Contents

39  Invited Review: Milestones Series
    Milestones in laboratory procedures and techniques
    M.E. Reid

44  Case Report
    Anti-Kpα–induced severe delayed hemolytic transfusion reaction
    R. Koshy, B. Patel, and J.S. Harrison

48  Review
    The ABO blood group system revisited: a review and update
    J.R. Storry and M.L. Olsson

60  Case Report
    Clinical evaluation for lymphoproliferative disease prompted by
    finding of IgM warm autoanti-I^T in two cases

63  Original Report
    Southeast Asian ovalocytosis is associated with increased expression
    of Duffy antigen receptor for chemokines (DARC)
    I.J. Woolley, P. Hutchinson, J.C. Reeder, J.W. Kazura, and A. Cortés

67  Review
    Sickle cell disease: a review
    S.D. Roseff

75  Report
    Consortium for Blood Group Genes (CBGG): 2008 report
    G.A. Denomme, C.M. Westhoff, L. Castilho, and M.E. Reid for the CBGG

81  Special Dedication
    W. John Judd, FIBMS, MIBiol
    D. Mallory

82  Letter from the Editors

83  Announcements

84  Advertisements

88  Instructions for Authors
Milestones in laboratory procedures and techniques

M.E. Reid

When I left England in 1969, hemagglutination was done by sedimentation. In New York, I was introduced to centrifugation as a method to speed the process. Both approaches accomplished similar goals, but one was more time efficient. Use of other techniques, such as Western immunoblotting, cloning genes, and polymerase chain reaction, made it possible to gain much knowledge about blood group antigens.

Immunohematology Beginnings

In 1975, Sandy Ellisor and I from Central California Region of the American Red Cross (ARC) and Helen Glidden from ARC Headquarters volunteered to co-edit the first American Red Cross Reference Laboratory Newsletter. The purpose of this newsletter, which was for the technologists and by the technologists, was to share techniques and technical tips, educate, and mentor in technical writing. The first year (1976), the newsletter was low-tech; it was simply photocopied. That year, the ARC Reference Laboratory Committee ran a competition and by popular vote the newsletter was renamed the Red Cell Free Press. And to improve its appearance it was printed. This informal newsletter was published for 8 years, until in 1984 it evolved into Immunohematology under the able editorship of Delores Mallory. During the 25-year life of Immunohematology, techniques evolved that allowed the gathering of an amazing amount of knowledge about blood groups and the membrane components on which they are carried. Despite this evolution, to this day we still mainly rely on hemagglutination (direct and indirect) for detection and identification of antibodies to blood group antigens and for testing for incompatibility. This amazing technique is hard to beat in terms of sensitivity and specificity that are appropriate for safe transfusions.

Early Immunohematology Testing Practices

In the era before the ARC Newsletter, it was commonplace to perform excessive testing (e.g., testing at room temperature, use of enzyme-treated RBCs at temperatures below 37°C, absorbing eluates from autoimmune hemolytic anemia cases, and performing minor crossmatches) on a single blood sample. Fortunately, Eloise Giblett in the early 1960s advocated stopping unnecessary testing; she believed testing should be done as close to physiologic conditions as possible, i.e., testing at 37°C using plasma-suspended RBCs. Her reasoning was scholarly and logical and changed the way testing was (and is) done. Giblett’s laboratory used plasma-suspended RBCs, which neutralized most antibodies to Lewis and Ch/Rg antigens and thus they did not spend time identifying these clinically insignificant antibodies. Abandonment of unnecessary testing dramatically improved the efficiency of testing by manual hemagglutination and did not have a noticeable negative impact on the efficacy of transfusion. Other changes, which occurred with the same goal, include using anti-IgG instead of the broad-spectrum reagent in routine antiglobulin tests, eliminating the routine direct antiglobulin test (DAT), and not preparing eluates from all samples whose RBCs were positive in the DAT.

Also in the late 1970s, an extraordinary number of crossmatches were performed. Indeed, the number was excessive. The concepts of a “type and screen” and a “maximum surgical blood order schedule” were introduced. These approaches were quickly adopted, and they reduced not only the number of crossmatches but also the number of RBC components reserved for a particular patient. Soon after, the suggestion to drop the antiglobulin crossmatch for patients who had a negative antibody screen was unleashed. Despite concerns that antibodies to antigens not expressed on screening RBCs (e.g., anti-Wr) would be missed, this practice is now commonplace. Indeed, the physical immediate-spin crossmatch is now frequently replaced by a computer crossmatch.

Use of Enhancement Reagents and Nonserologic Techniques

Old standby techniques such as saline and albumin fell from favor and were largely replaced by low-ionic-strength solution (LISS) methods in the mid-1970s and by LISS-additive solutions shortly thereafter. LISS rapidly gained popularity because it simultaneously reduced incubation time and increased sensitivity. In 1984, the same year that the first volume of Immunohematology appeared, a solid-phase adherence assay for detection of antibody-antigen reactions was introduced. For a review of this technology, see Beck et al. Subsequently, other techniques were reported and quickly became popular; two examples are the use of polyethylene glycol (PEG) as a potentiator of antibody-antigen reactions in 1987 and the column agglutination technology, using a gel as the support medium, in 1990. Automated assays based on hemagglutination and manual or automated solid-phase adherence assays are now in use, but they have not replaced hemagglutination tests in tubes in many settings.
Many reagents became commercially available, and as compliance requirements became more stringent, laboratories reduced the number of reagents they prepared in-house. Safety concerns prompted many changes. Favorite methods fell from grace, such as the use of ether—as a result of concerns about its low flash point. Also, teratogenic, carcinogenic, and neurotoxic chemicals became less readily available. Awareness of the potential hazards of working with certain chemicals and with blood samples that may contain various pathogens led to the strict requirement to wear personal protective equipment such as gloves, laboratory coats, eye protection, and closed-toed shoes. The longstanding practice of drinking, eating, and smoking in the laboratory was abolished.

The value of proteolytic enzymes, notably ficin, for enhancing the reactivity of some antibody-antigen reactions while weakening or ablating others has been appreciated for many years. Other red cell modifications such as di-thiothreitol (DTT), trypsin, and α-chymotrypsin were introduced. However, it took some time before the full power of using the combination (i.e., testing RBCs treated with ficin or papain, trypsin, α-chymotrypsin, or DTT) of these methods to aid in antibody identification was appreciated. Although classic antibody identification approaches (and appropriate controls) are still necessary to name an antibody specificity positively, a system of parallel testing of the same RBCs untreated and treated with different reagents provides a valuable tool in helping to guide us in the right direction, without requiring a vast library of rare reagents. When using enzymes for this purpose, it is important to include a negative (or auto) control, as a proportion of human sera contain antibodies that attach to RBCs treated with a proteolytic enzyme.

**Use of Monoclonal Reagents**

For many years, we were dependent on polyclonal antibodies from human or rabbit sera or from plant lectins such as *Vicia graminea*, *Ulex europaeus*, and *Dolichos biflorus* for blood grouping reagents. In 1975, Köhler and Milstein described a technique to fuse murine myeloma cells with antibody-secreting B cells to produce monoclonal antibodies. This technique was embraced, and monoclonal antibodies were produced to highly immunogenic antigens such as to carbohydrate human blood group antigens (A and B, Lewis, and P1) and high copy number glycoproteins (M, N). It took several years before human B cells were used in place of mouse cells. With this advancement came the production of monoclonal anti-D in 1983. Monoclonal antibodies with other specificities have been made in the last 25 years. They are now the predominant source of typing reagents, and three international workshops have been held to study reaction characteristics of numerous monoclonal antibodies. Monoclonal antibodies have had a major impact on our ability to define variant RBCs, especially partial D antigens, and to optimize techniques such as flow cytometry and Western immunoblotting. Molecular biology techniques have made it possible to convert murine IgG monoclonal antibodies to human IgG or directly agglutinating IgM.

**Techniques to Predict Clinical Significance of Antibodies**

Hemagglutination detects antibodies, but in itself does not predict their clinical significance. Criteria such as titer, phase of reactivity, Ig class, IgG subclass, and specificity (and the historic knowledge of the clinical significance of an antibody in other patients) are useful indicators but are not unequivocally good predictors of clinical significance. The monocyte monolayer assay (MMA) is as close to conditions in vivo as is possible to establish in vitro and provides insight into the potential clinical significance of an antibody. It has been applied to predicting the clinical relevance of antibodies in transfusion and hemolytic disease of the newborn, but is a highly specialized and technically difficult test that belongs in a small number of laboratories. Another technique, flow cytometry, has been used to detect minor RBC populations in a fetal maternal hemorrhage and to follow the survival of transfused RBCs to determine the dosage of a blood group antigen on RBCs, and quantitation of RBC-bound IgG.

Numerous techniques have been used to identify alloantibodies underlying autoantibodies. All are time-consuming, as are methods to separate patient RBCs from those of transfused donor RBCs so that the patient’s RBCs can be phenotyped. Washing peripheral blood RBCs from transfused patients with sickle cell disease with a hypotonic solution is a simple and clever technique to obtain the patient’s RBCs for typing by hemagglutination. Although these techniques have value, they also have limitations.

**Molecular Testing**

Although hemagglutination remains the predominant test for detecting antibody-antigen interactions, unrelated techniques have provided insight into RBC components that carry blood group antigens. In the decade before *Immunohematology* first appeared, the Western immunoblotting method was used extensively to study characteristics of components carrying blood groups. This approach, together with cloning and sequencing of the genes encoding blood group antigens, has revealed sequence homology to known proteins in other cell types and provided insights into the structure and function of the RBC membrane components carrying blood groups (Table 1).

The cloning and sequencing of genes laid the groundwork for analysis of many alleles encoding variant blood group antigens and phenotypes and, thus, our ability to test DNA by the polymerase chain reaction (PCR) to predict a blood group. Subsequent manufacturing of machines to automatically perform PCR amplification made it possible to perform this technique in a clinical laboratory setting. PCR-based tests on DNA to predict a blood type have several applications that provide a substitute for phenotyping.
presence of donor RBCs in a patient’s blood sample or IgG on RBCs (positive DAT) does not interfere with the tests. RBCs are not required to type a fetus at risk for hemolytic disease of the newborn (Table 2). Thus, during the life of Immunohematology, we have had a reversal of phenotype and genotype. We used to phenotype RBCs to predict the genotype; now we test DNA to predict the phenotype, both with the understanding that variant and null alleles exist.

If high-throughput platforms were inexpensive enough, DNA testing and prediction of antigen types could be used as a screening method to increase antigen-negative inventories. Where antisera are available, negative DNA screening results could be confirmed by hemagglutination methods, thereby conserving reagents. When antisera are not available, (e.g., anti-Do\textsuperscript{b}, anti-Hy) the predicted type is often more reliable than the crossmatch. The availability of increased inventories of RBC components with various combinations of antigen-negativity would make it possible to more precisely match a patient’s phenotype, before the patient was immunized. The technique could be used to essentially eliminate repetitive time-consuming techniques needed to identify underlying alloantibodies in cases of warm autoimmune hemolytic anemia and antibodies to high-prevalence antigens.

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### Table 1. Structure and function of RBC membrane components carrying blood group antigens

<table>
<thead>
<tr>
<th>Function</th>
<th>Carbohydrate</th>
<th>Single-pass</th>
<th>Multi-pass</th>
<th>GPI-linked</th>
<th>Adsorbed</th>
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<tbody>
<tr>
<td>Glycocalyx</td>
<td>ABO</td>
<td>MNS</td>
<td></td>
<td></td>
<td>LE</td>
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<td></td>
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<td>GLOB</td>
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<tr>
<td>Structural</td>
<td>GE</td>
<td>DI</td>
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<td>RH</td>
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<td>XK</td>
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<tr>
<td>Transport</td>
<td>CO</td>
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<td></td>
<td>DI</td>
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<td></td>
<td>GIL</td>
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<tr>
<td></td>
<td>XK</td>
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<tr>
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<td></td>
<td>FY</td>
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<td>JMH</td>
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<td>SC</td>
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<td></td>
<td>XG</td>
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<tr>
<td>Enzyme</td>
<td>KEL</td>
<td></td>
<td>DO</td>
<td></td>
<td>YT</td>
</tr>
<tr>
<td>Complement regulator</td>
<td>KN</td>
<td></td>
<td>CR</td>
<td></td>
<td>CH/RG</td>
</tr>
</tbody>
</table>

### Table 2. Potential uses of DNA-based assays

- To type patients who have been recently transfused
- To identify a fetus at risk for hemolytic disease of the newborn
- To type patients whose RBCs are coated with immunoglobulin (positive DAT)
- To type patients with AIHA\textsuperscript{*} to select antigen-negative RBCs for absorption of autoantibodies when searching for underlying alloantibodies
- To type donors, including mass screening for antigen-negative donors, when appropriate antisera are not readily available
- To type donors for use on antibody identification panels when antisera are not available
- To type patients who have an antigen that is expressed weakly on RBCs
- To resolve blood group A, B, and D discrepancies
- To study unusual and novel serologic reactions

\*AIHA = autoimmune hemolytic anemia

Modifications of classic hemagglutination, which increased its sensitivity and specificity and reduced the time it takes to perform the test, have contributed to the safe transfusion practice as we know it today. Although Western immunoblotting and PCR-based assays have led to an understanding of the function and structure of RBC membrane components carrying blood groups, hemagglutination remains the workhorse of immunohematology testing. However, despite all our advances, errors in identification and clerical errors are still the big problem, and ABO incompatibility remains a primary cause of preventable transfusion-related fatalities.\textsuperscript{38–40} Approximately half of the errors occur when blood is transfused to the wrong patient. Sample collection error, i.e., drawing blood from the wrong patient or mislabeling the sample, is also responsible for the blood being transfused to the wrong patient.\textsuperscript{41} Hemagglutination in tubes does not require sophisticated equipment. It is simple, inexpensive (although antibodies are becoming expensive and technologist costs constantly rise), sensitive, specific, and reproducible. It remains the basic method for detecting reactions between RBCs and antibodies.

**Acknowledgments**

I thank Steve Pierce for helpful suggestions and Robert Ratner for help in preparing the manuscript.
References

4. Oberman HA, Barnes BA, Friedman BA. The risk of abbreviating the major crossmatch in urgent or massive transfusion. Transfusion 1978;18:137–41.

Notice to Readers

Immunohematology, Journal of Blood Group Serology and Education, is printed on acid-free paper.

Attention: State Blood Bank Meeting Organizers

If you are planning a state meeting and would like copies of Immunohematology for distribution, please send request, 4 months in advance, to immuno@usa.redcross.org.
Anti-Kp\textsuperscript{a}–induced severe delayed hemolytic transfusion reaction

R. Koshy, B. Patel, and J.S. Harrison

Kp\textsuperscript{a} is a low-frequency antigen occurring in less than 2 percent of the Caucasian population. Mild to moderate delayed hemolytic transfusion reactions (DTHR) and hemolytic disease of the fetus and newborn attributable to anti-Kp\textsuperscript{a} have been reported. Severe overt DTHR attributed to anti-Kp\textsuperscript{a} after multiple RBC transfusions is being reported. A 52-year-old Caucasian woman received multiple units of RBCs for a lower gastrointestinal bleed. She was referred to our institution for hepatic and renal failure, which was supported by laboratory findings of peak LDH, bilirubin, BUN, and creatinine elevations. Hemoglobin had dropped on Day 10 after transfusion. The DAT and antibody screen (ABS) were negative. Initial workup and subsequent ABS were negative. Anti-Kp\textsuperscript{a} was identified when an additional RBC panel was tested. One of the RBC units transfused was incompatible by antihuman globulin (AHG) crossmatch with the patient’s plasma and typed positive for Kp\textsuperscript{a}. DTHR was confirmed after extensive workup. The patient responded to supportive therapy and experienced an uneventful recovery. DTHR may not be considered when DAT and ABS are negative. However, correlation of recent transfusion with signs and symptoms should alert the clinician to entertain and investigate a DTHR that should include the AHG crossmatch of all implicated RBC units. The severity of the reaction also raises concerns as to when and what antigen specificity should be considered for inclusion in the antibody screening cells.


Fig. 1 Hemoglobin (Hb; g/dL) and hematocrit (Hct; %) values for day 0 to day 13 are shown.

Fig. 2 Alkaline phosphatase (Alk Phos), lactate dehydrogenase (LDH), and creatine phosphokinase (CPK) values for day 0 to day 14 are shown.

Case Report

A 52-year-old Caucasian woman presented to the emergency room complaining of being light-headed and having rectal bleeding, nausea, weakness, and dizziness. Rectal examination confirmed the rectal fissures and bleeding. There were no other remarkable findings noted on physical examination. ECG showed sinus tachycardia at 106 beats/min. She was admitted for further workup and for RBC transfusions.

Past history was negative for prior transfusions. Patient has an adult son who was listed as a contact person, and no other pregnancy history was available. She had left renal angioplasty several years ago for left renal artery stenosis. She had a history of rectal fissures. She is a smoker (2 packs per day) and drinks occasionally. She has a history of allergy to amitriptyline.
Admission Hb was 6.1 g/dL. She was transfused with 5 units of type-specific, leukoreduced or washed (as per hospital policy), immediate-spin, crossmatch-compatible RBC during the course of 24 hours of admission, which raised her Hb to 12 g/dL. She remained hospitalized for further evaluation for gastrointestinal bleed. On the 10th posttransfusion day, her Hb dropped to 8.7 g/dL and her Hct was 26.9%. Her reticulocyte count was 4.1% (reference range, 0.9 to 1.9%) on Day 11. There was no evidence of continued rectal bleed. There was a gradual increase of several chemistry results, which peaked on the 10th and 11th posttransfusion days (Table 1; Figs. 1–4). The patient appeared severely jaundiced with signs of acute liver and renal failure, which was supported by her laboratory values. Investigation of adjacent areas, reported as suspicious for cholecystitis. Liver, spleen, and pancreas were within normal limits. There was also evidence of atrophic left kidney and hypertrophic right kidney with severe stenosis of a segment of the right renal artery close to its origin. The patient received 3 additional units of RBCs between Days 10 and 11 for the drop in hemoglobin.

On the 13th day of her hospital course, she was admitted to the referring hospital, our institution, for investigation of acute liver and renal failure. DHTR was suspected, and a workup was ordered by the hematologist. Pigment nephropathy attributable to intravascular hemolysis was considered as the cause for the acute renal failure. The cause of the severe hepatotoxicity could not be ascertained.

### Materials and Methods

At the referral hospital, ABO and D typings were performed using the Ortho A/B/D Monoclonal and Reverse Grouping Card (Ortho-Clinical Diagnostics, Inc., Raritan, NJ). Affirmagen reagent RBCs (pooled cells), 0.8%, were used for reverse typing, and antibody screen was done by IgG gel card, 0.8% Surgiscreen (Johnson & Johnson, New Brunswick, NJ). Antibody panel was performed using 0.8% Resolve Panel A (Ortho-Clinical Diagnostics, Inc.) and Panocell 16, by Immucor, Inc. (Norcross, GA). The DAT was performed using anti-IgG-C3d with check cells, anti-IgG and check cells, and anti-C3b/C3d with complement check cells (Immucor, Inc.). Reagents used for phenotyping were from Immucor, Inc. Elution was performed with Gamma ELU-KIT II (Gamma Biologicals, Inc., Houston, TX) following initial saline wash (Fisher Diagnostics, Middleton, VA) of patient cells.

ABO and D typings and a three-cell antibody screen were performed by gel technique and read through the MTS reader SA (Ortho-Clinical Diagnostics, Inc.). DAT was performed with polyclonal AHG, anti-IgG, and anti-C3d by tube method and eluate panel by gel method using Ortho and Immucor panel cells.

### Table 1. Laboratory data from the referring hospital

<table>
<thead>
<tr>
<th>Tests</th>
<th>Normal range</th>
<th>Admision</th>
<th>Day 2</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 11</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>4.5–11 g/dL</td>
<td>6.1</td>
<td>12</td>
<td>—</td>
<td>8.7</td>
<td>10.2</td>
<td>9.9</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>36%–48%</td>
<td>19.6</td>
<td>—</td>
<td>—</td>
<td>26.9</td>
<td>31.2</td>
<td>29.3</td>
</tr>
<tr>
<td>WBC</td>
<td>4.5–11.0 x 10(^3)/dL</td>
<td>9.9</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Platelets</td>
<td>120,000–450,000/µL</td>
<td>628,000</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Reticulocytes</td>
<td>0.9%–1.9%</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>PT</td>
<td>11.5–14.7 s</td>
<td>13.8</td>
<td>—</td>
<td>—</td>
<td>13.5</td>
<td>15.2</td>
<td>18.1</td>
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<td>Glucose</td>
<td>65–100 mg/dL</td>
<td>125</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Alkaline phosphatase</td>
<td>30–133 U/L</td>
<td>138</td>
<td>142</td>
<td>203</td>
<td>211</td>
<td>422</td>
<td>393</td>
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<td>BUN</td>
<td>7–21 mg/dL</td>
<td>18</td>
<td>—</td>
<td>18</td>
<td>36</td>
<td>46</td>
<td>51</td>
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<tr>
<td>Creatinine</td>
<td>0.5–1.4 mg/dL</td>
<td>0.8</td>
<td>—</td>
<td>1.0</td>
<td>4.2</td>
<td>5.6</td>
<td>6.2</td>
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<td>AST</td>
<td>5–39 U/L</td>
<td>31</td>
<td>—</td>
<td>—</td>
<td>161</td>
<td>529</td>
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<td>ALT</td>
<td>7–56 U/L</td>
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<td>—</td>
<td>113</td>
<td>168</td>
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<td>LDH</td>
<td>333–699 U/L</td>
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<td>521</td>
<td>3983</td>
<td>7637</td>
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<td>7905</td>
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<td>CPK</td>
<td>35–230 U/L</td>
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<td>425</td>
<td>—</td>
<td>—</td>
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<td>Total bilirubin</td>
<td>0.2–1.3 mg/dL</td>
<td>0.3</td>
<td>—</td>
<td>—</td>
<td>1.8</td>
<td>23.5</td>
<td>18.3</td>
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<td>Direct bilirubin</td>
<td>0.0–0.4 mg/dL</td>
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<td>—</td>
<td>—</td>
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<td>ABO, D</td>
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</tbody>
</table>

HBsAg, Hbc antibody, HBs antibody, and hepatitis C antibody—all nonreactive

Urinalysis

<table>
<thead>
<tr>
<th>Tests</th>
<th>Results</th>
<th>Day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>&lt;1 mg/dL</td>
<td></td>
</tr>
<tr>
<td>Free hemoglobin</td>
<td>Negative</td>
<td></td>
</tr>
</tbody>
</table>

ALT = alanine aminotransferase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; CPK = creatine phosphokinase; HBC antibody = hepatitis B core antibody; HBs antibody = hepatitis B surface antibody; HBsAg = hepatitis B surface antigen; PT = prothrombin time; WBC = white blood cell count.
Results

The DHTR investigation on the day of admission at the referral hospital revealed a negative antibody screen with the three-cell antibody screen. The DAT was negative (including a 15-minute incubation) with polyspecific, IgG, and C3b/C3d AHG. At the referral hospital, tests with a 16-cell reagent RBC panel (Immucor, Inc.) revealed the presence of anti-Kp\(a\). All other clinically significant antibodies were ruled out. Eluate prepared from DAT-negative RBCs was negative against Kp(a+) RBC on Immucor Panocell 16. The patient typed negative for Kp\(a\). AHG crossmatch was done from samples obtained from donor segments of the transfused RBC units received from the blood supplier. One of the five RBC units transfused on Day 1 of admission was incompatible with the patient’s plasma and typed positive for Kp\(a\). Haptoglobin was 1 mg/dL (reference range, 34–200 mg/dL). A diagnosis of severe DHTR with intravascular hemolysis attributable to anti-Kp\(a\) was confirmed. As the patient’s renal and liver functions improved, she did not undergo dialysis or consideration for further liver or renal evaluation.

The patient was monitored and maintained on supportive therapy. Her hospital course was uneventful. All laboratory results returned to normal values (Figs. 1–4). She was discharged 10 days after her admission to the referral hospital.

Table 2 presents the patient’s laboratory data on admission to the referral hospital on Day 13.

Table 2. Laboratory data on admission at the referral hospital, Day 13

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haptoglobin (reference range 34–200 mg/dL)</td>
<td>1 mg/dL</td>
</tr>
<tr>
<td>ABO, D (by Ortho gel)</td>
<td>O, D positive</td>
</tr>
<tr>
<td>Antibody screen (0.8% Surgiscreen)</td>
<td>Negative</td>
</tr>
<tr>
<td>Panel cells, A</td>
<td>Negative</td>
</tr>
<tr>
<td>Panocell 16</td>
<td>Positive (anti-Kp(a) identified)</td>
</tr>
<tr>
<td>Kp(a) typing of patient’s RBCs</td>
<td>Negative</td>
</tr>
<tr>
<td>DAT—using polyclonal, IgG, and C3b/C3d AHG</td>
<td>Negative</td>
</tr>
<tr>
<td>Panel with eluate</td>
<td>Negative</td>
</tr>
<tr>
<td>Crossmatch with 1 of 5 units transfused at referring hospital</td>
<td>Incompatible, Kp(a+)</td>
</tr>
<tr>
<td>Kp(a) typing of incompatible unit</td>
<td>Kp(a+): 1+ by tube and 2+ by gel techniques</td>
</tr>
</tbody>
</table>

Discussion

Kp\(a\) was first described by Allen and Lewis in 1957.\(^2\) The Kp\(a\) antigen is a member of the Kell blood group system and is carried on the Kell glycoprotein. The prototypical gene for the Kell protein family was cloned and characterized in the early 1990s.\(^9\) As discussed by Lee and colleagues,\(^9\) the antigens of the Kell blood group system result from single nucleotide changes in the Kell protein. Most Kell antigens reside on the C-terminal domain of Kell in the structural sequence N-terminal to the zinc-binding catalytic motif, which is the major site for endothelin-3–converting enzyme activity.

Kell antigens are important in transfusion medicine owing to their strong immunogenicity, and the corresponding antibodies are clinically significant.\(^9\) Anti-Kp\(a\) has been implicated in mild to moderate HDFN and DHTR. Severe DHTR as presented in this case has not been reported. Clinical signs and the laboratory values presenting on Day 7 with peak laboratory values at Days 10 and 11 after transfusion of a unit of RBCs positive for Kp\(a\) support a diagnosis in this case of a severe DHTR with intravascular hemolysis attributable to anti-Kp\(a\). The cause of the primary Kp\(a\) exposure could not be determined from the patient’s history. AHG crossmatch of all implicated RBC units despite the negative DAT or antibody screen would have positively identified the incompatible Kp(a+) RBC as the implicated unit for the DHTR.

The patient under discussion experienced unusually severe hepatotoxicity, and the reasons for this remain unclear: there was no prior history of liver disease. Severe hemolysis with resultant high levels of free heme can cause undesirable toxicity, leading to organ, tissue, and cellular injury. The mechanism of action is through free heme that catalyzes the oxidation, covalent crosslinking, and aggregate formation of protein and its degradation to small
peptides. It also catalyzes the formation of cytotoxic lipid peroxide via lipid peroxidation and damages DNA through oxidative stress. Heme, being a lipophilic molecule, intercalates in the membrane and impairs lipid bilayers and organelles, such as mitochondria and nuclei, and destabilizes the cytoskeleton. It also causes endothelial cell injury, leading to vascular inflammation, and stimulates the expression of intracellular adhesion molecules. As a proinflammatory molecule, heme induces inflammation that results in toxic effects on the kidney, liver, central nervous system, and cardiac tissue. The severe heme toxicity may also be attributable to the markedly compromised renal condition owing to the severe stenosis of the right renal artery.

The severity of the DHTR in this case may warrant consideration for inclusion of clinically significant low-frequency antigens that may be missed by current screening cells for detection of clinically significant RBC antibodies. However, the risk of overt DHTRs to low-incidence antigens is estimated at 1 per 650,000 crossmatches. The calculation was based on report of probability of acute overt hemolytic transfusion reaction at 1 per 260,000 with immediate-spin crossmatches of 1.3 million negative antibody screens. As the risk of overt DHTR is extremely small, the cost benefit should be considered for inclusion of low-frequency antigens such as Wr, Cw, and Kpa in the antibody screening cells. We concur with the previous observation. AHG phase crossmatch must be included in the investigation of any suspected DHTR irrespective of a negative DAT or negative antibody screen. Appropriate and timely patient care to avert further patient harm depends on a thorough investigation.

References
5. Garratty G. How concerned should we be about missing antibodies to low incidence antigens? Transfusion 2003;43:844–47.

Ranie Koshy, MD (corresponding author), Director, Blood Bank and Transfusion Services, and Bhishma Patel, SBB, Blood Bank and Transfusion Safety Officer, Department of Pathology and Laboratory Medicine, NJMS/UMDNJ, C101, University Hospital, Newark, NJ 07103; and Jonathan S. Harrison, MD, Department of Medicine, Hematology, Robert Wood Johnson Medical School—Pisc/New Brunswick, New Brunswick, NJ.
The ABO blood group system revisited: a review and update

J.R. Storry and M.L. Olsson

The antigens of the ABO system were the first to be recognized as blood groups and actually the first human genetic markers known. Their presence and the realization of naturally occurring antibodies to those antigens lacking from the cells made sense of the erratic failure of blood transfusion hitherto and opened up the possibility of a safe treatment practice in life-threatening blood loss. Although initially apparently simple, the ABO system has come to grow in complexity over the years. The mass of knowledge relating to carbohydrate chemistry, enzymology, molecular genetics, and structural and evolutionary biology is now enormous thanks to more than a century of research using ABO as a principal model. This has provided us with data to form a solid platform of evidence-based transfusion and transplantation medicine used every day in laboratories and clinics around the globe. This review aims to summarize key findings and recent progress made toward further understanding of this surprisingly polymorphic system. *Immunohematology* 2009;25:48–59.

**Key Words:** ABO, blood group, antigen, allele, genotype

There have been many reviews of the ABO blood group system written throughout the years, covering different aspects of this fascinating topic. A limited selection of such reviews can be found in the reference list, particularly for readers who want to focus more on the discovery,1 biochemistry,2,3 enzyme structure,4 and molecular genetics.5,6 Our intention is not to reproduce them but to follow the guidelines of this new series of blood group systematic reviews in *Immunohematology* to provide a brief introduction to this amazingly complex blood group system.

**History**

The discovery of the ABO blood group system ranges from myth and folk legend all the way to the Nobel Prize. Karl Landsteiner, a Viennese pathologist, made the observation that when his serum and that of five colleagues were mixed individually with their saline-suspended RBCs, agglutination was observed with some mixtures but not with others. He reported this as a footnote to a paper published in 1900 but followed by a more comprehensive paper in 1901.8 Translations of both papers can be found in the review by Camp and Ellis.1 In these early studies, it was shown that two each of the six sera discriminated among three blood groups: A, B, and C (later renamed O from the German ohne, meaning *without*). Thus, Landsteiner demonstrated that a person’s serum contained antibodies to the antigen(s) lacking from their RBCs. The fourth blood group, AB, was described a year later by Decastello and Sturli9 in four individuals in a larger study of 34 healthy subjects and 121 patients.

Variation in A antigen expression was also recognized very early in the twentieth century (reviewed in Race and Sanger10), and the A blood group was divided into A1 and A2.1,11 Other descriptions of weakened A antigen expression followed, and the A blood group was subdivided further based on characteristic reactivity with human polyclonal antisera, i.e., strength of reactivity and presence of mixed field agglutination; presence of anti-A; and whether A or H blood group substance was present in the saliva of secretor subjects (Table 1). Weak forms of B antigen were also found but are typically more difficult to define serologically into specific categories although some subgroups (e.g., B- or B0) are analogous to their A counterparts (A1).

The frequency of the common ABO phenotypes (A1, A2, B, A B, A B, and O) varies greatly among different populations.12,13 Populations with a high frequency of A phenotype are found mainly in Northern and Central Europe, and the

**Table 1.** Subgroups of A—agglutination reaction patterns adapted from textbooks

<table>
<thead>
<tr>
<th>Subgroup of A</th>
<th>Anti-A</th>
<th>Anti-A,B</th>
<th>Anti-A1</th>
<th>Anti-H</th>
<th>Substances in saliva†</th>
<th>Anti-A1 in serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>0</td>
<td>A, H</td>
<td>No</td>
</tr>
<tr>
<td>A2</td>
<td>++++</td>
<td>++++</td>
<td>0</td>
<td>++++</td>
<td>A, H</td>
<td>Sometimes</td>
</tr>
<tr>
<td>A1a</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>A, H</td>
<td>No</td>
</tr>
<tr>
<td>A2a</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>A, H</td>
<td>Sometimes</td>
</tr>
<tr>
<td>A1a</td>
<td>0/+</td>
<td>0/+(+)</td>
<td>0</td>
<td>++++</td>
<td>H</td>
<td>Often</td>
</tr>
<tr>
<td>A1a</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>++++</td>
<td>H</td>
<td>Sometimes</td>
</tr>
<tr>
<td>A2a</td>
<td>0/+</td>
<td>0/+(+)</td>
<td>0</td>
<td>++++</td>
<td>A, H</td>
<td>No</td>
</tr>
<tr>
<td>A1a</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>++++</td>
<td>H</td>
<td>Yes</td>
</tr>
<tr>
<td>A2a</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>++++</td>
<td>H</td>
<td>Yes</td>
</tr>
<tr>
<td>A1a</td>
<td>0°</td>
<td>0</td>
<td>++</td>
<td>++++</td>
<td>H</td>
<td>Yes**</td>
</tr>
<tr>
<td>A2a</td>
<td>0°</td>
<td>0</td>
<td>0</td>
<td>++++</td>
<td>A, H</td>
<td>No</td>
</tr>
<tr>
<td>A1a</td>
<td>0°</td>
<td>0</td>
<td>0</td>
<td>++++</td>
<td>H</td>
<td>Sometimes</td>
</tr>
<tr>
<td>A2a</td>
<td>0°</td>
<td>0</td>
<td>0</td>
<td>++++</td>
<td>H</td>
<td>Sometimes</td>
</tr>
</tbody>
</table>

* A negative reaction is denoted by 0. Positive reactions are denoted as from + (weak agglutination) to ++++ (maximal agglutination).

**Dolichos biflorus** only.

***Serum reactivity against both A1 and A2 RBC.

†Blood group ABO substances in saliva and other body fluids of secretors.

‡Despite lack of agglutination, anti-A can be adsorbed to and eluted from cells in this subgroup.

mf = mixed field agglutination.
A\textsubscript{1} phenotype reaches its peak among the Lapps in Northern Scandinavia but is very rare in Asia. The B phenotype is most frequent in Central Asia and almost absent in Amerindians. Blood group O is the most frequent phenotype in a global perspective, with Native American Indians being almost exclusively blood group O. Parts of Africa and Australia also show high frequencies of blood group O. The reason for the differences observed among populations is not fully understood although several theories have arisen. The concept of evolutionary selection based on pathogen-driven blood group changes will be discussed later (see section on pathogen interactions). The early importance of ABO diversity is supported by reports in which the group O-defining single base pair deletion at nucleotide position 261 (see section on molecular genetics) has been found in both Neandertal people\textsuperscript{44} and ancient Egyptian mummies,\textsuperscript{15} suggesting that selection pressure and survival of the fittest were indeed early features during our co-evolution with pathogens like malaria parasites and many others.

**Nomenclature**

The ABO system was the first to be discovered and has therefore been given the number 001 in the official International Society of Blood Transfusion (ISBT) terminology. Nomenclature for the ABO antigens is actually straightforward since the antigen status is determined by the presence or absence of specific carbohydrate molecules. This is particularly true for the A and B antigens but may be less clear for the two other antigens (A,B and A\textsubscript{1}) given official antigen status by the ISBT. The A,B,antigen is thought to be an epitope not involving the A versus B–differentiating surfaces, but consists of a common recognition motif\textsuperscript{46} found when either the A or B antigens are present. The debate surrounding the real identity of the A\textsubscript{1} antigen is still ongoing,\textsuperscript{17,18} but the A\textsubscript{1} phenotype reveals many differences compared with A\textsubscript{2}, both quantitative and qualitative, so the question is quite complex (see section on biochemistry). Table 2 shows both the numerical and traditional nomenclature according to the ISBT Working Party on Terminology for Red Cell Surface Antigens.\textsuperscript{19}

<table>
<thead>
<tr>
<th>System</th>
<th>ABO antigen notation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISBT number</td>
<td>001</td>
</tr>
<tr>
<td>ISBT name</td>
<td>ABO</td>
</tr>
</tbody>
</table>

Blood group allele nomenclature including ABO is under consideration by a subcommittee of the aforementioned ISBT Working Party. In the absence of an officially agreed-on terminology, several different variants have evolved (see Table 7 in Chester and Olsson\textsuperscript{10}). Typically, alleles are referred to by their serologic activity and a number. An unofficial but often used terminology is found at the Blood Group Antigen Gene Mutation Database, also known as the dbRBC Web site (http://www.ncbi.nlm.nih.gov/projects/gv/rbc\textsuperscript{30}). In this review, alleles will be referred to in the traditional way but with dbRBC terminology given in brackets, e.g., A\textsuperscript{1} [A101] and O\textsuperscript{1} [O01].

It can be specifically noted that it is important for clarity and consistency to use subscripts, superscripts, and italics appropriately. For example, A\textsubscript{1}, A\textsubscript{2}, and A\textsuperscript{1} mean the antigen, the phenotype, and the allele, respectively.

**Inheritance and Molecular Genetics**

The A and B antigens are inherited as Mendelian characteristics in a codominant autosomal fashion. In 1908, Epstein and Ottenberg\textsuperscript{21} were the first to suggest in a short case report that ABO blood groups might be inherited. This was later proved by von Dungern and Hirsfeld in 1910 (translated by Pohlmann\textsuperscript{22}). In fact, ABO inheritance was one of the first genetic markers to be used in paternity testing and in forensic medicine.\textsuperscript{23,24}

Unlike the majority of blood groups, the antigens of the six currently known carbohydrate systems are not coded by genes directly. Instead, these blood group genes encode glycosyltransferases that in turn synthesize the oligosaccharide epitopes (Fig. 1). Thus, the A and B antigens are made by A and B glycosyltransferase, respectively, encoded by the ABO gene carried on the long arm of chromosome 9 (9q34). As with many of the blood group genes, the position of the ABO locus was known for many years before the gene was cloned.\textsuperscript{25} The genes encoding the A-synthesizing 3-\alpha-N-acetylgalactosaminy transferase and the B-synthesizing 3-\alpha-N-galactosaminytransferase were cloned by Yamamoto and colleagues in 1990\textsuperscript{26,27} after purification and partial amino acid sequencing of A transferase from lung tissue.\textsuperscript{28} Probing cDNA libraries obtained from human adenocarcinoma cell lines of different ABO types, the main alleles were defined.\textsuperscript{29} They determined that the B–specific mRNA differed from the A-specific gene by only 7 of 1062 coding nucleotides, of which 4 result in amino acid differences in the enzyme product. The difference between the A\textsuperscript{1} [A101] and O\textsuperscript{1} [O01] genes was shown to be a single guanosine (G) deletion, which alters and severely truncates the open reading frame (ORF) as shown in Figure 2. The A\textsubscript{2} phenotype was shown to depend on a cytosine deletion in the 5’ end of the gene, resulting in elongation of the ORF.\textsuperscript{29} The organization of the ABO locus\textsuperscript{30,31} is shown in Figure 3. The gene consists
of seven exons (plus an alternative exon 1a located upstream of exon 1\textsuperscript{39}), with the majority of the catalytic domain of the enzyme encoded by exon 7.

Since the groundbreaking cloning paper, 215 ABO sequence entries have been submitted to the dbRBC Web site\textsuperscript{29} (accessed on April 15, 2009) and curated by the dbRBC staff and experts, but new alleles are constantly being identified. Figure 4 shows a breakdown of the 181 alleles in the dbRBC categorized by their association with normal or altered phenotypes: these include 65 different A alleles, 47 B alleles, 58 O alleles, and 11 “AB” alleles. The remaining 34 sequences listed in the current dbRBC consist of 20 sequences encompassing the 5’ noncoding region including the repetitive and polymorphic CCAAT box binding factor/nuclear factor Y (CBF/NF-Y) motif and 14 intronic or overlap sequences. Of the A alleles, 6 and 11 encode normal A\textsubscript{o} or A\textsubscript{\textgamma} phenotypes on RBCs, respectively, whereas 48 describe mutated A alleles (A\textsubscript{weak}) associated with different variants of weak A antigen expression (Table 1); the B alleles include 9 normal and 38 weak alleles; and 11 alleles have been described that encode glycosyltransferases capable of synthesizing easily detectable levels of both A and B antigens. This group includes alleles conveying the two unusual phenotypes cisAB and B(A). Fifty-eight alleles are predicted to give rise to proteins without enzymatic activity of which 45 contain 261delG, the mutation that alters the translational ORF and predicts a shortened protein product with no transferase activity. This large group of O alleles includes four principally important evolutionary lineages: O\textsuperscript{[001]}, O\textsuperscript{[002]}, O\textsubscript{tlse09} or O\textsuperscript{[467T;318T]} [009], and O\textsubscript{banatu} [O54].\textsuperscript{33-34} Numerous minor variants of these main alleles as well as hybrid O alleles combining A\textsuperscript{\textgamma} or B with different 261delG-containing O sequences\textsuperscript{35} have also been found, most notably in individuals of African ancestry. Of the remaining 13 “nondeleitional” alleles, 3 suffer similar fates to that caused by 261delG because they contain nonsense mutations resulting in altered ORFs caused by nucleotide insertions. Another 3 have nonsense mutations introducing immediate stop codons, thereby truncating the ORF at the same codon where they occur. Finally, 7 recorded nondeleitional O alleles are crippled by at least one missense mutation each, giving rise to critical amino acid substitutions, the most famous example of which is 802G\textsuperscript{[A]} resulting in 268Arg. For a recent review about this latter group of interesting O alleles designated O\textsuperscript{[003]}, see Yazer and Olsson.\textsuperscript{37} What is most intriguing about these nondeleitional O alleles is that they are all attributable to mutations in A allele backbone sequences. This has two main practical consequences: first, virtually all ABO genotyping methods will signal the

![Graphic representation showing the number of ABO alleles currently listed in the dbRBC database. The black bars represent alleles that encode a glycosyltransferase with normal activity; the white bars represent alleles encoding glycosyltransferases that have altered activity or specificity. The AB bar includes alleles encoding glycosyltransferases capable of synthesizing both A and B antigens, i.e., cisAB and B(A). The group O bar has been divided into the 45 O alleles that contain the mutation 261delG (black) and the 13 “nondeleitional” alleles without it (white). The latter group is often associated with weak expression of A antigen.](image-url)
presence of \(A^1\) or \(A^2\) alleles unless specifically designed to detect rare \(O\) alleles, which will result in the potentially catastrophic prediction of group A in a group \(O\) person. Second, the phenotype actually conveyed by inheritance of one of these so-called \(O\) alleles is not always \(O\) but often weak \(A\) or \(O\)-like but without anti-\(A\) present in plasma. The reason for this is currently unclear, but there has recently been a series of papers investigating the serologic pattern and mechanisms behind this interesting phenomenon.\(^{38-43}\)

There is currently no \(O\) allele described that is based on a \(B\) sequence, but we predict that when such an allele is found, it will show the opposite serology, i.e., either weak \(B\) expression or \(O\)-like without anti-\(B\) in plasma.

It is the molecular genetics that make this system so fascinating because mutations ranging from single nucleotide changes to more complex hybrid gene formations can alter the specificity of the enzyme, the efficacy of the enzyme, or both; changes that, at the phenotypic level, are manifested simply by altered antigen expression. Good examples are the various molecular bases of the \(A\) phenotype, which can either result from a single missense mutation in exon 7 of an \(A\) \([A101]\) allele or be based on different hybrids, for instance between the 5' end of \(B\) and the 3' end of \(O\) alleles.\(^{43-44}\)

The \(B\) phenotype in the Taiwanese population has also been shown to result from different molecular backgrounds, one a missense mutation but more commonly, a principally interesting mutation that was the first one shown to affect splicing of \(ABO\) mRNA and cause exon skipping.\(^{46}\) In addition to altered specificity or enzymatic efficacy, aberrant intracellular trafficking of \(ABO\) transferase may cause weak subgroup phenotypes.\(^{46}\)

As alluded to previously, this added layer of complexity, in which mutations affect enzyme activity and not the antigen directly, provides difficulties in designing genotyping assays that will detect rare variants, which if misinterpreted can have serious consequences in \(ABO\) group determination.\(^{47}\)

There are problems associated with all the more than 30 \(ABO\) genotype screening methods published or commercially available so far (reviewed in Olsson and Chester\(^{48}\)). The vast majority is only designed to determine between three and six of the common alleles, although additional alleles can often surface if their polymorphisms interfere with the detection system. However, virtually all methods will fail to predict the phenotype of a sample in the presence of most \(A/B\) subgroup alleles, rare nondeletional null alleles, cis\(AB\) and \(B(A)\) alleles, or hybrid alleles, which can lead to serious consequences. Because this is particularly dangerous if blood or transplant recipients are typed (as suggested, e.g., by Procter et al.\(^{49}\)) we recently developed and implemented an improved \(ABO\) genotype screening method that addresses these problems and can be used in a clinical laboratory setting.\(^{49}\) In summary, the complex genetics of \(ABO\) is a major challenge for \(ABO\) genotyping efforts, not least because of the disturbing fact that a certain allele can lead to more than one phenotype and seemingly identical phenotypes can have more than one molecular genetic background.

The regulatory mechanism of the \(ABO\) gene has been investigated extensively. An enhancer element located approximately 4 kbp upstream of exon \(^{1949}\) was found to contain four 43-bp repeats in all alleles except \(A^1\) \([A101]\) and the infrequent \(O^*\) \([O03]\), which have only one copy.\(^{51}\) This may play a role in expression.\(^{49}\) The enhancer region contains a CBF/NF-Y binding site; mutations in this site decrease enhancer activity in a gastric cancer cell line,\(^{39}\) and alterations in this region may even cause rare \(B\) subgroup phenotypes.\(^{52}\) However, it was recently shown that \(A^1\) \([A101]\) or \(A^2\) \([A201]\) transcripts are virtually undetectable in peripheral blood whereas \(B\) and \(O\) (including \(O^*\) \([O03]\)) mRNA is readily found.\(^{54,55}\) This appears to speak against a critical role for CBF repeats in erythroid \(ABO\) regulation, but more work is required. There is also an Sp1-binding site in the proximal promoter that may be important for erythroid \(ABO\) regulation.\(^{56}\)

Although much is understood regarding the cause of weak \(A/B\) antigen expression on RBCs when it comes to inherited weak \(ABO\) subgroups,\(^{5,6}\) less is known about altered \(ABO\) expression in hematologic disorders. Erythrocytes losing \(A\), \(B\), or \(H\) antigen have been noted in patients with hematologic malignancy, especially in the myeloid lineage.\(^{57,58}\) Very recently it was suggested that methylation of the \(ABO\) proximal promoter is the reason for such leukemia-associated downregulation.\(^{59}\) Reduced \(A\) and \(B\) antigen expression in bladder and oral carcinomas is partially attributable to loss of heterozygosity or hypermethylation.\(^{60,61}\) Furthermore, urothelial tumor tissue contains decreased amounts of \(A/B\) antigens that correlated to decreased levels of \(ABO\) mRNA compared with cells from normal tissue.\(^{62}\) Transient depression of \(A\) antigens has also been observed in some pregnant women,\(^{44}\) but the reason for this is still unknown.

**Biochemistry**

The biochemistry of the \(A\) and \(B\) antigens was elucidated by the astonishingly early and brilliant work from the groups of Morgan and Watkins, and Kabat (reviewed by Watkins\(^2\) and Kabat\(^63\)). The \(A\), \(B\), and \(H\) determinants were hypothesized to reside on water-soluble glycoproteins able to inhibit agglutination of RBCs by antibodies or lectins. A precursor substance, \(H\), was hypothesized as a building block for \(A\) and \(B\), and the terms \(O\)- (or \(H\)-) substance and anti-\(H\) were introduced in 1948.\(^{64}\)

Owing to difficulties in obtaining sufficient quantities of blood group active material after extraction from RBCs, most of the early studies were performed on ovarian cyst or animal mucin, rich in secreted blood group substances. Before isolation and chemical identification of these substances, inhibition with simple sugars indicated \(N\)-acetyl-\(\beta\)-galactosamine (GalNAc), \(d\)-galactose (Gal), and \(L\)-fucose (Fuc) as the defining sugars in blood groups \(A\), \(B\), and \(O\), respectively.\(^{55,66}\)

Independent of the blood group of the individual investigated, a surprisingly similar composition of carbo-
hydrate residues (mainly Fuc, Gal, GalNAc and N-acetyl-D-glucosamine [GlcNAc]) was found. This was taken as a sign of large similar precursor molecules carrying small residues that differentiate blood groups.67 The minimal determinant structures were subsequently shown to be trisaccharides as shown in Figure 1. The important concept of precursor-product relationship between H and A/B was formulated,68 and the critical role of nucleotide-bound sugars as substrates in oligosaccharide synthesis was appreciated.69 The biosynthetic pathway of the ABO blood group structures is as outlined in Figure 5, and the presence of A and B glycosyltransferases was first predicted70 and then experimentally established.71-73 Thus, the A and B glycosyltransferases use UDP-GalNAc and UDP-Gal, respectively, as substrates. Both require the H determinant as acceptor. The O protein is nonfunctional, leaving the H-defining terminal Fuc unaltered.

Although the composition of the A and B antigens is apparently straightforward, the biochemistry behind the shared A,B antigen recognized by many group O plasmas has only recently been elucidated experimentally. Bovin and his colleagues66 synthesized a deacetylated A trisaccharide structure and bound it to the precursor in such a way as to expose what they hypothesized would be the common A,B epitope. They were able to demonstrate binding of both monoclonal and polyclonal anti-A,B to the synthetic structure.

Although it is well known that there are approximately five times fewer A antigens on A\(_2\) than on A\(_1\) RBCs, the underlying basis for the qualitative differences between them is less well defined. The A\(_2\) transferase is 10 times less efficient than the A\(_1\) transferase and has a different pH optimum and pI.74-76 The A\(_2\) enzyme is also less able to use chains other than type 1 or 2 carbohydrate precursors like the extended type 3 (repetitive A) and type 4 (globo-A) chains on RBC glycolipids.77,78 More recently, Svensson et al.18 have produced somewhat contradictory data indicating that although the A\(_2\) transferase can readily use H type 3 chains to synthesize A antigen, the low levels of type 4 chains remain unconverted.

The ABH sugars are found on glycolipids (approximately 10%) and glycoproteins (approximately 90%) on the RBC as well as on many different tissues and cell types, including epithelial cells that line the lumen of the gastrointestinal, respiratory, and reproductive tracts as well as in salivary glands and skin. This wide distribution is a common feature for many of the carbohydrate blood groups, which has resulted in the term histo-blood group often being used to reflect this wide distribution. A and B antigen synthesis occurs during normal glycosylation of proteins and lipids in the Golgi compartment.77 The precursor H substance is synthesized by one of two fucosyltransferases depending on the acceptor substrate used. The FUT1 gene that encodes the 2-α-fucosyltransferase (α2FucT1) is responsible mainly for the synthesis of the H antigen on type 2 (and type 4) carbohydrate precursors found on RBCs.78 The closely related FUT2 gene encodes a very similar 2-α-fucosyltransferase (α2FucT2) that is expressed in epithelial cells and synthesizes H antigen mainly on type 1 and type 3 chains.77 The major precursor types present in different tissues and secretions are shown in Table 3.

![Fig. 5. Schematic depiction of the biosynthetic pathways for conversion of H determinants to A or B determinants. R represents the core structure. See text for enzyme abbreviations.](image)

### Table 3. Peripheral core structures and their principal tissue distribution (modified from Clausen and Hakomori²)

<table>
<thead>
<tr>
<th>Peripheral core type</th>
<th>Structure</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>Gal[1→3GlcNAc]1→R</td>
<td>Endodermal, secretions, plasma</td>
</tr>
<tr>
<td>Type 2</td>
<td>Gal[1→4GlcNAc]1→R</td>
<td>Ecto- and mesodermal (e.g., erythrocytes)</td>
</tr>
<tr>
<td>Type 3</td>
<td>Gal[1→3GlcNAc]1→R</td>
<td>O-linked mucin-type, erythrocytes</td>
</tr>
<tr>
<td>Type 4</td>
<td>Gal[1→3GlcNAc]1→R</td>
<td>Glycolipids in kidneys</td>
</tr>
</tbody>
</table>

R = inner core structure or linkage.

In the Bombay phenotype (O\(_h\)), a silenced FUT1 gene is present together with a silenced FUT2 gene. Because H antigen is the precursor substrate for both A and B antigens, neither antigen can be synthesized without α2FucT1 activity, independent of the ABO genotype. The para-Bombay phenotype results from either (1) a silenced FUT1 gene present together with an active FUT2 gene, which permits the synthesis of H type 1 (and therefore A/B antigens) that may be adsorbed onto the RBC from the plasma; or (2) a mutated FUT1 gene in which the encoded enzyme activity is greatly diminished, so that very low amounts of H antigen (and A/B antigen) are produced. It may be present with or without an active FUT2 gene. In both cases, H antigen (and A/B antigen) is very weakly expressed and is often only detected by adsorption and elution tests with the appropriate blood group reagents. The H blood group system (ISBT 018) will be the subject of another review later in this Immunohematology series.

The A and B glycosyltransferases are type II membrane proteins located in the Golgi compartment,79,80 although soluble forms are found in plasma and other body fluids. The enzyme consists of a short transmembrane domain, a stem region, and a catalytic domain that extends into the Golgi lumen (Fig. 6). The crystal structure elucidated by
The authors concluded that the absence of dietary exposure to bacteria prevented the production of anti-B despite normal immunoglobulin levels in the patients’ sera. Recognition of self prevents an individual from making antibodies to antigens shared by the bacteria and may also partially explain disease susceptibility of one blood group over another (discussed briefly in the next section).

Anti-A and anti-B are predominantly IgM antibodies although IgG or IgA components are often found. Class-switching to IgG does not occur unless there is a “hyperimmunizing” event such as an ABO-incompatible pregnancy or transfusion. Absence of the expected antibodies occurs rarely, although antibody titers vary considerably among individuals and have been shown to diminish with age. Patients who have immunoglobulin deficiencies will also lack anti-A or anti-B. Otherwise, most often the absence of an agglutinin in a healthy person should be taken as an indication that there may be a weak antigen or even (micro) chimerism present, perhaps detectable by adsorption and elution or by flow cytometry.

Individuals of the A_ and A_B phenotypes as well as those whose RBCs carry certain A subgroups (Table 1) can also produce anti-A_1, generally reactive at room temperature and below. Although ABO antibodies of IgG type can cross the placenta, severe ABO-related hemolytic disease of the newborn is not common. The mechanisms behind this are discussed in more detail in the section on clinical consequences.

**Pathogen Interactions**

Bacteria, viruses, and parasites have been proposed as important driving forces for the geographic distribution of ABO blood group phenotypes because different pathogens demonstrate blood group—identical or—cross-reactive molecules on their surfaces. These are the probable targets for “blood group” antibodies, and their existence is the leading hypothesis as to why we make naturally occurring antibodies against the carbohydrate blood groups we lack. In addition, many pathogens show selective binding to blood group carbohydrate moieties via lectins (reviewed by Garratty). More recent but perhaps anecdotal evidence for this theory was provided after severe hemolytic transfusion reactions in two group B patients receiving apheresis platelets from the same group A platelet donor. The donor had donated regularly for a period of 20 years, during which time no adverse effects of transfusion had been observed, but had recently begun to supplement his diet with high-dose probiotics and his anti-B titer was shown to be greater than 8000. Inhibition studies performed with plasma from random group A donors and solubilized probiotic tablets demonstrated a reduction in titer, hinting at a role for ABO antibodies in neutralizing pathogens. This line of thinking is supported further by data from studies on viral glycosylation in host cells. HIV cultured in vitro with peripheral blood mononuclear cells (PBMC) from donors of different
ABO groups demonstrated specific neutralization with anti-A of those isolates grown in group A PBMCs but not those cultured with group B or group O cells.\textsuperscript{89} Measles virus, when cocultured in a system expressing ABH glycosyltransferases (to mimic an in vivo environment), expressed A or B epitopes, or not, according to the enzymes expressed. Viral particles could then be neutralized by normal polyclonal anti-A or anti-B sera in the presence of complement if the corresponding glycans were present.\textsuperscript{90} Conversely, the A and B (and H) antigens are also used as receptors for pathogen invasion by attachment to host cells as mentioned previously, so the equilibrium between invasion and evasion is a fine balance on both sides.

Furthermore, growing evidence for a leading role of \textit{Plasmodium falciparum} as the major force shaping the human genome including the distribution of the blood groups has emerged.\textsuperscript{91,92} The parasite-induced RBC surface protein pfEMP1 is known to bind to the A antigen trisaccharide,\textsuperscript{93} and it has been shown that the severity, including mortality, of malarial disease is significantly lower in group O children than in other groups, mainly through the mechanism of reduced rosetting.\textsuperscript{94-95} It is the focus on pediatric disease that is thought to make this pathogen particularly effective in exerting a selection pressure on our genome.

The general theme here is the concept of herd immunity, i.e., the fact that differences in the population will keep at least a fraction of all individuals protected from most if not all pathogens for humanity to survive. ABO differences as part of our innate immunity appear to be one of the better examples of this idea.\textsuperscript{96}

\section*{Clinical Significance}

Of all antibodies to RBC blood group antigens, anti-A and anti-B are arguably the most clinically important. On the other hand anti-A1 and anti-H are very seldom found to correlate with hemolytic adverse events but often show lower thermal optima. Before the discovery of ABO, transfusion between humans had been attempted with mixed success. In some cases, the patient survived and got better. In others the patient died rapidly, which we now realize was attributable to rapid intravascular hemolysis of ABO-incompatible RBCs (reviewed by Mollison et al.\textsuperscript{97}). It has been reported that transfusion of as little as 30 mL of ABO-mismatched blood can result in a rapid fatal intravascular transfusion reaction (reviewed in Issitt and Anstee\textsuperscript{98}). Together with transfusion-related acute lung injury (TRALI) and bacterial contamination of blood components, ABO incompatibility is still one of the main risks for major morbidity and mortality associated with transfusions, after erroneous transfusions.\textsuperscript{99,100} The latest FDA report on transfusion-associated fatalities (found at http://www.fda.gov/cber/blood/fatal08.htm) shows that blood group incompatibility was judged as the cause of death in 37 percent of all cases during 2008, with TRALI at 35 percent. Among the hemolytic fatalities ABO was the cause in 59 percent. Surprisingly, however, many reports show that approximately 50 percent of patients who are inadvertently transfused with a major ABO-mismatched unit of blood tolerate the blood without any apparent signs of a transfusion reaction.\textsuperscript{101} The mechanisms underlying the ability of some individuals to tolerate ABO-mismatched blood are not well understood but are very interesting as identification of resistance markers could be exploited in transplantation therapy. Despite our relative sophistication in providing appropriately ABO-matched blood for patients, there is recent clinical evidence that even the concept of ABO-compatible (as opposed to ABO-identical) products may not be as safe as it would appear. A study that followed the posttransfusion mortality among more than 86,000 patients receiving plasma showed that exposure to ABO-compatible but non-ABO-identical plasma was associated with an increased risk of death.\textsuperscript{102} Whether this is associated with immune complexes between anti-A/B and soluble A/B antigen in AB plasma, for instance, remains to be studied. Furthermore, reports of the usage of platelet concentrates in cardiac surgery has also demonstrated less favorable outcomes in those patients receiving ABO-mismatched products.\textsuperscript{103} The authors hypothesized that the formation of immune complexes may trigger cellular and inflammatory changes that adversely affect patient outcome.

Hemolytic disease of the fetus and newborn (HDFN) as a result of ABO incompatibility between mother and baby is a relatively common event in group O mothers carrying a group A or group B fetus. However, the disease is generally mild and rarely requires treatment, most often by phototherapy,\textsuperscript{7} although inhibition of antibodies by administration of soluble A or B trisaccharides has been used successfully.\textsuperscript{104} Mild HDFN is a consequence of the comparatively low levels of IgG ABO antibodies (which are often mainly IgG2 or IgG4) capable of crossing the placenta and also is related to the immaturity of the glycosylated structures on fetal RBCs. In addition, ABH antigens are present on many other cell types so the antibody concentration on RBCs is limited and the DAT often only weakly positive.

The clinical significance of anti-A and anti-B extends beyond transfusion medicine and is important in both solid organ and hematopoietic transplantation. Although ABO-mismatched hematopoietic stem cell transplantation is standard practice with a favorable outcome in most cases, transplantation of ABO-incompatible solid organs has been established only relatively recently with moderately successful outcome.\textsuperscript{105} Children younger than 3 years of age have been shown to tolerate mismatched organs better, and West and colleagues have demonstrated good posttransplant survival in infants undergoing ABO-incompatible heart transplants,\textsuperscript{106} showing that the relatively immature B-cell response in these patients can be exploited.\textsuperscript{107} In a study of 46 patients undergoing ABO-mismatched renal transplants, Tobian et al.\textsuperscript{108} demonstrated that therapeutic apheresis to reduce anti-A and anti-B titers to less than
16 together with standard immunosuppression protocols results in successful and stable engraftment. Progress continues in this field, driven by a shortage of appropriate organs for transplant. Yazer and Triulzi conclude that immune hemolysis remains a major complication in ABO-mismatched transplantation of solid organs and to a lesser extent hematopoietic progenitor cells. However, passenger lymphocyte syndrome attributable to ABO or other blood group antibodies can now often be ameliorated by monoclonal antibody therapy, once clinicians realize the problem.

**Summary and Future Perspectives**

Despite the relative simplicity of the A and B antigens, perhaps especially considering the minor biochemical difference between them, the ABO blood group system remains one of the most interesting, both clinically and scientifically, dividing the world’s population including patients and donors into four groups irrespective of origin or creed. Figure 7 summarizes the four principal levels at which the ABO system can be considered and shows how immunohematologists have been able to exploit the steadily increasing knowledge of this system by devising tests taking advantage of each of the different levels. It also shows some of the natural consequences generated and their relation to the microbes surrounding us.

A whole array of ABO-related research and other projects is currently in progress. Even if a surprising number of ABO alleles has been discovered already, no doubt there are more to come. ISBT is currently working to facilitate communication and reporting by introducing an official allele nomenclature. Although ABO genotyping cannot yet be used as a stand-alone analysis to support clinical decisions, improvement of the current status is highly desirable for its use as an independent addition to serology in the reference laboratory. Together with colleagues across Europe, we recently introduced the first microarray-based ABO typing system that takes the first steps toward this goal on a higher throughput system. Efforts to renew methods for serologic typing are also ongoing. For instance, the young field of microfluidics holds promise for alternative blood grouping technology being more rapid, using smaller volumes of reactants, and being applicable to broader testing platforms.

The temptation to create ABO-universal blood for transfusion purposes has kept scientists busy since the early 1980s when the first successful report of deliberate transfusion with modified RBCs across the ABO barrier was published. Progress from those early days, using a poorly effective B-converting exoglycosidase from green coffee beans, has continued with the aims of eliminating ABO-associated hemolytic transfusion reactions and simplifying blood logistics and inventory management. Not only has the process taken important strides toward becoming a reality with both A- and B-degrading enzymes recombinantly available and necessary clinical trials in progress or being designed, but the project has also brought with it the scientifically exciting discovery of a large new class of bacterially derived exoglycosidases enzymes unknown hitherto and without clear homology or resemblance to any other group of molecules. It is unknown at this point why so many different bacterial species have developed these blood group–converting enzymes. Speculation includes that it would simply be a means of digesting potential nutrient saccharides, but it also may be yet another way for microbes to make sure they are able to attach to the host cell surface, even if the histo-blood group of the current host happens not to fit the bacterial lectins initially. It is actually quite likely that yet other glycosidase specificities will be found in this extended arsenal of newly discovered enzymes. A recently exploited example is the GalαβGal (also known as the straight B, fucose-less B, or Galli antigen) -converting enzyme subfamily of galactosidases that can, for instance, be used to degrade xenograft antigenicity in pig tendons for use in knee surgery.

Finally, it is easy to make the mistake of sitting back and looking at the ABO system as close to complete when it comes to knowledge and discovery. We predict that both the antigenic diversity and the way we look at antibody specificities in this and related systems will change dramatically during the next few years based on recent and future findings made possible by new technologies. ABO has for a long time served as a great model for genetics, enzymology, and biochemistry thanks to our deep understanding of the interindividual variation in this system, and there is nothing to suggest that it will stop now when high-throughput genetics and glycotpe arrays are here to help us. On the contrary, it should be expected that there are still more surprises waiting around the corner.

**Fig. 7** Principles of the ABO system at four molecular levels and their modes of interaction are schematically represented by arrows in black. On the left side, the laboratory analytical modalities currently exploited at each level are listed; the right side shows some of the innate mechanisms used between host and pathogen.
Acknowledgments

Some of the content and figures in this review have been modified from the doctoral theses of M.L.O. (Molecular Genetic Studies of the Blood Group ABO Locus in Man; Lund University, 1997) and Dr. Bahram Hosseini-Maaf (Genetic Characterisation of Human ABO Blood Group Variants with a Focus on Subgroups and Hybrid Alleles; Lund University, 2007).

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Clinical evaluation for lymphoproliferative disease prompted by finding of IgM warm autoanti-I\(^\text{T}\) in two cases


Anti-I\(^\text{T}\) is an unusual specificity originally described as a naturally occurring cold agglutinin. The antibody reacts strongly with cord RBCs, weakly with adult I RBCs, and most weakly with the rare adult i RBCs. IgG anti-I\(^\text{T}\) in patients with hemolytic anemia has been associated with Hodgkin’s lymphoma. Difficulties in blood grouping tests and the presence of a warm reactive agglutinin in samples from two patients with hemolytic anemia led to further serologic studies and the identification of anti-I\(^\text{T}\). In both cases, the anti-I\(^\text{T}\) was a rarely encountered IgM warm reactive agglutinin; in one case, the IgG component was also anti-I\(^\text{T}\), whereas in the second case the IgG antibody was broadly reactive. The unusual serologic finding of anti-I\(^\text{T}\) prompted further clinical evaluation for lymphoproliferative disease in these two patients. *Immunohematology* 2009;25:60–62.

**Key Words:** IgM warm autoantibody, autoanti-I\(^\text{T}\), hemolytic anemia

Anti-I\(^\text{T}\) was originally described as a benign, naturally occurring, IgM cold agglutinin in Melanesians\(^1\) and later in Venezuela Indians.\(^2\) The anti-I\(^\text{T}\) agglutinin did not demonstrate classical I or i specificity but reacted strongly with cord RBCs, weakly with normal adult I RBCs, and most weakly with the rare adult i RBCs. It was thought the agglutinin recognized a transition state of i to I, thus the designation I\(^\text{T}\) (T for transition).\(^3\) Subsequent data do not support this hypothesis; I\(^\text{T}\) is expressed nearly as well or as well on fetal RBCs ranging in age from 11 to 16 weeks as on cord RBCs.\(^4\) The biochemical structure of I\(^\text{T}\) is still unclear.

The first four examples of IgG anti-I\(^\text{T}\) were in sera of patients with autoimmune hemolytic anemia (AIHA) and Hodgkin’s lymphoma.\(^5,6\) Others reported a similar association.\(^7\) Halliegh et al.\(^8\) found three examples of IgG autoanti-I\(^\text{T}\) in patients who did not have Hodgkin’s disease or AIHA. One example of IgM warm anti-I\(^\text{T}\) and one IgM cold plus IgG anti-I\(^\text{T}\) in patients with AIHA were not associated with Hodgkin’s disease, but one of the cases was associated with non-Hodgkin’s lymphoma.\(^8,9\) We report an unusual 37\(^\circ\)C reactive IgM agglutinin plus an IgG anti-I\(^\text{vo}\) and a 37\(^\circ\)C reactive IgM agglutinating anti-I\(^\text{T}\) plus broadly reactive IgG autoantibody in two patients with hemolytic anemia who were subsequently evaluated for lymphoproliferative disease owing to the specificity of the antibodies reported.

**Case Reports**

**Case 1**

A 69-year-old Hispanic woman presented with pancytopenia (WBC = 2.3 \(\times\) \(10^9\)/L; Hb = 6.9 g/dL; Hct = 19.1%; platelets = 64,000/µL), splenomegaly but no lymphadenopathy, and hemolytic anemia (spherocytosis; LDH = 672 U/L [normal 355–630]; total bilirubin = 2.8 mg/dL [normal 0.1–1.2]; direct bilirubin = 0.7 mg/dL [normal 0–0.4]). The direct antiglobulin test (DAT) was positive. Lymphoma was not suspected until after the anti-I\(^\text{T}\) was identified. Computed tomography (CT) scans showed evidence of large lymphoid infiltrates composed of a mixture of small and large lymphocytes. Bilateral bone marrow aspiration showed increased lymphocytes but was not diagnostic of lymphoma. The patient was empirically treated with steroids and had improved hemoglobin and sense of well-being. Steroids were tapered. Two and a half months later the patient returned for a splenectomy, which was pathologically negative for lymphoma. Sections showed lymphoid hyperplasia with a benign phenotype consisting of T and B cells with no aberrant expression of CD5, CD23, or bcl-2. Serologic studies at that time showed similar reactivity of anti-I\(^\text{T}\). Three months later, the patient exhibited mild thrombocytopenia with slightly elevated LDH. A repeat bone marrow showed lymphocytosis suggestive of low-grade B-cell lymphoproliferative disease. A year later, after rituximab and other chemotherapy, the bone marrow showed atypical lymphoid infiltrates. The infiltrates were composed primarily of small mature lymphocytes with occasional larger lymphocytes noted, most likely representing a lymphoproliferative disorder. Flow cytometry showed an entire B-cell population that did not express CD20. Less than 3 years after original presentation, the patient was transfusion-dependant and diagnosed with aplastic anemia. She was discharged to hospice care with end-stage liver disease, lymphoproliferative disorder, and hemolytic anemia.

**Case 2**

A previously healthy 19-year-old Korean man presented to the emergency room with insidious onset of fatigue, shortness of breath, dyspnea on exertion, and pallor. Laboratory data revealed thrombocytopenia and hemolytic anemia:
Materials and Methods

The DAT was performed with anti-human IgG (Ortho Clinical Diagnostics, Raritan, NJ) and anti-human C3 (in-house reagent). A 6% bovine albumin control was tested in parallel. The in-house anti-C3 was prepared by injecting rabbits with purified proteins and standardized as previously described. Before testing, the patients’ RBCs were treated with 0.01 M dithiothreitol (DTT) to break IgM bonds that cause spontaneous agglutination. Eluates were prepared from the patients’ RBCs using a commercial acid elution kit (Gamma Elu-Kit II, Gamma Biologicals, Houston, TX); cold LISS was substituted for the kit wash solution.

For immunoglobulin classification, samples were treated with 0.01 M DTT. The agglutinin titer and thermal amplitude was determined at 37°C (prewarmed), 30°C, 22°C, and 4°C with group O adult I, cord, and adult i RBCs, and for Case 1, with DTT-treated autologous RBCS. The tests at each temperature were set up separately (i.e., using separate sets of dilutions) to eliminate potential carryover of agglutination. Patients’ sera were also tested by a previously described serum screen method used to characterize antibodies in AIHA. Briefly, serum was tested with and without acidification and the addition of fresh normal serum as a source of complement, against untreated and enzyme-treated RBCS at 37°C (prewarmed) and 20°C. Agglutination results were graded and scored as previously described.

Results

Case 1

The DAT on DTT-treated RBCs was 3+ with anti-IgG and anti-C3; the 6% albumin control was nonreactive. The initial titer and thermal amplitude studies showed a 37°C agglutinin that reacted to a higher titer with cord RBCS than with adult I RBCS. Titration results with adult I, cord i, and adult i RBCs (to determine specificity) are shown in Table 1. Anomalous results (unexplained high titer) were obtained with DTT-treated autologous RBCS: titers of 512 at 37°C, 1024 at 30°C, 2048 at 22°C, and 8000 at 4°C. An apparent anti-I was detected at 4°C (adult I RBC titer of 256, cord RBC titer of 64). An acid eluate prepared from the patient’s RBCS reacted more strongly with cord RBCS than with adult I RBCS at 37°C. After treatment with DTT, the patient’s serum and the eluate did not agglutinate cord RBCS, but reacted 1+ and 4+, respectively, at the antiglobulin test with anti-IgG. The dilution control (for the abolished agglutination) reacted 3½ to 4+ with the serum and 1½ with the eluate. Thus, IgM plus IgG anti-I1 was demonstrated in both the serum and the eluate. In the AIHA serum screen, agglutination was observed at 22°C and 37°C similar to that in the titration and thermal amplitude studies; a hemolysin was also observed with enzyme-treated RBCs at both temperatures, but without a clear preference for either temperature.

Case 2

The DAT on DTT-treated RBCs was 4+ with anti-IgG and anti-C3; the 6% albumin control was nonreactive. In the initial titer and thermal amplitude studies, an agglutinin reacted at 37°C with cord RBCS; adult I RBCs did not react at 37°C or 30°C. Titration results with adult I, cord i, and adult i RBCs are shown in Table 1. Sufficient autologous RBCS were not available. DTT treatment of the patient’s serum abolished agglutination with cord RBCS; however, dilutions of DTT-treated serum tested against adult I and cord RBCS did not show a difference in reaction strength of the IgG component. Thus, an IgM anti-I1 plus a broadly reactive IgG antibody was demonstrated in the serum. The eluate contained an IgG antibody suggestive of anti-I1 (titer/score versus adult I RBCS was 32/42 and versus cord RBCS was 128/55). In the AIHA serum screen, untreated RBCS were weakly agglutinated at 22°C and weakly sensitized at 37°C; enzyme-treated RBCS were agglutinated and hemolyzed at both temperatures.

Discussion

Table 1. Titer and thermal amplitude of two examples of anti-I1

<table>
<thead>
<tr>
<th>RBCS</th>
<th>Case 1 titer (score) at:</th>
<th>Case 2 titer (score) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
<td>30°C</td>
</tr>
<tr>
<td>Adult I</td>
<td>1 (4)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Cord I</td>
<td>32 (37)</td>
<td>128 (61)</td>
</tr>
<tr>
<td>Adult i</td>
<td>1 (6)</td>
<td>2 (10)</td>
</tr>
</tbody>
</table>

*NT = not tested
IgM warm autoagglutinins reactive at 37°C are an unusual subcategory of warm autoimmune hemolytic anemia. Typically, the patient’s RBCs are spontaneously agglutinated, requiring treatment with a sulphhydryl reagent such as DTT to resolve ABO and Rh typing and to interpret the DAT. The two cases presented here were referred to the immunohematology research laboratory for further diagnostic testing and characterization of the agglutinin when these characteristics were noted by our reference laboratory. In both cases the warm agglutinin demonstrated I\(^{\text{a}}\) specificity. Both of our cases also demonstrated an IgG autoantibody component; in Case 1, the IgG autoantibody was anti-I\(^{\text{a}}\).

We have no explanation for the high titers obtained after DTT-treatment of the group O autologous RBCs in Case 1. This may indicate that the patient’s RBCs had greatly increased I\(^{\text{a}}\) expression. When group O untreated, DTT-treated, and ficin-treated cord RBCs were tested in parallel with dilutions of this patient’s anti-I\(^{\text{a}}\) and another sample of anti-I\(^{\text{a}}\), no enhancement of reactivity was observed with DTT treatment whereas reactivity with ficin-treated RBCs was increased as expected by three to four dilutions (data not shown). Thus, the DTT treatment should not have affected the autologous RBC test results. The previous sample of warm IgM anti-I\(^{\text{a}}\) associated with AIHA did not agglutinate that patient’s untreated autologous RBCs at 37°C (the optimal temperature of reactivity).\(^7\)

Anti-I\(^{\text{a}}\) is an unusual specificity and may not even be suspected unless dilutions of the sera are tested with cord i and adult I RBCs in parallel with adult I RBCs. In our cases, additional titrations to identify the specificity were only pursued after the initial increased reactivity with cord RBCs was observed. Examples of anti-I\(^{\text{a}}\) have been reported to be IgM cold agglutinins or IgG antibodies optimally reactive at 37°C. We are only aware of one reported example of IgM warm anti-I\(^{\text{a}}\), and that case was not associated with Hodgkin’s disease or lymphoma.\(^7\) In more than 20 years, we have encountered only two other examples of IgM warm reactive anti-I\(^{\text{a}}\); neither had an IgG component, but both were associated with a history of lymphoma before the serologic workup.

Determining the specificity of autoantibodies is typically of academic interest only. In general, serologic results do not necessarily prompt a clinical evaluation for lymphoma. In both of these cases, the unusual RBC antibody and the historic association of this specificity with lymphoma, together with the presenting findings of AIHA, led to the lymphoma workup. For patients who do not progress to lymphoma, one might speculate that rituximab (anti-CD20) given for the AIHA could have eradicated a malignant clone of B cells.

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Regina M. Leger, MSQA, MT(ASCP)SBB, Research Associate, American Red Cross Blood Services, Southern California, 100 Red Cross Circle, Pomona, CA 91768; Frederick Lowder, CLS,MT(ASCP)SBB, Olive View–UCLA Medical Center, Sylmar, CA; Maria C. Dungo, MD, Assistant Clinical Professor, David Geffen School of Medicine at UCLA, Harbor–UCLA Medical Center, Torrance, CA; Weip Chen, MD, Harbor–UCLA Medical Center, Torrance, CA; Holli M. Mason, MD, Assistant Clinical Professor of Pathology, David Geffen School of Medicine at UCLA, Director of Transfusion Medicine and Serology, Harbor–UCLA Medical Center, Torrance, CA; and George Garratty, PhD, FRCPath, Scientific Director, American Red Cross Blood Services, Southern California, Pomona, CA.
Southeast Asian ovalocytosis is associated with increased expression of Duffy antigen receptor for chemokines (DARC)

I.J. Woolley, P. Hutchinson, J.C. Reeder, J.W. Kazura, and A. Cortés

The Duffy antigen receptor for chemokines (DARC or Fy glycoprotein) carries antigens that are important in blood transfusion and is the main receptor used by Plasmodium vivax to invade reticulocytes. Southeast Asian ovalocytosis (SAO) results from an alteration in RBC membrane protein band 3 and is thought to mitigate susceptibility to falciparum malaria. Expression of some RBC antigens is suppressed by SAO, and we hypothesized that SAO may also reduce Fy expression, potentially leading to reduced susceptibility to vivax malaria. Blood samples were collected from individuals living in the Madang Province of Papua New Guinea. Samples were assayed using a flow cytometry assay for expression of Fy on the surface of RBC and reticulocytes by measuring the attachment of a phycoerythrin-labeled Fy6 antibody. Reticulocytes were detected using thiazole orange. The presence of the SAO mutation was confirmed by PCR. There was a small (approximately 10%) but statistically significant (p=0.049, Mann-Whitney U test) increase in Fy expression on SAO RBC compared with RBC from individuals without this polymorphism: mean Fy expression (mean fluorescence intensity [MFI]) was 10.12 ± 1.22 for SAO heterozygotes versus an MFI of 8.95 ± 1.1 for individuals without SAO. For reticulocytes the MFI values were 27.61 ± 19.12 for SAO heterozygotes and 16.47 ± 3.81 for controls. SAO is associated with increased and not decreased Fy6 expression so that susceptibility to P. vivax infection is unlikely to be affected.


Key Words: Duffy, Southeast Asian ovalocytosis, antigen expression

In 1949 Haldane proposed that malaria had a dramatic influence on the human genome, by selecting for genetic polymorphisms that were known or suspected to protect against malaria. Although some of these polymorphisms can be detrimental when individuals are homozygous, e.g., sickle cell anemia, their apparent selection in human populations living in malaria-endemic areas is evidence that they offer some protection against death from malaria, an infectious disease that continues to kill more than 1 million persons annually. Critical to this theory is the concept of heterozygote advantage. That is, carriage of the mutant gene on one member of a chromosomal pair is beneficial whereas homozygosity is deleterious. An extreme example of heterozygote advantage in Melanesian populations is Southeast Asian ovalocytosis (SAO), a hereditary form of ovalocytosis that is caused by a 27-base pair deletion in the gene encoding the major integral RBC membrane protein band 3 (AE1, SLC4A1), leading to a distinctive change in RBC morphology. SAO is widespread in several populations of Papua New Guinea (PNG), where its prevalence correlates with malaria endemicity and altitude. In these populations, homozygosity is apparently incompatible with full development of the fetus inasmuch as no persons homozygous for the band 3 mutation have yet been described, but heterozygosity confers strong protection against cerebral malaria. Although early studies had suggested that the SAO trait may afford some protection against vivax or falciparum parasitemia, other studies did not support this hypothesis. The mechanism of protection against cerebral malaria is not completely understood, but the specific protection against cerebral malaria suggests that it may operate by means of alterations in sequestration of infected RBC. However, SAO confers limited or no protection against placental malaria, which is also linked to sequestration of infected RBCs.

Individuals whose RBCs are negative for expression of Duffy antigens illustrate homozygote advantage for vivax malaria in that their RBCs are completely refractory to invasion by Plasmodium vivax. Historic serologic data suggest expression of some antigens on human RBCs was suppressed by the SAO trait, but expression of Duffy antigens did not appear suppressed as determined by the less sensitive serologic instruments available at that time. In those studies, antibodies of varying specificities were placed in tubes using serial dilution, and results were read macroscopically with the aid of an eyepiece using a standardized scoring system. In this study, we used molecular and cytometric techniques to diagnose SAO and to reexamine whether there was a difference in expression of Duffy antigen receptor for chemokines (DARC) between SAO and control individuals in the context of possible differential susceptibility to infection with different Plasmodium species.

Materials and Methods

Blood was collected by venipuncture in CPD anticoagulant or by finger prick using a BD Microtainer (Becton Dickinson, San Jose, CA) with EDTA anticoagulant from healthy volunteers from two villages, Sempi and Bemlon, on the north coast of the Madang Province, PNG. Oral informed consent was obtained after approval for the study by the Medical Research Advisory Committee of the PNG...
Ministry of Health. After collection, samples were kept on ice or refrigerated at 4°C. Within 1 week, flow cytometric studies were undertaken with antibody to Fy6 (kindly provided by John Barnwell, CDC, Atlanta, GA). This is a mouse monoclonal antibody that attaches to the first extracellular domain of the Fy glycoprotein, in an area thought to correspond to the binding site of \( P. \) \textit{vivax} merozoites.\textsuperscript{17,18} The antibody was conjugated directly to a phycoerythrin (PE) label (Prozyme, San Leandro, CA) according to the manufacturer's instructions. A 5-µL aliquot of blood was washed three times in 50 µL of PBS and pelleted. RBCs were incubated with 50 µL of a 1 in 50 dilution of Fy6 antibody for 15 minutes at 37°C. Cells were then washed twice in PBS and resuspended in thiazole orange (TO) solution according to the manufacturer's specifications (Becton Dickinson). The samples were analyzed on a flow cytometer (Mo-Flo, Da-kOcytometry, Fort Collins, CO) equipped with a 70-mW air-cooled argon ion laser at 488 nm. TO fluorescence was read with a 525-nm bandpass optical filter and PE fluorescence with a 570-nm bandpass filter. Compensation was applied, and negative fluorescence signals were adjusted until they were orthogonal. Forward-scatter, side-scatter, and fluorescence data were analyzed. A count of 5000 TO-positive cells was taken per sample. This was approximately 1 to 2 percent of the total number of cells assayed for each individual. Beads of known immunofluorescence (Immuno-Brite, Coulter, Puerto Rico) were used to standardize experiments undertaken at different times. Detection of the SAO mutation was undertaken using previously described PCR methods.\textsuperscript{19} Experienced microscopists from the PNG Institute of Medical Research performed light microscopic inspection of Giemsa-stained blood films for malaria parasites. Statistical analysis was undertaken by using SPSS (Statview 4.51, Abacus Concepts, Berkeley, CA). We have used nonparametric testing because we do not have evidence for normal distribution of DARC abundance in the surface of SAO erythrocytes.

**Results**

Samples from 19 SAO heterozygotes and 20 non-SAO donors were examined. Mean age of donors did not differ significantly between the two groups (mean age, 29.32 ± 13.70 years for SAO heterozygotes versus 26.91 ± 11.80 years for non-SAO individuals). Four individuals had malaria infection based on inspection of blood smears. One SAO and one non-SAO individual had \textit{Plasmodium malari-ae}, and two non-SAO donors had \textit{Plasmodium falciparum}. SAO RBC showed increased expression of Fy glycoprotein relative to non-SAO RBC as measured by binding of the Fy6 monoclonal antibody (Table 1). The level of Fy6 antigen was also higher in SAO reticulocytes compared with control reticulocytes, but the difference was not statistically significant (Table 1). Subsequent analysis of the data did not suggest the differences were caused by a systemic bias attributable to sex, village of origin, or method of obtaining blood (data not shown).

**Table 1.** Mean fluorescence intensity (± standard deviation) of Fy6 antibody binding to erythrocytes and reticulocytes

<table>
<thead>
<tr>
<th></th>
<th>All Subjects (Reticulocytes)</th>
<th>All Subjects (Erythrocytes)</th>
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</thead>
<tbody>
<tr>
<td>SAO heterozygotes (N = 19)</td>
<td>27.61 ± 19.12</td>
<td>10.12 ± 1.22</td>
</tr>
<tr>
<td>Non-SAO individuals (N = 20)</td>
<td>16.47 ± 3.81</td>
<td>8.95 ± 1.1</td>
</tr>
<tr>
<td>P value for difference (Mann-Whitney U test)</td>
<td>0.65</td>
<td>0.0487</td>
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**Discussion**

The main finding of this study was that the SAO trait does not result in reduced expression of Fy antigen. In fact, we observed a 10 percent increase (p=0.049) in Fy antigen expression by SAO RBCs when compared with RBCs from non-SAO individuals from the same area. This difference was significant when expression by all RBC populations was evaluated but not when only SAO reticulocytes were compared with non-SAO reticulocytes (although a suggestive trend existed). As anticipated, reticulocyte expression of Fy6 antigen exceeded RBC expression.\textsuperscript{20} The reasons for the increased RBC Fy6 antigen expression in SAO are unclear at present, but it may reflect either a decreased rate of loss of Fy6 antigen from the surface of SAO RBC relative to control RBC, perhaps in relation to the increased rigidity of the SAO cell wall, or differences in the surface area of ovalocytes versus non-SAO RBC. Because there was only a small increase in Fy6 antigen expression on SAO RBC and there was no statistically significant increase in the reticulocyte subpopulation, which is the only subpopulation susceptible to infection by \( P. \) \textit{vivax}, it is unlikely that this alteration affects susceptibility to this parasite, although there is other in vitro evidence that alterations in numbers of Fy antigens expressed may alter that susceptibility.\textsuperscript{21} It is not surprising that the difference in Fy levels was not shown in the paper of Booth et al.\textsuperscript{16} because they were using less sensitive serologic methods or because Fy6 antigen is more available or more expressed in SAO ovalocytosis over the antigens tested in the previous study. However, their findings with respect to other antigens, including Rh polypeptides, have subsequently been confirmed by molecular techniques.\textsuperscript{22}

Fy antigen negativity caused by homozygosity for a promoter mutation that silences transcription of the \( FY \) gene\textsuperscript{23} is observed in many ethnic groups in sub-Saharan Africa, and presumably accounts for the relative lack of vivax malaria in this part of the world. The apparently independent emergence of heterozygosity for this same promoter mutation has also been described in the Wosera area of PNG, where SAO is rare or nonexistent. There is a need for further evaluation of whether other common RBC polymorphisms affect Fy antigen expression, especially those that are associated with changes in RBC shape and surface area. For example, \( \alpha \)-thalassemia is common in PNG, and in many areas, including the Madang Province, nearly all individuals have the typical \( \alpha \)-globin mutation seen in Melanesians.\textsuperscript{24} It
seems unlikely that this common polymorphism would act as a confounding factor in our study on the grounds of biologic plausibility, low likelihood of linkage disequilibrium, and very high prevalence in the region (assuming therefore more or less equal distribution between SAO and control individuals within the population studied). Finally, it will be important to determine whether environmental variables extant in malaria-endemic populations might change Fy antigen expression. Another example would be iron deficiency, which is prevalent in PNG and many other malaria-endemic regions of the world.

There is evidence that the SAO trait affects several steps of the biologic cycle of *P. falciparum* that may be relevant for the selective advantage conferred by this trait against malaria. In particular, SAO RBCs are partly resistant to invasion in culture by merozoites of *Plasmodium knowlesi* and several lines of *P. falciparum*, and the SAO trait also results in altered adhesion of infected RBCs to CD36. As with ABO, it is likely that the evolutionary pressure exerted by survival with SAO is related to survival of children from cerebral malaria before reproductive age. Although Fy expression probably affects *P. vivax* infection, this evolutionary pressure is almost certainly caused by *P. falciparum* through its relationship to RBC adhesion.

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**References**


I.J. Woolley (corresponding author), Department of Infectious Diseases, Monash Medical Centre and Monash University, Clayton, Victoria, Australia, Paul Hutchinson, Centre for Inflammatory Diseases, Monash Medical Centre, Clayton, Victoria, Australia, John C. Reeder, Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea (current affiliation: Burnet Institute, Prahran, Victoria, Australia), James W. Kazura, Center for Global Health & Diseases, Case Western Reserve University, Cleveland, Ohio, USA, and Alfred Cortés, Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea (current affiliation: ICREA and Institute for Research in Biomedicine, Barcelona, Spain).

Manuscripts
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Sickle cell disease (SCD) is described as the first identified “molecular” disease since its manifestations stem from a substitution of valine for glutamic acid in the structure of the β chain hemoglobin molecule. As a result of this change, RBCs form characteristic “sickle” shapes and the surface of these RBCs attract each other, polymerizing when in a low oxygen environment. This seemingly “small” variation in the structure of the RBC causing polymerization leads to manifestations such as chronic occlusion of blood vessels (vaso-occlusion), reduced blood flow to vital organs (ischemia), and alterations of the immune system. In addition, the abnormal sickle cells are prematurely removed from circulation, resulting in hemolytic anemia. Transfusion is a vital component of the treatment of some of the complications of SCD. It is also a modality used to prevent some of these complications from occurring. Patients with SCD are unique because those who are transfused usually require chronic transfusion, resulting in exposure to many different blood donors over the course of treatment. In addition, most patients with SCD in the United States are African American, and most donors are Caucasian from Western European descent. As a result of this difference, patients with SCD are exposed to RBC antigens that they lack, putting them at risk for forming alloantibodies, defined as RBC alloimmunization. Therefore, it is important to understand the issues involved in the safe and effective transfusion of patients with SCD.

Defining Hemoglobinopathies

Hemoglobin (Hgb) is made up of iron (heme) and 4 globin chains. The type of globin chains determines the type of hemoglobin (see Table 1). Since SCD is characterized by the presence of an abnormal or variant hemoglobin, hemoglobin S, it is characterized as a hemoglobinopathy. The composition of hemoglobin for patients with homozygous SS is presented in Table 2.

There are a variety of other abnormal hemoglobins that may be present with or without Hgb S. The type of hemoglobin a person has is based on patterns of inheritance. If each parent contributes hemoglobin S, the child can inherit two copies and is designated as Hgb SS, or is homozygous for hemoglobin S. If a child only inherits one copy from one parent and a copy of normal hemoglobin, Hgb A, they are designated as Hgb AS, or heterozygous. These individuals are usually asymptomatic, and only develop manifestations under rare circumstances, where they become hypoxic, such as at high altitudes. Therefore, not every person with an abnormal hemoglobin develops or exhibits signs and symptoms. Although Hgb S is found worldwide, it is most commonly found in western Africa. About one in every 400–500 African Americans, or 80,000, has SCD. About 9000 African Americans, or one in 12, have sickle cell trait.

On the other hand, patients who have manifestations of their sickle hemoglobin are considered to have SCD. This includes patients who are homozygous for Hgb SS, as described previously. In addition, some patients who inherit Hgb S from one parent and another abnormal hemoglobin from the other parent can also have SCD. Common examples are designated as Hgb SC and Sβ-thalassemia. These individuals can have a milder clinical course than that of individuals who are homozygous for Hgb S.

Since RBCs with Hgb S are abnormal, they are removed from circulation in the spleen more rapidly than normal RBCs. This leads to a reduced life in the circulation of 16–20 days in comparison to 120 days for normal RBCs. The premature destruction of RBCs, with the accompanying decrease in hemoglobin, is classified as a hemolytic anemia.

### Table 1. Composition of normal adult hemoglobin

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Composition</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hgb A</td>
<td>α2β2</td>
<td>96–98</td>
</tr>
<tr>
<td>Hgb A2</td>
<td>α2β2</td>
<td>1.5–3.5</td>
</tr>
<tr>
<td>Hgb F</td>
<td>α2γ2</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

### Table 2. Hemoglobin SS (α2β2)

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hgb S</td>
<td>80</td>
</tr>
<tr>
<td>Hgb A2</td>
<td>2–4.5</td>
</tr>
<tr>
<td>Hgb F</td>
<td>120</td>
</tr>
</tbody>
</table>

### Typical Laboratory Findings in Sickle Cell Disease

Patients with SCD are commonly diagnosed after newborn screening. In the past, young children presented with painful swellings of the feet and hands, a consequence of vaso-occlusion (see discussion on Clinical Manifestations of Disease).

Many patients with SCD have anemia, with hemoglobin levels between 6 and 8 g/dL. Characteristic sickle shaped cells are seen on peripheral smear (Fig. 1). Patients do not always have symptomatic anemia because the body compensates for the peripheral RBC destruction by increasing the rate of RBC production (erythropoiesis) in the bone marrow. Consequently, the bone marrow contains an increase in the number of RBC precursors. As a result, immature
precursors, suggestive of reticulocytes, are released from the marrow prematurely. Therefore, the peripheral smear will also show these immature RBCs, which are larger than mature RBCs and have a slightly blue color since they are not fully hemoglobinized. The term reticulocytes will be used in this education activity, and refers to these RBCs.

Another consequence of the high rate of hemolysis and erythropoiesis, or increased RBC turnover, is the accumulation of unconjugated or indirect bilirubin. As a result, some patients will have yellowing of the white of the eyes, scleral icterus, and skin, or jaundice. The increase in bilirubin also puts patients at increased risk for gallstones, and the patient may have to have their gallbladder removed. Table 3 lists the common laboratory findings in SCD and their etiologies.

Early diagnosis is essential in order to treat and even prevent some of the complications of SCD. In the past, approximately 25% of children between the ages of four months and five years with SCD died of pneumonia. The use of prophylactic penicillin in children with SCD in the past 14–16 weeks of gestation in pregnancies where there is a chance that the baby will inherit Hgb S from both parents. More commonly, newborn infants are screened through mandatory programs required by most states, soon after birth. Several methods exist for detecting abnormal hemoglobins. These include:

- High performance liquid chromatography (HPLC)—Considered one of the best methods to be able to detect abnormal hemoglobins and to be able to differentiate them from normal hemoglobin. HPLC uses reactions in columns of reagents. This technique also measures the quantity of the various hemoglobins.
- Hemoglobin electrophoresis—Separates different hemoglobins according to their characteristic migration across a gel of either cellulose acetate at an alkaline pH or citrate agar at an acid pH, based on charge. The different types of hemoglobin will migrate at different rates, occupying a characteristic position on the gel.
- Isoelectric focusing—If further separation is necessary, then isoelectric focusing can be performed. An electric current is passed through the gel that has areas of different pH. The identity of the hemoglobin can be made by its pattern of migration.
- Solubility test—Taking advantage of the fact that reduced Hgb S is insoluble in certain solutions, a simple solubility test can be used to detect the presence of Hgb S. After lysis, RBCs are reduced by sodium hydrosulphite. If Hgb S is present, the solution will be turbid; if not present, then the hemoglobin remains in solution and the suspension is clear. Since this test requires a certain amount of whole blood, it is not routinely used for newborn screening. Note that this test will just detect the presence of Hgb S, so patients who have sickle cell trait (heterozygous for Hgb S) will also be positive.

When performing patient testing, it is always important to follow the package insert and ensure that proficiency testing is performed in compliance with the Clinical Laboratory Improvement Amendments of 1988 (CLIA). CLIA regulations require that laboratories enroll in a Centers for Medicare and Medicaid Services (CMS) approved proficiency testing (PT) program for all regulated tests that the laboratory performs.

### Table 3. Common laboratory findings in sickle cell disease

<table>
<thead>
<tr>
<th>Laboratory value</th>
<th>Etiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low hemoglobin and hematocrit</td>
<td>Hemolysis</td>
</tr>
<tr>
<td>High mean cellular volume (MCV)</td>
<td>Reticulocytes</td>
</tr>
<tr>
<td>High white blood cell (WBC) count</td>
<td>Inflammation and increased marrow production</td>
</tr>
<tr>
<td>High platelet count</td>
<td>Increased marrow production</td>
</tr>
<tr>
<td>High lactate dehydrogenase (LDH)</td>
<td>Hemolysis</td>
</tr>
<tr>
<td>Low haptoglobin</td>
<td>Hemolysis</td>
</tr>
<tr>
<td>High total and indirect bilirubin</td>
<td>Hemolysis</td>
</tr>
<tr>
<td>High alkaline phosphatase until puberty</td>
<td>Elevated bone marrow activity</td>
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</table>

Clinical Manifestations of Sickle Cell Disease

The function of a normal RBC is dependent upon its ability to flow freely through blood vessels and to be flexible enough to move in and out of vessels and tissue where oxygen delivery occurs. Due to the rigidity of the sickle RBC, these properties are lost. Interestingly, when a patient with SCD has a higher percentage of hemoglobin F, the course of their
disease is milder and their life expectancy is longer. This is probably the result of the lower percentage of Hgb S in these patients, and that RBCs with Hgb F do not form polymers.\textsuperscript{9} These chronic physiologic changes can lead to problems in all organ systems; heart, lungs, and kidneys. SCD can be seen as a disease based on vaso-occlusion (VOC), chronic hemolytic anemia, and immunologic impairment.

**Occlusive Disease**

There are a variety of ways by which sickle cells cause obstruction. One minor mechanism is whereby irreversibly sickled cells obstruct small capillaries. More commonly, sickle cells are trapped in small venules and capillaries.\textsuperscript{1,10} The process of sickling and occlusion involves a complex interaction between chemical mediators, as well as elements other than the RBC itself. There is increasing understanding that the walls of blood vessels are involved. There appears to be increased adherence of sickle cells to the vascular walls, due to properties of the RBCs, as well as changes in the endothelial cells of blood vessels. The lipid bilayer of RBCs is disrupted in sickle cells, causing their early removal from circulation. Additionally, changes in their interactions with other cells within the blood stream, such as sticky white blood cells (WBCs) and activated platelets, contribute to vaso-occlusion.\textsuperscript{10,11} These changes can also cause the activation of coagulation, leading to an increased risk of deep venous thrombosis.\textsuperscript{10,12}

Vaso-occlusion manifests itself as one of the most common findings in a patient with SCD, pain crisis. Prior to routine screening of newborns, children were commonly diagnosed with SCD prior to the age of two, with occlusion in the hands and feet presenting as painful swelling, or dactylitis.\textsuperscript{4,5} This would not occur until six months of age because the higher level of fetal hemoglobin prior to six months is protective. Most patients will often have severe pain due to occlusion of blood flow to bones, bone marrow, muscles, or organs, and in their arms, legs, back, abdomen, and chest. Treatment consists of analgesia (many patients with SCD require chronic medication) and hydration, since dehydrated RBCs are more likely to sickle. The cause of crisis can vary between patients and between episodes in the same patient, but may be related to infection, extremes of temperature, medication, or any other physiologic changes. Occlusion is also associated with bony infarcts leading to osteonecrosis, skin ulcers, organ occlusion (including the spleen, resulting in functional asplenia or autosplenectomy), acute chest syndrome, and cerebrovascular accidents or strokes.

**Stroke**

Stroke in any patient is a devastating event, with approximately 11 percent of patients with Hgb SS having a stroke by the age of 20.\textsuperscript{13} As blood vessels become occluded, their diameter gets smaller and the rate of blood flow increases, thereby increasing the risk of stroke. Using a type of ultrasound, transcranial Doppler (TCD), to measure blood flow through the internal carotid and middle cerebral arteries of the brain, a study found that one could predict patients at risk for a first stroke.\textsuperscript{14} Chronic transfusion therapy has been used successfully to prevent primary stroke and then subsequent stroke (see Treatment of Sickle Cell Disease).\textsuperscript{15,16}

**Acute Chest Syndrome**

Acute chest syndrome (ACS) is the leading cause of death in adults with SCD and a major cause of morbidity in patients of all ages. It is characterized by the onset of worsening respiratory status, fever, and new infiltrate on chest x-ray. The inciting event can be respiratory infection; however, since a majority of cases of acute chest syndrome in adults follow pain crisis, it is believed that an embolus of fatty tissue from the bone marrow (the site of the initial vaso-occlusion) into the lungs is the culprit. Patients need enhanced oxygenation, so supplemental oxygen is administered. Transfusion is an essential component in the treatment of acute chest syndrome, as will be discussed in more detail later.\textsuperscript{17}

Of interest, there are many investigators looking at the role of inflammatory chemicals in the pathophysiology of SCD, as well as their potential for developing new treatments. For example, there is a reduction in levels of an adhesion molecule (VCAM-1) in patients with SCD on chronic transfusion protocols.\textsuperscript{18} Therefore, chronic transfusion protocols might prevent vaso-occlusion. Another recent study found that the increases in the inflammatory mediator, secretory phospholipase \(A_2\), can predict acute chest syndrome. Transfusing when levels are elevated can prevent acute chest syndrome.\textsuperscript{19}

**Hemolytic Anemia**

The abnormal shape and surface of sickle cells result in their shortened life span in the circulation and lead to the characteristic anemia. As RBCs are cycled through the spleen, the site of their ultimate destruction, they also injure the tissue of the spleen. Their rigidity impairs their ability to flow smoothly through the sinusoids, and their sharp edges cause them to be stuck, and to damage splenic tissue. In children under the age of five, blood can pool in the spleen, which becomes a site of sequestration of RBCs. When this occurs, the child has a painful, enlarged spleen accompanied by a drop in hemoglobin. Spleenic sequestration can be life-threatening since there is also a drop in blood volume in the vasculature, or hypovolemia. Transfusion management is essential in these cases. Many of these patients eventually need to have their spleen removed. As a patient gets older, the damage to the spleen is chronic, and the spleen actually becomes smaller, eventually shrinking and losing function.\textsuperscript{1,4,5}

In order to keep up with this chronic destruction, the body attempts to compensate by increasing RBC production. This chronic hemolysis may also create an inflammatory state.\textsuperscript{1,10,11} Also, the free hemoglobin that is released
from hemolyzed RBCs causes additional damage to the lining of blood vessels.\textsuperscript{10,13} The marrow is very active, as evidenced by immature RBCs or reticulocytes in the peripheral blood. As the marrow increases production of RBCs, platelet and WBC production also increases. As a consequence of increased erythropoesis, many patients do not suffer from signs and symptoms of anemia, even though they may have a low hemoglobin. As long as the individual can continue to compensate, the anemia does not present a problem. When the marrow cannot keep up with production (i.e., a lack of folic acid), or has its production impaired by viral illness, such as parvovirus B-19, an aplastic crisis may occur. Parvovirus B-19 is a common childhood virus responsible for Fifth’s Disease. This disease causes a rash, the classic “slapped-cheek” appearance, and high fevers. Parvovirus can suppress bone marrow production by destroying the RBC precursor cells in the bone marrow. In these cases of severe anemia, transfusion therapy is necessary.\textsuperscript{5}

\textbf{Immunologic and Infectious Manifestations}

The spleen can be affected in SCD due to occlusive forces, but it is also damaged by the pointed, inflexible sickle cells that travel through it and are stuck. The spleen is an important immunologic organ, helping to fight infections with encapsulated organisms (\textit{S. pneumoniae}, \textit{H. influenzae}, and \textit{N. meningitidis}). Therefore, it is important to recognize children with SCD and immunize them. As stated earlier, the use of prophylactic penicillin has been found to reduce death in children.\textsuperscript{5,20} All infections should be treated aggressively.

\textbf{Treatment of Sickle Cell Disease}

Prevention of early complications, such as infection, is important in the overall treatment plan for patients with SCD. As more is being learned about the basic mechanisms of the disease, new treatments are being developed. Analgesics, including opioids, are a mainstay of treatment for people suffering with pain crisis. In addition, it is important to give patients with SCD vitamins, such as folic acid, which are essential to the production of RBCs. Hydration is also key, since RBCs sickle when dehydrated. In addition, hydration improves the viscosity of the patient’s blood and allows the RBCs to move more easily. As hemoglobin rises, the viscosity of the bloodstream increases and there is an increased risk of occlusive disorders, as well as an increased risk of pain crisis.\textsuperscript{21} There is no data to show that transfusion should be part of the routine treatment for crisis.\textsuperscript{5,20} The role of nitric oxide is also being investigated as a therapeutic modality, since nitric oxide plays an important role in the physiology of RBCs.\textsuperscript{14}

Patients of all ages who have higher concentrations of Hgb F have milder disease and lower mortality than patients with lower levels of Hgb F. Increasing the production of Hgb F seems like a reasonable therapeutic intervention. In the laboratory, Hgb F has been shown to interfere with the polymerization of deoxygenated Hgb S.

The chemotherapeutic agent, hydroxyurea (HU), increases the amount of Hgb F that is made by the bone marrow. RBCs with Hgb F lack the \(\beta\) chain, which is responsible for the sickling seen in Hgb S RBCs. Therefore, these RBCs do not sickle, nor can they polymerize. This is one of the mechanisms considered to be responsible for the improved outcomes. In addition, HU has been found to reduce WBC production, thereby reducing the number of circulating inflammatory cells, capable of adhering to blood vessel walls. Platelet counts also drop and this may inhibit one of the factors responsible for vaso-occlusion. HU also influences nitric oxide metabolism. Nitric oxide leads to the dilation of blood vessels and seems to reduce the adhesion between RBCs and blood vessel walls.\textsuperscript{22}

HU has been found to reduce mortality due to an induction of Hgb F and a reduction in vaso-occlusive events. In addition, it reduces the incidence of acute chest syndrome, transfusion requirements, episodes of hospitalization, pain crisis, and reduces blood flow through intracranial blood vessels, as measured by transcranial Doppler in children.\textsuperscript{22–24} Currently, there is controversy about whether or not HU can be used to prevent repeat strokes as effectively as chronic transfusion in children.\textsuperscript{3,24–25} Since HU is a chemotherapeutic agent, there is concern that it will have long term side effects. It is not used in women who are pregnant or planning to become pregnant. In addition, some patients have to discontinue therapy because their WBC count becomes too low. The effects of hydroxyurea are not immediate, generally taking a few months to be effective.\textsuperscript{23} Therefore, it does not provide rapid response during acute illness.

Bone marrow transplant, peripheral blood stem cell transplant, and umbilical cord blood transplant are all strategies that can cure SCD. Due to the morbidity and mortality secondary to transplant, it is important to carefully choose patients with severe disease with a high risk of morbidity and mortality, such as those with recurrent strokes. Therefore, trials have centered on treating children or young adults, and there are some encouraging results. Unfortunately, one impediment is the low availability of matched sibling donors.\textsuperscript{20–28}

\textbf{Transfusion Management}

Transfusion therapy should only be initiated in patients with signs and symptoms of anemia. As mentioned, most patients with SCD, though anemic, do not generally have daily signs and symptoms of anemia. Therefore, RBC transfusion should only be used for specific indications. RBC transfusion, when necessary in patients with SCD, provides some additional benefits. While increasing the patient’s hemoglobin, transfusion dilutes the Hgb S with Hgb A. The RBCs with Hgb A have longer survival than the RBCs with Hgb S and neither sickle nor polymerize. In addition, transfusion will suppress the patient’s own erythropoiesis, or RBC production. As a consequence, they will produce less of their own Hgb S RBCs.\textsuperscript{4,5,29}
The decision to transfuse should take into account the known benefits in the face of risks. All blood products are capable of transmitting certain infectious diseases and causing transfusion reactions. In addition, the recipient is exposed to certain inflammatory mediators that accumulate in transfused blood and to foreign antigens from the donor. Exposure to foreign RBC antigens has important implications for patients with sickle cell disease.

RBCs can be transfused as a simple transfusion or via exchange transfusion (erythrocytopheresis).\textsuperscript{5,29} During simple transfusion, one or two units of RBCs are transfused through a peripheral IV. Exchange transfusion is usually performed using an automated machine that is designed to remove whole blood from the patient, separate into its various components, and then discard the patient’s Hgb S RBCs. RBCs from the blood bank are then used as replacement. Typically, an RBC exchange exchanges one to two RBC volumes (total blood volume × hematocrit = 1 blood volume). A larger bore, stiff-walled central venous catheter is usually required due to the flow requirements of the automated instrument. In the absence of automated equipment, manual exchange transfusion can be done. This is not optimal, since this can create blood pressure and volume changes during the alternating removal of whole blood through a peripheral vein with subsequent reinfusion of banked RBCs. Each type of transfusion has its own risks and benefits, as outlined in Table 4 and in Table 5.

### Table 4. The benefits and risks of simple transfusion

<table>
<thead>
<tr>
<th>Benefits</th>
<th>Risks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technical ease—requires only peripheral IV access</td>
<td>Increases viscosity</td>
</tr>
<tr>
<td>Low donor exposure—only 1 or 2 units of RBCs necessary</td>
<td>Risk of iron overload</td>
</tr>
<tr>
<td>Dilution of Hgb S</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5. The benefits and risks of exchange transfusion

<table>
<thead>
<tr>
<th>Benefits</th>
<th>Risks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Produces rapid reduction in Hgb S</td>
<td>Requires large gauge IV, usually central venous catheter</td>
</tr>
<tr>
<td>No increase in viscosity</td>
<td>Requires expertise and special equipment—may require transfer of patient to another facility</td>
</tr>
<tr>
<td>No risk of iron overload—some observe reductions in serum ferritin over time</td>
<td>Higher donor exposure—at least 4 units of RBCs for an adult, usually more</td>
</tr>
<tr>
<td>Increased risk of transfusion reactions</td>
<td>Higher expense</td>
</tr>
</tbody>
</table>

### Indications for Transfusion in Sickle Cell Disease

Indications for transfusion in SCD include:

- **Aplastic Crisis:** When RBC production in the bone marrow is interrupted, the delicate balance to maintain RBC production during chronic hemolysis is disrupted. Therefore, if the patient has a drop in their hemoglobin and becomes symptomatic, transfusion is required until the underlying process abates.\textsuperscript{5,29}

- **Acute Chest Syndrome (ACS):** Transfusion, either simple or exchange, implemented early in the course, improves oxygenation and alleviates organ dysfunction. For patients who are stable, simple transfusion should be performed. If the patient deteriorates, does not improve, or has a rapidly evolving course, exchange transfusion is recommended.\textsuperscript{5,29,32} Simple transfusion should only be performed until the hemoglobin reaches about 10 g/dL. Beyond that, there is concern for vaso-occlusion.\textsuperscript{5,29}

- **Priapism:** Priapism is the painful engorgement of the penis, as a result of vaso-occlusion. Supportive therapy consisting of hydration and analgesia is recommended, and a urologist should be consulted. When priapism does not resolve, simple transfusion or exchange transfusion should be considered. There are no studies to direct transfusion therapy. In addition, exchange transfusion has been associated with adverse neurologic sequelae, such as seizures and increases in intracranial pressure, so there is a reluctance to perform exchange until the patient is refractory to other treatments.\textsuperscript{5,29,31}

- **Presurgical Prophylaxis:** Patients with SCD are at high risk for complications when undergoing major surgery. Currently, some practitioners recommend that patients be transfused to a hemoglobin of 10 g/dL prior to surgery. A study done comparing exchange transfusion to simple transfusion found that exchange transfusion is unnecessary.\textsuperscript{5,29,32} There are investigations underway to determine whether or not a hemoglobin lower than 10 g/dL is safe for certain surgeries.\textsuperscript{33}

- **Splenic Sequestration:** When a child’s hemoglobin drops and they become symptomatic, transfusion is necessary, in this setting. Of interest, transfusion increases the hemoglobin beyond what’s expected, so it is important to transfuse slowly to avoid over-transfusion. Hepatic sequestration can also occur.\textsuperscript{5,29}

- **Pregnancy:** In uncomplicated pregnancies, there is no improvement in outcomes in women who are transfused. Patients with other complications of SCD should be transfused accordingly.\textsuperscript{5,29,30}

- **Stroke:** Due to the ease of simple transfusion in pediatric patients, they usually undergo simple transfusion. For patients who have an initial stroke, exchange transfusion is used to rapidly reduce the amount of Hgb S that can be recruited and extend the immediate damage to the brain. Once a patient has a stroke, they are at risk for additional strokes. By performing a monthly transfusion, either simple or exchange, this risk of recurrence is reduced. A recent study showed that stopping monthly transfusion after the return of normal flow by transcranial Doppler results in recurrent strokes and the return of abnormal TCDs.\textsuperscript{13,15,16,33} Of interest, during studies to reduce the risk of first stroke, patients on a chronic transfusion protocol also had a reduction in the risk of acute chest syndrome and a reduction in the number of pain crises.\textsuperscript{15,16}
Adverse Consequences of Transfusion of Patients with Sickle Cell Disease

Adverse consequences of transfusion of patients with SCD include:

Alloimmunization: Due to the disparity between donors and patients with SCD in the United States, patients with SCD are among those most frequently alloimmunized. Studies have shown that the most common antibodies formed in this population are C, E, and K1.36–38 There are also other antibodies that are formed due to donor–recipient disparity. Therefore, in order to prevent alloimmunization, some centers routinely perform RBC phenotypes on patients with SCD and only transfuse RBCs that lack C, E, and K1, if the patient is negative for the antigen. This strategy reduces the rate of antibody formation in these at-risk patients.39 Despite these findings, practice is variable. Analyzing the results of the 2003 J-C C College of American Pathologists Proficiency Testing Survey, Osby and Shulman found that only 37 percent of North American hospitals routinely perform antigen typing on the RBCs of non-alloimmunized patients with SCD. In the 439 laboratories that do perform RBC phenotyping, C, E, and K1 were the most frequent antigens that were matched.57 In a survey of 50 academic medical centers in the US and Canada, 73 percent of centers reported that they performed routine phenotyping with 89% of those centers also matching for C, E, and K1.58 There is controversy about providing RBCs that lack additional antigens. As you match for additional antigens, it becomes more difficult to find compatible units. It is argued that it is a better use of resources to save those rare units for patients with existing antibodies.39 Another strategy has been to use RBCs from donors who are ethnically similar to the patient, thereby creating a better chance that the donor and patient will match more closely.40 Whenever a patient develops an antibody, they will also receive RBCs lacking the corresponding antigen.41

Hyperhemolytic Syndrome: A serious type of hemolytic transfusion reaction, called the “hyperhemolytic syndrome,” can occur in the setting of transfusion. During these episodes, the patients are typically being transfused, and instead of rising, their hemoglobin falls with subsequent transfusion. It is felt that there is a “bystander” hemolysis of the patient’s own RBCs, as well as destruction of transfused RBCs. Of interest, the units transfused are crossmatch compatible, and no new alloantibodies are identified at the time of transfusion. Further transfusion compounds the problem. Therefore, it is important to recognize the syndrome and, if possible, stop transfusing. If transfusion is necessary, intravenous immunoglobulin (IVIG) and intravenous steroids have been found to be effective. If transfusion is required because of life-threatening anemia, it should be done cautiously, using IVIG and steroids concurrently.32–44

Iron Overload: Each unit of transfused RBCs contains about 200–250 mg of iron. With chronic transfusion, this iron accumulates and can be deposited into organs such as the heart, liver, and endocrine glands. In order to prevent this, medications are used to remove, or chelate, iron. Intravenous chelators have been used, but their efficacy is hindered by its half-life and poor patient compliance. A new generation of oral chelators is effective since there is increased compliance, and mode of actions results in a more continuous chelation. Serum ferritin is monitored in patients on chronic transfusion protocols who have SCD.45 The aim of treatment is to reduce serum ferritin and to remove iron from organs.

Transfusion Recommendations

When a patient develops complications from SCD, it also makes sense not to transfuse them with RBCs with additional Hgb S. Therefore, many laboratories will do a simple solubility test, as described earlier, and select units that lack Hgb S. Realize that individuals with SCD are anemic and cannot serve as blood donors, however, there are active donors with sickle cell trait.41

Leukoreduction (LR) of blood products has been proven to reduce the risk of cytomegalovirus (CMV) transmission, reduce the risk of febrile non-hemolytic transfusion reactions, and reduce the risk of human leukocyte antigen (HLA) alloimmunization.41 There are also studies, though controversial, that show there are deleterious immunologic sequelae of transfusion that are prevented by reducing the load of WBCs in transfused blood products. Since patients with SCD are chronically transfused, many believe that these patients should receive LR blood products. Of note, there is also a study that shows reduced rates of RBC alloimmunization in patients who receive LR blood products.46 Transfusion recommendations for patients with SCD are:

• Blood products that are sickle hemoglobin negative
• Blood products that are leukoreduced
• Blood products that are negative for C, E, and K1 antigens if the patient lacks these antigens
• Blood products that are negative for any additional antigens against which the patient has antibody
• Blood products that are from African American donors, if a program exists

Summary

The substitution of one amino acid in the hemoglobin molecule results in sickle hemoglobin. As a result, RBCs sickle in low oxygen states causing occlusion of blood vessels, increased viscosity, and inflammation. These RBCs are prematurely removed from the circulation, resulting in a chronic hemolytic anemia. With newborn screening and early treatment, the death rate among children with SCD has declined. In addition, a variety of treatments are being introduced to help manage the various manifestations of disease. Transfusion, simple or exchange, is a mainstay of therapy, since it reduces the amount of Hgb S in circulation and suppresses erythropoiesis. Transfusion is indicated for symptomatic anemia and specifically to prevent stroke (first or recurrent), during acute stroke, and for acute chest syndrome. Unfortunately, transfusion carries risks
for infectious disease transmission, as well as immunologic and inflammatory sequelae. For patients with SCD who may be chronically transfused, iron overload occurs frequently. In addition, due to differences in RBC antigens between donors and recipients, these patients are at increased risk for development of RBC alloantibodies, which can complicate further transfusion. It is, therefore, important to prevent alloimmunization by transfusing leukoreduced RBCs that match the patient for the C, E, and K1 antigens. Human progenitor cell (from bone marrow, peripheral blood stem cells, or umbilical blood) transplant can cure the disease, and is used for patients with severe disease for whom conventional therapy may not be effective.

References
39. Ness PM. To match or not to match: the question for chronically transfused patients with sickle cell anemia. Transfusion 1994;34:558–60.
46. Blumberg N, Heal JM, Gettings KF. Leukoreduction of red cell transfusion is associated with a decreased incidence of red cell alloimmunization. Transfusion 2003;43:945–52.

Additional Resources

Susan D. Roseff, MD, Medical Director, Transfusion Medicine, Professor, Virginia Commonwealth University, Department of Pathology, PO Box 980662, Richmond VA 23298-0662.

Attention SBB and BB Students
You are eligible for a free 1-year subscription to Immunohematology.
Ask your education supervisor to submit the name and complete address for each student and the inclusive dates of the training period to immuno@usa.redcross.org.

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The Consortium for Blood Group Genes (CBGG) is a nonprofit organization whose mission is “To establish guidelines, to provide education, and to provide a proficiency exchange for laboratories involved in DNA or RNA testing for the determination of blood group, platelet, and neutrophil antigens.” The consortium was established at the inaugural meeting on October 23, 2004, in Baltimore, Maryland, by a group of people with scientific or industry experience and interest in the field. The consortium is coordinated by Marion Reid with assistance of country coordinators: Lilian Castilho for Brazil, Gregory Denomme (with Maryse St. Louis as successor in 2009) for Canada, and Connie Westhoff for the United States. All members are expected to interact and participate. New members are encouraged to refer to the previously published information for background and progress information.1–4

The CBGG Document

The CBGG Document outlines the function of the CBGG. This document contains information on the structure, organizational rules and bylaws, regulatory compliance plan, preferred terminology, and progress on the working parties including proficiency exchange program, guidelines of practice, DNA repository, funding, forms and disclaimers, and proposed Web site. It is highly recommended that CBGG members refer to this document for the aforementioned duties and activities. Meetings are held annually, in part, for members to discuss outstanding issues and to provide the opportunity to give input and accept amendments to the document. The CBGG Document is distributed to members and is available to nonmembers on request.

Template Disclaimers

CBGG members continue to recognize the importance of appropriately worded reports of molecular analyses for both blood donors and transfusion recipients. Members continue to support the current recommendation that blood components should not be labeled with molecular test results as the sole means of antigen identification. Molecular data continue to be a source of information in the resolution of complex serologic problems, and are not intended as the sole means for patient transfusion management decisions.

Target Alleles

The list of target alleles prepared by the CBGG after meeting in 2007 was adopted. The preferred current terminology is listed in Table 1 of this report under the heading Target antigen (target allele). However, the final naming of alleles relies on the decision of the International Society for Blood Transfusion. When referring to a particular single-nucleotide polymorphism, nucleotide changes follow the designated position, e.g., 125G>A; intronic nucleotide changes represented in the lower case, e.g., −33t>c. The associated amino acid substitutions (not shown) flank the designated position, e.g., Pro103Ser or P103S. Because gene numbering systems vary, and to avoid ambiguity in the location of nucleotide changes, the CBGG has adopted GenBank gene reference sequences (RefSeqGene) and reference SNP numbers (rs#) for blood group genes and nucleotides (Table 1). These numbers provide the set of common references used to communicate or report molecular testing results.

Guidelines for Molecular Testing

The CBGG has developed guidelines for free and general distribution. In 2007, the CBGG guidelines were shared with the AABB Molecular Testing Standards Program Unit (SPU). In addition, five CBGG members are committee members of the AABB Molecular Testing SPU, and one member (MER) holds liaison status with the committee. The AABB Standards for Molecular Testing for Red Cell, Platelet, and Neutrophil Antigens was published in 2008.5 The intent of the CBGG guidelines is to maintain an independent forum and voice for proposed molecular testing standards in ISO format for use by international laboratories. The members will update, modify, or otherwise amend the CBGG guidelines by process of discussion and consensus. The CBGG guidelines will not become standards as such to reflect the fact that the CBGG is not responsible for laboratory inspection or accreditation.

* Full listing of the members of the CBGG can be found in the appendix.
Serologic confirmation is not mandated by this group owing to the lack of regulated antisera. The first exchange for platelet genotyping was coordinated by Vagner Castro (Platelet Immunology Laboratory of Hematology and Hemotherapy Center of the State University of Campinas, UNICAMP). This sample was previously genotyped by PCR-RFLP and PCR-SSP to the two HPA systems most frequently involved in platelet alloimmunization: HPA-1 and HPA-5.

Conclusions
The CBGG is a self-help, not-for-profit organization designed as an interactive collaborative for members to learn from each other and to strive to achieve excellence in molecular testing of blood group, platelet, and neutrophil antigens. Anyone interested and willing to contribute intellectually is welcome to join. To become a member contact Marion Reid (mreid@nybloodcenter.org), Lilian Castilho (castilho@unicamp.br), Maryse St. Louis (maryse.st-louis@hema-quebec.qc.ca), Greg Denomme (greg.denomme@bcw.edu), or Connie Westhoff (WesthoffC@usa.redcross.org).

Acknowledgments
We thank Robert Ratner for help in the preparation of this manuscript and Lakshmi Gaur and Tanya Kerrigan for their help assembling the GenBank gene and SNP reference accession codes. The findings and conclusions in the article should not be construed to represent any agency determination or policy.

References
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<th>ISBT system name (symbol) number</th>
<th>Target antigen (target allele)</th>
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<th>Target nucleotide number (GeneBank SNP rs#)</th>
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<td>† (see note below)</td>
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| Colton (CO) 015    | Co<sup>a</sup> | Co<sup>b</sup> | nt 134C>T (rs28362692) *
|                    | LW<sup>a</sup> | LW<sup>b</sup> | nt 308A>G *
| Cromton (CR) 021   | Cr<sup>a</sup> |         | nt 679G>C (rs60822373) *
| Knops (KN) 022     | Kn<sup>a</sup> | Kn<sup>b</sup> | nt 4681G>A (rs41274768) *
|                    | McC<sup>a</sup> | McC<sup>b</sup> | nt 4768A>G (rs17047660) *
|                    | S<sup>a</sup> | V<sup>b</sup> | nt 4801A>G (rs17047661) *
| Indian (IN) 023    | In<sup>a</sup> | In<sup>b</sup> | nt 252C>G *
| OK (OK) 024        | Ok<sup>a</sup> |         | nt 274G>A Heterozygote not required |

* NG_number.n represent the GenBank reference gene sequence (RefSeqGene) accession code for each blood group system gene.
# The rs number in brackets represents the GenBank SNP reference sequence (SNP rs) accession code for the given nucleotide (nt).
† Homozygous rare alleles are not required for molecular testing.

Appendix

Members of CBGG at end of 2008:

**Coordinator:**
**Marion E. Reid, PhD**  
Laboratory of Immunochemistry and Laboratory of Immunohematology  
New York Blood Center  
310 East 67th Street  
New York, NY 10065

**Country Coordinators:**
Connie Westhoff, PhD  
Molecular Blood Group and Platelet Antigen Testing Laboratory  
American Red Cross-Penn-Jersey Region  
700 Spring Garden Street  
Philadelphia, PA 19123

Maryse St-Louis, PhD  
Operational Research, Hema-Quebec  
1070, Avenue des Sciences-de-la-Vie  
Quebec, Quebec G1V 5C3, Canada

Lilian Maria de Castilho, PhD  
Laboratory of Immunohematology  
Hemocentro-State University of Campinas (UNICAMP)  
Rua Carlos Chagas 480 Barão Geraldo  
Campinas, São Paulo, 15081-970, Brazil

**Members:**
Bai Yu, MD, PhD  
Department of Pathology and Laboratory Medicine  
University of Texas Health Science Center at Houston  
6431 Fannin Street, MSB 2.222  
Houston, TX 77030

Daniel B. Bellissimo, PhD  
Molecular Diagnostics Laboratory  
BloodCenter of Wisconsin  
638 N. 18th Street  
Milwaukee, WI 53233

Imelda M. Bromilow  
DIAMED SA  
Cressier-sur-Morat, Switzerland

Tony S. Casina  
Ortho-Clinical Diagnostics, Inc.  
1001 Highway 202  
Raritan, NJ 08869

Vagner Castro  
Hematology and Hemotherapy Center  
Hemocentro-State University of Campinas (UNICAMP)  
Rua Carlos Chagas 480 Barão Geraldo  
Campinas, São Paulo, 15081-970, Brazil

Laura Cooling, MD  
University of Michigan Hospitals  
1500 East Medical Center Drive  
Ann Arbor, MI 48109-0054

Ana Paula Cozac, MD  
Centro Regional de Hemoterapia de Ribeirão Preto  
Ribeirão Preto, S.P. CEP 14051-140, Brazil

Daniel de la Vega, PhD  
Servicio de Medicina Transfusional  
Hospital Italiano Garibaldi  
Virasoro 1249 Rosario (2000)  
Argentina
Gregory Denomme, PhD
Immunohematology Reference Laboratory
Blood Center of Wisconsin
638 N 18th Street
Milwaukee, WI 53201-72178

Helene M. DePalma
Immunohematology Laboratory
New York Blood Center
310 East 67th Street
New York, NY 10065

Lakshmi K. Gaur, MSc, PhD
Puget Sound Blood Center
921 Terry Avenue
Seattle, WA 98104-1256

Ghazala P. Hashmi, PhD
Bioarray Solutions
35 Technology Drive, Suite 100
Warren, NJ 07059

Kim Hue-Roye
Laboratory of Immunochemistry
New York Blood Center
310 East 67th Street
New York, NY 10065

Susan T. Johnson, MSTM, MT(ASCP)SBB
Manager, Immunohematology Services
BloodCenter of Wisconsin
638 North 18th Street
Milwaukee, WI 53233

Hallie Lee-Stroka
Department of Transfusion Medicine
Clinical Center
National Institutes of Health
10 Center Drive MSC 1184
Bethesda, MD 20892

Christine Lomas-Francis, MS, FIBMS
Immunohematology Laboratory
New York Blood Center
45-01 Vernon Blvd.
Long Island City, NY 11101

Tanya Kanigan
BioTrove Incorporated
12 Gill Street
Woburn, MA 01801

Kirk D. Kitchen
Clinical Laboratories
Blood Systems Laboratories
2424 West Erie Drive
Tempe, AZ 85282

Gandhi J. Manish, MD
Laboratory Medicine and Pathology
Mayo Clinic
200 First Street, S.W.
Rochester, MN 55905

Mariza A. Mota, PhD
Departamento de Hemoterapia
Hospital Israelita Albert Einstein
Av. Albert Einstein, 627 - 4º andar, Morumbi
São Paulo, SP CEP 05651-901 Brazil

Joann M. Moulds, PhD
Clinical Immunogenetics
LifeShare Blood Centers
8910 Linwood Avenue
Shreveport, LA 71106

John J. Moulds, MT(ASCP)SBB
Scientific Support Services
LifeShare Blood Centers
8910 Linwood Avenue
Shreveport, LA 71106

Sandra Nance, MS, MT(ASCP)SBB
Technical Services
American Red Cross Penn-Jersey Region
700 Spring Garden Street
Philadelphia, PA 19123

Marcia Zago Novaretti, MD, PhD
Division of Immunohematology and Transfusion
Fundação Pro-Sangue/Hemocentro de Sangue
Av. Angélica, 2-261
Sao Paulo, Brazil

Joseph Ong
Blood Centers of the Pacific
270 Masonic Avenue
San Francisco, CA 94118

Jordão Pellegrino, Jr.
Rua Sampaio Ferraz 750 ap 41 B Campinas, São Paulo, Brazil CEP 13024-431

Maria Rios, PhD
DETTD/OBRR/CBER
29 Lincoln Drive
NIH Campus Building 29, Room B24
Bethesda, MD 20892

Dennis Roscetti
GTI Diagnostics
20925 Crossroads Circle, Suite 200
Waukesha, WI 53186

Dawn M. Rumsey, ART(CSMLT)
PEPFAR Team, Global Development
American Association of Blood Banks
8101 Glenbrook Road
Bethesda, MD 20814

Gayle Teramura
Puget Sound Blood Center
921 Terry Avenue
Seattle, WA 98104-1256
G.A. Denomme et al.

Rebecca Thomas  
Memorial Blood Centers  
737 Pelham Blvd  
St. Paul, MN 55114

Antonio Sergio Torloni, MD  
Mayo Clinic  
13400 East Shea Boulevard  
Scottsdale, AZ 85259

Sunitha Vege, MS  
Molecular Blood Group and Platelet Antigen Testing Laboratory  
American Red Cross  
700 Spring Garden Street  
Philadelphia, PA 19123

Rita Fontão Wendel  
Instituto de Hemoterapia Sirio-Libanes  
Rua D. Adma Jafet, 91 - 2 Andar  
Sao Paulo, CEP 01308-050 Brazil

Mark Yazer, MD  
RBC Serology Reference Laboratory  
Centralized Transfusion Service  
University of Pittsburgh  
3636 Boulevard of the Allies  
Pittsburgh, PA 15213

Liaison Members:  
Sheryl A. Kochman  
Devices Review Branch  
Center for Biologics Evaluation and Research  
Office of Blood Research and Review  
Division of Blood Applications  
Food and Drug Administration  
1401 Rockville Pike, HFM-390  
Rockville, MD 20852-1448

Jill Storry, PhD  
Blodcentralen Skåne-University Hospital  
SE-221 85  
Lund, Sweden

Free Classified Ads and Announcements

*Immunohematology* will publish classified ads and announcements (SBB schools, meetings, symposia, etc.) without charge. Deadlines for receipt of these items are as follows:

- **Deadlines**
  - 1st week in January for the March issue
  - 1st week in April for the June issue
  - 1st week in July for the September issue
  - 1st week in October for the December issue

E-mail or fax these items to immuno@usa.redcross.org or (215) 451-2538.
W. John Judd, FIBMS, MIBiol

W. John Judd, FIBMS, MIBiol, is being honored by Immunohematology, Journal of Blood Group Serology and Education for his many contributions to the journal and to the field of blood banking. John served on the editorial board of Immunohematology for 7 years, from 2002 through 2008. We thank him for giving generously of his time and ideas to improve the scope and concept of the journal. This included contributing nine articles published between 1979 and 2005 that were significant to the interest of the readers of Immunohematology. He also was a dedicated, knowledgeable, and responsive peer reviewer of submitted articles from the beginning of the publication and during the length of his very important career.

John retired in 2008 after 34 years as director of a first-rate AABB-accredited reference laboratory at the University of Michigan. His career is a tribute to his determination to investigate the unusual and the undiscovered. In 1972, he studied at the Sir John Cass College in London, England, where he was elected a Fellow of the Institute of Biomedical Sciences (FIBMS). He came to the University of Michigan in 1974 and was the director of the reference laboratory and professor of immunohematology in the Department of Pathology until his retirement in 2008. He is now emeritus professor of immunohematology, Department of Pathology, at the University of Michigan. John was awarded the usual blue and gold embossed chair at retirement to prove it! John and his wife, Jane, then moved to North Carolina to relax and watch the golfers go by.

John has received many awards, including the Ivor Dunsford Memorial Award and the John Elliott Memorial Award from the American Association of Blood Banks (AABB); the Founders Award and Kay Beattie Lecture from the Michigan Association of Blood Banks (MABB); the Pettaway-Sheppard Award of the North Carolina Association of Blood Banks; L. Jean Stubbins Memorial Lecture of the UTMB Medical Branch; Ronald Dubin Memorial Lecturer, NYABB; and Suzanne Leiden Memorial Lecturer, CABB Society. In North Carolina, the extra room over the garage is called the FROG (furnished room over garage); however, in John’s case, it is called a TROG (trophy room over garage) for all of his well-deserved awards!

John received these awards for his many investigative studies, research reports, review papers, presentations, and publications. In 2008, John coauthored the third edition of Judd’s Methods in Immunohematology, a major reference methods manual for laboratory workers. This edition contains more than 150 methods and procedures documents. During the span of his career, he has had more than 80 scientific papers published in peer-reviewed journals. Additionally, John is the author of review articles on lectins, polyagglutination, elution studies, pretransfusion studies, Rh blood groups, MNS-system antibodies, and investigation of the positive DAT. These are just some of the topics that he studied and investigated in depth.

A popular speaker, John gave numerous presentations at local, state, national, and international meetings. One could always count on John to talk! In fact, he and the late John Case, of Gamma Biologicals, Inc., were famous for their “chats” on the AABB SSCC forum and were well known for debating, in depth, many questions concerning blood group serology and its complexities.

In addition to this impressive scientific career, John also managed to be active on many committees of the AABB, MABB, and International Society of Blood Transfusion. He was also on the board of the AABB for 4 years and was on the board and president of the MABB.

The editors, authors, and reviewers of Immunohematology, Journal of Blood Group Serology and Education, would like to thank John for his contributions to the field of blood banking during his 34 years at the bench as an investigator, and as an author, educator, and mentor. We would particularly like to acknowledge his contributions during the past several years as an editor, peer reviewer, and contributing author to Immunohematology. John, you may be gone from the field, but you cannot be forgotten. Like the important scientists before you, you have left a large and lasting legacy of knowledge and respect. Thank you, and may you and Jane have a long, healthy, and happy retirement.

Delores Mallory, MT(ASCP)SBB
Emeritus Editorial Board
Immunohematology
Blood Group Reviews—A New Feature

The editors of *Immunohematology* present a new feature that is extremely exciting. Starting with this issue, *Immunohematology* will publish a series of 28 “Blood Group Reviews” in upcoming issues. Experts in the field have agreed to put their considerable knowledge and energy into writing a thorough review of each of the 28 selected areas. Readers will then have comprehensive reviews of all the major blood group systems and other topics at their fingertips. Of additional value will be the references for each article to which the reader can refer if interested in reading the original work. The authors will present each blood group review in the following format: history, nomenclature, genetics, molecular basis, biochemistry, antibodies in system, and clinical significance. The topics that will appear in sequential issues are detailed in the table below.

When all the topics have been published, *Immunohematology* will publish the entire collection as a stand-alone resource. This series provides an excellent opportunity for experienced staff to obtain continuing education, and it will be an educational tool for new staff. The editors envision a copy of the collected reviews as a must-have for transfusion medicine facilities, libraries, and personal bookshelves everywhere.

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Sandra Nance
Connie M. Westhoff
Editors-in-Chief
*Immunohematology*
Applications are invited from medical or science graduates for the Master of Science (MSc) degree in Transfusion and Transplantation Sciences at the University of Bristol. The course starts in October 2009 and will last for 1 year. A part-time option lasting 2 or 3 years is also available. There may also be opportunities to continue studies for PhD or MD following the MSc. The syllabus is organized jointly by The Bristol Institute for Transfusion Sciences and the University of Bristol, Department of Pathology and Microbiology. It includes:

- Scientific principles of transfusion and transplantation
- Clinical applications of these principles
- Practical techniques in transfusion and transplantation
- Principles of study design and biostatistics
- An original research project

Application can also be made for Diploma in Transfusion and Transplantation Science or a Certificate in Transfusion and Transplantation Science.

**The course is accredited by the Institute of Biomedical Sciences.**

Further information can be obtained from the Web site:
http://www.blood.co.uk/ibgrl/MscHome.htm

For further details and application forms please contact:

Dr Patricia Denning-Kendall
University of Bristol, Paul O’Gorman Lifeline Centre, Department of Pathology and Microbiology, Southmead Hospital, Westbury-on-Trym, Bristol BS10 5NB, England
Fax +44 1179 595 342, Telephone +44 1779 595 455, e-mail: p.a.denning-kendall@bristol.ac.uk.
Announcements, cont.

Meetings

September 25
Illinois Association of Blood Banks (ILABB)
The Illinois Association of Blood Banks (ILABB) Fall Meeting will be held September 25, 2009, at the Lisle/Naperville Hilton in Lisle, Illinois. For additional details please visit the Web site at www.ilabb.org or contact Kristi Williams at (309) 745-8999 or WilliaKr@usa.redcross.org.

Specialist in Blood Bank (SBB) Program
The Department of Transfusion Medicine, National Institutes of Health, is accepting applications for its 1-year Specialist in Blood Bank Technology Program. Students are federal employees who work 32 hours/week. This program introduces students to all areas of transfusion medicine, including reference serology, cell processing, HLA, and infectious disease testing. Students also design and conduct a research project. NIH is an Equal Opportunity Organization. Application deadline is December 31, 2009, for the July 2010 class. See www.cc.nih.gov/dtm > education for brochure and application. For further information contact Karen M. Byrne at (301) 451-8645 or KByrne@mail.cc.nih.gov.

Monoclonal antibodies available at no charge
The New York Blood Center has developed a wide range of monoclonal antibodies (both murine and humanized) that are useful for donor screening and for typing RBCs with a positive DAT. These include anti-A1, -M, -S, -U, -D, -Rh17, -K, -k, -Kp*, -Js*, -Fya*, -Fyb*, -Fy6*, -Wr*, -Xg*, -CD99, -Do*, -H, -Ge2, -Ge3, -CD55 (both SCR2/3 and SCR4), -Ok*, -I, and anti-CD59. Most of the antibodies are murine IgG and require the use of anti-mouse IgG for detection (anti-K, k, and -Kp*). Some are directly agglutinating (anti-A1, -M, -Wr* and -Rh17) and a few have been humanized into the IgM isoform (anti-Js*). The antibodies are available at no charge to anyone who requests them. Please visit our Web site for a complete list of available monoclonal antibodies and the procedures for obtaining them.

For additional information, contact: Gregory Halverson, New York Blood Center, 310 East 67th Street, New York, NY 10021; e-mail: ghalverson@nybloodcenter.org; phone: (212) 570-3026; fax: (212) 737-4935; or visit the web site at http://www.nybloodcenter.org > research > immunochemistry > current list of monoclonal antibodies available.

Advertisements

National Platelet Serology Reference Laboratory

Diagnostic testing for:
- Neonatal alloimmune thrombocytopenia (NAIT)
- Posttransfusion purpura (PTP)
- Refractoriness to platelet transfusion
- Heparin-induced thrombocytopenia (HIT)
- Alloimmune idiopathic thrombocytopenia purpura (AITP)

Medical consultation available

Test methods:
- GTI systems tests
- Detection of glycoprotein-specific platelet antibodies
- Detection of heparin-induced antibodies (PF4 ELISA)
- Platelet suspension immunofluorescence test (PSIFT)
- Solid phase red cell adherence (SPRCA) assay
- Monoclonal immobilization of platelet antigens (MAIPA)
- Molecular analysis for HPA-1a/1b

For further information, contact

Platelet Serology Laboratory (215) 451-4205
Maryann Keashen-Schnell (215) 451-4041 office
mschnell@usa.redcross.org
Sandra Nance (215) 451-4362
snance@usa.redcross.org

American Red Cross Blood Services
Musser Blood Center
700 Spring Garden Street
Philadelphia, PA 19123-3594

National Neutrophil Serology Reference Laboratory

Our laboratory specializes in granulocyte antibody detection and granulocyte antigen typing.

Indications for granulocyte serology testing include:
- Alloimmune neonatal neutropenia (ANN)
- Autoimmune neutropenia (AIN)
- Transfusion related acute lung injury (TRALI)

Methodologies employed:
- Granulocyte agglutination (GA)
- Granulocyte immunofluorescence by flow cytometry (GIF)
- Monoclonal antibody immobilization of neutrophil antigens (MAINA)

TRALI investigations also include:
- HLA (PRA) Class I and Class II antibody detection

For further information contact:

Neutrophil Serology Laboratory (651) 291-6797
Randy Schuller (651) 291-6758
schullerr@usa.redcross.org

American Red Cross Blood Services
Neutrophil Serology Laboratory
100 South Robert Street
St. Paul, MN 55107

American Red Cross
CLIA licensed
Reference and Consultation Services

Antibody identification and problem resolution
- HLA-A, B, C, and DR typing
- HLA-disease association typing
- Paternity testing/DNA

For information, contact
Mehdizadeh Kashi at (503) 280-0210, or write to:
Pacific Northwest Regional Blood Services
ATTENTION: Tissue Typing Laboratory
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IgA/Anti-IgA Testing

IgA and anti-IgA testing is available to do the following:
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Our ELISA for IgA detects protein to 0.05 mg/dL.

For additional information contact Cindy Flickinger at (215) 451-4909, or e-mail: flickingerc@usa.redcross.org,
or write to:
American Red Cross Blood Services
Musser Blood Center
700 Spring Garden Street
Philadelphia, PA 19123-3594
ATTN: Cindy Flickinger

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National Reference Laboratory for Blood Group Serology

Immunohematology Reference Laboratory
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(215) 451-2538—Fax

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(215) 451-2538—Fax
ardp@usa.redcross.org

Immunohematology
Journal of Blood Group Serology and Education
(215) 451-4902—Phone, business hours
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- Effective tool for screening large volumes of donors
- Gel diffusion test that has a 15-year proven track record:
  Approximately 90 percent of all donors identified as IgA deficient by are confirmed by the more sensitive testing methods

For additional information, call Kathy Kaherl at:
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or write to:
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American Red Cross Biomedical Services
Connecticut Region
209 Farmington Ave.
Farmington, CT 06032

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Blood Group Antigens & Antibodies

A guide to clinical relevance & technical tips

by Marion E. Reid & Christine Lomas-Francis

This compact “pocketbook” from the authors of the Blood Group Antigen FactsBook is a must for anyone who is involved in the laboratory or bedside care of patients with blood group alloantibodies.

The book contains clinical and technical information about the nearly 300 ISBT recognized blood group antigens and their corresponding antibodies. The information is listed in alphabetical order for ease of finding—even in the middle of the night. Included in the book is information relating to:

- Clinical significance of antibodies in transfusion and HDN.
- Number of compatible donors that would be expected to be found in testing 100 donors. Variations in different ethnic groups are given.
- Characteristics of the antibodies and optimal technique(s) for their detection.
- Technical tips to aid their identification.
- Whether the antibody has been found as an autoantibody.

Pocketbook Education Fund

The authors are using royalties generated from the sale of this pocketbook for educational purposes to mentor people in the joys of immunohematology as a career. They will accomplish this in the following ways:

- Sponsor workshops, seminars, and lectures
- Sponsor students to attend a meeting
- Provide copies of the pocketbook

Ordering Information

The book, which costs $25, can be ordered in two ways:

- Order online from the publisher at: www.sbbpocketbook.com
- Order from the authors, who will sign the book. Send a check, made payable to “New York Blood Center” and indicate “Pocketbook” on the memo line, to:
  Marion Reid
  Laboratory of Immunochemistry
  New York Blood Center
  310 East 67th Street
  New York, NY 10065

Please include the recipient’s complete mailing address.
Becoming a Specialist in Blood Banking (SBB)

What is a certified Specialist in Blood Banking (SBB)?
• Someone with educational and work experience qualifications who successfully passes the American Society for Clinical Pathology (ASCP) board of registry (BOR) examination for the Specialist in Blood Banking.
• This person will have advanced knowledge, skills, and abilities in the field of transfusion medicine and blood banking.

Individuals who have an SBB certification serve in many areas of transfusion medicine:
• Serve as regulatory, technical, procedural and research advisors
• Perform and direct administrative functions
• Develop, validate, implement, and perform laboratory procedures
• Analyze quality issues preparing and implementing corrective actions to prevent and document issues
• Design and present educational programs
• Provide technical and scientific training in blood transfusion medicine
• Conduct research in transfusion medicine

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

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

However: In recent years, a greater percentage of individuals 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:

<table>
<thead>
<tr>
<th>Program</th>
<th>Contact Name</th>
<th>Phone Contact</th>
<th>Email Contact</th>
<th>Website</th>
<th>On site or On line</th>
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<tr>
<td>Walter Reed Army Medical Center</td>
<td>William Turcan</td>
<td>202-782-6210</td>
<td><a href="mailto:William.Turcan@NAAMEDD.ARMY.MIL">William.Turcan@NAAMEDD.ARMY.MIL</a></td>
<td><a href="http://www.militaryblood.dod.mil">www.militaryblood.dod.mil</a></td>
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<tr>
<td>American Red Cross, Southern California Region</td>
<td>Michael Cooper</td>
<td>909-859-7946</td>
<td><a href="mailto:CooverM@usa.redcross.org">CooverM@usa.redcross.org</a></td>
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<tr>
<td>ARC-Central OH Region</td>
<td>Joanne Kosanke</td>
<td>614-253-2740 x 2270</td>
<td><a href="mailto:kosankej@usa.redcross.org">kosankej@usa.redcross.org</a></td>
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<tr>
<td>Blood Center of Southeastern Wisconsin</td>
<td>Lynne LeMense</td>
<td>414-937-6403</td>
<td><a href="mailto:Lynne.Lemense@bcw.edu">Lynne.Lemense@bcw.edu</a></td>
<td><a href="http://www.bcw.edu">www.bcw.edu</a></td>
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<tr>
<td>Community Blood Center/CTS Dayton, Ohio</td>
<td>Nancy Lang</td>
<td>937-461-3293</td>
<td><a href="mailto:nlang@cbctcs.org">nlang@cbctcs.org</a></td>
<td><a href="http://www.cbcctcs.org/education/sbb.htm">http://www.cbcctcs.org/education/sbb.htm</a></td>
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<td>Gulf Coast School of Blood Bank Technology</td>
<td>Clare Wong</td>
<td>713-791-6201</td>
<td><a href="mailto:cwong@giveblood.org">cwong@giveblood.org</a></td>
<td><a href="http://www.giveblood.org/education/distance/htm">www.giveblood.org/education/distance/htm</a></td>
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<td>Howsworth Blood Center, Univ. of Cincinnati</td>
<td>Susan Wilkinson</td>
<td>513-558-1275</td>
<td><a href="mailto:susanwilkinson@uc.edu">susanwilkinson@uc.edu</a></td>
<td><a href="http://www.howsworth.org">www.howsworth.org</a></td>
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<tr>
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<td>Jayanna Slayten</td>
<td>317-916-5186</td>
<td><a href="mailto:jslayten@indianablood.org">jslayten@indianablood.org</a></td>
<td><a href="http://www.indianablood.org">www.indianablood.org</a></td>
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<td>Johns Hopkins Hospital</td>
<td>Jan Light</td>
<td>410-955-6580</td>
<td><a href="mailto:jlight5@jhmi.edu">jlight5@jhmi.edu</a></td>
<td><a href="http://pathology2.jhu/department/divisions/tmfusion/sbb.cfm">http://pathology2.jhu/department/divisions/tmfusion/sbb.cfm</a></td>
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<tr>
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<td>Karen Kirkley</td>
<td>504-903-3954</td>
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<td>NIH Clinical Center Dept. of Transfusion Medicine</td>
<td>Karen Byrne</td>
<td>301-496-8335</td>
<td><a href="mailto:Kbyrne@mail.cc.nih.gov">Kbyrne@mail.cc.nih.gov</a></td>
<td><a href="http://www.cc.nih.gov/dtm">www.cc.nih.gov/dtm</a></td>
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<td>Rush University</td>
<td>Veronica Lewis</td>
<td>312-942-2402</td>
<td><a href="mailto:Veronica_Lewis@rush.edu">Veronica_Lewis@rush.edu</a></td>
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<tr>
<td>Transfusion Medicine Center at Florida Blood</td>
<td>Marjorie Doty</td>
<td>727-568-5433 x 1514</td>
<td><a href="mailto:mdoty@fbssblood.org">mdoty@fbssblood.org</a></td>
<td><a href="http://www.fbssblood.org">www.fbssblood.org</a></td>
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<td>Linda Myers</td>
<td>210-731-5526</td>
<td><a href="mailto:lmyers@bloodntissue.org">lmyers@bloodntissue.org</a></td>
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<td>Univ. of Texas Medical Branch at Galveston</td>
<td>Janet Vincent</td>
<td>409-772-3055</td>
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<td><a href="http://www.utmb.edu/sbb">www.utmb.edu/sbb</a></td>
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<td>Univ. of Texas SW Medical Center</td>
<td>Barbara Laird-Fyer</td>
<td>214-648-1785</td>
<td><a href="mailto:barbara.fryer@UTSouthwestern.edu">barbara.fryer@UTSouthwestern.edu</a></td>
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Advertisements, cont.
I. GENERAL INSTRUCTIONS
Before submitting a manuscript, consult current issues of Immunohematology for style. Double-space throughout the manuscript. Number the pages consecutively in the upper right-hand corner, beginning with the title page.

II. SCIENTIFIC ARTICLE, REVIEW, OR CASE REPORT WITH LITERATURE REVIEW
A. Each component of the manuscript must start on a new page in the following order:
1. Title page
2. Abstract
3. Text
4. Acknowledgments
5. References
6. Author information
7. Tables
8. Figures

B. Preparation of manuscript
1. Title page
   a. Full title of manuscript with only first letter of first word capitalized (bold title)
   b. Initials and last name of each author (no degrees; all CAPS), e.g., M.T. JONES, J.H. BROWN, AND S.R. SMITH
   c. Running title of ≤40 characters, including spaces
   d. Three to ten key words
2. Abstract
   a. One paragraph, no longer than 300 words
   b. Purpose, methods, findings, and conclusion of study
   c. When plotting points on a figure, use the following symbols if possible: ○ ● △ ▲ □ ■
3. Key words
   a. List under abstract
4. Text (serial pages): Most manuscripts can usually, but not necessarily, be divided into sections (as described below). Survey results and review papers may need individualized sections
   a. Introduction
      Purpose and rationale for study, including pertinent background references
   b. Case Report (if indicated by study)
      Clinical and/or hematologic data and background serology/molecular data
   c. Materials and Methods
      Selection and number of subjects, samples, items, etc. studied and description of appropriate controls, procedures, methods, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer's name, city, and state.
      Do not use patient's names or hospital numbers.
   d. Results
      Presentation of concise and sequential results, referring to pertinent tables and/or figures, if applicable
   e. Discussion
      Implication and limitations of the study, links to other studies; if appropriate, link conclusions to purpose of study as stated in introduction
5. Acknowledgments: Acknowledge those who have made substantial contributions to the study, including secretarial assistance; list any grants.
6. References
   a. In text, use superscript, Arabic numbers.
   b. Number references consecutively in the order they occur in the text.
7. Tables
   a. Head each with a brief title; capitalize the first letter of first word (e.g., Table 1. Results of . . .) use no punctuation at the end of the title.
   b. Use short headings for each column needed and capitalize first letter of first word. Omit vertical lines.
   c. Place explanation in footnotes (sequence: *, †, ‡, §, ¶, **, ††).
8. Figures
   a. Figures can be submitted either by e-mail or as photographs (5" × 7" glossy).
   b. Place caption for a figure on a separate page (e.g., Fig. 1 Results of . . .), ending with a period. If figure is submitted as a glossy, place first author's name and figure number on back of each glossy submitted.
   c. When plotting points on a figure, use the following symbols if possible: ○ ● △ ▲ □ ■
9. Author information
   a. List first name, middle initial, last name, highest degree, position held, institution and department, and complete address (including ZIP code) for all authors. List country when applicable.

III. EDUCATIONAL FORUM
A. All submitted manuscripts should be approximately 2000 to 2500 words with pertinent references. Submissions may include:
   1. An immunohematologic case that illustrates a sound investigative approach with clinical correlation, reflecting appropriate collaboration to sharpen problem solving skills
   2. Annotated conference proceedings

B. Preparation of manuscript
1. Title page
   a. Capitalize first word of title.
   b. Initials and last name of each author (no degrees; all CAPS)
2. Text
   a. Case should be written as progressive disclosure and may include the following headings, as appropriate
      i. Clinical Case Presentation: Clinical information and differential diagnosis
      ii. Immunohematologic Evaluation and Results: Serology and molecular testing
      iii. Interpretation: Include interpretation of laboratory results, correlating with clinical findings
      iv. Recommended Therapy: Include both transfusion and nontransfusion-based therapies
      v. Discussion: Brief review of literature with unique features of this case
      vi. Reference: Limited to those directly pertinent
      vii. Author information (see II.B.9.)
      viii. Tables (see II.B.7.)

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

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