induced thrombocytopenia (i.e., drug antibodies bind with the drug and then the drug–antibody complex is adsorbed nonspecifically by platelets) and was then later extended to apply to RBCs.¹⁰,¹¹ Criticisms of this mechanism started appearing in the 1970s to 1980s. Some findings that do not support the immune complex mechanism include the following: (1) drug–antibody immune complexes (e.g., RBC-bound or in plasma) have never been demonstrated experimentally; (2) some drug-dependent antibodies bind to their target cells by their Fab domain, e.g., quinidine antibodies with platelets¹²,¹³ and tolmetin antibodies with RBCs;¹⁴ if the binding was nonspecific, one would expect binding through both the Fab and the Fc domains; (3) drug-dependent antibodies appear to be highly specific for certain cell lines, e.g., either platelets or RBCs (this would not be expected if immune complexes were attaching nonspecifically to cells); (4) some drug-dependent antibodies react with specific antigens on target cells, e.g., anti-quinidine with glycoprotein Ib/IX or IIb/IIIa on platelets;¹⁵ (see Table 1 for reported specificities of drug-dependent antibodies reactive with RBCs); and (5) many patients have been described with antibodies reacting by more than one mechanism, e.g., drug-independent autoantibodies plus drug-dependent antibodies reacting by the drug adsorption or immune complex method (reviewed in Petz and Garratty¹⁶ and in Garratty¹⁷).

Some alternative hypotheses to the immune complex mechanism have been proposed to explain observations with drug-induced antibodies. Ackroyd proposed that for one type of drug-induced thrombocytopenia, the drug and cell formed
a loose complex, and the antibody was specific for only the combined drug–cell antigen. This drug–cell complex has been referred to as a “neoantigen.” Another type of neoantigen could occur if a drug induced a chemical change to a membrane to create a new antigen (changed membrane only). To explain why some patients with DIIHA have antibodies working by more than one mechanism, e.g., the immune complex method in addition to autoantibodies, Habibi suggested a single mechanism whereby the formation of a drug–cell conjugate initiates the immune response. Mueller-Eckhardt and Salama proposed a unifying hypothesis similar to the proposals of Ackroyd and Habibi. They proposed that the drug (or drug metabolite) first interacts with the cell membrane; this drug–membrane structure then provokes the production of two types of antibodies: drug-dependent or drug-independent antibodies.

**Table 1.** Reported blood group specificities of drug antibodies; specificities varied from simple (e.g., anti-e) to broad (e.g., antibody reactive with all cells except null cells)

<table>
<thead>
<tr>
<th>Blood group</th>
<th>Drugs (and earliest reference to note specificity)</th>
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<tbody>
<tr>
<td>Rh</td>
<td>catechins,16 diclofenac,17 glafenine,18 hydrochlorothiazide,18 ibuprofen,18 mexitilene,31</td>
</tr>
<tr>
<td></td>
<td>sulfamethoxazole,18 tolemin,27 clindamycin,21 norfloxacin,21</td>
</tr>
<tr>
<td>I</td>
<td>dexamethasone maleate,26 fluorouracil,26 metrizoic acid,26 nitrofurantoin,26 norfloxacin,26 rifampin,26</td>
</tr>
<tr>
<td></td>
<td>norfloxacin,26 thiopental23</td>
</tr>
<tr>
<td>Lutheran</td>
<td>ellipitinum,19 rifampin,18 piperaclilin,23 sulfamethoxazole18,29,30</td>
</tr>
<tr>
<td>P</td>
<td>ellipitinum,19 meglumine iothalamate,19 nomifensin31</td>
</tr>
<tr>
<td>Kell</td>
<td>glafenine,18 trimethoprim18,20</td>
</tr>
<tr>
<td>MNS</td>
<td>streptomycin,24 sulfamethoxazole33</td>
</tr>
<tr>
<td>Kidd</td>
<td>chlorpropamide35</td>
</tr>
<tr>
<td>H</td>
<td>sulfamethoxazole34</td>
</tr>
</tbody>
</table>

Shown above, there is evidence to dispute this mechanism. When describing this mechanism, this uncertainty can be indicated by adding quotes around the words “immune complex” or by using the phrase “so-called immune complex mechanism.” It is more accurate to describe the method used to detect these antibodies as testing in the presence of a solution of drug instead of the immune complex method.

**Cephalosporins**

The cephalosporins are a class of β-lactam antibiotics grouped into generations based on their antimicrobial properties. In the 1970s to early 1980s, DIIHA was described only a handful of times as being associated with first and second cephalosporins (cephalothin, cefazolin, and cefamandole; reviewed in Garratty). These cephalosporins, like penicillin (another β-lactam antibiotic), formed covalent bonds with RBC membranes; thus, drug-treated RBCs could be prepared in the laboratory. The characteristics of these early cephalosporin DIIHA cases were (1) the DAT was positive for IgG, sometimes also C3; (2) the serum and RBC eluate reacted with drug-treated RBCs; (3) preincubation of the cephalosporin with the antibody caused inhibition of reactivity with drug-treated RBCs; and (4) in vivo hemolysis was extravascular. Thus, up to the mid-1980s there was a general assumption that antibodies to cephalosporins reacted by the drug adsorption mechanism, and the best way to detect them was with drug-treated RBCs. Then things started to change.
Cefotaxime
In 1987, Salama et al. described a patient with immune complex-mediated intravascular hemolysis caused by an antibody to cefotaxime (a third-generation cephalosporin). The DAT was positive for C3 only, the serum was nonreactive with drug-treated RBCs but did react by the immune complex method (serum plus RBCs in the presence of a solution of drug), and preincubation of drug with the antibody did not cause inhibition of its reactivity.

Ceftriaxone
A year later, our laboratory reported, in an abstract at the 1988 AABB meeting, a fatal case of DIIHA attributable to ceftriaxone (another third-generation cephalosporin). The clinical and serologic characteristics were those associated with the immune complex mechanism. The DAT was positive for C3 only, and the serum reacted only by the immune complex method (serum plus RBCs in the presence of a solution of drug); drug-treated RBCs were nonreactive.

Cefotetan
In 1989, there was one AABB abstract and, in 1990, there were four AABB abstracts describing DIIHA attributable to cefotetan (reviewed by Garratty). These antibodies reacted by more than one method: all five reacted with cefotetan-treated RBCs, three of four reacted in the presence of a solution of cefotetan, and two of four reacted with untreated RBCs (no cefotetan added). Table 2 lists reasons for reactivity of serum in tests when the drug was not added in vitro. In cases of DIIHA caused by cefotetan, we believe this reactivity with untreated RBCs is a result of drug-independent autoantibodies that are found in combination with drug-dependent antibodies (possibly represented by the "mainly membrane" epitope in Fig. 1).

Thus, it was clear by the late 1980s that the best way to detect cephalosporin antibodies was not always by testing drug-treated RBCs. Since then, there have been numerous reports of DIIHA caused by different cephalosporins. Table 3 summarizes how these cephalosporin antibodies were detected. Antibodies to first- and second-generation cephalosporins were primarily detected using drug-treated RBCs with the exception of cefotetan-dependent antibodies, which were detected equally by two methods: drug-treated RBCs and in the presence of a solution of drug. Antibodies to third-generation cephalosporins were primarily detected by testing in the presence of a solution of drug (ceftriaxone antibodies are only detected by this method). Reactivity in the absence of the in vitro drug was primarily detected in patients with DIIHA caused by cefotetan but was found with some other cephalosporins as well. Our current approach for detection of drug-dependent antibodies of any kind is as follows: (1) if the drug antibody has been well studied, use the previously described method(s) for detection, or (2) if the drug has not been previously described or well studied, test by both methods: (a) serum and RBC eluate with drug-treated RBCs (and untreated RBCs) and (b) serum with untreated or enzyme-treated RBCs in the presence of soluble drug (or saline as a dilution control).

Table 2. Reasons for serum to be reactive in drug-dependent antibody tests when drug has not been added in vitro (e.g., controls)

<table>
<thead>
<tr>
<th>Reason for Reactivity</th>
<th>Drug-treated RBCs</th>
<th>Serum + RBCs + Drug</th>
<th>No in vitro drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alloantibody</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autoantibody, two types</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Autoantibody alone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Idiopathic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. Drug-independent antibody (e.g., methyldopa or fludarabine)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Autoantibody found in the presence of drug-dependent antibody</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Circulating drug or drug plus antibody immune complexes present in the blood sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of drug in reagent (e.g., low-ionic-strength saline [LISS])</td>
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<td></td>
</tr>
</tbody>
</table>

Table 3. Drug-induced immune hemolytic anemia caused by cephalosporins: summary of methods used and results (number positive/number tested) in references in which data on methods were given

<table>
<thead>
<tr>
<th>Cephalosporin</th>
<th>Reference</th>
<th>Drug-treated RBCs</th>
<th>Serum + RBCs + Drug</th>
<th>No in vitro drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>First-generation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cephalothin</td>
<td>5</td>
<td>5/5</td>
<td>1/1</td>
<td>0/5</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>2</td>
<td>2/2</td>
<td>0/1</td>
<td>0/2</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>1</td>
<td>1/1</td>
<td>0/0</td>
<td>?</td>
</tr>
<tr>
<td>Second-generation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>4</td>
<td>4/4</td>
<td>1/2</td>
<td>0/3</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>1</td>
<td>1/1</td>
<td>0/0</td>
<td>0/1</td>
</tr>
<tr>
<td>Cefotetan</td>
<td>31</td>
<td>31/31</td>
<td>20/22</td>
<td>12/25</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>2</td>
<td>2/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>4</td>
<td>3/4</td>
<td>3/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Third-generation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>4</td>
<td>2/4</td>
<td>3/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>28</td>
<td>0/12</td>
<td>28/28</td>
<td>5/19</td>
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<tr>
<td>Ceftizoxime</td>
<td>4</td>
<td>2/4</td>
<td>3/4</td>
<td>2/4</td>
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<tr>
<td>Cefixime</td>
<td>1</td>
<td>1/1</td>
<td>1/1</td>
<td>0/1</td>
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</table>

RBCs = red blood cells.
CASE STUDIES

In the 1970s, the most commonly seen drug-induced antibodies in Dr. Garratty’s laboratory were caused by methyldopa and penicillin (Table 4). This scenario changed in the 1980s, and for the last several decades, cephalosporins, particularly cefotetan and ceftriaxone, have been the most common causes of DIIHA.56,57 Two cases illustrate common features of DIIHA caused by ceftriaxone or cefotetan.

Table 4. Causes of drug-induced immune hemolytic anemia seen in Dr. Garratty’s laboratories during two different 10-year periods

<table>
<thead>
<tr>
<th>Drug</th>
<th>Number (percentage) of cases</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1969–78</td>
</tr>
<tr>
<td>Methyldopa</td>
<td>29 (67%)</td>
</tr>
<tr>
<td>Penicillin</td>
<td>10 (23%)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>17 (19%)</td>
</tr>
<tr>
<td>Cefotetan</td>
<td>24 (28%)</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>32 (37%)</td>
</tr>
<tr>
<td>Platinum-based chemotherapies</td>
<td>6 (7%)</td>
</tr>
<tr>
<td>Others</td>
<td>4 (9%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>43</strong></td>
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</tbody>
</table>

Patient 1 was a 10-year-old boy with juvenile idiopathic arthritis who had previously received ceftriaxone. He was admitted to the emergency room with fever and vomiting. At 1409 hours, his hemoglobin (Hb) was 10.9 g/dL. Ceftriaxone (tradename Rocephin) was started at 1542 hours. At 1615 hours, his blood pressure dropped and a change in consciousness was noted; therefore, the ceftriaxone was stopped, but it was then restarted at 1745 hours. At 1825 hours, his Hb had plummeted to 0.5 g/dL; transfusions were unsuccessfully attempted, and he was pronounced dead at 1834 hours. Postmortem samples were sent to our laboratory to determine whether the patient had fatal DIIHA caused by ceftriaxone. RBC-bound IgM and C3 were detected on the patient’s RBCs (no IgG or IgA). Ceftriaxone-treated RBCs cannot be prepared in vitro (we and others have tried several methods without success), so the only way to test for ceftriaxone antibodies is in the presence of a solution of ceftriaxone. The patient’s serum plus PBS also caused agglutination (2+) of ficin-treated RBCs. This reactivity without adding the drug in vitro was presumably because of the presence of ceftriaxone in the patient’s circulation at the time that the blood sample was drawn but could also have been the result of autoantibody. Two ways to distinguish an autoantibody (which should be persistent) from circulating drug (which would be transient; the time it takes for a drug to clear the circulation depends on the half-life of the drug and the patient’s renal status) are to (1) obtain a new blood sample from the patient after the drug has cleared (e.g., a few days after the drug is stopped); an autoantibody would be expected to still react but a drug antibody should no longer react; and (2) dialyze the sample to remove the drug.58,59

Thus, Patient 1 had fatal DIIHA caused by ceftriaxone. Although DIIHA caused by ceftriaxone occurs in adults, it is usually more acute and severe in children. Often in children, the time from receipt of drug to reaction is 1 hour or less and the nadir Hb is 5 g/dL or less. Twelve of 18 reported ceftriaxone-induced fatalities were in children. Typically, patients with DIIHA caused by ceftriaxone have a history of previous ceftriaxone therapy and signs of intravascular hemolysis (hemoglobinemia, hemoglobinuria, and renal failure). One study performed to determine the prevalence of ceftriaxone antibodies in 64 pediatric patients with HIV or sickle cell disease detected antibodies in 8 patients (12.5%); 1 patient had fatal DIIHA minutes after receiving ceftriaxone.60

Serologic results in DIIHA caused by ceftriaxone include the following: (1) positive DAT (C3, sometimes C3 plus IgG) and (2) drug-dependent antibody (primarily IgM) that can only be detected in the presence of ceftriaxone (reactions include agglutination, hemolysis, and positive antiglobulin test). About two-thirds of the patients’ samples we have tested reacted without the addition of in vitro ceftriaxone; this was most likely because of the presence of circulating drug in the blood samples.61 There have been three reports of DIIHA caused by ceftriaxone in which antibodies were only detected by a gel method using ex vivo samples (e.g., urine from people receiving ceftriaxone).62–64

Patient 2 had cefotetan-induced IHA; details of this case were previously reported in Immunohematology.65 In brief, a woman received two doses of cefotetan (trade name Cefotan) at the time of a cesarean section. Five days later, she had signs of IHA (Hb = 8.7 g/dL, reticulocytes = 10.5%, lactate dehydrogenase [LDH] = 503 U/L, haptoglobin <6 mg/dL) and a positive DAT (IgG and C3). Her serum contained a high-titer drug-dependent antibody that reacted with cefotetan-treated RBCs (hemolysin titer = 128, agglutination titer = 1024, and IAT titer = 128,000) and a weak autoantibody.
that reacted with untreated RBCs (IAT titer = 1). An eluate prepared from her RBCs reacted with cefotetan-treated RBCs (3+ IAT); the last wash control was weakly reactive (1+ IAT), which is not an unusual finding in cefotetan-induced IHA. The serum antibody also reacted when tests were performed in the presence of a solution of cefotetan (1 mg/mL) but to a much lower titer (IAT titer vs. untreated RBCs = 512) than with cefotetan-treated RBCs (prepared using cefotetan at a concentration of 40 mg/mL). It is possible that cefotetan antibodies are reacting with weakly cefotetan-coated RBCs and not by the immune complex mechanism when tested in the presence of soluble cefotetan. This woman had received cefotetan 3 years earlier at the time of another cesarean section and at that time had signs of IHA after 13 days (Hb = 5 g/dL, reticulocytes = 15%, haptoglobin <6 mg/dL). Serum drawn just before the current surgery demonstrated preexisting anti-cefotetan (IAT titer vs. cefotetan-treated RBCs = 512). Thus, it was likely that she had two episodes of cefotetan-induced IHA: a primary response 3 years earlier (which was not recognized at the time) and then a later secondary response.

Cefotetan is commonly used prophylactically with surgery, e.g., cesarean sections. The hemolytic anemia becomes apparent 1–2 weeks after receipt of the drug, and the patients return to the emergency room where the differential diagnosis includes sepsis and they are sometimes given more cefotetan with disastrous results. Only one dose of cefotetan can result in dramatic hemolysis; this could be explained by prior environmental exposure (e.g., in food or water), which primes the immune system. The hemolytic anemia sometimes lasts longer than expected; this could be because cefotetan has been shown to remain RBC-bound for a median of 67.5 days. In cefotetan-induced IHA, the Hb drops to a mean of about 5 g/dL, signs of intravascular hemolysis are often present (hemoglobinemia, hemoglobinuria, and renal failure), and fatalities occur. Patients with anti-cefotetan should be warned to never receive cefotetan again. Can these patients receive other cephalosporins? Serologic studies of anti-cefotetan with other cephalosporins showed little in vitro cross-reactivity, but there are no data to determine whether these in vitro data relate to in vivo reactivity.

Serologic results in cefotetan-induced IHA include the following: (1) the DAT is positive (usually both IgG and C3 are present); (2) serum contains a drug-dependent antibody that may react to very high titers with, and completely hemolyze, cefotetan-treated RBCs; (3) the serum drug-dependent antibody also reacts with untreated RBCs in the presence of a solution of cefotetan; (4) serum may also contain a low-titer drug-independent autoantibody (enhanced by sensitive methods, e.g., ficin, polyethylene glycol [PEG]); and (5) an eluate reacts strongly with cefotetan-treated RBCs; the last wash control is often reactive as well. Some other issues that arise when testing cefotetan-treated RBCs are that (1) cefotetan causes NIPA, which can lead to positive IATs when testing undiluted serum, and (2) about 80 percent of normal sera contain low-titer IgM anti-cefotetan agglutinins. Testing patients’ and control sera at a 1/100 dilution should overcome both of these problems when testing cefotetan-treated RBCs; patients with DIIHA caused by cefotetan have very high titer antibodies (median IAT titer = 16,000) that would not be missed by testing serum at a 1/100 dilution.

Conclusion

In the early 1980s, it was generally well accepted that cephalosporin antibodies, like penicillin antibodies, would be expected to cause extravascular hemolysis in vivo and that cephalosporin antibodies would be detected using drug-treated RBCs. Today we know that cephalosporin antibodies can be associated with quite severe (e.g., fatal) intravascular hemolysis in vivo and that these antibodies may react with drug-treated RBCs, or in the presence of a solution of drug, or by both methods.

Nonimmunologic Protein Adsorption

In the 1960s to 1980s, NIPA was described with cephalosporins, diglycoaldehyde, suramin, and cisplatin. There were no recognized cases of hemolytic anemia; thus, the assumption was that NIPA only caused positive DATs and IATs, not decreased RBC survival. Then things started to change.

β-Lactamase Inhibitors

In 1985, clavulanic acid was reported to cause positive DATs (45% of 23 patients) as a result of NIPA. In 1992, a high incidence of positive DATs (39%) was reported in 59 patients taking Unasyn (a combination of ampicillin and sulbactam). None of the patients in either of these reports showed signs of hemolytic anemia. Clavulanic acid (clavulanate) and sulbactam are both β-lactamase inhibitors used in combination with β-lactam antibiotics (e.g., ampicillin and ticarcillin). In 1998, our laboratory reported that both sulbactam and clavulanate caused NIPA. In this same report, we also described 3 patients who had temporal relationships of drug administration (after receipt of either Unasyn, which contains sulbactam, or Timentin, which contains clavulanate) with positive DATs and hemolytic anemia; no drug-dependent antibodies were detected. In 2000, we showed that sulbactam-
and clavulanate-treated RBCs, after incubation with normal plasma, showed increased reactivity with monocytes in an MMA, an in vitro method used to predict decreased RBC survival in vivo. A third β-lactam antibiotic, tazobactam, has also been associated with NIPA, positive MMAs, and hemolytic anemia, possibly as a result of NIPA in a patient with high plasma IgG level.

**Conclusion**

In the early 1980s, NIPA was thought to be a cause of positive DATs and IATs but not hemolytic anemia. Today, there is in vitro and in vivo evidence that NIPA may also be the cause of hemolytic anemia.

**Penicillins**

Numerous cases of DIIHA as a result of the β-lactam antibiotic penicillin have been reported since the 1960s. Characteristics included the following: (1) the DAT was positive for IgG with or without C3, (2) the patient’s serum and an eluate contained an IgG anti-penicillin that reacted with penicillin-treated RBCs (serum titer ≥1000), (3) incubation of the anti-penicillin with soluble penicillin inhibited reactivity with penicillin-coated RBCs, and (4) the hemolysis was extravascular in vivo. These characteristics also applied to other drugs in the penicillin family (e.g., ampicillin, amoxicillin, nafcillin, and ticarcillin). Thus, it was assumed that the best way to detect antibodies to penicillins was to test drug-treated RBCs. Because some people without hemolytic anemia had low-titer IgM penicillin antibodies in their serum, it was important when diagnosing DIIHA caused by penicillin to demonstrate high-titer IgG anti-penicillin in the patient’s serum or anti-penicillin in an eluate from the patient’s RBCs. But then in the 1990s things started to change.

**Piperacillin**

In 1994, Johnson et al. reported the first serologically well-documented case of DIIHA caused by piperacillin, a semisynthetic penicillin. The DAT was positive (IgG and C3), the serum reacted with both piperacillin-treated RBCs and with untreated RBCs in the presence of soluble piperacillin, and an RBC eluate was nonreactive by both methods. In addition, the piperacillin antibody had relative anti-e specificity. In 2002, our laboratory reported two patients with DIIHA caused by piperacillin; one of the patients’ samples (serum and eluate) reacted only in the presence of soluble piperacillin (piperacillin-treated RBCs were nonreactive).

In 2008, our laboratory reported that the best way to detect piperacillin antibodies was by the immune complex method; this differed from the best way to detect antibodies to other penicillins, which was by testing drug-treated RBCs. The immune complex method was recommended because the sera of 91 percent of 100 normal donors and 49 percent of 35 random patients directly agglutinated piperacillin-treated RBCs; two donors’ plasma reacted weakly by antiglobulin test only. Samples from donors were nonreactive when testing ficin-treated RBCs in the presence of piperacillin (the immune complex method). Antibodies to piperacillin found in donors were determined to be IgM, and reactivity with piperacillin-treated RBCs could be inhibited by preincubation with piperacillin or penicillin. In contrast, four patients with DIIHA caused by piperacillin had piperacillin antibodies reactive by the immune complex method that were not inhibited by preincubation with solutions of piperacillin.

In addition to piperacillin-treated RBCs, we have found sera from donors and random patients to directly agglutinate some other drug-treated RBCs; Table 5 shows data from our laboratory. It is interesting to ponder why this occurs. We know that in the United States, our food sources are exposed to drugs (e.g., antibiotics added to cattle feed). Small amounts of drugs can also be detected in tap water because drugs may be discarded down the drain or sewer or excreted in patients’ urine and unfortunately water treatment does not remove drug residue. There is also an increasing environmental presence of some drugs (e.g., platinum) in the air, soil, and water as a byproduct of automobile catalytic converters. There may be other environmental sources. Thus, we may all be exposed to drugs for decades without knowing it. The presence of drug antibodies in normal donors and random patients is one reason why it is important to test normal sera as a control when testing drug-treated RBCs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Blood donors</th>
<th>Random patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin (unpublished results)</td>
<td>5%</td>
<td>6%</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>39%</td>
<td>NT</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>33%</td>
<td>NT</td>
</tr>
<tr>
<td>Cefotetan (and unpublished results)</td>
<td>80%</td>
<td>78%</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>91%</td>
<td>49%</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>16%</td>
<td>4%</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>7%</td>
<td>NT</td>
</tr>
<tr>
<td>Meropenem</td>
<td>93%</td>
<td>60%</td>
</tr>
</tbody>
</table>

*It is interesting to note that, for some drugs, fewer patients than donors have antibodies. NT = not tested.
Thus, because many normal donors and random patients may react with piperacillin-treated RBCs, which can confuse the interpretation of a DIIHA workup, testing with piperacillin-treated RBCs is not the best way to detect piperacillin antibodies. Luckily, piperacillin antibodies found in patients with DIIHA also react by the immune complex method (serum plus RBCs in the presence of a solution of drug), making this the preferred method of testing. Table 6 lists the different penicillins associated with DIIHA and how the antibodies were detected. Piperacillin was the only one with reactivity by all three methods; the reactivity seen when no in vitro drug was added was attributable to the presence of the circulating drug at the time the patient’s sample was collected.

Table 6. Drug-induced immune hemolytic anemia caused by penicillins: summary of methods used and results (number positive/number tested) in reviewed references in which data on methods were given

<table>
<thead>
<tr>
<th>Drug</th>
<th>Reference</th>
<th>Drug-treated RBCs</th>
<th>Serum + RBCs + drug</th>
<th>No in vitro drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>15</td>
<td>15/15</td>
<td>1/1</td>
<td>0/10</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>4</td>
<td>4/4</td>
<td>0/1</td>
<td>0/4</td>
</tr>
<tr>
<td>Nafcillin</td>
<td>3</td>
<td>3/3</td>
<td>0/1</td>
<td>0/3</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>2</td>
<td>2/2</td>
<td>0/0</td>
<td>1/2</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>2</td>
<td>2/2</td>
<td>0/0</td>
<td>0/2</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>18</td>
<td>4/5</td>
<td>17/17</td>
<td>11/15</td>
</tr>
</tbody>
</table>

RBCs = red blood cells.

CASE STUDY

In the 1990s, cefotetan was the single most common drug seen by our laboratory to cause DIIHA.48 In the last decade, this has changed so that now piperacillin is the single most common drug to cause DIIHA seen in our laboratory (Table 4). The following case illustrates some features of DIIHA caused by piperacillin.

Patient 3 had cystic fibrosis and had undergone bilateral lung transplantations. Laboratory data indicated that he was exhibiting hemolysis—the Hb dropped from 11.9 to 6.4 g/dL, the LDH was 1014 U/L, and the total bilirubin was elevated. The patient’s DAT was positive (3+ IgG only); his serum was reactive with all cells tested by saline, low-ionic-strength saline (LISS), PEG, and gel IAT methods (1–3+); and an eluate from his RBCs reacted 2–3+ by PEG-IAT with all RBCs, including the patient’s ethylenediaminetetraacetic acid (EDTA) glycine acid–treated RBCs. Adsorptions removed the serum antibody, and no underlying alloantibodies were detected. Thus, serologically it appeared that the patient had a warm autoantibody but the patient’s physician did not think that he had warm AIHA (the hemolytic anemia was too severe). The patient had been started on Zosyn (a combination of piperacillin and tazobactam) 2 days before the start of hemolysis and the positive serology. Thus, the Zosyn was stopped, the patient was transfused with 4 units of RBCs, and samples were sent to our laboratory for a DIIHA workup.

The two components of Zosyn are piperacillin, a β-lactam antibiotic, and tazobactam, a β-lactamase inhibitor. We believe that when a drug consists of multiple components, each component should be tested separately if possible to avoid misinterpretations.6 Piperacillin and tazobactam can both be obtained commercially (Sigma Chemical Co., St. Louis, MO). Numerous cases of DIIHA caused by piperacillin antibodies have been reported, but there have been no reports of antibodies to tazobactam. Tazobactam does cause NIPA,69 but there are no commonly available serologic tests to prove NIPA as a cause of DIIHA. Thus, in the laboratory, we usually only test for antibodies to piperacillin. Piperacillin-treated RBCs can be prepared using a high-pH buffer,72 but we do not use them when testing patients’ sera for the presence of piperacillin antibodies because (1) plasma from donors and random patients without IHA agglutinate piperacillin-treated RBCs,72 (2) piperacillin antibodies in patients with DIIHA do not have high titers so testing a dilution (as is done in cefotetan antibody workups) will not help (unpublished results), and (3) sera from some patients with DIIHA caused by piperacillin are nonreactive with piperacillin-treated RBCs.71 Thus, our preferred method for testing sera for piperacillin antibodies in patients with IHA is to test RBCs in the presence of a solution of piperacillin. Patient 3’s serum reacted weakly (1+ IAT) with untreated RBCs in the presence of piperacillin; the PBS control was negative. The patient’s serum caused strong agglutination (3+) with ficin-treated RBCs in the presence of piperacillin; the IAT was also positive (3+). The control of the patient’s serum (obtained 1 day after the Zosyn was stopped) plus PBS also reacted weakly (1+ IAT) with ficin-treated RBCs, presumably because of the presence of the circulating drug. No in vitro hemolysis was observed, even in tests with fresh normal serum added.

Thus, Patient 3, with cystic fibrosis, had DIIHA caused by piperacillin. There are several other reports of piperacillin-induced IHA in patients with cystic fibrosis who receive antibiotics for lung infections.73 This patient’s initial serology was consistent with warm AIHA, but the clinical picture did not fit (the hemolysis was too severe). Some patients with DIIHA caused by piperacillin have been noted to have intravascular hemolysis, and some fatalities have been reported. Most patients with DIIHA caused by piperacillin have received the combination of piperacillin plus tazobactam (tradename

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Zosyn in the United States). Typical expected serologic results in DIIHA are a positive DAT with a nonreactive eluate, but this patient had a strongly reactive eluate. This has been observed in some other cases of DIIHA caused by piperacillin and with other drugs (e.g., cefotetan), usually when the drug is present in the patient’s circulation. Several days after the drug is stopped, the apparent autoantibody in the serum and in an RBC eluate should no longer be detectable.

Serologic results in DIIHA caused by piperacillin include (1) a positive DAT, usually with both anti-IgG and anti-C3, and (2) reactivity of serum plus RBCs in the presence of soluble piperacillin (agglutination and positive IAT). Seventy percent of the patients we studied with DIIHA caused by piperacillin presented with apparent warm autoantibodies (some with autoanti-e specificity); these were most likely piperacillin antibodies reacting with the circulating drug present in the patients’ sera. This result has been reported by others.

**Conclusion**

Up through the 1980s, it was assumed that antibodies to the penicillins could only be detected by testing drug-treated RBCs and that these patients had primarily extravascular hemolysis. Penicillin antibodies in sera had high titers (≥1000), and RBC eluates reacted with penicillin-treated RBCs. Now we know that antibodies to at least one semisynthetic penicillin, piperacillin, react with both drug-treated RBCs and in the presence of soluble drug. In patients we studied with DIIHA caused by piperacillin, serum antibodies had low to moderate titers (50% of titers were less than 20; unpublished results), and eluates were often nonreactive when tested with piperacillin-treated RBCs. Anti-piperacillin in sera from patients we studied with DIIHA caused by piperacillin also had low titers when tested in the presence of a solution of piperacillin (median titers were 4 with untreated RBCs and 32 with ficin-treated RBCs; unpublished results); 50 percent of eluates reacted in the presence of piperacillin.

**Closing Remarks**

In closing, it is important to remember that DIIHA is rare. Millions of doses of these drugs are given to patients without IHA occurring. Drug-dependent antibodies, when they are present, may react by multiple methods: (1) drug-treated RBCs, (2) untreated or enzyme-treated RBCs in the presence of a solution of drug, or (3) untreated RBCs (no drug added in vitro). NIPA may be a cause of DIIHA; the only proof is temporal (e.g., if a patient redevelops hemolytic anemia after rechallenge with the drug). Finally, DIIHA may be confused with AIHA (e.g., see Patient 3) or hemolytic transfusion reactions (e.g., if a patient receives drug and transfusion around the same time) and thus we are most likely missing some cases (e.g., see Patient 2).

**Acknowledgments**

I would like to acknowledge and thank the blood bankers who encouraged or inspired me to learn more about blood banking and attend an SBB program: LaVonna Hasz, Ron Simpson, Ira Shulman, and Claire McGrath. I would also like to acknowledge and thank my coworkers in the Research Laboratory: Nina Postoway, Sandy Nance, Gina Leger, and of course, my boss and mentor for over 30 years, George Garratty.

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Drug-induced immune thrombocytopenia (DIIT) is a relatively uncommon adverse reaction caused by drug-dependent antibodies (DDAbs) that react with platelet membrane glycoproteins only when the implicated drug is present. Although more than 100 drugs have been associated with causing DIIT, recent reviews of available data show that carbamazepine, eptifibatide, ibuprofen, quinidine, quinine, oxaliplatin, rifampin, sulfamethoxazole, trimethoprim, and vancomycin are probably the most frequently implicated. Patients with DIIT typically present with petechiae, bruising, and epistaxis caused by an acute, severe drop in platelet count (often to <20,000 platelets/µL). Diagnosis of DIIT is complicated by its similarity to other non–drug-induced immune thrombocytopenias, including autoimmune thrombocytopenia, posttransfusion purpura, and platelet transfusion refractoriness, and must be differentiated by temporal association of exposure to a candidate drug with an acute, severe drop in platelet count. Treatment consists of immediate withdrawal of the implicated drug. Criteria for strong evidence of DIIT include (1) exposure to candidate drug–preceded thrombocytopenia; (2) sustained normal platelet levels after discontinuing candidate drug; (3) candidate drug was only drug used before onset of thrombocytopenia or other drugs were continued or reintroduced after resolution of thrombocytopenia, and other causes for thrombocytopenia were excluded; and (4) reexposure to the candidate drug resulted in recurrent thrombocytopenia. Flow cytometry testing for DDAbs can be useful in confirmation of a clinical diagnosis, and monoclonal antibody enzyme-linked immunosorbent assay testing can be used to determine the platelet glycoprotein target(s), usually GPIIb/IIIa or GPIb/IX/V, but testing is not widely available. Several pathogenic mechanisms for DIIT have been proposed, including hapten, autoantibody, neoepitope, drug-specific, and quinine-type drug mechanisms. A recent proposal suggests weakly reactive platelet autoantibodies that develop greatly increased affinity for platelet glycoprotein epitopes through bridging interactions facilitated by the drug is a possible mechanism for the formation and reactivity of quinine-type drug antibodies.

**Incidence and Drugs Most Frequently Implicated**

The exact frequency of DIIT is not known, but the overall incidence is estimated to be 10 cases per million population per year. Higher incidence is reported in specific patient populations, including 25 percent of critically ill patients, the elderly, and patients taking the drugs quinine and sulfamethoxazole-trimethoprim who were reported to be at higher risk of developing DIIT—38 and 26 cases per million exposed, respectively. Thrombocytopenia associated with the GPIIb/IIIa inhibitors, abciximab, eptifibatide, and tirofiban occurs in approximately 1 to 2 percent of patients exposed to these drugs and as many as 10 percent of patients exposed to abciximab for the second time.

Recently, several groups have mined data from available sources to determine those drugs most frequently associated with thrombocytopenia.
with DIIT. Reese et al.11 used published case reports, reference laboratory testing of patients’ sera for DDAs over a 13-year period, and data mining of 41 years’ worth of the U.S. Food and Drug Administration’s Adverse Event Reporting System (AERS) to identify those drugs most frequently implicated in causing DIIT. They identified 1468 drugs suspected of causing thrombocytopenia, and 102 of these were evaluated by all three methods. Only 23 of the 102 drugs demonstrated a causal association with thrombocytopenia by all three methods (Table 1). In a separate analysis focused on children (<18 years old) of the same published case reports and DDAb test data over a 5-year period (2008–2012), 34 drugs showed a causal association with thrombocytopenia (Table 1).12 Garbe et al.14 used active surveillance of patients in 50 German hospitals (Berlin Case-Control Surveillance Study) to identify drugs that might cause DIIT. They identified 90 cases of acute immune thrombocytopenia, in which a drug was possibly associated. A total of 85 different drugs were involved, and 31 drugs were determined to be definitely (n = 7) or probably (n = 24) related to the patients’ thrombocytopenia. Tirofiban, abciximab, sulfamethoxazole-trimethoprim, and flu vaccine were the most frequently implicated drugs. Arnold et al.13 analyzed data from the same case reports database used by Reese et al.,11 combined with a search of MEDLINE and EMBASE, of more than 72 years of DIIT case reports for only those who had been tested for DDAb by two or more laboratories. Using these criteria and four independent assessors, only 16 drugs met the criteria for a definite association with DIIT (Table 1).

Analysis of the most current data (2012) from our reference laboratory (Platelet and Neutrophil Immunology Lab, BloodCenter of Wisconsin, Milwaukee, WI) of all serum tests for DDAs from patients with suspected DIIT showed that 101 different drugs were tested during this period (Table 2). Vancomycin (n = 127), piperacillin-tazobactam (n = 34), sulfamethoxazole-trimethoprim (n = 43), cefepime (n = 24), and ceftriaxone (n = 15) were the five most frequently tested drugs, and there were 18 drugs for which DDAs against

Table 1. Drugs frequently associated with drug-induced immune thrombocytopenia from literature sources

<table>
<thead>
<tr>
<th>Reese et al.11</th>
<th>Arnold et al.13</th>
<th>Reese et al.13</th>
<th>Garbe et al.14</th>
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<tr>
<td></td>
<td></td>
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<td>trimethoprim/sulfamethoxazole</td>
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*Heparin was excluded from the other reports because its pathogenic mechanism differs significantly from nonheparin drugs.
platelets were detected using a flow cytometry assay (Table 2). Carbamazepine, etoposide, ibuprofen, quinidine, quinine, oxaliplatin, rifampin, sulfamethoxazole, trimethoprim, and vancomycin are all represented in data from both Tables 1 and 2, suggesting that these 10 drugs are the most frequently associated with DIIT caused by DDABs. Although the drugs listed in Tables 1 and 2 are the most frequently reported, it should be pointed out that there are numerous other drugs and even herbal remedies,\textsuperscript{15–17} foods,\textsuperscript{17–20} and beverages\textsuperscript{21,22} that have been implicated in DIIT. Illicit drug use has also been associated with DIIT. Cocaine and heroin are sometimes cut or mixed with drugs known to cause DIIT, like quinine\textsuperscript{23,24} or levamisole.\textsuperscript{25} Testing for DDABs can be helpful in making a diagnosis in this setting and should include testing with quinine to determine whether it has been added as an adulterant.

The rates of thrombocytopenia observed with chemotherapeutic agents are quite high, ranging from 21 to 70 percent.\textsuperscript{26,27} Bone marrow suppression is the major cause of thrombocytopenia associated with this class of drugs.\textsuperscript{28} However, DIIT has been reported in a number of cases involving various chemotherapeutic compounds.\textsuperscript{29–32} Oxaliplatin, a widely used platinum compound for the treatment of colorectal cancer, has been implicated in an increasing number of DIIT cases.\textsuperscript{26,29–32–34}

The incidence data just described are useful in identifying drugs that are the top candidates for consideration in a thrombocytopenic patient with compatible temporal association and could be useful in hastening diagnosis and treatment. Clearly, good medical practice requires that the clinical criteria established for DIIT, together with laboratory confirmation, be applied to all patients suspected of DIIT, including exclusion or inclusion of all drugs to which the patient has been recently exposed. Another issue worth mentioning about the identification of drugs causing DIIT is that to have good available data, physicians, pharmacists, testing laboratories, and investigators must regularly report identified cases of DIIT. Unfortunately, when an accurate diagnosis is made, the case is often not reported to national databases (e.g., AERS) or is not published in the medical literature. It is my opinion that this is partly the result of the increasing difficulty clinicians and investigators face trying to publish a single clinical case report of a novel finding such as DIIT because of the reluctance of scientific journal editors. This trend continues at the risk that investigators will no longer bother to submit such cases, and this important information will be lost.

**Clinical Features, Diagnosis, and Treatment**

Patients with DIIT typically present with petechiae, bruising, and epistaxis caused by an acute, often-severe drop in platelet count. When thrombocytopenia is severe (i.e., ≤20,000 platelets/µL), mucosal bleeding can occur

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**Table 2. Drugs (n = 101) tested for drug-dependent platelet antibodies in patient sera 2011–12**

<table>
<thead>
<tr>
<th>acetaminophen</th>
<th>clopidogrel</th>
<th>ketorolac</th>
<th>phenytoin (2)‡</th>
</tr>
</thead>
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<tr>
<td>albuterol</td>
<td>cyclosporine</td>
<td>lanosaprazole</td>
<td>piperacillin-tazobactam (34)† (5 piperacillin)</td>
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<td>digoxin</td>
<td>levetiracetam</td>
<td>pravastatin</td>
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<td>linezolid</td>
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<td>lisinopril</td>
<td>quinidine</td>
</tr>
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<td>argatroban</td>
<td>epitiabide (2)‡</td>
<td>loratadine</td>
<td>quinine (1)‡</td>
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<td>fluoxetine</td>
<td>minirone</td>
<td>spironolactone (3)‡</td>
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<tr>
<td>cefepine (24)†, (1)‡</td>
<td>furosemide (1)‡</td>
<td>nafcillin (1)‡</td>
<td>sulfamethoxazole-trimethoprim (22)†‡ (11/22</td>
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<td>gabapentin</td>
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<td>phenobarbital (2)‡</td>
<td>voriconazole</td>
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\*Drugs (n = 101) for which sera from patients with suspicion of drug-induced immune thrombocytopenia were tested in flow cytometry assay by Platelet & Neutrophil Immunology Lab, BloodCenter of Wisconsin, Milwaukee.

\†Five most frequently tested drugs (number of patient samples tested).

\‡Drugs (n = 18) for which drug-dependent antibodies (DDAbs) were detected (number of DDAbs detected).
in the gastrointestinal or genitourinary tracts (so-called wet purpura). In extreme cases, intracranial or pulmonary hemorrhage resulting in fatalities has been reported. Platelet counts usually fall rapidly from normal or near-normal levels to nadirs in the single digits within several hours of a repeat drug exposure. For patients receiving a drug for the first time, 5 to 7 days of exposure is usually required for sensitization. Patients previously sensitized can experience rapid drops in platelet counts within 1 to 2 hours of repeat exposure. DIIT is a diagnosis of exclusion, i.e., the exclusion of the many other causes of thrombocytopenia. Therefore, the diagnosis is complex and is often mistaken for other immune platelet disorders such as immune thrombocytopenia (ITP), posttransfusion purpura (PTP), or platelet transfusion refractoriness (PTR), especially for patients receiving multiple medications.

ITP usually presents as isolated thrombocytopenia most commonly occurring in response to an unknown stimulus and is caused by nondrug autoantibodies that bind and destroy autologous platelets. ITP can also develop secondarily to other autoimmune disorders, viral infections, and certain drugs (e.g., procainamide and gold salts); however, in this form of DIIT, true autoantibodies develop, and also do not require the presence of drug for their detection in laboratory tests. Distinguishing DIIT from ITP can be quite challenging; however, it is important to do so to prevent patients with DIIT from undergoing unnecessary, invasive treatments.

PTP is a rare disorder, occurring approximately 5 to 10 days after a blood transfusion, in which there is an acute, severe drop in platelet count very similar in timing and severity of the thrombocytopenia that occurs in DIIT. PTP is distinguishable from DIIT by its association with a blood transfusion rather than medication, but this distinction can be more difficult when a patient receives both. Another difference is that platelet-specific antibodies, most often targeting human platelet alloantigen (HPA)-1a, are often detected in the sera of patients with PTP, whereas DD Abs specific for the suspected drug, and not HPA antibodies, are detected in DIIT.

PTR develops most often in oncology patients, and occurs when posttransfusion platelet increments do not achieve expected levels despite multiple platelet transfusions. Although the majority of PTR is attributable to nonimmune causes (e.g., bone marrow suppression, fever, sepsis, etc.), immune forms caused by class I HLA antibodies and, rarely, platelet-specific antibodies also occur. Distinguishing thrombocytopenia caused by DIIT from that caused by PTR can be particularly challenging. A major complicating factor is that many patients at risk of developing PTR also receive drugs reported to have caused DIIT, so a detailed review of platelet counts and timing of drug exposures is critical in this setting. Testing of a patient’s serum for HLA and platelet-specific antibodies can be helpful in identifying possible immune causes of PTR. However, discontinuing candidate drugs, when possible, with careful observation for a rise in platelet count over the course of 3 to 4 days may be required in exceptionally difficult cases.

Clinical criteria that have been established to implicate a suspected drug include the following:

1. Exposure to the candidate drug preceded thrombocytopenia.
2. Recovery from thrombocytopenia was complete and sustained after discontinuing candidate drug.
3. Candidate drug was the only drug used before the onset of thrombocytopenia, or other drugs were continued or reintroduced after discontinuation of the candidate drug with a sustained normal platelet count.
4. Other causes for thrombocytopenia were excluded.
5. Reexposure to the candidate drug resulted in recurrent thrombocytopenia.

Evidence is considered Definite if criteria 1, 2, 3, and 4 are met; Probable if criteria 1, 2, and 3 are met; Possible if criterion 1 only is met; and Unlikely if criterion 1 is not met.

For patients receiving chemotherapy who exhibit thrombocytopenia, it is common to attribute this complication to suppression of megakaryocytopoiesis, a recognized side effect of these classes of drugs. However, as mentioned previously, DIIT has been reported to develop from exposure to these drugs. Accordingly, it is important to consider DIIT in patients undergoing chemotherapy who have a sudden, isolated drop in platelet levels.

Once a clinical diagnosis of DIIT is established, treatment consists of immediate withdrawal of the implicated drug, and platelet counts should be monitored until they reach normal, sustained levels. Patients with severe thrombocytopenia or hemorrhage may require platelet transfusions. Treatment with corticosteroids has not been shown to be an effective treatment in DIIT. Platelet levels typically begin to recover in 3 to 4 days, as the drug is cleared from the circulation, and attain normal levels by 5 to 7 days after stoppage of the offending drug. It is possible for drug clearance and resolution of thrombocytopenia to be prolonged in patients with renal impairment.
Laboratory Testing for Drug-Dependent Platelet Antibodies

Detection of serum antibodies that react with normal platelets only in the presence of the suspected drug is helpful in confirming a clinical diagnosis of DIIT. Several different methods have been used during the years for detection of DDAbs, but use of flow cytometry with intact platelets is one of the most sensitive assays currently in use. A typical assay involves incubation of serum with group O platelets and buffer or drug in the well of a microtiter plate, followed by washing of the platelets to remove unbound immunoglobulins. The test well incubated with buffer is washed in buffer, and the well with drug is washed with buffer containing drug, usually at the therapeutic concentration. Platelets are then incubated with a combination of fluorescent-labeled anti-immunoglobulin reagents: fluorescein-isothiocyanate–labeled anti-human immunoglobulin (Ig) G and phycoerythrin-labeled anti-human IgM. Fluorescent events are acquired on a flow cytometer with simple forward scatter and side-scatter gates set to identify platelets. Stronger median fluorescence intensity values obtained with samples in the presence of drug compared with those without drug are considered positive for DDAbs (Fig. 1). Sera from normal, healthy individuals previously tested and shown not to have DDAbs and sera from patients previously shown to have drug-dependent antibodies should be tested in each assay as important negative and positive controls, respectively. Although this testing is available in the United States, unfortunately, it is not more widely available because it requires an experienced laboratory with access to sufficient rare control samples and a large inventory of various pharmaceuticals. There has been a recent call to create broader access to laboratory testing.

Several issues encountered with flow cytometry testing for DDAbs include insolubility of some drugs, non-DDAbs present in patient sera, and the fact that some DDAbs recognize only drug metabolites. Because most drugs have affinity for albumin in circulation, drugs that are poorly soluble in buffer can often be adequately solubilized in a buffer containing 5 percent bovine serum albumin. Non-DDAbs, e.g., autoantibodies and class I HLA antibodies, are easily identified because they bind to platelets in both the presence and absence of drug, and strong non-DDAbs can mask the presence of a DDAb in testing. Absorption of sera with platelets in the absence of drug before testing will remove non-DDAbs and reveal DDAbs present. DDAbs are encountered that show reactivity only with metabolites of the native drug. In these cases, metabolites can sometimes be obtained from pharmaceutical companies or other commercial sources, and as a last resort, urine collected from someone having taken the drug for several days can be

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**Fig. 1** Fluorescence histograms from immunofluorescence detection of IgG drug-dependent platelet antibodies by flow cytometry assay. Normal isolated platelets were incubated with sera and washed, and platelet-bound antibodies were detected with fluorescent anti-human IgG. 

(A) Normal serum incubated with platelets in the presence of either sulfamethoxazole (black histograms) or buffer (white histograms) shows low IgG fluorescence, indicating no antibodies are present. 

(B) Serum from a patient with suspected drug-induced thrombocytopenia after exposure to sulfamethoxazole-trimethoprim incubated with platelets showing high IgG fluorescence (MFI = 453.2) only with sulfamethoxazole, indicating the presence of sulfamethoxazole drug-dependent antibodies. Median fluorescence intensity (MFI) values are shown for each histogram.
used as a source of metabolites for testing after adjusting the pH and salt concentration.

Enzyme-linked immunosorbent assays (ELISAs) like the modified antigen-capture ELISA (MACE)\(^{43}\) and immunoblotting procedures\(^{48–50}\) are primarily used to identify the platelet GPs that DDABs target. In the MACE, platelet sensitization with patient's serum and drug is the same as that just described for flow cytometry. Sensitized platelets are then lysed in detergent, the platelet GP–drug–antibody complexes are captured in a microtiter plate well with specific monoclonal antibodies, and DDABs are detected with enzyme-labeled anti-human IgG reagents. The majority of DDABs bind to GPIIb/IIIa,\(^{29,42,43,51}\) the major fibrinogen receptor, or GPIb/IX/V\(^{52,53}\) a receptor for von Willebrand factor on platelets. There has been a single report of a carbimazole-dependent platelet antibody targeting platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31).\(^{54}\) Flow cytometry assays using platelets from patients with the platelet function disorders Glanzmann thrombasthenia (platelets lack GPIIb/IIIa) and Bernard-Soulier syndrome (platelets lack GPIb/IXV) have also been used successfully to identify the platelet GP targets of DDABs.\(^{29,55}\)

An in vivo mouse model was recently developed in which normal human platelets and human serum containing strong quinine-dependent platelet antibodies are transfused into nonobese diabetic/severe combined immunodeficient (NOD/scid) mice and allowed to circulate. Rapid clearance of platelets occurred after intraperitoneal injection of quinine.\(^{56}\)

This model might be useful in detecting low-affinity DDABs that wash off platelets and go undetected in conventional laboratory tests or those induced by drug metabolites, which the authors showed could be produced by the mice after intraperitoneal injection of the native drug.\(^{56}\)

The clinical sensitivity and specificity of the different assays is unknown because it is extremely difficult to obtain the large numbers of samples that would be required both from patients with a clinical diagnosis of DIIT and from patients taking the drugs who did not exhibit thrombocytopenia. In a single study of 59 patients exposed to vancomycin, 29 of 31 who met the criteria for vancomycin-induced thrombocytopenia tested positive for vancomycin-dependent platelet antibodies by flow cytometry, and all 25 who did not develop thrombocytopenia gave negative results. In addition, 10 samples from patients not exposed to vancomycin but with quinine-dependent antibodies tested negative for vancomycin DDABs, and only 1 of 451 sera from normal healthy subjects never exposed to vancomycin had weak IgM-positive reactivity.\(^{51}\) Altogether, these results indicate that the flow cytometry assay had high sensitivity (93.5%) and specificity (99.8%) for detection of DDABs. It has been suggested that tests for DDABs are not practical for use in clinical decision making because the testing is available in only a handful of laboratories in the world, DDABs are not always detected, and there are claims of suboptimal test turnaround times. However, it certainly can be argued that for cases with a difficult diagnosis that knowing the results of DDAB testing, even within 2 to 3 days, is probably more clinically useful than continuing to expose a patient with undiagnosed DIIT to the offending drug.

Pathogenic Mechanisms

DDABs that are the cause of DIIT are exquisitely unique in their ability to bind to specific platelet membrane GPs only in the presence of the drug that induced them. In fact, the drug is so critical to the reactivity of these antibodies that, during the performance of DDAB assays, they will easily and rapidly wash off the platelets if drug is not present throughout testing. Several mechanisms have been proposed to explain the formation of these most interesting antibodies (Fig. 2).\(^{6,57–60}\)

Hapten Mechanism

Haptens are low-molecular-weight (usually <5000 daltons) molecules that are not capable of eliciting an immune response unless they are coupled to a larger carrier protein. Drugs such as penicillin and some cephalosporin drugs when covalently linked to cell surface proteins can elicit drug-specific antibodies. This mechanism is well described for DDABs targeting red blood cells that cause immune hemolytic anemia.\(^{61}\) Although it is a potential mechanism to explain DIIT, attempts in our laboratory to detect DDABs with drug-coated platelets have not been successful to date, and there are no known reports in which hapten-induced antibodies have been detected in DIIT.\(^{1,59}\)

Autoantibody Mechanism

There are reports of patients who exhibited acute, severe thrombocytopenia after exposure to specific drugs, but no DDABs can be detected in their serum. However, autoantibodies that are not dependent on drug can be detected in these patients' sera, suggesting that the drug induced a true autoantibody, but this is very difficult to prove.\(^{62}\)

Neoepitope Mechanism

The most popular mechanism espoused for DDAB formation is the neoepitope mechanism (Fig. 2). It is well established that most drugs in circulation make low-affinity
Drug-induced immune thrombocytopenia

Fig. 2 Proposed mechanisms for drug-induced antibody formation and platelet destruction. (1) Hapten-dependent antibody: Covalent binding of drug/hapten to platelet membrane protein(s) induces antibody specific to drug/hapten. (2) Autoantibody: Drug induces formation of a true platelet autoantibody. (3) Fiban-type drug-dependent antibody: Drug binding to GPIIb/IIIa induces neoepitopes that are recognized by drug-dependent antibody. (4) Drug-specific antibody: Antibody recognizes mouse components of mouse/human chimeric Fab fragments of drug bound to platelet GPIIb/IIIa. (5) Quinine-type drug-dependent antibody: Drug and platelet glycoprotein together form a combinatorial epitope recognized by drug-dependent antibody. (6) Quinine-type drug-dependent antibody: Drug induces antibody that binds more tightly to platelet glycoprotein in the presence of a soluble drug. Figure created by Rachel Kubsh.
interactions with many different proteins in the plasma, especially albumin, but also probably platelet GPs, e.g., GPIIb/IIIa. These interactions are speculated to induce conformational changes in the protein structures that elicit DDAsbs in certain individuals. However, when drug is cleared from the circulation and the conformation of the platelet protein reverts to its native state, DDAsbs, although still present, can no longer bind and destroy the platelets (Fig. 2). It is proposed that the platelet GPIIb/IIIa inhibitors, tirofiban and eptifibatide, small-molecule fibrinogen receptor antagonists (fibans), induce DDAsbs by this mechanism. Fibans are a widely used class of drugs capable of preventing restenosis in patients undergoing percutaneous transluminal coronary angioplasty (PTCA) procedures. Interestingly, an unusual aspect of fiban-dependent antibodies is that they can occur naturally in approximately 2 percent of normal people.

**Drug-Specific Mechanism**

Abciximab (Reopro) is a chimeric (mouse-human) monoclonal antibody Fab fragment specific for GPIIa used primarily in PTCA procedures to prevent thrombus formation. Like the previously mentioned fibans, it blocks interactions of fibrinogen with GPIb/IIIa to prevent platelet aggregation. Approximately 1 to 2 percent of patients receiving abciximab exhibit acute thrombocytopenia within a few hours of receiving the drug. Although abciximab binds tightly to platelets, it does not cause thrombocytopenia by macrophage clearance in the RES because it lacks the immunoglobulin Fe; rather, affected patients make drug-specific antibodies that appear to recognize murine sequences in the complimentary determining region 3 (CDR3) region of abciximab (Fig. 2). Like DIIT induced by fibans, some patients affected with abciximab-induced immune thrombocytopenia also have preexisting antibodies in their serum. Many normal, healthy people never exposed to abciximab also have IgG antibodies that react with abciximab-coated platelets; however, these antibodies recognize the cleaved end of the Fab fragment that is created when the drug is treated with papain. It is unknown whether Fab-reactive antibodies seen in healthy people can cause DIIT should these individuals ever be exposed to the drug.

**Quinine-Type Drug Mechanism**

The classic DDAsbs induced by drugs such as quinine, quinidine, sulfamethoxazole, and vancomycin attach tightly to platelets only in the presence of the sensitizing drug, and most often target GPIIb/IIIa or GPIb/IX/V. These antibodies bind their epitopes by their Fab domains, indicating that their reactivity with platelets is not the result of drug–antibody immune complexes engaging platelet Fc receptors, as is the case with heparin-PF4 antibodies that cause HIT. Early mechanisms proposed to explain platelet destruction by quinine-type DDAsbs were (1) the previously described hapten mechanism; however, quinine-type antibodies still react with platelets in the presence of large concentrations of soluble drug that should block their binding if they were produced by this mechanism; (2) drug-induced conformational changes in the GPs that are targeted by DDAsbs; and (3) compound epitopes consisting of both drug and platelet protein form that are recognized by antibodies. However, none of these proposed mechanisms have been proven.

The mechanisms by which quinine-type DDAsbs form and react with platelets have been intensely studied in the laboratory of Dr. Richard Aster at the BloodCenter of Wisconsin in Milwaukee. Using chimeric human/rat GPIIia and targeted mutants of human GPIIia, they identified a 17–amino acid (aa 50–66) stretch spanning the hybrid and plexin-semaphorin-integrin (PSI) domains of GPIIia that was recognized by all three antibodies only in the presence of quinine. Substitution in human GPIIIia of only three amino acids (Ala50, Arg62, or Asp66) abolished DDAb reactivity, as did reduction of wild-type GPIIIia with 2-mercaptoethanol, indicating that disulfide linkages are also important in formation of this epitope. Burgess et al. previously identified a peptide sequence (aa 282–293) in GPIba that was also required for reactivity of a single quinine DDAb, and studies by Asvadi et al. demonstrated that amino acid residues Arg110 and Gln115 in GPIX were critical for binding of six quinine DDAsbs.

Later studies again by the Aster group showed that 14 quinine DDAsbs, including the three originally studied, all recognized a small 29-kD recombinant fragment of GPIIia that included the three amino acids (Ala50, Arg62, or Asp66) previously shown to be critical for DDAb binding. Additional studies with blocking monoclonal antibodies and overlapping mutant GPIIia constructs showed that as many as eight different overlapping epitopes were recognized by the 14 different quinine DDAsbs, leading this group to propose that DDAsbs induced by quinine, and presumably other drugs, do not recognize a single distinct epitope on a GP. This result further indicates that mechanisms proposing DDAsbs recognize drug-induced conformational changes elsewhere in platelet membrane GPs are not viable explanations for DDAb formation.

In other studies, the Aster lab found both the classic drug-dependent platelet-reactive antibodies and drug-specific antibodies in plasma from 6 of 7 patients who exhibited DIIT after exposure to quinine. DDAsbs reacted with GPIIib/IIIa...
or GPIb/IX/V on platelets only in the presence of quinine. Drug-specific antibodies bound directly to quinine that was covalently linked to human serum albumin, and this reactivity was inhibited by excess soluble quinine. Despite recognizing different targets, both types of antibodies were identical in their drug specificity, and this suggests that both are produced against a similar antigen as part of the same B-cell immune response. Based on these findings, the authors proposed a novel model in which patients have autoantibodies with only weak reactivity for platelet GP epitopes in the absence of drug, but that this reactivity is significantly enhanced through bridging interactions by drug binding between both the protein and antibody; the resultant sandwich entraps a drug at the antigen–antibody interface and greatly increases antibody binding energy for the GP autoepitope (Fig. 2).5,8,9 Two quinine-dependent mouse monoclonal antibodies recently developed by Aster’s group with specificity for epitopes on human GPIIb will be valuable tools to help further elucidate the complex mechanisms responsible for DIIT.70

Summary

DIIT is a relatively uncommon, but often-undiagnosed, disorder that can occur after exposure to numerous drugs. Several recent large analyses of data from multiple sources indicate that the drugs carbamazepine, eptifibatide, ibuprofen, quinidine, quinine, oxaliplatin, rifampin, sulfamethoxazole, trimethoprim, and vancomycin are most often implicated. Diagnosis is complex and involves excluding other causes of thrombocytopenia and ascertainment of the patient’s complete medication history. Laboratory testing of patient’s serum for DDAbs can be very useful in confirming a clinical diagnosis and, with broader access, will be used more often. The hallmark of treatment is immediate cessation of the offending drug(s) and platelet transfusions for patients with severe bleeding symptoms. DD Abs usually target GPIIb/IIIa and GPIb/IX/V on the platelet membrane surface. Several mechanisms have been proposed to explain the development and reactivity of DD Abs, and a recent hypothesis suggests that many are weakly reactive platelet autoantibodies that develop greatly increased affinity for platelet GP epitopes through bridging interactions facilitated by the drug. Data obtained from studies using newly developed drug-dependent monoclonal antibodies and mouse models of DIIT should yield important new knowledge about this fascinating disorder.

Acknowledgments

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References


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Drugs that have been shown to cause drug-induced immune hemolytic anemia or positive direct antiglobulin tests: some interesting findings since 2007

G. Garratty and P.A. Arndt

This review updates new findings in drug-induced immune-hemolytic anemia (DIIHA) since the 2007 review in *Immunohematology* by these authors. Twelve additional drugs have been added to the three tables listing drugs associated with drug-dependent antibodies, drugs associated with drug-independent antibodies, and drugs associated with nonimmunologic protein adsorption. Other updated findings include (1) piperacillin is currently the most commonly encountered cause of DIIHA, (2) new data on blood group specificity of drug-dependent antibodies, (3) drug-dependent antibodies detected in healthy donors, (4) DIIHA associated with transplantation, and (5) DIIHA associated with chemotherapeutic drugs. *Immunohematology 2014;30:66–79.*

**Key Words:** drugs, immune hemolytic anemia, autoantibody, drug-dependent antibody, nonimmunologic protein adsorption

Dr. Garratty has published five previous reviews on drug-induced immune hemolytic anemia (DIIHA) in *Immunohematology;* the last one in 2007 was coauthored by Pat Arndt.1–5 This issue is devoted to drug-induced immune cytopenias (red blood cells [RBCs], platelets, and white blood cells [WBCs]). If one reads the DIIHA reviews previously published in *Immunohematology,* you will get a view of how many drugs were found to be responsible over each 5-year period (1985–2007), which ones were the most common causes of DIIHA, and what concepts were most commonly suggested to explain various types of DIIHA. The reviews by Arndt6 and Leger et al.7 in this issue cover many of these areas in great detail and add new data that were not fully covered in the previous *Immunohematology* reviews. Some other recent reviews by Garratty can update you further.8–12 For readers who do not have access to these publications,8–12 we will summarize the most important information that has emerged in the last 10 years, with emphasis on the period since 2007.

**Drugs Causing DIIHA**

In our 2007 review, we included tables containing 125 drugs that we believed had reasonable evidence of causing DIIHA.6 Indisputable evidence would require the following findings:

1. A well-defined hemolytic anemia (HA).
2. A temporal relationship to drug therapy and the start of the HA.
3. A positive direct antiglobulin test (DAT) after drug therapy (preferably with a negative DAT result preceding therapy).
4. Results of testing for drug antibodies (e.g., using drug-treated RBCs or testing serum in the presence of soluble drug7,8). These results must be accompanied by pertinent controls.7,8 Laboratory tests cannot prove that autoantibodies are drug-induced, but it is important to show that apparent autoantibodies are truly autoantibodies and not drug-dependent antibodies that are reacting without the in vitro addition of drug because enough drug is present in the patient’s plasma or serum.5–7,13
5. Patient responds hematologically after drug cessation. Unfortunately, this is not easy to define, as patients are often simultaneously given steroids.

Since 2007, we have added seven new drugs to Table 1 (drug-dependent antibodies): hydrocortisone (2008), pemetrexed (2008), cimetidine (2010), etoricoxib (2013), iomeprol (2013), puerarin (2013), and vincristine (2013). Of these 115 drugs, five (carbromal, cefpirome, furosemide, methadone, and norfloxacin) may have caused positive DATs but no HA. Twenty-seven (23%) drugs were associated with drug antibodies reacting with drug-treated RBCs; 44 (38%) drugs were associated with antibodies reacting in the presence of soluble drug; 41 (36%) drugs were associated...
with antibodies detected by both methods. Fifty (43%) drugs were associated with in vitro reactions against untreated cells without any drug being added. These could be associated with drug-independent antibodies, but we believe that many may have been caused by drug or drug complexes being present in the patient’s plasma or serum (see footnote in Table 1). It will probably be no surprise that not all of the drugs listed in Table 1 satisfy 100% of our criteria for perfection. Hence, we can only say that there is reasonable evidence that these drugs were the cause of DIIHA.

We added four new drugs to Table 2 that appeared to cause autoimmune hemolytic anemia (drug-independent antibodies): rituximab (2003), alemtuzumab (2007), weidean (2009), and bendamustine (2013). All but one of the drugs in Table 2 (chaparral) may have caused a DAT-positive autoimmune HA (AIHA). It should be noted that 16 of 21 drugs in this group were classified as needing more evidence to support that they belong in this group. One drug (cephaloridine) was added to the nonimmunologic protein adsorption group (Table 3); this was observed in 1967 but was unfortunately left off our 2007 Table 3.

**Classification of Drug Antibodies**

There are two main types of drug antibodies: drug-dependent antibodies and drug-independent antibodies.

Drug-dependent antibodies need the drug to be present for in vitro reactions; the drug can be bound to test RBCs or soluble drug can be added to patient’s plasma or serum (source of antibody) and allogeneic group O RBCs; after 37°C incubation, the tests are inspected for lysis, direct agglutination, and by the indirect antiglobulin test (IAT). Enzyme-treated RBCs should be used in addition to untreated RBCs. Test results are only valid when appropriate controls are used (see Leger et al. for complete details). Salama’s group does not advise using enzyme-treated RBCs because of problems with nonspecific reactions, but we believe these can usually be overcome with experience and pertinent controls.

Drug-independent antibodies react without any drug being added in vitro. The results are indistinguishable from RBC autoantibodies. It is thought that such drugs affect the immune system so that true autoantibodies and sometimes AIHA are produced. There are no special in vitro tests to define that a particular drug caused the AIHA. The diagnosis rests on the physician stopping the drug and finding that the anemia resolves, but the serology (e.g., DAT) can remain positive for some time longer.

**Positive DATs and DIIHA Caused by Nonimmune Protein Adsorption**

Drug-induced positive antiglobulin tests (e.g., DATs) and sometimes HA can occur with no drug antibodies involved, and yet RBCs are destroyed by macrophages in the spleen and liver. Thus, the drug interaction with the RBCs does not involve drug-induced antibodies, but the shortened life of the RBCs is by a cellular immune mechanism. The drugs that can be involved in this mechanism are listed in Table 3. These drugs can change the RBC membrane so that proteins (e.g., immunoglobulins, complement, albumin, etc.) attach to the RBCs, leading to positive antiglobulin tests. This may involve the patient’s RBCs in vivo (positive DAT), or may occur in vitro (positive IAT) when the patient’s serum or plasma is incubated with drug-coated RBCs (e.g., when testing for drug antibodies). This phenomenon was first observed with the first-generation cephalosporin, cephalothin, and was later found to occur with other cephalosporins (e.g., cefotetan). It was termed the “membrane modification mechanism” or “nonimmunological protein adsorption (NIPA).” It was thought for many years to be an interesting phenomenon that could interfere with interpretation of laboratory tests but had no clinical significance; then β-lactamase inhibitors, which cause NIPA, were shown to possibly cause DIIHA. Other drugs (see Table 3) can also cause NIPA and DIIHA (e.g., cisplatin and oxaliplatin). The involvement of NIPA is sometimes hard to define, as drug antibodies (e.g., anti-oxaliplatin) may also be involved. The nonimmunologically adsorbed immunoglobulin (Ig) G (and possibly C3) can react with receptors on macrophages, even though it is not an antibody to the drug or the RBCs. The most useful test to indicate that NIPA is occurring is to show that albumin is present on the RBCs by testing with anti-human albumin by the antiglobulin test. Such antisera have to be standardized carefully in-house, as no U.S. Food and Drug Administration–licensed reagent is available.

**New Findings Since Our Last Review in Immunohematology (2007)**

In the period covered by our 2007 review (2005–2007), the most common drugs to cause DIIHA in patients’ samples submitted to our laboratory were cefotetan, ceftriaxone, and piperacillin, in that order. For the period 2008–2013, piperacillin, rose to number 1, followed by cefotetan and ceftriaxone (Table 4). We are starting to see a small increase in DIIHA caused by drugs in the platinum family.
<table>
<thead>
<tr>
<th>Drug (alternative name)</th>
<th>Reference</th>
<th>Therapeutic category</th>
<th>Number of references (single [year] vs. multiple [&lt;5, &lt;10, or ≥10])</th>
<th>HA</th>
<th>Positive DAT</th>
<th>Method detecting serum antibody</th>
<th>Reactive without drug added in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen (Paracetamol)</td>
<td>15,16</td>
<td>NSAID</td>
<td>Multiple (&lt;10)</td>
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<td>✓</td>
<td>Serum + drug + RBCs</td>
<td>✓</td>
</tr>
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<td>Acyclovir</td>
<td>17</td>
<td>Antiviral</td>
<td>Single (2003)</td>
<td>✓</td>
<td>✓</td>
<td>Not reported</td>
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</tr>
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<td>Aminopyrine</td>
<td>18</td>
<td>NSAID</td>
<td>Single (1981)</td>
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<td>✓</td>
<td></td>
<td>✓</td>
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<td>Antimicrobial</td>
<td>Multiple (&lt;5)</td>
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<td>✓</td>
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<td>✓</td>
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<td>Antimicrobial</td>
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<td></td>
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<td>Azapropazone (Apazone)</td>
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<td>✓</td>
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<td>✓</td>
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<td>Single (1984)</td>
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<td>✓</td>
<td></td>
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<td>26</td>
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<td>✓</td>
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<td>Carboplatin†</td>
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<td>✓</td>
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<td>Carbromal</td>
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<td>Sedative, hypnotic</td>
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<td>✓</td>
<td></td>
<td>✓</td>
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<td>✓</td>
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<td>✓</td>
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<td>Antimicrobial</td>
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<td>✓</td>
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<td>Multiple (≥10)</td>
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<td>✓</td>
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<td>Antimicrobial</td>
<td>Multiple (&lt;10)</td>
<td>✓</td>
<td>✓</td>
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<td>✓</td>
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<tr>
<td>Cefpirome</td>
<td>40</td>
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<td>Single (2005)</td>
<td>✓</td>
<td>✓</td>
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<td>Ceftizoxime</td>
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<td>Multiple (&lt;5)</td>
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<td>✓</td>
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<tr>
<td>Ceftriaxone†</td>
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<td>Antimicrobial</td>
<td>Multiple (≥10)</td>
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<td>✓</td>
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<td>Cefuroxime</td>
<td>45</td>
<td>Antibacterial</td>
<td>Multiple (&lt;5)</td>
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<td>Cephalexin</td>
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<td>Antimicrobial</td>
<td>Multiple (5)</td>
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<td>✓</td>
<td></td>
<td>✓</td>
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<td>Cephalothin†</td>
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<td>Antimicrobial</td>
<td>Multiple (≥10)</td>
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<td>✓</td>
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<td>✓</td>
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<tr>
<td>Chloramphenicol</td>
<td>50</td>
<td>Antibacterial</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Chlorinated hydrocarbons</td>
<td>51</td>
<td>Insecticides</td>
<td>Multiple (&lt;10)</td>
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<td>✓</td>
<td></td>
<td>✓</td>
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<td>Antiemetic, antipsychotic</td>
<td>Multiple (&lt;10)</td>
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<td>✓</td>
<td></td>
<td>✓</td>
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<tr>
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<td>Antidiabetic</td>
<td>Multiple (&lt;10)</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
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<td>Cimetidine†</td>
<td>55</td>
<td>Antulcerative</td>
<td>Single (2010)</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
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<td>Ciprofloxacin</td>
<td>56</td>
<td>Antibacterial</td>
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<td>✓</td>
<td>✓</td>
<td></td>
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</tr>
<tr>
<td>Cisplatin (Cisdiamino-dichloroplatinum)</td>
<td>57,58</td>
<td>Antineoplastic</td>
<td>Multiple (&lt;10)</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
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<tr>
<td>Cloxacillin</td>
<td>59</td>
<td>Antibacterial</td>
<td>Single (1980)</td>
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<td>✓</td>
<td></td>
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<tr>
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<td>60</td>
<td>Gonad-stimulating principle</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td></td>
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</tbody>
</table>

Table 1. Drugs associated with cases of IHA or positive DAT, or both, in which drug-dependent antibodies were detected*
Table 1. Drugs associated with cases of IHA or positive DAT, or both, in which drug-dependent antibodies were detected* (continued)

<table>
<thead>
<tr>
<th>Drug (alternative name)</th>
<th>Reference</th>
<th>Therapeutic category</th>
<th>Number of references (single [year] vs. multiple [&lt;5, &lt;10, or ≥10])</th>
<th>HA</th>
<th>Positive DAT</th>
<th>Drug-coated RBCs</th>
<th>Serum + drug + RBCs</th>
<th>Not reported</th>
<th>Reactive without drug added in vitro</th>
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</thead>
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<tr>
<td>Cyclosporin (Cyclosporine)</td>
<td>61</td>
<td>Immunosuppressant</td>
<td>Multiple (&lt;5)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Dextchlorpheniramine maleate (Chlorpheniramine)</td>
<td>62</td>
<td>Antihistaminic</td>
<td>Single (1981)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Diclofenac†</td>
<td>63–66</td>
<td>NSAID</td>
<td>Multiple (≥10)</td>
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<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Diethylstilbestrol (Stilboestrol)</td>
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<td>Estrogen</td>
<td>Multiple (&lt;5)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<td>NSAID</td>
<td>Multiple (&lt;5)</td>
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<td>✔</td>
<td>✔</td>
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<td>Erythromycin‡</td>
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<td>Antimicrobial</td>
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<td>✔</td>
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<td>NSAID</td>
<td>Single (2000)</td>
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<td>✔</td>
<td>✔</td>
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<tr>
<td>Etoricoxib</td>
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<td>NSAID</td>
<td>Single (2013)</td>
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<td>✔</td>
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<tr>
<td>Fenoprofen</td>
<td>73</td>
<td>Injectable dye</td>
<td>Single (1993)</td>
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<td>77</td>
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<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<td>80</td>
<td>Antineoplastic</td>
<td>Multiple (&lt;5)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Hydrocortisone</td>
<td>81</td>
<td>Glucocorticoid</td>
<td>Single (2008)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Ibuprofen</td>
<td>82</td>
<td>Antineoplastic</td>
<td>Multiple (&lt;5)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Imatinib mesylate</td>
<td>83</td>
<td>Antidiabetic</td>
<td>Multiple (&lt;5)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Insulin</td>
<td>84</td>
<td>Radiopaque medium</td>
<td>Single (2013)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Isoniazid</td>
<td>85</td>
<td>Antimicrobial</td>
<td>Multiple (&lt;10)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Latamoxef (Moxalactam)</td>
<td>86</td>
<td>Antimicrobial</td>
<td>Single (1985)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Levofloxacin (Ofloxacin)</td>
<td>87</td>
<td>Antibacterial</td>
<td>Multiple (&lt;5)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Melphalan</td>
<td>88</td>
<td>Antineoplastic</td>
<td>Single (1967)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>89</td>
<td>Antineoplastic</td>
<td>Single (2000)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Methadone</td>
<td>90</td>
<td>Analgesic</td>
<td>Multiple (&lt;5)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Methotrexate</td>
<td>91</td>
<td>Antineoplastic, antirheumatic</td>
<td>Multiple (&lt;5)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<td>✔</td>
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<tr>
<td>Metrizoic acid</td>
<td>92</td>
<td>Radiopaque medium</td>
<td>Single (1991)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Minocycline</td>
<td>93</td>
<td>Antibacterial</td>
<td>Single (1994)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Nabumetone</td>
<td>94</td>
<td>Anti-inflammatory, analgesic</td>
<td>Single (2003)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔†</td>
<td>✔</td>
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<tr>
<td>Nafcin‡</td>
<td>95</td>
<td>Antimicrobial</td>
<td>Multiple (&lt;10)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Naproxen</td>
<td>96</td>
<td>Anti-inflammatory, antipyretic</td>
<td>Multiple (&lt;5)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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<td>✔</td>
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<tr>
<td>Nitrofurantoin</td>
<td>97</td>
<td>Antibacterial</td>
<td>Single (1981)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
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</table>
Table 1. Drugs associated with cases of IHA or positive DAT, or both, in which drug-dependent antibodies were detected* (continued)

<table>
<thead>
<tr>
<th>Drug (alternative name)</th>
<th>Reference</th>
<th>Therapeutic category</th>
<th>Number of references (single [year] vs. multiple [&lt;5, &lt;10, or ≥10])</th>
<th>HA</th>
<th>Positive DAT</th>
<th>Method detecting serum antibody</th>
<th>Drug-coated RBCs</th>
<th>Serum + drug + RBCs</th>
<th>Not reported</th>
<th>Reactive without drug added in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nomifensine§</td>
<td>98</td>
<td>Antidepressant</td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
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<td></td>
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<tr>
<td>Oxa-löfloxacin#</td>
<td>100, 101</td>
<td>Antineoplastic</td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>p-Aminosalicylic acid (PAS)</td>
<td>para-aminosalicylsäure</td>
<td></td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Pemêtrexed</td>
<td>103</td>
<td>Antineoplastic</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
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<td>Penicillin G§</td>
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<td>Antimicrobial</td>
<td>Multiple (≥10)</td>
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<td>✓</td>
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<tr>
<td>Phénacetin§ (Acetophenetidin)</td>
<td>106</td>
<td>NSAID</td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Phénytoïne (Fenitoïne)</td>
<td>17</td>
<td>Anticonvulsant, antiarrhythmic</td>
<td>Single (2003)</td>
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<td>✓</td>
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<td>Pipéracilline#</td>
<td>107</td>
<td>Antimicrobial</td>
<td>Multiple (≥10)</td>
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<td>✓</td>
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<td>Puerarin</td>
<td>110</td>
<td>Chinese herb</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td></td>
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<td></td>
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<tr>
<td>Pyrimethamine (Pirimetamine)</td>
<td>17</td>
<td>Antimicrobial</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Quinidine</td>
<td>111</td>
<td>Antiarrhythmic, Antimicrobial</td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
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<td>Quinine</td>
<td>106</td>
<td>Antimicrobial</td>
<td>Multiple (&lt;10)</td>
<td>✓</td>
<td>✓</td>
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<td>Ranitidine</td>
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<td>Antulcerative</td>
<td>Multiple (&lt;5)</td>
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<td>✓</td>
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<tr>
<td>Rifampicin (Rifampicin)</td>
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<td>Antibacterial</td>
<td>Multiple (≥10)</td>
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<td>✓</td>
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<tr>
<td>Stibophénen</td>
<td>116</td>
<td>Antimicrobial</td>
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<td>✓</td>
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<td>Streptomyccin</td>
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<td>✓</td>
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<tr>
<td>Sulfasalazine</td>
<td>121</td>
<td>Anti-inflammatory</td>
<td>Multiple (&lt;5)</td>
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<td>✓</td>
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<tr>
<td>Sulindac</td>
<td>122</td>
<td>Anti-inflammatory</td>
<td>Multiple (&lt;10)</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Suprofen</td>
<td>123</td>
<td>NSAID</td>
<td>Single (1989)</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Tartrazine</td>
<td>124</td>
<td>Colorant</td>
<td>Single (1979)</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Teicloplatin</td>
<td>125</td>
<td>Antimicrobial</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Temafloxicin§</td>
<td>126</td>
<td>Antimicrobial</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Teniposide</td>
<td>127</td>
<td>Antineoplastic</td>
<td>Single (1982)</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Tetracycline</td>
<td>128</td>
<td>Antimicrobial</td>
<td>Multiple (&lt;10)</td>
<td>✓</td>
<td>✓</td>
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</tr>
<tr>
<td>Thiopental sodium</td>
<td>129</td>
<td>Anesthetic</td>
<td>Single (1985)</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Ticarcillin§</td>
<td>130</td>
<td>Antimicrobial</td>
<td>Multiple (&lt;5)</td>
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<td>✓</td>
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<tr>
<td>Tolbutamide</td>
<td>131</td>
<td>Antidiabetic</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
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<td></td>
</tr>
<tr>
<td>Tolmetin§</td>
<td>132</td>
<td>NSAID</td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
<td></td>
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<td></td>
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<tr>
<td>Triamterene</td>
<td>133</td>
<td>Diuretic</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trimellitic anhydride</td>
<td>134</td>
<td>Used in prep of resins, dyes, adhesives, etc.</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>
Table 1. Drugs associated with cases of IHA or positive DAT, or both, in which drug-dependent antibodies were detected* (continued)

<table>
<thead>
<tr>
<th>Drug (alternative name)</th>
<th>Reference</th>
<th>Therapeutic category</th>
<th>Number of references (single [year] vs. multiple [&lt;5, &lt;10, or ≥10])</th>
<th>HA</th>
<th>Positive DAT</th>
<th>Method detecting serum antibody</th>
<th>Reactive without drug added in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimethoprim and Sulfamethoxazole†</td>
<td>135</td>
<td>Antibacterial</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td>Drug-coated RBCs Serum + drug + RBCs Not reported</td>
<td>✓</td>
</tr>
<tr>
<td>Vincristine</td>
<td>136</td>
<td>Antineoplastic</td>
<td>Single (2013)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Zomepirac</td>
<td>137</td>
<td>NSAID</td>
<td>Single (1983)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

IHA = immune hemolytic anemia; DAT = direct antiglobulin test; HA = hemolytic anemia; RBCs = red blood cells; NSAID = nonsteroidal anti-inflammatory drug.

* When a drug antibody is indicated to be reactive by two methods, e.g., vs. drug-treated RBCs and when serum + drug + RBCs are mixed together, not all cases necessarily had drug antibodies reactive by both methods. Using ampicillin, for example, four reported antibodies reacted with drug-treated RBCs and were either nonreactive (n = 1) or not tested (n = 3) by the serum + drug + RBCs method, and two antibodies reacted when serum + drug + RBCs were tested but were nonreactive with drug-treated RBCs.

† One or more samples only positive or strongest reactions seen with ex vivo (urine or serum) or metabolite.

‡ Cases of DIIHA or positive DAT caused by these drugs have been identified in Dr. Garratty's laboratory.

§ No longer manufactured.

‖ Associated with nonimmunologic protein adsorption (NIPA).

**One or more samples positive, possibly owing to the presence of circulating drug or drug-antibody immune complexes.

Table 2. Drugs associated with cases of IHA or positive DAT or both in which only drug-independent antibodies (autoantibodies) were detected

<table>
<thead>
<tr>
<th>Drug (alternative name)</th>
<th>Reference</th>
<th>Therapeutic category</th>
<th>Number of references (single [year] vs. multiple [&lt;5, &lt;10, or ≥10])</th>
<th>HA</th>
<th>Positive DAT</th>
<th>More evidence needed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alemtuzumab</td>
<td>138</td>
<td>Antineoplastic; immunosuppressant</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Bendamustine</td>
<td>139</td>
<td>Antineoplastic</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Captopril</td>
<td>140</td>
<td>Antihypertensive</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Chaparral</td>
<td>141</td>
<td>Herbal</td>
<td>Single (1980)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>142</td>
<td>Antilulcerative</td>
<td>Multiple (&lt;10)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Cladribine (2-chloro-deoxyadenosine)</td>
<td>143</td>
<td>Antineoplastic</td>
<td>Multiple (&lt;10)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Fenfluramine</td>
<td>144</td>
<td>Anorexic</td>
<td>Single (1973)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Fludarabine*</td>
<td>145,146</td>
<td>Antineoplastic</td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Interferon</td>
<td>147</td>
<td>Antineoplastic, antiviral</td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Interleukin-2</td>
<td>148</td>
<td>Antineoplastic</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
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<tr>
<td>Ketoconazole</td>
<td>149</td>
<td>Antifungal</td>
<td>Single (1987)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Levodopa (L-dopa)</td>
<td>151</td>
<td>Antiparkinsonian</td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Mefenamic acid</td>
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<td>NSAID</td>
<td>Multiple (≥10)</td>
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<td>✓</td>
<td>✓</td>
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<tr>
<td>Mesantoin (Mephenytoin)</td>
<td>153</td>
<td>Anticonvulsant</td>
<td>Single (1953)</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Methyldopa*</td>
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<td>Antihypertensive</td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>155</td>
<td>Antibacterial</td>
<td>Multiple (&lt;10)</td>
<td>✓</td>
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<tr>
<td>Procainamide*</td>
<td>156,157</td>
<td>Antiarrhythmic</td>
<td>Multiple (&lt;10)</td>
<td>✓</td>
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<tr>
<td>Tacrolimus</td>
<td>159</td>
<td>Immunosuppressant</td>
<td>Multiple (&lt;10)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

IHA = immune hemolytic anemia; DAT = direct antiglobulin test; HA = hemolytic anemia; NSAID = nonsteroidal anti-inflammatory drug.

*Cases of drug-induced immune hemolytic anemia or positive DAT caused by these drugs have been identified in Dr. Garratty’s laboratory.
Table 3. Drugs associated with the detection of nonimmunologic protein adsorption onto RBCs

<table>
<thead>
<tr>
<th>Drug (alternative name)</th>
<th>Reference</th>
<th>Therapeutic category</th>
<th>Number of references (single [year] vs. multiple [&lt;5, &lt;10, or &gt;10])</th>
<th>HA</th>
<th>Positive DAT</th>
<th>Drug-dependent antibody(ies) also detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefotetan*</td>
<td>35,36</td>
<td>Antimicrobial</td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>48</td>
<td>Antimicrobial</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Cephalothin*</td>
<td>47,48</td>
<td>Antimicrobial</td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>58</td>
<td>Antineoplastic</td>
<td>Multiple (&lt;10)</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Clavulanate potassium*</td>
<td>161,162</td>
<td>β-Lactamase inhibitor</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Diglycoaldehyde (INOX)</td>
<td>163,164</td>
<td>Antineoplastic</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Oxaliplatin*</td>
<td>101</td>
<td>Antineoplastic</td>
<td>Multiple (≥10)</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Sulbactam*</td>
<td>161,165</td>
<td>β-Lactamase inhibitor</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Suramin</td>
<td>166</td>
<td>Antihelminthic, antiprotozoal</td>
<td>Single (1988)</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Tazobactam*</td>
<td>167,168</td>
<td>β-Lactamase inhibitor</td>
<td>Multiple (&lt;5)</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

RBCs = red blood cells; HA = hemolytic anemia; DAT = direct antiglobulin test; IHA = immune hemolytic anemia.
*Cases of drug-induced immune hemolytic anemia or positive DAT caused by these drugs have been identified in Dr. Garratty’s laboratory.

Table 4. Drug antibodies detected by the Research Lab at the American Red Cross in Pomona, CA, from 1978 to 2013

<table>
<thead>
<tr>
<th>Years</th>
<th>Ceftriaxone</th>
<th>Cefotetan</th>
<th>Piperacillin</th>
<th>Platinum-based drugs</th>
<th>Other drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1978–83</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>1984–89</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>1990–95</td>
<td>2</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>1996–2001</td>
<td>6</td>
<td>45</td>
<td>2</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>2002–07</td>
<td>7</td>
<td>15</td>
<td>6</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>2008–13</td>
<td>14</td>
<td>13</td>
<td>30</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>93</td>
<td>38</td>
<td>8</td>
<td>37</td>
</tr>
</tbody>
</table>

Drug Antibodies Showing Blood Group Specificity

I (G.G.) have been interested in this area for more than 30 years. The first report (in 1977) concerned a streptomycin antibody having Rh specificity. In 1981, Duran-Suarez et al. described antibodies to three drugs with I specificity. In 1984, we described a chlorpropamide-induced autoanti-Jka.4 In 1983 and 1985, Habibi et al. and, in 1986, Salama and Mueller-Eckhardt added more drugs showing this phenomenon. The list has grown quite long (see Table 1 of Arndt’s), but this is still not a common finding. There are many piperacillin antibodies showing blood group antigen specificity that have been described since 1984. Most of these were Rh specificities (especially anti-e or “relative” anti-e specificity). Of 37 piperacillin antibodies we were sent to investigate, the referring laboratories detected 8 anti-e, 1 anti-e-like antibody, and 1 possible anti-f when testing serum without piperacillin added in vitro (in vivo circulating piperacillin was most likely present). Twenty-seven of these were tested with R1,R1, and R2,R2, RBCs in the presence of in vitro added piperacillin; 16 of 27 (59%) were nonreactive (7) or weaker (9) with R1,R2 RBCs (e.g., e or relative e specificity), and 2 (8%) were weaker with R1,R2 RBCs (relative c specificity).172

In 2013, we reported results of tests from our own laboratory during the last 30 years on 53 antibodies to 13 different drugs. We found no blood group specificity in single examples of antibodies to acetaminophen, phenacetin, probenecid, quinidine, teniposide, cefotaxime, cimetidine, or diclofenac, four examples of anti-cefotaxime, or ten examples of anti-cefotetan. We found some blood group antigen specificities associated with sulfamethoxazole (MNS and Lutheran) and piperacillin (Rh and Lutheran). Some specificities only became apparent when the antibodies were diluted.

Drug Antibodies Detected in Healthy Blood Donors

We have known for many years that healthy humans can have drug antibodies in their plasma or serum. Low-titer, often IgM, penicillin antibodies were detected in blood donors (5%) or patients with DIIHA (6%). This finding did not confuse the serologic diagnosis because patients with penicillin DIIHA had very high titer (>1000), usually IgG, penicillin antibodies. About 30 to 40 percent of blood donors were found to have antibodies to cephalothin and ticarcillin. Problems were encountered when it was noticed that 80 percent of donors and patients with no DIIHA had cefotetan antibodies; luckily, patients with DIIHA had very high titers of cefotetan antibodies compared with patients with no HA. The most interesting results in this area involved piperacillin. In 2008, we reported...
that 91 percent of donors’ sera and 49 percent of random patients’ sera, contained piperacillin antibodies reactive against piperacillin-coated RBCs.\textsuperscript{173} Luckily, we determined that antibodies to piperacillin (unlike antibodies to other penicillins) react well by adding soluble drug solution to the patients’ sera, and this reactivity correlated with the DIIHA. We no longer use piperacillin-coated RBCs in screening tests.

A few other drug antibodies have been detected in healthy and sick individuals with no DIIHA (oxaliplatin,\textsuperscript{174} cisplatin,\textsuperscript{176} and meropenem\textsuperscript{175}).

It is not known why some of the preceding drug antibodies are found in healthy donors or why they sometimes appear more commonly in healthy donors than in random patients. For instance, 91 percent of donors and only 49 percent of random patients have piperacillin antibodies; 16 percent of donors and only 4 percent of patients have oxaliplatin antibodies.

It has been suggested that environmental factors may be responsible for the production of antibodies. For instance, automobile catalytic convertors release platinum into the atmosphere and may explain the production of antibodies to drugs that belong to the platinum family (oxaliplatin, carboplatin, and cisplatin). Another major concept to explain the production of antibodies to antibiotics in individuals with no DIIHA is that, in the United States, antibiotics are routinely fed to cattle and chickens, so we are continually exposed to drugs such as the cephalosporins and penicillins. Many countries in Europe have banned this practice mainly to prevent problems with treatment resistance of many organisms.

**DIIHA Associated With Transplantation**

Many of the drugs used for transplantation are designed to affect the immune system, so we should not be surprised to see true AIHA develop sometimes. Trying to prove the cause of HA or production of RBC autoantibodies (e.g., positive DAT or IAT) is a difficult task, but DIIHA has to be considered. Examples of drugs said to cause DIIHA after transplantation are cyclosporine, tacrolimus, and the combination of alemtuzumab, mycophenolate mofetil (MMF), and daclizumab (anti-CD52). Mycophenolate mofetil (MMF), and daclizumab (anti-CD52) in pancreas transplant patients. Data from a 2-year period were reported for 357 pancreas transplants. AIHA was detected in 16 patients (7 of these also had RCA; 3 had RCA only). All were DAT+ (all but one had RBC-bound IgG + C3). When MMF was discontinued in the RCA/AIHA group, seven patients recovered from RCA, but only three also recovered from the HA. In the AIHA-alone group (nine patients), MMF was discontinued in two patients with no effect on the HA. Other patients in this group were treated with steroids, rituximab, intravenous IgG, splenectomy, or plasma exchange, with remission seen in only two of the nine patients. The authors suggested that a combination of the drugs may have been involved in the AIHA. It is interesting that the RCA, which is thought to be caused by autoantibody to early RBC precursors, appeared to be caused by MMF.

**Drug-Induced AIHA Associated With Chemotherapeutic Drugs**

Although several chemotherapeutics have been suggested to cause DIIHA, similar problems to those of the transplant associations make the diagnosis difficult. Often several drugs are involved and no drug-dependent antibodies are detectable; the antibodies are usually drug-independent and the patient appears to have developed a true AIHA. The best examples of such drugs are the purine analogs (e.g., fludarabine and cladribine) used to treat patients with chronic lymphocytic leukemia (CLL). Hemolytic anemia after fludarabine therapy occurred in 14 of 66 (22%),\textsuperscript{176} 9 of 52 (17%),\textsuperscript{177} 5 of 36 (14%),\textsuperscript{178} and 5 of 104 (5%)\textsuperscript{146} patients. The analyses of patients with CLL are more complex, with many more confounding factors than the data accumulated in the 1970s on the prototype drug (methyldopa), which includes RBC autoantibodies and AIHA in patients taking the drug for hypertension. Patients with CLL are a very different group of patients, as CLL is known to be associated with positive DATs and AIHA without any drug involvement. Nevertheless, there are reports of exacerbation...
of AIHA that was present before fludarabine therapy. There are fewer reports of AIHA caused by fludarabine in de novo CLL patients receiving fludarabine for the first time compared with patients who have had multiple courses of alkylating agents among whom the prevalence is about 20 percent. There are reports of catastrophic hemolysis (some fatalities) after fludarabine therapy.

In 2008, an interesting study of 777 patients with CLL randomly assigned to receive chlorambucil (C) or fludarabine (F) alone or with cyclophosphamide (FCy) was published. Fourteen percent of these patients had a pretreatment positive DAT. Only 28 percent of patients with positive DATs had an associated HA. Of 249 patients, those treated with F were most likely to become DAT-positive. Patients treated with F or C were twice as likely as those treated with FCy to have AIHA. The authors concluded that a positive DAT was a good prognostic indicator and that FCy combination therapy may protect against AIHA. There is an excellent review on AIHA in patients with CLL by Hamblin.

In one publication, 300 patients with CLL taking fludarabine, cyclophosphamide, and rituximab were followed. Nineteen (6.5%) developed HA. The authors considered these to be AIHA even though 82 percent of the DATs were negative. Such cases of DAT-negative AIHA have been reported in other publications on fludarabine-induced HA.

Recently, there has been an interesting association between fludarabine and bendamustine, a chemotherapeutic drug that is also used for CLL and lymphoma. Several cases of bendamustine-associated AIHA (involving drug-independent autoantibodies) have been described. Goldschmidt et al reported on five cases of AIHA in 31 CLL patients treated with bendamustine; none of the non-CLL patients developed AIHA; and two of five cases were DAT-negative. All five cases had received fludarabine previously. One single case of bendamustine-associated AIHA was DAT-negative and had received no previous fludarabine. Although fludarabine is a purine nucleoside and bendamustine is an alkylating agent, it contains a purine-like benzimidazole ring. This might explain why the bendamustine appeared to give a more severe “secondary” response in patients who had sometimes had a less severe HA caused by fludarabine.

Closing

As new therapeutic drugs appear, we will need to be aware of their potential for inducing drug-dependent antibodies, drug-independent antibodies, or NIPA. The diagnosis is not always easy, and as stated in previous summaries, when studying DIIHA, we typically end up with more questions than answers!

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Diagnostic pitfalls of drug-induced immune hemolytic anemia

A. Salama and B. Mayer

Immune hemolytic anemia (IHA) is a rare complication of drug administration. However, its true incidence remains obscure, as there are a number of factors that may lead to misdiagnosis. The clinical and serologic pictures are variable, and there is a great deal of unawareness that certain drugs can cause IHA. Furthermore, serologic results can be easily misinterpreted, resulting in a wrong diagnosis. *Immunohematology* 2014;30:80–84.

**Key Words:** drug-induced antibody, immune hemolytic anemia, drug-dependent antibody, autoantibodies, immune hemolysis, hemoglobinuria, hemoglobinemia, intravascular hemolysis

Although drug-induced immune hemolytic anemia (DIIHA) is a well-known complication of drug administration, a number of diagnostic pitfalls still occur on the patient’s side, at patient bedside, and in laboratories.

The key to a correct diagnosis is a sophisticated history of drug administration and sufficient clinical as well as serologic experience in this field. As demonstrated in this issue, the list of drugs implicated in DIIHA continues to grow; not only by a variety of new drugs but also by known drugs. However, some published data rely solely on temporal relationships between hemolysis and drug administration without confirmation by serologic testing. On the other hand, there is evidence that DIIHA is far more common than has yet been estimated. Ultimately, serologic testing is frequently inadequate or even lacking. In addition, some cases remain unrecognized or unreported. In this review, we attempt to highlight the main errors leading to false diagnosis or a lack of diagnosis. To provide clarity to such errors, we first describe a few representative cases.

**Representative Cases**

**Cases 1 and 2**

A 54-year-old woman was admitted for hematuria and anemia. She rapidly recovered during clinical observation, presumably as a result of unintended discontinuation of all medications. Serologic testing revealed an only C3d-positive direct antiglobulin test (DAT), and her plasma was observed to be reddish. Based on these findings, DIIHA was suspected. However, the patient was discharged from the hospital without diagnosis. The patient was readmitted on the following day with massive hemolysis, thrombocytopenia, and shock, and subsequently died because of complications resulting from massive hemolysis. From a clinical viewpoint, an acute Evans syndrome was initially suggested. However, we highly suspected a DIIHA. The patient was revealed to have received at irregular intervals antihypertensive drugs including nifedipine and a combination of buthiazide, reserpine, rescinnamine, rauwolfa, and potassium chloride. After several weeks of uninformative serologic testing, the drugs were ingested by a volunteer. Serologic testing in the presence of serum samples collected after drug ingestion (ex vivo antigen) disclosed the buthiazide as the causative drug. In that study, we also described a second patient (Case 2) who received an intramuscular injection containing a drug mixture because of a prolapsed disk. The patient later experienced shock and renal failure that could not be clinically explained. We further suspected DIIHA, but serologic testing was unsuccessful, even in the presence of ex vivo antigens of the injected drugs. On repeat questioning of the patient several months later, she declared that she had ingested nomifensine (antidepressant) before the onset of lumbar pain. She had not previously declared this owing to feelings of shame because of her depression and therapy.

**Case 3**

A 79-year-old woman had abdominal pain and her urine was reddish. Because of suspected hematuria and urinary tract infection, treatment with an antibiotic drug was started; subsequently her condition abruptly deteriorated, eventually resulting in shock. The patient was immediately transferred to the hospital, where urosepsis, shock, and hemolysis were suspected. A blood sample was referred to our laboratory as all crossmatched packed red blood cell (RBC) units were positive at the hospital providing treatment. On the basis of this history, we suspected DIIHA and advised an immediate transfusion. Unfortunately, the patient died, and the question as to which drug she had received before the development of abdominal
pain and hematuria could not be answered. On further investigation, evidence of vitamin E was found in her handbag. The drug prescribed from her physician was, we believe, harmless, as hemolysis was present before deterioration of her condition. In an attempt to determine the causative drug, the patient’s belongings were searched and more than 40 drugs were found (Fig. 1). Thus, it was impossible to identify the causative drug. Similar cases may remain unrecognized either owing to DIIHA not being suspected or owing to inadequate serologic testing or a lack in its implementation.

Case 4

In 2010, we described a patient with fatal immune hemolysis caused by specific antibodies to 5-fluorouracil (5-FU). The patient had cholangiocarcinoma and was receiving chemotherapy including oxaliplatin and 5-FU. Before admission, the patient had been repeatedly treated (15 times) with the same combination of drugs without relevant side effects. She experienced fatal hemolysis that was initially concealed by a blood transfusion. Furthermore, serologic findings (positive antibody screening test and positive C3d-DAT) were misinterpreted as an in vitro phenomenon caused by cold agglutinins. After thorough clinical and serologic reinvestigation of the case, it became clear that hemolysis was related to drug administration. Initially, oxaliplatin, a drug frequently resulting in immune hemolysis, was suspected. Only after dialysis of the patient’s serum and intensive serologic retesting with several ex vivo metabolites was 5-FU determined to be the cause of the hemolysis.

Pitfalls and Limitations

Pitfalls on the Patient’s Side

As described above in our case reports, information concerning the patient plays a key role in clinical and serologic aspects. The vast majority of patients are not informed about symptoms of hemolysis and that drugs may, although rarely, result in hemolysis.

The clinical picture of DIIHA is extremely variable, and the affected patients are usually unable to recognize a relationship between the drugs and secondary symptoms of hemolysis, i.e., tachycardia or dyspnea caused by anemia, abdominal pain, lumbar pain, and red urine (hemoglobinuria) caused by intravascular hemolysis. Depending on the patient’s information and the most prominent symptoms, the following errors may occur before or after consideration of DIIHA as a possible diagnosis:

1. Some patients may recover without further investigations, with the diagnosis remaining unclear.
2. Isolated patients deny drug ingestion, i.e., antidepressant drugs (Case 2), herbal remedies, or other drugs.
3. The dilemma commonly faced is that some patients infrequently or simultaneously ingest various drugs; therefore, it is sometimes impossible to identify what drug(s) could have caused the hemolysis and drug testing is not possible, even when DIIHA is suggested. We are aware of several cases in which we were unable to identify the causative drug because of these factors.
4. Some patients, as well as physicians, automatically exclude long-term administered drugs that have not presented with prior signs of complications.
5. It is largely unknown that drug administration does not result in significant primary immunization within the first 5 days if the patient has not previously received the drug. Thereafter, immune reaction may occur at any time during continuous or intermittent administration or after reexposure of the causative drug.

Pitfalls at Patient Bedside

The clue to correct diagnosis of DIIHA primarily remains in the hands of the primary physician. If this physician is not familiar with all aspects of acquired immune hemolytic anemias, this may then result in numerous errors.

1. Unfortunately, many physicians are not familiar with DIIHA. They are unaware that clinical manifestations and pictures are variable. Furthermore, there remains a lack of knowledge from a clinical or serologic viewpoint concerning the difference between DIIHA by...
autoantibodies (aAbs) alone and that induced by drug-dependent antibodies (ddAbs). These deficiencies are the reasons behind the various failures at the clinical bedside and in laboratories (see subsequent discussion).

2. Patients in addition to physicians seem to exclude drugs as a cause of acute reactions when they have been tolerated for a long time. Many physicians do, in fact, consider DIIHA in many patients with hemolysis. However, they seem to suspect, in general, only newly administered drugs. Most frequently, patients are asked whether they have altered their medications or received a new drug. Detailed information including drug history, onset and type of hemolysis, clinical picture, and course are rarely documented.

3. Inconclusive patient or drug history may lead to confusion with autoimmune hemolytic anemia (AIHA) of the warm type, particularly if the drug has led to the production of ddAbs or aAbs, leading to positive immunoglobulin G (IgG) DAT. Only a few drugs are known to cause the production of the latter antibodies alone (e.g., methyldopa, fludarabine), and some drugs have been reported to cause both types (e.g., diclofenac, cefotetan). It is unknown how many cases of warm AIHA are in fact not idiopathic but related to drugs. On physical examination, there are no characteristic findings of AIHA related to drugs. Autoantibodies induced by drugs are also serologically indistinguishable from “idiopathic” autoantibodies. Unfortunately, hemolysis related to drug-induced aAbs may persist for several weeks or even months after discontinuation of the causative drug. In such cases, recovery could be attributed to treatment rather than to stopping the drug. Thus, these cases remain unrecognizable as DIIHA.

4. On admission, almost all patients who exhibit immune hemolysis automatically receive high doses (at least 0.5–1.0 mg/kg body weight) of steroids. In the case of DIIHA, an improvement caused by intentional or unintentional cessation of the causative drug might be attributed to treatment and not to discontinuation of the drug(s). The incidence of these cases remains obscure.

5. Confusion with acute hemolytic transfusion reactions is also relatively common. Some patients may receive drugs that cause acute hemolysis before or simultaneously with blood transfusion. In most of these cases, a hemolytic transfusion reaction (HTR) will primarily be suspected rather than DIIHA (Case 4). The correct diagnosis can only be established if laboratory testing does not confirm either HTR or AIHA. This result is supported by several reported\textsuperscript{16–11} and unreported cases.

6. A fatal failure is the administration of the causative drug during the hemolytic attack. This failure may occur as long as the true diagnosis is unclear or when true AIHA has been suggested. Most dangerous is when the main symptom of the underlying disease is similar to that of intravascular hemolysis. We were aware of some patients who received diclofenac because of chronic lumbar pain and were administered the drug again after development of acute lumbar pain related to massive intravascular hemolysis rather than to the chronic affliction.

7. Infrequently, only secondary symptoms of DIIHA may initially be significant, leading to misdiagnosis or no diagnosis at all. During observation, the causative drugs are often automatically ceased. If these patients do not exhibit complications, they may quickly recover and be discharged without a final diagnosis (see described cases).

**Pitfalls in the Laboratory**

Similar to the situation at the clinical bedside, many serologists are not well informed about all the serologic and clinical aspects of DIIHA. This makes correct testing and interpretation of serologic findings often impossible. Unfortunately, to date, there remain no standard tests or guidelines to confirm serologic findings by certified laboratories similar to those for blood cell antigens and antibodies. These deficiencies represent risks of pitfalls leading to false-positive and false-negative results, misdiagnosis, or no diagnosis.

**False-Positive Results**

1. Use of enzyme-treated RBCs may result in nonspecific agglutination in the presence of abnormal patient’s serum but not in the presence of normal serum. Indeed, the samples are not identical, and in vitro–treated RBCs are sometimes more susceptible to patient’s serum samples than to normal serum samples. We have not observed a reaction between specific ddAbs and enzyme-treated RBCs, and not with native cells. However, some investigators have found that the use of enzyme-treated RBCs can be important for enhancing the detection of ddAbs. In these cases, it is indispensable to use an appropriate negative control (saline instead of the drug; if both react similarly, then a ddAb is not present).

2. Use of in vitro–treated RBCs may result in nonspecific reactions (nonimmunologic protein adsorption) similar to those observed by using enzyme-treated RBCs. This phenomenon has been described for different drugs including β-lactamase inhibitors, platinum, and some cephalosporins.\textsuperscript{12}
FALSE-NEGATIVE RESULTS

Different reasons may lead to false-negative results.

1. Testing of other drugs but not the causative drug may occur in cases in which a number of drugs are suspected or when only an innocent drug is suspected (see described cases).

2. Some laboratories still use test systems requiring washing procedures. Because the vast majority of drugs do not bind tightly to RBCs, ddAbs may escape detection if the wash solution used does not contain the causative drug or metabolites (1 mg/mL).13

3. Some ddAbs recognize, in the presence of the drug, RBC antigens and do not react with cells lacking the corresponding antigen.14–17 This phenomenon must be considered by using different or pooled RBCs and, most importantly, the patient’s RBCs.

4. Some ddAbs react with RBCs only in the presence of drug metabolites and not with the native drug (Cases 1 and 2). Because drug metabolites are usually not available or remain unknown, the use of ex vivo preparations, e.g., urine or serum from persons receiving the suspected drug, is obligatory in all suspected cases that demonstrate negative results in the presence of the native drug.14,15 In such cases, we recommend the use of different ex vivo preparations (urine from the patient and urine or serum from different persons taking the drug) to include rare or even private metabolites.

5. Some ddAbs may become, within a relatively short time, undetectable in the patient’s serum. Thus, in such cases, negative results do not exclude DIIHA. Unfortunately, there is little information regarding this aspect. However, we are aware of isolated cases in which the causative antibody became undetectable within a 2-week period or less of the event occurring, i.e., diclofenac-dependent antibodies and one iohexol-dependent antibody. Initially, these antibodies were detectable in the presence of the drug or its metabolites.

MISDIAGNOSIS

The most common misdiagnoses are as follows:

1. Warm AIHA in cases in which aAbs are detectable. In addition to the phenomenon that drugs may lead to the production of aAbs or ddAbs, some drugs also result in the production of both types in the same patient. Thus, the presence of aAbs does not exclude ddAbs. The latter antibodies must be suspected in all cases in which intravascular hemolysis abruptly develops. In all these cases, a C3d-positive DAT (with or without IgG) is a cardinal finding.3

2. Panagglutinating antibodies that may be attributed to cold agglutinins of high thermal amplitude, agglutinating IgM warm aAbs, unspecific reactions, or IgG warm aAbs, sometimes with specificity. This phenomenon is characteristic in many cases during the acute phase of hemolysis and when the drug and its metabolite are still present in the circulation.

3. Acute HTRs are primarily suspected in nearly all cases in which blood transfusion is administered before or during hemolysis.2,6–11 If serologic testing remains inconclusive, a misdiagnosis will result even when alloantibodies are not detectable.

4. Infrequently, toxic reactions might be suggested if ddAbs escape serologic testing, e.g., contrast media.18

NO DIAGNOSIS

Some patients have a history of DIIHA but have not been investigated at the appropriate stage. We are aware of several cases in which DIIHA appears to have developed, but could not be excluded or proven.

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How we investigate drug-induced immune hemolytic anemia

R.M. Leger, P.A. Arndt, and G. Garratty

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.

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Key Words: immune hemolytic anemia, drugs, antibodies, drug-treated red blood cells, testing in presence of drug, penicillin, cephalosporin, antibiotics

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
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• 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
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. NIPA is independent of antibody production, but may be a cause of hemolytic anemia. 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). The antihuman albumin is not licensed for RBC indirect antiglobulin testing, but 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. 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. 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.

**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],...
Preparation of 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. 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, but we have found that 6 percent albumin decreases the binding of some drugs (e.g., cefotetan, cephalothin) to RBCs. 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.

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\(^ {22}\)). 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

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

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

*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.

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**Table 3. Interpretation of tests with drug-treated RBCs**

<table>
<thead>
<tr>
<th>Patient’s serum</th>
<th>Eluate</th>
<th>Last wash</th>
<th>Normal sera/plasma</th>
<th>Normal sera/plasma 1/20</th>
<th>PBS</th>
<th>Positive control</th>
<th>Patient’s serum</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>NT</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>Drug antibody</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>Drug antibody</td>
</tr>
<tr>
<td>+</td>
<td>NT</td>
<td>+</td>
<td>0</td>
<td>NT</td>
<td>0</td>
<td>NA</td>
<td>0</td>
<td>Drug antibody</td>
</tr>
<tr>
<td>0</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>No drug antibody detected</td>
</tr>
<tr>
<td>0</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>NT</td>
<td>0</td>
<td>NA</td>
<td>0</td>
<td>No drug antibody detected; ?drug bound to RBCs; no positive control</td>
</tr>
<tr>
<td>+ (AGT only)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+ (AGT only)</td>
<td>0</td>
<td>0</td>
<td>+ at ≥1/20*</td>
<td>NIPA but no drug antibody detected</td>
</tr>
<tr>
<td>+ direct aggl, + or 0 AGT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+ direct aggl, + or 0 AGT</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>Not DIIHA; environmental exposure?</td>
</tr>
<tr>
<td>+</td>
<td>NT</td>
<td>+/0</td>
<td>0</td>
<td>0</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*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. Widespread use of antibiotics in agriculture and environmental contamination have been suggested as the source of exposure to many drugs. 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. 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>
<thead>
<tr>
<th>Table 4. Inhibition test: reactivity of plasma (diluted) plus solution of oxaliplatin or PBS versus oxaliplatin-treated red blood cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution added</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Oxaliplatin (mg/mL)</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>PBS</td>
</tr>
</tbody>
</table>

| 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., ceftiraxone) 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

Table 5. Testing in the presence of a drug solution

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
</table>
| 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.

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-ceftiraxone 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., R₁R₂ and R₁R₃ 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).

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
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.

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

<table>
<thead>
<tr>
<th>Sample tested</th>
<th>Untreated RBCs</th>
<th>Enzyme-treated RBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 min at</td>
<td>60 min at</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>37°C</td>
</tr>
<tr>
<td></td>
<td>AHG</td>
<td>AHG</td>
</tr>
<tr>
<td>Patient’s serum + drug</td>
<td>+/0</td>
<td>+/0</td>
</tr>
<tr>
<td>Patient’s serum + PBS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Patient’s serum + C + drug</td>
<td>+/0</td>
<td>+/0</td>
</tr>
<tr>
<td>Patient’s serum + C + PBS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Positive control + drug</td>
<td>+/0</td>
<td>+/0</td>
</tr>
<tr>
<td>Positive control + PBS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C + drug</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C + PBS</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Test</th>
<th>Untreated RBCs</th>
<th>Enzyme-treated RBCs</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
<td>37°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AHG</td>
<td>AHG</td>
<td></td>
</tr>
<tr>
<td>Patient’s serum + drug</td>
<td>0</td>
<td>3+</td>
<td>Positive (drug antibody)</td>
</tr>
<tr>
<td>Patient’s serum + PBS</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Patient’s serum + C + drug</td>
<td>0</td>
<td>4+</td>
<td>Positive (drug antibody)</td>
</tr>
<tr>
<td>Patient’s serum + C + PBS</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Patient’s serum + drug</td>
<td>2+H/3+</td>
<td>4+H</td>
<td>Positive (drug antibody)</td>
</tr>
<tr>
<td>Patient’s serum + PBS</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Patient’s serum + C + drug</td>
<td>2+H/3+</td>
<td>4+H</td>
<td>Positive (drug antibody)</td>
</tr>
<tr>
<td>Patient’s serum + C + PBS</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Patient’s serum + drug</td>
<td>0</td>
<td>4+</td>
<td>Positive (drug antibody) and circulating drug or drug–anti-drug complexes</td>
</tr>
<tr>
<td>Patient’s serum + PBS</td>
<td>0</td>
<td>m+</td>
<td>Positive (drug antibody) and circulating drug or drug–anti-drug complexes</td>
</tr>
<tr>
<td>Patient’s serum + C + drug</td>
<td>0</td>
<td>4+</td>
<td>Positive (drug antibody) and circulating drug or drug–anti-drug complexes</td>
</tr>
<tr>
<td>Patient’s serum + C + PBS</td>
<td>0</td>
<td>1+</td>
<td>Not interpretable as a result of autoantibody or alloantibody; adsorb and retest</td>
</tr>
<tr>
<td>Patient’s serum + drug</td>
<td>3+s</td>
<td>2+</td>
<td>Positive (drug antibody) and circulating drug or drug–anti-drug complexes</td>
</tr>
<tr>
<td>Patient’s serum + PBS</td>
<td>0</td>
<td>3+</td>
<td>Positive (drug antibody) and circulating drug or drug–anti-drug complexes</td>
</tr>
<tr>
<td>Patient’s serum + C + drug</td>
<td>2+s</td>
<td>2+H/4+</td>
<td>Positive (drug antibody) and circulating drug or drug–anti-drug complexes</td>
</tr>
<tr>
<td>Patient’s serum + C + PBS</td>
<td>0</td>
<td>3+</td>
<td>Positive (drug antibody) and circulating drug or drug–anti-drug complexes</td>
</tr>
<tr>
<td>Patient’s serum + drug</td>
<td>0</td>
<td>4+</td>
<td>Positive (drug antibody) and circulating drug or drug–anti-drug complexes</td>
</tr>
<tr>
<td>Patient’s serum + PBS</td>
<td>0</td>
<td>4+</td>
<td>Positive (drug antibody) and circulating drug or drug–anti-drug complexes</td>
</tr>
<tr>
<td>Patient’s serum + C + drug</td>
<td>0</td>
<td>4+</td>
<td>Positive (drug antibody) and circulating drug or drug–anti-drug complexes</td>
</tr>
<tr>
<td>Patient’s serum + C + PBS</td>
<td>0</td>
<td>4+</td>
<td>Positive (drug antibody) and circulating drug or drug–anti-drug complexes</td>
</tr>
</tbody>
</table>

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)\(^{16}\), wash for the antiglobulin test using a solution of drug (e.g., 1 mg/mL), 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; 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.\(^{33}\) 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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Drug-induced immune neutropenia/agranulocytosis

Neutrophils are the most abundant white blood cell in blood and play a critical role in preventing infections as part of the innate immune system. Reduction in neutrophils below an absolute count of 500 cells/μL is termed severe neutropenia or agranulocytosis. Drug-induced immune neutropenia (DIIN) occurs when drug-dependent antibodies form against neutrophil membrane glycoproteins and cause neutrophil destruction. Affected patients have fever, chills, and infections; severe infections left untreated can result in death. Treatment with granulocyte colony-stimulating factor can hasten neutrophil recovery. Cumulative data show that severe neutropenia or agranulocytosis associated with exposure to nonchemotherapy drugs ranges from approximately 1.6 to 15.4 cases per million population per year. Drugs most often associated with neutropenia or agranulocytosis include dipyrone, diclofenac, ticlopidine, calcium dobesilate, spironolactone, antithyroid drugs (e.g., propylthiouracil), carbamazepine, sulfamethoxazole-trimethoprim, β-lactam antibiotics, clozapine, levamisole, and vancomycin. Assays used for detection of neutrophil drug-dependent antibodies (DDAbs) include flow cytometry, monoclonal antibody immobilization of granulocyte antigens, enzyme-linked immunosorbent assay, immunoblotting, granulocyte agglutination, and granulocytotoxicity. However, testing for neutrophil DDAbs is rarely performed owing to its complexity and lack of availability. Mechanisms proposed for DIIN have not been rigorously studied, but those that have been studied include drug- or hapten-induced antibody formation and autoantibody production against drug metabolite or protein adducts covalently attached to neutrophil membrane proteins. This review will address acute, severe neutropenia caused by neutrophil-reactive antibodies induced by nonchemotherapy drugs—DIIN. Immunohematology 2014;30:95–101.

Key Words: drug-dependent neutrophil antibodies, drug-induced immune neutropenia, agranulocytosis

Drug-Induced Immune Neutropenia

Neutrophils (polymorphonuclear leukocytes [PMNs]) are a type of granulocyte and are the major class of white blood cells (WBCs) in human peripheral blood. The circulation of a healthy human adult contains 4500 to 10,000 WBCs/μL with approximately 60 percent or more being neutrophils. A healthy adult produces $1 \times 10^{11}$ neutrophils each day, each of which survives about 6 to 8 hours in the circulation. Neutrophils play an important role in the innate immune response and are critical to host defense against infectious disease. They circulate in the blood until recruited by chemical signals to sites of infection, where they function as phagocytes that engulf and kill extracellular pathogens with their microbicidal arsenal. Macrophages also contribute significantly to this process. Because of their important role in preventing infections, disorders that result in a significant lack of neutrophils put the host at risk of severe illness and even death. A reduction of neutrophils (segmented and band cells) in the blood circulation to less than an absolute neutrophil count (ANC) of 1000 to 1500/μL is considered neutropenia. An ANC less than 500/μL is considered severe neutropenia or agranulocytosis, and individuals with an ANC less than 100/μL are at severe risk of morbidity and mortality from infections. The terms “agranulocytosis” and “neutropenia” are often used interchangeably, but some argue that use of the term “agranulocytosis” should be reserved for conditions in which the bone marrow cannot make sufficient granulocytes, and “neutropenia” when just neutrophils fall below normal levels. For the purposes of this review, the terms will be used synonymously, with agranulocytosis representing cases of severe neutropenia in which the ANC is less than 100/μL.

Like thrombocytopenia and anemia, neutropenia can be induced after exposure to myriad drugs and results from either decreased production or increased destruction of neutrophils. Decreased production is usually a consequence of exposure to chemotherapeutic drugs causing immune suppression of bone marrow myeloid precursors. Idiosyncratic or acute neutropenia resulting from increased neutrophil destruction is commonly caused by adverse reactions to nonchemotherapy drugs.

Clinical Features, Diagnosis, and Treatment

Drug-induced immune neutropenia (DIIN) is typically suspected when a sudden severe drop in neutrophils (ANC <500/μL) occurs shortly after repeat exposure to a drug or 5 to 7 days after first exposure. Patients’ symptoms include, but are not limited to, fever, chills, nonspecific sore throat, and myalgia or arthralgia. Diagnosis is difficult because patients often are asymptomatic before severe neutropenia.
is discovered, usually from a blood count performed for another reason. Without treatment, patients can experience severe infections, septicemia, and septic shock that can lead to death in 2 to 10 percent of cases. In DIIN, the bone marrow typically shows normal or mild hypocellularity with decreased or absent myeloid precursor cells, or it can be hypercellular with neutrophilic maturation arrest.

Criteria for drug imputability are the same as those applied in drug-induced immune thrombocytopenia or anemia: (1) exposure to the candidate drug preceded neutropenia, (2) recovery from neutropenia was complete and sustained after discontinuing candidate drug, (3) candidate drug was the only drug used before the onset of neutropenia or other drugs were continued or reintroduced after discontinuation of candidate drug with a sustained neutrophil count, and (4) other causes of neutropenia were excluded.

Treatment of patients with DIIN begins with immediate withdrawal of the implicated drug. Further treatment consists of supportive care for complications such as fever and infections, which may include analgesics and antibiotics for severe infections (ANC <100/μL) and antifungal agents for fungal infections. Treatment with recombinant granulocyte colony-stimulating factor (G-CSF) in severe infections can hasten neutrophil recovery and shorten neutropenic fever, but is more often used in neutropenia induced by chemotherapy. Granulocyte transfusions are typically not required in DIIN, and the risk–benefit is not favorable.

### Incidence and Drugs Most Frequently Implicated

In 1980, the International Agranulocytosis and Aplastic Anemia Study (IAAAS), a population-based case-controlled surveillance program, was coordinated, which involved Israel and seven regions of Europe. The most recent analysis of these data shows that the frequency of agranulocytosis in patients receiving nonchemotherapy drugs is approximately 5 cases/million population per year. Cumulative data from IAAAS, case reports, and clinical trials show that agranulocytosis associated with drug exposure ranges from approximately 1.6 to 15.4 cases per million population per year. Drug-associated neutropenia frequency increases with age, being highest in people older than 65 years, and the incidence is also higher in women. Drugs most often associated with neutropenia include dipyrone (metamizole), diclofenac, ticlopidine, calcium dobesilate, spironolactone, antithyroid drugs (e.g., propylthiouracil), carbamazepine, quinine, sulfamethoxazole-trimethoprim, β-lactam antibiotics, clozapine, levamisole, and vancomycin (Table 1). Neutropenia incidence is higher for a select number of drugs.

Propylthiouracil is used to treat hyperthyroidism (Graves disease) by decreasing the levels of thyroid hormone. Mild leukopenia (WBCs <4000/μL) occurs in 12 percent of adults and 25 percent of children receiving propylthiouracil, but severe neutropenia occurs less frequently (0.31%).

A careful review of a 7-year period of 114 patients receiving home intravenous vancomycin therapy in New Mexico found 12 percent developed vancomycin-induced neutropenia and 3.5 percent had drops in ANC to 500/μL or less.

The antihelmintic agent levamisole has been known to cause agranulocytosis or neutropenia in exposed patients since the 1970s, primarily in individuals using cocaine adulterated with levamisole, but also when used as adjuvant chemotherapy in patients with colorectal cancer. Drug Enforcement Administration (DEA) reports in 2009 showed 69 percent of seized cocaine was contaminated with as much as 10 percent levamisole. Neutropenia has been reported to occur in as many as 13 percent of patients exposed to levamisole. In cancer trials, agranulocytosis associated with levamisole showed dose dependence, with agranulocytosis developing in 3.1 percent of patients receiving 2.5 mg/kg for 2 consecutive days every week compared with 0.1 percent receiving the same dosage for 3 consecutive days but every other week.

Clozapine-induce neutropenia occurs in about 1 percent of patients during the first 3 months of treatment. Clozapine, a dibenzodiazepine, is an atypical antipsychotic medication used in the treatment of schizophrenia resistant to conventional neuroleptics. Clozapine is perhaps the drug most often associated with agranulocytosis or neutropenia, and it carries five black box warnings alerting physicians to that risk and several other adverse effects of the drug. However, the mechanism responsible for severe neutropenia after exposure to clozapine is not immune mediated, but is the result of ATP depletion through formation of an active nitrenium metabolite that causes apoptotic destruction of neutrophils.

Whereas the overall incidence of drug-induced neutropenia or agranulocytosis has been examined, the incidence of specifically DIIN is not well defined. This is primarily related to the difficulty in distinguishing drug-induced neutropenia caused by myelosuppression and direct neutrophil cytotoxicity from immune mechanisms, and poor sensitivity and availability of laboratory testing for detection of drug-dependent neutrophil antibodies. Table 1 lists those drugs that have most often been reported to cause DIIN.
Drug-induced immune neutropenia

Laboratory Testing for Drug-Dependent Neutrophil Antibodies

As just mentioned, unlike for drug-induced immune thrombocytopenia and anemia, laboratory testing for neutrophil drug-dependent antibodies (DDAbs) is not as productive, and testing for neutrophil antibodies is not widely available. The reasons for this are that neutrophil antibody testing is technically complex, labor-intensive, and expensive. In addition, the inability to maintain the integrity of neutrophils

Table 1. Nonchemotherapy drugs reported to be associated with drug-induced neutropenia

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug</th>
<th>Drug</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>aminopyrine</td>
<td>carbamazepine</td>
<td>alimemazine</td>
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</tr>
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*Drugs reported to be associated with drug-induced immune neutropenia.
†Drugs reported to be highly associated with agranulocytosis.
‡Drugs in 2012 for which sera from patients with suspicion of drug-induced immune neutropenia were tested for antibodies in a flow cytometry assay by Platelet & Neutrophil Immunology Lab, BloodCenter of Wisconsin, Milwaukee. No drug-dependent antibodies involving these drugs were detected in any of the patients’ sera.
IVIG = intravenous immunoglobulin G.
for testing by storage at 4°C or by cryopreservation requires that cells be isolated from fresh blood each day. This demands the regular availability of large numbers of blood donors. For these reasons, it is critical that granulocyte antibody and antigen testing be performed by an experienced laboratory using appropriate controls. When neutrophil DDAb testing is performed, methods used are very similar to those used for testing of platelet DDAbs with substitution of freshly isolated neutrophils for platelets. A typical assay consists of incubation of isolated neutrophils with patient’s serum in the presence or absence of the implicated drug, followed by washing of cells incubated with drug with washes containing drug and those not incubated with drug, just with buffer. Neutrophils are then incubated with fluorescent or enzyme-labeled anti-human immunoglobulin (Ig)G or anti-human IgM for detection of bound DD Abs. Flow cytometry for immunofluorescent detection of DD Abs (Fig. 1) and monoclonal antibody immobilization of granulocyte antigens (MAIGA) assay for enzyme-linked immunosorbent assay (ELISA) detection and determination of the neutrophil glycoproteins targeted by DD Abs are methods in current use.20,24–27 Other methods that have been used are immunoblotting, granulocyte agglutination test (GAT), and granulocytotoxicity.8 Despite the availability of these various methods, neutrophil DD Abs, with the exception of those induced by quinine,8,25,26,28,29 are rarely detected. The paucity of neutrophil DD Abs detected suggests that currently used tests lack sensitivity or that mechanisms responsible for drug-induced neutropenia often do not involve antibody formation (see next section on Pathogenic Mechanisms).

Like some platelet and red blood cell DD Abs,30,31 neutrophil DD Abs that can only be detected in the presence of drug metabolites have also been reported.32 Another issue is that, in the small number of cases in which patient sera have been tested, often the sera were tested without the addition of drug so that only nondrug antibodies were detected. The presence of non-DD Abs in suspected cases of DIIN suggests a drug-induced autoantibody mechanism is responsible for the patient’s neutropenia. In vitro assays demonstrating DD Abs that inhibit the growth of myeloid progenitor cells have also been reported.33

The neutrophil glycoprotein targets of DD Abs have not been well studied for most drugs. Stroncek et al.,28 using immunoprecipitation, showed that a quinine-dependent antibody targeted the neutrophil-specific protein CD177 and another unidentified glycosphatidylinositol-linked membrane glycoprotein. Meyer et al.27 found a cefotaxime DD Ab targeted CD11b and CD35 and a metamizole DD Ab directed against CD16.

**Fig. 1** Fluorescence histograms generated from immunofluorescence detection of immunoglobulin G (IgG) drug-dependent neutrophil antibodies by flow cytometry. Normal isolated neutrophils were incubated with sera and washed, and neutrophil-bound antibodies were detected with fluorescent anti-human IgG. (A) Normal serum incubated with neutrophils in the presence of either quinine (black histograms) or buffer (white histograms) shows low IgG fluorescence, indicating no antibodies are present. (B) Serum from a patient exposed to quinine with drug-induced neutropenia incubated with neutrophils showing high IgG fluorescence (MFI = 69.8) only with quinine, indicating the presence of quinine drug-dependent antibodies. Median fluorescence intensity (MFI) values are shown for each histogram.
**Pathogenic Mechanisms**

Mechanisms of drug-dependent antibody formation and binding to neutrophils have not been rigorously studied, and proposed mechanisms are largely unsubstantiated. However, some of the mechanisms proposed to explain DDABS formed against platelets (see article in this issue of the journal) probably also apply to those against neutrophils, but this has not been proven. Greater study has been made of mechanisms to explain nonimmunologic drug-induced neutropenia or agranulocytosis. Proposed mechanisms of immunologic and nonimmunologic drug-induced neutropenia include oxidation of drugs by neutrophil reactive oxygen species (ROS), causing the formation of toxic metabolites or haptens, cytotoxicity by large granular T lymphocytes (T-LGL), disruptions of granulopoiesis and egress of neutrophils from the bone marrow, hapten- or drug-specific antibodies, formation of drug-induced autoantibodies, and genetic and epigenetic modifications that predispose an individual to drug sensitivity. Mechanisms specific for DIIN will be discussed next.

**Drug Oxidation**

It is essential that drugs undergo biotransformation, primarily in the liver, into water-soluble metabolites that can be excreted in the urine to prevent cellular accumulation and toxicity. Biotransformation gives rise to reactive intermediates and metabolites that can readily make covalent linkages with cellular proteins, including neutrophil membrane glycoproteins. It is also possible that neutrophil enzymes such as myeloperoxidase (MPO) could be capable of producing reactive drug metabolites. Once formed and attached to neutrophil proteins, these molecules could elicit drug-specific antibody production through a hapten mechanism or by production of autoantibodies targeting drug–protein adducts. Several drugs associated with a significant incidence of agranulocytosis (e.g., methimazole, propylthiouracil, captopril, levalamisole, clozapine) have been reported to form drug–protein adducts in vitro, although in contrast, structurally related drugs are rarely associated with agranulocytosis.

**Hapten Mechanism**

Haptons are low-molecular-weight (usually <5000 daltons) molecules that are not capable of eliciting an immune response unless they are coupled to a larger carrier protein. Drugs like penicillin and some cephalosporin drugs when covalently linked to cell surface proteins can elicit drug-specific/hapten antibodies. This mechanism is well described for DDABS targeting red blood cells (RBCs), which cause immune hemolytic anemia, but has not been proven to occur in drug-induced thrombocytopenia. As mentioned in the previous section, several drugs highly associated with agranulocytosis do form drug–protein adducts with neutrophil membrane glycoproteins that could elicit hapten-specific (drug-specific) antibodies that destroy neutrophils. Hapten antibodies were reported to cause DIIN in patients exposed to flecanide and dipyrylene.

**Immune Complex Mechanism**

Fc-receptors (FcR) are a family of related protein molecules expressed on various cells in the body that engage the Fc portion of immunoglobulins (antibodies) and regulate activation or inhibition of various cellular functions important in the immune response, including phagocytosis, immunoglobulin transport, and prevention of IgG catabolism. Resting neutrophils express FcyRIIib and FcyRIIa receptors and express FcyRI receptors when induced by various neutrophil-activating stimuli. Therefore, neutrophils are capable of binding immune complexes of DDABS and drug, but this mechanism has not been proven for DIIN.

**Autoantibody Mechanism**

Autoantibodies produced against platelets and RBCs have been reported after exposure to gold salts and α-methylidopa, respectively. In some cases of suspected DIIN, neutrophil-reactive antibodies are detected that do not depend on the presence of drug. It is possible that these non–drug-dependent antibodies could be true autoantibodies induced by the drug. Demonstration that serum from such patients reacts with autologous neutrophils collected once their ANCs normalize would be one way to confirm these are true autoantibodies, but there are no known reports in which such testing has been performed.

**Summary**

DIIN can occur in susceptible individuals 5 to 7 days after first exposure to a drug. Those affected typically experience acute, severe neutropenia or agranulocytosis (ANC <500/μL) and symptoms of fever, chills, sore throat, and muscle and joint pain. Although difficult to diagnosis, it is important to identify DIIN because, if left untreated, mortality is as high as 10 percent in cases exhibiting severe infections. Idiosyncratic drug-induced neutropenia or agranulocytosis occurs with a frequency of approximately 1.6 to 15.4 cases per million population per year, and is higher in the elderly and women.
The classes of drugs most commonly involved in DIIN include analgesics, antiarrhythmic agents, antibiotics, antimalarials, and antithyroid agents. Laboratory testing for neutrophil DDAbs as confirmation of DIIN is rarely performed owing to the difficulty and low sensitivity of tests used and the lack of testing availability. Flow cytometry, MAIGA, GAT, immunoblotting, and granulocyte cytoxicity methods have been used to detect DDAbs. Some DDAbs target myeloid progenitor cells and cause neutropenia by inhibiting neutrophil production. Neutrophil glycoprotein targets that have been described for DDAbs include CD11b, CD16, CD35, and CD177. Mechanisms of drug-dependent antibody formation and binding to neutrophils have not been rigorously studied, and proposed mechanisms are largely unsubstantiated. Mechanisms that have been proposed include destruction of neutrophils by hapten/drug-specific antibodies, and autoantibodies that bind adducts of drug metabolites bound to neutrophil membrane proteins formed after biotransformation of drugs, although several of the other mechanisms of drug-dependent antibody formation that have been described as causative of drug-induced immune thrombocytopenia could also possibly be involved in DIIN.

References


34. Dekant W. The role of biotransformation and bioactivation in toxicity. EXS 2009;99:57–86.


The Johns Hopkins Hospital Specialist in Blood Bank Technology Program

The Johns Hopkins Hospital was founded in 1889. It is located in Baltimore, Maryland, on the original founding site, just 45 minutes from Washington, DC. There are approximately 1,000 inpatient beds and another 1,200 outpatient visits daily; nearly 600,000 patients are treated each year.

The Johns Hopkins Hospital Transfusion Medicine Division is one of the busiest in the country and can provide opportunities to perform tasks that represent the entire spectrum of immunohematology and transfusion medicine practice. It provides comprehensive support to all routine and specialized areas of care for surgery, oncology, cardiac, obstetrics, neonatal and pediatric, solid organ and bone marrow transplant, therapeutic apheresis, and patients with hematological disorders to name a few. Our intradepartment immunohematology reference laboratory provides resolution of complex serologic problems, transfusion management, platelet antibody, and molecular genotype testing.

The Johns Hopkins Hospital Specialist in Blood Bank Technology Program is an onsite work-study, graduate-level training program for certified medical technologists, medical laboratory scientists, and technologists in blood banking with at least two years of full-time blood bank experience.

The variety of patients, the size, and the general intellectual environment of the hospital provide excellent opportunities for training in blood banking. The program is a challenging one that will prepare competent and knowledgeable graduates who will be able to effectively apply practical and theoretical skills in a variety of employment settings. The Johns Hopkins Hospital Specialist in Blood Bank Technology Program is accredited by the Commission on Accreditation of Allied Health Education Programs (CAAHEP). Please visit our Web site at http://pathology.jhu.edu/department/divisions/transfusion/sbb.cfm for additional information.

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- Develop, validate, implement, and perform laboratory procedures
- Analyze quality issues preparing and implementing corrective actions to prevent and document nonconformances
- Design and present educational programs
- Provide technical and scientific training in transfusion medicine
- Conduct research in transfusion medicine

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Why become an SBB?

- Professional growth
- Job placement
- Job satisfaction
- Career advancement

How does one become an SBB?

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Which approach are you more compatible with?

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Additional information can be found by visiting the following Web sites: www.ascp.org, www.caahep.org, and www.aabb.org

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<td><a href="http://www.utsouthwestern.edu/education/school-of-health-professions/programs/certificate-programs/medical-laboratory-sciences/index.html">www.utsouthwestern.edu/education/school-of-health-professions/programs/certificate-programs/medical-laboratory-sciences/index.html</a></td>
</tr>
</tbody>
</table>

Revised May 2012
**Immunohematology**

**Instructions for Authors**

I. **GENERAL INSTRUCTIONS**

Before submitting a manuscript, consult current issues of *Immunohematology* for style. Number the pages consecutively, beginning with the title page.

II. **SCIENTIFIC ARTICLE, REVIEW, OR CASE REPORT WITH LITERATURE REVIEW**

A. Each component of the manuscript must start on a new page in the following order:

1. Title page
2. Abstract
3. Text
4. Acknowledgments
5. References
6. Author information
7. Tables
8. Figures

B. Preparation of manuscript

1. Title page
   a. Full title of manuscript with only first letter of first word capitalized (bold title)
   b. Initials and last name of each author (no degrees; all CAPS), e.g., M.T. JONES, J.H. BROWN, AND S.R. SMITH
   c. Running title of ≤ 40 characters, including spaces
   d. Three to ten key words

2. Abstract
   a. One paragraph, no longer than 300 words
   b. Purpose, methods, findings, and conclusion of study

3. Key words

4. Text (serial pages): Most manuscripts can usually, but not necessarily, be divided into sections (as described below). Survey results and review papers may need individualized sections

   a. Introduction — Purpose and rationale for study, including pertinent background references
   b. Case Report (if indicated by study) — Clinical and/or hematologic data and background serology/molecular
   c. Materials and Methods — Selection and number of subjects, samples, items, etc. studied and description of appropriate controls, procedures, methods, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer’s name, city, and state. Do not use patient’s names or hospital numbers.
   d. Results — Presentation of concise and sequential results, referring to pertinent tables and/or figures, if applicable
   e. Discussion — Implication and limitations of the study, links to other studies; if appropriate, link conclusions to purpose of study as stated in introduction

5. Acknowledgments: Acknowledge those who have made substantial contributions to the study, including secretarial assistance; list any grants.

6. References
   a. In text, use superscript, Arabic numbers.
   b. Number references consecutively in the order they occur in the text.

7. Tables
   a. Head each with a brief title; capitalize the first letter of first word (e.g., Table 1. Results of…) use no punctuation at the end of the title.

   b. Use short headings for each column needed and capitalize first letter of first word. Omit vertical lines.

   c. Place explanation in footnotes (sequence: *, †, ‡, §, ¶, †*, ††).

8. Figures
   a. Figures can be submitted either by e-mail or as photographs (5 × 7” glossy).
   b. Place caption for a figure on a separate page (e.g. Fig. 1 Results of…), ending with a period. If figure is submitted as a glossy, place first author’s name and figure number on back of each glossy submitted.
   c. When plotting points on a figure, use the following symbols if possible: l, l, s, s, n, n.

9. Author information

   a. List first name, middle initial, last name, highest degree, position held, institution and department, and complete address (including ZIP code) for all authors. List country when applicable. Provide e-mail addresses of all authors.

III. **EDUCATIONAL FORUM**

A. All submitted manuscripts should be approximately 2000 to 2500 words with pertinent references. Submissions may include:

1. An immunohematologic case that illustrates a sound investigative approach with clinical correlation, reflecting appropriate collaboration to sharpen problem solving skills
2. Annotated conference proceedings

B. Preparation of manuscript

1. Title page
   a. Capitalize first word of title.
   b. Initials and last name of each author (no degrees; all CAPs)

2. Text
   a. Case should be written as progressive disclosure and may include the following headings, as appropriate
      i. Clinical Case Presentation: Clinical information and differential diagnosis
      ii. Immunohematologic Evaluation and Results: Serology and molecular testing
      iii. Interpretation: Include interpretation of laboratory results, correlating with clinical findings
      iv. Recommended Therapy: Include both transfusion and nontransfusion-based therapies
      v. Discussion: Brief review of literature with unique features of this case
      vi. Reference: Limited to those directly pertinent
      vii. Author information (see II.B.9.)
      viii. Tables (see II.B.7.)

IV. **LETTER TO THE EDITOR**

A. Preparation

1. Heading (To the Editor)
2. Title (first word capitalized)
3. Text (written in letter [paragraph] format)
4. Author(s) (type flush right; for first author: name, degree, institution, address [including city, state, Zip code and country]; for other authors: name, degree, institution, city and state)
5. References (limited to ten)
6. Table or figure (limited to one)

Send all manuscripts by e-mail to immuno@redcross.org
A. For describing an allele which has not been described in a peer-reviewed publication and for which an allele name or provisional allele name has been assigned by the ISBT Working Party on Blood Group Allele Terminology (http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/blood-group-terminology/blood-group-allele-terminology/)

B. Preparation
1. Title: Allele Name (Allele Detail)
   ex. RHCE*01.01 (RHCE*ce48C)
2. Author Names (initials and last name of each (no degrees, ALL CAPS)

C. Text
1. Case Report
   i. Clinical and immunohematologic data
   ii. Race/ethnicity and country of origin of proband, if known
2. Materials and Methods
   Description of appropriate controls, procedures, methods, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer’s name, city, and state. Do not use patient names or hospital numbers.
3. Results
   Complete the Table Below:

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Allele Name</th>
<th>Nucleotide(s)</th>
<th>Exon(s)</th>
<th>Amino Acid(s)</th>
<th>Allele Detail</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>e weak</td>
<td>RHCE*01.01</td>
<td>48G&gt;C</td>
<td>1</td>
<td>Trp16Cys</td>
<td>RHCE*ce48C</td>
<td>1</td>
</tr>
</tbody>
</table>

   Column 1: Describe the immunohematologic phenotype (ex. weak or negative for an antigen).
   Column 2: List the allele name or provisional allele name.
   Column 3: List the nucleotide number and the change, using the reference sequence (see ISBT Blood Group Allele Terminology Pages for reference sequence ID).
   Column 4: List the exons where changes in nucleotide sequence were detected.
   Column 5: List the amino acids that are predicted to be changed, using the three-letter amino acid code.
   Column 6: List the non-consensus nucleotides after the gene name and asterisk.
   Column 7: If this allele was described in a meeting abstract, please assign a reference number and list in the Reference section.

4. Additional Information
   i. Indicate whether the variant is listed in the dbSNP database (http://www.ncbi.nlm.nih.gov/snp/); if so, provide rs number and any population frequency information, if available.
   ii. Indicate whether the authors performed any population screening and if so, what the allele and genotype frequencies were.
   iii. Indicate whether the authors developed a genotyping assay to screen for this variant and if so, describe in detail here.
   iv. Indicate whether this variant was found associated with other variants already reported (ex. RHCE*ce48C,1025T is often linked to RHD*DIVa-2)

D. Acknowledgments

E. References

F. Author Information
List first name, middle initial, last name, highest degree, position held, institution and department, and complete address (including ZIP code) for all authors. List country when applicable.
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