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CONTENTS

137
Letter to the readers
Introduction to the review articles
S.T. NANCE

138
Review: drug-induced immune hemolytic anemia—the last decade
G. GARRATTY

147
Review: what to do when all RBCs are incompatible—serologic aspects
S.T. NANCE AND P.A. ARNDT

161
Review: transfusing incompatible RBCs—clinical aspects
G. MENY

167
Review: evaluation of patients with immune hemolysis
L.D. PETZ

177
Case report: exacerbation of hemolytic anemia requiring multiple incompatible RBC transfusions
A.M. SVENSSON, S. BUSHOR, AND M.K. FUNG

184
Delayed hemolytic transfusion reaction due to anti-Fy\(^b\) caused by a primary immune response: a case study and a review of the literature

187
Maternal alloanti-hr\(^a\) — an absence of HDN
R. KAKAIYA, J. CSERI, B. JOCHUM, L. GILLARD, AND S. SILBERMAN

190 COMMUNICATIONS
Letter to the Editors
HAMA (Human Anti-Mouse Antibodies) do not Cause False Positive Results in PAKPLUS
L.A. TIDEY, S. CHANCE, M. CLARKE, AND R.H. ASTER
Reply to letter
M.F. LEACH AND J.P. AU Buchon

195
Letters From the Editor-in-Chief
Ortho dedication
The final 20th anniversary issue

198 ANNOUNCEMENTS

193
Letter to the Editor-in-Chief
Immunohematology to be listed in Index Medicus and MEDLINE
S.G. SANDLER

196 SPECIAL SECTION
Excerpts from the American Red Cross Reference Laboratory Newsletter—1976

199 ADVERTISEMENTS

202 INSTRUCTIONS FOR AUTHORS
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Introduction to the review articles

Welcome to the third issue of 2004, celebrating Immunohematology’s 20 years of publication. As with the first two issues of 2004, it contains four invited review articles. This issue’s focus is on diagnostic uses of immunohematology serologic testing.

The first review is a recurring favorite, “Drug-Induced Immune Hemolytic Anemia—The Last Decade,” by George Garratty, PhD. Over the years, the editors of Immunohematology have invited Dr. Garratty to write review articles on drug-induced hemolytic anemia and he has kept our readers current. This review continues the tradition with another excellent review.

The second and third reviews focus on what to do when all units of blood are incompatible. Serologic aspects are covered by Sandra Nance and Patricia Arndt; these aspects include the testing often performed in the immunohematology reference laboratory (IRL) and monocyte monolayer assays performed as a specialty test in two IRLs in the United States. This second review also includes cases that demonstrate the points under discussion. Dr. Geralyn Meny reviews clinical responses when all units of blood are incompatible and reminds us of all the elements that should be considered when this situation arises in your facility.

Dr. Larry Petz completes the invited review section with “Evaluation of Patients With Immune Hemolysis.” This review takes the reader through the differential diagnoses of a patient that presents with immune hemolysis and discusses the importance of the precise diagnosis in prognosis and therapy.

This third issue is a good mix of reviews that discuss serologic testing and clinical use of results for diagnostic and therapeutic measures. These reviews are written by authors with a great deal of experience in their field. I sincerely hope you enjoy the issue as much as I have enjoyed being the guest editor.

Sandra T. Nance, MS, MT(ASCP)SBB
Member of the Editorial Board and
Guest Editor of this issue,
American Red Cross Blood Services
Penn-Jersey Region
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Philadelphia, PA 19123
Review: drug-induced immune hemolytic anemia—the last decade

G. Garratty

I have written three previous reviews on drug-induced immune hemolytic anemia (DIIHA) for this journal.1–3 The last one was written in 1994.3 This year, I would like to review what has happened in the last decade.

When Dr. Petz and I published the first edition of our book (Acquired Immune Hemolytic Anemias) in 1980,4 we found that there was reasonable evidence to support that about 30 drugs could cause DIIHA. One drug, methyldopa, was by far the most common drug to do this. Almost 70 percent of DIIHAs referred to our laboratory in the 1970s were associated with methyldopa. Antibodies associated with methyldopa were drug-independent and the patients showed all the serologic and hematologic characteristics of “warm type” autoimmune hemolytic anemia (WAIHA).

In the next 20 years, methyldopa and high dose IV penicillin were used less and less; we have not seen a case of DIIHA associated with these two drugs for many years. By 1994 about 71 drugs had been implicated in DIIHA.5 Recently published results reflect a changing picture in the spectrum of DIIHA in the last 25 years.6,7 There are now approximately 100 drugs associated with DIIHA (see Table 1), and methyldopa and penicillin have been replaced by a single group of drugs, the cephalosporins (93% of cases), with cefotetan alone accounting for 83 percent of the DIIHAs we have encountered in the last 10 years. Table 2 shows the drugs causing DIIHA that we have encountered in the past 26 years (1978–2003). Methyldopa is probably underrepresented as, by 1978, cases of autoimmune hemolytic anemia (AIHA) in patients taking methyldopa were not usually sent to specialist laboratories such as ours for investigation.

Because of these statistics, I will be emphasizing the cephalosporins in this review.

Cephalosporin-Induced Immune Hemolytic Anemia

There are about 70 individual published case reports of cephalosporin-induced immune hemolytic anemia (CIIHA),8–64 but many more are contained in reviews or tables without case histories65–67 (see Table 3). Most patients have had severe hemolytic anemia (HA), often with intravascular lysis, and 40 percent were associated with fatal HA. It is not known if this is the tip of the iceberg and there are many more cases of milder HA or positive DATs that are not reported; the same questions apply to cephalosporin-induced thrombocytopenia. Tables 4 and 5 summarize the clinical and serologic findings associated with cefotetan- and ceftriaxone-induced immune HA. It should be emphasized that cefotetan antibodies always react with cefotetan-coated RBCs and almost always react with untreated RBCs in the presence of cefotetan (“immune complex” method), and about one-third will react with RBCs without the presence of drug (i.e., will appear to be autoantibodies). The latter findings can lead to problems in the blood transfusion service. If a patient receives cefotetan prophylactically for surgery, receives a blood transfusion during or after surgery, and then develops HA 7 to 10 days afterwards, a delayed hemolytic transfusion reaction is often suspected. The hematologic findings can also mimic AIHA. If the HA is due to cefotetan, the DAT will be positive (although we have reported one case where the DAT was negative).68 Sometimes the serum will react with all untreated RBCs, mimicking an alloantibody to a high-frequency antigen, or a mixture of alloantibodies or autoantibody, and many hours may be wasted investigating these possibilities. If there is a history of cefotetan
administration, I recommend testing an eluate from the patient’s RBCs against untreated and cefotetan-coated RBCs to help with the differential diagnosis. If the eluate is reactive with cefotetan-treated RBCs and not the same untreated RBCs, the diagnosis is obvious. Unfortunately, in about 15 percent of cases we have studied, the eluate also reacted with untreated RBCs.

Table 1. Drugs causing immune hemolytic anemia and/or positive DATs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Number of case reports</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>aceclofenac</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>diethylstilbestrol</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>nomifensine</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>acetaminophen/paracetamol</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>diglycoaldehyde (INOX)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>oxalaplatin</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>aminopyrine/pyramidon</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>dipropylyldantoin</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>penicillin G</td>
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<td></td>
</tr>
<tr>
<td>ampicillin</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>erythromycin</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>phenacetin</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>antazoline</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>etodolac</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>phenytoin</td>
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<td></td>
</tr>
<tr>
<td>apazone/azapropazone</td>
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<td></td>
</tr>
<tr>
<td>fenfluramine</td>
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</tr>
<tr>
<td>piperacillin</td>
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<td>apronalide</td>
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<tr>
<td>butizide</td>
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<tr>
<td>fludarabine</td>
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<tr>
<td>procainamide</td>
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<tr>
<td>carbencillin</td>
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<td></td>
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<tr>
<td>fluorescein</td>
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<td>propyphenazone</td>
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<td>carbimazole</td>
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</tr>
<tr>
<td>carboplatin</td>
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<td>fluorouracil (5-FU)</td>
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<td>quinine</td>
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<td>carbromal</td>
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</tr>
<tr>
<td>rituximab</td>
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<td>cefazolin</td>
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<tr>
<td>hydrochlorothiazide</td>
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</tr>
<tr>
<td>sodium pentothal/thiopental</td>
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<td>cefotaxime</td>
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<tr>
<td>9-hydroxy-methyl-ellipticinium</td>
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<td>subephen</td>
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<tr>
<td>cefotetan</td>
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<tr>
<td>indene derivatives (e.g., sulindac)</td>
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<td></td>
</tr>
<tr>
<td>sulbactam sodium (e.g., in Unasyn)</td>
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</tr>
<tr>
<td>sulindac</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>cefoxitin</td>
<td>1</td>
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</tr>
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<td>insulin</td>
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<tr>
<td>sulfonamides</td>
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<tr>
<td>ceftazidime</td>
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<tr>
<td>interferon</td>
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<td>sulfonylurea derivatives (e.g., chlorpromazine)</td>
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<tr>
<td>latamoxef</td>
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<tr>
<td>tazobactam (e.g., in Zosyn)</td>
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<tr>
<td>cephalordine</td>
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<td>levodopa</td>
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<td>cyclophosphamide</td>
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<td>chlorambucil</td>
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<td>methotrexate</td>
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<tr>
<td>trimethoprim/hydrochloroquine</td>
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<td>ciapranol</td>
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<td>methotrexate</td>
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<tr>
<td>diclofenac</td>
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<td></td>
</tr>
<tr>
<td>nafillin</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*Drugs in publications containing reasonable evidence for an immune etiology were the only drugs included (many more are in the literature).

Table 2. Drugs associated with DIIHA investigated at American Red Cross Blood Services, Los Angeles, in the last 26 yrs. (1978–2003)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Number of patients</th>
</tr>
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<tbody>
<tr>
<td>cefotetan</td>
<td>74</td>
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<tr>
<td>ceftriaxone</td>
<td>12</td>
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<tr>
<td>piperacillin</td>
<td>5</td>
</tr>
<tr>
<td>clavulanate</td>
<td>3</td>
</tr>
<tr>
<td>fludarabine</td>
<td>2</td>
</tr>
<tr>
<td>penicillin</td>
<td>2</td>
</tr>
<tr>
<td>probenecid</td>
<td>2</td>
</tr>
<tr>
<td>rifampicin</td>
<td>2</td>
</tr>
<tr>
<td>cefotaxime</td>
<td>1</td>
</tr>
<tr>
<td>sulbactam</td>
<td>1</td>
</tr>
<tr>
<td>ticarcillin</td>
<td>1</td>
</tr>
<tr>
<td>mefloquine</td>
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<td>cefoxitin</td>
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<tr>
<td>chlorpropamide</td>
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<td>nafillin</td>
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<td>phenacetin</td>
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</tr>
<tr>
<td>procarbamide</td>
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<td>erythromycin</td>
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<td>tolmetin</td>
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</tr>
<tr>
<td>oxalaplatin</td>
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</table>

Total 119

Table 3. Cephalosporins reported to cause immune hemolytic anemia (up to 2003)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Number of case reports</th>
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<tr>
<td>cefazolin</td>
<td>1</td>
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<tr>
<td>cefepime</td>
<td>2</td>
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<tr>
<td>cefamandole</td>
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<tr>
<td>cefoxitin</td>
<td>2</td>
</tr>
<tr>
<td>cefotaxime</td>
<td>1</td>
</tr>
<tr>
<td>ceftriaxone</td>
<td>1</td>
</tr>
<tr>
<td>cefotetan</td>
<td>5*</td>
</tr>
<tr>
<td>ceftepizole</td>
<td>1</td>
</tr>
<tr>
<td>cefixime</td>
<td>1</td>
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<tr>
<td>cefizoxime</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
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</table>

*Many other cases are reported, without individual case reports, in references 65 (43 cases) and 66 (85 cases)
Clinical and serologic findings associated with cefotetan-induced IHA

- Patients always had a positive DAT:
  - 100% had RBC-bound IgG
  - 86% had RBC-bound C3
  - 44% had RBC-bound IgA
  - 7.4% had RBC-bound IgM
- All sera agglutinated cefotetan-treated RBCs (median titer 512) and reacted by IAT (median titer 16,000); all normal sera also reacted with the cefotetan-treated RBCs, but were nonreactive when diluted ≥ 1 in 100.
- As with penicillin, there is evidence of drug reactivity of undiluted sera.
- Ceftriaxone-coated RBCs cannot be made, thus the "immune-complex" mechanism.
- Patients' nadir Hb after receiving cefotetan = 2.6 g/dL (mean = 4.8 g/dL).
- Fatal HA and renal failure occurred in 19 percent of patients.
- Patients' nadir Hb after receiving ceftriaxone = 6.7 g/dL (mean = 8.4 g/dL).
- All but one of the sera reacted with untreated RBCs in the presence of drug, but were nonreactive when diluted ≥ 1 in 100.
- Fatal HA occurred in 67 percent of 9 children and 30 percent of ten adults.
- There is a history of previous ceftriaxone therapy.
- Positive DATs associated with complement are present in all cases and additional IgG is present in 75 percent; none had detectable RBC-bound IgA or IgM.
- Antibodies are detected only by immune complex method (serum + drug + RBCs). Some patients' sera only react with a metabolite of cefotetan (ex vivo antigen).
- Antibodies in their sera that can directly agglutinate cefotetan-coated RBCs.
- Clinical data from references 38–40, 42–49, 50–53, 56, 57
- Serologic data from reference 65

Table 4. Clinical* and serologic** findings associated with cefotetan-induced IHA

- Approx. 80 percent of patients received cefotetan for surgery; usually a single dose of 2 g was used.
- A history of previous cefotetan therapy was not common.
- HA was obvious in less than 1 day to 13 days after receiving cefotetan. Only two patients had HA in < 1 day; the mean of the other 29 was 9 days.
- Patients' nadir Hb after receiving cefotetan = 2.6 g/dL (mean = 4.8 g/dL).
- Most patients had signs of intravascular lysis (hemoglobinemia/hemoglobinuria).
- Fatal HA and renal failure occurred in 19 percent of patients.
- Patients always had a positive DAT:
  - 100% had RBC-bound IgG
  - 86% had RBC-bound C3
  - 44% had RBC-bound IgA
  - 7.4% had RBC-bound IgM
- All sera agglutinated cefotetan-treated RBCs (median titer 512) and reacted by IAT (median titer 16,000); all normal sera also reacted with the cefotetan-treated RBCs, but were nonreactive when diluted ≥ 1 in 100.
- All but one of the sera reacted with untreated RBCs in the presence of cefotetan ("immune complex" mechanism).
- 33 percent and 40 percent of sera reacted with cefotetan-coated RBCs without drug being present, with saline-suspended RBCs or in the presence of PEG, respectively.65

Table 5. Clinical and serologic findings associated with ceftriaxone-induced IHA

- HA is more acute and severe in children compared to adults. In children, HA started 5 minutes to 7 days after receiving drug; for six children there was a mean of 27 minutes and for three children a mean of 6 days. In adults, HA started 30 minutes to 34 days. One patient started after 30 minutes; the mean for nine others was 9 days.
- Fatal HA occurred in 67 percent of 9 children and 30 percent of ten adults.
- There is a history of previous ceftriaxone therapy.
- Positive DATs associated with complement are present in all cases and additional IgG is present in 75 percent; none had detectable RBC-bound IgA or IgM.
- Antibodies are detected only by immune complex method (serum + drug + RBCs). Some patients' sera only react with a metabolite of ceftriaxone (ex vivo antigen).

(Arndt, Leger, Garratty, unpublished observations). The diagnosis may then rest on the presence of a high-titer (> 100) cefotetan antibody in the patient's serum. Some illustrative case histories associated with such problems have been published in this journal.36

Technical hints when investigating CIIHA

1. When preparing cefotetan-coated RBCs, use a buffer with a pH of 6 to 7 (or normal saline) instead of the high pH buffer (pH 8 to 10) used for preparation of penicillin-coated RBCs.69 The lower pH does not reduce sensitivity but does reduce nonspecific uptake of protein leading to falsely positive antihuman globulin (AHG) tests. Some investigators have suggested that drugs can be solubilized more efficiently in 1% albumin.70 We do not advise this when testing for cefotetan antibodies, as albumin can cause reduced binding of drug to drug-treated RBCs.71

2. Most individuals, including healthy donors, have antibodies in their sera that can directly agglutinate cefotetan-coated RBCs.72 In addition, almost all sera (including those from healthy individuals) will yield positive IATs with cefotetan-treated RBCs. When testing a patient's serum with cefotetan-coated RBCs, dilute the serum 1 in 100 in saline.36 This will avoid agglutination and nonspecific protein uptake onto cefotetan-treated RBCs; clinically significant antibodies always have titers > 100 (e.g., in the thousands). Some texts still recommend a dilution of 1 in 20, which is what we recommended when using cephalothin-coated RBCs. We have found that 1 in 20 is sufficient to exclude nonspecific adsorption of proteins by cefotetan-treated RBCs, but is not sufficient to exclude agglutination of cefotetan-treated RBCs by about 10 percent of sera (even from healthy donors).72

3. The lesson to be learned by the above is: never report cefotetan antibodies to be present based on reactions of undiluted sera. As with penicillin-induced immune HA, the diagnosis should be based on an eluate from the patient's DAT-positive RBCs reacting with cefotetan-coated RBCs, but not untreated RBCs; unlike penicillin, the cefotetan antibody in the eluate may sometimes react with untreated RBCs (see reference 65). Another problem is that the last wash of the patient's RBCs may be reactive.73 The presence of a high titer (> 100) cefotetan antibody in the serum confirms the diagnosis.

4. Ceftriaxone-coated RBCs cannot be made; thus the serologic diagnosis is based on the results of the "immune-complex" method. Nonspecific adsorption of proteins is not a problem by this method, thus undiluted sera can be used. Sometimes, positive reactions are only obtained by using enzyme-treated RBCs, or metabolites of the drug (e.g., in the presence of urine from a patient taking ceftriaxone).20,32,35
**DIIHA Associated With Nonimmunologic Adsorption of Proteins Onto RBCs**

RBCs treated with the first-generation cephalosporin, cephalothin, were found to adsorb many proteins nonimmunologically when incubated in vitro with normal plasma/sera. Such adsorbed proteins led to a positive AHG test, but it was thought that this was clinically insignificant, and indeed there were only five cases of DIIHA due to cephalothin reported in more than 30 years of use. Some other drugs have been found to show a similar phenomenon (see Table 6), but some of these drugs have been thought to cause DIIHA more commonly than cephalothin.

**Table 6.** Drugs associated with nonimmunologic adsorption of proteins onto RBCs

<table>
<thead>
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<th>Drugs</th>
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<tr>
<td>cephalosporins</td>
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<tr>
<td>cisplatin/oxaliplatin/carboplatin</td>
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<tr>
<td>diglycoaldehyde (INOX)</td>
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<tr>
<td>suramin</td>
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<tr>
<td>sulbactam (contained in Unasyn)</td>
</tr>
<tr>
<td>clavulanate (contained in Augmentin and Timentin)</td>
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<tr>
<td>tazobactam (contained in Zosyn)</td>
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Causes for the uptake of protein are not always clear. Garratty and Petz suggested that cephalothin changed the RBC membrane in a way that created sites for protein binding. Later this was challenged by Branch and Petz, who suggested that no membrane change occurred and that protein attached to free protein binding sites on the cephalothin that had covalently bonded to proteins on the RBC membrane. Garratty and Leger were stimulated by an old finding, that cefotetan-treated RBCs were “PNH-like” (e.g., had a positive acidified serum test). To reexamine the issue, Garratty and Leger confirmed these old findings by showing, using flow cytometry, that cephalothin- and cefotetan-treated RBCs had markedly decreased amounts of CD55 and CD58 on the RBC membrane; CD59 was only slightly decreased. Thus, the results were different from the changes associated with PNH but confirmed the original hypothesis of Garratty and Petz that cephalosporins can modify the RBC membrane. Whether this is the cause of the nonimmunologic uptake of protein remains to be proved.

Other drugs have been found to cause nonimmunologic adsorption of proteins onto RBCs (Table 6). Some drugs containing aldehyde groups can bind to RBC proteins and adsorb proteins from the plasma, leading to positive AHG tests, but HA has not been associated with such drugs.

Drugs belonging to the platinum family (e.g., cisplatin, carboplatin, and oxaliplatin) have been associated with positive DATs and HA. Some investigators have suggested that cisplatin causes nonimmunologic adsorption of protein and that the HA is coincidental; others believe the drug can cause DIIHA. We believe that some patients can develop antibodies to the drug and that the DIIHA may occur because of this, regardless of nonimmunologic protein adsorption. We have described a patient who had antibodies to oxaliplatin but no HA.

Nevertheless, I now believe that nonimmunologic adsorption of protein may lead to HA. This is mainly based on work we have performed on another family of drugs, the beta lactamase inhibitors. Examples of these commonly used drugs are sulbactam (contained in Unasyn), clavulanate (contained in Augmentin and Timentin), and tazobactam (contained in Zosyn). They are used together with the beta lactam antibiotics ampicillin, ticarcillin, and piperacillin, respectively.

Lutz and Dzik reported that 39 percent of patients receiving Unasyn developed weakly positive DATs but no HA. We showed later that RBCs treated in vitro with the beta lactamase inhibitors contained in Unasyn, Augmentin, Timentin, and Zosyn would adsorb proteins nonimmunologically, leading to positive IATs, and that this may be the cause of positive DATs associated with such drugs. It should be mentioned that patients may make antibodies to the antibiotics also present in the drug (e.g., ampicillin, piperacillin), and these antibodies can cause positive AHG tests and HA. Garratty and Arndt suggested that HA could also occur in patients who have no antibodies to the antibiotics and that this may be associated with nonimmunologic adsorption of protein. This suggestion was supported by case histories and in vitro experiments where it was shown that RBCs treated with the beta lactamase inhibitors, washed, and incubated in normal plasma, washed, and then added to a monocyte monolayer yielded results suggesting that the RBCs would have shortened survival. Broadberry et al. described a DIIHA associated with tazobactam; the HA was thought to be due to nonimmunologic adsorption of protein.

Unfortunately, we cannot prove in the laboratory that this nonimmunologic adsorption of proteins is causing the HA in a particular patient; one can only suggest it to the physicians and refer them to the appropriate literature.
Mechanisms of DIHA

There are two well-accepted mechanisms for DIHA, namely, that individuals can produce either drug-dependent antibodies or drug-independent antibodies (Table 7). The latter can be classic autoantibodies in that they do not require drug to be present for their reaction with RBCs in vitro or in vivo; drug is only needed to initiate the production of antibodies. It is still unclear how this occurs, but the most popular concept is that certain drugs given to certain patients somehow affect the immune system to produce pathogenic autoantibodies, as do certain infections in certain patients. The mechanisms may turn out to be similar. The AIHA often continues for some time after the drug is discontinued. Some drug antibodies (e.g., cefotetan) sometimes react with RBCs without drug being present, but I do not think that these are “classic” autoantibodies (in contrast to those associated with methyldopa). Drug-independent antibodies (e.g., cefotetan antibodies) are never seen without the presence of drug-dependent antibodies and when the drug is stopped, the “autoantibodies” become weaker, eventually disappearing (see Table 7). I believe that these antibodies are not produced because of the drug’s effect on the immune system, but rather are part of the spectrum of antibodies produced as a result of the immune response to a hapten (e.g., antibodies to the drug, carrier [RBC protein], and drug + carrier). See Figure 1.

Table 7. Drugs that have been reported to induce RBC drug-independent antibodies (i.e., autoantibodies)*

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<tr>
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<th>Group 2</th>
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<td>rituximab</td>
<td>nomifensine</td>
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*Drugs in groups 1 and 1a have been reported to induce drug-dependent antibodies only. More evidence is needed to prove that drugs in group 1a really can induce RBC autoantibodies. Drugs in group 2 induce drug-independent antibodies together with antibodies reacting by different mechanisms.

Drug-dependent antibodies can be of two types. One type of antibody can be inhibited by the drug (hapten inhibition) and reacts only with washed, drug-coated RBCs (prototype drug is penicillin). The other type, which relates to most drugs, is not inhibited by the drug and reacts with untreated RBCs in the presence of the drug. We still do not know why. There have been two main suggestions:

- The drug antibody combines with the drug, forming immune complexes which attach to the RBC (transiently) and activate complement.
- The drug changes the RBC membrane, producing a new antigen (“neoantigen”) as a new epitope. This neoantigen may be a chemically modified membrane protein or part drug, part RBC membrane protein (“compound” epitope).

The latter part of the latter concept is the basis of the unifying hypothesis originally suggested by Habibi,69 supported by Mueller-Eckhardt and Salama,70 which I feel has much merit. Unfortunately, like other concepts, it has some failings (these are discussed in references 5, 6, and 99). Although the immune complex hypothesis is out of favor, the clinical findings and some serologic data fit much better with the “immune complex” hypothesis. Shulman and Reid100 tried to take some of the best of both hypotheses, and suggest some explanations for the discrepancies, but personally I do not think that, in 2004, we have any better explanations that we did in 1994!
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A serologic workup to investigate the presence of irregular antibodies in a patient's sample includes testing the patient's serum (or plasma) against a panel of reagent RBCs and the patient's autologous RBCs. If all panel RBCs and the autologous RBCs are equally reactive, then the most likely conclusion is that the patient's serum contains a warm autoantibody and/or a cold autoantibody. If all panel RBCs are reactive and the autologous RBCs are nonreactive, then the patient has either multiple alloantibodies and/or an antibody directed against a high-frequency antigen. Using a case study approach, the first part of this review will cover what steps can be used to detect and identify any alloantibodies underlying autoantibodies and how to differentiate multiple alloantibodies from antibodies directed against high-frequency antigens. If it is determined that an alloantibody is directed against a high-frequency antigen, then it becomes important to know the likelihood of that antibody being clinically significant, because antigen-negative RBCs may be difficult to obtain for transfusion. The second part of this review will focus on in vitro cellular methods used to determine the clinical significance of alloantibodies.

It is 2 a.m. on the overnight shift and you are alone in the blood bank with a specimen drawn from a patient in the emergency department who has a gunshot wound. This patient has a history of previous admissions and transfusions at your hospital. The antibody screen is 3+ with screening cells I, II, and III by the gel method. The emergency department is notified of the positive screen and gives an indication that blood is needed for transfusion; the patient's hemoglobin is holding at 5g/dL but he is symptomatic.

The antibody's specificity should be identified; how this is done depends on the resources available at the facility. The patient's sample should be tested against a panel of reagent RBCs, either at the facility or at an immunohematology reference laboratory (IRL). Since this was a three-cell screen and all RBCs tested were reactive, the chance of this being a simple antibody is low and reactions of the three cells on the gel card should be reviewed to see if there are differences in the appearance of the reactions with each of the three RBC suspensions. If there are differences, then multiple antibodies might be present; if all reactions appear the same, then there may be an autoantibody or an antibody to a high-frequency antigen present.

A panel is performed and all RBCs are reactive; the autologous RBCs are nonreactive. The gel cards should be reviewed to determine if the reactions are equal. In this case, the gel reactions are equal. The case is referred to the local IRL and an anti-Yta is identified. In the interim period between sample submission and resolution, the patient receives four units of incompatible blood. Over the next 3 weeks, the Hb gradually falls and the patient receives two units of Yt(a−) blood.

This review will focus on further investigation of situations involving cases that present serologically with all reagent RBCs reactive, including or not including the autologous RBCs. The starting point will be in the hospital to highlight when, in each circumstance, it would be advisable to send the sample to the IRL.

Autoantibodies

The presence of an autoantibody is usually determined by a reactive autologous control in a patient who has not been recently transfused. This reactivity could be primarily present at room temperature (probable cold autoantibody) or primarily present at 37°C and detected at the antihuman globulin
(AHG) phase of testing (probable warm autoantibody). It is “best practice” to confirm that the reactivity is due to an autoantibody by performing an autoadsorption and showing that the reactivity is removed; the autoadsorbed sample can then be tested to determine if alloantibodies are also present. Autoadsorptions are performed using autologous RBCs that have been treated (e.g., with heat, enzyme, or ZZAP [a combination of enzyme and DTT]) to remove some of the globulin coating on the RBCs and/or to increase the capacity of the RBCs to adsorb autoantibody from the serum. If the patient has been transfused recently, e.g., in the last 3 or 4 months, then autologous RBCs must be obtained to determine if the reactivity is autoantibody prior to reporting it as such. Two methods used to obtain autologous RBCs from posttransfusion samples are reticulocyte separation by weight-based gradient separation1 and separation of hemoglobin S positive RBCs by hypotonic wash.2 Once the autologous RBCs are obtained, they may be insufficient in volume to use for routine adsorption but they can be cleared of coating globulins and tested with the patient’s serum and eluate to determine if there is reactivity directed against the patient’s RBCs.

If the patient has been transfused in the last 3 to 4 months, then an autoadsorption should not be performed to determine if underlying alloantibodies are present, because a small amount of transfused RBCs can adsorb autoantibody.3 One method that can be used to determine if alloantibodies are present in a transfused patient with autoantibodies is allogeneic adsorption (adsorption of the patient’s serum/plasma with allogeneic RBCs).4 The selection of RBCs for the adsorptions is critical; there must be adsorption of autoantibody onto RBCs expressing some antigens and lacking others so that the commonly encountered, potentially clinically significant antibodies can be identified in the adsorbed serum. The inherent risk with allogeneic adsorptions is that, if there is an antibody to a high-frequency antigen, it will be adsorbed by the allogeneic RBCs and not be detectable. Usually, the following RBC samples are chosen: R1, R2, and rr; at least one sample lacking K; randomly selected RBCs negative for Jkα, Jkβ, S, s; and RBCs treated with enzyme or ZZAP so that they are effectively negative for Fyα, Fyβ, M, and N. In general, consideration is usually not given to Leα, Leβ, or P1 unless there is a suspicion that antibodies to those antigens are present. Variations of allogeneic adsorptions include utilizing untreated cells (if the autoantibody is directed at an enzyme-sensitive antigen) or adding LISS or PEG to the serum-RBC mixture.5–11 The use of PEG for adsorptions has been the topic of controversy because, in some studies, loss of alloantibody reactivity has been reported.7,8,12 Adsorptions can be performed at 37°C or at 4°C to remove primarily warm or cold autoantibodies. A combination of temperatures is often used for those antibodies showing reactivity in room temperature (RT) and 37°C phases (sometimes referred to as warm/cold or combination autoantibodies). The selection of the number of adsorptions that one performs varies between IRLs; some do one more adsorption than the strength of the serum reactivity (e.g., two adsorptions for an antibody reacting 1+; some use more adsorptions if testing is performed with PEG), and some use the strength of the DAT to determine the number of adsorptions. Others monitor the strength of the DAT on the adsorbing cells or test the adsorbed serum against fresh adsorbing cells to determine if more adsorptions need to be done. This has the disadvantage of delaying adsorptions and makes the turnaround time longer.

37°C reactive autoantibodies

Case #1: A 68-year-old African American man is admitted to the emergency room (ER) with a Hb of 4.5 g/dL. He is pale and short of breath. The admitting resident requests four units of leukoreduced RBCs as soon as possible. The antibody screen is 3+ positive at the AHG phase with all three screening RBCs. Table 1 shows the initial panel results, with reactivity with a few RBCs at the 37°C phase, corresponding to anti-E specificity, and reactivity with all RBCs at the AHG phase, including the autologous control. The hospital refers the sample to the local blood center IRL. The ER later calls to say that the patient was transfused a month ago at another hospital. The hospital blood bank calls the technologist at the IRL, who is just finishing the testing on the autoadsorbed serum. Anti-E is present in LISS at 37°C and AHG phases (see results in Table 2). Because the patient has been recently transfused, the IRL repeats the adsorptions using enzyme-treated allogeneic RBCs. Four allogeneic adsorptions are performed using the RBC samples listed in the bottom of Table 3. The results of the allogeneic adsorptions are shown in Table 3. The results show that the patient has an antibody reactive with all RBCs tested (from the initial panel, Table 1), the suspected anti-E, and something else, possibly anti-K (reactive against
Another possibility is that this reactivity is due to an HLA antibody. Further tests show that this is an anti-K. The anti-K was not present in the autoadsorbed serum, perhaps due to remaining transfused RBCs in the patient's sample adsorbing the weak reactivity. The reactivity with all RBCs tested (Table 1) is presumed to be due to an autoantibody even though autoadsorption was not valid due to the presence of transfused RBCs.

**Case Comments:** Results in this case suggest that the patient's broadly reactive antibody has autospecificity; the proof of an autoantibody would be adsorption of the antibody onto autologous RBCs. In the case of a transfused patient, autologous cell

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**Table 1. Initial panel results—case #1**

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**Table 2. IRL testing on the autoadsorbed serum—case #1**

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<th>37°C LISS</th>
<th>Anti-IgG</th>
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**Table 3. The results of four allogeneic adsorptions—case #1**

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<th>37°C LISS</th>
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</table>

*enzyme-treated RBCs used for the allogenic adsorptions

RBC suspension #2). Another possibility is that this reactivity is due to an HLA antibody. Further tests show that this is an anti-K. The anti-K was not present in the autoadsorbed serum, perhaps due to remaining transfused RBCs in the patient's sample adsorbing the weak reactivity. The reactivity with all RBCs tested (Table 1) is presumed to be due to an autoantibody even though autoadsorption was not valid due to the presence of transfused RBCs.
Cold-reactive autoantibodies

Some laboratories approach the detection of underlying alloantibodies, in the presence of suspected cold-reactive antibodies, by using a prewarming method. This is not recommended until it has been proved by autoadsorption that the antibody is an autoantibody, as incorrect use of the technique has been reported to yield incorrect results.13 Excellent discussions of the pros and cons of prewarming were published in 1995 and 1996.14–16 Once the autoreactivity of the broadly reactive antibody is defined, prewarmed testing may be of value in the assessment of underlying alloantibodies and in the characterization of the clinical significance of the reactivity. Caution is advised, however, as the loss of potentially clinically significant alloantibody reactivity in prewarmed testing has been described.17–19 Autoadsorption at cold temperatures (4°C) is the method of choice to resolve cases of suspected cold autoantibodies. If the patient has been transfused, allogeneic adsorption in cold temperatures as well as use of rabbit RBCs for adsorption can be used. It is essential to read package inserts for use of rabbit RBCs or stroma, as other antibodies may also be adsorbed.

Case #2: A sample is received in the blood bank from a patient scheduled for cardiac surgery the next day. The antibody screen by gel shows strong reactivity with many RBCs in the top layers of the gel and some RBCs streaming through the gel in all tests. The panel gives similar results (all RBCs are reactive) and the sample is sent to the local IRL, as the hospital does not test in tubes and has exhausted its resources. The IRL’s routine approach involves tube testing in albumin (immediate spin [IS], RT for 15 minutes, 37°C for 30 minutes with albumin, and AHG phase using anti-IgG) and against ficin-treated cells (37°C for 15 minutes, AHG phase with anti-IgG). The IRL observes 1+ reactivity at IS, 3+ at RT, 1+ agglutination and a trace of hemolysis at 37°C, and + reactivity at the AHG phase that could reflect carryover from 37°C (see Table 4). The ficin-treated RBC panel was completely hemolyzed at 37°C and no RBCs were left to read at the AHG phase. The DAT was 3+ with anti-C3. Four adsorptions with autologous RBCs treated with ZZAP were performed at 4°C. In this case, the patient’s sample was sufficient to allow four aliquots of treated autologous RBCs to be

| Panel cells | D | C | E | c | e | f | K | k | Fyα | Fyβ | Jkα | Jkβ | M | N | S | s | P1 | IS | RT | 37°C | 37°C |
|-------------|---|---|---|---|---|---|---|---|-----|-----|-----|-----|---|---|---|---|---|---|---|---|---|---|
| 1           | + | + | 0 | 0 | + | 0 | 0 | + | + | + | + | 0 | + | 0 | + | 0 | 1+ | 3+ | 1+th* | +w | h1 | nc |
| 2           | + | 0 | 0 | + | + | + | 0 | + | + | 0 | 0 | + | 0 | 0 | + | 0 | 1+ | 3+ | 1+th | +w | h | nc |
| 3           | + | 0 | 0 | + | + | 0 | 0 | 0 | + | + | 0 | + | + | + | + | + | 0 | 1+ | 3+ | 1+th | +w | h | nc |
| 4           | 0 | 0 | 0 | 0 | + | + | + | 0 | 0 | + | + | 0 | 0 | 0 | + | 0 | + | 1+ | 3+ | 1+th | +w | h | nc |
| 5           | 0 | 0 | 0 | + | + | + | 0 | 0 | + | 0 | + | + | 0 | + | 0 | + | + | 1+ | 3+ | 1+th | +w | h | nc |
| 6           | 0 | 0 | 0 | + | + | + | 0 | 0 | + | 0 | + | + | + | 0 | 0 | + | 0 | 1+ | 3+ | 1+th | +w | h | nc |
| 7           | 0 | 0 | 0 | + | + | + | 0 | 0 | + | 0 | + | + | + | 0 | 0 | + | 0 | 1+ | 3+ | 1+th | +w | h | nc |
| 8           | 0 | 0 | 0 | + | + | + | 0 | 0 | + | 0 | + | + | + | 0 | 0 | + | 0 | 1+ | 3+ | 1+th | +w | h | nc |
| 9           | 0 | 0 | 0 | + | + | + | 0 | 0 | + | 0 | + | + | + | 0 | 0 | + | 0 | 1+ | 3+ | 1+th | +w | h | nc |
| 10          | 0 | 0 | 0 | + | + | + | 0 | 0 | + | 0 | + | + | 0 | 0 | + | 0 | 1+ | 3+ | 1+th | +w | h | nc |
| 11          | 0 | 0 | 0 | + | + | + | 0 | 0 | + | 0 | + | + | 0 | 0 | + | 0 | 1+ | 3+ | 1+th | +w | h | nc |

*Trace hemolysis
†Completely hemolyzed
‡No cells remaining

Table 4. IRL results of panels using serum from case #2
Review: all RBCs incompatible—serologic aspects

Prepared. In some cases, insufficient RBCs are available, due either to the patient's Hb or to the sample size submitted. In those cases, it may be necessary to use the same aliquot of autologous RBCs over and over and re-treat the aliquot between adsorptions. The autoadsorbed serum was nonreactive when tested with a set of three antibody screening cells. When the report of a cold autoantibody was given to the hospital, the cardiac surgeon requested a cold agglutinin titer and thermal amplitude study to determine if this would interfere with the surgery and the plans to use cell salvage techniques. A thermal amplitude study was performed with dilutions of the patient's serum in saline with the following results (titers): 30°C = 8, 22°C = 64, 4°C > 2048. The surgery could not be postponed. After consultation with the medical director of the IRL, hospital blood bank physician, and surgeon, it was decided that the apparent cold agglutinin might be a problem with cell salvage at RT and cold cardioplegia might result in agglutinated autologous RBCs in the coronary arteries. In the surgery, the blood was first drained from the heart and then cold was applied. Cell salvage was not used and the temperature was not lowered below normal RT. The patient did well and received two units of blood, which were given through a blood warmer.

Case Comments: This patient had serologic results consistent with a diagnosis of cold agglutinin syndrome; the patient's Hct was 33%. This had not been detected prior to scheduling surgery. The patient's pathologic cold agglutinin was more of a concern for cardiac surgery than for other surgeries because the temperature of the patient is lowered in cardiac surgery.20

Multiple Antibody Specificities

Often the presence of multiple antibodies can be discerned by the differences seen in serologic phases or strengths of reactivity with panel RBCs in tube testing and in the visual appearance of positive reactions in gel testing. Depending upon the configuration of the panel and the antibodies present in the patient's sample, nonreactive cells may be obtained or reactivity indicating dosage effects or separate antibodies may be discerned. These features can be used to differentiate the presence of multiple antibody specificities from an antibody to a high-frequency antigen. The use of enzyme-treated RBCs can also be helpful in differentiating antibody specificities.

Case #3: A sample from a patient with sickle cell disease was received with a request for two units of blood for outpatient transfusion the next day. The antibody screen was positive. Cell I was 2+, cell II was 1+, and cell III was 3+ in the AHG phase. A panel was tested and there was variable reactivity with 16 of 16 RBC suspensions tested. The blood bank requested that the IRL evaluate the sample, as it was apparent the hospital would not have sufficient RBC panels to evaluate the sample, even though they saved the most recently expired panels. At the IRL, the patient's RBCs typed as D+C−E−c+c+e+ in the routine Rh phenotype that is performed on all new patients.

Table 5. IRL special panel configured for case #3

| Panel cells | D | C | E | c | e | f | K | k | Fyα | Fyβ | Jkα | Jkβ | M | N | s | s | Luα | Luβ | P1 | IS | 37°C | Anti-IgG | 37°C | Anti-IgG |
|-------------|---|---|---|---|---|---|---|---|-----|-----|-----|-----|---|---|---|---|-----|-----|---|----|-----|-----|-----|
| 1           | + | 0 | 0 | + | + | + | 0 | + | 0 | + | 0 | + | + | + | + | 0 | 0 | 0 | 0 | 2+ | 0 | 0 | 3+ |
| 2           | + | 0 | 0 | + | + | + | 0 | + | 0 | + | 0 | + | + | + | 0 | 0 | 0 | 0 | 2+ | 0 | 0 | 3+ |
| 3           | 0 | 0 | 0 | + | + | + | 0 | + | 0 | + | + | 0 | 0 | + | 0 | 0 | + | 0 | 0 | 1+ | 0 | 0 | 3+ |
| 4           | 0 | 0 | 0 | + | + | + | 0 | + | 0 | + | 0 | + | + | 0 | 0 | 0 | + | + | 0 | 0 | 1+ | 0 | 0 | 3+ |
| 5           | 0 | 0 | 0 | + | + | + | 0 | + | 0 | 0 | 0 | + | + | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 3+ | 0 | 0 | 3+ |
| 6           | + | 0 | 0 | + | + | + | 0 | + | 0 | 0 | 0 | + | + | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 3+ | 0 | 0 | 3+ |
| 7           | + | 0 | 0 | + | + | + | 0 | 0 | 0 | + | + | 0 | + | 0 | + | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3+ | 0 | 0 | 3+ |
| 8           | 0 | 0 | 0 | + | + | + | 0 | 0 | 0 | + | 0 | + | + | 0 | 0 | + | 0 | 0 | 0 | 0 | 3+ | 0 | 0 | 0 | 0 | 3+ | 0 | 0 | 3+ |
| 9           | + | + | 0 | 0 | + | 0 | 0 | + | 0 | 0 | 0 | + | + | + | 0 | + | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3+ | 0 | 0 | 3+ |
| 10          | + | + | 0 | 0 | + | 0 | 0 | + | 0 | 0 | 0 | + | + | + | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3+ | 0 | 0 | 3+ |
| 11          | + | + | 0 | 0 | + | 0 | 0 | + | 0 | 0 | 0 | + | + | + | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3+ | 0 | 0 | 3+ |
| 12          | + | + | 0 | 0 | + | 0 | 0 | + | 0 | 0 | 0 | + | + | + | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3+ | 0 | 0 | 3+ |

Case Comments: This patient had serologic results consistent with a diagnosis of cold agglutinin syndrome; the patient's Hct was 33%. This had not been detected prior to scheduling surgery. The patient's pathologic cold agglutinin was more of a concern for cardiac surgery than for other surgeries because the temperature of the patient is lowered in cardiac surgery.20

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The IRL panel was configured by the staff so that the first six RBC suspensions were C-E-K- and the rest of the panel (six RBC suspensions) was designed to rule in anti-C, -E, or -K if they were present. The routine testing at the IRL included a LISS panel and a ficin-treated RBC panel run in parallel. The results are shown in Table 5. The use of enzyme-treated RBCs demonstrated the likelihood of anti-Fy\(^a\). Subsequent studies proved that anti-C, -E, -K, -Fy\(^a\), and -Jk\(^a\) were present in this patient's sample.

**Case Comments:** Utilizing information obtained from the patient's Rh phenotype made antibody identification easier. Additionally, the IRL studied the panel that had been performed at the hospital and saw that there was variation in reactivity. With the knowledge that the first antibodies formed often have Rh specificity, that the K antigen is very immunogenic, and that the hospital panel indicated those specificities could cause the reactivities seen, a custom panel was designed. Since the IRL obtains panels from all manufacturers, it was easy to make a “custom” panel to perform the testing. Custom panel configuration is facilitated with computer software called Antigen Plus Ab-ID (Rowny Systems, Inc., Gaithersburg, MD), which allows the user to select the RBCs (by phenotype) for testing. This program utilizes electronic information from the panel manufacturer (Immucor/Gamma, Norcross, GA; Olympus America, Melville, NY), which allows mistake-free entry into Antigen Plus. Ortho-Clinical Diagnostics (Raritan, NJ) does not yet provide this information; the user must manually enter the phenotype information from their panels. Then the user requests the desired phenotype of RBCs and the software peruses the database and presents choices to the user. In this way, only RBCs that are of the needed phenotype are tested, thus conserving serum (and the reagent RBCs); the phenotype information on the selected RBCs is made into a panel worksheet by the software, thus preventing handwriting and associated errors. The information is presented in a panel format with space for recording test results.

By using all the resources available, the IRL was able to resolve the case by testing two panels in about 3 hours. If the IRL does not have access to RBCs with the phenotypes needed for testing, allogeneic adsorptions of the patient's serum may require high numbers of adsorptions to adsorb out some specificities and leave others behind. It should be remembered that very strongly reactive antibodies may require high numbers of adsorptions to be completely removed. Increasing the number of adsorptions does increase the risk of diluting the serum and thus missing weak antibodies.

**Antibodies to High-Frequency Antigens**

**Case #4:** A 59-year-old Caucasian man was on dialysis for kidney failure. Two units of blood were requested for transfusion with his next dialysis procedure (EPO was not yet available). The antibody screen was positive (3+ with all cells by the IAT). A panel was tested and all RBCs were reactive (3+); autologous RBCs were nonreactive. Samples were sent to the IRL.

**Case #5:** A 36-year-old Hispanic woman had a history of six pregnancies and four transfusions (associated with two of the deliveries). There was no history of HDN. She was admitted with a diagnosis of spontaneous abortion. The antibody screen and panel were positive (2+ with all cells by IAT); autologous RBCs were nonreactive. Samples were sent to the IRL.

When antibodies to antigens of high frequency are present, the resolution can be time- and resource-consuming. The first sign that an antibody to a high-frequency antigen is present is usually uniform reactivity with all reagent RBCs tested; commonly, this reactivity is only at the antiglobulin phase of testing. In the majority of cases, the autologous RBCs are nonreactive, but sometimes they are reactive due to the presence of recently transfused RBCs (the reaction may have a mixed-field appearance) or due to another unrelated cause (medication, nonspecific, etc.). Approaches to resolution of the antibody's specificity include testing RBCs that have been treated with different agents, e.g., enzyme or DTT, and looking for enhancement or diminution of reactivity. Additionally, even though the use of a high titer value to steer the investigation toward a Knops (KN) system antibody is downplayed by some experts, one of the authors (STN) still finds it valuable. If the antibody's titer is equal to or greater than 32, DTT-treated RBCs are nonreactive, and the KN null cell is nonreactive, STN's laboratory reports the antibody as a probable KN system antibody and stops the investigation. This approach was approved by the Technical Advisory Committee (customers).

In the evaluation of an antibody to a high-frequency antigen, the IRL's frozen rare RBC and serum resources are usually utilized to the full extent to identify the antibody. If the IRL does not have ABO-compatible RBCs negative for all high-frequency
antigens for testing against the patient’s serum, then the presence or absence of those antigens on the patient’s RBCs becomes important. It is helpful to know the patient’s race when choosing which antigens to test for and it is important to know the patient’s transfusion history when interpreting the RBC typing results. Although IRLs are continually seeking to freeze more rare sera and RBCs, these are generally not available commercially and are obtained through resource sharing groups like SCARF and an exchange program in the American Red Cross IRLs known as TRANSFERASE. Some of the “more common” high-frequency, antigen-negative RBCs that may be available on commercial panels are k–, I–, Yt(a–), Js(b–), and Kp(b–), especially if a review of recently outdated panels is done. If expired panel RBCs are used and a negative reaction is obtained, then additional testing using in-date RBCs or frozen/thawed RBCs is indicated.

If there is a suspicion that a sample contains an antibody to a high-frequency antigen and underlying alloantibodies, it is advisable to identify the antibodies to common antigens first. Test results of RBCs negative for high-frequency antigens may be unproductive if the RBCs are positive for a common antigen that the patient also has an antibody against. Underlying alloantibodies can be identified by first phenotyping the patient for common antigens, then selecting a phenotype-similar RBC sample for adsorptions. If untreated RBCs are used for the first adsorption, then an eluate can be prepared from the adsorbing RBCs, and a pure source of the antibody to the high-frequency antigen will be available. The adsorbed serum should be tested back against the adsorbing RBCs for evidence of complete adsorption, as alloantibodies are more variable in their strength (in comparison to most autoantibodies, which have relatively low titers). The adsorbed serum can be used for identification of antibodies against common antigens that the patient’s RBCs lack, and the eluate from the first aliquot of adsorbing RBCs can be used to test for the antibody to the high-frequency antigen, once any antibodies to common antigens are identified.

The investigation may be assisted through the use of different media (e.g., enzymes). Frequently, the use of a combination of phenotyping the patient’s RBCs (e.g., for Js\(^b\), S, s, Kp\(^b\), I, Jk\(^c\), Jk\(^b\), Lu\(^b\), H) and testing the patient’s serum against “null” RBCs (e.g., cord, O\(_n\), or DTT-treated [K\(_n\)] RBCs) is valuable. Knowledge of the race of the patient may be helpful early in the investigation to choose the more common RBCs negative for high-frequency antigens known to exist in that ethnic group (e.g., U–, Sl[a–], Js[b–] RBCs for African Americans). Other clues include reactivity at RT (e.g., anti-H, -PP, P\(^b\)) or hemolysis of RBCs (e.g., anti-Vel, -P). See Table 6 for a list of antibodies to high-frequency antigens that are suggested by some of the clues mentioned above. This is not a complete list and the reader is referred to the Blood Group Antigen FactsBook. If such investigation is unproductive, testing of the entire collection of RBCs negative for high-frequency antigens or molecular studies are often the solution.

Once the specificity of an antibody to a high-frequency antigen is identified, then the clinical significance must be determined so the hospital can know what to transfuse. There are excellent texts that review the literature on this. For antibodies that are known not to be clinically significant, by either specificity or phase of reactivity, transfusion of random RBCs is recommended. After transfusion, serologic reevaluation of the patient’s sample should be performed for exclusion of other newly developed alloantibodies. Re-identification of existing antibodies is optional.

If the antibody is known to be clinically significant and blood availability is usually not a problem (e.g., k– or Kp[b–] RBCs), then a request can be made to the local blood supplier and blood can be obtained. If the

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**Table 6. Some antibodies to high-frequency antigens to consider**

<table>
<thead>
<tr>
<th>Room temperature reactivity</th>
<th>Hemolysis seen</th>
<th>Decreased reactivity in enzyme</th>
<th>African American patient</th>
<th>Caucasian patient</th>
<th>Asian, Hispanic, Native American patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-H</td>
<td>Anti-Vel</td>
<td>Anti-Ch</td>
<td>Anti-U</td>
<td>Anti-Kp(^b)</td>
<td>Anti-Dl(^b)</td>
</tr>
<tr>
<td>Anti-I</td>
<td>Anti-PP, P(^b)</td>
<td>Anti-Rg</td>
<td>Anti-Js(^b)</td>
<td></td>
<td>Anti-Yt(^a)</td>
</tr>
<tr>
<td>Anti-PP, P(^b)</td>
<td>Anti-P</td>
<td>Anti-In(^b)</td>
<td>Anti-Jo(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Vel</td>
<td>Anti-Jk(^b)</td>
<td>Anti-JMH</td>
<td>Anti-Hy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Sd(^b)</td>
<td>Anti-H</td>
<td>Anti-En(^a)TS</td>
<td>Anti-Cr(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-P</td>
<td>Anti-I</td>
<td>Anti-En(^a)FS</td>
<td>Anti-Tc(^a)</td>
<td></td>
<td>Anti-At(^b)</td>
</tr>
</tbody>
</table>

---
blood is not immediately available from the local blood supplier, the request can go to an American Rare Donor Program member facility. The reader is referred to the next issue of Immunohematology for a discussion of the American Rare Donor Program and the ISBT Rare Donor Program.

If the antibody is directed against an antigen of variable clinical significance, and/or the blood is not immediately available, then in vitro assays to predict in vivo clinical significance should be used. These studies are valuable; if results are negative, blood may be available as liquid random units, thus reducing the logistics and time needed for transfusion, and the rare units can be saved for those patients who cannot receive random units. Patients who should not receive random units are those who have had either a positive in vitro test, a transfusion reaction to random units, or both.

Methods Used to Determine the Clinical Significance of Alloantibodies

Before describing methods used to determine the clinical significance of alloantibodies, it is important to discuss what is meant by the term “clinically significant” in relation to alloantibodies and RBCs. Everyone would agree that an antibody that causes an obvious, clinical hemolytic transfusion reaction (fever, chills, hemoglobinemia, hemoglobinuria, etc.) is a clinically significant antibody and that antigen-negative RBCs are needed for transfusion. What about an antibody that does not cause any overt clinical symptoms, but is associated only with laboratory signs of hemolysis (increased bilirubin, decreased haptoglobin, etc.)? Or an antibody that is not associated with any clinical or laboratory signs of hemolysis, but RBCs incompatible with it survive less than their normal lifespan? The definition of “clinical significance” may vary depending upon the needs of the patient. For a patient with a short-term requirement for blood, e.g., a surgical patient, it may not be important for the transfused RBCs to have completely normal survival. On the other hand, for a patient with a hematological disorder and long-term requirements for blood, it would be more important that the blood survive as long as possible, to limit the total number of transfusions.

The methods used to determine the clinical significance of RBC alloantibodies have been reviewed elsewhere. They include routine serologic methods (which give information about an antibody’s specificity and thermal range), IgG subclassing methods, in vivo survival studies (e.g., $^{51}$Cr and the “biological crossmatch”), and in vitro cellular assays. This review will concentrate only on the latter, the in vitro cellular assays. These include the antibody-dependent cellular cytotoxicity (ADCC) assay (which has only been used to assess the clinical significance of antibodies in the setting of HDFN), the chemiluminescence test (CLT), and the monocyte monolayer assay (MMA); most of this review will focus on the MMA.

The premise of all in vitro cellular assays is to mimic in the laboratory what occurs in vivo when antigen-positive RBCs are transfused into a patient with the corresponding antibody. If the antibody-coated RBCs are destroyed, it will be by one of two mechanisms: intravascular or extravascular. Intra-vascular RBC destruction (hemolysis of RBCs directly in the bloodstream) is a rare event due to the action of complement on the RBC membrane. Most antibodies do not cause RBC destruction via this mechanism, but rather by the extravascular mechanism, which involves destruction of RBCs via macrophages in the liver or spleen. Macrophages recognize and attach to RBCs that are coated with IgG and/or C3 via specific receptors on the macrophage. The attached RBCs are then destroyed in three ways: the RBCs can be completely engulfed (phagocytized) by the macrophage, the RBCs can be partially phagocytized and the resulting spherocytes trapped and destroyed in the spleen, or the RBCs can be lysed extracellularly by enzymes secreted by the macrophage. In vitro cellular assays attempt to mimic extravascular RBC destruction.

In all in vitro cellular assays, RBCs are sensitized with the antibody in question and are then incubated with mononuclear cells (usually monocytes, the precursors of macrophages). The in vitro reactions of the monocytes with the antibody-coated RBCs are then ascertained by (1) visual inspection for attachment and/or phagocytosis of RBCs by monocytes (MMA), (2) measurement of oxygen radicals released by monocytes during phagocytosis (CLT), or (3) measurement of direct hemolysis of $^{51}$Cr-labeled RBCs (ADCC). The MMA and CLT assays, which have been used to determine clinical significance of alloantibodies for transfusion purposes, have a cutpoint, or value above which the test is considered to be positive. When a positive result is obtained, it is recommended that antigen-negative blood be transfused in order to avoid an overt transfusion reaction.
A number of variations of the MMA have been performed by different investigators over the past 25+ years. Many of the variables involved in the MMA have been reviewed; they include the source of monocytes, the RBC sensitization procedure (including whether or not fresh normal serum as a source of complement is added), the "culture" conditions (e.g., a CO₂ atmosphere versus ambient air), and how the results are expressed. The assay currently used by the American Red Cross (Southern California and Penn-Jersey Regions) is as follows: mononuclear cells from one or more donors are isolated using Ficoll-Hypaque and incubated in ambient air on a glass slide, the nonadherent lymphocytes are suctioned off, the sensitized RBCs (sensitized with and without fresh complement present) are added to the monocyte monolayer on the slide and incubated in ambient air; the nonadherent RBCs are removed (via suctioning and washing), and the slide is stained and examined microscopically for the percentage of monocytes with RBCs adhering and/or phagocytized.

When the assay currently used by the American Red Cross was originally set up, a cutpoint of 3 percent monocyte reactivity was selected based on the results of unsensitized RBCs in the assay. The Southern California Region later used statistical methods and data from patients who had received incompatible transfusions to determine that a more appropriate cutpoint for their assay was 5 percent reactivity. Using this 5 percent cutpoint, the sensitivity of the MMA is 100 percent (no false negatives) and the specificity is 61 percent (there are false positives).

If a result greater than the cutpoint is obtained when testing an antibody, the MMA is considered to be positive, and to avoid an overt transfusion reaction, antigen-negative blood is recommended as the optimal choice for transfusion. When a result less than the cutpoint is obtained when testing an antibody, the MMA is considered to be negative; antibody should not cause acute hemolysis of transfused antigen-positive RBCs at that time. A negative MMA, however, does not guarantee that transfused, sensitized RBCs will have normal long-term survival. If a patient with a negative MMA is transfused with antigen-positive RBCs, the exposure to more antigen may cause the antibody to change its characteristics (e.g., strength/titer or subclass composition). Thus, the MMA must be repeated in these patients prior to transfusion of more antigen-positive RBCs. There are reports of negative MMAs becoming positive after transfusion, as well as of positive MMAs becoming negative after transfusion. In general, once a positive MMA result is obtained, further MMAs are not performed.

**Case #4 (continued):** The antibody detected in the Caucasian patient's serum was identified as anti-Ge. The patient received Ge- units for 10 years, until the supply of Ge- RBCs in the country was almost depleted. An MMA was requested and found to be negative (2.8%). The patient received two Ge+ units during dialysis with no untoward reaction noted. The Hb rose as expected and there was no rise in bilirubin. One week after the transfusion, the MMA was repeated and was still negative (2.7%); the antibody reactivity was 2+. The patient received two more Ge+ units without an obvious reaction and there was an expected rise in Hb. The MMA was repeated three more times over the next several months. Each time the MMA was negative and the patient was able to successfully receive Ge+ RBCs. The strength of the anti-Ge weakened; it was only microscopically reactive in the last sample. The patient was then started on EPO and required no further transfusions.

**Case #5 (continued):** The antibody in the Hispanic woman's serum was identified as anti-Ytá. An MMA was requested and found to be negative (0%). Two months later, during an emergency, the patient was transfused with two Ytá(+) units of blood. Her DAT became transiently positive but no clinical symptoms of a reaction were observed. A month later another MMA was requested in anticipation of a scheduled hysterectomy. The MMA was now positive (23%) and the patient received two Ytá(--) units during her surgery.

These two patients demonstrated different outcomes after transfusion of incompatible RBCs. Patient #4's antibody became progressively weaker and the MMAs remained negative. Patient #5's antibody changed its characteristics so that antigen-negative blood became the better choice for transfusion.

Tables 7 and 8 summarize data from published reports on alloantibodies to high-frequency antigens that may be of variable or unknown clinical significance. In Table 7, MMA results from 29 such antibodies are compared with the results of 51Cr survival studies. 51Cr survival study results are commonly expressed as the percentage of incompatible RBCs surviving at 1 hour and 24 hours.
after transfusion of a small dose of RBCs and/or as the half-life of those RBCs ($T_{50\text{Cr}}$). The International Committee for Standardization in Haematology published the following guideline with respect to using the $^{51}\text{Cr}$ survival study as a compatibility test: “In cases of urgency or when there is great difficulty in finding donor red cells may be transfused with minimal hazard when, following a test with 0.5 ml of the donor’s red cells, . . . the red-cell survival at 60 minutes is not less than 70%.” It is known that a normal 1-hour $^{51}\text{Cr}$ survival result does not mean that the transfused incompatible RBCs will survive normally (i.e., the 24-hour survival or $T_{50\text{Cr}}$ may be abnormal). As it can be difficult to have $^{51}\text{Cr}$ survival studies performed, in vitro assays (e.g., the MMA) were developed to obtain the same information as a 1-hour $^{51}\text{Cr}$ survival. Of the 29 reports in Table 7, only 16 included the 1-hour $^{51}\text{Cr}$ survival data. Results in Table 7 are divided into those which had 1-hour $^{51}\text{Cr}$ survival data available and those that did not (e.g., data were available from other times posttransfusion or the authors summarized the interpretation of the $^{51}\text{Cr}$ survival study without giving actual data).

Although most MMA and $^{51}\text{Cr}$ results concurred, there are eight results in Table 7 that appear to be discrepant: one antibody with a negative MMA had an abnormal 1-hour $^{51}\text{Cr}$ survival study and seven antibodies with positive MMAs had normal 1-hour $^{51}\text{Cr}$ survival results (six had normal 1-hour results). The other antibody (anti-Lu6) with a positive MMA and a normal 51Cr result was associated with a median survival of 26 days, and thus appears to be falsely positive.

Table 8 shows data on 56 antibodies where MMAs were performed and the patients were transfused with incompatible RBCs (unfortunately, in many cases it is not

<table>
<thead>
<tr>
<th>Antibody Specificity (references)</th>
<th>MMA = Negative Hemolytic txn reaction</th>
<th>MMA = Positive Hemolytic txn reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yta§,34,35,37,43,44,51,52,53</td>
<td>22 0</td>
<td>5 4 (1/3/0)</td>
</tr>
<tr>
<td>Ge34,36,37,39,46</td>
<td>6  5 0</td>
<td>0 1 (0/0/1)</td>
</tr>
<tr>
<td>Cr47,48</td>
<td>4  2 0</td>
<td>1 1 (0/1/0)</td>
</tr>
<tr>
<td>Tca8,9</td>
<td>1  0 0</td>
<td>0 1 (0/1/0)</td>
</tr>
<tr>
<td>Lu6,7</td>
<td>1  1 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Lu88</td>
<td>1  0 0</td>
<td>0 1 (1/0/0)</td>
</tr>
<tr>
<td>Hy65</td>
<td>1  0 0</td>
<td>0 1 (0/0/1)</td>
</tr>
<tr>
<td>Gv40</td>
<td>1  0 0</td>
<td>0 1 (0/1/0)</td>
</tr>
<tr>
<td>Jo52</td>
<td>1  0 0</td>
<td>1 0</td>
</tr>
<tr>
<td>Jr55,71,72</td>
<td>4  3 0</td>
<td>0 1 (1/0/0)</td>
</tr>
<tr>
<td>Lan7</td>
<td>1  1 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Dr73</td>
<td>1  0 0</td>
<td>0 1 (0/1/0)</td>
</tr>
<tr>
<td>Dr74,75</td>
<td>1  1 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Er75</td>
<td>1  1 0</td>
<td>0 0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>56 35 0</td>
<td>7 13 (3/8/2)</td>
</tr>
</tbody>
</table>
known if the MMA was performed before or after the transfusion). In these cases, information was given as to whether or not there was evidence for a hemolytic transfusion reaction, but often not many details were given. The results in Table 8 listed under the “Hemolytic transfusion reaction” heading are subdivided into those said to have an immediate reaction or clinical signs; those with a mild, delayed reaction or only laboratory signs of a reaction; and those with no detailed information. Although most MMA results agreed with the results of transfusion, there appear to be seven discrepant results in Table 8: seven patients with positive MMAs had no signs of a transfusion reaction and the MMA results appear to be falsely positive.

There are other single-case reports in the literature, on patients with antibodies to high-frequency antigens with variable or unknown significance, where MMAs were performed but the patients were not transfused with incompatible blood. One study reported MMA data on 251 alloantibodies; 192 of these alloantibodies were directed against high-frequency antigens of variable or unknown clinical significance. The majority (68%) of these 192 alloantibodies were positive in the MMA. Thus, one should presume that most alloantibodies to high-frequency antigens have the potential to be clinically significant, unless proven otherwise by MMA or ⁵¹Cr survival studies.

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Review: transfusing incompatible RBCs—clinical aspects

G. Meny

Introduction

“As soon as the blood began to enter into his veins, he felt the heat along his arm and under his armpits. His pulse rose and soon after we observed a plentiful sweat over his face. His pulse varied extremely at this instant and he complained of great pains in his kidneys, and that he was not well in his stomach, and that he was ready to choke unless given his liberty. He was made to lie down and fell asleep, and slept all night without awakening until morning. When he awakened he made a great glass full of urine, of a color as black as if it had been mixed with the soot of chimneys.”

The above description of the signs and symptoms following a blood transfusion were published by Jean Denis, a physician working for King Louis XIV in 1668. We can thank Dr. Denis for providing a vivid and classic description of what is now known to be a hemolytic transfusion reaction (HTR). Various in vitro (or, less frequently, in vivo) immunohematologic techniques which attempt to prevent HTRs have evolved over the years. The “goal” is to predict compatibility and transfuse RBCs which will survive and function as well as the recipient’s own RBCs. One is more comfortable transfusing “compatible” RBCs. Nonetheless, there are times when “incompatible” RBCs must be transfused. This paper will review clinical aspects of this decision.

Issues for Consideration

Issues that should be considered when caring for a patient requiring incompatible blood are: “Is transfusion really necessary?” “Is the antibody(ies) clinically significant?” and “How should the transfusion be administered?”

When to transfuse incompatible RBCs

It can be difficult to state with certainty when to transfuse incompatible RBCs to an anemic patient as there are many variables which play a role in individuals’ responses to their Hb (or Hct) levels. Most physicians still utilize the Hb level as the “trigger” for transfusion. There is no absolute Hb level below which a patient will not survive and that makes it difficult when considering transfusing incompatible RBCs.

The signs and symptoms experienced by a patient are usually consistent with the rate at which the anemia developed. Weiskopf et al. found that acute isovolemic anemia to a Hb level of < 6 gm/dL resulted in mild, reversible changes in memory reaction time, while < 5 gm/dL impaired immediate and delayed memory. Cardiovascular condition and age are also important factors to consider when deciding if (and when) transfusion is necessary. Cardiovascular compensatory mechanisms to anemia, such as increase in heart rate, increase in stroke volume, and decrease in peripheral vascular resistance, are impaired in patients with cardiovascular diseases and (usually) in the elderly. Carson et al. retrospectively analyzed the 30-day mortality of 1958 surgical patients >18 years of age who refused transfusion for religious reasons. They compared outcome in those with and without cardiovascular disease (history of angina, myocardial infarction, congestive heart failure, or peripheral vascular disease). Mortality was 1.3 percent (0.8% to 2.0%) in patients with a preoperative Hb of 12 g/dL or greater and 33.3 percent (18.6% to 51.0%) in patients with a preoperative Hb < 6 g/dL. A correlation noted between serious morbidity and blood loss in the low preoperative Hb group was more apparent in patients with cardiovascular disease than those without cardiovascular disease. Hebert et al. published a multicenter, randomized, controlled study examining whether a restrictive or liberal transfusion strategy had an effect on mortality in critically ill patients with euovia. Patients were randomly assigned to maintain their Hb to either between 7.0 and 9.0 g/dL (restrictive transfusion strategy) or between 10.0 and 12.0 g/dL (liberal transfusion strategy). Interestingly, 30-day mortality rates were similar in the two groups and, in fact, were significantly less in the restrictive group in
individuals < 55 years of age and in those with an Acute Physiology and Chronic Health Evaluation II score of < 20. This benefit was lost, however, in critically ill patients with acute myocardial infarction and unstable angina.

Tachycardia and postural hypotension, which are usually present in acute blood loss, are not often seen with chronic anemia. A patient with chronic anemia may have an increased blood volume due to an overexpanded plasma volume. Rapidly administering blood to a patient with severe chronic anemia may precipitate cardiopulmonary decompensation (or circulatory overload). Indeed, circulatory overload is an important and underrecognized complication of transfusion, particularly in the elderly. See Popovsky for a review on this transfusion complication.

Thus, in summary, while otherwise healthy patients usually will require a transfusion when their Hb declines to < ~ 6 to 7 g/dL, these patients may be able to tolerate and compensate at these low Hb levels without transfusion, particularly if the anemia has slowly evolved. However, this does not apply to the patient with cardiovascular disease.

Note that the preceding discussion applies to patients for whom compatible or incompatible RBCs are available for transfusion. Decisions to transfuse compatible blood in urgent situations are frequently made with little hesitation. However, transfusing incompatible blood is frequently delayed until the patient’s life is in serious jeopardy. Transfusion of incompatible blood must occur where there is an urgent, life-threatening clinical need, and transfusion should not be withheld because of the serologic findings.

Is the antibody clinically significant?

As one is evaluating the patient’s clinical status and determining the urgency of the RBC transfusion requirement, frequently asked simultaneous questions include: “How clinically significant is this RBC antibody?” and “What will happen to the RBCs if transfused—will they be destroyed intravascularly? extravascularly? how rapidly?” Some antibodies are known to be clinically significant (i.e., cause hemolysis), such as anti-A, -B, -D, and -K. Others are usually not considered clinically significant, such as anti-Le4. M.E. Reid and C. Lomas-Francis have written an excellent source book on blood group antibodies for use by transfusionists. This book, as well as a literature search (e.g., the National Library of Medicine’s PubMed at http://www.ncbi.nlm.nih.gov/PubMed) can provide information on general “tendencies” of an antibody’s clinical significance. Thus, if time is available, in vitro testing of the patient’s antibody may be desirable and help predict, for example, which RBC units (antigen positive or antigen negative) should be transfused. The paper in this issue by Nance and Arndt provides additional information on useful laboratory assays, such as the monocyte monolayer assay (MMA).

In vivo testing is rarely performed, although the 1-hour 51Cr RBC survival study recommended by the International Committee for Standardization in Hematology is the gold standard for predicting clinical significance of a patient’s antibody. A radiolabeled 0.5 mL sample of incompatible RBCs is injected into the patient and samples are obtained at 10 and 60 minutes. If radioactivity in the plasma at 10 and 60 minutes is < 3% of the total injected and the RBC survival at 60 minutes is at least 70%, then transfusion of the incompatible blood carries minimal risk. Most facilities are not equipped to perform this type of in vivo testing.

Another in vivo test which may be performed is known as the “in vivo crossmatch” or “biological crossmatch.” 10 to 50 mL of unlabeled incompatible RBCs is transfused and a sample is collected from the patient posttransfusion to check for hemoglobinemia. This test only detects intravascular hemolysis. Extravascular RBC destruction cannot be predicted.

The transfusion

If, and when, the decision is made to transfuse, how can the process be made as free of adverse events as possible? It is best to always transfuse using leukocyte-reduced blood components. Although the patient’s clinical condition may not warrant their use, leukocyte-reduced blood components can prevent febrile nonhemolytic transfusion reactions, which are complications of transfusion and can mimic a HTR. For example, one does not want to discontinue a RBC transfusion to a patient with multiple alloantibodies and autoantibodies because of fever (due to a febrile nonhemolytic transfusion reaction). Premedication with antipyretics may mask the fever of a HTR, but other signs and symptoms of hemolysis will still be present. Premedication with an antipyretic or antihistamine (for patients with a history of allergic reactions) may be administered immediately prior to transfusion if administered intravenously. They should be given 30 to 60 minutes prior to the start of the
transfusion if given orally. Some patients may benefit from receiving corticosteroids or IVIG (see Case Studies section of this paper).

If possible, transfuse the patient during a time when adequately trained staff is available to monitor the transfusion. This includes laboratory staff available to interpret any adverse reactions, should they develop. The most knowledgeable individuals for all aspects of a transfusion are usually available during the day, rather than at night.

Systems must be in place to select the unit and issue and match it to its intended recipient. As with any transfusion, make certain that an informed consent which complies with applicable laws is obtained. Vital signs, including blood pressure, pulse, and temperature, should be recorded prior to initiating the transfusion. The patient should be kept well hydrated during the transfusion. Once the transfusion is started, the transfusionist should remain with and observe the patient for at least the first 15 minutes. Vital signs should be documented at that time. The patient should be observed at frequent intervals. Vital signs should be recorded at the end of the transfusion.

Davenport summarized the most frequent clinical signs and symptoms observed in 90 cases of intravascular HTRs and 101 cases of extravascular HTRs (Table 1). Intravascular hemolysis, for example, could be observed when transfusing Vel+ RBCs to a patient with anti-Vel. During extravascular reactions, the membrane attack complex is not generated. RBCs become sensitized with IgG antibodies, complement, or both, and are removed by macrophages within the reticuloendothelial system. Extravascular hemolysis, for example, could be observed when transfusing e+ RBCs to a patient with anti-e.

When a patient has multiple antibodies and transfusion of incompatible blood is required, incompatible blood is chosen which will survive the longest and with minimal adverse effects. For example, a patient in need of urgent surgery has “antibody 1” and “antibody 2.” Blood compatible with both cannot be obtained without importing it from out of the country through the American Rare Donor Program and surgery must be performed. “Antibody 1” frequently destroys RBCs intravascularly, while “antibody 2” frequently destroys RBCs extravascularly. Both antibodies are detectable in the patient’s serum. MMAs show that both are clinically significant. If compatible blood can be found for only one of the antibodies, which should be selected? Blood compatible with “antibody 1” (incompatible with “antibody 2”) should be selected because otherwise intravascular lysis is likely to occur, and it should be avoided.

What laboratory tests can be performed after transfusing incompatible blood to assist in the clinical management of the patient? Tests which can evaluate the presence of both intravascular and extravascular hemolysis (Table 2) should be performed. Many of these tests are the same as those used in the evaluation of a HTR workup when “compatible” blood is transfused. In addition to the Hb and Hct, examination

<table>
<thead>
<tr>
<th>Table 1. Signs and symptoms of a HTR at initial presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravascular HTR (%)</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Fever, chills, or both</td>
</tr>
<tr>
<td>Pain</td>
</tr>
<tr>
<td>Hypotension, tachycardia, or both</td>
</tr>
<tr>
<td>Nausea, vomiting, or both</td>
</tr>
<tr>
<td>Jaundice</td>
</tr>
<tr>
<td>Dyspnea</td>
</tr>
</tbody>
</table>

The timing and location of any RBC destruction are important to note when transfusing incompatible blood. Immune-mediated RBC destruction can be categorized by the time of the reaction—acute or delayed—and by the location of the hemolysis—intravascular or extravascular. Acute hemolysis takes place within minutes to hours (up to 24 hours after transfusion) and delayed hemolysis occurs within several days following transfusion. During intravascular reactions, antigen-antibody interactions result in binding of complement and generation of the membrane attack complex (C5b–9). This causes osmotic lysis of RBCs, resulting in hemoglobinemia and hemoglobinuria. Either IgG or IgM antibodies are capable of causing intravascular lysis. Intravascular hemolysis, for example, could be observed when transfusing Vel+ RBCs to a patient with anti-Vel. During extravascular reactions, the membrane attack complex is not generated. RBCs become sensitized with IgG antibodies, complement, or both, and are removed by macrophages within the reticuloendothelial system.
of the peripheral blood smear is important. For example, RBCs may appear in aggregates in cold agglutinin disease. Spherocytes and microspherocytes are usually observed in immune hemolytic anemia. RBC fragments and helmet cells, though, are suggestive of a microangiopathic hemolytic anemia, not an immune-mediated process.

Other laboratory tests can be used to indicate the presence of intravascular or extravascular hemolysis. An acute intravascular hemolytic event tends to be followed by low serum haptoglobin, hemoglobinemia, and hemoglobinuria. Langley et al. described two patients who developed very low serum haptoglobin levels which persisted for several days after HTRs. In contrast, there was no consistent change in haptoglobin levels in 21 patients following compatible blood transfusion. Haptoglobin is an acute-phase reactant and comorbid conditions such as infection may mask the diagnosis by somewhat elevating haptoglobin levels. However, in classic acute intravascular hemolysis, the haptoglobin level is usually < 25 mg/dL and may approach 0 mg/dL.

Hemoglobinemia can be detected by inspecting a sample of the patient’s plasma. A centrifuged urine sample is examined for hemoglobinuria. Care must be taken to differentiate intact RBCs from free hemoglobin and free myoglobin, if rhabdomyolysis is suspected. Both hemoglobinemia and hemoglobinuria are rather transient and may be cleared within a matter of hours. The urine should be examined for hemosiderin if it is suspected that the acute intravascular hemolytic episode took place a few days previously or is chronic in nature. Hemosiderin is iron-containing granules in the urine which are first shed a few days after hemolysis begins. Hemosiderin, however, may be found in any condition which causes deposition of iron in the renal parenchyma, such as hemochromatosis or multiple transfusions.

Markedly elevated levels of lactate dehydrogenase (LD) are observed with acute intravascular hemolysis due to RBC lysis. Although rarely tested for, a LD1:LD2 flip may be observed in hemolytic anemia if LD isoenzymes are examined. The LD1:LD2 flip is also observed in patients with myocardial infarction.

An elevated reticulocyte count is indicative of but not specific for hemolysis. Patients with sickle cell disease have a reticulocytosis as a means of compensating for shortened RBC survival. If a reticulocytopenia is present and hemolysis is occurring, these patients are at risk for developing life-threatening anemia. The unique challenges of transfusing patients with sickle cell disease are briefly discussed later in this paper.

Extravascular immune-mediated RBC destruction occurs when immunoglobulin- and/or complement-sensitized RBCs are destroyed by macrophages within the reticuloendothelial system. Hemoglobinemia and hemoglobinuria typically do not occur. While serum haptoglobin levels may decrease, they do not reach the very low levels seen with intravascular hemolysis. Usually, total serum bilirubin increases only slightly (from the normal < 1.5 mg/dL to < 6 mg/dL) during a hemolytic episode in patients with normal liver function. An increase in indirect serum bilirubin and urine urobilinogen will also be observed. If the elevated total serum bilirubin is composed predominantly of indirect bilirubin, hemolysis or Gilbert’s disease is likely to be present.

### Case Studies

In a patient with multiple antibodies or an antibody to a high-frequency antigen, one may anticipate the need to transfuse “incompatible” blood. The following two case studies, and a paper in this issue by A.M. Svensson et al., illustrate how a decision is reached.

A 77-year-old woman with a 6.7 g/dL Hb required transfusion (i.e., she was clinically symptomatic), but no compatible blood was available in the United States.

---

**Table 2.** A diagnostic approach to hemolysis

<table>
<thead>
<tr>
<th>Test</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete blood count</td>
<td>Determine Hb, Hct, and RBC indices</td>
</tr>
<tr>
<td>Platelet count usually normal</td>
<td>with intra- and extravascular hemolytic anemia</td>
</tr>
<tr>
<td>Thrombocytopenia with positive DAT</td>
<td>Evans syndrome</td>
</tr>
<tr>
<td>Peripheral smear</td>
<td>Spherocytes suggest hemolytic anemia (e.g., schistocytes, malignancy, hemoglobinopathy, iron deficiency)</td>
</tr>
<tr>
<td>Serum haptoglobin</td>
<td>Low in acute intravascular hemolysis</td>
</tr>
<tr>
<td></td>
<td>Acute-phase reactant</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>Elevated in intravascular hemolysis</td>
</tr>
<tr>
<td></td>
<td>Elevated in other damaged tissue or neoplastic cells</td>
</tr>
<tr>
<td>Serum bilirubin</td>
<td>Elevated total and indirect in extravascular hemolysis</td>
</tr>
<tr>
<td></td>
<td>Slight increase in total and indirect in intravascular hemolysis</td>
</tr>
<tr>
<td></td>
<td>Higher levels seen in patients with compromised liver function or Gilbert’s disease</td>
</tr>
<tr>
<td>Urinalysis</td>
<td>Hemoglobinuria seen in intravascular hemolysis</td>
</tr>
<tr>
<td></td>
<td>Rule out hematuria, myoglobinuria, porphyria, which can also cause “red urine”</td>
</tr>
<tr>
<td></td>
<td>Hemosiderinuria seen in intravascular hemolysis</td>
</tr>
</tbody>
</table>
She was group O, D−, with anti-hr\textsuperscript{a}, -E, and -S. MMAs were performed by the American Red Cross National Reference Laboratory for Blood Group Serology and the results were interpreted as “clinically significant,” (i.e., these antibodies would result in less than normal RBC survival). Blood was available from another country, but it would take several days to arrive.

How could this patient be managed until compatible blood was available? Initially, it was noted that RBCs negative for her “formed” antibodies, but D+, were available. Two units of D+, hr\textsuperscript{a}−, E−, S− RBCs were transfused and her Hb increased to 10 g/dL. However, as an anti-D developed, the Hb declined to 5.1 g/dL over the next 6 days. Three units of “incompatible” D−, E−, S−, hr\textsuperscript{a}+ RBCs were then transfused, raising the patient’s Hb to 8.6 g/dL. These units were destroyed extravascularly, and Hb declined to 6.7 g/dL over a few days until compatible blood arrived from South Africa. Two units of group O, D−, hr\textsuperscript{a}−, E−, S− RBCs were transfused and the Hb increased to 9.6 g/dL, where it remained stable until the patient was discharged.

To summarize this patient’s transfusion management, multiple alloantibodies were identified. Initially, time was available, which permitted performing an in vitro assay (MMA) to assist in predicting the clinical significance of the patient’s antibodies. Her clinical condition dictated that transfusion be performed. This is important—transfusion should never be withheld from a patient with a clinical need based on a serologic incompatibility. Until compatible units could be located, D+ but otherwise-compatible RBCs were transfused (which were eventually destroyed by the formation of anti-D), followed by transfusion of incompatible D−, E−, S−, hr\textsuperscript{a}+ RBCs (also eventually destroyed via extravascular mechanisms). Both sets of transfusions permitted proper clinical management of the patient without serious morbidity and mortality that could have occurred while awaiting arrival of compatible blood.

While the above case illustrates how one may transfuse against different “incompatibilities” until compatible blood arrives, this approach may not work well in every clinical situation. One such scenario to keep in mind is the patient with sickle cell disease who is developing a progressively more severe anemia with each transfusion. The following case illustrates this point.

A 21-year-old woman with sickle cell anemia was admitted with pneumonia in December. Her medical history is significant for Burkitt’s lymphoma, successfully treated, at age 15, and pregnancy, at age 19. RBC alloantibodies previously identified included anti-F\textsubscript{y}\textsuperscript{a}, -S, and -Le\textsuperscript{b}. She received two units of group O, D−, F\textsubscript{y}\textsuperscript{a}−, S−, Le\textsuperscript{b}− RBCs and was discharged. Several days later, she was readmitted with declining Hb (4.5 g/dL). She was transfused with one unit of group O, D−, F\textsubscript{y}\textsuperscript{a}−, S−, Le\textsuperscript{b}− RBCs, but her Hb declined the next day to 3.1 g/dL. Additional antibodies could not be identified as an explanation for the apparent HTR. While one may feel the need to continue transfusing this patient, this case illustrates many of the features of the “sickle cell hemolytic transfusion reaction,” i.e., manifestation of a delayed HTR, development of a more severe anemia after transfusion than was present before, and subsequent transfusions that further exacerbate anemia that may become life-threatening; both RBC alloantibodies and autoantibodies can be present; serologic studies may not explain the HTR; and administration of corticosteroids may lead to a gradual improvement in some patients’ Hb.\textsuperscript{18–20} This patient’s Hb improved to 7.8 g/dL after receiving 60 mg/day of prednisone without additional transfusion. In a case report described by Petz et al.,\textsuperscript{20} their patient with sickle cell anemia and anti-E, -C, -K, -S, -F\textsubscript{y}\textsuperscript{a}, and -Jk\textsuperscript{b} received a total of 15 units of RBCs and her Hct dropped from 13.2% to 9.3%. Prednisone (60 mg/day) was started, two units of RBCs were transfused, and the Hct increased to 22.4% prior to discharge 1-week later. While this patient responded to steroid medication, consideration can be given to also treating with IVIG to prevent RBC destruction.\textsuperscript{21}

**Summary**

As with any transfusion, the risks must be weighed against the benefits for each individual patient prior to transfusion. Transfusion should not be withheld from the patient with an urgent, life-threatening clinical need on the basis of serologic test results. If incompatible blood must be transfused, it is important for clinical care staff and laboratory staff to communicate frequently to determine which RBC units should be selected for transfusion, when the transfusion will be administered, and how the patient will be monitored.

**References**

G. Meny


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REMEMBER: THE PASSWORD IS “2000” For www.redcross.org/pubs/immuno

Now, as a subscriber, you can enter the password, 2000, and access the back issues. That means cover to cover! You will receive every article in total, every letter to the editor, every review, every ad, every notice, and every literature review! All of the other services will continue to be available on the Web site, including sending a letter to the editor, subscribing with a credit card on the secure order site, performing a literature search, reviewing instructions for authors, and linking to other important sites. Log on now to see this great service!
The presence of hemolysis is usually easily confirmed by simple tests such as reticulocyte count, review of the peripheral blood film, serum bilirubin (direct and indirect), LDH, and haptoglobin. When it has been established that the patient has a hemolytic anemia, the cause of the hemolysis should be sought next. The DAT should be performed on the RBCs of every patient in whom the presence of hemolysis has been established, and a positive DAT in a patient with hemolytic anemia does, of course, indicate that the most likely diagnosis is one of the immune hemolytic anemias (IHAs). However, there are numerous causes of IHA and the clinical manifestations, prognosis, and therapy vary so that defining the precise diagnosis is of considerable importance.

Classification of IHAs

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

Distinctive Clinical Signs and Routine Laboratory Procedures

Association with exposure to cold

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

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

Autoagglutination

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

Table 1. Differential diagnosis of immune hemolytic anemias

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

Note: Tables 1, 2, 4, 5, and 6 were taken from Petz LD, Garraty G. Immune hemolytic anemias. 2nd ed. Philadelphia: Churchill Livingstone, 2004.
the autoagglutination caused by cold agglutinins is often 2+ to 4+, it almost always completely disperses after a few minutes of incubation at 37°C, whereas that caused by warm autoantibodies is usually much weaker and will not disperse at 37°C. If the blood sample has been obtained from a patient known to have hemolytic anemia, such simple observations offer an important clue to the correct diagnosis.

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

Drug ingestion

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

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

Alloantibody-induced IHA

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

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

Hemoglobinemia and hemoglobinuria

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

<table>
<thead>
<tr>
<th>Table 2. Causes of hemoglobinuria</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute Hemoglobinuria</strong></td>
</tr>
<tr>
<td>Incompatible blood transfusion</td>
</tr>
<tr>
<td>Transfusion of damaged blood (overheating or freezing, bacterial contamination, pump-oxygenation)</td>
</tr>
<tr>
<td>Drugs and chemical agents (immune or nonimmune mechanisms)</td>
</tr>
<tr>
<td>Paroxysmal cold hemoglobinuria</td>
</tr>
<tr>
<td>Acute severe warm-antibody AIHA</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em> infection</td>
</tr>
<tr>
<td>Malaria (blackwater fever)</td>
</tr>
<tr>
<td>Bartonellosis, babesiosis, leptocephalosiosis, toxoplasmosis</td>
</tr>
<tr>
<td>Peritoneal hemorrhage</td>
</tr>
<tr>
<td>Severe hypophosphatemia</td>
</tr>
<tr>
<td>Snake and spider bites</td>
</tr>
<tr>
<td>Cold agglutinin syndrome*</td>
</tr>
<tr>
<td>March hemoglobinuria</td>
</tr>
<tr>
<td>Microangiopathic hemolytic anemia</td>
</tr>
<tr>
<td>Hypotonic bladder irrigation during prostatic surgery</td>
</tr>
<tr>
<td>Mistaken intravenous administration of water</td>
</tr>
<tr>
<td><strong>Chronic Hemoglobinuria</strong></td>
</tr>
<tr>
<td>Paroxysmal nocturnal hemoglobinuria**</td>
</tr>
<tr>
<td>Prosthetic cardiovascular materials</td>
</tr>
</tbody>
</table>

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

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

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

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

Association with Mycoplasma pneumoniae

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

Association with underlying disorders (secondary IHAs)

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

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

Still another reason for suspecting a relationship between the occurrence of AIHA and an associated disease consists of evidence of immunologic aberration as part of the underlying disorder, especially if the associated disease is thought to have an autoimmune pathogenesis. In general, the evidence for a relationship between immunologic disorders, including immune deficiency states, and AIHA is strong.15-19

Although the pathogenetic basis for the association between cytopenias and congenital immune deficiency is unclear, defects in T-cell regulation, cytokine defects, abnormal apoptosis, and abnormal production of immunoglobulins with autoimmune features are potential mechanisms.15

Laboratory Diagnosis of IHAs

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

Significance of the DAT in the diagnosis of IHAs

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

<table>
<thead>
<tr>
<th>Table 3. Secondary autoimmune hemolytic anemias</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian tumors</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>Hodgkin’s disease</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>Other lymphoproliferative disorders</td>
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<tr>
<td>Systemic lupus erythematosus (SLE)</td>
</tr>
<tr>
<td>Antiphospholipid syndrome</td>
</tr>
<tr>
<td>Collagen disorders other than SLE</td>
</tr>
<tr>
<td>Thymoma</td>
</tr>
<tr>
<td>Carcinomas</td>
</tr>
<tr>
<td>Primary immunodeficiency diseases</td>
</tr>
<tr>
<td>Autoimmune lymphoproliferative syndrome (ALPS)</td>
</tr>
<tr>
<td>Miscellaneous disorders</td>
</tr>
<tr>
<td>AIHA associated with infectious agents</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
</tr>
<tr>
<td>Infectious mononucleosis (Epstein-Barr Virus)</td>
</tr>
<tr>
<td>Cytomegalovirus (CMV)</td>
</tr>
<tr>
<td>Human immunodeficiency virus (HIV)</td>
</tr>
<tr>
<td>Varicella (chickenpox)</td>
</tr>
<tr>
<td>Rubella</td>
</tr>
<tr>
<td>Parvovirus B19</td>
</tr>
<tr>
<td>Hepatitis</td>
</tr>
<tr>
<td>Malaria and other blood parasites</td>
</tr>
<tr>
<td>Bacterial infections</td>
</tr>
</tbody>
</table>

IMMUNOHEMATOLOGY, VOLUME 20, NUMBER 3, 2004
autoantibody; furthermore, even if autoantibody is present, the patient may or may not have a hemolytic anemia. Thus, an independent assessment must be made to determine the presence or absence of hemolytic anemia, and the role of the DAT is to aid in the evaluation of the etiology of hemolysis when present. The results of DATs in patients with various types of IHAs are indicated in Table 4.

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

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

The AIHA serum screen test

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

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

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

**Characteristic serology of WAIHA**

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

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

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

Characterization of antibodies in CAS

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

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

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

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

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

<table>
<thead>
<tr>
<th>Results</th>
<th>Untreated serum</th>
<th>Acidified serum plus acidified complement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serologic reactions</td>
<td>(% Positive reactions)</td>
<td>(% Positive reactions)</td>
</tr>
<tr>
<td>20°C Untreated RBCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysis</td>
<td>2.0</td>
<td>14.3</td>
</tr>
<tr>
<td>Agglutination</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>20°C Enzyme-treated RBCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysis</td>
<td>24.5</td>
<td>93.8</td>
</tr>
<tr>
<td>Agglutination</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>37°C Untreated RBCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Agglutination</td>
<td>10.7</td>
<td>10.7</td>
</tr>
<tr>
<td>Indirect anti-globulin test</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>37°C Enzyme-treated RBCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysis</td>
<td>12.2</td>
<td>22.5</td>
</tr>
<tr>
<td>Agglutination</td>
<td>28.6</td>
<td>28.6</td>
</tr>
</tbody>
</table>

Patients who have warm and cold autoantibodies

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

PCH

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

The autoantibody associated with PCH is termed a biphasic hemolysin, that is to say, it sensitizes RBCs in the cold but only hemolyzes them when the RBCs reach 37°C. The diagnostic test is the DL test, wherein RBCs are incubated with the patient’s serum at 0°C (e.g., melting ice) and then moved to 37°C for a further incubation. No lysis occurs following the incubation at 0°C, and no lysis occurs if the incubation is carried out only at 37°C. The thermal amplitude of this antibody is usually less than 20°C, that is to say, the antibody will give a positive DL test only when the initial incubation is < 20°C; stronger results will occur as the temperature of the initial incubation is lowered. Rare patients have been described, in whom the DL test is positive when the first incubation phase is as high as 32°C, or their DL antibody sensitized RBCs up to 37°C, as detected by the IAT.30–31

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

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

Cautions regarding the interpretation of the DL test

The DL test is essentially diagnostic for PCH, but one must be cautious when using serum from patients with CAS. This is true because about 15 percent of sera from patients with CAS contain monophasic cold hemolysins that will hemolyze untreated RBCs at around 20°C. Up to 95 percent of such sera will cause direct lysis of enzyme-treated RBCs at 20°C. During the performance of the DL test, there is a brief period of time when cells and serum are at room temperature after being moved from the ice bath to a 37°C water bath. In about 15 percent of patients with CAS, falsely positive (weak) DL tests will occur on this basis. If enzyme-treated RBCs are used for the DL test, as suggested by some investigators,3 the falsely positive rate is likely to be higher. Therefore, if enzyme-treated
RBCs are used for the DL test, a control for monophasic lysis set up in parallel should be mandatory.

**Performing the DL test in patients with hemoglobinemia**

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

**Differentiating Delayed Hemolytic Transfusion Reactions From Autoimmune Hemolytic Anemia**

Delayed hemolytic transfusion reactions (DHTRs) are a recognized risk of blood transfusion. The reaction is caused by the reappearance of an antibody, presumably first stimulated by pregnancy or a previous transfusion. Unlike immediate transfusion reactions, which are usually caused by human error, delayed reactions are usually not avoidable. Since hemolysis is delayed in onset (typically 3 to 14 days after transfusion), the relationship of hemolytic anemia to prior transfusion may not be suspected, and a diagnosis of AIHA may seem more appropriate. Further, laboratory tests are likely to reveal the presence of a positive DAT and IAT, spherocytosis, and reticulocytosis. If multiple alloantibodies or an alloantibody against a high-frequency antigen is formed, the findings may be difficult to differentiate from AIHA. The diagnostic problem is compounded by the fact that, in some cases, AIHA may actually develop as a result of transfused RBCs. This is true even though the DAT may remain weakly positive for several months following a DHTR. In contrast, a rapid diminution in the strength of the DAT would not be expected in AIHA except as a result of treatment, as with corticosteroids, immunosuppressive drugs, or splenectomy.

**Antibody specificity**

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

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

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

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

Additional approaches

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

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

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

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

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Case report: exacerbation of hemolytic anemia requiring multiple incompatible RBC transfusions

A.M. Svensson, S. Bushor, and M.K. Fung

RBC transfusions in a patient with a history of autoimmune hemolytic anemia (AIHA) can represent both a laboratory and a clinical challenge. The development of high-titer low-avidity antibodies and antibodies to high-frequency antigens may further impair the ability to identify compatible donor RBCs. Not infrequently, incompatible RBCs must be used and the desire to increase oxygen carrying capacity conflicts with the desire to avoid exacerbating the autoimmune hemolytic process with RBC transfusions. A 66-year-old Caucasian female with coronary artery disease and a history of refractory AIHA had recently developed anemia and required multiple RBC transfusions. The patient had maintained adequate RBC counts with erythropoietin and prednisone therapy for the previous 16 months. With the recent worsening of her hemolytic anemia, she had developed angina that was treated with RBC transfusions in an outpatient setting. However, her angina increased as her RBC counts decreased, leading to hospital admission for further management of her hemolytic anemia and angina. She subsequently required multiple incompatible RBC transfusions despite increased prednisone therapy and did not improve until after coronary artery stent placement and high dose IVIG therapy. This case demonstrates the usefulness of early patient phenotyping in a case of accelerating hemolytic anemia to aid in donor RBC selection, the value of communicating with clinicians and the patient regarding the use of least-incompatible RBCs, and the importance of optimizing the patient's clinical condition to avoid ischemia. In addition, it demonstrates the value of repeated attempts with IVIG treatment despite previous refractoriness to this treatment. Immunohematology 2004;20:177–183.

Key Words: autoimmune hemolytic anemia, multiple incompatible transfusions, IVIG therapy

Indications for RBC transfusion of patients with warm autoimmune hemolytic anemia (AIHA) include the risk for cardiac or cerebral ischemia. Transfusion of these patients may confer a risk of accelerated hemolysis due to stimulation of the autoimmune process, although this is a controversial notion (see reference 1 for a comprehensive review). However, there is also the risk for intravascular or extravascular hemolytic transfusion reactions due to the presence of alloantibodies in these often multitransfused patients. These alloantibodies may be very difficult to distinguish from the background of warm autoantibodies, and in cases of high-titer, low-avidity (HTLA) antibodies or antibodies to high-frequency antigens, the workup may be referred to a reference laboratory with access to rare cells and antisera.

It is not possible to define a critical Hb level below which transfusion should be administered during an exacerbation of hemolysis. The individual's capacity to adjust to anemia depends not only on the level of Hb measured, but also on the rate of decline in Hb levels and the capacity for cardiac compensation. In cases with a background of cardiovascular disease, clinical symptoms and signs of cardiac ischemia guide the threshold for transfusion on an individual-case basis. The decision to transfuse such a patient thus depends on the clinical situation and extensive communication with the clinical staff.

Materials and Methods

ABO and Rh grouping, and screening for RBC antibodies, were performed by standard tube techniques. Similarly, RBC phenotyping was performed by standard tube techniques with commercially available typing sera. Antibody screening and panel cells were from multiple sources, including Immucor, Inc., Norcross, Georgia; Gamma Biologicals, Inc., Houston, Texas; and Ortho-Clinical Diagnostics, Raritan, New Jersey. All samples were tested with at least 11 different panel cells plus an autocontrol. DATs were performed using polyclonal anti-IgG, C3d (Bioclon Ortho), rabbit anti-IgG (Immucor), and anti-C3b, C3d (Bioclon, Ortho)-specific antisera.
Eluates were prepared by a rapid acid elution technique (Elu-Kit II, Gamma). Antibody enhancement was achieved using a PEG-based method (PEP, GTI, Inc., Waukesha, WI) or treatment of RBCs with freeze-dried papain (Immucor). PEG autoadsorption was performed as previously described. Alloadsorption of serum was performed using untreated RBCs. Other methods performed by the American Red Cross National Reference Laboratory are as described in the case report.

Incompatible units were transfused using the in vivo crossmatch procedure, in which 30 to 50 mL of donor blood was slowly transfused into the patient, then the transfusion was temporarily stopped and a sample drawn from the patient and examined for visible evidence of hemolysis. In the absence of hemolysis, the remainder of the donor unit was transfused. A second sample was drawn from the patient at the end of the transfusion to look for visible hemolysis. This procedure was repeated with each unit transfused.

Case Report

A 66-year-old Caucasian woman, previously diagnosed with refractory AIHA, presented to the emergency department with increasingly severe hemolysis and angina on exertion, following about 3 weeks of increasing transfusion needs on an outpatient basis. Her Hct had dropped to 22.5% from 33% 4 days prior to admission. The Hb was 7.5 g/dL and the WBC count was 22,630/µL, with 88% neutrophils, 4% monocytes, 5% lymphocytes, 1% basophils, 2% metamyelocytes, and 6 nucleated RBCs/100 WBCs. The platelet count was 513,000/µL, and the reticulocyte count was > 30%. Total bilirubin was 4.5 mg/dL.

Past history

The patient was group O, D+ and had refractory AIHA diagnosed 6 years previously that required treatments including splenectomy, cyclophosphamide, rituximab, cyclosporine, and high-dose IVIG. A bone marrow biopsy performed at the initial presentation of disease showed no evidence of malignancy. For the previous 16 months she had not required blood transfusions and antibody screening tests had been negative for the previous 2 years. Additional medical history included coronary artery disease requiring bypass graft surgery more than 10 years previously, two minor strokes 2 years previously, hypertension, hyperlipidemia, and four uneventful pregnancies. On admission her medications included clonidine, diltiazem, prednisone at a dose of 15 mg/day, weekly erythropoietin injections, dietary iron supplementation, and opioid analgesics for chronic lower back and lower extremity pain. There had been no recent changes in her medication or any infectious prodrome prior to this most recent exacerbation of her hemolytic anemia.

Recent history

Three weeks prior to admission to the emergency department, the patient had an office visit for complaints of fatigue and mild angina. She had previously experienced chest pain when her Hct had fallen below 30%. At this time, the Hb was 10.6 g/dL and the Hct was 32.7%. On the basis of her mild angina symptoms, a RBC transfusion in an outpatient setting was ordered. The blood bank workup demonstrated strong microscopic reactivity with all antibody screening cells in the antiglobulin phase (but no reactivity at the immediate spin phase). The autocontrol was negative. Serum reactivity was positive to a dilution of 1:16 and could not be neutralized with plasma. A screen for cold agglutinins was negative. The use of papain-treated RBCs resulted in enhanced reactivity to 1+ with all antibody screening cells. No distinct specificity was apparent. An attempt to remove possible autoreactive antibodies by PEG autologous adsorption also generated strong to 2+ microscopic reactivity with all antibody screening cells. The serum also reacted with RBCs negative for Rg, Ch, Kp, Js, and U.

The DAT was negative and since the patient had not been transfused for more than 1 year, phenotyping was performed. The patient was negative for Cw, Fya, K, S, and P1. Crossmatching with random RBC units showed strong microscopic reactivity. The interpretation of these serologic findings was that the antibodies detected were most consistent with nonneutralizable HTLA antibodies, but the possibility of antibodies to clinically significant, high-frequency antigens could not be excluded. Following discussions with the ordering physician, the patient, and her husband, the patient received the two least-incompatible K- units by in vivo crossmatch, without complications.

Before admission to the emergency department, the patient had received transfusions of six least-incompatible K-, Fya(-) units by in vivo crossmatch on three occasions without complications, but at
increasingly shorter intervals between transfusions. Furthermore, the reactions to antibody screening cells and crossmatches had increased to macroscopic levels (1+). The DAT and autologous control cells remained negative. However, RBCs from the patient were noted to weakly autoagglutinate in the saline control. This effect could be removed with warm 37°C saline washing of the cells, suggesting the development of a cold agglutinin. Two allogeneic adsorptions using antibody screening cells, both with and without PEG enhancement, failed to completely remove reactivity that persisted against the cells used in the adsorption procedure. Antibodies against preservatives in the screening cells were ruled out by using multiply-washed screening cells. No correlation between the strength of reactivity and the age of crossmatched RBC units was identified. An extensive review of her medication list found no drugs reported to induce hemolytic anemia, although the patient’s husband did report a temporal association with the use of the antidepressant mirtazapine in the past. However, a search of the literature showed no reported association.

Extensive serologic workup

Given the increasing strength of reactivity against allogeneic cells and the shortened RBC survival, there was a concern that the antibodies detected were against a clinically significant, high-frequency antigen. A more extensive serologic workup was requested from the American Red Cross (ARC) Blood Services—New England Region Reference Laboratory in Burlington, Vermont. Briefly, it was concluded that no specificity could be identified, but evidence of weak complement binding (microscopic) was detected using an enhanced DAT (5 minute incubation at 37°C), which suggested the presence of a warm autoantibody.

It was further concluded that a nonneutralizable HTLA antibody was present. RBCs lacking the following high-frequency antigens were tested and found to be reactive with the patient’s serum: k, I, Js, Kp, Yt, U, Rg, Ch, Lan, AnWj, Ge2, Ge3, Jr, Vel, PP1P, Co, Jo, Gy, Hy, Yk, Kn, McCa, McCh, Cs, Lu, and JMH. A new specimen was then sent to the ARC National Reference Laboratory for Blood Group Serology in Philadelphia, Pennsylvania, for a more extensive evaluation.

Testing performed by the ARC National Reference Laboratory was similarly unable to identify an antibody specificity. The sample received by their laboratory was a later sample. The DAT was 1+ with polyspecific anti-human globulin, negative with monospecific anti-IgG, and 1+ with monospecific anti-C3d, and a saline control using warm saline 37°C washed RBCs was negative. Weak autoagglutination of the patient’s RBCs could be removed by warm 37°C saline washing. The patient’s serum reacted with all RBCs, including autologous cells tested by 37°C albumin, IgG antoglobulin test (AGT), and ficin IgG-AGT. RBCs treated with DTT were still reactive. Furthermore, DTT treatment of the patient’s serum changed reactivity from weak reactivity to 3+. The serum was reactive to a dilution of 1:256. Five adsorptions with rr, r’r”, or r”r’ RBCs did not remove this reactivity. However, after the fifth adsorption, the remaining reactivity was uniformly weakly positive against panel cells, suggesting that no strongly reactive antibodies existed against major RBC antigens. RBCs with the following phenotypes were found reactive at the albumin-IgG-AGT phase: JMH-, Di(b–), At(a–), Cs(a–), Yk(a–), Jk(a–b–), I–, PP1Pb, -D–, Rhnull, Cde/Cde, cdE/cdE, Lu(a–b–), Yt(a–), Co(a–), Lan–, Vel–, Kp(b–), McLeod, Sc–1, and Jo(a–). Finally, the antibody reactivity was assessed for clinical significance with the monocyte monolayer assay using group O, c–, E–, P1–, S–, K–, Fy (a–); group O, C–, e–, P1–, S–, K–, Fy (a–); and group O, C–, E–, P1–, S–, K–, Fy (a–) RBCs, with and without fresh complement, and using pooled monocytes from two donors. The reactivity of the patient’s serum was 21.5 percent to 34 percent reactive monocytes, consistent with a clinically significant antibody (normal range is 0–3%).

On the present admission to the emergency department, the patient had angina on exertion. There were no ischemic EKG changes and Troponin I was < 0.15 ng/mL (normal < 0.15, indeterminate 0.15–1.50, positive > 1.50 ng/mL). The blood bank workup at that point showed increased panel cell reactivity of 1 to 2+. Furthermore, the DAT was 1+ with broad-spectrum antioglobulin reagent, 1+ with IgG monospecific serum, and 1+ with anti-C3 monospecific serum. However, the saline control was now strongly positive microscopically, suggestive of autoagglutination. With warm 37°C saline washing of the patient’s RBCs, only the broad spectrum and anti-C3–specific reactions remained reactive at 1+. An eluate was performed and found to have nonspecific reactivity at the antiglobulin phase (weak to 1+ microscopic) for all RBCs tested. The autologous cell control at 4°C was also 1+. All crossmatched C“, Fy (a–), K–, S– RBC units showed 1+ to 2+ reactivity.

At this point the risk for ischemic damage to the heart due to severe anemia was weighed against the
risk of exacerbating the autoimmune process underlying the hemolytic anemia with the transfusion of incompatible RBCs. Because at this point there were no clear clinical signs of a developing myocardial infarction, it was felt that transfusion should be held off, if possible, pending further testing results. However, the following night the patient started to complain of moderate chest pain at rest. Troponin I had increased to 0.29, Hb was 8.5 g/dL, and the Hct was 25.6%. The patient subsequently received two 1+ crossmatch-incompatible units by in vivo crossmatch without complications, following a bolus dose of 60 mg prednisone. She continued on 60 mg of prednisone per day. On day 3, Troponin I peaked at 0.44. Hb had decreased to 7.7 g/dL, the Hct was 23%, and the patient’s chest pain persisted at the same level, necessitating further transfusions. Only 2+ incompatible units could be found and the patient received a unit of C\(^{w-}\), Fy (a–), K–, S–, P1– RBCs by in vivo procedure without complications.

On day 4, Hb was 9.3 g/dL and Hct was 27.8%. It was decided that the patient would undergo cardiac catheterization and coronary artery stents were placed, with immediate relief of symptoms. On day 5, Hb had decreased to 8.0 g/dL, the Hct was 23.9%, and total bilirubin had increased to 5.8 mg/dL, confirming increasing hemolytic activity. Reactivity of crossmatched phenotype matched units (C\(^{w-}\), Fy (a–), K–, S–, P1–) was generally increased to 1+ to 3+; however, two microscopically reactive units were found. Over the following 5 days, the patient continued to receive least-incompatible phenotype-matched units without immediate complications or evidence of intravascular hemolysis. However, there was an increase in the total bilirubin (7.7 mg/dL on day 6), an increase in the strength of reactivity against crossmatched units, and increasing frequency of transfusion requirements (two RBC units every 2–3 days). Reactivity to crossmatched units continued to increase to up to 4+ on day 7.

Following 10 days of high-dose prednisone treatment without discernible improvement, high dose IVIG therapy was attempted with a one-time dose of 1g/kg body weight. Three days later, Hb had reached 10.6 g/dL, Hct was 31.8%, and total bilirubin was 2.1 mg/dL. The patient was no longer in need of transfusions and could be transferred to a rehabilitation unit, where she continued to maintain Hb values around 11 g/dL and Hct well above 30% (range of 33%–37%). A bone marrow biopsy was scheduled and then canceled due to the patient’s dramatic improvement. The patient’s medication at time of transfer included prednisone 60 mg/day, erythropoietin, folic acid, and clopidogrel.

**Discussion**

Differentiation between a preexisting warm-reactive autoantibody and an antibody against a high-frequency antigen appearing after a transfusion is difficult. However, critical results in this patient’s evaluation were a negative DAT and a negative autologous control. However, with continued transfusion, positive results with gradually increasing strength were seen in these tests. With these findings, warm-reactive autoantibodies were considered less likely, but could not be completely excluded, and more thought was given to the possibility of a HTLA antibody or an antibody against a high-frequency antigen. A HTLA antibody was initially favored given the weak reactivity that persisted to dilutions of 1:16. However, concern for a clinically significant antibody, especially against a high-frequency antigen, began to grow as the patient began to require more RBC transfusions at shorter time intervals, and the reactions obtained with panel cells and crossmatches increased to macroscopic reactivity, considered unusual for a HTLA antibody. Although the DAT and autologous cell control were initially negative, the patient likely had a chronic immune hemolytic anemia that was previously kept under control with erythropoietin and prednisone therapy. Therefore, the confluence of an autoimmune hemolytic process, HTLA antibody, and possible antibody against a high-frequency antigen rendered the decision to transfuse crossmatch-incompatible RBCs that much more difficult.

To aid in the selection of RBC units for transfusion of patients with unidentifiable specificities, phenotyping for common antigens of the Rh, K, Jk, Fy, and MNS systems should be performed on the patient’s RBCs at the first opportunity prior to RBC transfusion. In this case, the patient had not been transfused in more than a year and presented with a negative DAT. While difficult, even in patients who have been transfused over the past 3 months, a phenotype can be obtained based on either molecular \(^{1-9}\) or serologic techniques.\(^{10,11}\) With the knowledge of which major antigens are lacking in the patient, decisions can then be made in identifying appropriately phenotype-matched units that would be least likely to generate a hemolytic transfusion reaction and would survive longer in the patient’s circulation.
When searching for RBC units that are antigen negative, the difficulties increase as multiple antigens are required to be simultaneously absent. The need for using only partially matched or incompatible units arises and choices must be made as to which antigens to ignore in screening for antigen-negative RBC units to transfuse. The degree of immunogenicity of each antigen must then be taken into account, as well as the severity of the possible consequences of transfusing each antigen. Certain combinations of antigen-negative RBC units are more easily found than others and must also be taken into account in a search. Furthermore, results of alloadsorption studies may indicate the absence of any antibodies against major RBC antigens and help in guiding which antigens could be more comfortably ignored. In this patient’s situation, alloadsorptions performed at the reference laboratory failed to completely adsorb all antibodies but reduced the reactivity to only microscopic reactivity with all cells tested. The inference from this finding was that while no statement could be made regarding reactivity against high-frequency antigens, no strongly reacting antibodies against major RBC antigens were identified. However, alloadsorptions would need to be performed at 3 to 4 day intervals to rule out their emergence if the patient continued to be transfused with units positive for antigens that the patient lacked.

In the absence of the ability to identify antibody specificities and compatible RBC units by routinely used serologic tube-based methods, alternative methods exist for determining if an antibody may be clinically relevant. The monocyte monolayer assay quantifies rosetting or phagocytosis of antibody-sensitized cells by monocytes. In this case it indicated that the antibodies in the patient’s serum had clinical significance, i.e., would be likely to cause decreased survival of transfused RBCs. In this particular case, the monocyte monolayer assay might have been more useful if donor cell phenotypes were identified that predicted increased RBC survival. Other reported methods that have been used include °Cr labeling of RBCs and flow cytometry. The results of such assays may help evaluate the current immunization status and help reveal alloimmunization by comparing the survival of autologous RBCs with allogeneic RBCs. However, because an antibody may develop at any time during a period of repeated transfusions, the results cannot be used to exclude the existence of clinically significant alloantibodies in the clinical setting beyond the point of the next transfusion.

An alternative option that may be available in the future is the use of blood substitutes. Hemoglobin-based blood substitutes are currently in phase III clinical trials for various indications and have been used successfully in at least one instance for AIHA. The most effective use of hemoglobin-based blood substitutes would be to temporarily stabilize the patient until difficult-to-find or rare donor blood units could be obtained or until more aggressive immunosuppressive regimens have had a chance to take effect. In both instances however, there would be a prolonged requirement for the use of blood substitutes and the introduction of free hemoglobin into the patient’s circulation would interfere with the visual assessment of hemolysis.

The balance between the risks of inadequate oxygenation and subsequent ischemic damage on the one hand, and the risks of transfusing incompatible RBCs on the other hand, represents a clinical challenge. In situations where there is a critical need to optimize oxygen delivery by increasing RBC volume but for which no compatible units can be found, transfusion by in vivo crossmatch is unavoidable. In these instances, such as in this case, close communication with the clinicians is essential to give adequate input into the decision-making process. In this context it should be emphasized that the in vivo crossmatch has a limited value in predicting survival of the transfused RBCs, since one is only looking for acute gross intravascular hemolysis during the transfusion. Therefore, a negative in vivo crossmatch result only indicates that the likelihood of acute intravascular hemolysis and its attendant morbidity and mortality is lessened. Furthermore, in situations where the in vivo crossmatch procedure has been repeated multiple times with negative results, continuous awareness of the increased risks of transfusing otherwise incompatible RBCs is imperative for both the physicians who order the transfusions and the nurses who administer them. Finally, it is important to communicate with the clinicians and the patient the exact nature of “least-incompatible blood” and its increased risks, to address any concerns regarding the in vivo crossmatch procedure, and to further explain why no compatible blood could be found.

Apart from identifying least-incompatible RBC units for transfusion, therapeutic options for patients with AIHA are generally aimed at reducing the clearance of RBCs and reducing the production of antibodies. Corticosteroids usually constitute the first
line of treatment. Splenectomy is used when the patient is refractory to treatment with corticosteroids. Immunosuppressive therapy, such as cyclophosphamide and azathioprine and short-term use of cyclosporine, is sometimes successful in inducing remission in these otherwise refractory patients. The use of rituximab in AIHA, a monoclonal antibody that causes specific B-cell depletion by targeting the B-cell CD20 antigen, has been reported. For patients who are critically anemic and who are refractory to transfusion, plasma exchange may be considered, but it is highly inefficient at the removal of IgG antibodies, and better suited for patients with hemolysis due to IgM antibodies.

The immunomodulatory action of IVIG therapy, although not well understood, has been used successfully for a myriad of autoimmune disorders. Although unresponsive to this therapy in the past, our patient responded promptly to administration of IVIG. There are substantial differences in IVIG, based on differences in purification and chemical stabilization by manufacturers as well as lot-to-lot variations due to variation in the donor pool, even with the same manufacturing process. Such differences have been noted in the use of IVIG as an immunomodulatory therapy in solid organ transplantation and may explain the different responses to IVIG in this patient.

In conclusion, this case illustrates the management of accelerating AIHA with critical levels of Hb in association with threatened cardiac ischemia and possible alloantibodies. It demonstrates the usefulness of performing phenotyping in these patients at first opportunity and the necessity to thoroughly work up the antibody reactivity in the presence of a negative autologous cell control. Furthermore, it exemplifies the importance of optimization of clinical conditions, in this case performing cardiac catheterization and stent placement to maximize oxygen delivery to the heart. In addition, it shows that IVIG may be useful and should be attempted in these patients even if prior treatments with IVIG have been unsuccessful.

Acknowledgments

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Hemolytic anemia and multiple incompatible RBC transfusions


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Delayed hemolytic transfusion reaction due to anti-Fy\(^b\) caused by a primary immune response: a case study and a review of the literature

H.H. Kim, T.S. Park, S.H. Oh, C.L. Chang, E.Y. Lee, and H.C. Son

Delayed hemolytic transfusion reactions (DHTRs) usually occur between 3 and 14 days posttransfusion as a result of a secondary immune response, with a drop in Hb level, fever, jaundice, or hemoglobinuria. DHTRs caused by a primary immune response are particularly rare events, and only a few reports have been known. In this report, we describe an unusual case of a DHTR caused by anti-Fy\(^b\) in a 42-year-old man, who had no prior history of transfusion. Although it seems to be a rare phenomenon, we suggest that DHTRs by a primary immune response may be considered even in the case of the patient who had typical evidence of hemolysis but who had no previous transfusion history. *Immunohematology* 2004;20:184–186.

**Key Words:** delayed hemolytic transfusion reactions, anti-Fy\(^b\), primary immune response

The most important Duffy antigens in routine blood bank serology are Fy\(^a\) and Fy\(^b\).\(^1\) Because Duffy antigens are only moderate immunogens, anti-Fy\(^a\) occurs with one-third the frequency of anti-K. In addition, anti-Fy\(^b\) antibodies have an incidence rate of 1 to 20 compared to anti-Fy\(^a\).\(^2\) However, these alloantibodies are usually IgG; react best at the antiglobulin phase; and are known as clinically significant, unexpected antibodies, related to acute hemolytic transfusion reactions (HTRs) and delayed hemolytic transfusion reactions (DHTRs).\(^3,4\) IgM Duffy antibodies usually occur as a primary immune response. IgG Duffy antibodies form via a secondary immune response after another exposure to Duffy antigens.\(^4,5\) DHTRs usually become apparent between 3 and 14 days posttransfusion and are generally attributed to an anamnestic immune response. HTRs caused by a primary immune response are extremely rare events, and only a few reports have been published.\(^6,9\)

**Case Report**

A 42-year-old man suffering from multiple fractures was admitted to the emergency room of Pusan National University Hospital on January 15, 2003, after falling while he was on duty at a construction site. He was scheduled for emergency surgery since an L-Spine MRI revealed an unstable bursting fracture (L1) with cord compression. Right distal radius fracture and right calcaneal fracture were also present. His RBCs typed as group O,D+. Antibody screening was negative. During surgery, the patient received three units of packed RBCs and two units of FFP. Postsurgical bleeding caused the patient’s Hb level to continuously fall. Over the following 7 days, he received an additional 18 units of saline crossmatch-compatible packed RBCs until the Hb level stabilized at 11.5g/dL. The prothrombin time was mildly increased to 18.7 seconds (reference range 11–14.1). On the 11th hospital day, jaundice and an unexpected fall in Hb (9.9 g/dL) occurred. Antibody screening was performed again. Anti-Fy\(^b\) was identified and confirmed by gel test using LISS/Coombs and NaCl/Enzyme cards (DiaMed AG, Cressier Sur Morat, Switzerland). The patient’s RBCs were negative in the DAT. The patient had no previous history of transfusion nor history of any particular medical or surgical illnesses, except that he had received minor surgery on one of his right toes 5 years previously. The laboratory findings on the 11th hospital day were as follows: total bilirubin 2.15 mg/dL (reference range 0.3–1.3 mg/dL), direct bilirubin 0.87 mg/dL (reference range 0.05–0.40 mg/dL), LDH 1146 IU/L (reference range 218–472 IU/L), C3 121 mg/dL (reference range 50–90 mg/dL),
Primary response: anti-Fyb and delayed transfusion reaction

C4 23.5 mg/dL (reference range 10–40 mg/dL), and C-reactive protein (CRP) 5.17 mg/dL (reference range 0–0.5 mg/dL). The haptoglobin was below detectable levels (< 10.0 mg/dL), but the level of alpha-1-acid glycoprotein was 171.0 mg/dL (reference range 45–98 mg/dL). The patient was diagnosed with a DHTR caused by anti-Fyb. Three compatible units of Fy(b−) packed RBCs were issued after crossmatching using the antiglobulin method with LISS/Coombs card (DiaMed AG). No further hemolytic reactions occurred, and the unexpected antibody, present in his serum for 46 days after antibody detection, could no longer be detected.

Materials and Methods

For detection of RBC antibodies, column agglutination methods were used. The DAT was performed on the patient's RBCs from an EDTA-anticoagulated sample using the polyspecific LISS/Coombs card (DiaMed AG). Duffy phenotypes were determined on RBCs from EDTA-anticoagulated samples using ID-Antigen profile III (DiaMed AG). The RBCs used as screening and identification panels were from commercially prepared reagent panel cells (DiaMed AG). Antibody screening and identification tests were performed with LISS/Coombs and NaCl/Enzyme cards (DiaMed AG) at 37°C for 15 minutes. DTT (Sigma Chemical Co., St. Louis, MO) treatment was also performed to determine the immunoglobulin class of the alloantibody in the patient's serum.

Results

The serum of the patient was reactive with all Fy(b+) RBCs and negative with all Fy(b−) RBCs. Anti-Fyb was not detectable in the enzyme phase. The patient's RBCs were Fy(a+b−) and were negative in the DAT when anti-Fyb was detected. DTT treatment of the patient's serum did not alter reactions, suggesting that the anti-Fyb was an IgG alloantibody.

Discussion

A study of 11 cases related to DHTRs by a primary immune response in burned children, who had never been transfused, was reported by Bacon and co-workers. All 11 patients had a negative DAT and IAT before transfusion. In this study, anti-K and anti-E were the most frequently identified antibodies. The authors also reported DHTRs due to anti-S, -C, and -Jka, respectively. Another case of DHTR due to anti-C by a primary immune response was reported by Patten et al. Solanki et al. described three patients with no transfusion history who had DHTRs caused by a primary immune response. Recently, a DHTR due to a primary immune response that stimulated anti-Jka and K in a 24-week-old female was reported. However, no known report has yet been published on a DHTR by a primary immune response involving Fyb, an antigen which has relatively low immunogenecity.

Haptoglobin depletion is usually the most sensitive laboratory indicator of hemolysis. On the other hand, haptoglobin synthesis is increased in the presence of acute inflammatory processes. In our patient, we observed an undetectable haptoglobin level but high alpha-1-acid glycoprotein and CRP, so the possibility of extravascular hemolysis plus an acute phase response must be considered. Unfortunately, we could not test the urine or test for plasma Hb because of the presence of hematuria caused by trauma and because a plasma specimen was not available.

Conclusion

On the basis of clinical and laboratory findings, as well as the patient's past history, DHTR by a primary immune response was strongly suspected, even though DHTRs caused by a primary immune response are extraordinarily rare events. It is thought that such an atypical hemolytic reaction must be related to the transfusion of the large amount of packed RBCs within a short period of time. Thus, although it is a very rare phenomenon, we suggest that DHTR by a primary immune response should be considered, even in the case of the patient who had typical evidence of hemolysis but no previous transfusion history.

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Maternal alloanti-hr₅—an absence of HDN


A 24-year-old female, gravida III, para III, delivered a full-term infant by cesarean section. A maternal blood sample at the time of admission showed antibody in her serum that had apparent anti-e specificity and that her RBCs were e+. Further studies determined that the antibody was anti-hr₅. Cord RBCs had a negative DAT and a normal Hb level. There was no clinical evidence for increased hemolysis in the infant. We describe an hr₅+ infant with no evidence of HDN due to anti-hr₅. Immunohematology 2004;20:187–189.

Key Words: anti-hr₅, variant e antigen, HDN due to anti-hr₅

The e antigen is present in about 98 percent of the Caucasian population. The hr₅ antigen, a variant form of the e antigen, is a high-frequency Rh antigen present on all cells except for e– RBCs and rare e+ RBCs that lack hr₅. Alloanti-e–like antibodies may be made by those individuals with e+ RBCs lacking this variant antigen. The hr₅ antigen was first reported in 1960 by Shapiro, in a Bantu patient whose serum reacted with all cells bearing E or e.¹ Shapiro estimated that about 6 percent of Bantu Rh haplotypes were Dce or dce encoding no hr₅ antigen. Following adsorption of the patient’s serum with R₂R₂ RBCs, the serum no longer reacted with E+e– RBCs but still reacted with most e+ RBCs.² The antigen, defined by the antibody reacting only with e+ RBCs (except for a rare e antigen detected in black South Africans) was named hr₅ and the broader specificity reacting with E+e+ RBCs was named anti-Hr (later, anti-Rh18). Thus, anti-hr₅ should be suspected in patients with apparent alloanti-e specificity whose RBCs are positive for the e antigen. This antibody has been described as analogous to the anti-D made by D+ individuals.

Some anecdotal reports suggest that antibodies to portions of the e antigen may be clinically significant.² According to Reid and Lomas-Francis,³ the available data for the clinical significance of this alloantibody in HDN are limited. We describe an hr₅+ infant with no evidence of HDN due to anti-hr₅ and review some of the available information concerning the heterogeneity of the antibody response in individuals whose RBCs express a variant e antigen or lack some epitopes of the e antigen.

Case Report

A 24-year-old African American female, gravida III, para III, presented for elective cesarean section in October 2003. Maternal history included two previous cesarean sections with the last delivery in February 2000, at which time an anti-e was detected in the maternal serum. The second child was not affected with HDN. No information was available for the first pregnancy and delivery. The father’s ethnic origin is unknown and he was not available for testing.

Results

Maternal sample

A maternal blood sample obtained before the cesarean section was tested during the present admission. The RBCs were A+, C–, E+, c+, e+. A DAT was negative. Serum tested in a routine LISS panel (Immucor-Gamma, Houston, TX) showed no reactions at immediate spin, a weak reaction with eight of eleven cells at 37°C, and micro to weak reactions with seven of eleven cells at the antihuman globulin (AHG) phase. A routine PEG panel (Immucor-Gamma) revealed apparent anti-e specificity with 1+ to 2+ reactions at the AHG phase. An autocontrol run with both panels was negative. Serum was also tested against a seven-member select-cell PEG panel consisting of e– RBCs. The e– PEG panel was negative.

Because the patient’s RBCs were e+, the possibility that the maternal antibody might be anti-hr₅ and not anti-e was considered. To confirm this possibility, the serum was tested against two RBC samples that were e+, hr₅–. This testing showed negative reactions with both cells. In addition, positive reactions were seen with the maternal serum against two RBC samples that were e+ and hr₅–, ruling out the presence of anti-hr₅ in...
the maternal serum. At the time of these tests, an anti-hr^3 was not available to confirm the patient’s RBCs as hr^-,. The titer of the maternal serum versus rr RBCs without the addition of enhancement media was as follows: undiluted, 2+; 1:2, 1+; 1:4 and 1:8, microscopic reactions.

**Child’s sample**

A cesarean section was performed and a healthy baby girl was delivered. The cord blood was O+ with a negative DAT. Antibody screen on the cord serum was negative. At birth, the child’s Hb level was 18.8 gm/dL and the Hct was 56.5%. The total bilirubin levels on the first day of life ranged between 2.5 and 4.0 mg/dL. Clinically and serologically, the baby did not show signs of hemolysis; she was discharged on the third day.

At 2 months of age, the child’s blood sample was obtained and tested. The child tested positive for e and hr^e antigens. (At this time an anti-hr^e from a patient was available to test the child’s RBCs). A complete Rh phenotype was determined with the following results: D+, C–, c+, E+, e+, and hr^3+.

**Discussion**

In our case, the maternal antibody in the serum appeared to have anti-e specificity on routine testing using LISS and PEG panels. The reactions were weak with a LISS panel, but stronger with a PEG panel. The maternal RBCs were e+. Because an apparent alloantibody was present in the serum of a patient who was e+, we suspected the possibility that the patient may have an alloantibody to the e-variant hr^3. Therefore, we undertook further testing using RBCs that were e+, hr^-.. Using these rare RBCs, we were able to confirm the presence of anti-hr^e in the maternal serum, suggesting that the patient’s RBCs were hr^-+, a variant form of the e antigen.

Testing of the infant revealed an absence of serologic or clinical signs of hemolysis, including a negative DAT on the cord blood sample. At 2 months of age, the child’s RBCs were tested and shown to be e+ hr^-+. These findings show that the maternal anti-hr^e failed to cause HDN.

Unpublished observations suggest that some antibodies produced in people with a e-variant phenotype can be significant in vivo and sometimes effect clearance of small labeled aliquots of incompatible RBCs. However, recent data from Noizat-Pirenne et al.° show that anti-Rh18 (containing anti-hr^3) caused fatal transfusion reactions in two patients after transfusions of incompatible RBCs. Therefore, transfusion in patients with these antibodies should be approached with caution. According to Moores, the immune response of hr^- people is highly variable. The results of the cord blood DAT in her report varied from microscopic positive to 4+ due to anti-Rh18. Seven affected infants received successful exchange transfusions, although one died later. In addition, three infants suffered mild hemolytic disease, and seven other infants either were miscarried or were delivered elsewhere. Grobbelaar and Moores° described an anti-hr^3 which would now be considered an anti-Rh18 in the serum of a Bantu woman whose newborn suffered from mild HDN. In our case, the maternal antibody was anti-hr^e (not anti-Rh18) and was not clinically significant. This was our patient’s third pregnancy and at least the last two of her infants were unaffected. The antibody tested only weakly with LISS and was stronger with PEG. Moreover, this antibody did not react with RBCs that are both E+ and e+, the child’s phenotype. The reasons for the absence of HDN in our case are unclear but could be related to the nature or strength of maternal alloantibody and/or a decreased antigen density on fetal RBCs.

Noizat-Pirenne et al.° determined that several genetic events give rise to the absence of hr^3 in Black individuals. Specifically, she determined that loss of certain amino acid residues in a transmembrane domain might alter the protein configuration and the immune response to the antigen.

The ability to identify alleles responsible for variable expression of the e antigen likely will have practical application. For instance, fetal DNA obtained from maternal plasma during pregnancy can be examined to determine if the allele of interest was transmitted to the fetus.

**References**


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Letter to the Editors

HAMA (Human Anti-Mouse Antibodies) do not Cause False Positive Results in PAKPLUS

Regrettfully, we wish to inform readers that a recent article which appeared in *Immunohematology* (False reactivity in GTI PAKPLUS ELISA kits due to the presence of anti-mouse antibody in patient's samples, MF Leach, JP AuBuchon, Volume 19, No.4, p 112.) contained several significant errors.

In Figures 1 and 2 the authors provide illustrations of how a human anti-mouse antibody (HAMA) can cause both false positive and false negative results in testing done with the GTI kits. On the one hand, they claim that a false positive result can be caused by binding of a HAMA to the mouse monoclonal antibody used to immobilize platelet antigens in the well. On the other hand, they suggest that a false negative result can be caused by binding of a HAMA to the secondary antibody-enzyme conjugate, thereby preventing it from recognizing a patient antibody bound to the target antigen (resulting in “neutralization” of the secondary antibody-enzyme conjugate).

We wish to point out that these suggestions are flawed for the following reasons:

1. The PAKPLUS kit is not a simultaneous sandwich immunoassay (i.e., the sample is not simultaneously incubated with both the capture and the detection antibody reagents). As stated in the PAKPLUS direction insert, there is a wash step between the addition of the patient sample and the addition of the secondary antibody-enzyme conjugate. Thus, any unbound antibody, (including HAMA) would be removed from the test well prior to the addition of the secondary antibody-enzyme conjugate. Therefore, a false negative result due to HAMA antibodies could not occur in the way described by the authors in Figure 2.

2. The authors claim to have identified HAMA antibodies that cause false positive results with each of the five glycoproteins immobilized in the wells. However, as described in the package insert, only two glycoproteins in PAKPLUS are captured in the wells by the use of immobilized monoclonal antibodies. The other three glycoproteins are affinity purified and immobilized directly. Therefore, the suggestion that a HAMA can cause false positive results in the GPIb/IX, GIV, and HLA wells is untenable.

3. Lastly, false positive results due to HAMA are very unlikely because the specimen diluent provided with the GTI PAKPLUS kit already contains mouse immunoglobulin. The amount of immunoglobulin present in the diluent was optimized to prevent interference from HAMA. The use of this diluent in PAKPLUS has been tested using well-characterized samples containing HAMA. None was found to give a positive result in the PAKPLUS kit. This includes HAMA induced by treatment with therapeutic reagents containing mouse monoclonal antibodies and those naturally occurring in human populations.

We would also like to add that, although commercial ELISA assays are available for confirming the presence of HAMA antibodies, none of the samples in this study were tested by the authors to confirm the HAMA specificity.

The authors recommended that mouse IgG should be added routinely to all patient samples prior to testing. **We strongly disagree with them on this point.** Users are generally not aware of all of the complexities that underlie a commercial assay. Manipulating the components without a full understanding of these complexities can easily lead to erroneous results. Finally, the mouse IgG used by the authors was not knowingly provided by GTI for the type of study done by the authors.

Leigh Ann Tidey, MS, MT(ASCP)SBB  
Director of Operations  
QA Manager  
GTI

Suzette Chance, PhD  
Director of Product Development  
GTI
COMMUNICATIONS CONT’D

Marilyn Clarke, PhD
Production/Process Scientist
GTI

RH Aster, MD
Senior Investigator
Blood Research Institute
The Blood Center of Southeast Wisconsin
Milwaukee WI

The above letter was sent to Leach and AuBuchon. They offered the following reply.

We appreciate the interest of Tidey et al.¹ in our recent studies of the effect of heterophile antibodies in enzyme immunoassays for platelet alloantibodies.² We were frankly surprised at many of their comments inasmuch as it was a staffmember of their company that first led us to consider the presence of this phenomenon as an explanation for some anomalous results we had experienced and provided (on June 9, 2000) mouse immunoglobulin and instructions for its use to allow us to investigate this problem.

The authors of the letter contend that the washing step before the addition of the conjugate precludes a heterophile antibody from remaining in the well. However, the capability of heterophile antibodies to bind with sufficient avidity such that they remain in the test system to be recognized by the secondary antibody-antigen conjugate has been seen in many other test systems. Thus, when we encountered unexplained reactivity in samples that were not expected to contain platelet alloantibodies, and when GTI’s suggestion to add (additional) mouse immunoglobulin removed this reactivity, we felt it was not unreasonable to conclude that heterophile antibodies had been the cause. The authors of the letter also stated that the antigens in some wells are immobilized such that an anti-mouse antibody would have no target. While we are not privy to the details of the manufacture of the kit and did not directly analyze samples for the presence of anti-mouse antibodies, our observation that the addition of murine immunoglobulin blocks otherwise inexplicable activity is prima facie evidence of the presence of anti-mouse antibody reactivity and an obvious and simple solution to the problem we encountered. We thank GTI for suggesting this approach to us.

The presence of mouse immunoglobulin in the specimen diluent of this kit as routinely supplied by the manufacturer undoubtedly helps reduce the frequency of this problem. This amount apparently may not be sufficient to neutralize anti-mouse reactivity in some samples. We attempted to identify results that might make the user of the test kit suspicious for the unexpected effect of the heterophile antibodies that eluded neutralization in order to prompt the use of additional mouse immunoglobulin to obtain an accurate result. As this and many other laboratories depend on these test results to detect and characterize alloantibodies in order to provide effective platelet hemotherapy, we hope that others may find this supplemental technique useful in certain situations.

Researchers in our laboratory have enjoyed successful collaborations with many manufacturers in developing and validating a wide variety of techniques and equipment. We have found open communications essential in these efforts to improve our collaborative service to patients, and our communications to GTI in investigating the situation we were encountering were no different. The letter’s implication of nefarious conduct is unwarranted and unsubstantiated. We appreciate GTI’s provision of mouse IgG to investigate this problem after their staff had alerted us to its possibility, and we offered GTI review of an abstract of the work³ (on April 9, 2002) at the same time that we requested additional mouse immunoglobulin to continue our investigations. We regret that the manufacturer did not pursue this opportunity.

Enzyme immunoassays remain an important tool for the detection of platelet alloantibodies. As additional labs report their experiences with using these techniques in “real life” situations, further knowledge may accrue that facilitates improvement of their useful attributes.
References

1. Tidey LA, Chance S, Clarke M, Aster RH. HAMA (human anti-mouse antibodies) do not cause false positive results in PAKPLUS. Immunohematology 2004;20:


3. Leach MF, AuBuchon JP. False positive results in GTI-Pak Plus ELISA kits due to the presence of anti-mouse antibodies in patient samples. Transfusion 2002;42:64S.

Miriam Fogg Leach, MS, MT(ASCP)SBB
James P. AuBuchon, MD
Department of Pathology
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COMMUNICATIONS CONT’D

Letter to the Editor-in-Chief

Immunohematology to be listed in Index Medicus and MEDLINE

S. Gerald Sandler, MD

In February 2004, I was informed by the National Library of Medicine (NLM) that its Literature Section Technical Review Committee had completed a favorable review of Immunohematology and that the NLM would begin to cite and index the journal in Index Medicus and MEDLINE. The NLM’s decision is a key milestone in the history of Immunohematology. It means that articles in Immunohematology—past, present, and future—will be listed on electronic databases, making them readily accessible to researchers, authors, and readers worldwide. Preparing the application for the NLM’s review of Immunohematology was an informative and inspiring experience. I would like to share some of my observations of that experience with you.

The NLM’s Technical Review Committee had declined three prior applications for Immunohematology to be listed in Index Medicus and MEDLINE. In 2003, I approached Delores Mallory, Editor-in-Chief, and volunteered to initiate a fourth attempt. I was convinced that Immunohematology, as well as its distinguished contributors, deserved this recognition and status. I drafted an extensively referenced appeal to the NLM’s Technical Review Committee, basing arguments on the following three observations:

(1) Immunohematology serves a unique niche among scientific publications. It is the only journal worldwide that limits its focus to blood group serology. No other journal, including Transfusion, Blood, or Vox Sanguinis, targets a readership of blood group serologists and immunohematologists to provide up-to-date practical information.

(2) Immunohematology’s editors and editorial board are the international leaders in blood group serology. I listed the members of the editorial board and, one by one, cited examples of each member’s scientific contributions and expertise in the field of blood group immunohematology.

(3) I provided an article-by-article critique of the three most recent issues of Immunohematology, explaining to the reviewers (I assumed none were “blood bankers”) what each article contributed to our discipline. I identified each of the authors and, I must admit, the list of contributors was truly distinguished and impressive.

Compiling this information to illustrate Immunohematology’s high standards and quality was both an inspiring and a sentimental experience. I recall, as though it were only yesterday, that Delores Mallory arrived at the National Headquarters of the American Red Cross to direct our newly relocated National Reference Laboratories in Bethesda, MD. Her responsibilities included coordination of the National Reference Laboratory Committee, which published the Red Cell Free Press. Delores had a vision for the Red Cell Free Press. She believed that there was a need and an opportunity to dress up the newsletter and establish a quarterly journal to be named Immunohematology, Journal of Blood Group Serology and Education. In 1986, she recruited Mary McGinniss, who had recently retired from a distinguished career at the National Institutes of Health, to assist in scientific editing. Within a few years, Mary was appointed Managing Editor of Immunohematology. Volume 1, number 1 of Immunohematology appeared in September 1984. Delores announced the change and her vision in a page 1 editorial, as follows:

“The title of our newsletter—Red Cell Free Press—has been put to rest and in its place is a new name—Immunohematology—and a new direction. There will be more articles relevant to blood group serology and education . . .”

 Contributors to this first issue included Rebecca H. Buckley, MD; Herbert A. Perkins, MD; Peter Issitt, PhD; Kay Beattie, MT(ASCP)SBB; Tabbie Bolk, MT(ASCP)SBB;
Judy Robinson; Mary N. Crawford, MD; Denise A. Valko, MS, MT(ASCP)SBB; Joan Barker, MT(ASCP)SBB; Roger Collins; and Dorothy C. Malamut, SBB(ASCP). The journal’s standards were set high from the beginning and these high standards have been maintained for 80 quarterly issues during the past 20 years. Happy 20th birthday, *Immunohematology!* Those of us who have had an opportunity to share in your progress are proud of your achievements and progress.

*S. Gerald Sandler, MD*
Associate Medical Editor, *Immunohematology*
Professor of Medicine and Pathology
Georgetown University Medical Center
Washington, DC

**IMPORTANT NOTICE ABOUT MANUSCRIPTS**

**FOR**

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**Mail** all manuscripts (original and 2 copies) to Mary H. McGinniss, Managing Editor, 10262 Arizona Circle, Bethesda, MD 20817. In addition, please **e-mail** a copy to Marge Manigly at mmanigly@usa.redcross.org
Letters From the Editor-in-Chief

Ortho dedication

14 years! Thank you Ortho-Clinical Diagnostics for the financial support needed to publish the September issue of *Immunohematology* for the 14th year in a row! It demonstrates once again the commitment Ortho-Clinical Diagnostics has to the support of education and to the advancement of scientific knowledge and original ideas.

Please let your Ortho-Clinical Diagnostics representatives know how much you appreciate their support of *Immunohematology*. It means a great deal to the publication of this journal! Thank you!

The final 20th anniversary issue

The final 20th anniversary issue is a grand finale that you should not miss! Ralph R. Vassallo, MD, Senior Medical Director of the American Red Cross, Penn-Jersey Region, is the final guest editor and he has a four-star, two-thumbs-up issue to finish the year. There will be five invited reviews: The Function of Blood Groups by Jill Storry, PhD, FIBMS; Immunohematologic Aspects of Transplantation by Jeffrey McCullough, MD, et al.; IgA Anaphylactic Transfusion Reactions: I. Laboratory Diagnosis, Incidence, and Supply of IgA-Deficient Products by Ralph R. Vassallo, MD; IgA Anaphylactic Transfusion Reactions: II. Clinical Diagnosis and Management by S. Gerald Sandler, MD; and The American Rare Donor Program by Ann Church, Cynthia Flickinger, and Tammy Petrone. In addition, there will be three interesting and informative original papers. This will be an issue for you to read and reread!

In addition, a letter that Dr. Tibor Greenwald graciously sent to me describes some of his memories of the early days of the reference laboratory at American Red Cross National Headquarters and the start of two rare donor programs in the United States. The first program was the Rare Donor File of the American Association of Blood Banks and the second was the Rare Donor Registry of the American Red Cross. Dr. Greenwald started both programs. The two programs were combined into the American Rare Donor Program which is now housed in the Penn-Jersey American Red Cross. One of the invited review articles in the December issue is a report of the American Rare Donor Program by Ann Church and co-authors.

Dr. Greenwald was honored this year by the American Association of Blood Banks with the Karl Landsteiner Memorial Award, the highest award given by the association. Congratulations, Dr. Greenwald. Well done and thank you for your contributions to transfusion medicine and the American Rare Donor Program.

Finally, the December issue will contain some reprints from early issues of the Red Cell Free Press, including a poem by a very famous blood banker. It may be a good thing he concentrated on immunohematology! He might be very thin now if he had kept to poetry!

Delores Mallory
Editor-in-Chief
Elution of Anti-S

A patient whose serum contained multiple alloantibodies including anti-S was transfused with 2 units of blood that lacked all appropriate antigens except S. The direct antiglobulin test post transfusion was weakly positive with broad spectrum and anti-IgG antiglobulin serum but negative with anti-complement reagent. The anti-S was IgG in nature, as determined by monospecific (anti-IgG and anti-IgM) antiglobulin sera.

An ether eluate prepared from the patient’s post transfusion cell sample possessed no blood group antibody activity; however a heat eluate contained clear cut anti-S specificity. Has anyone else experience difficulty in recovering cell-bound anti-S by the ether elution method? We are interested in your findings.

M. Reid
Reference Laboratory
Central California Regional Red Cross
Blood Program
San Jose, California


Hemolytic Transfusion Reaction

A patient with hereditary hemorrhagic telangiectasia was admitted to a local hospital because of G.I. bleeding. The patient had a ten year history of hemoglobinuria post transfusion. Our pretransfusion samples were negative by standard and enzyme techniques. The patient received 3 units of packed cells uneventfully. Seven days later he experienced hemoglobinuria. Serum samples tested within the next week demonstrated weak unidentified albumin-antiglobulin reactivity. In an idle conversation with a technologist from another Blood Center in the area, I learned that a trypsin-only anti-hr”(e) was demonstrated in the patient’s serum 3 years previously. The report had gone to the clinician who neglected to pass that information on to the Blood Center or transfusion service. The patient had not been transfused since that time.

The patient eventually developed an identifiable anti-Chido. The serum was tested in numerous laboratories and the anti-hr”(e) could not be detected by standard techniques which included many different enzyme techniques. The anti-hr”(e) has been detected using the microtiter technique and using the autoanalyzer. The patient has received approximately 40 units of hr”(e) negative, Chido positive blood in the last three years without any hemoglobinuria or other signs of a transfusion reaction.

S. Ellisor
Reference Laboratory


Notice to Readers: All articles published, including communications and book reviews, reflect the opinions of the authors and do not necessarily reflect the official policy of the American Red Cross.
Controls for Enzyme Premodification

The following controls have proven useful in our laboratory for demonstrating adequate enzyme premodification of red cells.

1. Using an antibody/antigen system known to be enhanced by enzyme treatment. A weakly reactive antibody, with no saline reactivity, is selected and tested against cells possessing the weakest available form of the appropriate antigen.
   For example:
   - anti-c and heterozygous – positive cells
   - anti-D and R,r cells
   - anti-Lea and Le(a+b-) cells
   - anti-Leb and Le(a-b+) cells

2. Using an antibody/antigen system known to be denatured by enzyme treatment. A strongly reacting antibody is selected and tested against cells possessing the strongest available form of the appropriate antigen.
   For example:
   - anti-M and MM cells
   - anti-Fya and Fy(a+b-) cells

3. Using a substance that demonstrates reduction in red cell sialic acid levels.
   For example:
   - Polybrene – untreated cells aggregate
     Enzyme premodified cells do not aggregate
   - Soybean extract – untreated cells do not aggregate
     Enzyme premodified cells aggregate

We do not use milk flocculation or X-ray paper digestion methods for quality control of enzyme activity since they only measure proteolytic activity.

M. Reid
Reference Laboratory
Central California Regional Red Cross
Blood Program
San Jose, California

Monoclonal antibodies available at no cost. The Laboratory of Immunochemistry at the New York Blood Center has developed a wide range of monoclonal antibodies (both murine and humanized) that are useful for screening for antigen-negative donors and for typing patients' RBCs with a positive DAT. Monoclonal antibodies available include anti-M, -Fya, -Fyb, -K, -k, -Kpa, -Jsb, -Dob, -Wrb, and -Rh17. For a complete list of available monoclonal antibodies, please see our Web site at www.nybloodcenter.org/framesets/FS-4C7.htm. Most of those antibodies are murine IgG and, thus, require the use of anti-mouse IgG for detection, i.e., anti-K, -k, and -Kpa. Some are directly agglutinating (anti-M, -Wrb, and -Rh17), and a few have been humanized into the IgM isoform and are directly agglutinating (anti-Jsb and -Fya). The monoclonal antibodies are available at no charge to anyone who requests them. Contact: Marion Reid (mreid@nybloodcenter.org) or Gregory Halverson (ghalverson@nybloodcenter.org), New York Blood Center, 310 East 67th Street, New York, NY 10021.

Announcements

HEMATOLOGÍA HABANA’ 2005—First Announcement.
The 5th National Congress and the 7th Latin American Meeting in Hematology, Immunology, and Transfusion Medicine will present a scientific program at the International Conference Center, Havana, Cuba, May 16–20, 2005. A preliminary program lists malignant hemopathies, disorders of RBC membranes, immunotherapy, histocompatibility, immunohematology, hemolytic disease of the newborn, regenerative medicine, and blood components as some of the topics. For more information contact: Prof. José M. Ballester, President, Organizing Committee, Hematology Habana’ 2005, Apartado 8070, Ciudad de la Habana, CP 10800, Cuba, e-mail: ihidir@hemato.sld.cu; Web site: www.loseventos.cu/hematologia2005.

Annual Symposium. The National Institutes of Health, Department of Transfusion Medicine, will hold their 24th annual symposium, Immunohematology and Blood Transfusion, on September 23, 2004. The symposium is co-hosted by the Greater Chesapeake and Potomac Region of the American Red Cross and is free of charge. Advance registration is encouraged. For more information and registration, Contact: Karen Byrne, NIH/CC/DTM, Bldg. 10/Rm. 1C711, 10 Center Drive, MSC 1184, Bethesda, MD 20892-1184; e-mail: kbyrne@mail.cc.nih.gov; or visit our Web site: www.cc.nih.gov/dtm>education.
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Further information can be obtained from the Web site:

http://www.bloodnet.nbs.nhs.uk/ibgrl/MSc/MScHome.htm

For further details and application forms please contact:

Professor Ben Bradley
University of Bristol, Department of Transplantation Sciences
Southmead Hospital, Westbury-on-Trym, Bristol BS10 5NB, England
FAX +44 1179 595 342, TELEPHONE +44 1779 595 455, E-MAIL: ben.bradley@bristol.ac.uk
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• Platelet suspension immunofluorescence test (PSIFT)
• Solid phase red cell adherence (SPRCA) assay
• Monoclonal antibody immobilization of platelet antigens (MAIPA)

For information, e-mail: immuno@usa.redcross.org or call:
Maryann Keashen-Schnell
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(215) 451-4205 laboratory
Sandra Nance
(215) 451-4362
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SCIENTIFIC ARTICLES, REVIEWS, AND CASE REPORTS

Before submitting a manuscript, consult current issues of *Immunohematology* for style. Type the manuscript on white bond paper (8.5" × 11") and double-space throughout. Number the pages consecutively in the upper right-hand corner, beginning with the title page. 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—see 6 under Preparation
8. Figures—see 7 under Preparation

Preparation of manuscripts

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 and J.H. BROWN
   C. Running title of ≤ 40 characters, including spaces
   D. 3 to 10 key words
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   A. One paragraph, no longer than 300 words
   B. Purpose, methods, findings, and conclusions of study
3. Key words—list under abstract
4. Text (serial pages)
   Most manuscripts can usually, but not necessarily, be divided into sections (as described below). Results of surveys and review papers are examples that may need individualized sections.
   A. Introduction
      Purpose and rationale for study, including pertinent background references.
   B. Case Report (if study calls for one)
      Clinical and/or hematologic data and background serology.
   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 patients' names or hospital numbers.
   D. Results
      Presentation of concise and sequential results, referring to pertinent tables and/or figures, if applicable.
   E. Discussion
      Implications 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.
   C. Use inclusive pages of cited references, e.g., 1431–7.
   D. Refer to current issues of *Immunohematology* for style.
7. Tables
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   B. Use short headings for each column needed and capitalize first letter of first word. Omit vertical lines.
   C. Place explanations 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: ○ ● △ ▲ ■
9. Author information
   A. List first name, middle initial, last name, highest academic degree, position held, institution and department, and complete address (including zip code) for all authors. List country when applicable.

SCIENTIFIC ARTICLES AND CASE REPORTS SUBMITTED AS LETTERS TO THE EDITOR

Preparation

1. Heading—To the Editor:
2. Under heading—title with first letter capitalized.
3. Text—write in letter format (paragraphs).
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. One table and/or figure allowed.
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- Conduct research in transfusion medicine

**Who are SBBs?**

- Supervisors of Transfusion Services
- Managers of Blood Centers
- LIS Coordinators
- Educators
- Managers of Blood Centers
- LIS Coordinators
- Educators
- Supervisors of Reference Laboratories
- Research Scientists
- Consumer Safety Officers
- Quality Assurance Officers
- Technical Representatives
- Reference Lab Specialist

**Why be an SBB?**

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

**How does one become an SBB?**

- Attend a CAAHEP-accredited Specialist in Blood Bank Technology Program OR
- Sit for the examination based on criteria established by ASCP for education and experience

**Fact #1:** In recent years, the average SBB exam pass rate is only 38%.

**Fact #2:** In recent years, greater than 73% of people who graduate from CAAHEP-accredited programs pass the SBB exam.

**Conclusion:**

The **BEST** route for obtaining an SBB certification is to attend a CAAHEP-accredited Specialist in Blood Bank Technology Program.

**Contact the following programs for more information:**

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<td>727-568-5433 x 1514; <a href="mailto:mdoty@fbsblood.org">mdoty@fbsblood.org</a></td>
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