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Immunohematology is published quarterly (March, June, September, and December) by the American Red Cross, National Headquarters, Washington, DC 20006.

Immunohematology is indexed and included in Index Medicus and MEDLINE on the MEDLARS system. The contents are also cited in the EBASE/Excerpta Medica and Elsevier BIOBASE/Current Awareness in Biological Sciences (CABS) databases.

The subscription price is $40.00 (U.S.) and $50.00 (foreign) per year.

Subscriptions, Change of Address, and Extra Copies:
Immunohematology, P.O. Box 40325
Philadelphia, PA 19106
Or call (215) 451-4902
Web site: www.redcross.org/immunohematology
Copyright 2010 by The American National Red Cross
ISSN 0894-203X

On Our Cover
“Les yeux clos” (1889–1905), Odilon Redon

Odilon Redon created several versions of Les yeux clos (Closed Eyes) between 1889 and 1905. The striking radiance of the woman’s face hints at both the mystery and the significance of inner vision. With her eyes shut, she is unable to see the world, but because the eyes are so central to human identity, the world is also unable to truly see her. Is she asleep or dead? What are her dreams, her thoughts? The subject of the painting was Redon’s wife, although he may have also been inspired by a Michelangelo sculpture of a dying Roman slave. The II blood group is the subject of a review article in this issue, which provides insight into the clinical and basic science of polylactosamines.

——David Moolten, MD
Polylactosamines, there’s more than meets the “Ii”: a review of the I system

L. Cooling

Key Words: polylactosamine, I blood group, cold agglutinins

History

The unofficial birth of the I blood group system can be traced to a seminal case report in 1956.1 Wiener and colleagues at New York University and Jewish Hospital reported a fatal case of cold agglutinin syndrome caused by a potent autoantibody they named “anti-I” for “individuality.” The patient was a 62-year-old woman with a 5-year history of anemia and episodes of acute life-threatening hemolysis. Although the patient’s serum was compatible with donor RBCs at 37°C, she had periodic acute hemolytic transfusion reactions with “compatible” RBCs, even after warming the blood before transfusion. In an attempt to provide compatible blood, the patient’s serum was eventually tested against family members, 22,000 blood donors, and an assortment of animals including rabbit, sheep, horse, and cow. A total of five compatible donors were eventually identified, and their RBCs were successfully transfused to the patient during the next 2 years. Because these donors appeared to lack I, their RBCs were designated i phenotype. In a desperate moment when “compatible” human blood was unavailable, the patient was transfused with cow blood, which had demonstrated only weak activity at 4°C. Not surprisingly, the transfusion was aborted within 10 minutes, after the patient developed anxiety, acute dyspnea, and feelings of “impending doom!”

The serologic relationship between I and i phenotypes was solidified with the identification of an anti-i by Dr. Lawrence Marsh.2 The antibody was strongly reactive with cord RBCs and iadult RBCs but not normal adult RBCs. More importantly, Marsh was able to demonstrate a progressive age-dependent decrease in i on infant RBCs, suggesting a developmental relationship between I and i.

Ii System

As noted by Marsh, Ii and i are developmentally regulated antigens on RBCs. At birth, cord RBCs are serologically type as I-i+. A discernible increase in I expression is observed by 3 months, accompanied by parallel decreases in i expression, with an adult-type I+ phenotype observed by 18 months.3 Increases in I mirror increases in ABO expression, which typically reach adult levels by 2 to 3 years of age (see section on HDFN).3 Using human anti-I sera, the number of available I sites is estimated to be anywhere from 30,000 to 140,000 on untreated adult RBCs,4-6 but increases nearly threefold after enzyme treatment (see section on Ii antibodies).6

The iadult phenotype is a rare, autosomal-recessive trait characterized by an absence of I on RBCs as a result of mutations in the I gene, IGnt (GCNT2).7-10 Serologic studies of family members, who presumably are heterozygous, can have an intermediate I+i phenotype with elevated i and weakened I expression relative to normal controls.6,11

In certain kindreds, iadult is associated with congenital cataracts.12-14 Based on older serologic studies, the incidence of iadult ranges from 1 in 4400 to 1 in 17,000.1,15 The prevalence of mutant IGnt alleles in the general population is unknown but is presumably rare.

Increased i expression can be observed with several inherited and acquired hemolytic disorders as a sign of stressed erythropoiesis. Increased i expression is found on reticulocytes, and also occurs in megaloblastic anemia, erythroleukemia, thalassemia, paroxysmal nocturnal hemoglobinuria, and other hemolytic disorders.16-18 Increased i is also a common finding in hereditary erythroblastic multinuclearity with positive acidified serum lysis test (HEMPAS) disease, a rare hematopoietic disorder characterized by dyserythropoiesis, altered RBC glycosylation, and hemolysis.19 Unlike other hemolytic disorders, the increased i observed on HEMPAS RBCs reflects abnormal Golgi trafficking and glycosylation.20,21

Biochemistry

Ii Structure

I and i are structurally and biosynthetically related oligosaccharide chains, composed of repeating units of N-acetyllactosamine (Fig. 1; [Galβ1-4GlcNAcβ1-], LacNAc). I and i are ubiquitously expressed on human tissues and membrane structures. On RBC glycoproteins, polylactosamines are expressed as asparagine- or N-linked glycans, ranging from biantennary to large, polyvalent tetraantennary structures (Fig. 1B). On gut, leukocytes, and other tissues, polylactosamine may also be expressed as O-linked glycans via covalent linkages on serine and threonine residues (Fig. 1C). Polylactosamines are also found on membrane glycosphingolipids (Fig. 1A), including massive polyglycosylceramides ranging from 20 to 50 carbohydrate residues in size.22

Structurally, i is defined as a linear, unbranched type 2 chain structure bearing at least two successive LacNAc residues (Fig. 1A).22 I is a branched polylactosamine derived from i by the addition of a β1,6-linked polylactosamine side chain.23 Antibodies against I, therefore, must recognize GlcNAcβ1-6Galβ-R as part of the immune epitope.23-24 Although I is often depicted with binary, terminal β1-3 and β1-6 LacNAc epitopes, in reality, β1,6 branching can occur
anywhere along the polylactosamine backbone, assuming the presence of at least two successive LacNAc residues (see later section). The best example of the latter is the massive N-glycan on Band 3 of adult RBCs, which contains 40 to 50 oligosaccharides including five, short β1,6 LacNAc side chains (see section on HDFN).

Ii expression can be modified by enzyme treatment of RBCs. Endo-β-galactosidase from *Bacteroides fragilis* and *Escherichia freundii* cleave internal, unsubstituted Galβ1\(^4\)R linkages. The enzyme will readily cleave i-active oligosaccharides in the absence of branching or modification. Internal galactose residues bearing substitutions, such as fucose (ABH, LeX; Fig. 1D) or β1,6 GlcNAc (I) are resistant to enzyme cleavage. As a result, endo-β-galactosidase will destroy i reactivity and reduce, but not eliminate, I because of the presence of substituted galactose at β1,6 branch points (Fig. 1A). In contrast, Ii expression tends to increase after digestion with proteases and neuraminidase.

Polylactosamine Biosynthesis

The Golgi and Glycosyltransferases

Proteins and lipids are initially synthesized in the endoplasmic reticulum (ER, Fig. 2A), followed by posttranslation glycosylation in the Golgi. The regulatory mechanisms directing the passage and modification of substrates through the Golgi is still not entirely understood owing to its inherent dynamic and structural complexity. Grossly, the Golgi is composed of stacked membranous cisternae arranged into three regions; cis-, medial-, and trans-Golgi. Cargo (proteins, lipids) is shuttled between the ER and various Golgi compartments by membrane budding, followed by targeting and fusion of vesicles to another Golgi compartment or plasma membrane. The process is dependent on GTP, coat complex proteins (COPI, COPII), SNARE proteins, membrane tethering proteins, and lipid transfer proteins.
have recently been linked to altered glycosylation in HEMPAS disease.\(^{20}\)

Glycosyltransferases and other Golgi processing enzymes are typically localized within specific regions of the Golgi, with many glycosyltransferases co-localizing to the same Golgi region and forming heterodimeric complexes. C2GnT, a β1,6 glucosaminyltransferase responsible for branched polylactosamines on O-glycans, is located in the cis-medial Golgi as a dimer.\(^{38}\) β3GalT1 (iGnT) and β4GalT1 specifically colocalize to the trans-Golgi and may synergistically participate in polylactosamine regulation.\(^{31}\) β3GnT2 and β3GnT8, two β3 glucosaminyltransferases important in polylactosamine synthesis on complex N-glycans, preferentially exist as a heterodimer.\(^{32}\)

**Synthesis of Ii on Glycolipids and Glycoproteins**

As carbohydrate antigens, Ii are synthesized by a regulated, stepwise addition of sugars by a series of glycosyltransferases, many of which are tissue-specific. This is particularly true for the β1,3 glucosaminyltransferases (β3GnT), which initiate or elongate polylactosamine chains. As shown in Table 1, up to eight different β1,3 glucosaminyltransferases have been identified capable of either initiating or elongating polylactosamine-type glycans. The spectrum of polylactosamine structures ultimately synthesized by any cell will reflect the complexity of tissue-specific transcription, enzyme kinetics, substrate specificity, and acceptor availability.

An example of how substrate specificity and transcriptional regulation can direct polylactosamine synthesis is the synthesis of type 2 chain glycosphingolipids. Synthesis of i-active glycosphingolipids proceeds from lactosylceramide (LacCer; CDH) by the addition of a β1→3 GlcNAc residue by β3GnT5, a rate-limiting, gateway enzyme regulating type 2 chain glycolipid synthesis (Fig. 2B).\(^{35–37}\) Once formed, Lac3 is rapidly galactosylated by β4GalT1 to form paragloboside (nLc4),\(^{37}\) which then is further extended by β3GnT1 (iGnT), or possibly β3GnT5,\(^{35–36}\) to form nLc5. The latter is followed by β4GalT1 to form nLc6, the first i-active glycolipid bearing two successive LacNAc motifs.\(^{22}\) Because β3GnT5 is specific for short-chain glycolipid substrates,\(^{35–36}\) further elongation of nLc6 would be initiated by either β3GnT1 or, possibly, β3GnT2 (Table 1).

The formation of I proceeds from the addition of a β1→6 GlcNAc to i by IgNt (GCNT2 by Human Genome Organisation [HuGO] nomenclature).\(^{2,38}\) Enzyme studies with purified oligosaccharides indicate the enzyme requires at least two successive LacNAc motifs for binding and activity.\(^{27}\) This is also confirmed by detailed analysis of I-active oligosaccharides, which typically demonstrate at least two LacNAc epitopes per β1,6 branch (Fig. 2C).\(^{22}\) The enzyme can form branches on both distal and centrally placed galactose, as long as the prerequisite for two adjacent LacNAc motifs is satisfied.\(^{27}\) The enzyme will not recognize LacNAc motifs replaced with fucose, neuraminic acid, or α-linked galactose. As a result, sialylation and fucosylation can be considered regulators of polylactosamine synthesis, dictating both the length and potential number of β1,6 branch points (Fig. 1D). Detailed studies have shown an inverse reciprocal relationship between sialylation and polylactosamine chain length in immortalized cell lines.\(^{39}\)

There have been attempts to dissect the contributions of enzyme activity, substrate specificity, and Golgi localization in regulating polylactosamine synthesis. Kinetic studies with purified enzyme extracts and defined oligosaccharides

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**Table 1. Human β1,3 and β1,6 N-acetylglosaminyltransferases (GnT)**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Alias</th>
<th>Chromosome</th>
<th>Total</th>
<th>Size (aa)*</th>
<th>N-glycan sites</th>
<th>Substrate</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>β3GnT1</td>
<td>iGnT</td>
<td>11q13.2</td>
<td>415</td>
<td>28</td>
<td>2</td>
<td>Galβ1→4GlcNAc</td>
<td>with LARGE, 84GalT1 in trans-Golgi</td>
</tr>
<tr>
<td>β3GnT2</td>
<td>β3GnT1*</td>
<td>2p15</td>
<td>397</td>
<td>11</td>
<td>5</td>
<td>[Galβ1→4GlcNAc]_n→5</td>
<td>N-glycans; GSLs?</td>
</tr>
<tr>
<td>β3GnT3</td>
<td></td>
<td>13p13.1</td>
<td>372</td>
<td>21</td>
<td>3</td>
<td>Galβ1→3GlcNAc-O-Ser/Thr</td>
<td>core 1 extension</td>
</tr>
<tr>
<td>β3GnT4</td>
<td></td>
<td>12q24</td>
<td>378</td>
<td>20</td>
<td>3</td>
<td>Galβ1→3GlcNAc-O-Ser/Thr</td>
<td>core 3 synthesis</td>
</tr>
<tr>
<td>β3GnT5</td>
<td>Lc3 synthase</td>
<td>9q28</td>
<td>378</td>
<td>12</td>
<td>4</td>
<td>Galβ1→4Glc-Cer</td>
<td>Lc3, ± Lc5 synthesis</td>
</tr>
<tr>
<td>β3GnT6</td>
<td>C3GnT</td>
<td>11q13.4</td>
<td>353</td>
<td>19</td>
<td>3</td>
<td>GalNAc-O-Ser/Thr</td>
<td>keratan sulfate</td>
</tr>
<tr>
<td>β3GnT7</td>
<td></td>
<td>2q37.1</td>
<td>401</td>
<td>24</td>
<td>1</td>
<td>[Galβ1→4(SO)]_nGlcNAc81→R</td>
<td>tetraantennary N-glycans; activates β3GnT2 in WBC</td>
</tr>
<tr>
<td>β3GnT8</td>
<td></td>
<td>19q13.31</td>
<td>397</td>
<td>19</td>
<td>1</td>
<td>Galβ1→4GlcNAc81→R</td>
<td>tetraantennary N-glycans; activates β3GnT2 in WBC</td>
</tr>
</tbody>
</table>

* Amino acid (aa) length of the total protein and transmembrane domain (TM).

†83GnT1\(^{35}\) was renamed 83GnT2 after resequencing and comparison of the two enzymes revealed they encoded the same enzyme.\(^{34}\)
substrates are mixed. It is clear that the synthesis of $\beta_1 \rightarrow 3$ GlcNAc by $\beta_3$GnT is a critical rate-limiting step, with many $\beta_3$GnT displaying specific substrate specificities (Table 1). On $N$-glycans, LacNAc synthesis requires the involvement of at least three different $\beta_3$GnTs: $\beta_3$GnT1, $\beta_3$GnT2, and $\beta_3$GnT8. $\beta_3$GnT1 reacts well with linear and branched acceptors although the recombinant enzyme may have slightly higher activity with $\beta_1,6$ branched acceptors. $\beta_3$GnT2, like $\beta_3$GnT1, has broad acceptor reactivity, whereas $\beta_3$GnT8 is quite specific for large tetraantennary substrates on $N$-glycans (Fig. 1). $\beta_3$GnT8 and $\beta_3$GnT2 may cooperatively enhance polylactosamine synthesis, particularly on leukocytes. In HL60 cells, $\beta_3$GnT8 has been shown to activate $\beta_3$GnT2, increasing the $V_{\text{max}}/K_m$ ratio tenfold with a fourfold increase in $\beta_3$GnT2 activity.

$\beta_3$GnT1 and $\beta_4$GalT1 colocalize within the trans-Golgi, suggesting a possible coregulatory role for $\beta_4$GalT1. Although $\beta_4$GalT1 is not a rate-limiting step, studies indicate subtle enzyme preferences that may influence the length and degree of branching. With simple trisaccharide substrates, $\beta_4$GalT1 preferentially reacts with GlcNAc$\beta_1-3$LacNAc (i type) over GlcNAc$\beta_1-6$LacNAc (I type), suggesting a concerted effort by $\beta_3$GnT1 and $\beta_4$GalT1 toward synthesis of long i-type chains. Slightly different results are obtained when $\beta_4$GalT1 is tested against branched substrates offering both $\beta_1,3$ GlcNAc and $\beta_1,6$ GlcNAc termini. Unlike linear substrates, $\beta_4$GalT1 will preferentially recognize the $\beta_1,6$ GlcNAc branch early in the reaction, although this tended to inhibit further galactosylation of the remaining $\beta_1,3$ branch. In contrast, initial galactosylation of the $\beta_1,3$ GlcNAc permitted a second galactosylation to form an I-type structure with both $\beta_1,3$ and $\beta_1,6$ LacNAC termini. Like sialylation and fucosylation, a distal $\beta_1,6$ branch near the nonreducing galactose terminus may inhibit further chain elongation.

**Molecular Biology**

**The i Gene**

The gene generally referred to as $iGnT$ is $\beta_3$GnT1, a $\beta_1,3$-N-acetylgalcosaminyltransferase, which transfers a GlcNAc $\beta_1 \rightarrow 3$ to lactose and LacNAc termini. The gene resides on chromosome 11q13.2 and encodes a 415-amino acid, 47-kDa type II transmembrane protein. The enzyme contains two potential $N$-glycosylation sites and an unusually long, 28-amino acid transmembrane domain. The gene shares little sequence homology with other $\beta_1,3$ glucosaminyltransferases.
The enzyme is reportedly capable of both initiating the synthesis of poly-N-acetyllactosamines and elongating existing poly-N-acetyllactosamine oligosaccharides. As discussed earlier, β3GnT1 colocalizes with β4GalT1 in the trans-Golgi, where β4GalT1 may co-associate and stabilize β3GnT1 retention.31 β3GnT1 also complexes with LARGE, an unusual glucosaminyltransferase implicated in O-linked glycans on α-dystrophin.45 By Northern blot, the enzyme is ubiquitously expressed in most human tissue tested; however, expression is extremely weak in WBCs, thymus, neural tissue, lung, and liver.40 The relative absence of detectable RNA in WBCs is noteworthy: granulocytes and monocytes express primarily type 2 chain glycans on both glycoproteins and glycolipids.46,47

Seven additional β3GnTs have been cloned and characterized (Table 1). The literature regarding these β3GnTs is a bit confusing as a result of duplicate publication and name changes. All seven β3GnTs share significant homology with each other and are related to a larger family of β1,3 galactosyltransferases.34,35 β3GnT2 (originally named β3GnT7)33 to distinguish it from iGnT), was isolated from human uterus and bladder.33,34,44 Like β3GnT1, β3GnT2 was able to initiate and elongate poly-N-acetyllactosaminoglycans on a variety of substrates and displays wide expression.33 Data from β3GnT2 knockout mice indicate a major role for β3GnT2 in poly-N-acetyllactosamine synthesis on N-linked glycans.33-35 β3GnT2 appears to physically associate with β3GnT8,32 an elongating β3GnT specific for the β1,6 branch on tetraantennary N-glycans.44 In HL60 cells, myeloid differentiation is accompanied by a parallel increase in β3GnT8 mRNA and poly-N-acetyllactosamine chain length.37 β3GnT8 is highly expressed in bone marrow, spleen, pancreas, and small intestine with lower level expression in other tissues.44

β3GnT3 is specific for initiating type 2 chain synthesis on glycolipids,35-36 which contributes significantly to II and sialo agglutinin expression on human RBCs. β3GnT5 is upregulated during myeloid differentiation,26 coincident with the synthesis of type 2 chain glycolipids with LeX, sLeX, and VIM activity (Fig. 1D).46-48 Polylactosamine synthesis on O-linked glycans and keratan is directed by β3GnT3, β3GnT6, C2GnT, and β3GnT7 (Fig. 1C, Table 1).34-49,50 β3GnT3 and β3GnT6 are related glycosyltransferases responsible for initiating core 2 and core 3 O-glycan synthesis and are highly expressed in gastrointestinal tissues (Fig. 1C).34-49 Core 3 O-glycans can be further modified by C2GnT (Fig. 1C), an I-like β1,6 branching enzyme located in the cis-medial Golgi.30,31 β3GnT7 recognizes and elongates sulfated LacNAc oligosaccharides found on keratan sulfate and is highly expressed in placenta, colon, stomach, and small intestine.50,52

The I Gene

I (IgNT, GCNT2) is located on chromosome 6p24.2 (Fig. 3A).38 It consists of five exons (E1A, E1B, E1C, E2, E3), which give rise to three related isoforms of the enzyme depending on which exon 1 is used (Fig. 3B, 3C).7,8 IgNTA (GCNT2A, IgNT2), the most common transcript found in human tissues, is encoded by exons E1A, E2, and E3. The IgNT2B (GCNT2B) uses exon E1B and is the only mRNA transcript identified in purified human lens epithelium.8 It is also highly expressed in fetal brain and adult cerebellum.

IgNTC (GCNT2C), on the other hand, is nearly unique to human reticulocytes and is responsible for I antigen expression on RBCs.7,8 This has been confirmed by studies in hematopoietic stem cells.53 IgNT expression is extremely low in both adult and cord CD34+ cells. On erythroid differentiation, a parallel increase in IgNTC and I antigen expression is observed.53 Mutations in exons E1C, E2, and E3 have all been associated with the iadult RBC phenotype.7-10

Although encoded by the same gene, IgNTA, IgNTB, and IgNTGNTC share only 66 to 73 percent homology because of sequence differences in exon 1, which may account for differences in enzyme activity among the three enzyme isoforms.8 In enzyme assays, IgNTC has nearly twofold higher activity than either the IgNTA or IgNTB...
isoform. Exon E1 encodes nearly 77 percent of the active enzyme (Fig. 4), including the transmembrane domain, stem region, and part of the catalytic domain containing the nucleotide binding site. E2 and E3, which are shared by all three isoforms, encode the carboxy-terminal end of the enzyme.

**Transcriptional Control**

The identification of three tissue-specific IGnT/GCNT2 isoforms strongly suggests transcriptional regulation of exon 1 by cis-regulatory sequences in the 5′ untranslated region (5′UTR) or promoter region. This possibility is also supported by the structure of IGnT, in which exons E1A, E1B, and E1C are each separated by relatively large intronic sequences that may each harbor binding sites for tissue-specific transcription factors. This was recently confirmed by studies in K562 cells, which identified a promoter sequence –318 to –251 base pairs upstream of the translation initiation site. As shown, the IGnTC promoter in erythroid cells contains consensus sequences for Oct-2, Sp1, and C/EBPα transcription factors (Fig. 3D).

Although both Oct-2 and Sp1 bind the IGnTC promoter, C/EBPα binding is critical for IGnTC transcriptional activation. How C/EBPα regulates IGnTC is complex, apparently involving posttranslational phosphorylation of C/EBPα protein. In K562 erythroleukemia cells, butyrate induces changes in C/EBPα phosphorylation, a prerequisite for functional C/EBPα binding to the IGnTC promoter (Fig. 3D). Once bound, the phosphorylated-C/EBPα drives IGnTC transcription irrespective of Oct-2 and Sp1 binding. Similar results can be observed during in vitro differentiation of adult and cord stem cells. Loss of phosphoserine residues may be critical for C/EBPα-mediated transcription activation: serine phosphorylation has been shown to repress C/EBPα during early hematopoiesis.

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**Fig. 4** Structure-function of IGnT. The IGnTC isoform, expressed in reticulocytes, is a 402–amino acid glycoprotein protein, possessing up to five N-glycosylation sites and nine conserved cysteine residues. Exon 1, which shows only 65 percent homology among the three IGnT isoforms, encodes nearly 76 percent of the enzyme. Like all glycosyltransferases, the molecule contains a short 19-amino acid hydrophobic transmembrane region (TM domain) that anchors the molecule within the Golgi membrane, a stem region, and a large catalytic domain. IGnT shares three regions of significant homology with C2GnT, a related β-1,6-N-acetylglucosaminyltransferase (G) whose three-dimensional structure has been characterized. Using C2GnT as a template, the location of four potential disulfide bonds (---), nucleotide-binding site, and acceptor-binding sites were extrapolated. IGnTC and C2GnT share significant homology in β1, β2, β3, and β4 strands that incorporate the UDP or nucleotide donor–binding site. Of six nucleotides critical for acceptor binding by C2GnT (E243, K251, R254, E320, W356, Y358; ↓, bold), only two homologous sequences were identified in IGnT (E298, W328). Conserved sequences, including a lysine, along the β7 strand may play a role in binding phosphate of the UDP donor.
IGNt (GCNT2) Protein Structure-Function

The translated protein varies from 400 (IGNtB) to 402 amino acids (IGNtA, IGNtC). Like all glycosyltransferases, the enzyme is a type 2 transmembrane glycoprotein possessing several potential N-glycosylation sites and a 16- to 19-amino acid hydrophobic transmembrane domain near the amino terminus (Fig. 4). It is presumed to possess significant tertiary structure based on exhibiting nine conserved cysteine residues.

IGNt shares sequence homology with C2Gnt, a related β-6-N-acetylgalcosaminyltransferase that transfers UDP-GlcNAc to Galβ-3GalNAc-O-Ser/Thr, transforming simple core 1 and core 3 O-glycans to branched, core 2 and core 4 O-glycans, respectively (Fig. 1C). Mucin-type C2Gnt (C2Gnt-M) also displays IGNt-like activity with synthesis of I antigen on N-glycans.

The x-ray crystallography structure of mouse C2Gnt was recently determined and identified C2Gnt as a novel metal ion–independent GT-A glycosyltransferase. Like most GT-A type glycosyltransferases, C2Gnt has an a/β/α structure with a mixed β-sheet containing seven topological β strands. Strands β1 through β4 bind the nucleotide (UDP) of the UDP-Glcnac donor: acceptor binding occurs downstream of the UDP site and involves amino acids located within the β5, β6, α5, and β7 regions (Fig. 4).

The i adult Phenotype and Cataracts

The i adult phenotype is a rare, autosomal-recessive phenotype caused by a mutation in the IGNt/GCNT2 gene. The linkage of i adult, with congenital cataracts was originally reported by Ogata et al. and primarily limited to Asian kindreds. The etiology behind both i adult and congenital cataracts was finally resolved after mapping of the IGNt gene and identification of three distinct IGNt isoforms (Fig. 3). Mutations in exon E1C, which is the predominant isoform expressed in erythroblasts and reticulocytes, leads to an i adult RBC phenotype without congenital cataracts. Although IGNt activity is missing in RBCs, IGNt activity is intact in lens and other tissues via IGNtA and IGNtB (Fig. 3C). In contrast, mutations in exons E2 and E3, which are common to all three IGNt isoforms, result in a tissue-wide loss of IGNt activity including RBCs and lens. Mutations in E2 and E3 are responsible for i adult, with congenital cataracts. A summary of the specific mutations and phenotypes is listed in Table 2.

An I+i+ phenotype might occur in some heterozygous carriers of mutant IGNt alleles. Early family studies of i adult individuals reported decreased I expression among many first-degree family members. Although these relatives still typed as I+, titration scores with anti-I and i sera were intermediate between those of normal and i adult RBCs, resulting in an “I+i–like” phenotype. Examples of haploinsufficiency and weak antigen expression have been observed with other glycosyltransferases, including the related C2Gnt. Heterozygosity for a single missense mutation in C2Gnt (S158C) results in a marked decrease in core 2 O-glycans on T cells, accompanied by resistance to galectin-mediated cell apoptosis.

Table 2. IGNt mutations associated with i adult phenotype

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>i adult RBC</th>
<th>Cataracts</th>
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</thead>
<tbody>
<tr>
<td>E1C</td>
<td>243T&gt;A</td>
<td>N81L</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>E1C</td>
<td>505G&gt;A</td>
<td>A169T</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>E1C</td>
<td>683G&gt;A</td>
<td>R228Q</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>E2</td>
<td>983G&gt;A</td>
<td>W328X</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E2</td>
<td>1005G&gt;A</td>
<td>G336R</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E3</td>
<td>1049G&gt;A</td>
<td>G350E</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E3</td>
<td>1154G&gt;A</td>
<td>R385H</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Antibodies to i

Anti-I

Naturally occurring antibodies against I are common and have even been reported in cord blood samples. Anti-I increases progressively during childhood, with peak titers around 10 years of age. In adults, titers are generally low, even at 4°C (<64). Titers can increase in the setting of infection, particularly after infection with *Mycoplasma pneumoniae* or *Streptococcus pneumoniae* or with rubella.

By definition, anti-I reacts strongly with adult RBCs but not cord or *i*<sub>adult</sub> RBCs. Some examples of anti-I can display complex reactivity, reacting more strongly with RBCs of specific ABO or Pt types. Adult Bombay (O<sub>b</sub>) cells, which lack ABH, have the strongest reactivity with anti-I. Anti-I reactivity can be enhanced by protease and neuraminidase but is resistant to endo-β-galactosidase treatment. The amount of antibody binding sufficient to induce hemagglutination is clone- and temperature-dependent. At 4°C, as few as 50 to 100 molecules per RBC are required for hemagglutination but that number can increase to more than 10,000 per RBC at 37°C.

The exact epitope recognized by anti-I is clone-specific, although nearly all clones examined recognize a GlcNAcβ1-6Gal as part of the epitope. Feizi et al. were able to identify at least three distinct epitopes by testing a series of human monoclonal anti-I against a panel of oligosaccharides (Fig. 5). However, even among clones sharing a common binding pattern, subtle differences were evident. The heterogeneity of binding epitopes, and therefore physiologic receptors, is also inferred by the range in antigen binding sites when different anti-I clones are tested (Table 3). Anti-I displaying both I and ABH activity (e.g., anti-HI) recognize a branched epitope bearing both LacNAC and ABH epitopes (Fig. 5). Loss of the ABH epitope is associated with loss of antibody reactivity.

Anti-i

Naturally occurring and monoclonal antibodies (MAbs) against i are relatively uncommon. As described by Marsh, anti-i reacts strongly with cord and *i*<sub>adult</sub> but not adult RBCs. The minimal epitopes necessary for anti-i binding are two repeating LacNAc motifs (Figs. 1A, 5). Like anti-I, different anti-i clones may recognize slightly different antigens based on the number of antigen sites per RBC, particularly when cord and *i*<sub>adult</sub> RBCs are compared (Table 3). Anti-i is sensitive to endo-β-galactosidase (Fig. 1) but can be enhanced by protease and neuraminidase digestion. Bombay cells do not display enhanced reactivity with anti-i.

Infectious mononucleosis is commonly associated with increased anti-i titers and can occasionally result in immune-mediated hemolysis. Lymphoid cell lines derived from Burkitt’s lymphoma patients, and after EBV transformation in vitro, can produce MAbs with apparent anti-i specificity. When tested against specific glycolipids, some of these “anti-i” were sialo agglutinins, reactive with sialylated linear type 2 chain structures (see later section).

Anti-II

Monoclonal antibodies strongly reactive with both adult and cord cells have been described (Fig. 5). Some investigators have referred to these antibodies as “anti-j” (because j is next in the alphabet). Roelcke et al. described two cases (ZI, BR) that reacted equally well with adult and cord cells. Hemagglutination was reduced or inhibited by linear and branched type 2 chain oligosaccharides, as well as endo-β-galactosidase. Dube et al. also investigated a monoclonal cold agglutinin with I and i reactivity. Inhibition studies indicated that the active epitope included both GlcNAcβ1-6Gal and linear, i-active sequences.

Table 3. Difference in antigen sites recognized by monoclonal anti-I/i

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Name*</th>
<th>Untreated</th>
<th>Protease†</th>
<th><em>i</em>&lt;sub&gt;adult&lt;/sub&gt;</th>
<th>Cord</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-I</td>
<td>Kau</td>
<td>30,000 ± 10,000</td>
<td>90,000 ± 20,000</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Fla</td>
<td>130,000 ± 10,000</td>
<td>ND</td>
<td>&lt;1000</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Loi-1*</td>
<td>115,000 ± 10,000</td>
<td>ND</td>
<td>&lt;1000</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Loi-2*</td>
<td>50,000 ± 5,000</td>
<td>ND</td>
<td>&lt;1000</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>AJ</td>
<td>32,000</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-i</td>
<td>Fou</td>
<td>&lt;1000</td>
<td>ND</td>
<td>30,000 ± 5000</td>
<td>55,000 ± 10,000</td>
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<tr>
<td></td>
<td>Min</td>
<td>0</td>
<td>ND</td>
<td>60,000 ± 10,000</td>
<td>25,000 ± 5000</td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>30,000</td>
<td>ND</td>
<td>110,000</td>
<td>75,000</td>
</tr>
<tr>
<td>Anti-II</td>
<td>Abg</td>
<td>100,000 ± 20,000</td>
<td>ND</td>
<td>45,000 ± 10,000</td>
<td>40,000 ± 5000</td>
</tr>
<tr>
<td>Anti-A</td>
<td></td>
<td>0.8–1.7 × 10⁶</td>
<td>250–370,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND = not done.
*Name of human monoclonal antibody.
†O<sub>b</sub> adult RBCs digested with papain.
‡Two different antibody binding sites and densities were observed with the Loi antibody.
### Fig. 5 Epitopes for examples of anti-Ii. Anti-I requires at least two LacNAc residues. Several different epitopes have been identified among anti-I (lines), although all include a GlcNAc\(\beta_1\rightarrow 6\)Gal epitope. Examples of anti-Ii with HI and sialo agglutinin activity are also shown. Cer = ceramide; Fuc = fucose; Gal = galactose; GlcNAc = N-acetylglucosamine; NeuAc = neuraminic acid.

<table>
<thead>
<tr>
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<tr>
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</tr>
<tr>
<td>i</td>
<td>Den</td>
</tr>
<tr>
<td></td>
<td>Gal(\alpha),1\rightarrow 4\text{GlcNAc}</td>
</tr>
<tr>
<td></td>
<td>Gal(\beta_1\rightarrow 4\text{GlcNAc}),\beta_1</td>
</tr>
<tr>
<td>I</td>
<td>Ma, Woj</td>
</tr>
<tr>
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<td>Gal(\alpha,1\rightarrow 4\text{GlcNAc}),\beta_1\rightarrow 3\text{Gal},\alpha,1\rightarrow 4\text{GlcNAc}/\text{Glc-R}</td>
</tr>
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</tr>
<tr>
<td>I</td>
<td>Step, Gra Ver,Ful</td>
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<tr>
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<td>Phi, Da Sch, Low</td>
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<td>Gal(\beta_1\rightarrow 4\text{GlcNAc}),\beta_1</td>
</tr>
<tr>
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<td>Hy</td>
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**Li and ABO activity**

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<tr>
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<tr>
<td>Hi</td>
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<tr>
<td>Hi</td>
<td>Britton</td>
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<tr>
<td>Hi</td>
<td>s22</td>
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**Li and Sialic acid activity**

<table>
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<th>Sialic Acid Activity</th>
</tr>
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<tr>
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<td>NeuAcc(\beta,2\rightarrow 3\text{Gal}),\alpha,1\rightarrow 4\text{GlcNAc}),\beta_1\rightarrow 3[\text{Gal},\alpha,1\rightarrow 4\text{GlcNAc}],\beta_1\rightarrow 3\text{Gal},\alpha,1\rightarrow 4\text{GlcNAc}/\text{Glc-R}</td>
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</tr>
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<td>I</td>
<td>NeuAcc(\beta,2\rightarrow 3\text{Gal}),\alpha,1\rightarrow 4\text{GlcNAc}),\beta_1\rightarrow 3\text{Gal},\alpha,1\rightarrow 4\text{GlcNAc}/\text{Glc-R}</td>
</tr>
<tr>
<td></td>
<td>Fuc(\alpha,1\rightarrow 2\text{Gal}),\alpha,1\rightarrow 4\text{GlcNAc}),\beta_1</td>
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**Glycolipid-Dependent (Gd)**

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<tr>
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</tr>
<tr>
<td>I</td>
<td>NeuAcc(\beta,2\rightarrow 3\text{Gal}),\alpha,1\rightarrow 4\text{GlcNAc}),\beta_1\rightarrow 3\text{Gal},\alpha,1\rightarrow 4\text{GlcNAc}/\text{Glc-R}</td>
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<td>NeuAcc(\beta,2\rightarrow 3\text{Gal}),\alpha,1\rightarrow 4\text{GlcNAc}),\beta_1\rightarrow 3\text{Gal},\alpha,1\rightarrow 4\text{GlcNAc}/\text{Glc-R}</td>
</tr>
<tr>
<td></td>
<td>NeuAcc(\beta,2\rightarrow 3\text{Gal}),\alpha,1\rightarrow 4\text{GlcNAc}),\beta_1\rightarrow 3\text{Gal},\alpha,1\rightarrow 4\text{GlcNAc}/\text{Glc-R}</td>
</tr>
</tbody>
</table>

**Notes:**

- Cer = ceramide; Fuc = fucose; Gal = galactose; GlcNAc = N-acetylglucosamine; NeuAc = neuraminic acid.
Sialo Agglutinins

Sialo agglutinins represent a subgroup of I/i antibodies that require a terminal sialic acid for activity. They are distinguished from anti-I and anti-i by their sensitivity to neuraminidase. Dieter Roelcke26 broadly subclassified the sialo agglutinins into two major classes, based on whether the antibodies recognize linear (Sia-i1) or branched (Sia-b1) sialooligosaccharides. Like anti-I, Sial-ib includes antibodies against branched structures with ABH activity (anti-Fl).67 Sialo agglutinins with Sia-ib reactivity have been linked with both CMV and Mycoplasma infections.47-75

A third category of sialo agglutinins are Sia-lb1 and Sia-lb2, which recognize sialylated glycosphingolipids or gangliosides (Gd, or glycolipid-dependent).26 Sia-lb1,2 bind linear and branched type 2 chain gangliosides and are resistant to protease digestion. Inhibition studies indicate the minimal epitope for Sia-lb1,2 is the terminal mono-disaccharide incorporating sialic acid with or without galactose (Fig. 5). Not surprisingly, some Sia-lb1,2 antibodies also bind ganglioside GM3 (sialyl-lactose) and sialylparagloboside.26 As discussed earlier, anti-i produced by EBV-transformed human lymphocytes may recognize linear type 2 gangliosides.68,69

Sialylated lactosamines have been shown to be receptors for M. pneumoniae.70 Mycoplasma has been shown to bind sialyl-polyolactosamines along the apical aspect of ciliated bronchial epithelium and microvilli.27 The latter could serve as a trigger for autoantibody formation and may contribute to the prevalence of sialo-I (Sia-ib) after Mycoplasma infection.74-78

Antibody Structure-Function

Cold Agglutinin Disease

MAbs from patients with cold agglutinin disease have been invaluable in dissecting the pathologic autoimmune response to II. Multiple independent investigators have confirmed that IgM binding to II is primarily mediated by the IgM heavy chain via the VH or variable heavy domain. Of the 123 to 129 heavy chain VH genes available, the VH4-34 gene segment (VH4-21 or VH4-21 in older papers) is almost exclusively used by monoclonal cold agglutinins with II autoreactivity (Fig. 6).79 VH4-34 is also overrepresented in many, but not all, B-cell lymphoproliferative disorders including follicular lymphoma, mantle cell lymphoma, and Waldenström’s macroglobulinemia.80 In contrast, VH4-34 is unusual in chronic lymphocytic leukemia.80,81 In normal individuals, VH4-34 is identified in 3 to 6 percent of adult and fetal B cells but constitutes less than 0.5 percent of the total circulating IgM and IgG.80 Antibodies bearing the VH4-34 gene segment can be identified with the antidiidiotype antibody MAb 9G4.79,82

Detailed sequence analysis, coupled with construction of recombinant immunoglobulins, has uncovered the basis of II binding by VH4-34. Although only 110 to 120 amino acids in length, the VH region has distinct subdomains determined to be important in antigen binding. As shown in Figure 6D, three hypervariable or complementarity determining regions (CDR) are flanked by so-called framework regions (FR). Among VH4-34 monoclonal cold agglutinins, binding to either I or i is mediated by the N-terminal framework region 1 (FR1). This has been verified by recombinant IgH clones using an FR1 from a different VH4 subgroup.79 The 9G4 epitope is located in FR1 (amino acids 23–25).79 Although MAb 9G4 can inhibit RBC agglutination,77 the 9G4-binding site lies outside the antigen-binding site.83 It is theorized MAb 9G4 may inhibit

![Fig. 6 Structure-function of anti-II antibodies. (A) A schematic of human IgM showing five antigen binding sites per molecule. (B) The structure of an IgM heavy chain and light chain pair showing constant (C, C) and variable region (V, V) gene segments. The V and V regions encode the antigen binding site. The hinge region along C2 is a site for Fab or Fab antibodies. (C) Autoantibodies against II tend to use V gene segments from specific families. Polyclonal benign anti-II tend to use V gene segments from the V3 and V4 families, whereas the VH4-34 gene segment (historically VH4-21) accounts for the majority of monoclonal cold agglutinins. VH4-34 is also common among IgM anti-Rh monoclonal sera. (D) The V gene segment consists of four framework regions (FR) and three complementarity determining regions (CDR). For VH4-34, FR1 is required for II binding. Sequence variation in CDR3 may contribute to the fine specificity of the antibody (l or i). MAb 9G4 antibody recognizes an epitope in FR1 of VH4-34.](image-url)
binding by steric hindrance or induced conformational change.\textsuperscript{83}

Recombinant IgH clones also revealed a role for CDR domains, mutational “hot spots” known to direct antigen binding and specificity. Of three CDR domains, evidence suggests that CDR3,\textsubscript{H}, in concert with light chains, may define the fine specificity (anti-I or anti-i) of the antibody.\textsuperscript{79}\textsuperscript{80} The ability of CDR3 to distinguish I from i, however, is not mediated by a specific sequence motif: individual B-cell clones show significant sequence variability in CDR3.\textsuperscript{79,82} Crystallography studies of a human monoclonal anti-I show that CDR\textsubscript{\lambda}3 and two light chain variable regions (V\textsubscript{\lambda}CDR\textsubscript{\lambda}1 and CDR\textsubscript{\lambda}3) line the antigen-binding pocket of the antibody.\textsuperscript{83}

The role of light chains in Ii specificity is less clear-cut. There is a preference for V\textsubscript{\kappa}, particularly V\textsubscript{\kappa}3, among examples of human monoclonal anti-I.\textsuperscript{82} Kappa light chain restriction is also observed among monoclonal anti-Pr cold sialo agglutinins, with V\textsubscript{\kappa}4 and V\textsubscript{\kappa}3 accounting for 61 percent of clones.\textsuperscript{84} Interestingly, V\textsubscript{\kappa}4 was linked to recognition of sialo agglutinins bearing an α2→3 neuraminic acid.\textsuperscript{84} Monoclonal anti-i uses both V\textsubscript{\kappa} and V\textsubscript{\lambda}, with higher V\textsubscript{\lambda} usage relative to polyclonal antibodies.\textsuperscript{86} V\textsubscript{\lambda} usage is also relatively common in other autoimmune diseases, including antibodies against dsDNA, phospholipid, rheumatoid factor, and histone.\textsuperscript{85}

**Infection and Naturally Occurring Autoantibodies**

Unlike monoclonal anti-I/i, polyclonal anti-I/i does not display light or heavy chain restriction. Early studies using the 9G4 MAb showed that only a fraction of naturally occurring polyclonal anti-I/i carried the V\textsubscript{\kappa}4-34 gene segment.\textsuperscript{82,86} This was also supported by the inability of MAb 9G4 to inhibit hemagglutination with polyclonal antibodies.\textsuperscript{86} Naturally occurring anti-I/i uses a mix of V\textsubscript{\kappa} segments from the V\textsubscript{\kappa}3 and V\textsubscript{\kappa}4 families.\textsuperscript{86} V\textsubscript{\kappa}3 and V\textsubscript{\kappa}4 family genes account for 65 percent of the total B-cell repertoire and nearly 78.7 percent of all circulating immunoglobulin.\textsuperscript{85}

Infection may increase the concentration of V\textsubscript{\kappa}4-34 antibody.\textsuperscript{79} In patients with infectious mononucleosis and *M. pneumoniae*, there was an increase in V\textsubscript{\kappa}4-34 antibody in the majority of patients. Antibody binding to RBCs was directly associated with total circulating V\textsubscript{\kappa}4-34 antibody levels. When individual B-cell clones from infected patients were examined, only clones using the V\textsubscript{\kappa}4-34 gene segment secreted anti-i IgM capable of RBC hemagglutination.\textsuperscript{79} There was no association between hemagglutination and light chain (\kappa, \lambda) usage.

The ability of infection to stimulate anti-I/i has been studied in a transgenic mouse model of cold agglutinin disease carrying the sequence for a human sialo agglutinin (Sia-ib).\textsuperscript{80,88} Normally, transgenic mice did not demonstrate autoimmune hemolytic anemia attributable to a depletion of “autoreactive” B cells in the bone marrow and periphery.\textsuperscript{87} Low-level anti-Siab1 was only produced by rare B cells in the peritoneal cavity. Infection of mice with a murine *Mycoplasma* strain resulted in B-cell activation and loss of tolerance with markedly increased cold agglutinin synthesis, room temperature hemagglutination, and increased reticulocytosis.\textsuperscript{88}

Cross-reactivity and molecular mimicry may also contribute to a rise in cold agglutinins after infection. The polysaccharide of *S. pneumoniae* is a linear trisaccharide sequence of Gal, GlcNAc, and Glc, with short, β1,4-linked Gal side chains that is similar to the I and i epitopes (Fig. 7).\textsuperscript{23} Lactosamine epitopes are also expressed on the lipooligosaccharide (LOS) of some *Neisseria* gonococcus and meningococcus strains and can stimulate antibodies capable of cross-reacting with Ii glycolipids on RBCs (Fig. 7).\textsuperscript{89} As discussed earlier, *M. pneumoniae* binding to branched sialo agglutinins on bronchial epithelium may stimulate cross-reactive antibodies.\textsuperscript{77,78}

**Monoclonal IgM Rh and Other Antibodies**

Not surprisingly, other IgM MAbs using the V\textsubscript{\kappa}4-34 gene segment often display anti-i activity.\textsuperscript{90,91} This has been well documented with several IgM anti-D MAbs. Thorpe et al.\textsuperscript{90} showed that 59 percent of all IgM anti-D MAbs carried the V\textsubscript{\kappa}4-34 gene segment.\textsuperscript{90} Of these, 84 to 94 percent demonstrated anti-i activity when tested against papain-treated RBCs at 4°C.\textsuperscript{90} Similar “cold-agglutinin” activity has been demonstrated with other V\textsubscript{\kappa}4-34–derived MAbs against endotoxin lipid A, DNA, and IgG (rheumatoid factor). Sequence analysis suggests that the apparent I/i reactivity of many V\textsubscript{\kappa}4-34 anti-Rh MAbs is, in fact, charge-dependent.\textsuperscript{91} Most clones display a strong net positive charge at pH 7.5 and will react with RBCs, vimentin, and even cell nuclei at neutral pH and low ionic strength.

**Pneumococcus Polysaccharide**

\[
[-6\text{GlcNAc}]_1 \rightarrow 3\text{Gal}]_1 \rightarrow 4\text{Glc}]_1 \rightarrow 4\text{GlcNAc}]_1 \rightarrow 3\text{Gal}]_1 \rightarrow 4\text{Glc}]_1 \rightarrow \ldots
\]

Gal\textsubscript{p1} → Gal\textsubscript{p1}

**Neisseria Lipo oligosaccharide**

Gal\textsubscript{j1} \rightarrow 4\text{GlcNAc}]_1 \rightarrow 3\text{Gal}]_1 \rightarrow 4\text{Glc}]_1 \rightarrow 4\text{Hep}]_1 \rightarrow 5\text{R (KDO)}

\textsubscript{1}\textsuperscript{5}

GlcNAc\textsubscript{\alpha1} \rightarrow 2\text{Hep}]_1

**Fig. 7** Structure of the oligosaccharides on *Streptococcus pneumoniae*\textsuperscript{23} and *Neisseria* species\textsuperscript{89} (*N. gonorrhea* and *N. meningitidis*). Note that *S. pneumoniae*, a linear repeating trisaccharide, has cross-reactive motifs with structural similarity to i and I.\textsuperscript{23} Gal = galactose; Glc = glucose; GlcNAc = N-acetylglucosamine; Hep = heptose; KDO = 2-keto-3-deoxyoctulosonic acid.
Regulatory Autoantibodies to Cold Agglutinins

Cold autoantibodies may be autoregulated by naturally occurring anti-F(ab′)2 IgG antibodies directed against a short peptide epitope in the hinge-region. The Roelke laboratory demonstrated an inverse correlation between anti-F(ab′)2/hinge antibodies and monoclonal cold agglutinin titers. There was no correlation with hinge antibodies and polyclonal cold agglutinins. It is hypothesized that these hinge antibodies may suppress B-cell and autoantibody production. The relatively poor response of cold agglutinin disease to IVIG suggests that any downregulation by circulating hinge antibodies is moderate at best.

Reagents Against Lactosamine and Related Epitopes

Monoclonal Antibodies

Anti-II

MAbs IB2, FeA5, and H11 are three murine IgM MAbs that recognize unmodified, terminal lactosamine residues on either linear or branched, I-active structures (Table 4). MAb H11 and IB2 were raised against paragloboside and i-active glycolipids, whereas FeA5 was stimulated against murine testicular germ cells. In hemagglutination assays, MAbs FeA5 and IB2 behave as an anti-I with little or no reactivity against unmodified cord cells. Both MAbs will strongly agglutinate adult and cord cells after neuraminidase digestion. No hemagglutination data are available for MAb H11. MAb IB2 is available as cells from the American Type Culture Collection (ATCC, Manassas, VA). MAb FeA5 is available as a hybridoma supernatant from the Developmental Studies Hybridoma Bank held at the University of Iowa.

In addition to these antibodies, two MAbs developed against Neisseria gonococcus and meningococcus LOS also have lactosamine activity (Fig. 7). MAb 3F11 and 06B4 recognize a paragloboside epitope expressed on most gonococcal LOS. In addition to paragloboside, both antibodies will recognize linear and branched polylactosamine epitopes on RBC glycolipids. MAbs 3F11 and 06B4 preferentially agglutinate adult RBCs at 4°C. Hemagglutination with both adult and cord RBCs can be observed after neuraminidase or trypsin digestion.

We have successfully used both IB2 and FeA5 against glycoproteins and glycolipids by Western blotting, high-performance thin-layer chromatography (HPTLC), and flow cytometry. Although these antibodies are specific for Galβ1-4GlcNAcβ-R termini, they can be used to identify sialylated polylactosamines when coupled with neuraminidase.

Anti-i

Two MAbs with anti-i specificity have been isolated by heterohybridization of human B cells with murine myeloma cells. MAb NCC-1004 is a human IgMλ produced from the B cells of a patient with metastatic adenocarcinoma of the lung. The antibody strongly and preferentially agglutinated cord RBCs at 4°C with no observable agglutination at 37°C. Typical of anti-i, epitope mapping confirmed that the antibody was specific for a linear oligosaccharide containing at least two LacNAc residues. The antibody would tolerate terminal substitutions, binding sialo-i and long-chain, linear A-active glycolipids (nor-A'). When tested against human tissues, NCC-1004 recognized carcinoma of the thyroid, esophagus, lung, embryonal carcinoma, basal cell epithelium, mantle cell lymphocytes, and endothelium.

MAb MH21-134 is a human monoclonal IgG antibody derived from a patient with squamous cell carcinoma of the lung. The antibody specifically recognized glycolipids possessing a linear, polylactosamine structure. Unlike NCC-1004, MH21-134 will not bind sialylated i. In hemagglutination assays, MH21-134 will only weakly agglutinate neuraminidase-treated cord cells in the antiglobulin test; no agglutination was observed with unmodified cord and adult RBCs by immediate-spin. Among normal tissues, MH21-134 only reacted with granulocytes and bronchial and pancreatic epithelial cells. MH21-134 reacted with several gastrointestinal tract malignancies.

Anti-I

Three anti-I murine MAbs are reported in the literature; Fe-C6, M18.3, and M39.6. Initially identified as a stage-specific antigen on murine embryos, MAb Fe-C6 was subsequently shown to recognize a binary structure, requiring both an intact β1-3 and β1-6 terminal lactosamine epitope as shown in Table 4. Its reactivity is similar to the human MAbs Phi, Da, Sch, and Low (Fig. 5). MAb Fe-C6 is available as a hybridoma supernatant from the University of Iowa.

MAbs M18.3 and M39.6 were raised against human milk globule membranes as potential immune markers for breast tissue. Epitope mapping indicates that MAbs M18.3 and M39.6 primarily recognize the Galβ1-4GlcNAcβ-R termini, with evidence of β1-6 branching. Its reactivity is reminiscent of the human Step, Gra, Ver, and Ful antibodies (Fig. 5). MAb Fe-C6 is available as a hybridoma supernatant from the University of Iowa.

Anti-N-acetylgalcosamine

MAb J1 was developed against murine testicular cells and recognizes a stage-specific differentiation antigen expressed on high molecular weight polylactosaminyl-glycans on sperm. Using a panel of glycolipids, J1 was shown to recognize terminal anti-N-acetylgalcosamine (GlcNAc) residues on growing type 2 chain oligosaccharides (GlcNAcβ1-3Gal). J1 preferentially binds long-chain and multivalent epitopes. We have tested MAb J1 against...
neutrophil glycolipids, which express lactotriaosylceramide (Lc3; Fig. 2B) as well as longer type 2 chain intermediates. Although Lc3 reportedly binds J1 by ELISA, we could not demonstrate J1 binding to isolated Lc3, Lc5, or any other neutrophil glycolipids by HPTLC (L. Cooling, unpublished observations). MAb J1 is available as a hybridoma supernatant from the University of Iowa.

Holmes et al. and Hu et al. have also produced a panel of murine MAbs with GlcNAcβ1-R activity using the pentaosylceramide Lc5 as an immunogen (Fig. 2B). TE4 and TE7 have disaccharide and trisaccharide epitopes, respectively, on the termini of type 2 chain oligosaccharides (Table 4). MAbs TE5 and TE6 recognize terminal βGlcNAc on the growing chains of type 2 chain oligosaccharides and, possibly, GlcNAcβ1-Man structures on N-glycan intermediates. All four antibodies recognize linear type 2 chain oligosaccharides, whereas only MAb TE4 shows strong reactivity to branched lactosaminyl structures. All four antibodies will react with glycoproteins and glycolipids. MAb TE5 has been very useful to identify Lc3, Lc5, and other intermediate structures in tumor lines and human leukemia. MAbs TE4, TE5, and TE7 are murine IgM: MAb TE6 is an IgG3.

Lectins

Tomato Lectin

The tomato lectin, Lycopersicon esculentum (LEA) can hemagglutinate group O RBCs and all neuraminidase-treated RBCs, regardless of ABO group. Hemagglutination is reportedly inhibited by oligomers containing β-linked N-acetylgalactosamine. The lectin has proved quite useful in the isolation and characterization of glycoproteins, where it preferentially binds long-chain linear polyolactosamines containing at least three or more repeating lactosamine residues. It has also been used to detect increased lactosamine on cells by flow cytometry.
**Erythrina cristagalli**

*Erythrina cristagalli* (ECA) is a lactosamine-specific lectin from the seeds of the South American coral bean tree. It will agglutinate RBCs, regardless of ABO group. Agglutination is enhanced when tested against trypsin- or neuraminidase-treated RBCs. By thin-layer chromatography, ECA specifically recognizes glycolipids with terminal, unsubstituted LacNAc epitopes. Among I-active glycolipids, ECA will not bind sialo lactosamines, even if one terminal branch bears a bare, nonsialylated lactosamine terminus. Like *E. coralloidendron* lectin (later), ECA can also bind type 2 chain H structures, although ECA does not display obvious HI activity when tested against RBCs.

**Erythrina coralloidendron**

*Erythrina coralloidendron* (EcorL) is a galactophilic lectin isolated from the seeds of the West Indian coral tree. In hemagglutination assays, the lectin displays both I and HI activity. EcorL strongly agglutinates adult O RBCs, with weaker activity against group O cord, i\(^{-}\)-active, and Bombay (O\(^{-}\)) RBCs. EcorL has little or no activity when tested against AB and Bombay (O\(^{+}\)) cells. The absence of agglutination with Bombay cells is a conundrum because Bombay cells should have the highest expression of lactosamine and sialo lactosamine glycans.

The I and HI-like activity have been confirmed by structural studies using well-defined oligosaccharide receptors, x-ray crystallography, and recombinant lectin mutants. Like ECL, EcorL will bind terminal LacNAc epitopes on paragloboside, type 2 chain H, and branched polylactosamine glycolipids. Fucose, however, is the only terminal modification accepted: EcorL will not recognize type 2 chain structures with a terminal sialic acid, Galα1\(^{-}\)-4 (P antigen), A or B epitope. In solid phase assays, EcorL binds the H-active glycolipid with higher affinity than paragloboside; however, no comparative binding studies were performed with i\(^{-}\)-or I-active, polylactosaminyl glycolipids. Crystallography indicates that LacNAc, not fucose, is the active ligand.

**Aplysia Gonad Lectin**

Aplysia gonad lectin (AGL) is a galactophilic lectin from the gonads of *Aplysia depilans*, a fairly large sea slug found in the northeast Atlantic and Mediterranean Sea. In early studies, AGL was shown to agglutinate RBCs of humans and animals, particularly after papain digestion. A more recent study compared AGL, EcorL, and human anti-I against adult, cord, and Bombay (O\(^{+}\)) RBCs. AGL reactivity was similar to human anti-I, preferentially binding adult RBCs. The strongest agglutination was observed with Bombay RBCs. Unfortunately, no information is available regarding the reactivity of AGL with neuraminidase-treated RBCs.

**Maackia amurensis**

*Maackia amurensis* (MAA) is isolated from the seed of the Amur maackia tree, a short, hardy tree common to the midwest United States and Northeast China. The lectin has sialo agglutinin activity, recognizing a terminal NeuAcα2\(^{-}\)-3 lactosamine. It has been used successfully for the isolation of glycoproteins by immunoprecipitation and lectin affinity chromatography. It has also been used to identify sialylated polylactosamines by Western blotting, thin-layer chromatography, and flow cytometry. Of note, MAA does not bind glycophorin A and only weakly agglutinates human RBCs. We have used fluorescein-labeled MAA with ficin-treated RBCs when testing by flow cytometry.

**Viscum album**

*Viscum album* agglutinin (VAA) or mistletoe lectin recognizes sialylated polylactosamines bearing a terminal α2\(^{-}\)-6 neuraminic acid. VAA has been used for Western blotting, thin-layer chromatography, and tissue culture. VAA strongly binds granulocytes and lymphocytes and has been shown to enhance phagocytic activity, stimulate cytokine secretion, and increase natural killer cells. VAA is a potent cytotoxin and has been studied as an adjuvant agent in cancer trials.

**Polyporus squamosus**

PSL agglutinin is a product of the polypore mushroom, *Polyporus squamosus*, and is similar in activity to VAA. Unlike SNA lectin (later), PSL is specific for sialylated lactosamines expressed on N-linked glycans: PSL will not bind Tn-like epitopes on O-linked glycans. In hemagglutination assays, PSL agglutinated formalin-fixed human and rabbit RBCs, irrespective of blood type. Hemagglutination was specifically inhibited by the trisaccharide NeuAcα2\(^{-}\)-6Galβ1\(^{-}\)-4GlcnAc, but not N-acetyllactosamine or its α2\(^{-}\)-3 sialo-derivative. PSL has been used for immunohistochemistry, Western blotting, and thin-layer chromatography.

**Sambucus nigra**

*Sambucus nigra* agglutinin (SNA) is derived from the bark of the elderberry tree. The lectin also recognizes α2\(^{-}\)-6 sialylated glycoconjugates, but has a broader acceptor profile than PSL or VAA. SNA readily precipitates both glycophorin A and human erythrocytic, indicating an ability to recognize NeuAcα2\(^{-}\)-6GalNac and NeuAcα2\(^{-}\)-6Gal epitopes on O- and N-linked glycans, respectively. Hemagglutination assays, SNA agglutinates human RBCs regardless of ABO group or enzyme modification. SNA has been used for glycoprotein isolation, Western blotting, HPTLC-immunostaining, immunohistochemistry, and flow cytometry.

**Trichosanthes japonica**

TJA-I is one of two lectins isolated from the roots of the snakegourd or Chinese cucumber plant, *Trichosanthes japonica*. TJA-II is specific for H-like epitopes, whereas
TJA-1 preferentially recognizes sialolactosamines.\textsuperscript{199} Like PSL and VAA, TJA-1 binds N-glycans bearing a terminal NeuAcα2→6Galβ1→4GlcNAc/Glc epitope. It has been used for the isolation and characterization of glycoproteins.\textsuperscript{199}

**Biologic Role**

**Hemolytic Disease of the Fetus and Newborn**

The delay in I synthesis until several months of age may play a protective role against hemolytic disease of the fetus and newborn (HDFN) caused by ABO incompatibility. Major maternal-fetal ABO incompatibility is relatively common, with up to 30 percent of infants demonstrating a positive DAT at birth.\textsuperscript{48} Nonetheless, the incidence of severe HDFN requiring exchange transfusion is quite rare (12 of 29,200 or 0.04%).\textsuperscript{48} The infrequency of clinically significant ABO-HDFN is believed to reflect low ABH levels on cord cells, which are 25 percent of adult levels (Table 3). The low density of ABH epitopes on fetal RBCs may be insufficient to support efficient complement activation by maternal IgG antibodies.\textsuperscript{48}

A comparison of i, I, and A strength shows that there is a parallel increase in I and A in the first few months of life, with I and A reaching adult levels by 2 to 3 years of age (Fig. 8A–C).\textsuperscript{2,3} These results are consistent with detailed carbohydrate analysis of the lactosaminyl, biantennary N-glycan present on Band 3, which accounts for more than 50 percent of all ABH on RBCs (Fig 8D). On fetal RBCs, only a linear, i-active polylactosamine is synthesized, with approximately 25 percent bearing a terminal ABH epitope (fucose) on the long, α1-6 mannosyl branch.\textsuperscript{120} On adult RBCs, there is elongation and β1,6 branching along both α1,6 and α1,3 mannosyl branches, with up to four distal ABH epitopes.\textsuperscript{25} At one million molecules per RBC, Band 3 is capable of displaying 2 to 4 million ABH epitopes per adult RBC.

**Animal Models**

β3GnT2

Two strains of mice lacking β3GnT2 have been bred (β3GnT1 in some papers using the original citation by Zhou et al.).\textsuperscript{42-43} β3GnT2−/− were shown to have altered neural development. There was a loss of olfactory sensory neurons early in embryonic development,\textsuperscript{44} decreased migration of gonadotropin-releasing hormone into the ventral forebrain,\textsuperscript{121} and decreased reproduction, possibly attributable to poor olfactory recognition of estrous females.\textsuperscript{122} β3GnT2−/− also altered expected immune responses. There was a marked decrease in polylactosamine on N-glycans of CD28 and CD19, accompanied by evidence of T-cell, B-cell, and macrophage hyperresponsiveness. The results suggest a role for polylactosamines in regulating basal levels of immune reactivity.\textsuperscript{43}

**IGNT**

A murine model of IGNt (GCNT2) deficiency has been constructed by Chen and colleagues.\textsuperscript{123} To produce an IGNt-deficient strain, the investigators deleted exon III of the murine \textit{IGNT} gene. As discussed earlier, mutations affecting exons E2 or E3 are associated with i phenotype and cataracts in humans (Table 2).

Contrary to expectations, IGNt-deficient mice had normal fertility and development, despite a decrease in embryoglycan and laminin adhesion on embryonic stem cells.\textsuperscript{123-124} More surprisingly, there was no increase in either the onset or incidence of cataracts in IGNt-deficient mice.\textsuperscript{123} IGNt-deficient mice did display subtle abnormalities, including a decrease in B cells, increased epidermoid cyst formation, and mild renal dysfunction, as evidenced by elevated blood urea nitrogen and creatinine and increased renal tubular vacuolization. The latter appears to represent an accumulation of autophagocytic vacuoles and diminished membrane repair as a consequence of decreases in N-glycosylated lysosomal proteins (LAMP-1, LAMP-2, synaptotagmin II, synaptotagmin IV). It is hypothesized that branched poly lactosamines may play a role in stabilizing these lysoprotein proteins: inhibition of LAMP-1 glycosylation by tunicamycin shortens LAMP-1 half-life.\textsuperscript{123,125}

**Cataracts**

The association between the \textit{i}_\text{adult} phenotype and congenital cataracts was initially described by Ogata et al.\textsuperscript{12} in 1979, who reported cataracts in 17 of 18 \textit{i}_\text{adult} individuals from 10 different Japanese families. Subsequent studies by other investigators, however, were conflicting, indicating that the \textit{i}_\text{adult} phenotype did not always predispose to congenital cataracts, particularly in non-Asian populations.\textsuperscript{13,14} The apparent discrepancy was finally resolved with cloning of the \textit{IGNT} gene,\textsuperscript{38} followed by sequencing analysis of \textit{i}_\text{adult} individuals.\textsuperscript{7-10} As already described, the \textit{i}_\text{adult} phenotype without cataracts is the consequence of mutations affecting exon ExC, leading to an isolated loss of IGNt activity in erythroid cells (Table 2, Fig. 3). Conversely, mutations affecting E2 or E3 result in loss of IGNt activity in all tissues, including human lens.

It has been presumed that loss of branched type 2 glycans in human lens is responsible for congenital cataracts. Human lens epithelial cells do synthesize type 2 chain glycosphingolipids, including high molecular weight, long-chain sLeX gangliosides with repeating LeX motifs (Fig. 1D).\textsuperscript{46,126} In senile cataractous lens, there is an increase in LeX glycolipids, presumably caused by desialylation of sLeX structures.\textsuperscript{127} It is likely, therefore, that similar polylactosamines exist on lens glycoproteins. Although there are no published studies of glycoprotein oligosaccharides on human lens epithelial cells, lens tissue does express several N-linked glycoproteins, including α- and β-integrins, capable of I antigen expression.\textsuperscript{128,129} Altered glycosylation of β-integrins frequently accompanies cell differentiation, activation, and oncogenesis with changes in cell adhesion, motility, and signaling.\textsuperscript{130}

The absence of congenital cataracts in IGNt-deficient mice,\textsuperscript{123} however, is perplexing and raises old questions...
Fig. 8 Correlation between I, i, A, and Band 3 N-glycan structure on fetal and adult erythrocytes. (A) i and I strength of expression on RBCs by age.² (B) A strength of expression on RBCs, as determined by hemolysis.³ (C) Correlation between I and A expression by age.² ³ (D) Structure of Band 3 N-glycan on cord cells.¹²⁰ (E) Structure of Band 3 N-glycan on adult RBCs.²⁵
regarding the causal link between cataract formation and i<sub>thal</sub> phenotype. It is quite possible that IGnT deficiency plays no direct role in cataract formation but is closely linked to an unrelated mutant gene. Alternatively, there may be important species-specific differences in glycan expression on lens epithelial cells, making mice a poor animal model for studying the effect of IGnT deficiency on lens development. Indirect evidence for the latter explanation comes from knockout mice lacking α<sub>1</sub>-3 galactosyltransferase (α3GT-KO),<sup>131</sup> an animal-specific glycosyltransferase responsible for linear B (Galα1-3Galβ1-R), a xenoantigen absent in humans and Old World apes.<sup>132-133</sup> αGTKO mice develop congenital cataracts by day 36, suggesting a key role for α1,3-galactose in murine lens differentiation.<sup>131</sup>

**HEMPAS**

HEMPAS disease, or congenital dyserythropoietic anemia type 2 (CAD2), is a congenital and acquired disorder of hematopoiesis. Patients with CAD2 have a chronic mild to severe hemolytic anemia, jaundice, and splenomegaly.<sup>19</sup> Their bone marrow is striking for erythroid hyperplasia with 20 to 45 percent binucleated and multinucleated erythroblasts. Electron microscopy of marrow erythroblasts and erythrocytes shows “membrane duplication” as a result of accumulation and retention of fragmented endoplasmic reticulum.<sup>19</sup> In the blood bank, CDAI or HEMPAS RBCs show increased i expression and hemolysis with acidified donor sera. The amount of i expressed on HEMPAS RBCs is significantly higher than that observed on cord RBCs.<sup>134</sup>

Detailed analysis of HEMPAS RBCs has shown altered glycosylation on RBC glycoproteins (ex, band 3, glycoporphin) and glycolipids.<sup>20-21</sup> Some aspects of HEMPAS can be duplicated in vitro by inhibiting N-glycan processing, leading to speculation that HEMPAS was a glycosyltransferase defect.<sup>21</sup> In late 2009, the molecular basis for HEMPAS was finally resolved after extensive genetic studies in 33 individuals from 28 unrelated families.<sup>22</sup> Schwarz and colleagues<sup>22</sup> in Germany were able to show that all HEMPAS patients were heterozygous for mutations in SEC23B, a Golgi COP II component protein. COP II proteins facilitate and direct cargo vesicles among different Golgi compartments.<sup>29</sup> It is possible that the defect in HEMPAS leads to disordered trafficking in the Golgi, with prolonged retention in some compartments and decreased trafficking to others. It is interesting to note that βGalT1 and β3GnT1, which drive linear lactosamine synthesis, are colocalized in the trans-Golgi.<sup>31</sup> Although the Golgi location for IGnT is unknown, C2GnT, which catalyzes β1,6 branching on O-glycans, is located within the cis-medial Golgi.<sup>30</sup>

**Embryogenesis and Neoplasia**

Many carbohydrate antigens are critical to normal embryonic development and serve as oncofetal antigens during neoplastic transformation.<sup>135-136</sup> In mice, I is expressed on unfertilized ovum up to the morulae.<sup>137</sup> With subsequent differentiation, strong expression of I can be observed in embryonic endoderm with apical polarization along differentiating epithelial cells.<sup>138</sup> Increased Ii is also observed in murine teratocarcinomas and some human choriocarcinoma cell lines.<sup>137</sup> Disseminated choriocarcinomatosis has been associated with elevated anti-i in some patients.<sup>139</sup>

Changes in Ii or their associated glycosyltransferases have been described in several tissues, but this topic is too extensive for a full discussion here. Among published reports are increased sialylation with masking of I expression in breast cancer.<sup>101,102</sup> An increase in polylactosamines, β3GnT8 and GnTV, which catalyze synthesis of tetravalent N-glycans, is reported in colon cancer.<sup>44,100</sup> Conversely, transitional cell carcinoma of the bladder is associated with an 11-fold decrease in β3GnT2.<sup>41</sup> Particularly interesting is the possible role of β3GnT1 in promoting tumor metastasis.<sup>45</sup> β3GnT1, in conjunction with LARGE glycosyltransferase, regulates the synthesis of laminin-binding glycans on α-dystroglycan. Decreased βGnT1 could facilitate tumor migration and metastatic tumor formation in breast and prostate cancers.<sup>45</sup>

**Gene Array Data**

For several years, commercial “gene-chips” have been available for high-throughput screening of tissues for thousands of genes, including many glycosyltransferases involved in blood group expression. Transcriptome studies are probably the most common analysis performed. In these studies total tissue mRNA is analyzed for global differences in gene expression. At this time, the NIH requires investigators to upload their findings into GenBank, a public access database, allowing individual gene expression to be queried directly by other investigators. The data are located in Entrez under GEO Profiles or the Gene Expression Omnibus.<sup>140</sup> Of note, the system does support Boolean logic, limiting the search to human tissue. The latter is a useful trick to know: an initial search of GEO Profiles for IGnT resulted in 5654 entries, covering mouse, rat, and human arrays.

Among human studies, IGnT and iGnT array data are available for a wide range of studies, including these:

- Cancer
- Cell differentiation
- Organ transplantation
- Heart failure, atrial fibrillation
- Infection
- Rheumatoid arthritis
- Emphysema and cigarette smoking
- Hormone replacement
- Chemotherapy
- Immunosuppression
- Heavy metal exposure
- Growth factors
- Bipolar disorder
- Social isolation (i.e., loneliness!)
A brief review did not identify any clear disease association between IGnT and β3GalT1 levels in the studies available. Interestingly, many tissues displayed significant normal variation in glycosyltransferase expression between individuals. An example of IGnT and β3GalT1 expression in fetal and adult human reticulocytes is shown in Figure 9.141

Fig. 9 |GnT expression arrays. Individual data for β3GnT1 (|GnT1) (A) and IGnT (C2GnT) (B) expression in human fetal and adult reticulocytes. Array data from Goh et al.142 as extracted and displayed on NCBI GEO Profiles.140

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Attempts to support an immune etiology in 800 patients with direct antiglobulin test–negative hemolytic anemia

R.M. Leger, A. Co, P. Hunt, and G. Garratty

Clinical and hematologic evidence of warm autoimmune hemolytic anemia (AIHA) is present in some patients whose direct antiglobulin test (DAT) is negative. The most common causes for AIHA associated with a negative DAT are RBC-bound IgG below the sensitivity threshold of the DAT, RBC-bound IgA and IgM not detectable by routine reagents, and low-affinity IgG that dissociates during the testing process. Samples submitted from 800 patients with hemolytic anemia and a negative DAT were tested by an antiglobulin sera (AGS) panel of anti-IgG, anti-C3, anti-IgM, and anti-IgA by a routine DAT. Additional tests included a direct Polybrene test to detect small amounts of RBC-bound IgG, a cold-wash technique to detect low-affinity IgG, and a DAT by gel test with anti-IgG. A positive result was obtained with at least one method for 431 (54%) of 800 specimens tested. The AGS panel was positive for 400 (50%) of samples, with IgG or C3 or both accounting for reactivity in 48 percent. IgA alone was found on 2 percent of samples; IgM was never found alone. Low-affinity IgG was found on 37 (5%) samples. The direct Polybrene test was the only positive test for 15 (2%) samples. The gel anti-IgG test was never the only positive test. Clinical correlations for these data were not available; however, previously published correlations suggest a positive predictive value for tests that extend routine DAT methods in patients with DAT-negative AIHA. Immunohematology 2010;26:156–60.

Key Words: autoimmune hemolytic anemia, DAT-negative, Coombs-negative

Most patients with autoimmune hemolytic anemia (AIHA) have IgG or complement, or both, on their RBCs detectable by the routine DAT. However, approximately 2 to 11 percent of patients with hematologic and clinical findings suggestive of warm AIHA have a negative DAT by routine tube technique and have no detectable serum antibodies.1 Hematologists seeking evidence supporting immunemediated hemolysis may request nonroutine and more sensitive tests for these patients.

It has been suggested that there are at least three causes for AIHA associated with a negative DAT: RBC-bound IgG below the threshold of detection of the routine antiglobulin test, low-affinity IgG that dissociates from RBCs during saline washes for the DAT, and RBC-bound IgM and IgA that are not detectable by routine reagents.1,2 The routine tube DAT can detect about 150 to 200 molecules of IgG per RBC. Gilliland and coworkers3–4 suggested that the inability to detect IgG on the RBCs of some patients with AIHA was caused by low levels of RBC-bound IgG that were below the threshold of detection by the routine DAT. They developed a method, the complement-fixing antibody consumption (CFAC) test, capable of detecting this low level of RBC-bound IgG. The authors reported that RBCs from five of six patients with acquired hemolytic anemia and a negative DAT were found to have greater quantities of IgG per RBC (70–434 molecules) compared with 15 to 35 molecules of IgG per RBC found on the RBCs obtained from normal persons. Gilliland4 extended these results with an additional nine patients; six of these nine responded to corticosteroid therapy.

Petz and Garratty5 performed extensive tests, including the CFAC, on specimens obtained from 27 patients with a diagnosis of immune hemolytic anemia with a negative DAT, confirming that all of these patients had RBC-bound IgG detectable by the CFAC; 11 (41%) patients had RBC-bound C3 detectable by a potent anti-C3 prepared in their laboratory but not detected by the referring hospital. Because the CFAC is difficult to standardize and to perform consistently, other tests have been used to detect RBC-bound IgG, including enzyme-linked antiglobulin test (ELAT), radiolabeled anti-IgG, flow cytometry, monocyte monolayer assay (MMA), concentrated eluates, the direct Polybrene test, the direct polyethylene glycol test, solid phase, column agglutination, and the DAT using cold washes.2 When hematologists or referring laboratories contact our laboratory, they often ask for a “Super Coombs Test.” There is no such test. We believe the term originated as jargon to cover tests such as the ELAT and radiolabeled anti-IgG previously used to detect small amounts of RBC-bound IgG.

No one test has been shown to be optimal for investigating DAT-negative AIHA; we and others have found that a battery of tests appears to be the best approach.1,6–9 By 1995 we had decided that there was no cost benefit in performing time-consuming assays such as the ELAT, so we concentrated on a serologic approach. We transferred DAT-negative AIHA workups from our Immunohematology Research Laboratory to the Immunohematology Reference Laboratory (IRL). All samples that are submitted for a DAT-negative AIHA investigation are tested by tube test with an antiglobulin sera (AGS) panel consisting of anti-IgG, anti-C3, anti-IgM, and anti-IgA. When the AGS panel is not informative, additional tests performed include a direct Polybrene test to detect low amounts of immunoglobulin, a cold (4°C) LISS wash technique for preparation of RBCs to detect low-affinity IgG, and a column agglutination (gel) test for IgG.
We report the serologic results for a large series of patients’ samples that were submitted for a DAT-negative AIHA evaluation.

**Material and Methods**

The AGS panel consisted of a rabbit anti-IgG (American National Red Cross, Washington, DC; or Ortho Clinical Diagnostics, Raritan, NJ), anti-C3 (reagent prepared by injecting a rabbit with purified C3), rabbit anti-human IgM (CLB, distributed by Research Diagnostics, Flanders, NJ; or Sanquin, distributed by Accurate Chemical & Scientific Corporation, Westbury, NY), goat anti-human IgA (Tago, Burlingame, CA), and a 6% bovine serum albumin control. The anti-C3, anti-IgM, and anti-IgA were standardized for RBC agglutination in tube tests as previously described.1 Specimens were also tested from April through December 2008 with Gamma-clone Anti-IgG (murine monoclonal, Immunogenics, Norcross, GA). For the DAT, RBCs were washed four times with PBS (pH 7.0–7.4) and resuspended to achieve a 3 to 5% suspension; 1 drop was added to 2 drops of the antiagglutinin reagent or control, centrifuged, and read immediately. Centrifugation and reading was repeated after incubation at room temperature if indicated by the standardization. A positive result with the 6% albumin control invalidated the test. When a positive result with the albumin control was obtained, repeat testing may have been performed after washing a fresh aliquot of RBCs with warm (37°C) saline to remove cold autoagglutinins, or after treatment with 0.01 M dithiothreitol (DTT) to disperse IgM autoagglutinins.10

The cold LISS wash technique was performed by washing the RBCs four times with ice-cold (4°C) LISS using a refrigerated centrifuge.1 The RBCs were resuspended to a 3 to 5% suspension in cold LISS; 1 drop was added to 2 drops of cold anti-IgG or cold 6% albumin, centrifuged in a serologic centrifuge, and read immediately. The 6% albumin served as a control for the presence of a cold autoagglutinin; a positive result with the 6% albumin control invalidated the cold LISS wash test with anti-IgG.

The direct Polybrene test,1 a modification of the indirect Polybrene test,12 was performed as previously described. Briefly, 1 drop of 3 to 5% washed RBCs was added to 2 drops of 5% group AB inert plasma. In parallel, 3 drops of a 1.5% suspension of the patient’s unwashed RBCs suspended in autologous plasma were tested. One milliliter of the low-ionic strength medium was added to each tube, and the test was incubated at room temperature for 1 minute. Two drops of 0.05% Polybrene (hexadimethrine bromide, Sigma Chemical Co., St. Louis, MO) in unbuffered saline were added, and the contents were mixed. After 15 seconds, the tubes were centrifuged at 1000 x g for 10 seconds. The supernatant was decanted, and 2 drops of sodium citrate resuspending solution were added. The tubes were gently shaken at a 45-degree angle and examined for agglutination. Results were compared with concurrent positive and negative controls to ascertain the persistence of agglutination. One additional drop of resuspending solution was added plus 2 drops of group AB plasma (undiluted). After mixing, the tubes were washed three times and converted to an antiglobulin test with anti-IgG.

The gel test DAT was performed as described by the manufacturer (ID-MTS Anti-IgG Card, Ortho). Control tests were performed in parallel with the ID-MTS Buffered Gel Card.

**Results**

Eight hundred specimens submitted January 1997 through December 2008 (12 years) for suspected DAT-negative AIHA were evaluated. All 800 samples were tested by the AGS panel; 761 (95%) were tested by the 4°C LISS wash technique, and 667 (83%) were tested by the direct Polybrene test. All three techniques were performed on 635 (79%) of the 800 specimens tested; the gel test was performed on 434 (54%) samples. When positive results were obtained by the AGS panel, especially with anti-IgG, the 4°C LISS wash or direct Polybrene test was not always performed. Positive results were obtained with at least one of the methods for 431 (54%) of the 800 specimens tested. Some specimens were reactive by one method but not by another; others were reactive by more than one method. Results for the battery of serologic tests used in the DAT-negative AIHA workup are shown in Table 1. Specimens that yielded an invalid result, i.e., a reactive negative control, were not included in the number of specimens tested for that method. The number of invalid results obtained for each method was as follows: AGS panel = 2, direct Polybrene test = 2, 4°C LISS wash technique = 11, and DAT by gel test = 2. An initially reactive control for the AGS panel was resolved for 10 samples by washing the RBCs with warm (37°C) saline (n = 7) or by treating them with DTT (n = 3).13

Our AGS panel provided a positive DAT result in 50 percent of cases. The distribution of the AGS panel reactivity is shown in Figure 1. Reactivity with anti-IgG or anti-C3 accounted for positive results in 48 percent of cases; most of this reactivity was weak (≤1+). All eight specimens that reacted with anti-IgM also reacted with anti-C3 or with anti-IgG plus anti-C3. IgA was detected on 13 (2%) specimens that were nonreactive with anti-IgG and anti-C3.

<table>
<thead>
<tr>
<th>Results</th>
<th>No. tested*</th>
<th>No. positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any positive test</td>
<td>800</td>
<td>431 (54%)</td>
</tr>
<tr>
<td>Antiglobulin sera panel positive</td>
<td>800</td>
<td>400 (50%)</td>
</tr>
<tr>
<td>4°C LISS wash anti-IgG positive, room temp wash anti-IgG negative</td>
<td>761</td>
<td>37 (4.9%)</td>
</tr>
<tr>
<td>Direct Polybrene test only positive</td>
<td>667</td>
<td>15 (2.2%)</td>
</tr>
<tr>
<td>Gel anti-IgG test only positive</td>
<td>434</td>
<td>0</td>
</tr>
</tbody>
</table>

*Number tested does not include invalid results.
The direct Polybrene test was positive for 67 of 667 (10%) specimens tested, and was the only positive test for 15 specimens. The 4°C LISS wash technique was used for 761 samples. Of these, low-affinity IgG was detected on 37 (4.9%) samples that did not react with anti-IgG using the routine room temperature wash in the DAT AGS panel; 21 of these 37 samples reacted with anti-C3 in the routine test. The anti-IgG reactivity after the 4°C LISS wash varied from weak to 4+. Interestingly, three of these were readily detected (1+ to 2+) by the murine monoclonal anti-IgG (Immucor), but were nonreactive (n = 2) or only very weakly (microscopically, n = 1) reactive with the rabbit anti-IgG (Ortho).

The anti-IgG gel test was positive in 57 of 434 (13%) tests, but was never the only positive test. The reactivity of ten samples that were positive by gel test (weak to 1+) but negative with anti-IgG by room temperature tube method is shown in Table 2. RBC-bound IgG was detected by the gel test in four samples that were nonreactive with anti-IgG by tube test using room temperature or 4°C LISS wash; these RBCs also reacted with anti-C3. The direct Polybrene test was also positive in the three samples that were tested. Eighteen of the 37 (49%) low-affinity IgG autoantibodies were tested by the gel test: five were reactive and 13 were nonreactive. We discontinued using the gel test in April 2005 as part of the DAT-negative AIHA workup.

### Discussion

We have previously reported on a 10-year series of 259 cases of hemolytic anemia in which an immune basis was suspected but the DAT was reported as negative. Similar to the results in the current series, 10 percent of the cases had positive DATs with commercial anti-IgG (e.g., used by hospitals) when tested in our laboratory. A battery of tests (the direct Polybrene test, direct polyethylene glycol test, DAT with cold washes, flow cytometry with fluorescein-labeled anti-IgG, direct ELAT, a concentrated eluate, and the MMA) was used to detect RBC-bound IgG on the remaining samples. Thirty-four percent of the 234 samples were positive by at least one of the additional tests. Although it was found that the direct Polybrene test was as efficient as the more-complex flow cytometry or ELAT methods, some samples that were positive by the latter were not positive by the direct Polybrene test and vice versa. It was concluded that no one test is optimal, and a battery of tests would be the most efficient approach for these DAT-negative AIHA cases. Subsequently, a battery of serologic tests that could be performed as a routine investigation in our IRL was adopted. In the current series of 12 years of data generated from this battery of tests, we were able to provide some serologic evidence of an immune etiology in slightly more than half of the cases submitted.

Our panel of AGS detected immunoglobulin or complement on RBCs of 50 percent of the samples that were DAT negative at the hospital. The 13 percent positive for IgG probably reflects differences in performing the DAT between the referring and the reference laboratory; weak agglutination can be easily shaken out and missed by less experienced technologists. Some of the 45 percent positive anti-C3 results may reflect a difference in technique plus the use of a stronger rabbit anti-C3. Most of the anti-C3 reactions were weak (≤1+), but 47 (13%) were moderately to strongly reactive. RBC-bound IgM can be difficult to detect by the antiglobulin test; fortunately, IgM antibodies associated with AIHA characteristically bind complement. IgA autoantibodies are uncommon, and for them to occur as the only detectable immunoglobulin in a patient with AIHA occurs in only about 2 percent of AIHA.

Low-affinity autoantibodies are uncommon, but they may sometimes cause a false-negative DAT result because of a loss of reactivity during the test process. These antibodies are susceptible to "elution" when the RBCs are washed with room temperature or 37°C saline. Washing RBCs with cold (4°C) saline or LISS has been shown to enhance detection of low-affinity antibodies. In the current series, washing

### Table 2. Reactivity of ten samples of RBCs using anti-IgG by gel test but nonreactive with anti-IgG in the routine tube test

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gel anti-IgG</th>
<th>AGS panel</th>
<th>Anti-IgG by 4°C LISS wash</th>
<th>Direct Polybrene test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1+</td>
<td>C3 only</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>1+</td>
<td>C3 only</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>1+</td>
<td>C3 only</td>
<td>1+</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>1+</td>
<td>C3 only</td>
<td>4+</td>
<td>NT</td>
</tr>
<tr>
<td>5</td>
<td>1+</td>
<td>C3 only</td>
<td>Micro</td>
<td>NT</td>
</tr>
<tr>
<td>6</td>
<td>1+</td>
<td>C3 only</td>
<td>Negative</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>1+</td>
<td>Negative</td>
<td>1+</td>
<td>NT</td>
</tr>
<tr>
<td>8</td>
<td>Weak</td>
<td>C3 only</td>
<td>Negative</td>
<td>NT</td>
</tr>
<tr>
<td>9</td>
<td>1+</td>
<td>C3 only</td>
<td>Invalid</td>
<td>Positive</td>
</tr>
<tr>
<td>10</td>
<td>1+</td>
<td>C3 only</td>
<td>1+</td>
<td>NT</td>
</tr>
</tbody>
</table>

AGS = antiglobulin sera; NT = not tested.
with 4°C LISS enabled detection of IgG in 37 (5%) samples when the routine DAT (i.e., washing with room temperature saline) was negative.

Column agglutination tests, e.g., gel test, have sometimes yielded a positive DAT for IgG on RBCs that are DAT-negative by routine tube test.16–20 This, however, may depend on the method and reagents used by the manufacturer as various techniques and antibody sources used in different countries are not identical. The increasing use of the gel test in the United States may change the failure rate of hospitals for detection of RBC-bound IgG because of poor technique when performing tube methods. Column agglutination tests theoretically would be helpful in detecting low-affinity antibodies as the washing phase has been eliminated.21 There have been a few reports of low-affinity IgG detected using a cold-wash technique that were also detected by column agglutination.17,22 In our study, two thirds of the low-affinity IgG detected using the 4°C LISS wash were not detected by the ID-MTS gel anti-IgG test.

The direct Polybrene test is rapid, but the test is technique-dependent. Polybrene (hexadimethrine bromide) causes nonspecific aggregation and close approximation of normal RBCs permitting cross-linking of antibody molecules. Aggregates are dispersed by the addition of a sodium citrate solution, but antibody-mediated agglutination will persist after neutralization of the Polybrene effect. The test can then be converted to an antiglobulin test (anti-IgG) if antibody is not detected after the aggregates are dispersed. The direct Polybrene test has previously been shown to be almost as sensitive as flow cytometry, and as sensitive as the ELAT, for detecting low levels of RBC-bound IgG.1,6,23 Others have also found this test to be useful for DAT-negative AIHA samples.8,24

We had limited hematologic data and follow-up of the patients in this study. The data reflect the results of tests on samples from patients who were suspected to have AIHA but the hospital laboratory had reported a negative DAT. The hematologists were seeking some support for their suspected diagnosis and therapy. The current study was not designed to confirm earlier publications showing that such tests are useful in the diagnosis of DAT-negative AIHA. The data were reviewed to see how many times in 12 years we found RBC-bound proteins not detected in routine DATs performed at hospitals. The results in this large series show that there is value in using an IRL experienced in reading weak reactions, where special AGS are available, and where tests to detect low-affinity and small amounts of RBC-bound IgG can be performed.

References

R.M. Leger et al.

Manuscripts
The editorial staff of Immunohematology welcomes manuscripts pertaining to blood group serology and education for consideration for publication. We are especially interested in case reports, papers on platelet and white cell serology, scientific articles covering original investigations, and papers on new methods for use in the blood bank. Deadlines for receipt of manuscripts for consideration for the March, June, September, and December issues are the first weeks in November, February, May, and August, respectively. For instructions for scientific articles, case reports, and review articles, see Instructions for Authors in every issue of Immunohematology or on the Web at www.redcross.org/immunohematology. Include fax and phone numbers and e-mail address with all articles and correspondence. E-mail all manuscripts to immuno@usa.redcross.org.
The pathophysiology and prevention of transfusion-related acute lung injury (TRALI): a review

D.C. Mair and T. Eastlund

Transfusion-related acute lung injury (TRALI) is a clinically important complication of transfusion that is often difficult to diagnose, and is probably underreported, and likely has a multifactorial origin that is incompletely understood, making it challenging to find effective treatments and preventative steps. The spectrum of its severity and clinical symptoms seems wide, but its pathogenesis is most likely associated with pulmonary damage from activated recipient neutrophils. Despite the pathogenesis of TRALI being unclear, many severe cases are related to transfusion of donor WBC antibodies, and preventive measures based on avoiding donations by multiparous donors have been implemented at some sites, with early reports showing benefits. This review will address some of the questions surrounding the etiology of this potentially fatal reaction and how measures, predicated on many severe cases being related to transfusion of plasma from multiparous donors, led to preventive steps to avoid these donations. Immunohematology 2010;26:161–73.

Transfusion-related acute lung injury (TRALI) has become one of the more frequently reported causes of transfusion-related morbidity and mortality in several countries including the United States. From 2005 to 2009, TRALI was the leading cause of patient death attributable to blood components, comprising 48 percent of all the fatal transfusion reactions reported to the Food and Drug Administration (FDA). Because TRALI is suspected to be underrecognized, underdiagnosed, and hence underreported, these documented cases likely represent just the tip of what has been called the TRALI iceberg. In other words, the true prevalence of TRALI is unknown, and consequently its mortality rate may be higher than the number of reports would suggest.

Because treatment for TRALI is basically supportive, and up to 10 percent of patients with it die even with aggressive therapy, implementation of effective preventive measures is urgently needed. However, preventing an illness usually requires a good understanding of its distinctive clinical characteristics and its causes, and the ready availability of diagnostic testing. At present the exact cause or causes of TRALI remain unclear. Its pathogenesis appears complicated, and its clinical presentation is similar to that of other illnesses and syndromes. In addition, there are no clear-cut diagnostic tests. Further studies into its pathogenesis are needed to develop more effective therapies than the general supportive measures that are available today.

There are two leading theories on the pathophysiology of TRALI, the antibody-mediated theory and a “two-event, or two-hit model.” The former has served as the basis for some recent preventive interventions that appear to be decreasing the prevalence of TRALI in the United States and internationally.

In this review we will address the definitions of TRALI and the two commonly proposed pathogenetic mechanisms of TRALI along with the central role the neutrophil plays in causing it, and we will specifically look at data from recent animal models and clinical research. Finally, we will touch on how our current understanding of TRALI formed the foundation of the preventive measures that have already been implemented.

TRALI: Background and Definition

A hallmark of TRALI is noncardiogenic pulmonary edema. When a transfusion is accompanied by severe shortness of breath, causes other than TRALI are often the first to be considered, including hemolytic reactions, bacterially contaminated components, circulatory volume overload, bronchospastic “anaphylactoid” allergic pulmonary reactions, and coincidental underlying conditions such as pulmonary emboli, asthma, and anxiety that can occur during a transfusion.

Reports of posttransfusion, noncardiogenic pulmonary edema first surfaced in the 1950s. Since then two seminal papers by Popovsky et al. in the 1980s described pulmonary reactions in patients from the Mayo Clinic and linked them with donor leukocyte antibodies in what we now identify as a clinical entity called TRALI. The 1985 article depicted 36 patients with acute respiratory distress and new infiltrates on chest x-ray lacking an obvious cause such as circulatory overload or pneumonia. All of the reactions occurred within 6 hours of administering the blood, and most of them took place within 2 hours. Two of the patients died. In 89 percent of the cases a WBC antibody was identified in a donor, and in 6 percent the antibody was in the patient.

Since these landmark papers, TRALI has generally been considered to be mediated by the transfusion of components containing a donor antibody with specificity to the recipient’s WBCs. Although happening rarely, cases have been reported that seem to be related to a recipient antibody with specificity to donor WBCs contained in the transfusion. Because antibodies have not been identified in all cases of TRALI, there has been much interest in TRALI cases that do not seem to be antibody mediated.
Studies have shown that other substances in stored blood components may also cause TRALI, and this has been called the two-event, or two-hit, model of TRALI. In this model, endogenous inflammatory mediators produced in certain patients may result in priming and sequestration of neutrophils in the lung. Substances that develop and accumulate during blood component storage can activate the sequestered neutrophils of transfused recipients, causing damage and alveolar permeability. This two-event theory was proposed as a major alternative to the antibody-mediated model of TRALI’s pathogenesis. Both antibody-mediated TRALI and the two-event model will be discussed in more detail in a later section.

Since the 1980s, with improvements in infectious disease screening of donors and reduction of transfusion-transmitted infections, TRALI gradually became recognized as a leading cause of transfusion-related morbidity and mortality. Partly in response to the increasing number of TRALI fatalities reported to agencies like the FDA, British Blood Services, etc., the National Heart, Lung, and Blood Institute (NHLBI) and a Canadian consensus conference each convened experts to analyze the TRALI literature with the ultimate goal of improving patient safety. Because the cause of TRALI remains somewhat unclear, both forums concluded that decreasing the number of TRALI cases posed potentially significant challenges given the current state of knowledge and that additional studies were needed. To advance research on TRALI, a commonly accepted clinical description was viewed as indispensable. NHLBI and the Canadian panel produced similar definitions based on the American-European Consensus Conference (AECC) definition of acute lung injury (ALI), with one difference being the Canadian group created a separate entity called “possible TRALI” for patients who might have other risk factors for lung damage besides transfusion (Table 1). Both groups selected the AECC definition for ALI versus the clinically more severe acute respiratory distress syndrome (ARDS), but added O₂ saturation as another way to measure hypoxemia. This gave physicians the flexibility of using pulse oximetry to aid in the diagnosis of TRALI when an arterial blood gas had not been performed. It is also noteworthy that these TRALI definitions rely on clinical information, and although the presence of WBC antibodies in the donor or patient may support the diagnosis, neither definition required that testing be performed.

Less severe forms of posttransfusion dyspnea are often not investigated for a diagnosis of TRALI, and the NHLBI and Canadian Conference definitions did not address this less established entity called “mild TRALI.” Palfi et al. demonstrated that plasma from multiparous donors, which was more likely to contain antibody (although the investigators did not test for this), induced a small, yet clinically significant, drop in the partial pressure of arterial oxygen to fraction of inspired oxygen (PaO₂/FIO₂) ratio of 100 patients when compared with control plasma. The authors suggested this slight hypoxia may represent a milder form of TRALI. In an abstract published 3 years later, Palfi and associates noted the majority of TRALI reactions reported in south Sweden were considered to be mild. They also acknowledged the need for an accurate consensus definition. As research and our understanding of TRALI’s pathophysiology and incidence improve, hopefully milder forms of transfusion-associated respiratory distress will also be addressed.

Retrospective “lookback” studies of recipients who received transfusions containing donor WBC antibodies have provided evidence that donor antibodies frequently do not cause symptoms in transfused recipients and also suggested there may be more subtle versions of the reaction that qualify as mild TRALI. In these reports, an

<table>
<thead>
<tr>
<th>American-European Consensus Conference definition of ARDS and ALI</th>
<th>National Heart, Lung, and Blood Institute definition of TRALI</th>
<th>Canadian Consensus Conference definition of TRALI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute onset</td>
<td>New-onset ALI (i.e., no ALI exists before transfusion) by AECC definition of ALI*</td>
<td>New-onset ALI (i.e., no ALI exists before transfusion) by AECC definition of ALI*</td>
</tr>
<tr>
<td>Hypoxemia</td>
<td>Clear temporal relationship to transfusion</td>
<td>Clear temporal relationship to transfusion</td>
</tr>
<tr>
<td>PaO₂/FIO₂ ≤ 200 mm Hg = ARDS</td>
<td>During or within 6 hours of completing the transfusion</td>
<td>During or within 6 hours of completing the transfusion</td>
</tr>
<tr>
<td>PaO₂/FIO₂ ≤ 300 mm Hg = ALI</td>
<td>Patients may have other risk factors for ALI†</td>
<td>No alternative risk factor for ALI</td>
</tr>
<tr>
<td>Bilateral infiltrates on chest radiograph</td>
<td>If risk factors other than transfusion are also present, patient’s clinical course may clarify whether ALI was caused by blood components or the alternative cause or both</td>
<td>Possible TRALI</td>
</tr>
<tr>
<td>PAOP ≤ 18 mm Hg when measured or no clinical evidence of left atrial hypertension</td>
<td></td>
<td>New onset ALI with a temporal relationship to a transfusion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung injury also has a clear temporal relationship to an alternative risk factor for ALI†</td>
</tr>
</tbody>
</table>

ALI = acute lung injury; ARDS = acute respiratory distress syndrome; FIO₂ = fraction of inspired oxygen; PaO₂ = partial pressure of arterial oxygen; PAOP = pulmonary artery occlusion pressure.

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* NHLBI and the Canadian group expanded the criteria for hypoxemia to include O₂ saturation of ≤ 90% on room air = ALI.

† Causes of ALI other than transfusion may include aspiration, severe sepsis, pneumonia, shock, toxic inhalation, multiple trauma, lung contusion, burn injury, near drowning, acute pancreatitis, cardiopulmonary bypass, and drug overdose.
antibody-positive donor was identified in the investigation of a severe or fatal TRALI. Evaluating prior recipients of components from these antibody-positive donors revealed previously unreported cases of milder respiratory distress. A minority of the recipients also had severe reactions that were suspicious for TRALI and should have been reported to the hospital's transfusion service, but were not. This latter finding supports the contention that TRALI is often unrecognized and probably underreported.

**The Pathophysiology of TRALI**

**The Role of the Neutrophil in TRALI**

The clinical presentation of ARDS/ALI has much in common with the features observed in TRALI patients. Beyond the signs and symptoms there is also similarity in the histopathology insofar as neutrophils are concerned. For example, microscopic examination of postmortem lung samples from TRALI victims has shown sequestration of neutrophils in the pulmonary vasculature and in the alveolar space, analogous to what has been observed in ARDS and ALI. Reviewing how the neutrophil functions in pulmonary inflammatory processes, such as infection or lung injury, would be beneficial in understanding its role in either the antibody-mediated or the two-event model.

The neutrophil is the first line of defense against bacterial infection, and it can generate a wide array of molecules that are bacteriocidal and initiate inflammation that can damage healthy cells and tissue in the same area. To more efficiently eradicate infectious agents, neutrophils ideally go through a stage of priming first. Primed neutrophils have enhanced microbial killing capabilities in response to a subsequent stimulus versus their unprimed counterparts. Priming agents such as platelet-activating factor (PAF) or tumor necrosis factor α (TNFα) potentiate both the oxidative and granular microbicidal arsenal when the neutrophil is later exposed to an activating substance such as N-formyl-methionine-leucine-phenyalanine; GM-CSF = granulocyte/macrophage colony stimulating factor; LPS = lipopolysaccharide; O₂⁻ = superoxide anion; PAF = platelet-activating factor; TNFα = tumor necrosis factor α. Reproduced with permission from Condliffe et al.

Priming starts when neutrophils home in on infected areas such as N-formyl-Met-Leu-Phe or fMLP (Figure 1). Priming starts when neutrophils home in on infected areas in response to chemoattractants that could be bacterial derived or released when local endothelial cells become activated. Increased expression of neutrophil adhesion molecules occurs on the surface of both the neutrophil and the endothelium. The neutrophils become less deformable, further slowing their transit through the narrow pulmonary vascular beds, allowing more time and increased contact between the leukocytes and the now more receptive endothelium, leading to further accumulation of neutrophils (sequestration) at the site of infection. At this point the neutrophils can be said to be primed. In an infection, the neutrophils then marginate into the tissue's interstitium, where they can phagocytize the pathogens, resulting in neutrophil activation and destruction of the microbe through the release of reactive oxidative species and proteases. In this way, a primed neutrophil can clear an infection more effectively than it could in its pre-primed state. In most cases, this response to the infection is short and limited to a level that is appropriate to remove the infecting agents without significant neutrophil-related damage to the surrounding tissues.

This same sequence of neutrophil priming, sequestration, and activation is believed to occur in the lung in TRALI, except the activated neutrophils release their toxic substances onto the pulmonary capillary, disrupting the barrier between the endothelium and lung epithelium, and ending with edema in the alveolar space. In healthy people the lung normally stores a relatively large amount of the body's available neutrophils (28%), and because this quantity increases even further with sequestration, the respiratory system may be more inclined to experience neutrophil-induced injury than other organs. Neutrophil priming and activating agents that cause TRALI may spare nonpulmonary tissue because of the relatively low neutrophil burden outside of the lung. These substances that trigger sequestration and cell damage for each of the two TRALI theories are discussed in a later section.

**The Role of Antibodies in TRALI**

A causal relationship between the transfusion of WBC antibodies and ALI has been suspected for more than 50 years. In 1957, Brittingham described a healthy research subject who exhibited symptoms of ALI after receiving a leukoagglutinin-containing blood component. Popovský and colleagues in 1983 and 1985 crafted the term TRALI and emphasized the association between TRALI and the passive transfusion of donor HLA or granulocyte-specific antibodies into recipients with corresponding antigens on
their leukocytes. In a minority of their cases, Popovsky’s team found the antibodies in the patient and the reactions were seemingly initiated by infusion of incompatible donor WBCs.

Whether the antibody originated in the donor or the patient, the interaction between the antibody and its neutrophil ligand can result in endothelial cell and alveolar damage with capillary leak into the alveoli. Animal models have provided more detailed insights into the antibody theory and have demonstrated some immunoglobulins are capable of both directly priming and activating neutrophils in TRALI (Figure 2A).

Silliman and colleagues demonstrated in vitro that donor antibodies to the human neutrophil antigen (HNA) -3a can prime and hence enhance an fMLP-activated respiratory burst in antigen-positive leukocytes. Similarly, in another experiment using a rat model, the release of reactive oxygen species from the animal’s isolated neutrophils was increased after they were incubated with antibodies that recognize regions of the rat MHC locus that are analogous to class I HLA A, B, and C loci in humans. This latter rodent experiment also required fMLP to induce granulocyte activation.

Several studies have suggested that antibodies can cause neutrophil activation and lung injury. In an ex vivo model, rabbit lungs developed evidence of edema (an increase in pulmonary vascular permeability and lung weight) after being injected with a combination of complement, human antibody to HNA-3a, and human neutrophils positive for

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**Fig. 2.** Three scenarios of how transfused antibodies might prime and activate the recipient’s neutrophils (A–C) to cause transfusion-related acute lung injury (TRALI). (A) Antibody directly binds to antigen on the neutrophil. (B) HLA class II antibody attaches to the patient’s monocytes, stimulating their release of inflammatory mediators, which ultimately affects neutrophils. (C) Antibody to HLA class I antigen binds to pulmonary endothelium in the recipient. The neutrophil’s Fc receptor adheres to the constant region of the antibody, leading to sequestration in the lungs and activation.
that antigen.\textsuperscript{25} No edema was seen in animals when HNA-3a–negative neutrophils were substituted or if complement was not added. In animals it seemed that anti-HNA-3a, in the presence of complement, activated antigen-positive human neutrophils and caused lung injury.

A study by Sachs et al.,\textsuperscript{26} using another ex vivo lung rodent model, showed the antibody-antigen reaction could cause lung injury independent of complement and that TRALI may be more complex than simply antibody-to-WBC antigen recognition alone. In these experiments, rats were infused with human HNA-2a–positive neutrophils and CD177 monoclonal antibody (an antibody that recognizes the HNA-2a antigen) without the addition of complement. A key variable here was that the quantity of HNA-2a antigen expression can vary dramatically among different antigen-positive persons. Using quantitative indirect immunofluorescence and flow cytometry, two different sources of neutrophils were identified for this study: one from individuals with a high percentage of HNA-2a–positive cells (defined as greater than 70% expression) and the second from low expressers (less than 30%). TRALI did not occur in these rats when the neutrophils came from individuals with a lower percentage of HNA-2a, unless it was followed by injection of fMLP as an activator. Conversely, HNA-2a antibody alone appeared to activate leukocytes and cause lung injury when cells from high expressers were used.\textsuperscript{26,27} On the basis of this study, neutrophil-activated TRALI reactions attributable to anti-HNA-2a appeared to be antigen dose–dependent (i.e., the amount of antigen present was also important to neutrophil activation), although this could be overcome by the later addition of another inflammatory mediator (e.g., fMLP).

**Infusion of Donor-Derived Antibody Without Causing TRALI**

The observation made by Sachs and colleagues,\textsuperscript{25} that infused antibody with specificity for recipient antigens may sometimes be insufficient to induce TRALI, has also been demonstrated in a number of lookback studies on transfused patients. In these publications donor antibody, directed against antigens that most patients would be expected to carry on their cells, was not harmful in the majority of recipients.

Toy et al.\textsuperscript{16} identified only one patient with TRALI from among 103 recipients of blood from a donor with multiple HLA antibodies. Fifty-four of these patients had a corresponding WBC antigen and no TRALI was observed. Forty-two of these 103 patients were neutropenic, an attribute that might have protected them against lung injury mediated by activated neutrophils. In another study, Win and associates\textsuperscript{28} found no cases of TRALI in a lookback analysis on 43 transfusions from six donors with leukocyte antibodies, again directed against either multiple HLA or neutrophil antigens having a relatively high frequency in the general population. Nicolle et al.\textsuperscript{29} reported two cases of TRALI as a result of donor HLA antibodies and evaluated 18 other recipients of blood from the same donors. Of five plasma recipients only one was found to have undiagnosed TRALI, whereas another had an HLA type matching the donor antibody specificity but had no reaction. Of 13 RBC recipients, none developed a reaction. Kopko et al.\textsuperscript{17} initiated a lookback investigation after a fatal TRALI reaction was attributed to a granulocyte antibody directed against HNA-3a. This antigen is common throughout the population as it has been found in 89 percent to 99 percent of tested individuals.\textsuperscript{30} The lookback study evaluated 36 patients who previously received blood from that donor and identified 15 transfusion reactions in only 13 (36.1%) of the recipients, and just 2 of these individuals were initially diagnosed with TRALI by the treating physicians.\textsuperscript{17} Although an additional 7 of these 13 reacting patients had severe respiratory problems that possibly should have been called TRALI, most of the 36 recipients had been transfused without incident. Muniz et al.\textsuperscript{31} also identified a donor with an anti-HNA-3a whose blood had been given previously to 11 patients. One experienced TRALI, another had chills, but the remaining nine had no reactions. Fadeyi et al.\textsuperscript{32} described a donor-derived anti-HNA-2a (the corresponding antigen is present in 95–99% of the population) that induced dyspnea or leukopenia in 18 of 39 transfusion events, but no episodes of TRALI were found. As was discussed earlier, variation in HNA-2a expression among antigen-positive individuals might explain why many of the recipients were asymptomatic or did not experience more severe pulmonary compromise.

Each of these lookback studies suggests that even in the setting of donor antibody with specificity for recipient antigen, TRALI usually does not occur. Even taking into account potential TRALI cases that were initially missed, most recipients were asymptomatic. The reasons for this wide range of clinical responses are unclear, but may be related to individual variations in antibody titers or avidity, concentration of corresponding antigens on recipient cells, underlying diseases in recipients, variability in neutrophil reactivity to activated complement and cytokines, concentration of neutrophil adhesion molecules, and reactivity of the recipient’s pulmonary vessels and alveoli.

**Antibodies to Class II HLA Antigens**

In addition to antibodies directed at class I HLA and HNA specificities, later work showed that donor antibodies to HLA class II antigens can also be associated with TRALI,\textsuperscript{33} and these antigens are not normally expressed on neutrophils.\textsuperscript{34} Granulocytes have exhibited HLA-DR (a class II antigen) on their surface after exposure to agents like granulocyte/macrophage colony-stimulating factor, interferon (IFN)-γ, and interleukin (IL)-3,\textsuperscript{35} but this antigen expression has not been documented in TRALI cases.\textsuperscript{22} Immunohistochemical stains performed on lung tissue from a TRALI fatality found HLA class II expression...
was limited only to pulmonary macrophages, despite granulocyte accumulation in the capillaries and evidence that the endothelium was activated.35 Activated pulmonary endothelium may also demonstrate HLA class II antigens, but pulmonary endothelium from the deceased TRALI patient did not express class II antigens despite being positive for CD34, an indicator of endothelial activation.35 Accordingly, some authors have postulated these antibodies act indirectly on neutrophils by first binding to monocytes, which in turn release cytokines that sequester and activate neutrophils to induce lung damage (Figure 2B).72 In an in vitro study in which Kopko et al.33 combined anti-HLA class II antibodies with human monocytes of a corresponding HLA type, the cells became activated and produced the inflammatory cytokines IL-1 β and TNFα, as well as tissue factor, and each of these inflammatory cytokines is capable of attracting and stimulating neutrophils.36 When Nishimura and colleagues37 incubated antibodies to HLA-DR15 with antigen-positive monocytes and cultured human lung microvascular endothelium, high levels of the cytokine leukotriene B4 (a potent neutrophil attractant) were produced. Endothelial cells were also needed to interact with the WBCs and antibody to generate leukotriene B4 in this model. From this it may be inferred that endothelium is also a necessary part of the inflammatory process in TRALI.

**Antibody Interaction With Lung Tissue and TRALI**

Presumably, the antigen-binding site of the offending antibody is targeting a matching substance on either the neutrophil or the monocyte in TRALI.4 However, recent work by Looney et al.38 using a murine model of lung injury, argued that the antigen binding sites may also adhere to HLA antigens on the endothelium instead and that the Fc receptor of the circulating neutrophil interacts with the antibody's constant region to alter the neutrophil's circulation, leading to its sequestration and activation (Figure 2C). The investigators were able to induce ALI (demonstrated by excess lung water and increased vascular permeability) in vivo by injecting the mice with a monoclonal antibody targeting an antigen in the murine class I MHC complex. Although flow cytometry showed that the monoclonal antibody recognized class I MHC antigen on neutrophils, this antibody to WBC antigen binding was not thought to have caused the lung injury because the antibody did not directly activate the cells. Microscopic examination of lung tissue from the affected mice confirmed there was marked pulmonary neutrophil sequestration, and immunohistochemistry revealed the monoclonal antibody preferentially bound to pulmonary endothelium over tissue in other organs. Despite other WBCs having Fc receptors, the mice did not exhibit lung injury if they were made neutropenic before being challenged with the class I MHC antibody. TRALI also did not occur when antigen-positive mice that lacked Fc receptors on their cells were given the antibody, but lung injury did transpire if they first were infused with normal Fc receptor–positive neutrophils and then received the monoclonal antibody. These authors hypothesized that the infused class I MHC monoclonal antibody preferentially bound to antigen present on the pulmonary endothelium, and the antibody then sequestered circulating neutrophils via the Fc receptor interaction, resulting in neutrophil activation and lung injury. The presence of Fc receptors on neutrophils appeared to be a requisite feature for TRALI in this experiment.

Clinical evidence that TRALI can develop from transfusion of antibody with specificity to antigens on lung tissue came from a patient who experienced unilateral TRALI.39 Ten weeks after a single-lung transplant, the patient became dyspneic and exhibited pulmonary infiltrates in the single transplanted lung but not in the native lung after receiving a unit of RBCs containing antibodies with HLA-B44 specificity. The recipient was HLA-B44 negative, but the transplanted lung was B44 antigen positive. Although the lung donor’s B44-positive leucocytes may have been circulating in small numbers, the transfused antibody was presumed to have bound to the B44-positive transplanted lung itself.

**The Two-Event Model and the Role of Biologically Reactive Mediators in TRALI**

Although much of the transfusion medicine literature has focused on the antibody-mediated cause of TRALI, the two-event model of ARDS has attempted to answer some of the questions that remain about TRALI's origin and especially how TRALI can develop in the absence of an antibody.40 The first event is a preexisting clinical condition in the patient (e.g., sepsis, recent surgery) whereby the patient's illness produces endogenous generalized inflammation that affects the pulmonary endothelium. The endothelium can then become activated, expressing adherence molecules on its surface, resulting in binding of the neutrophils to the pulmonary vasculature, sequestration, and the neutrophils becoming primed.20,21,22 Neutrophil priming agents may be released from patient-derived sources in the postoperative period or by invading infectious agents. Potential neutrophil priming substances, which may be produced by dying cells, WBCs, or activated pulmonary endothelium, include PAF, TNFα, and IL-8.41–43 Lipopolysaccharides (LPS) from bacterial cell walls may also serve in this capacity.44

The proposed second event is transfusion of a cellular blood component containing various exogenous cytokines or lipid-based biologically reactive mediators (also known as biologic response modifiers or BRMs) that stimulate and activate the adherent neutrophils to release proteases, which, as in the antibody-mediated model, causes endothelial cell and alveolar damage with noncardiogenic pulmonary edema.40,45

These lipid-based substances seem to accumulate only in cellular components during storage and are not
found in frozen plasma components, CD40 ligand, a platelet-derived inflammatory mediator, as well as lysophosphatidylcholines (Lyso-PCs), IL-6, IL-8, and others, have all been proposed to function as BRMs in this model. Older platelet components and packed RBCs contain greater amounts of these BRMs and thus appear to pose a greater risk of causing TRALI.

Wyman et al. exposed monolayers of human pulmonary endothelial to one potential priming agent: bacterial endotoxin (i.e., LPS). In response, the cells generated chemokines and expressed adhesion molecules. When neutrophils were later added, they became primed and adherent to the monolayer. Analogously to what is thought to happen in TRALI, the adherent neutrophils were then activated by biologically active lipids from stored packed RBCs (i.e., Lyso-PC) and damaged the endothelial cells. Blocking antibodies against the adhesion molecules on the neutrophils and the endothelium prevented injury to the monolayer. Also notable was that LPS and Lyso-PC used individually only primed the neutrophils, but adding them in sequence caused neutrophil activation and harm to the monolayer. This showed that using two priming agents together could result in activation.

BRMs have also been demonstrated in the posttransfusion serum of patients who exhibited TRALI reactions. When their post-TRALI serum was incubated with neutrophils, a significantly greater superoxide release was observed on activation than when neutrophils were incubated with the same patient’s pretransfusion serum or when the sera of recipients with febrile and urticarial reactions were used. This suggested the presence of a neutrophil priming agent in these TRALI patients that arose from the infusion of the blood component.

Although BRMs are not found in frozen plasma components, donor-derived antibodies can be found in these components. This explains the strong correlation between frozen plasma transfusion and the development of TRALI. Because antibodies can serve as the second event, they can play a causal role in either the antibody hypothesis or the two-event model, and hence these two TRALI theories are not mutually exclusive. A recent study using a rat model demonstrated that either antibodies or BRMs can cause TRALI. As the first event, rats were injected intraperitoneally with LPS versus control animals that were injected with an equal volume of saline solution. For the second event the rodents were injected with either an antibody directed against class I MHC antigens or the extract from a 42-day-old RBC. Both antibody and the RBC extract were able to produce pulmonary edema in the LPS-injected group but not in the control animals. Microscopic examination in the rats with pulmonary injury documented neutrophil infiltration in the lungs, again demonstrating the importance of neutrophils in ALI. Furthermore, inducing neutropenia in the LPS-primed rodents was protective against lung damage.

The investigators of a previously discussed murine model, which argued the importance of the Fc-receptor on neutrophils, surprisingly found in later work that the experiment was less likely to produce lung injury after the mice had been moved to barrier-protected, pathogen-free housing. The rodents from the prior study had been kept in a “dirtier” environment and had a higher neutrophil count than their counterparts in the more sterile setting, which the authors attributed to a more primed state of the immune system in the previous group. Exposing the animals from the pathogen-free environment to LPS (analogous to a first event) restored the model’s ability to cause TRALI. Looney et al. interpreted this as evidence their experiments also represented a two-event model of TRALI. Interestingly, they found not only that neutropenia was protective but that thrombocytopenia was as well. Mice pretreated with aspirin also did not experience lung injury, which suggests that anti-platelet therapy may have a role in the future treatment or prevention of TRALI.

One limitation of the two-event model is not all investigators accept the premise of TRALI being limited to the ill. For example, Brittingham’s healthy research subject, appearing to lack a first event, exhibited clinical findings consistent with TRALI (dyspnea, fever, cyanosis, hypotension, leukopenia, and marked bilateral infiltrates on chest x-ray) after receiving just 50 mL of blood from a patient with a strong leukoagglutinin. Recently, a theory known as the threshold model attempted to explain this discrepancy between the Brittingham case and the two-event theory, while proposing answers to other TRALI puzzles including the origin of mild TRALI and why antibody and corresponding WBC antigen does not produce TRALI in all cases.

The Threshold Model of TRALI

Many investigators now accept that TRALI appears to be multifactorial with neutrophil activation and lung injury representing a final common pathway that can be initiated by either an antibody or biologically active lipids. Along these lines, the threshold model put forth by Bux and Sachs attempts to merge the two TRALI theories. Consistent with its name, this model assumes neutrophils must exceed a certain hypothetical threshold of activation to cause lung injury. The first and second events (and again the latter can be passive donor antibody) have additive inflammatory effects toward surmounting this theoretical activation level needed to cause either a severe or a mild TRALI reaction (Figure 3). Presumably, a mild TRALI case has a lower threshold than a serious one and no reaction is observed if the degree of activation falls beneath the mild level.

This threshold model incorporates both of the leading theories and takes into account the observation that a broad variety of substances may be able to prime or activate neutrophils. The patient’s threshold could be reached by the transfusion of an extremely potent antibody from a
blood component (Figure 3: healthy research participant). Alternatively, the patient approaches the threshold by releasing his or her own endogenous inflammatory substances as a result of a preexisting disease state, and then transfusion of a less potent antibody or a moderate level of BRMs is sufficient to push the patient over the limit (Figure 3: patients 1 and 2).

This model also addresses some of the gaps in our understanding of this transfusion reaction. In the rare circumstance of a healthy person exhibiting TRALI, the recipient would be assumed to contribute no endogenously generated priming or activating agents to the process. Therefore, the blood component alone must have enough potency to push the recipient over the TRALI threshold without assistance from a preexisting illness. This may have caused the transfusion-associated respiratory distress in the research subject reported by Brittingham, and this uncommon event has been referred to by some as a "one-event" TRALI. From results of lookback studies, one could hypothesize that most patients did not experience TRALI as a result of some combination of their not being ill enough and the blood donor's antibody not being potent enough. The dose dependency of the HNA-2a antigen in causing TRALI, found in the rat model used by Sachs et al., may be another such example. When low expressers of HNA-2a antigen were used, antibody was insufficient to overcome the threshold and fMLP had to be added to push the neutrophils beyond the activation level needed to cause lung injury. Additional research would be needed to explore whether such variables truly play a role in the low reaction rates observed in these lookback cases.

**TRALI Prevention**

Because treatment of TRALI is currently supportive, actions taken to prevent the reaction become the paramount means of decreasing the morbidity and mortality associated with it. A very important first step for prevention of TRALI is to avoid unneeded transfusions. Monitoring of use, enforcement of guidelines, and education about the judicious use of blood components should not be ignored. This is especially important for transfusions of plasma and platelets that contain a larger portion of donor plasma than other components.

Some of the more specific recommended approaches for preventing TRALI have included changes to donor screening practices such as gender-based exclusions or implementing new tests. Others have suggested different manipulations to the final component (e.g., washing), but regardless, all of the proposed interventions are based on the two leading theories of TRALI.

**Preventive Measures for Frozen Plasma**

Based on the antibody-mediated mechanism, components with the most plasma and highest antibody content would be more likely to cause TRALI and thus would make a logical target for preventive action. Previously pregnant female donors are more likely to have WBC antibodies and hence pose a greater risk than male donors of being associated with TRALI reactions. Several studies have supported preventing TRALI by restricting the use of plasma from parous donors. From 1996–2003 the United Kingdom’s hemovigilance program (Serious Hazards of Transfusion, or SHOT) analyzed 93 reported TRALI cases and found frozen plasma and platelets were respectively about 7 and 8 times more likely to be the implicated component than a relatively low plasma-containing component such as a packed RBC. Densmore et al. found that 14.6 percent of female apheresis donors who reported one or two pregnancies had HLA antibodies, and this rate increased to 26.3 percent among multiparous donors, defined as women with three or more pregnancies. These authors’ work was also recently confirmed in the much larger leukocyte antibody prevalence study (LAPS), which enrolled approximately 8000 donors, including about 5800 females. LAPS found the prevalence of HLA antibodies in women with zero, one, two, three, and four or more pregnancies was 1.7 percent, 11.2 percent, 22.5 percent, 27.5 percent, and 32.2 percent, respectively.

In October 2003, the British implemented a predominantly male-donor plasma program. SHOT data published after taking this initiative showed that highly likely or probable cases of TRALI, attributed to plasma, decreased from their preprogram level of 29 to a total of 2. It should be noted, the editorial accompanying this publication commented that the SHOT description of a highly likely or probable case was not purely clinical and relied in part on the presence of a WBC antibody. This differed from the NHLBI and Canadian TRALI definitions, which did not require positive serology in the donor or patient. Accordingly, TRALI cases caused by the other mechanisms (e.g., the two-event model) and lacking antibody may not have been appropriately included or categorized. But given the reported success of the British male plasma initiative, and because TRALI was the leading cause of transfusion-related mortality in the United States, Americans considered adopting this strategy. Additionally, data from the American Red Cross was found to be consistent with the SHOT study’s findings. The American Red Cross, which provides more than 40 percent of the nation’s blood, analyzed 550 suspected TRALI cases, 72 of which were fatal, in the system for 2003 to 2005. In 71 percent of these fatalities, a female, WBC antibody–positive donor was believed to be the cause, and in 75 percent, plasma was the transfused component. AABB followed the United Kingdom’s lead in 2006 and instituted recommendations for TRALI reduction: blood collection facilities needed to implement processes that lessen the chance of high-plasma volume–containing components being collected from donors at higher risk of having WBC antibodies. These processes needed to be in place by the fall of 2007 for frozen plasma components (except cryoprecipitate, which was considered
Review of TRALI

As in Britain, preliminary data in the United States collected by the American Red Cross since establishment of a male-predominant plasma initiative revealed the project has already had a positive impact. Plasma distribution from male donors was 55 percent in 2006, 79 percent in 2007, and 95 percent in 2008, and the number of probable TRALI fatalities attributed to plasma in those years were 6, 5, and then 0, respectively, with the last year being a statistically significant drop from the preceding 2 years. The quantity of nonfatal TRALI cases attributable to plasma also decreased dramatically from 26 in 2006 to 7 in 2008.

An international forum published in 2007 revealed that most of its participants had implemented the production of plasma from males only or used a solvent detergent-treated (SD) component. The latter is suspected to diminish the risk of TRALI because it is manufactured from pools of more than a thousand donations, so theoretically any single donor with a potentially harmful WBC antibody should have the immunoglobulin diluted below the level of clinical significance for a recipient. SD-plasma is not available in the United States but has been used in Europe and no cases of TRALI have been reported after use of over 13 million units.

Preventive Measures for Platelets

Although some were considering it at the time, none of the countries with nationalized blood systems had yet implemented HLA or HNA antibody screening of their donors by the time of their participation in the 2007 international forum. Testing for granulocyte antibodies is generally considered to be labor intensive and does not readily lend itself to the high throughput needed for processing blood. On the other hand, solid-phase assays with shorter turnaround times and relevance to donor screening may become a reality in the near future now.

**Fig. 3.** The threshold model. Shown are three blood component recipients and the hypothetical levels of neutrophil activation needed to exceed their thresholds for a transfusion-related acute lung injury (TRALI) reaction. The gray bar represents the relative contribution of their own inflammatory mediators before transfusion, and the black bar is the contribution of mediators in the blood component, such as biologically reactive mediators (BRMs) or antibodies. In both patients 1 and 2, the blood component alone had insufficient inflammatory potential to exceed the threshold, and therefore the occurrence of either a severe (patient 1) or mild (patient 2) TRALI reaction relied on contributions from the recipients’ preexisting inflammatory mediators. The severity of the reaction is determined by the magnitude of neutrophil response. Unlike the first two, the third recipient was healthy before transfusion and thus had no endogenous mediators (i.e., lacked a first event). In this setting, all of the TRALI-producing mediators were provided by a potent blood component alone (e.g., as a strong leukoagglutinin), and hence this bar is completely black. The double arrow between the resting and primed state of the neutrophils indicates that the cells may deprime if activation does not occur.
that several neutrophil antigens have been characterized molecularly, including the clinically important HNA-3a.48,49 Platelet additive solutions, which have been licensed in some European countries, were also mentioned in the international forum, but because 100 mL of plasma may remain in the final component, the boon to TRALI reduction is still in question.5 Several countries admitted to a variety of different initiatives to decrease the risk of TRALI from this component, such as resuspending the pooled buffy coat platelets in the plasma from one of the male donors, whereas other countries restricted women with a history of pregnancy from donating whole blood–derived platelets (WBPs).5

Recently, a number of American blood centers opted to meet the AABB’s 2008 deadline for apheresis platelets by implementing HLA antibody screening, as this was technically less cumbersome than testing for granulocyte antibodies. Some use algorithms based on parity to more strategically select which donors to perform HLA antibody testing on, and this practice is supported by the LAPS paper that showed that asking about transfusion history brought no additional benefit as there was no statistical difference in the rate of antibody positivity among untransfused males, transfused males, and nontransfused females without a history of pregnancy.50 As a result, blood centers might decrease testing costs by choosing not to screen these three groups for HLA antibodies.50 The LAPS authors estimated that deferred previously pregnant, HLA antibody–positive donors from apheresis collections would result in a potentially tolerable 6 percent decrease in platelet apheresis donations.50 The AABB’s 2006 recommendations did not suggest interventions for WBPs because any antibody present was believed to be diluted by pooling.51

**Preventing TRALI as a Result of BRMs**

To date, most of the actions to avoid TRALI reactions have concentrated on preventing antibody-mediated reactions. Interventions for decreasing BRMs infused into recipients have also been described, although not as popularly implemented because of potential downsides. Washing is one way to remove BRMs; however, because of the preparation time involved, washed RBCs and platelets would likely be used only for planned transfusions and would be impractical for emergency or massive-transfusion settings.60 Furthermore, little data exist on the effectiveness of lipid removal by washing.60 Theoretically, in a nonemergent setting, severely ill patients believed to be at the highest risk for TRALI (e.g., in the intensive care unit), could be scheduled to receive washed components, but this potential benefit would have to be weighed against the reduction in component quality and shelf life that is caused by washing.60

Since cellular blood components develop higher levels of biologically active lipids and cytokines during storage,47–49 some investigators believe that patients with a preexisting illness might benefit from receiving fresher blood (e.g., RBCs that are less than 2 weeks old and platelets less than 3 days old).70,71 This might minimize accumulation of and exposure to BRMs that act as the second event in TRALI development. In the 2007 international forum, only Poland indicated they advise against giving blood older than 14 days to patients in severe clinical condition.2 However, concerns about blood availability and how to appropriately define fresh have likely limited this practice. None of the other participants, which included representatives from South America, Japan, the United States, and several other European countries, admitted to having interventions aimed at preventing TRALI as a result of BRMs. Continued research is needed to more fully understand the generation and accumulation of BRMs during storage of cellular blood components and to develop effective methods for their prevention or removal to reduce the risk of TRALI and other transfusion complications.

**Conclusions**

Many questions remain about the mechanism or likely mechanisms behind TRALI. Until recently, clinical research on this reaction was believed to be limited by the lack of a commonly accepted definition. Despite these challenges, current data suggest that TRALI has a pathogenesis that is multifactorial. It appears that transfusion of either BRMs or antibodies can end in a final common pathway of lung injury as a result of neutrophil activation. Why some patients may be more susceptible to TRALI than others remains a mystery, but potential explanations include severity of their preexisting illness or possibly the amount of antigen expressed and therefore available to certain infused antibodies. The apparent, early success of preventive measures aimed at avoiding donations by multiparous donors suggests our understanding of TRALI may be improving. Now that similar clinical definitions of TRALI are available, and new insights have been provided by recent animal models, we can hope that forthcoming clinical and laboratory research will provide even better preventive steps in the future, along with treatments that are curative instead of just supportive.

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Comparison of gel test and conventional tube test for antibody detection and titration in D-negative pregnant women: study from a tertiary-care hospital in North India


Conventional tube testing was used for antibody screening and titration in D– pregnant women in our hospital until the recent introduction of the gel test. In this study we assessed the sensitivity of the gel test in our setup and tried to establish a correlation between these tests for determining antibody titer. We collected 652 blood samples from 223 antenatal D– women during a span of 1 year. The samples were tested separately by the conventional tube technique and the gel test for antibody detection and titration. The tube test detected 84 (12.8%) positive samples as compared with 93 (14.2%) by gel test, indicating the latter to be more sensitive (p < 0.01). The gel test picked up weakly reactive anti-D that the tube test missed. We did not use any enhancing media such as LISS in titration studies performed by either method in an effort to establish a correlation. However, much higher titers (one- to fivefold) were obtained by the gel test with no clear correlation with the corresponding tube values. When comparing the titer values to the finding of hydrops on ultrasound and Liley's chart OD reading on amniocentesis, a value of less than 128 (i.e., 64) by gel test corresponded to normal results. Through this study, we thus conclude that the gel test is more sensitive for antibody detection, although a linear correlation could not be established for titers. Clinical correlation may point toward a critical titer of 64 for the gel test, but further studies need to be done to support this finding. Immunohematology 2010;26:174–77.

Key Words: alloantibodies, antibody screening, titration, gel test, D-negative women

Hemolytic disease of the fetus and newborn (HDFN) was first reported in 1609,1 although the discovery of the Rh system was made in 1939 and the implication of D antigen in its pathogenesis in 1940.2 However, to the present day, researchers and clinicians alike have worked to unravel its pathogenesis, effects, prevention, and treatment.

Anti-D is by far the most common cause of HDFN although its incidence has drastically fallen with antenatal D immunoglobulin use as prophylaxis. Other antibodies are also implicated, and their prevalence has been studied in the Western world but such data are lacking in the Indian population. The antibodies can be identified as well as semiquantitated from the mother’s serum. Titer determination of anti-D by tube test helps in the clinical decision to proceed with an invasive procedure such as amniocentesis.

The conventional tube test has stood the test of time in both antibody detection and titration. The gel test introduced by Lapierre et al.3 in 1990 has gained popularity as a result of its standardized performance, technical ease, stable end point, and versatility of methodology. There have been many studies to show its superiority versus the tube test for antibody detection. The blood transfusion services in our country are gradually introducing gel technology for grouping, compatibility testing, and alloantibody detection owing to the many advantages. However, in some situations such as HDFN not only the specificity of alloantibody but also its titer has a direct impact on the fetus. Therefore, we evaluated the gel test and the conventional tube test for antibody screening and titration to determine if there is any correlation between the results obtained by using both technologies and to find out whether critical levels of alloantibody could be determined. We further studied obstetric management such as ultrasonography and intrauterine transfusion. We tried to correlate the titers with these clinical interventions.

Material and Methods

The duration of study was from July 2005 to June 2006, during which a total of 652 clotted samples were collected from 223 antenatal D– women. The study was conducted after approval from the Institute Ethics Committee. A written informed consent was taken from the patients for the study.

Sample Collection

A 3-mL clotted sample was collected, which was then centrifuged and the serum separated. Serum was stored in a frozen state at –80°C in two aliquots each until further testing. The first sample was taken in the first trimester or at the time of the first visit. Subsequent samples were taken in every trimester and at 28 weeks before the administration of anti-D immunoglobulin. Immunized women were followed up every 3 to 4 weeks.

Antibody Detection

The serum was thawed to room temperature before being tested. Antibody detection was done in parallel by the conventional tube technique and the gel test. A commercially available three-cell screening panel (DiaScreen; DiaMed, Cressier sur Morat, Switzerland) was used. For tube testing,
a 3% suspension in normal saline was used, and for gel testing, a 0.8% suspension in LISS was used.

**Tube IAT**

One drop of RBC suspension was added to two drops of serum to be tested into labeled test tubes. The tubes were incubated in a water bath at 37°C for 60 minutes. After three washes with normal saline solution, polyspecific anti-human globulin was added to the RBC button and the tube was centrifuged at 3000 x g for 15 seconds in an appropriate centrifuge. The tubes were examined for agglutination. The reactions were graded and recorded as per the AABB Technical Manual.

**Gel Test**

We dispensed 50 µL of RBCs into labeled gel cards and added 25 µL of the serum to be tested. The cards were incubated for 15 minutes at 37°C in specially designed incubators. They were then centrifuged at 1050 rpm for 10 minutes. The reactions were graded and recorded. Antibody identification was done using commercially available panels (DiaMed) according to the manufacturer’s instructions.

**Antibody Titration**

Titration was performed on those samples that were positive on antibody screening. Serial twofold dilutions were made in normal saline solution in clean test tubes. The dilutions were tested in parallel for both tests. In-house prepared R,R, RBCs from a single donor were used for D antibodies, whereas RBCs with heterozygous expression were used for Lea and Leb antibodies. The RBCs were washed three times in normal saline solution and resuspended to a final concentration of 3% and 0.8% in normal saline solution for tube testing and gel testing, respectively. Critical titer was taken as 16 by the tube technique followed in our institute.

**Tube Test**

The method used was the same as that for antibody detection.

**Gel Test**

The RBCs used were suspended in normal saline solution to a final concentration of 0.8%, and the cards incubated at 37°C for 60 minutes. The remainder of the procedure was the same as for antibody detection. Titers were taken as the highest dilution that gave 1+ agglutination. The gel test had been modified from the manufacturer’s guidelines for titration. LISS was not used in the process, and the time of incubation was increased from 15 minutes to 60 minutes. We tested several samples in parallel with the standard method and found that deviating from the original method did not miss any antibodies.

Table 1. Comparison of antibodies detected by both methods

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<th>Sample tested</th>
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<tr>
<td>Total</td>
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<td>652</td>
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<tr>
<td>Tests positive</td>
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<td>93</td>
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<tr>
<td>Anti-D (all)</td>
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<td>83</td>
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<td>Anti-D (passive)</td>
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<td>Anti-Lea</td>
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<td>5</td>
</tr>
<tr>
<td>Anti-Leb</td>
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<td>5</td>
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</tbody>
</table>

*Anti-C was found in association with anti-D.

Table 2 compares the titer values obtained by both methods for D antibodies. The Lewis antibody titer values are compared in Table 3. The titers on gel were generally higher than those obtained by the tube technique, and as the tube titers increased, the gel titers also increased. The titer increase with the gel test was higher when compared with the tube test. The values varied from onefold to fivefold (mean, 1.6-fold). The observed differences were onefold in 21 sera, twofold in 32, threefold in 18, fourfold in 3, and fivefold in 1.

**Obstetric Studies**

**Ultrasonography**

The titers were compared with the fetal findings on ultrasonography, i.e., normal vs. hydropic. All the women with alloantibodies were investigated. The mean titer value for those who had normal ultrasonographic findings was 62.39 by tube technique with a standard deviation of 11.5 and titers ranging from 1 to 512. By the gel technique, the
The mean value was 271.04 with a standard deviation of 460.27 and a range of 2 to 2048. The mean titer value for those who had hydropic findings on ultrasound was 182.86 by tube technique with a standard deviation of 145.138 and titers ranging from 128 to 512. By the gel technique, the mean value was 841.04 with a standard deviation of 604.259 and a range of 256 to 2048.

When compared using nonparametric tests, the difference between the two groups was significant by the tube method (p = 0.02) and also by gel test (p = 0.03).

Table 4 shows the median values for normal and hydropic findings using both techniques.

Amniocentesis

The titer values by both methods were compared with the need for amniocentesis, which was performed on all women with a tube titer greater than 16. The values for the corresponding zones on Liley's chart were compared by both methods. The comparison is shown in Table 5.

Discussion

Rh antigens are by far the most common cause of alloimmunization in pregnant women. The D antigen is the most immunogenic; yet, alloimmunization caused by it has been virtually eliminated in the Western world. Use of antibody screening and titration studies aid the obstetrician in deciding among early preventive or treatment modalities to manage HDFN.

Risk of HDFN can be diagnosed early in pregnancy with noninvasive serologic methods such as the indirect antiglobulin test to detect the presence of irregular antibodies in the maternal serum. Usually the firstborn is the initiator of sensitization to antigens present on fetal RBCs. This occurs mostly at delivery. During subsequent pregnancy reexposure to the same antigen initiates a secondary immune response and with the potential for the pathogenesis of HDFN. Antibody that has been detected in maternal serum must be identified. The antibody can then be quantitated using laboratory methods such as titration studies. The titration studies are useful in guiding the timing of clinical intervention required in utero. The noninvasive serologic methods precede the more invasive methods such as amniocentesis, which is associated with fetal morbidity and mortality.
The higher sensitivity and versatility of the gel test compared with the tube test have been reported in various studies.\textsuperscript{6,7} A recent study comparing the conventional tube test with the gel technique for crossmatch also showed the latter to be more sensitive.\textsuperscript{8} Other positive points of the gel test include smaller volume of sample required, elimination of washing steps, stable results, and easy readability. The tube test in our study missed 10 examples of anti-D that the gel test detected. However, the anti-D in these samples were all attributable to antenatal anti-D immunoprophylaxis and they became undetectable after 4 to 6 weeks. Thus, the tube test did not miss any of the significant antibodies. Data are also presented that indicate the propensity of the gel test to miss clinically insignificant antibodies like Le\textsuperscript{a}.\textsuperscript{7} The gel test in our study missed one sample of anti-Le\textsuperscript{a}. Nine samples of anti-Le\textsuperscript{a} and anti-Le\textsuperscript{b} were, however, detected by both methods. The titration of antibodies by both methods showed variable results. Similar data have been published by Novaretti et al.\textsuperscript{9} in which they tested gel and tube titers for anti-D. They found the titers to be threefold to eightfold higher. Our study showed the titers to be one- to fivefold higher. The gel titers tended to be higher when compared with the tube titers. However, no correlation between the two methods could be found.

The recommended titration study by Judd\textsuperscript{10} is a saline antiglobulin procedure with 60-minute incubation at 37°C. In our study, we did not use LISS for gel titration, which is a known enhancing medium, in an attempt to establish a correlation, if any exists, between the conventional tube technique and the gel technique. Judd also stated that until substantial data are available that show correlation between gel microcolumn assay and saline tube antiglobulin titers IgG gel column technology should not be used for prenatal antibody titration. The critical tube titers of 16 corresponded to gel titers ranging from 32 to 128. Hence, it is difficult to arrive at a definitive conclusion of critical gel titers when a direct correlation is attempted. When the titers were compared with ultrasonography for hydrops and amniocentesis OD values as per Liley’s chart, a gel value of less than 128 (i.e., 64) corresponded with normal ultrasonography and low zone OD values. However, this value should be interpreted with caution until more studies support it.

Based on the above observations, we conclude that the gel test is a better method than the conventional tube test for antibody detection because of its higher sensitivity and technical safety. Titration by gel, however, should not be considered for antenatal HDFN management as gel titers do not show linear correlation with tube titers, which predict fetal outcome in RhD sensitized women. Developing countries work under resource constraints, and such studies would optimize cost-effective use of this technology.

References


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The Rh and RhAG blood group systems

S.T. Chou and C.M. Westhoff

History

Rh is the most well-recognized blood group system after ABO because of the immunogenicity of the principal antigen, D. Discovered more than 70 years ago, the D antigen is well known as the target in severe and potentially fatal hemolytic disease of the fetus and newborn (HDFN).1 Today, HDFN caused by anti-D is much less frequently observed in developed countries owing to prevention by administration of Rh immunoglobulin (RhIG; anti-D IgG) after delivery of a D+ infant.2-3 Children of women of European extraction are more likely affected as D— is most common in Caucasians (15–17%), less common in African Blacks (3–5%), and uncommon or rare in Asians (<0.1%).4 Thus, the D antigen status is not routinely tested for in some parts of Asia, principally China, where D— is considered a rare blood type. HDFN caused by anti-D is still seen where women have limited access to prenatal care and in countries where RhIG administration is not standard practice. Anti-D during pregnancy is also more often seen if RhIG is not administered during the third trimester of pregnancy in addition to after delivery. The policy of giving antepartum RhIG to all D— women has not been adopted in all countries.

The Rh system is a complex RBC antigen system that includes more than 50 different antigenic specificities (Table 1). “Rh-positive” and “Rh-negative” refer to the presence or absence of the D antigen. The five principal Rh antigens—D, C, c, E, and e—are responsible for the majority of clinically significant antibodies. Some of the others represent compound specificities, i.e., ce or f is an epitope on the Rhce protein that is not present on RBCs with RhC or RhCe. Many of the antigens are found primarily in a population group or specific ethnic group; for example C5 has the highest prevalence in Finns, and V and VS are found primarily in African Black ethnic backgrounds (summarized in Reid and Lomas-Francis5).

Our understanding of the Rh system has been greatly advanced since the genes were cloned in the early 1990s. The sequence of RHCE was first reported in 1990,6,7 and RHD was subsequently sequenced in 1992.6,8 The different RHCE alleles responsible for the C or c and E or e antigens were clarified in 1994.9 In the last decade, molecular genotyping has revealed that the genetic diversity of the RH locus greatly exceeds estimates predicted by serology. More than 170 RHD and more than 70 different RHCE alleles have been described, and new alleles are still being discovered. A directory of RHD alleles is maintained on the RhesusBase website, and RHCE alleles are found on the National Center for Biotechnology Information (NCBI) human blood group mutation Web site.10 In an effort to standardize terminology, the International Society of Blood Transfusion (ISBT) Working Party on Red Cell Immunogenetics and Blood Group Terminology maintains a list of alleles that encode blood group antigens (see Web Resources).

Terminology and Nomenclature

The term “Rh” for Rhesus resulted from work by Landsteiner and Wiener, who found that antiserum produced by immunizing guinea pigs and rabbits with RBCs of the Rhesus macaque agglutinated 85 percent of human RBCs. Initially, they believed that a common factor “Rh” had been identified in animals and humans.11 The antibody actually detected the LW antigen, which is present on D+ RBCs in greater amounts than on D— RBCs. Although misnomers, the terms Rh factor and anti-Rh continued in common usage to describe the human D antigen and the D antibody. Although the use of “Rhesus” is now discouraged, it has been pointed out (WA Flegel, personal communication) that “Rh” can be difficult to translate into other languages.

Over the years, four Rh system nomenclatures have been introduced (Table 1). Terminology established by Fisher-Race in England was based on the premise that three closely linked genes, C/c, E/e, and D, were responsible for the antigens, whereas the Wiener nomenclature (Rh-Hr) was based on the belief that a single gene encoded several blood group factors. The Rh system is encoded by two genes, RHD and RHCE, as predicted by Tippett.12 Rosenfeld gave each Rh antigen a number based on the order of its discovery or assignment to the Rh system. The ISBT Working Party on Terminology for Red Cell Surface Antigens adopted a six-digit number for each RBC antigen.13 The first three numbers represent the system (the number 004 was assigned to the Rh system), and the remaining three digits refer to the antigenic specificity (Table 1).

Current terminology distinguishes the genes and the proteins from the antigens. Capital letters, with or without italics, are used when referring to the RH genes, RHD and RHCE. Alleles of RHCE are designated after an asterisk, i.e. RHCE*ce, RHCE*Ce, RHCE*eE, RHCE*CE, according to which antigens they encode. Alleles of RHD are designated RHD*DVI, RHD*DIIIa, RHD*weak D type 2, and so on, according to the partial or weak D encoded, and the alleles have also been given numbers by the ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology (see Table 2 for examples and Web Resources). Lastly, the Rh proteins are indicated without italics and with the “h” in lowercase, as RhD or RhCE, or according to the specific antigens they carry, Rhce, RhCe, RhcE, or RhCE.
Table 1. Rh and RhAG blood group systems: antigen terminology/nomenclature

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**Genetics and Inheritance**

**RH Genes and Rh Proteins**

RH genes are inherited as codominant alleles. RHDA encodes the D antigen and RHCE encodes CE antigens (ce, Ce, Ce, or CE). Each gene has ten coding exons, and the RHDA and RHCE proteins differ by 32 to 35 amino acids (depending on the specific CE allele). The large number of differences explains why D, when seen by the immune system of a D- person who lacks the protein, often induces a robust immune response. The large number of amino acid differences also explains the numerous epitopes of the D antigen, estimated to range from 9 to more than 30. Although only nine or ten of the amino acid changes are predicted to be on the extracellular surface of the RBC membrane, changes located in the transmembrane and cytoplasmic regions also alter the topology and epitopes of the protein. Although early on it was assumed that only extracellular changes would prompt an immune response to Rh proteins, new antigens identified by an antibody response are also generated with intracellular changes. For example, the Leu245Val change in Rhce proteins is not extracellular, but this change results in expression of the novel RBC antigens V and VS, which are also serologic markers for an altered e antigen. Patients who are homozygous for altered Rhce alleles encoding partial e antigens type serologically as e+, but are at risk for anti-e production when exposed to conventional Rhce protein.

The RHCE gene encodes C/c and E/e antigens on a single protein. C and c differ by four amino acids, whereas the E and e antigens differ by only one amino acid. Although the common alleles are RHCE*ce, RHCE*Ce, and RHCE*eC, a large number of alleles with additional polymorphisms are found in different ethnic groups and are discussed in a later section.

**Molecular Basis of Rh Antigens**

**D Antigen**

**D Negative (Rh-Negative)**

Several genetic mechanisms are responsible for the D- phenotype, but deletion of the RHD gene is the primary background. Some exceptions include silenced RHD as a result of point mutations, nucleotide insertions, premature stop codons, or RHD-CE hybrids. In African Blacks, 66 percent of D- individuals have a 37-bp insertion in RHD causing a premature stop codon, whereas 15 percent carry a hybrid RHD-CE-D characterized by expression of weak C but no D antigen. The rare D- phenotype in Asians is often attributable to point mutations in RHD, although 10 to 30 percent of Asians who type as D- have very low levels of D; this phenotype is termed Del.17

**D Positive (Rh-Positive)**

Most people with D+ RBC phenotypes have conventional RhD protein, but more than 150 different alleles encoding changes in the amino acid sequence of the...
Table 2A. Examples of RHD alleles

<table>
<thead>
<tr>
<th>Phenotype(s) or category</th>
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**Partial D examples**

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<td>DIIla</td>
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<td>186G&gt;T</td>
<td>Leu62Phe</td>
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<td>RH:54 (DAK+)</td>
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<td>Ala137Val</td>
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<td>Asn152Thr</td>
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<td>Ser103Pro</td>
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</tbody>
</table>

**Weak D example**

<table>
<thead>
<tr>
<th>Type 1</th>
<th>Allele designation</th>
<th>Nucleotide changes</th>
<th>Amino acid changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHD*01W.1</td>
<td>RHD*weak D type 1</td>
<td>809T&gt;G</td>
<td>Val270Gly</td>
</tr>
</tbody>
</table>

**D– examples**

<table>
<thead>
<tr>
<th>D–</th>
<th>Allele designation</th>
<th>Nucleotide changes</th>
<th>Amino acid changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>D–</td>
<td>RHD*01N.01</td>
<td>Complete deletion</td>
<td>inactive</td>
</tr>
<tr>
<td>D–</td>
<td>RHD*04N.01</td>
<td>37-bp insert, 609G&gt;A,</td>
<td>inactive</td>
</tr>
<tr>
<td></td>
<td>RHD*Pseudogene</td>
<td>645G&gt;C, 677T&gt;G,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RHD*Ψ</td>
<td>674C&gt;T, 807T&gt;G</td>
<td></td>
</tr>
</tbody>
</table>

W in the allele name indicates a weak phenotype.
N in the allele name indicates a null phenotype.

**ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology**

protein, or changes in the conserved nucleotides at the ends of coding exons required for efficient splicing of pre-mRNA to generate mature transcripts, have been reported. These result in weak D, partial D, and D_d phenotypes.

**Weak D**

Although common D phenotypes have 10,000 to 30,000 antigen sites per RBC, weak D RBCs have less than 100 to 5000 sites (summarized in Reid and Lomas-Francis). Historically, weak D RBCs were defined as requiring the IAT for detection, but with the introduction of monoclonal antibodies this depends on the specific reagent used to test the RBCs. Weak D phenotypes are most often associated with single nucleotide changes in RHD. These encode amino acid changes that negatively affect insertion of the protein in the membrane or negatively affect efficient splicing of the coding exons, resulting in reduced expression of D antigen on the RBCs. To date, more than 70 different mutations causing weak D expression have been reported, the most common encoding a Val270Gly change called weak D type 1. The different weak D phenotypes have been given number designation, i.e., 1 through 73 to date. (summarized by Wagner, FF. RhesusBase Web site; see Web Resources)

Additionally, less D antigen is present in cells that also have C antigen, such that an R_R, individual (DeE/DeE) has more D antigen sites than an R_R, individual (DeC/DeC). A weak D phenotype can occur when Ce (r’) is present in trans to RHd, and depression of an already weak D type by Ce (r’) can result in an apparent D– (rr’, r’r’, or r”r’) or D_d phenotype in a sample that is in reality R (weak D)/r’(Ce).

**D_d**

RBCs with a D_d phenotype express very low levels of D antigen detected serologically only by adsorption and elution with anti-D. D_d is most commonly found in Asians, who comprise 10 to 30 percent of individuals who type as D– with standard serologic testing. D_d is less often found in Europeans (0.027%). In Asians, the D_d phenotype most often results from a nucleotide change 1227G>A, which does not affect the amino acid sequence but interferes with efficient splicing of exon 9, whereas a single amino change, Met295Ile, is the most common mutation in Europeans. D_d RBCs are primarily diagnosed by RHD genotyping or adsorption and elution studies because the RBCs type as D–.

**Partial D**

RBCs with partial D type serologically as D+, but individuals can produce anti-D when stimulated by transfusion or pregnancy. Because the D antigen consists of numerous conformation-dependent epitopes (epD), a person who lacks one or more D epitopes can form alloantibodies to the missing portion(s). More than 30 D epitopes have been defined with monoclonal antibodies, designated epD1 through epD9 with further subdivisions of each. Point mutations in RHD causing single amino acid changes, or more often hybrid genes in which portions of RHD are replaced by the corresponding regions of RHCE, cause partial D. The novel sequences of the hybrid protein resulting from regions of RhD joined to RhCE result in the loss of D epitopes and may generate new low prevalence or cause the loss of high prevalence antigens.

**Elevated D or D– – Haplotypes**

Deletion phenotypes, designated D–, Dc–, and Dc–, (the dashes represent missing antigens) can have enhanced expression of D antigen and no, weak, or altered C/c and E/e antigens. Many are caused by replacement of portions of RHCE by RHD, such that the additional RHD sequences in RHCE, along with a normal RHD, account for the increased D expression. However, some are caused by mutations that alter or silence RHCE. For example, a silenced RHCE*CE designated RHCE*CE(907del C) encodes a stop codon and is associated with the D– haplotype and the absence of c and E antigen expression in Hispanics.
C/c and E/e Antigens

The common RHCE alleles encode the C or c and E or e antigens. Of the four amino acid changes (Cys16Trp; Ile60Leu; Ser68Asn; Ser103Pro) associated with the C to c polymorphism, Ser103Pro correlates with C/c antigen expression. The E to e polymorphism is caused by a single amino acid substitution, Pro226Ala. Numerous changes in RHCE cause quantitative and qualitative changes in C/c or E/e antigen expression, with altered C and e most often encountered. More than 70 different RHCE alleles are associated with altered, weak, or no expression of the principal antigens.

Compound Antigens (ce, Ce, cE, and CE)

Compound antigens define epitopes that depend on conformational changes that result from amino acids associated with both C/c and E/e. These were previously referred to as cis products, to indicate the antigens were expressed from the same haplotype, but it is now known that these are expressed on a single protein. These include ce or f, Ce or rh, cE or Rh27, and the rare CE or Rh22. Serologically, RBCs with Dce/DCE or Dce/DCe haplotypes would be identical when tested with common Rh antisera, but only the former sample would react with anti-f.

Altered or Variant C and e Antigens

In Caucasians, altered C is not common but when encountered is primarily associated with amino acid changes Gln41Arg or Ala36Thr, and expression of the Cw and Cx antigens, respectively. RBCs that express Cw and Cx, in the absence of C in transfusion, can make anti-C when exposed to conventional C antigen. Altered C is also associated with RhCE proteins expressing novel antigens, including JAHK (Ser122Leu), and JAL (Arg114Trp). The RBCs type as C+, but patients can make anti-C when exposed to conventional C antigen.

Altered RHCE alleles are relatively common in individuals with African ancestry. In this population, the most common cause for altered C is an RHDIia-CE(4-7)-D hybrid gene, which does not encode a D antigen, but encodes altered C antigen on a hybrid protein background (Fig. 1). Approximately 25 percent of African Americans have this hybrid. In most cases, the RBCs type C+ with commercial monoclonal reagents, and the altered C goes undetected. Most of these patients coinherit an altered e antigen and have a V–VS+ phenotype; together this haplotype is referred to as (C)ceS. Because altered RHCE alleles are not distinguished by routine serologic tests, homozygous patients often make anti-C and anti-e alloantibodies after transfusion. Additionally, homozygous individuals typically lack high-prevalence antigens, designated hrB and hrS.

D and D-like Epitopes on Rhce

One further complication of serologic D status determination is the expression of D epitopes by the protein product of the RHCE gene in the absence of RHD. Two significant examples are D[rAR], found in individuals of German ancestry, and Crawford (ceCF), found in individuals of African ancestry. These two are notable because the RBCs show strong reactivity with some monoclonal anti-D reagents (3+ to 4+), but are nonreactive with others. Other amino acid changes in Rhce mimic the RHD protein, and individuals with them can be readily sensitized if transfused with D+ RBCs. RBCs with Dce/DCE or Dce/DCe haplotypes would be identical when tested with common Rh antisera, but only the former sample would react with anti-f.

Frequency of Altered RHD

Approximately 2 percent of Europeans carry altered RHD, and those most often encountered will vary by geographic location, which reflects population diversity in the region. In our experience, weak D is found primarily in European and Asian backgrounds. The incidence of partial RHD alleles in individuals with African backgrounds has not been determined but seems to be significantly higher than in European populations.
Anti-hr^b and anti-hr^s can be clinically significant, and finding compatible blood can be very difficult because of differing RH gene backgrounds. As an additional complication, altered RHCE^ce may lead to production of antibodies sometimes identified as anti-Hr^b (RH34) and anti-Hr (RH18).

**G Antigen**

The G antigen is found on RBCs expressing C or D with a 103Ser residue present on RhD, RhCe, or RhCE. Serologically, anti-G reacts as though it were anti-D plus anti-C. Anti-G explains the D− person transfused with D−/C+ blood, or the D− woman who delivered a D−/C+ child, who subsequently appears to have made anti-D. To distinguish anti-D, -C, and -G, adsorption and elution studies can be performed. Their results determine the need for RhIG prophylaxis for obstetric patients with anti-G to prevent additional immunization and formation of anti-D.

**Antibodies in the Rh System**

Most Rh antigens are immunogenic and have the potential to cause clinically significant HDFN or transfusion reactions. Rh alloimmunization occurs by exposure to foreign RBCs during pregnancy or after transfusion and may persist for many years. Even if serum antibody drops below detectable levels, subsequent exposure to the antigen can produce a rapid secondary immune response. Outside of the ABO system, the D antigen is the most immunogenic antigen, followed by c and E. Anti-c can cause severe HDFN, whereas anti-C, -E, and -e are associated with mild HDFN. Because Rh alloimmunization is IgG-mediated, transfusion reactions are primarily extravascular. The patient may have fever, jaundice, icteric sclera, dark urine, or a drop in hemoglobin level. Antigen-negative RBCs should be provided to any patient with a history of Rh antibody sensitization.

Rh antibodies are often found together. It is not unusual for a patient with a single Rh antibody to become alloimmunized to additional Rh antigens if further exposed. For example, a DCE/DCe (R,R,) patient with anti-E has usually been exposed to the c antigen as well. Anti-c may be present at the time anti-E is identified, but may be weak and undetectable. Therefore, to prevent delayed transfusion reactions, some advocate avoiding c+ blood for patients alloimmunized to the E antigen. In contrast, pursuing anti-E in serum containing anti-c is not necessary as the patient will likely have been exposed to c without being exposed to E. Moreover, the vast majority of c− donor blood will be negative for E.

**Rh Antibodies in Patients with Sickle Cell Disease**

Some sickle cell transfusion programs perform Rh typing to match patients and donors for D, C, and E (in addition to K) to prevent alloimmunization. The prevalence of RH alleles that encode altered or variant D, C, and e antigens in African ethnic groups often underlies the production of complex Rh alloantibody specificities in chronically transfused patients with sickle cell disease (SCD). Many altered Rh protein epitopes cannot be distinguished serologically, and are only identified by RH genotyping once a patient develops apparent autoantibodies to Rh antigens. Some common examples include the partial C antigen encoded by the r′S allele, i.e. (C)ceS, and partial DIIIa; the RBCs react 3+–4+ with monoclonal reagents and are not detected as altered. Anti-hr, -hr, -Hr, and -hr can be difficult to identify and can cause clinically significant hemolytic transfusion reactions including transfusion fatalities.

**Other Rh Antibodies**

In addition to the SCD population, D− and Rh null individuals also develop alloantibodies to high-prevalence Rh antigens with Rh17 and Rh29 specificity, respectively. Lastly, autoantibodies to high-prevalence Rh antigens often occur in the sera of patients with warm autoimmune hemolytic anemia and in some cases of drug-induced autoimmune hemolytic anemia. The biologic explanation for this apparent specificity or cross-reactivity remains to be determined.

**Clinical Considerations**

**Determination of D status**

The AABB Standards for Blood Banks and Transfusion Services requires donor blood to be tested for weak D expression and to be labeled as Rh-positive if the test is positive. Hospital transfusion services are also required to confirm the D-negative status before the unit is released for transfusion to avoid transfusion of D+ RBCs to D− recipients. It is recognized that some very weak D antigens are not detected, and standard typing procedures (including IAT) do not detect D− RBCs. Isolated cases have been reported in which very weak D and D^el donor units have caused anti-D alloimmunization in D− recipients.

When determining the D type of a patient, detecting weak D expression is not necessary unless testing RBCs of an infant of a D− mother at risk for D immunization. DVI is the most common partial D found in Caucasians, and anti-D produced by women with partial DVI has resulted in
Sickle Cell Disease and Altered RH Alleles

RBC alloimmunization remains a serious complication of transfusion for patients with SCD, with more than 25 to 30 percent of chronically transfused SCD patients developing RBC antibodies. Altered D, C, and e antigens often underlie the complex RH alloantibodies SCD patients exhibit after transfusion. Although randomized controlled trials have not been performed, extended RBC antigen-matching (including D, C, E, and K) has been shown in numerous single-institutional and prospective multicenter experiences to significantly reduce the incidence of alloantibody production in SCD. Therefore, many centers determine the pretransfusion RBC serologic phenotype and provide RBCs that are antigen-matched for D, C, E, and K. Because the high alloimmunization rates are also believed to be caused by antigenic disparity between African Americans and Caucasians, some programs actively recruit African American donors to supply blood specifically for patients with SCD. Although these approaches reduce the incidence of alloantibody production, some patients still become sensitized to Rh antigens (D, C, e, and hr), indicating they were not Rh antigen matched.

RH Genotyping

RBC genotyping methods were introduced to transfusion medicine a decade ago after cloning of the genes made genetic testing for blood groups possible. Genotyping methods include amplification of target gene sequences by PCR followed by analysis using RFLP, real-time PCR, or sequence-specific primer PCR. More recently, mass-scale genotyping technologies have been developed by several manufacturers to perform high-throughput blood group prediction. There is nearly complete concordance between the genotype and serologic phenotype, and studies have included samples from diverse backgrounds. However, the complexities and numerous alleles in the Rh blood group system have precluded high-throughput genotyping for RH other than for the common nonvariant forms of C/c and E/e antigens. The detection of numerous silencing mutations is required for accurate typing; several regions of the genes must be sampled to detect multiple alleles, and new alleles are continuously being identified.

Currently, RH genotyping is primarily restricted to specialized immunohematology laboratories. One application of RH genotyping is to find compatible donors in the American Rare Donor Program (ARDP) for patients with antibodies to high-prevalence Rh antigens. Genotyping can also be used to determine the RH status of a fetus by amniocentesis or chorionic villus sampling. More recently, noninvasive techniques have been used to collect cell-free, fetal-derived DNA from maternal plasma for RH genotype prediction with a high-throughput method. Once high-throughput platforms encompassing all the Rh variants are developed and are cost effective, RH genotyping can complement serologic testing for typing transfused patients, RH zygoty determination, fetal D typing, resolution of D status, and finding compatible blood for patients with SCD.

RhAG System

RHAG is the ancestral gene from which RHCE and RHD arose. It resides on chromosome 6 and encodes the Rh-associated glycoprotein and the antigens of the RhAG blood group system. The RhAG protein consists of 409 amino acids and associates with the Rh proteins in the membrane to form the Rh-core complex.

Antigens

The RhAG protein does not express the common Rh antigens, but it has recently been shown to carry several antigens, and it has been assigned system 30 by ISBT (Table 1). Two high-frequency antigens, Duclos and DSLK, and one low-frequency antigen, Ol, had serologic characteristics suggestive of expression on RhAG. This was confirmed by gene sequencing and expression of recombinant RhAG in HEK 293 cells. The Duclos-negative patient was homozygous for a nucleotide 316C>G change, encoding Glu106Glu. The DSLK-negative patient was homozygous for nucleotide 490A>C, encoding Lys164Gln. Two O(a+) family members of a Norwegian family were heterozygous for 680C>T encoding Ser227Leu. Duclos is RHAG1, Ol is RHAG2, and DSLK is provisionally RHAG3.

Rhnull

RhAG protein must be present for the expression of Rh antigens. Rhnull RBCs lack expression of Rh antigens, and the phenotype most often results from mutations in RHAG, previously termed “regulator” Rhnull. Less often, Rhnull individuals have inactivating or silencing mutations in RHCE and deletion of RHD previously referred to as “amorph” Rhnull.
The Rh-core complex also interacts with band 3, glycophorins A and B, LW, and CD47 and is believed to contribute to the RBC membrane structure because Rh-null RBCs demonstrate abnormal morphology. The function of the Rh blood group proteins is not known, but RhAG is an ammonia transporter, and the recent crystalization of the human RhAG homolog from the kidney, RhCG, confirms the relationship of human Rh proteins to ammonia transporters.\(^{36}\)

**Summary Perspective**

The Rh/RhAG blood group systems are some of the most complex, and in the past two decades major insights have been gained into the molecular basis of the Rh system. The genetic information has confirmed many of the predictions of the serologists, whose primary (and often only) tools were the antibodies made by immunized individuals. Exploiting adsorption and elution approaches, along with selected RBC testing strategies, they uncovered many of the details concerning the specificity and complexity of the Rh system. The prediction that Rh antigens are encoded by two genes, not one or three, was adeptly forecast by Patricia Tippett based solely on serologic observations.\(^{12}\) Their work is the foundation of our understanding today, as new genetic information builds on the serologic backbone. Serologic reactivity is still the basis for blood groups and blood transfusion practice because serology defines an antigen. That will not change as we use DNA-based testing methods because it is important to remember that without a serologic relationship, a variation in a blood group allele at the DNA level is just another SNP (single nucleotide polymorphism), and these occur once in every 100 to 300 bp in the human genome. These polymorphisms are only of academic interest until associated with a phenotype and found to be relevant to transfusion medicine by stimulation of an antibody.

Molecular testing will be a powerful adjunct to serologic methods and improve transfusion safety and outcomes, particularly for the chronically transfused population. Which of the numerous alleles in the Rh system are clinically significant is yet to be fully determined. Ultimately, with the development of mass-scale RH genotyping technology, genetically matching donor units with the patients could significantly reduce alloimmunization, and RHD genotyping will provide a definitive D classification for donors and patients. The availability of cost-effective, reliable high-throughput genotyping platforms is needed for this to become incorporated into clinical transfusion medicine practice.

**References**


Web Resources


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To the Editors:

Anti-Vel and cold-reactive autoantibody

In *Immunohematology* Volume 26, Number 1, 2010, Linz and colleagues present a case report titled “Role for serial prenatal anti-Vel quantitative serologic monitoring with 2-ME serum during pregnancy.” The report includes a statement that anti-Vel can simulate the serologic profile of a cold-reactive autoantibody. The authors did not quote a previous paper with similar conclusions that further support this premise. Mechanic et al. describe a study in which eight samples of stored anti-Vel were adsorbed with rabbit erythrocyte stroma (REST, Immucor/Gamma, Norcross GA). (Transfusion 2002;42(9):1180–3). The antibodies were reactive at various testing phases. The adsorbed samples were tested at phases selected based on the test results of the neat samples. The results indicated that anti-Vel was either partially or completely adsorbed by REST. These results further support the idea that one must be thorough when investigating these types of antibodies.

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**Manuscripts**

The editorial staff of *Immunohematology* welcomes manuscripts pertaining to blood group serology and education for consideration for publication. We are especially interested in case reports, papers on platelet and white cell serology, scientific articles covering original investigations, and papers on new methods for use in the blood bank. **Deadlines** for receipt of manuscripts for consideration for the March, June, September, and December issues are the first weeks in November, February, May, and August, respectively. For instructions for scientific articles, case reports, and review articles, see Instructions for Authors in every issue of *Immunohematology* or on the Web at www.redcross.org/immunohematology. **Include fax and phone numbers and e-mail address with all manuscripts and correspondence.** E-mail all manuscripts to immuno@usa.redcross.org

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**For information** concerning the National Reference Laboratory for Blood Group Serology, including the American Rare Donor Program, **contact** Sandra Nance, by phone at (215) 451-4362, by fax at (215) 451-2538, or by e-mail at snance@usa.redcross.org
COMMUNICATIONS

Letter from the Editors:

To Contributors to the 2010 Issues

The journal depends on readers, authors, editorial board, peer reviewers, and our Penn-Jersey staff. We wish we could thank all of you personally, but doing so is not practical. Instead, we thank each of you as members of an honored group.

First and foremost, we thank the authors for their reviews, scientific articles, case reports, book reviews, and letters to the editors that come not only from the United States but from many countries of the world. This has given the journal an international flavor.

Our editorial board is a prestigious one and we depend on its members, not only for peer reviews, but for guidance in policy and suggestions for improvements. Special thanks go to our medical editors, who review every article for medical content, and to our technical editors, who read every article for technical content. The current board is listed by name in the front of each issue of the journal.

Our peer reviewers did a wonderful job in 2010. They are listed below; our thanks to each.

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We also want to thank Marge Manigly, our production assistant, and Sheetal Patel, our editorial assistant, for their help in preparing the journal for press. We also thank Christine Lomas-Francis and Dawn Rumsey, our technical editors, Mary Tod, our copy editor, Lucy Oppenheim, our proofreader; and Wilson Tang, our electronic publisher.

Finally, thanks go to our readers, whose enthusiasm and interest in the journal make it all worthwhile.

Sandra Nance
Editor-in-Chief

Cindy Flickinger
Managing Editor
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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 information to immuno@usa.redcross.org or (215) 451-2538
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The Clinical and Laboratory Standards Institute (www.clsi.org) recently published the guideline “Validation of Automated Systems for Immunohematological Testing Before Implementation; Approved Guideline” (I/LA33-A). This document provides guidance to the end user and laboratory for validation of automated systems used in immunohematologic testing before implementation. The link to this document is:


The link to the press release is:


For information on an upcoming teleconference from CLSI-APHL titled:


For additional information, contact the Web site at www.clsi.org or Amanda Cushman Holm at (610) 688-0100 ext. 129 or aholm@clsi.org.

**Important Notice About Manuscripts for Immunohematology**

Please e-mail all manuscripts to immuno@usa.redcross.org

**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, -Rh17, -K, -k, -Kp\(\alpha\), -Js\(\beta\), -FY\(a\), -Fy\(3\), -Fy\(6\), Wr\(h\), -Xg\(a\), -CD99, -Do\(h\), -H, -Ge2, -Ge3, -CD55 (both SCR2/3 and SCR4), -Ok\(\alpha\), -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\(\alpha\)). Some are directly agglutinating (Anti-A1, -M, -Wr\(h\) and -Rh17) and a few have been humanized into the IgM isofrom (Anti-Js\(\beta\)). 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 procedure 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 >immunohematology >current list of monoclonal antibodies available.

**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, 2010 for the July 2011 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

**Notice to Readers**

*Immunohematology, Journal of Blood Group Serology and Education,* is printed on acid-free paper.
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 2011 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.
## 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  
American Red Cross  
3131 North Vancouver  
Portland, OR 97227

## IgA/Anti-IgA Testing

**IgA and anti-IgA testing is available to do the following:**

- Identify IgA-deficient patients
- Investigate anaphylactic reactions
- Confirm IgA-deficient donors

Our ELISA for IgA detects protein to 0.05 mg/dL.

**For additional information contact:**

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

## Donor IgA Screening

- 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:**

Kathy Kaherl  
(at (860)678-2764  
e-mail: kaherlk@usa.redcross.org  
write to:  
Reference Laboratory  
American Red Cross Biomedical Services  
Connecticut Region  
209 Farmington Ave.  
Farmington, CT 06032
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

Advertisements, cont.
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

(See www.sbbpocketbook.com for details to apply for funds)

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?**

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<th>Managers of Blood Centers</th>
<th>LIS Coordinators</th>
<th>Educators</th>
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<td>Supervisors of Reference Laboratories</td>
<td>Research Scientists</td>
<td>Consumer Safety Officers</td>
<td>Reference Lab Specialist</td>
</tr>
<tr>
<td>Quality Assurance Officers</td>
<td>Technical Representatives</td>
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**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:**

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<th>Phone Contact</th>
<th>Email Contact</th>
<th>Website</th>
<th>Onsite or Online Program</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walter Reed Army Medical Center</td>
<td>William Turcan</td>
<td>202-782-6210</td>
<td><a href="mailto:William.Turcan@NA.AMEDD.ARMY.MIL">William.Turcan@NA.AMEDD.ARMY.MIL</a></td>
<td><a href="http://www.militaryblood.dod.mil">www.militaryblood.dod.mil</a></td>
<td>On site</td>
</tr>
<tr>
<td>American Red Cross, Southern California Region</td>
<td>Michael Coover</td>
<td>909-859-7496</td>
<td><a href="mailto:CooverM@usa.redcross.org">CooverM@usa.redcross.org</a></td>
<td>none</td>
<td>On site</td>
</tr>
<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>
<td>none</td>
<td>On site</td>
</tr>
<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>
<td>On site</td>
</tr>
<tr>
<td>Community Blood Center/CTS Dayton, Ohio</td>
<td>Nancy Lang</td>
<td>937-461-3293</td>
<td><a href="mailto:nlang@cbctscts.org">nlang@cbctscts.org</a></td>
<td><a href="http://www.cbctscts.org/education/sbb.htm">http://www.cbctscts.org/education/sbb.htm</a></td>
<td>On line</td>
</tr>
<tr>
<td>Gulf Coast School of Blood Bank Technology</td>
<td>Clare Wong</td>
<td>713-791-6201</td>
<td><a href="mailto:crong@giveblood.org">crong@giveblood.org</a></td>
<td><a href="http://www.giveblood.org/education/distance/htm">www.giveblood.org/education/distance/htm</a></td>
<td>On line</td>
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<tr>
<td>Hoxworth Blood Center, University of Cincinnati</td>
<td>Susan Wilkinson</td>
<td>513-558-1275</td>
<td><a href="mailto:susan.wilkinson@uc.edu">susan.wilkinson@uc.edu</a></td>
<td><a href="http://www.hoxworth.org">www.hoxworth.org</a></td>
<td>On site</td>
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<td>Indiana Blood Center</td>
<td>Jayanna Slayten</td>
<td>317-916-5186</td>
<td><a href="mailto:jslayten@indianablood.org">jslayten@indianablood.org</a></td>
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<td>410-955-6580</td>
<td><a href="mailto:jilight5@jhmi.edu">jilight5@jhmi.edu</a></td>
<td><a href="http://pathology2.jhu/department/divisions/tranfusion/sbb.cfm">http://pathology2.jhu/department/divisions/tranfusion/sbb.cfm</a></td>
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<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>
<td><a href="http://www.rushu.rush.edu/health/dept.html">www.rushu.rush.edu/health/dept.html</a></td>
<td>On line</td>
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<tr>
<td>Transfusion Medicine Center at Florida Blood Services</td>
<td>Marjorie Doty</td>
<td>727-568-5433 x 1614</td>
<td><a href="mailto:mdoty@fbsblood.org">mdoty@fbsblood.org</a></td>
<td><a href="http://www.fbsblood.org">www.fbsblood.org</a></td>
<td>On line</td>
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<tr>
<td>University of Texas Health Science Center at San Antonio</td>
<td>Linda Myers</td>
<td>210-731-5526</td>
<td><a href="mailto:lmyers@bloodntissue.org">lmyers@bloodntissue.org</a></td>
<td><a href="http://www.utshscsa.edu">www.utshscsa.edu</a></td>
<td>On site</td>
</tr>
<tr>
<td>University of Texas Medical Branch at Galveston</td>
<td>Janet Vincent</td>
<td>409-772-3055</td>
<td><a href="mailto:jvincent@utmb.edu">jvincent@utmb.edu</a></td>
<td><a href="http://www.utmb.edu/sbb">www.utmb.edu/sbb</a></td>
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</tr>
<tr>
<td>University of Texas SW Medical Center</td>
<td>Barbara Laird-Fryer</td>
<td>214-648-1785</td>
<td><a href="mailto:barbara.fryer@UTSouthwestern.edu">barbara.fryer@UTSouthwestern.edu</a></td>
<td><a href="http://telecampus.utsystem.edu">http://telecampus.utsystem.edu</a></td>
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Additional Information can be found by visiting the following Web sites: www.ascp.org, www.caahep.org and www.aabb.org

Revised August 2007
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
   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 model or lot and manufacturer's name, city, and state. Do not use patient's names or hospital numbers.
      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, city and state)
   5. References (limited to ten)
   6. Table or figure (limited to one)

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