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G.M. Meny

This issue introduces an exciting change to the cover of *Immunohematology*. Images depicting a visual interpretation of a journal article or another timely aspect of blood banking will grace the cover of selected issues. I am pleased to announce that David Moolten, MD, award-winning poet and associate medical editor of *Immunohematology*, will provide a description of the cover art from both an artistic and a scientific perspective. I hope these images remind us of the art as well as the science of transfusion medicine. Your feedback is welcome.

Geralyn M. Meny, MD
Senior Medical Editor
*Immunohematology*

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On Our Cover

*El Acueducto de las Ferreras*, an aqueduct also known as *Puente del Diablo (Devil's Bridge)*, spans a ravine approximately 4 km north of the town to Tarragona in the Autonomous Community of Catalonia, Spain. The aqueduct has a maximum height of 26 m and a length of 249 m. Built by the Roman Empire to supply water to the ancient city of Tarraco, the aqueduct features a superimposed series of arches, and may represent the first aqueduct of its kind. The Colton blood group antigen (discussed in this issue) is of course found on aquaporin-1, a widely expressed water channel protein.

——David Moolten, MD
The Knops blood group system: a review

J.M. Moulds

The Knops blood group system finds its roots in the group of antibodies that were previously called high-titer, low-avidity, or HTLA, antibodies. This term was used to describe a group of alloantibodies that appeared to have many of the same characteristics, including very weak reactions at the anti-human globulin (AHG) phase of testing. However, as their specificities were more clearly defined, each was placed into a blood group system, and Knops became the 22nd system recognized by the ISBT Committee on Terminology for Red Cell Surface Antigens.1 Since that time, the protein bearing the Knops antigens has been identified, the gene has been cloned, and all of the known Knops antigens have been characterized at the molecular level. This has allowed investigators to more precisely study the role of the Knops blood group system in disease.

History

In 1965, the sera from three unrelated patients (Cope-land, Stirling, and Wainright) were reported as having an antibody with similar specificity.2 The antibody was named after the first two antibody producers, Co and St (Cost), and given the designation Csα. Ten years later a new antigen, York (Ykα) was reported.3 The York serum was initially believed to be an example of anti-Csα until the RBCs of the donor were found to be Cs(a+). This same study found that 12 of 1,246 Caucasian donors were both Cs(a−) and Yk(a−), suggesting that these antigens were at least phenotypically related. Neither, however, was given blood group status, and they became part of the HTLA group of antibodies. Even though Ykα was thought to be associated with Csα at this time, it would later be assigned to the Knops system.

Another similar antibody being investigated in the 1960s was anti-Knα. Anti-Knα was described in a transfused Caucasian woman who had a saline-reactive anti-K plus an unidentified antiglobulin-reactive antibody to a high-frequency antigen.4 The antithetical Knβ was later reported in the Hall serum, which contained a potent anti-Kpβ and was being used to screen Australian blood donors.5

The Knops blood group system began to expand when Molthan and Moulds6 described a new antigen, McCα, which seemed to be related to Knα. They reported that 53 percent of McC(a−) samples were also Kn(a−). Interestingly, a majority of McCα antibody producers were Black whereas most of those making anti-Knα were Caucasian, thus suggesting that ethnic differences might exist in their respective gene frequencies.

The next pair of alleles, Slα and Vil, was reported in separate abstracts with one author using the term McC for Slα and McCd for Vil.7,8 The Sl terminology came from the names of the first two antibody producers, i.e., Swain and Langley. It was preferred by these authors because they believed that this antigen was independent from McCoy. After identification of the protein bearing the Knops antigens, Slα was renamed S11 and Vil became S12.9

The last high-incidence antigen identified in the Knops system was KAM,10 later renamed KCAM by the ISBT Committee on Terminology for Red Cell Surface Antigens.11 The antibody producer was a Caucasian man who exhibited the Helgeson phenotype, i.e., serologic Kn null. Like Slα, this antigen showed a widely diverse frequency in Blacks vs. Caucasians (Table 1). Although KCAM is a high-frequency antigen in Caucasians, only 20 percent of African Blacks were KCAM+.

Table 1. Frequency of the Knops antigens in several ethnic groups

<table>
<thead>
<tr>
<th>Population</th>
<th>Knα</th>
<th>Knβ</th>
<th>McCcα</th>
<th>McCcβ</th>
<th>S11</th>
<th>S12</th>
<th>Ykα</th>
<th>KCAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasians</td>
<td>98%</td>
<td>4%</td>
<td>98%</td>
<td>1%</td>
<td>99%</td>
<td>&lt;1%</td>
<td>90%</td>
<td>98%</td>
</tr>
<tr>
<td>West Africans</td>
<td>100%</td>
<td>NT</td>
<td>89–92%</td>
<td>49–54%</td>
<td>30–38%</td>
<td>95%</td>
<td>NT</td>
<td>20%</td>
</tr>
<tr>
<td>African Americans</td>
<td>99%</td>
<td>&lt;1%*</td>
<td>90%</td>
<td>44%</td>
<td>51–61%</td>
<td>80%*</td>
<td>98%</td>
<td>NT</td>
</tr>
<tr>
<td>African Brazilians</td>
<td>98%</td>
<td>2%</td>
<td>93%</td>
<td>42%</td>
<td>70%</td>
<td>86%</td>
<td>NT</td>
<td>53%</td>
</tr>
<tr>
<td>Asian Brazilians</td>
<td>100%</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
<td>NT</td>
<td>95%</td>
</tr>
</tbody>
</table>

*Marilyn Moulds, personal communication.
Note: The publication by Covas et al.12 incorrectly lists 4870A (isoleucine) as being KCAM negative.
NT = not tested.
**Knops Protein**

In 1991, two groups identified complement receptor one (CR1) as the protein carrying the Kn\(^a\), McC\(^b\), and Sl\(^a\) blood group antigens.\(^{15,16}\) In addition, Moulds et al.\(^{17}\) also located Yk\(^a\) to CR1 and suggested that the Helgeson phenotype was attributable to low CR1 copy numbers on the erythrocytes (E-CR1). Several years later, Petty et al.\(^{18,19}\) confirmed these reports using monoclonal antibody-specific immobilization of erythrocyte antigens (MAIEA) analyses and also confirmed that Cs\(^a\) was not on CR1. Thus, Cs\(^a\) and Cs\(^b\) have remained a collection as defined by ISBT.

CR1 is a membrane-bound glycoprotein and, with the exception of platelets, is found on most human peripheral blood cells. Depending on the methods used, erythrocytes display approximately 300 to 800 CR1 molecules per cell whereas leukocytes display approximately 10,000 to 30,000 molecules per cell. Because erythrocytes are present in the peripheral circulation at concentrations 10^3-fold higher than the peripheral blood mononuclear cells (PBMCs), they account for greater than 85 percent of CR1 in the blood. E-CR1 binds immune complexes (ICs) that are shuttled to the liver or spleen for transfer to and ingestion by macrophages, leading to their elimination. IC-free erythrocytes return to the circulation, where they may continue participating in IC clearance. We have noted that individuals with high CR1 copy numbers may exhibit a weak false-positive DAT in the gel technique, most likely as a result of increased binding of ICs.

**Knops Antigen Characteristics**

The Knops antigens can vary greatly in strength (Table 2), and weakly reactive cells can be falsely phenotyped as negative if the cells are stored for some duration or have low E-CR1 numbers. Chemicals that can disrupt disulfide bonds, i.e., DTT and AET, can destroy Knops, McCoy, Swain-Langley, and York antigens because they destroy the conformational structure of the short consensus repeats (SCRs). There are no examples of these antibodies that are enhanced by enzyme treatment of RBCs, and most are still reactive (sometimes weaker) with either ficin- or papain-treated cells.\(^{18,19}\) However, all Knops system antibodies currently identified are nonreactive with trypsin-treated RBCs. It is known that a trypsin cleavage site exists in SCR 28 of the CR1 protein. Because the blood group antigens identified to date have been found in SCR 25, they are lost on trypsin treatment of the cells. This fact can be a useful tool not only in antibody identification but also for absorption to remove other antibodies from a sample.

Although the variability in antigen strength can be inherited, it may also be acquired. Diseases that have high levels of ICs being processed, e.g., systemic lupus erythematosus or HIV infection, can cause an increased loss of E-CR1, resulting in weakened Knops antigen strength.\(^{20}\) In addition, when RBCs are stored, vesicles are budded from the membrane, and these vesicles contain CR1.\(^{21}\) Therefore, the longer RBCs are stored, the weaker the Knops antigens become. This may explain why In(Lu) RBCs were initially reported to have weakened Knops antigens.\(^{22}\)

**Table 2. Characteristics of the Knops antigens**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Inherited as Mendelian-dominant traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-frequency RBC antigens (except Kn(^n) and McC(^b))</td>
<td>Developed on cord blood cells but may be weaker</td>
</tr>
<tr>
<td>Weaker on stored RBCs</td>
<td>Not destroyed by ficin or papain</td>
</tr>
<tr>
<td>Destroyed by trypsin treatment</td>
<td>Not found on platelets</td>
</tr>
<tr>
<td>Found in low levels in plasma but not in urine or saliva</td>
<td></td>
</tr>
</tbody>
</table>

**Knops Antibodies**

The Knops antibody characteristics are shown in Table 3. Two characteristics that were often attributed to Knops antibodies were (1) they were not neutralized with plasma, saliva, or urine, and (2) they were difficult to adsorb and elute. The latter most likely reflects the low density of the CR1 protein on the RBC membrane. However, Race and Sanger\(^{23}\) reported that adsorption performed with buffy coats (WBCs) were able to remove anti-Kn\(^n\) from serum. This led to the speculation that anti-Kn\(^n\) and related specificities were WBC antibodies. The ability of antigen-positive WBCs to adsorb Knops antibodies most likely relates to the fact that the CR1 copy number on WBCs is in the tens of thousands compared with a few hundred on RBCs.

**Table 3. Characteristics of Knops system antibodies**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Titer is dependent on E-CR1 levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Can demonstrate variable reactions</td>
<td></td>
</tr>
<tr>
<td>Not neutralized by pooled serum or other body fluids</td>
<td></td>
</tr>
<tr>
<td>Difficult to adsorb and elute from RBCs</td>
<td></td>
</tr>
<tr>
<td>IgG reacting by AHG technique</td>
<td></td>
</tr>
<tr>
<td>Do not bind complement</td>
<td></td>
</tr>
<tr>
<td>Usually not clinically significant</td>
<td></td>
</tr>
</tbody>
</table>

Although CR1 has not been found in the saliva, low levels have been found in both urine\(^{26}\) and plasma.\(^{27}\) These are believed to be the result of proteolytic cleavage of CR1 from leukocytes.\(^{28}\) Serum CR1 is present only in nanogram amounts,\(^{27}\) and therefore the levels are insufficient to neutralize Knops antibodies using routine serologic techniques. Hence, Moulds and Rowe\(^{29}\) developed an inhibition technique using recombinant, soluble CR1 (sCR1). Because their source of sCR1 was genetically positive for Kn\(^n\), McC\(^b\), Sl\(^a\), and Yk\(^a\), it would only inhibit these antibodies and not inhibit anti-Kn\(^n\) or McC\(^b\). It must be remembered that the Knops phenotype of the sCR1 will be dependent on the gene chosen for its production. More recently, these investigators...
have used mutated CR1 constructs to produce peptides capable of inhibiting anti-McCc and S12 (Vil).29

Clinical Importance

Because the Knops antibodies are found in the serum of multiply transfused individuals, these sera often contain additional alloantibodies directed at (for example) K, E, and Duffy antigens. Most of the Knops antibodies are not considered clinically significant because they do not cause overt hemolytic transfusion reactions or hemolytic disease of the fetus and newborn. Some examples of Knops system antibodies have been studied using in vitro tests such as the monocyte monolayer assay (MMA). Arndt and Garriott30 studied three anti-Knα, three anti-Ykα, and five anti-Kn/McC and found that only one had a macrophage index of greater than 20, suggesting increased RBC destruction. Other MMA studies of two anti-Knα, two anti-Slα, one anti-KCAM, and one anti-Yk/Cs all gave indexes of less than 0, indicating no enhanced RBC destruction (J.J. Moulds, unpublished data).

The CR1 Gene

The CR1 gene resides on chromosome 1 (1q32) and comprises 39 exons spread out over approximately 133 kilobase (kb) pairs of DNA.31 These exons encode SCRs of approximately 60 amino acids in the functional CR1 protein. Seven SCRs are organized into larger units called long homologous repeats (LHRs). The most common size protein product, CR1-1, is made up of four LHRs (A, B, C, D), a transmembrane region (TM), and a cytoplasmic tail (CYT), as shown in Figure 1. The binding sites for C3b and C4b have been localized to SCRs 8-9 and 15-16 (LHRs B and C) and to SCRs 1-2 (LHR-A), respectively.

Fig. 1. Schematic of the CR1-1 protein bearing the Knops antigens in SCR 25.

The CR1 gene also exhibits two other polymorphisms besides the Knops blood group. A structural, or size, polymorphism results from four different genes encoding four different molecular weight proteins: 190 kDa (CR1*3), 220 kDa (CR1*1), 250 kDa (CR1*2), and 280 kDa (CR1*4). Owing to the looping structure imparted by multiple disulfide bonds, the molecular weight shifts downward by approximately 30 kDa when the proteins are separated on SDS-PAGE using nonreducing conditions. It is now known that the molecular weight polymorphism is independent from the blood group polymorphism.

The third commonly recognized polymorphism is based on quantitative differences in E-CR1. A HindIII RFLP is detected by two allelic fragments of 7.4 or 6.9 kb on Southern blots. Homozygotes for the 7.4-kb fragment are high CR1 expressors (H), and homozygotes for the 6.9-kb fragment are low expressors (L) of CR1.32 Alternatively, a PCR-RFLP can be used that results in a band of 1.8 kb for H or 1.3 and 0.5 kb for L alleles.33 Although this RFLP correlates with RBC expression in Caucasian and Chinese individuals,34 there is no relationship between this polymorphism and CR1 expression in African Americans35 or West Africans.36 The exact molecular mechanism regulating CR1 RBC expression has been elusive but does not appear to be part of the promoter or 3′ untranslated portions of the CR1 gene.37

Molecular Basis of Knops Antigens

Using CR1 deletion constructs, Moulds et al.29 first localized the McCoy and S11 (Slα) antigens to LHR-D of CR1. By direct DNA sequencing they were then able to identify two separate mutations in SCR 25 that correlated with these two blood group antigens. The McCα/McCc polymorphism is at base pair 4795, where an A encodes proline (McCα) and a G encodes aspartic acid (McCCc). The S11/S12 mutation is only 11 amino acids away; at base pair 4828, an A encodes arginine and a G encodes glycine. Accordingly, the ISBT has now assigned these antigens to the Knops system with the numbers shown in Table 4.38

Table 4. Molecular basis of the Knops antigens

<table>
<thead>
<tr>
<th>ISBT number</th>
<th>Antigen</th>
<th>Nucleotide</th>
<th>Nucleotide‡</th>
<th>Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN1</td>
<td>Kna</td>
<td>4708G</td>
<td>4681G</td>
<td>Val11561</td>
</tr>
<tr>
<td>KN2</td>
<td>Knb</td>
<td>4708A</td>
<td>4681A</td>
<td>Met11561</td>
</tr>
<tr>
<td>KN3</td>
<td>McCα</td>
<td>4795A</td>
<td>4768A</td>
<td>Lys1590</td>
</tr>
<tr>
<td>KN4</td>
<td>S11</td>
<td>4828A</td>
<td>4801A</td>
<td>Arg1601</td>
</tr>
<tr>
<td>KN5</td>
<td>Ykα</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>KN6</td>
<td>McCα</td>
<td>4795G</td>
<td>4768G</td>
<td>Glu1590</td>
</tr>
<tr>
<td>KN7</td>
<td>S12</td>
<td>4828G</td>
<td>4801G</td>
<td>Gly1601</td>
</tr>
<tr>
<td>KN8</td>
<td>S13 (provisional)</td>
<td>4828A, 4855T*</td>
<td>4801A, 4828T*</td>
<td>Arg1601, Ser1610</td>
</tr>
<tr>
<td>KN9</td>
<td>KCAM</td>
<td>4870A</td>
<td>4843A</td>
<td>Ile1615</td>
</tr>
</tbody>
</table>

Note: The publication by Moulds et al.29 incorrectly lists the 4855 mutation as A>G. The correct substitution is T>A.

‡Nucleotide number when nucleotides are counted beginning with the A of the AUG start codon.

Two other mutations have been identified in SCR 25, one of which was found in a Caucasian and is related to S11. KMW was initially believed to have produced anti-Slα. However, after gene sequencing it was found that she was not only homozygous for S11 but was homozygous for another SNP at position 4855 that substituted threonine for serine at amino acid 1610. After extensive molecular studies, Moulds et al.39 proposed a model in which S13 was
a conformational epitope needing S11 (1601R) and S14 (1610S) to react. S14 and the antithetical antigen S15 are proposed epitopes awaiting the identification of the appropriate antibodies before they can be officially recognized. However, the related SNPs can be identified molecularly. Limited population studies of Caucasians, Blacks, and Asians predict that S14 may be a high-frequency antigen in all populations and that S15 predominantly occurs in Caucasians.

The final antigen assigned to the Knops system is KCAM (initially reported as KAM). Interestingly, the SNP producing KCAM was already known but had not been associated with a blood group specificity until a McCoy-like antibody was found in a Caucasian blood donor. On DNA sequencing, it was discovered that he was homozygous for an SNP at bp 4870 that substituted valine for isoleucine. Hence, it was assigned the number Kn9, and the antigen was renamed KCAM (KC for the city where the donor was found and AM for the donor’s initials).

**Disease Associations**

In 1997, Rowe et al. identified CR1 as a ligand for the rosetting of *Plasmodium falciparum*-infected RBCs with uninfected cells. The ability of erythrocytes infected with *P. falciparum* to form rosettes is a property shown by only some parasite isolates, but is of importance because it has been associated with severe malaria. These authors showed that CR1 on uninfected erythrocytes was required for the formation of rosettes by demonstrating that CR1-deficient erythrocytes (Helgeson phenotype) had reduced rosetting and soluble recombinant CR1 could inhibit rosetting. RBCs having the Sl:–1 phenotype showed reduced binding to the parasite rosetting ligand PfEMP1. Thus, the authors hypothesized that this polymorphism may have been selected for in malaria-endemic regions by providing protection against severe malaria. The hypothesis was then tested by studies of Africans in Mali and Kenya, where it was found that the combined Knops haplotype Kn(a+)/McC(a−)/McC(s−)/Sl:–1 appeared to be protective. CR1, as well as other complement receptors, has been identified as a receptor facilitating cell entry for a variety of pathogenic organisms. Pathogens using CR1 include *Leishmania major* (monocyte-macrophage), *Legionella pneumophila* (monocyte-macrophage), *Leishmania panamensis*, and *Mycobacterium tuberculosis* (monocyte-macrophage). In an AABB abstract, Noumsi et al. reported that individuals heterozygous for McC and McC were less likely to be infected by *M. tuberculosis* (odds ratio, 0.42; 95% confidence interval, 0.22 to 0.81; P = 0.007). These results suggested that McC may have evolved among African populations in the context of *M. tuberculosis* pressure, and conferred a survival advantage in its heterozygous form.

**Conclusions**

The molecular identification of the Knops blood group polymorphisms holds the promise for a better means of typing for these antigens, the only exception to this being the yet unidentified Yk*. Because of the inherited or acquired changes in RBC expression of CR1, i.e., Knops antigens, genotyping may become the method of choice for identifying these antigens, as it is for those of the Dombrock system. It is interesting to note that all of the known Knops polymorphisms are in SCR 25 of the CR1 protein. However, additional SNPs have been found in the CR1 gene, which suggests that the identification of new Knops blood group antigens is not yet at an end and will surely provide a challenge to serologists in the future.

**References**

37. Cockburn IA, Rowe JA. Erythrocyte complement receptor 1 (CR1) expression level is not associated with polymorphisms in the promoter or 3′ untranslated regions of the CR1 gene. Int J Immunogenet 2006;33:17–20.

Joann M. Moulds, PhD, MT(ASCP)SBB, Director, Clinical Immunogenetics, LifeShare Blood Centers, 8910 Linwood Avenue, Shreveport, LA 71106.

**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.

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Role for serial prenatal anti-Vel quantitative serologic monitoring with 2-ME serum treatment during pregnancy: case report


Anti-Vel is an uncommon antibody to a high-prevalence antigen. Its clinical significance and management in the prenatal setting are not well characterized. We present a case that demonstrates the utility of serial prenatal anti-Vel quantitative serologic monitoring with 2-ME serum treatment during pregnancy. The patient is a 23-year-old Hispanic woman with history of prior pregnancy and prior transfusion who was discovered to have an antibody to the high-prevalence Vel antigen in the first trimester (week 7) of her second pregnancy. Interval measurements of the serologic antibody titers were performed during the next 26 weeks. The untreated serum (IgM and IgG) titer increased from a baseline of 4 to 16 during that interval, while the 2-ME (presumed IgG component) titer remained stable at 4. Responding to ultrasound findings suspicious for fetal anemia, the child was delivered without complications at 34 weeks’ gestation. At birth, the DAT was negative and there was no evidence of HDN. Placed in the context of other similar reports, this case demonstrates the importance of separately reporting the IgG fraction (after either DTT treatment or 2-ME treatment) from the untreated (IgM and IgG) fraction and the importance of correlating the treated serum titer with potential clinical significance.

Key Words: Vel antigen collection, anti-Vel, prenatal serologic monitoring, 2-ME-treated serum, pregnancy

Vel is a high-prevalence antigen first described by Sussman et al. in 1952 in association with a transfusion reaction. Although several additional cases quickly confirmed these authors’ findings, our knowledge of the Vel antigen (collection) remains incomplete. The presence of the Vel-negative phenotype in the general population is estimated to be 1 in 4000 individuals. Vel alloimmunization can be mediated through IgG or IgM. Anti-Vel is thought to be associated rarely with hemolytic disease of the fetus and newborn (HDFN) because many examples of anti-Vel are mostly IgM and Vel is weakly expressed on the surface of fetal RBCs. Although the risk may be low, it is reasonable to monitor prenatal serologic titers serially during pregnancy. The findings published in a recent case report by van Gammeren et al. of severe HDFN from anti-Vel, support this approach. The van Gammeren case report describes discovery in a prenatal patient of an anti-Vel, which at initial presentation was determined to be composed exclusively of IgM. At the end of the pregnancy, an anti-Vel titer of 64 was demonstrated in untreated serum, whereas the DTT-treated serum (presumed IgG content) titer rose to 16. In their case, the infant exhibited severe jaundice and reticulocytosis. As a corollary to the van Gammeren case report, we present a case of an anti-Vel for which the IgG titer as determined by 2-ME treatment was stable through pregnancy despite a significant rise in the titer of the anti-Vel in the untreated serum. At birth, the child showed no evidence of HDFN. This case report supports the importance of serial determination of the prenatal IgG titer to help assess risk of HDFN.

Case Report

A 23-year-old Hispanic woman, with a history of prior blood transfusion and one successful pregnancy, presented initially early in the first trimester of the index pregnancy. Her RBCs were typed as group O, D+. Routine serologic testing demonstrated variable reactivity using gel technology. The autocontrol was negative. Further testing using tube technology with PEG as enhancement also demonstrated variable weak reactivity, again with no specificity. No reactivity was demonstrated when using tube technology with LISS. A repeat serologic evaluation was performed 4 weeks later to help clarify the nature of this reactivity. The subsequent serologic evaluation demonstrated reactivity with all RBCs tested whether using gel or test tube technology with LISS or PEG methods. The autocontrol was consistently negative. As an antibody to a high-prevalence antigen was suspected, the sample was referred to a large nationally recognized immunohematology reference laboratory. With use of rare RBCs lacking various high-prevalence antigens, reactivity was determined to be that of anti-Vel. The patient’s RBCs were shown to lack the Vel antigen. The initial quantitative serologic titer was 4 when tested against Vel+ RBCs using both 2-ME-treated and untreated serum. The Marsh score ranged from 14 with untreated serum to 4 with 2-ME-treated serum. The developing baby’s RBCs were presumed to be Vel+ as the father’s RBCs were demonstrated to express the Vel antigen. Subsequent quantitative serologic monitoring was performed in approximately 4- to 6-week intervals for the next 20 weeks of pregnancy (Table 1).

At 33 2/7 weeks’ gestation, Doppler ultrasonography showed an increase in middle cerebral artery velocity (>1.5 multiples of the median [MoM] for gestational age) suspicious for fetal anemia. This finding was a significant change from previous Doppler ultrasound studies and corresponded with the change in quantitative serologic titer of the
untreated serum from 4 at presentation to 16 by week 26 (Table 1). A multidisciplinary risk-benefit analysis, including consideration of potential maternal and fetal need for transfusion with an extremely limited blood supply (two available units), the unpredictable timing issues encountered with induction, and the limited time-availability for transfusion after thawing the available units, prompted the decision to move toward delivery in a manner that optimized the availability of blood for the newborn, if it was indeed anemic. The patient underwent cesarean delivery at 34 3/7 weeks’ gestational age, 2 days after the initial administration of Celestone to promote fetal pulmonary maturity. A 3088-g male newborn with Apgar scores of 7 at 1 minute and 9 at 5 minutes was born without complication. The child’s RBCs typed as group A, D+, and the antibody screen was negative. Initial laboratory data were also remarkable for a Hb level of 16.6 g/dL (normal range), reticulocyte count of 3.5% (normal range), and negative DAT. Approximately 1 week after delivery, a mild rise in bilirubin was observed, reaching a peak of 10 mg/dL. Given that the DAT and the initial screen were negative, the mild hyperbilirubinemia was thought not likely to have resulted from transplacental anti-Vel or a possible anti-A from a group O mother. Moreover, although laboratory data are a bit limited, there was no evidence of anemia or hemolysis. Importantly, the mean corpuscular hemoglobin was 18.4 (normal range), reticulocyte count 4.9 at admission and 9 at 5 minutes was born without complication. The patient’s serum was diluted in 6% albumin. A 30-minute 37°C incubation was performed, followed by a saline IAT (Anti-IgG, Immucor Gamma Inc.).6 Reaction scoring was performed using the system originally described by Marsh.7 The patient’s serum was treated with 2-ME (Fisher Scientific Co., Hanover Park, IL) and dialyzed overnight using dialysis membranes (Spectra/Por2, 12,000 to 14,000 dalton molecular weight cut-off [MWCO], Spectrum Laboratories, Inc., Rancho Dominguez, CA) in PBS, pH 7.3. Dialed serum was tested using the methods previously described.

### Discussion

This case adds to the limited published information regarding the natural history of anti-Vel in the prenatal setting, and it supports the value of sorting immunoglobulin classes as an aid to proper prenatal management. It is worth noting that at initial presentation, weak, variable serologic reactivity was demonstrated. Despite use of several standard methods, specificity could not be assigned to the serologic reactivity. In the initial serologic evaluation, reactivity was present using gel technology and tube technology with PEG as enhancement. Tube testing with LISS showed no reactivity. Although a LISS tube method is considered an appropriate antibody detection method in the prenatal setting,8 this method would not have detected the antibody. A repeat study approximately 7 weeks after the initial study did demonstrate the presence of strong serologic reactivity using tube testing with LISS and PEG as well as with gel. The titer of the untreated serum and 2-ME–treated serum was 4. Presumably, late in the first trimester of pregnancy the mother was exposed to fetal RBCs expressing the Vel antigen because by week 14 she demonstrated a specific serologic immune response. Interestingly, for the remainder of the pregnancy, the titer of the anti-Vel in the 2-ME (presumed IgG component)–treated serum remained stable although the titer in the untreated serum (presumed IgM and IgG) increased by two dilutions. This observation may be useful to laboratories without the capacity to treat patient serum with 2-ME or DTT. Namely, in this case anti-Vel titer in the untreated serum appeared to rise before the anti-Vel IgG titer in the 2-ME–treated serum.

The rise in antibody titer in the untreated serum combined with the abnormal Doppler velocimetry prompted the preterm delivery. At birth, the DAT and antibody screen were negative, and the hemoglobin and reticulocyte count were in the normal range. As the anti-Vel present in the maternal serum contained an IgG component, the negative DAT suggests that Vel antigen on the fetal RBCs may either be weakly or partially expressed9 or the antibody avidity to the antigen was poor. Regardless, in this case, an anti-Vel titer of 4 does not appear to be independently sufficient for HDFN, whereas a titer of 16 in the case reported by van Gammellen et al.5 was associated with HDFN. The cause of the rise in

### Methods

ABO and D testing and DAT were performed using commercially available reagents according to manufacturers’ protocols using tube method (Immucor Gamma Inc., Norcross, GA). Antibody detection and identification were performed using either gel technology according to the manufacturer’s protocol using reagent cells (RBCs 0.8% Surgiscreen and reagent RBCs 0.8% Resolve Antigram, ID-MTS Gel Test; Ortho-Clinical Diagnostics, Raritan, NJ) or tube method with commercially available cells and reagents (PEG and LISS, Immucor Gamma Inc.). Titration studies performed at the immunohematology reference laboratory used tube method. The patient’s serum was diluted in 6% albumin. A 30-minute 37°C incubation was performed, followed by a saline IAT (Anti-IgG, ImmucorGamma Inc.).6 Reaction scoring was performed using the system originally described by Marsh.7 The patient’s serum was treated with 2-ME (Fisher Scientific Co., Hanover Park, IL) and dialyzed overnight using dialysis membranes (Spectra/Por2, 12,000 to 14,000 dalton molecular weight cut-off [MWCO], Spectrum Laboratories, Inc., Rancho Dominguez, CA) in PBS, pH 7.3. Dialed serum was tested using the methods previously described.

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the bilirubin level after birth remains uncertain, although liver immaturity is the most likely explanation. Finally, the abnormal Doppler velocimetry in the absence of true fetal anemia reflects the inherent limitations of a screening test that is known to have a 12 percent rate of false-positive results.10

The Vel antigen (collection) and anti-Vel remain enigmatic and are in need of further study. To date, it is known that the Vel-negative phenotype is universally uncommon. The incidence of the Vel-negative phenotype in the Hispanic population is not known with certainty. Anti-Vel is famously associated with in vitro hemolysis, severe transfusion reactions, and shortened RBC survival,11 although there is at least one case report of Vel+ blood being successfully transfused to a recipient possessing anti-Vel.12 An auto-anti-Vel13 has been described, although it is uncertain to what degree the autoantibody contributed to the patient’s RBC destruction. Anti-Vel can be difficult to detect, and it can simulate the serologic profile of a cold-reactive autoantibody, with disastrous consequences if improper analytic methods are used.14 The Vel antigen appears to have a variable antigenic expression and appears to be best expressed on adult cells.

When viewed in concert with the recent case report by van Gammeren et al.,5 our case report further supports the need not only to properly identify anti-Vel in the prenatal setting but also to perform serial quantitative serologic monitoring using methods to clearly separate the IgG fraction (DTT treatment or 2-ME treatment) from the untreated (IgM and IgG) fraction. Although both the untreated and treated serum titer and score results may be reported, changes in the treated serum fraction, representing the IgG component, may be a better trigger to initiate additional clinical investigation. Additional cases may help identify the most appropriate critical 2-ME or DTT titer for anti-Vel.

References

Walter J. Linz, MD, MBA, Medical Director, Scott and White Transfusion Service and Donor Center, Assistant Professor of Pathology, Texas A&M UHSC, 2401 South 31st Street, Temple, TX 76508; Judith T. Fueger, MT(ASCP)SBB, Immunohematology Reference Laboratory, Diagnostic Laboratories, BloodCenter of Wisconsin, Milwaukee, WI; Steven Allen, MD, Associate Professor, Texas A&M UHSC/Scott and White Memorial Hospital, Chair, Department of Ob/Gyn, Director, Division of Obstetrics and Gynecology, Scott & White Healthcare and Texas A&M University Health Science Center College of Medicine, Temple, TX; and Susan T. Johnson, MSTM, MT(ASCP)SBB, Immunohematology Reference Laboratory, Diagnostic Laboratories, BloodCenter of Wisconsin, Milwaukee, WI.

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Granulocyte serology: current concepts and clinical significance

M.E. Clay, R.M. Schuller, and G.J. Bachowski

Applying serologic procedures to the detection of RBC and lymphocyte antigens has facilitated the identification of granulocyte antigens with established clinical significance, which are now classified in the human neutrophil antigen system. Granulocyte alloantibodies and autoantibodies have been implicated in a variety of clinical conditions including alloimmune neutropenia, autoimmune neutropenia, febrile and severe pulmonary transfusion reactions, drug-induced neutropenia, refractoriness to granulocyte transfusions, and immune neutropenia after hematopoietic stem cell transplantation. Although the intrinsically fragile nature of granulocytes contributes to the inherent challenges of granulocyte serology, several advances in laboratory procedures have improved detection of granulocyte antibodies. This review will provide a current perspective about the importance and use of granulocyte serology for detection of granulocyte antibodies that have significant medical effects.

**Key Words:** granulocyte, neutrophil, antibodies, HNA, alloimmune neonatal neutropenia, autoimmune neutropenia, transfusion-related acute lung injury, TRALI

**Clinical Significance**

**Alloimmune Neonatal Neutropenia**

Neutropenia observed in the neonate is primarily the result of neutrophil-specific alloantibodies transplacentally transferred to the fetus from the maternal circulation. The presence of these human neutrophil antigen (HNA) antibodies most likely is the result of prenatal maternal sensitization by fetal neutrophils crossing the placental barrier, although the antibodies may have also arisen in association with autoimmune diseases such as systemic lupus erythematosus or rheumatoid arthritis. Maternal alloimmunization can occur anytime after the first trimester of pregnancy. Once present in the fetal circulation, maternal HNA antibodies will bind to the mature fetal cells expressing the corresponding neutrophil alloantigens, as the neutrophil precursor cells are spared. Neonates affected by alloimmune neonatal neutropenia (ANN) are almost always neutropenic at birth, although cases have been reported in which the neutropenia has been delayed by 1 to 3 days. ANN is often asymptomatic and goes undiscovered unless the deficit of the neutrophils is profound enough (absolute neutrophil count < 1.5 × 10^9/L) that the newborn becomes susceptible and succumbs to infection caused by bacterial or fungal pathogens. These children commonly present with fever, malaise, lethargy, skin infections such as cellulitis and omphalitis, mucosal and respiratory infections (stomatitis, otitis media, and pneumonia), urinary infections, and very rarely septicemia. These infections are usually mild, but fatalities have been reported in 5 percent of cases. As there are numerous reasons for neonatal neutropenia (i.e., congenital, antibody-mediated destruction, or infection that decreases hemopoietic cell production), detection of these maternal neutrophil antibodies is very important for determining the mechanism causing neutropenia. This allows the physician to focus on the appropriate treatment. The presence of maternal antibodies can persist up to 6 months after birth although antibodies and clinical effects usually dissipate more quickly. The incidence of ANN has been estimated to be 0.1 to 0.2 percent. Currently, neutrophil antibodies with the following specificities have been implicated in cases of ANN: HNA-1a, HNA-1b, HNA-1c, FcγRIIIb (CD16), HNA-2a, HNA-3a, and HNA-4a.

It has now been half a century since Lalezari described the first case of neonatal neutropenia attributable to transplacental transfer of granulocyte-specific antibodies, thus initiating the field of granulocyte serology. This event fostered several decades of work directed at the identification of granulocyte-specific antigens (and antibodies), methods for their detection, and an appreciation for their clinical significance. In many ways, granulocyte serology developed along the laboratory footprints of RBC and human lymphocyte antigen (HLA) serology with one major exception—granulocytes could not be preserved for testing purposes. The requirement for fresh cells was and continues to be a major impediment for wide-scale implementation of granulocyte immunobiology studies. Although the number of laboratories worldwide that specialize in this technology is limited, the clinical significance of granulocyte antibodies has not diminished and is now supporting the development of new methodologies that have the potential to enhance the utility of testing and growth of this field. Therefore, as we move ahead, it is important to (1) focus on the clinical events that support the need for granulocyte serology, (2) understand the benefits and limitations of the current serologic assays, and (3) evaluate the application of new technologies for the detection of granulocyte antigens and antibodies.
Autoimmune Neutropenia
Autoimmune neutropenia (AIN) occurs in both infants and adults. It is the most common form of chronic neutropenia in infants. Severe neutropenia is usually recognized 4 to 7 months after birth. In the vast majority of AIN cases infants exhibit relatively mild clinical issues such as stomatitis, otitis, and respiratory infections. The presence of clinical symptoms lessens as the child becomes older. Neutropenia in infants is self-limited with complete resolution commonly observed within 7 to 24 months. However, this condition necessitates aggressive hygienic care of infants to prevent infection and vigorous therapy that may include severe antibiotic therapy along with treatment with recombinant G-CSF and IVIG when infection occurs. Cell destruction can occur in the peripheral circulation in addition to interference with myelopoiesis in the bone marrow. Complete blood counts demonstrate absolute neutropenia, although random fluctuations in the neutrophil count can range from zero to near normal.

AIN is also observed in adults. Monocytosis with or without lymphopenia may also be present. As in infants, the bone marrow commonly demonstrates an absence of mature cells with an increase in myeloid precursors. This apparent imbalance of precursors to mature segmented neutrophils is distinct in cause from the maturation arrest that occurs in congenital defects of neutrophils or hematologic malignancy. Unlike RBC autoantibodies, antibodies in AIN are often reported to have specificity that can include HNA-1a, HNA-1b, and HNA-2a.

Drug-Induced Neutropenia
Although neutrophil antibodies are believed to be involved in drug-induced neutropenia, neither the precise mechanisms nor the particular antigens on the cell surface have yet been clarified. Some cases of drug-induced granulocytopenias probably result from direct marrow toxicity, although immune-mediated processes also occur. Quinine, quinidine, ibuprofen, and psychotropic medications such as clozapine are recognized as potential causes of neutropenia. Drug-dependent antibodies have been found to react with both granulocytes and granulocyte precursors. The FeyRIIib and CD177 neutrophil glycoproteins have been reported to form neoantigens with drugs or their metabolites, which are recognized by drug-dependent neutrophil antibodies.

Transfusion Reactions
Antibodies to neutrophil and HLA antigens can result in a variety of transfusion reactions. The spectrum of severity can range from mild febrile reactions to death in the case of severe transfusion-related acute lung injury (TRALI). Recently TRALI has been shown to be the most common cause of transfusion-related fatalities in the United States. Febrile nonhemolytic transfusion reactions are common and can occur when blood products containing WBCs are transfused into patients who have leukocyte antibodies.

The occurrence of febrile reactions has been mitigated with the use of leukocyte-reduced RBCs and platelet products. As early as 1951 blood transfusions were implicated in noncardiogenic lung edema. The term TRALI was conceived in 1985 when Popovsky and Moore investigated a series of 36 patients with well-defined transfusion-related acute lung injury. They detected leukocyte (HLA and HNA) antibodies in 89 percent of the implicated donors. Seventy-two percent of these patients were treated with mechanical ventilation, and 6 percent died. The leading theories for the cause of TRALI involve the priming or activation of neutrophils with antigen-antibody interactions, inducing an acute inflammatory response. Antibodies to HLA class I and class II as well as HNAs have all been implicated in the development of TRALI.

Even though HLA class I alloantibodies are the most frequently encountered WBC antibodies in implicated blood donors, they may act as much weaker triggers of acute lung injury when compared with HLA class II and HNA antibodies. In a study designed to investigate leukocyte antibody specificities in severe TRALI reactions, Reil et al. reported that even though 73 percent of leukocyte antibodies identified in their donor population were HLA class I, HLA class II and HNA antibodies were associated with 81 percent of the antibody-mediated TRALI cases. Specifically, in their 36 reported cases, 17 were elicited by blood products containing HLA class II antibodies, and HNA antibodies were implicated in 10 cases. Of the 10 fatal outcomes, 6 were linked to HNA-3a antibodies in the transfused blood products, and HLA class II antibodies were associated with 3 fatalities.

Although leukocyte agglutinating (aggregating) antibodies have been frequently associated with TRALI, the ability of HNA-3a antibodies to also prime and activate neutrophils in the pulmonary vasculature is thought to be central to severe and fatal TRALI. Studies have now shown that in addition to HNA-3a antibodies, HNA-2a and HNA-4a antibodies also have the ability to prime neutrophils in vitro.

Although the pathophysiology of TRALI appears complex and remains under investigation, substantial clinical and scientific information suggests that HLA (especially class II) and HNA antibodies do play a significant role in this disorder. Efforts are now being focused on detecting HLA and HNA antibodies in donors both to retrospectively investigate the cause of acute lung injury in blood recipients and to serve as a valuable strategy in preventing TRALI by identifying donors who may be responsible for the transfusion reaction.

Granulocyte Antigens
Granulocyte-specific antigens are those with a tissue distribution restricted to granulocytes (neutrophils, eosinophils, and basophils), whereas neutrophil-specific antigens are only present on neutrophils. Because of the difficulty in characterizing antigens on basophils and eosinophils, many
Antigens described as neutrophil-specific have not been tested to determine whether they are present only on neutrophils or on all granulocytes. Although granulocyte (neutrophil) antigen systems were initially identified through studies of ANN and AIN, both terms, granulocyte and neutrophil, have been used to describe the alloantigens.

**Nomenclature**

Lalezari and colleagues\(^8\) identified the first granulocyte antigen during their investigation of a neonate with transient ANN and subsequently proposed the first nomenclature system for these antigens. Because their studies indicated that the antigen was neutrophil-specific, they designated it the N system and the antigens were named in chronologic order of discovery. The antigens were labeled alphabetically and the alleles were described numerically. The system eventually classified seven antigens: NA\(^1\), NA\(^2\), NB\(^1\), NB\(^2\), NC\(^1\), ND\(^1\), and NE\(^1\), which became the foundation for granulocyte serology.

During this same period and subsequently, several different granulocyte antigens were identified. Some were specific for granulocytes (or neutrophils): HGA-3, GA, GB, GC, Gr1, Gr2\(^{47–49}\); some were shared with other cells or tissues: HGA-1, 5a, 5b, 9, MART\(^{47,50–52}\); and some had unclassified distribution: CN\(^1\), KEN, LAN, LEA, SL\(^{53–56}\). All of these granulocyte antigens were detected with sera from patients with autoantibodies or alloantibodies directed against granulocytes, using traditional blood group serologic techniques such as agglutination, immunofluorescence, and cytotoxicity. Unfortunately, most of these antigens have not been further classified owing to the lack of sufficient biologic material for study.

Although this led to a consolidation of individuals working in this field, advances were made and efforts were directed at immunobiochemical, genetic, and structural or functional studies for a few of the well-classified granulocyte antigens. With the advent of this information came the need for a standardized granulocyte antigen nomenclature system based on the molecules carrying the antigens and the genes encoding each allele. In 1999, the Granulocyte Antigen Working Party of the International Society of Blood Transfusion (ISBT) established a new nomenclature system for the well-characterized granulocyte antigens, which was based on the glycoprotein location of the antigens.\(^57\) In this system the granulocyte antigens are called human neutrophil alloantigens to indicate they are expressed on neutrophils; however, this does not imply that they are neutrophil-specific. Each antigen group is assigned a number, and polymorphisms within the group are designated alphabetically in sequential order of detection. The HNA system currently includes seven antigens, which are restricted to five antigen groups. The key features of the HNA system are summarized in Table 1. An extensive amount of information is now known about these antigens, and the reader is referred to Bux’s recent and comprehensive review about the antigens that constitute this system.\(^58\)

HNA-1 is the best-characterized group, and it contains three antigens: HNA-1a, HNA-1b, and HNA-1c. The HNA-1 antigens are expressed only on neutrophils, and antibodies to these antigens are frequently implicated in cases of alloimmune or autoimmune neutropenia.\(^58\) The antigens are epitopes that occur on the neutrophil low-affinity FcγRIIIb receptor (CD16). This molecule binds the Fc region of IgG antibodies complexed to other antigens or immunoglobulins. The gene frequencies of HNA-1a, HNA-1b, and HNA-1c vary among different ethnic populations.\(^38\) Furthermore, individuals lacking FcγRIIIb have been identified. These individuals do not express the HNA-1 antigens on their neutrophils, thus presenting as an HNA-1 null phenotype.\(^54,62\)

The HNA-2 group has one characterized antigen—HNA-2a—which is located on a 58- to 64-kDa glycoprotein that is expressed only on neutrophils.\(^63\) Although HNA-2a is a high-frequency antigen phenotype (i.e., greater than 90% for most populations), it is unique in that it is expressed on subpopulations of neutrophils among antigen-positive people. The expression of HNA-2a is greater on neutrophils from women than men, and two or three subpopulations of expression are often detected: one population that lacks HNA-2a expression and one or two populations that express the antigen but with different intensities.\(^64\) The functional

### Table 1. Human neutrophil antigens†

<table>
<thead>
<tr>
<th>Antigen groups</th>
<th>Antigens</th>
<th>Former name</th>
<th>Antigen location/ glycoprotein CD number</th>
<th>Coding gene alleles‡</th>
<th>Clinical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNA-1</td>
<td>HNA-1a</td>
<td>NA1</td>
<td>FcγRIIb/CD16b</td>
<td>FCGR3B*01</td>
<td>ANN, AIN, TRALI§</td>
</tr>
<tr>
<td></td>
<td>HNA-1b</td>
<td>NA2</td>
<td>FcγRIIb/CD16b</td>
<td>FCGR3B*02</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>HNA-1c</td>
<td>SH</td>
<td>FcγRIIb/CD16b</td>
<td>FCGR3B*03</td>
<td>Unknown</td>
</tr>
<tr>
<td>HNA-2</td>
<td>HNA-2a</td>
<td>NB1</td>
<td>58–64 kDa/CD177</td>
<td>Unknown</td>
<td>ANN, AIN, TRALI, febrile transfusion reactions, drug-dependent neutropenia</td>
</tr>
<tr>
<td>HNA-3</td>
<td>HNA-3a</td>
<td>5b</td>
<td>CTL2/Unknown</td>
<td>FCGR3*03</td>
<td>ANN, TRALI, febrile transfusion reactions</td>
</tr>
<tr>
<td></td>
<td>HNA-3b</td>
<td>5a</td>
<td>CTL2/Unknown</td>
<td>Unknown</td>
<td>ANN, TRALI, febrile transfusion reactions</td>
</tr>
<tr>
<td>HNA-4</td>
<td>HNA-4a</td>
<td>MART</td>
<td>CR3/CD11b (a M subunit)</td>
<td>ITGAM*01 (230G)</td>
<td>ANN</td>
</tr>
<tr>
<td>HNA-5</td>
<td>HNA-5a</td>
<td>OND</td>
<td>LFA-1/CD11a (a L subunit)</td>
<td>ITGAL*01 (237G)</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

†Information from references 20, 58, 59, 60.
‡Alleles of the coding genes are named according to the ISGN Guidelines for Human Gene Nomenclature.\(^61\)
§Except HNA-1c.
AIN = autoimmune neutropenia; ANN = alloimmune neonatal neutropenia; CD = clusters of differentiation; CR3 = complement receptor 3; CTL2 = choline transporter-like protein 2; FcγRIIb = Fc gamma receptor IIb; HNA = human neutrophil antigen; LFA-1 = leucocyte function antigen-1; TRALI = transfusion-related acute lung injury.
role of HNA-2a is not known, and antibodies to this antigen have been associated with AIN, ANN, febrile transfusion reactions, TRALI, and drug-dependent neutropenia.

HNA-3a is expressed on granulocytes, lymphocytes, platelets, endothelial cells, kidney, spleen, and placental cells. It is a high-frequency antigen located on choline transporter-like protein 2 (CTL2), and its function is not known. Alloantibodies to HNA-3a have been associated with occasional cases of febrile transfusion reactions and one case of ANN. Although antibodies to HNA-3a are rare, their major clinical significance has been their association with serious and fatal TRALI events.

The HNA-4 and HNA-5 antigens are located on subunits of the β2 integrin family (CD11a and CD11b molecules). Integrins are a large family of adhesive receptors that are essential for cell-cell interactions and cell trafficking. HNA-4a is an epitope on the α M (CD11b) chain of CD11b/CD18 (the CR3 molecule), and HNA-5a is an epitope on the α L chain (CD11a) of CD11a/CD18 (the leukocyte function antigen-1 [LFA-1] molecule). HNA-4a is expressed on granulocytes, monocytes, and natural killer cells, whereas HNA-5a is expressed on all leukocytes. Alloantibodies to HNA-4a can cause ANN, and the CD11b/CD18 complex has been reported to be the target of autoantibodies. To date, HNA-5a antibodies have not been clinically associated with neutropenia.

A general consensus exists that granulocytes do not express ABO antigens or HLA class II antigens. Although the density of HLA class I antigens on granulocytes is fairly low, it can vary among individuals. Therefore, these antigens must be taken into consideration when patient samples are tested for granulocyte antibodies. HLA class I antigens may be of particular interest during granulocyte transfusion for neutropenic patients as these antigens, in granulocyte concentrates, may be introduced in high amounts to recipients with preformed class I antibodies. Transfusion reactions including TRALI have been observed in this situation, and the antigen-antibody interaction has been proposed to limit effectiveness of the transfusion.

**SeroLogic Testing**

The detection of HNA antibodies is labor intensive and technically challenging. Unlike HLA testing, commercial test kits are not readily available for the detection of granulocyte antibodies by solid-phase methodologies. Therefore, intact viable granulocytes are required for granulocyte antibody screening. Granulocytes are intrinsically designed to respond to physiologic priming signals, so they must be handled very carefully to prevent activation. Once activated it is impossible to interpret any serologic test results involving these cells, as false-positive test results abound. Granulocytes are also extremely labile and must be used within 24 hours of collection, necessitating ready access to panel cell donors with the needed antigen phenotypes. Although many have tried, attempts to develop short-term or long-term granulocyte preservation procedures have been unsuccessful, thus requiring that fresh suspensions of granulocytes be prepared daily for testing procedures. Finally, the presence of HLA antibodies in test sera makes identifying HNA antibodies difficult because granulocytes also express HLA class I antigens on their cell surface.

**Preparation of Pure Granulocyte Suspensions**

The isolation of pure granulocyte suspensions from peripheral blood was greatly facilitated and simplified with Bøyum’s introduction in 1968 of the Ficoll-Isopaque gradient technique. Double-density gradient centrifugation is now the most widely used approach for the isolation and purification of granulocytes for serologic testing. The procedure makes use of discontinuous Ficoll gradients, and numerous publications exist describing the methodology and the recovery, purity, functional integrity, and activity of the isolated granulocytes.

**Granulocyte Antibody Detection and Antigen Typing**

Laboratory approaches for the detection of granulocyte antibodies require procedures that are accurate, reproducible, and practical. Although numerous granulocyte serologic assays have been described, not all meet these criteria. In addition to the assay used, the ability to detect antibodies in test specimens or antigens on test cells may also depend on target cell antigen density and the concentration and immunoglobulin type of the antibody in the test reagent. Therefore, the successful detection of granulocyte antibodies often requires that a combination of methods be used.

Although several methods for the detection of granulocyte-specific antibodies are used by the few laboratories that perform this testing, the ISBT Working Party on Granulocyte Immunobiology, a worldwide consortium of 16 laboratories that participate in the annual International Granulocyte Immunology Workshop (IGIW), have recommended that granulocyte antibodies should be investigated using a minimum of two methods: the granulocyte immunofluorescence test (GIFT) and the granulocyte agglutination test (GAT). Multiple workshops have shown the sensitivity of GIFT to far exceed that of GAT; however, agglutinating antibodies such as HNA-1c, HNA-3a, and HNA-4a are much more readily detectable in GAT and in many cases it is the only reliable method for detecting these specificities. For example, the seventh IGIW included a test specimen that contained an HNA-3a antibody that could only be detected by GAT and the granulocyte chemiluminescence test (GCLT). Although GCLT has been useful in selective laboratory settings, it is not routinely used for clinical granulocyte antibody detection and is not discussed.
Antibody Detection Methods
Granulocyte Agglutination Test

Lalezari developed the basis of this assay in the early 1960s. Since that time, micro techniques and the use of pure granulocyte suspensions with optimized concentrations of EDTA have vastly improved the performance of this test. Typically, a panel of three to five donors is selected to include all known neutrophil antigens currently defined by the ISBT consortium. Purified granulocytes are isolated from EDTA-anticoagulated peripheral whole blood using a double-density gradient. These granulocytes are then washed in PBS before use. The test is biphasic and is based on the intrinsic response of granulocytes to aggregate when stimulated by antibodies reacting to corresponding cell surface antigens. The resulting agglutination is the consequence of the granulocyte activation that occurs during the sensitization phase, which induces the cells to form pseudopods and slowly migrate toward one another during the aggregation phase, until membrane contact is established. Typically, the serum or plasma from the specimen being investigated is incubated with the granulocyte suspension for 4.5 to 6 hours at 30°C. The reactions are evaluated using an inverted-phase microscope and graded from negative to 4+ on the basis of the percentage of cells that are agglutinated. Our laboratory has determined cutoff scores for both adult and pediatric patients (<6 years old) on the basis of the reaction grades established during microscopic evaluation. Both IgG and IgM antibodies are detected by this method.

Granulocyte Immunofluorescence Test

Developed by Verheugt et al. in the late 1970s, this fluorescent “antiglobulin” technique is used for the detection of granulocyte alloantibodies and autoantibodies that are circulating and cell bound. As with the GAT, an antibody screening panel of three to five donors is selected to include all currently defined HNA antigens. A purified granulocyte suspension is prepared and treated with 1% paraformaldehyde for a short time to prevent nonspecific immunoglobulin binding to the neutrophil Fc receptors and to stabilize the cell membrane. Serum or plasma from the patient is then incubated with an optimized concentration of granulocytes for 30 minutes at 37°C. After a wash step that removes unbound antibodies, the granulocytes are then incubated with F(ab′)_2 fragments of a fluorescent conjugated anti-human antibody for approximately 30 minutes at room temperature in a dark environment. The assay’s performance is optimized with the use of a fluorescent secondary probe that can detect both IgM and IgG antibodies (to ensure the detection of both primary and secondary immune responses) and the use of F(ab′)_2 Ig fragments to prevent the probe from binding to the high concentrations of Fc receptors on granulocyte surface membranes. The cells undergo another wash cycle, are resuspended, and then are analyzed. Detection of the immunofluorescence reactions can be accomplished by using either a fluorescence-detecting microscope or a flow cytometer. Evaluation by a flow cytometer has in most cases replaced the fluorescence microscope as more cells can be analyzed in a shorter time, resulting in improved assay sensitivity and reproducibility, and it does not require the expertise needed to evaluate the characteristic staining pattern seen with specific granulocyte antibodies by microscopic analysis.

Monoclonal Antibody Immobilization of Granulocyte Antigens

The monoclonal antibody immobilization of granulocyte antigens assay (MAIGA) is based on the platelet version (MAIPA) developed by Kiefel et al. in 1987. MAIGA relies on the capture of neutrophil-specific antigen-antibody complexes by a murine monoclonal antibody onto a solid-phase surface. The benefits of this test are twofold: first, this is currently the most sensitive assay for the detection of granulocyte antibodies, and second, the assay is designed to detect only HNA antibodies even when HLA antibodies are present in the test specimen. The disadvantage of this procedure is that it is very complex and requires highly skilled staff to perform the detailed techniques.

Granulocytes are incubated with the test serum or plasma for 30 minutes at 37°C. This granulocyte suspension is then washed to remove any unbound immunoglobulins and incubated with a murine monoclonal antibody to a specific neutrophil glycoprotein for an additional 30 minutes. After another wash step, the granulocyte membranes are disrupted in a mild detergent and centrifuged. The resulting lysate is then transferred to polystyrene microwells coated with anti-mouse immunoglobulins for incubation. The trimolecular neutrophil antigen–patient HNA antibody–murine monoclonal antibody complex present in the lysate is captured on the solid phase, whereas any HLA antibody-antigen complexes (if present) are removed by a subsequent wash step. Remaining complexes are then detected by the addition of anti-human IgG conjugated to horseradish peroxidase followed by a substrate (OPD dissolved in 30% H,O), and the reaction is analyzed with a spectrophotometer.

MAIGA can be used to detect antibodies specific to HNA-1a, HNA-1b, and HNA-1c antigens located on FcγRIIIb (CD16); HNA-2a present on gp 58- to 64-kDa (CD177); HNA-4a on the complement component receptor C3bi (CD11b); and HNA-5a on the LFA-1 receptor (CD11a). HNA-4a and HNA-5a antibodies can also be detected by a common CD18 monoclonal antibody.

Antigen Detection and Typing Methods

The same methodology that is used to detect antibodies in serum and plasma can be used to characterize the antigen phenotype of an individual’s granulocytes. Just as the short lifespan of granulocytes is a limitation to obtaining panel cells for antibody testing, it also restricts the ability to do antigen typing because granulocyte specimens must be tested
within 24 hours of collection to obtain reliable results. A sufficient volume of whole blood must be collected to obtain an adequate number of granulocytes for phenotyping. This can be a dilemma in pediatric patients or patients who are neutropenic. Because ample volume, freshness, and availability of granulocytes is critical, this also acts as a practical barrier to doing donor-recipient crossmatch testing during investigations of suspected granulocyte-antibody–mediated transfusion reactions such as TRALI. The highly characterized antisera used to phenotype granulocytes should be free of HLA class I antibodies as their presence can result in a false-positive HNA typing. At a minimum, the titer of the HLA antibody should be weaker than the HNA antibody as it can then be readily diluted to a nonreactive level. Another constraint to phenotyping granulocytes is the limited availability of qualified antisera for the currently defined HNA antigens.

Monoclonal antibodies specific to several HNA antigens are commercially available and have been used to type granulocytes by GIFT using the flow cytometer. An advantage to phenotyping by this method is that it can be done with whole blood instead of with isolated granulocytes.

Because phenotyping granulocytes cannot often be performed or can be unreliable if samples are not handled in a careful and timely manner, genotyping by PCR, which has less stringent sample age and handling restraints, can serve either as an alternative to or as confirmation of serologic results.

**Granulocyte Genotyping**

The discovery of the PCR in the 1980s facilitated the development of molecular methods that have now been applied to delineate the genetics and allelic variations of the HNAs. Initially, the characterization of the genes encoding the HNA-1 antigen system (HNA-1a, HNA-1b, and HNA-1c) allowed for the development of PCR assays using sequence-specific primers (SSP) to differentiate the alleles. To complicate matters there is a high degree of homology between the FCGR3A gene that encodes the FcγRIIIa granulocyte receptor and the FCGR3B gene where the three different HNA-1 polymorphisms reside. HNA-1a, HNA-1b, and HNA-1c are encoded by FCGR3B*1, FCGR3B*2, and FCGR3B*3 genes, respectively. FCGR3B*1 differs from FCGR3B*2 by five nucleotide bases, and a single nucleotide polymorphism (SNP) differentiates FCGR3B*2 from FCGR3B*3 (Table 2). FCGR3A differs from FCGR3B at five different nucleotide locations (Table 2).

Methods are also available to genotype the HNA-4a and HNA-5a alleles. Both of these polymorphisms are the result of SNPs. Even though the molecular sequence of HNA-2a has been identified, genotyping methods to detect the gene that encodes this alloantigen are not available. The HNA-2a negative phenotype is attributable to CD177 mRNA splicing defects, and no mutations have been detected in the CD177 introns or exons in these individuals. Because the gene encoding HNA-3a has only recently been identified, there has not been a genotyping procedure for this antigen, but the clinical importance of this antigen is supporting efforts to get a molecular method developed.

Genomic DNA (gDNA) can be isolated and purified from anticoagulated blood using any number of published methods. A unique set of sense and antisense oligonucleotide primers that border the DNA fragment to be amplified are added to a master mix of reagents that includes deoxyribonucleoside triphosphates (dNTPs), Taq polymerase, a buffer solution containing Mg²⁺, and the individual’s gDNA. The master mix is subjected to a series of 30 to 40 temperature changes that define the amplification cycles. Each cycle consists of three temperature steps that are critical in the amplification of the DNA target strands. The first step in the cycle typically involves heating the master mix to a temperature of 95°C. This denaturation step results in double-stranded DNA separating into single strands by disrupting the hydrogen bonds between each nucleotide base pair. The annealing step follows in which the reaction temperature is lowered to 50°C to 65°C. This allows the primers to hybridize to the opposing strands of the target DNA. The final extension step heats the master mix from 71°C to 72°C, which results in DNA synthesis in the presence of Taq polymerase and dNTPs complementary to the oligonucleotide primers. A final extension step is added after all cycles have been completed to ensure that any remaining single-stranded DNA is fully elongated. The amplified double-stranded DNA (amplicon) is separated by size using agarose gel electrophoresis, stained with ethidium bromide, and visualized by fluorescence using ultraviolet light. The sizes of the amplicons are determined by comparison with a DNA ladder, which contains DNA fragments of known sizes that were run alongside the PCR products during the agarose gel electrophoresis step.

**Future Directions**

**Solid-Phase Technology**

The complement-dependent lymphocyte cytotoxicity assay was the first platform, developed nearly 50 years ago, for identifying HLA antibodies and remains the standard for the development of new methodologies in this field.

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**Table 2.** Nucleotide differences for the three HNA-1 alleles (1a, 1b, and 1c) on FcγRIIIa and among the FCGR3A genes†

<table>
<thead>
<tr>
<th>HNA</th>
<th>Gene</th>
<th>Polymeric nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>FCGR3B*1</td>
<td>G C A C G A C G C T</td>
</tr>
<tr>
<td>1b</td>
<td>FCGR3B*2</td>
<td>C T G C A A C G C T</td>
</tr>
<tr>
<td>1c</td>
<td>FCGR3B*3</td>
<td>C T G A A A A C G C T</td>
</tr>
<tr>
<td></td>
<td>FCGR3A</td>
<td>G C G C G A G T T T C</td>
</tr>
</tbody>
</table>

†Information from references 91 and 92. 
NOTE: Boldface indicates substituted nucleotides. 
FcγRIIIa = Fc gamma receptor IIIa; HNA = human neutrophil antigen.
Multiple new technologies are now being used by HLA laboratories, and in the past few years solid-phase assays have been developed in which purified HLA molecules are bound to a well in a microtiter plate or to a bead. HLA antibody is then detected either using an enzyme-linked immunosorbent assay (ELISA) technique or by analyzing the beads with a flow cytometer, which uses a new technology developed by Luminex (Austin, TX).44 A critical advantage of this technology is its ability to measure multiple analytes simultaneously in a single reaction system. The commercially available test kits use purified HLA antigens bound to microbeads, which can be individually differentiated by varying ratios of internal fluorescent dye prepared during the manufacturing process. The test involves incubation of test sera with the panel of microbeads, and the bound HLA antibody is detected by a secondary R-phycoerythrin (PE)-conjugated IgG (or IgM)–specific antibody.

Analysis is done with the use of a Luminex flow analyzer in which the beads pass through two lasers, similarly to classic flow cytometry. One laser identifies the specific bead being analyzed, thereby identifying the unique HLA antigen. The other laser detects the presence of bound Ig on the surface of the microbead. The color signals are then detected and processed into data for each reaction. A significant fluorescence shift from the negative control range indicates the presence of specific antibody in the test sample.

The widespread use of Luminex technology by HLA laboratories has fostered commercial activity directed at laboratories has fostered commercial activity directed at the development of a new sensitive and specific platform for the detection of granulocyte antibodies, (2) to foster working relationships among laboratories involved in this field, and (3) to support the development of the science and technology for the detection of granulocyte antibodies.99

The Working Party was initially affiliated with the British Blood Transfusion Society but is now affiliated with the ISBT. The goals of the Second Workshop, in 1996, were the (1) establishment of typed granulocyte panels by well-defined typing sera, (2) proficiency testing of granulocyte antibody detection, (3) investigation of uncharacterized sera, (4) proficiency testing of HNA genotyping, and (5) exchange of information to establish standards in granulocyte serology.100

To date, the Working Party has conducted nine Granulocyte Serology Workshops and comprises 16 international member laboratories, which are listed in Table 3. Also, the American Red Cross Mid-America Blood Services Neutrophil & Platelet Immunology Laboratory has been a member of this consortium since its inception in 1989. The workshops continue to focus on the QA and standardization of the test systems, and the Working Party recently published its

**ISBT Working Party on Granulocyte Immunobiology**

In 1989, Professor Alan Waters organized the UK Platelet and Granulocyte Serology Working Group, and 11 international laboratories participated in the First International Workshop on Granulocyte Serology. The objectives of this first workshop were as follows: (1) to initiate an exchange of sera among laboratories to evaluate the techniques being used for the detection of granulocyte antibodies, (2) to foster working relationships among laboratories involved in this field, and (3) to support the development of the science and technology for the detection of granulocyte antibodies.

The Working Party is now affiliated with the ISBT. The goals of the Second Workshop, in 1996, were the (1) establishment of typed granulocyte panels by well-defined typing sera, (2) proficiency testing of granulocyte antibody detection, (3) investigation of uncharacterized sera, (4) proficiency testing of HNA genotyping, and (5) exchange of information to establish standards in granulocyte serology.
Serologic screening recommendations for the investigation and prevention of leukocyte antibody-mediated TRALI.102

**Conclusions**

Granulocyte antibody and antigen testing has played and continues to play a critical role for diagnosing and investigating relatively rare but potentially lethal clinical conditions such as TRALI and immune neutropenias. Although mitigation strategies are already being implemented nationwide, TRALI still appears to be a leading and preventable cause of transfusion fatalities. As advancing technology allows granulocyte antibody testing to be done on a less manual and larger scale with more standardized and reproducible results, it now has the potential to become even more valuable as a tool to increase blood safety by actually preventing transfusion reactions without compromising an adequate supply of blood.

**Acknowledgments**

The authors would like to thank Penny Milne and Bobbie Gibson for their helpful assistance with the preparation of the manuscript.

**Table 3.** Members of the ISBT Working Party on Granulocyte Immunobiology

- Platelet and Leucocyte Immunology Laboratory, EFS Ile de France, Hopital Henri Mondor, Creteil, France
- Leucocyte and Platelet Immunology Laboratory, Blood Service West of the German Red Cross, Hagen, Germany
- The BloodCenter of Wisconsin, Platelet and Neutrophil Immunology Laboratory, Milwaukee, WI, USA
- Institute of Transfusion Medicine, University of Schleswig-Holstein, Kiel, Germany
- Australian Red Cross Blood Service-Queensland, Brisbane, Australia
- Platelet and Granulocyte Immunology Laboratory, National Blood Service, Bristol, UK
- Blood Transfusion Center of Slovenia, Ljubljana, Slovenia
- Centre de Transfusio i Banc de Teixits, Hospital Vall d’Hebron, P. Vail d’Hebron, Barcelona, Spain
- Sanguin Diagnostics, Immunohaematology Diagnostic Department, Amsterdam, The Netherlands
- Institute for Clinical Immunology and Transfusion Medicine, Justus Liebig University, Giessen, Germany
- American Red Cross Mid-America Blood Services, Neutrophil & Platelet Immunology Laboratory, St. Paul, MN, USA
- Department of Transfusion Medicine, Graduate School of Medical Sciences, University of Tokyo, Tokyo, Japan
- Department of Immunohaematology and Transfusion Medicine, Institute of Haematology and Blood Transfusion, Warsaw, Poland
- Aberdeen and North East Scotland Blood Centre, Scottish National Blood Transfusion Service, Aberdeen, UK
- Platelet and Leucocyte Immunology Laboratory, Institute of Biology, Nantes, France
- Department of Transfusion Medicine, Karolinska University Hospital, Stockholm, Sweden

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Mary E. Clay, MS, MT(ASCP), (corresponding author) Director, Transfusion Medicine, Research and Development Program, University of Minnesota Medical School, Department of Laboratory Medicine and Pathology, MMC 198, D-251 Mayo Building, 420 Delaware Street, SE, Minneapolis, MN 55455, and Consultant, Neutrophil & Platelet Immunology Laboratory, American Red Cross, Mid-America Blood Services Region, 100 South Roberts Street, St. Paul, MN 55107, Randy M. Schuller, BS, MT(ASCP), Supervisor, and Gary J. Bachowski, MD, PhD, Assistant Medical Director, Neutrophil and Immunobiology Laboratory, American Red Cross, Mid-America Blood Services Region, St. Paul, MN.

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

G.R. Halverson and T. Peyrard

History

The idea that there must be openings in the cell membrane to permit the flow of water and salts to maintain cellular function and to eliminate the byproducts of cellular metabolism was recognized by the middle of the 19th century. Water is the major component of all living cells, including those of vertebrates, invertebrates, and unicellular organisms and plants. Salt water comprises approximately 70 percent of the human body. After debating for decades about the process of water transfer across cell membranes, most scientists finally decided that water must pass freely through biologic membranes by simple diffusion. Only a small number of scientists disagreed, and they had only indirect evidence that there were special channels for the transport of water. After all, there must be something to explain the high permeability of RBCs and renal tubules. Moreover, the water permeability of these tissues can be reversibly inhibited by mercuric ions.1

By 1950 it had been shown that water moves quickly into and out of cells through pores that are selective for water molecules only. Every second, billions of water molecules pass through a single channel. By the mid-1980s scientists had found an RBC membrane protein whose 28-kDa N-terminal amino acid sequence was related to the 26-kDa major intrinsic protein (MIP26) of bovine lens fibers.2–4 The 28-kDa protein was shown to be a unique molecule that is abundant on erythrocytes and on renal tubules. Preston and Agre4 succeeded in 1991 in isolating the same membrane protein from RBCs, which in 1997, was officially named aquaporin-1 (AQP1) by the Human Genome Organization, for the water channel. AQP1 has been found in many tissues including several parts of the kidney, liver, gall bladder, eye, choroid plexus, hepatobiliary epithelium, and capillary endothelium. AQP1 turned out to be the first of the family of major intrinsic proteins (MIP), which are highly conserved membrane proteins that regulate small molecule transport. The MIP family of proteins is believed to date back 2.5 to 3 billion years in evolutionary time.5 It is believed that the MIP family evolved from two basal lineages: AqpZ-like water channels and GlpF-like glycerol facilitators. These divergent lineages probably originated from an archaeal (AqpM-like) aquaporin.5 The MIP family is now divided into two groups: aquaporins (AqpZ, AqpO, Aqp1, Aqp2, Aqp4, Aqp5, Aqp6, and Aqp8) and aquaglyceroporins (GlpF, Aqp3, Aqp7, Aqp9, and Aqp10).6–10 In 1994, AQP1 protein was characterized as carrying the Colton blood group antigens.11

Genetics and Inheritance

In 1965 in Oslo, Norway, the discovery of an antibody that detected a “public” (or high prevalence) antigen was linked to two other cases discovered earlier by workers in Minneapolis, Oxford, and London. The antigen was named Coa (Colton1) in 1967 for the first of the three producers of anti-Coa, who was from Minneapolis; it should actually have been named “Calton,” but the handwriting on the tube apparently was misread.11–13 Coa was shown to be an inherited characteristic.14 In 1970, the antithetical antigen, Cob, was reported by Giles et al (Table 1).15 In analysis of unrelated people of European extraction, the occurrences of the three major Colton phenotypes were as follows: Co(a+b–) 0.914, Co(a+b+) 0.084, and Co(a–b+) 0.002 (Table 2).16 In 1971, the genetic independence of the Colton blood group system from Kell and Lutheran was reported, and it was confirmed that it is independent from MNSs, P, Rh, Duffy, Dombrock, and sex.17 Coa and Cob are codominant alleles that respectively encode the Coa and Cob antigens at the RBC surface. In 1974 the Co(a–b–) phenotype of three persons in a French Canadian family was reported.18 The serum from a patient in 1964 (Swarts) was eventually found to be negative only with these Co(a–b–) family members.19 Colton is the 15th human blood group system recognized by the ISBT (ISBT 015).

Molecular Basis

The AQP1 locus was mapped to human chromosome 7p14 by fluorescent in situ hybridization.19 The Colton (Co) blood group antigens had been previously linked to the short arm of chromosome 7.20 Immunoprecipitation studies

**Table 1. Antigens of the Colton blood group system: their prevalence, molecular basis, and sensitivity to enzyme and DTT treatment**

<table>
<thead>
<tr>
<th>Name</th>
<th>ISBT symbol</th>
<th>ISBT number</th>
<th>Prevalence</th>
<th>Molecular basis</th>
<th>Effect of enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coa</td>
<td>Co1</td>
<td>015001</td>
<td>99.8%</td>
<td>Ala 45</td>
<td>PR TR CR NR DTTR</td>
</tr>
<tr>
<td>Cob</td>
<td>Co2</td>
<td>015002</td>
<td>8.6%</td>
<td>Val 45</td>
<td>PR TR CR NR DTTR</td>
</tr>
<tr>
<td>Coa</td>
<td>Co3</td>
<td>015003</td>
<td>&gt;99.99%</td>
<td>unknown</td>
<td>PR TR CR NR DTTR</td>
</tr>
</tbody>
</table>

CR = α-chymotrypsin resistant; DTTR = dithiothreitol resistant; NR = neuraminidase resistant; PR = papain resistant; TR = trypsin resistant.

**Table 2. Phenotypes (% occurrence)**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>% Occurrence (most populations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co(a+b–)</td>
<td>91.4</td>
</tr>
<tr>
<td>Co(a–b+)</td>
<td>0.2</td>
</tr>
<tr>
<td>Co(a+b+)</td>
<td>8.4</td>
</tr>
<tr>
<td>Co(a–b–)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
and DNA sequencing showed that the Colton antigens are the result of a polymorphism in AQP1 at the extracellular site of loop A, which connects the first and second spanning domains. Figure 1 depicts the structure of the protein as it passes through the membrane, and indicated are the Co<sup>a</sup>/Co<sup>b</sup> polymorphic site at position 45 and the only N-glycan site at position 42. The Co<sup>a</sup>/Co<sup>b</sup> polymorphism arises from a single amino acid change of Ala45Val (alanine for Co<sup>a</sup>, valine for Co<sup>b</sup>) caused by a nucleotide change of C>T at position 134 in exon 1 of AQP1 gene, named CO according to the ISBT nomenclature and AQP1 according to the Human Genome Organization (HUGO). The Colton gene consists of four exons distributed over 11.6 kbp of genomic DNA. The amino acid sequence of the wild-type AQP1 protein is shown in Table 3. Also known as the channel-forming integral protein (CHIP), the Colton glycoprotein is a multipass protein consisting of three external loops and six membrane-spanning regions, as well as 2 cytoplasmic loops, with both the NH<sub>2</sub> and COOH terminus located on the cytoplasmic side of the membrane. Common to all MIP superfamily proteins is the occurrence of the asparagine-proline-alanine (NPA) motifs, two being present in each half of the protein. An hourglass structural model has been proposed by Agre and colleagues.

Table 3. Amino acid sequence of wild-type AQP1 protein<sup>13</sup>

<table>
<thead>
<tr>
<th>Amino acid sequence of wild-type AQP1 protein&lt;sup&gt;13&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MASEFKKLFL WRRAVVEFALL TLFVFSISIG SALGFKYPVG NQQTAVGDNV</td>
<td>50</td>
</tr>
<tr>
<td>KVSFLAGFLSI ATLAQSVGHI SGAIHNQVAT LLGLLSCQIS IFRAMLMIYA</td>
<td>100</td>
</tr>
<tr>
<td>QCQGVAIATA ILSGTSSLT GNSSLGRNDLA DGVNSQGQLG IEIGTLOLV</td>
<td>150</td>
</tr>
<tr>
<td>LCVLATDDRR RRDGGASAPL AIGLIALGH LLADYTTGGC INPARSFGSA</td>
<td>200</td>
</tr>
<tr>
<td>VITHFNISHW IFVGGPFGGG ALAVLIDFI LAPRSSDLTD RKVWTSQGV</td>
<td>250</td>
</tr>
<tr>
<td>EEYDDLADDI NSRVMKPK</td>
<td>269</td>
</tr>
</tbody>
</table>

Amino acid sequence taken from GenBank, accession # M77829

The molecular basis of the null Co<sup>a</sup>–<sup>b</sup> phenotype has been attributed to several different molecular backgrounds; these are depicted in Table 4. Other changes in the Co protein have been reported to cause decreased or no expression of the Colton antigens. An amino acid change of Glu47Arg was observed to cause a Co<sup>a</sup>–<sup>b</sup>– phenotype. The same amino acid substitution was recently fully investigated in a Turkish female with the Co<sup>a</sup>–<sup>b</sup>– phenotype. Her RBCs were found by molecular studies to have normal water permeability by stopped-flow analysis and a normal amount of AQP1 protein in the RBC membrane. (See section on clinical significance). By PCR-RFLP the specimen was genotyped as Co<sup>a</sup>/Co<sup>b</sup>, but full sequencing revealed a 140A>G mutation leading to a Glu47Arg amino acid substitution. This change in amino acids is close to the Co<sup>a</sup>/Co<sup>b</sup> polymorphism, and thus, the probable cause of the apparent discrepancy between phenotype and genotype. When using a specifically designed K-562 cell expression system for Colton antigens, the Glu47Arg substitution was shown to inhibit both Co<sup>a</sup> and Co<sup>b</sup> antigen expression. The antibody produced by these Co<sup>a</sup>–<sup>b</sup>– individuals with a Glu47Arg substitution is not a mixture of anti-Co<sup>a</sup> and -Co<sup>b</sup>, rather it is “anti-Co<sup>b</sup>–like,” reacting with both Co<sup>a</sup>–<sup>b</sup>– and Co<sup>a</sup>–<sup>b</sup>+, Co<sup>a</sup>–<sup>b</sup>+, but more weakly than anti-Co<sup>b</sup> produced by the true null phenotype. This suggests the existence of a new high-prevalence antigen in the Colton blood group system, with a corresponding antibody that Arnaud et al. proposed to name anti-Co<sup>4</sup>. Therefore, it is possible that some of the Co<sup>a</sup>–<sup>b</sup>– people known throughout the world, who would have not been fully investigated by molecular techniques, may actually not be “true” Co<sup>a</sup>–<sup>b</sup>– but “Co<sup>a</sup>–<sup>b</sup>–,–2,3,–4.” This may also be why some antibodies directed against a high-prevalence Colton antigen, mistakenly considered as anti-Co<sup>3</sup>, were unexpectedly weakly reactive with Co<sup>a</sup>–3 RBCs.

**Biochemical Physiology of the Colton Glycoprotein**

Protons are necessary for the bioenergetics of the living cell. The proton gradient drives many transport functions, membrane fusions, and the synthesis of ATP. Maintaining the proton gradient is essential, and indiscriminate proton leakage across cellular membranes would be fatal to the living cell. At the same time, cells must maintain the balance of water’s ability to permeate the membrane bilayer. Aquaporins are strictly selective water channels that have evolved for that purpose. AQP1 was found to have a configuration with intracellular N and C terminals, similar to that of other ion channel proteins. Protein modeling from the cDNA sequences predicts a two-tandem repeat of three membrane-spanning helices that exist as tetramers within the cell membrane (Fig. 2). An electrostatic field created by the highly conserved amino acid sequence NPA forms the water channel, which prevents proton loss while allowing water to permeate the membrane. The most prominent feature is the association of loop B with loop E driven by their positive N-terminal ends that brings together the NPA motifs at the center of the pore. This causes the formation of strong electrostatic fields that block...
the permeation of protons as well as H\textsubscript{3}O\textsuperscript{+} and other cations. When *Xenopus laevis* oocytes were transfected with cRNA, they were found to have markedly increased water permeability, causing the oocytes to swell and burst.\textsuperscript{32} This permeability could be reversibly inhibited by Hg\textsubscript{2+}.\textsuperscript{33} When the same cRNA transcript was reconstituted into lipid vesicles, the same observations were made. This confirmed that membranes exhibit water permeability and showed that the water permeability of membranes with AQP1 was up to 100 times greater than that of those without it.

### Colton Antigen Typing

To date, anti-Co\textsuperscript{a} and anti-Co\textsuperscript{3} reagents, either monoclonal or polyclonal, are not commercialized. As a result, human polyclonal reagents are only available in immunohematology reference laboratories, but usually in small amounts. However, a human polyclonal anti-Co\textsuperscript{b} is available in Europe as a CE-marked in vitro diagnostic reagent (DiaMed, a division of Bio-Rad, Cressier sur Morat, Switzerland). The two major high-throughput molecular genotyping platforms, a commercialized DNA glass array by Progenika (Barcelona, Spain) and a DNA bead array by BioArray/Immucor (Norcross, GA) allow Co\textsuperscript{a} and Co\textsuperscript{b} allele typing by searching for the 134C>T polymorphism in exon 1, with subsequent prediction of Colton phenotype. It is important, however, to be aware that prediction of the Co phenotype in Conull and Covariant people would be a source of misinterpretation with these DNA-based techniques.

### Clinical Significance

Antibodies to Colton blood group antigens are generally IgG and react best by the antiglobulin test, especially when protease-treated RBCs are used.\textsuperscript{34,35} Some anti-Co\textsuperscript{a}, -Co\textsuperscript{b} and -Co\textsuperscript{3} may bind complement.\textsuperscript{36} Few reports of significant delayed or acute transfusion reactions or hemolytic disease of the fetus and newborn (HDFN) attributable to anti-Co\textsuperscript{a} have been reported, although both are known to occur with severe morbidity.\textsuperscript{27,37–40}

Anti-Co\textsuperscript{b} is relatively rare and often found in patient’s sera containing other alloantibodies.\textsuperscript{34,35} Two reports of in

### Table 4. Molecular bases of the Colton-null [Co(a–b–)] phenotype: effect of the change at the DNA and protein level, and ancestry of the probands

<table>
<thead>
<tr>
<th>Molecular alteration</th>
<th>Effect</th>
<th>Observed result on AQP1 protein</th>
<th>Presence of anti-Co3</th>
<th>HDFN reported</th>
<th>Ancestry</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co\textsubscript{null} phenotypes</td>
<td>Large deletion comprising most of exon 1&lt;br&gt;c.309insT</td>
<td>Frameshift mutation&lt;br&gt;AQP1 not detected in RBC membrane extracts</td>
<td>Not tested</td>
<td>No</td>
<td>Northern European</td>
<td>Proband 1 in Preston et al.\textsuperscript{29}</td>
</tr>
<tr>
<td></td>
<td>c.113C&gt;T</td>
<td>Missense mutation (p.Pro38Leu)&lt;br&gt;AQP1 not detected in RBC membrane extracts</td>
<td>Not reported</td>
<td>No</td>
<td>Northern European</td>
<td>Proband 3 in Preston et al.\textsuperscript{29}</td>
</tr>
<tr>
<td></td>
<td>c.576C&gt;A</td>
<td>Missense mutation (p.Asn192Lys)&lt;br&gt;Not tested</td>
<td>No</td>
<td>No</td>
<td>Portuguese</td>
<td>Chrétien et al.\textsuperscript{26}</td>
</tr>
<tr>
<td></td>
<td>c.232delG</td>
<td>Frameshift mutation&lt;br&gt;Not tested</td>
<td>Yes</td>
<td>Yes</td>
<td>Indian</td>
<td>Joshi et al.\textsuperscript{27}</td>
</tr>
<tr>
<td></td>
<td>c.601delC</td>
<td>Frameshift mutation&lt;br&gt;Not tested</td>
<td>Yes</td>
<td>No</td>
<td>Caucasian</td>
<td>Nance et al.\textsuperscript{29}; Reid and Lomas-Francis\textsuperscript{13}</td>
</tr>
<tr>
<td>Altered Co phenotypes</td>
<td>c.140A&gt;G</td>
<td>Missense mutation (p.Gln47Arg)&lt;br&gt;Not tested</td>
<td>No</td>
<td>No</td>
<td>Not reported, presumably Caucasian</td>
<td>Wagner and Flegel\textsuperscript{29}</td>
</tr>
<tr>
<td></td>
<td>c.140A&gt;G</td>
<td>Missense mutation (p.Gln47Arg)&lt;br&gt;Normal AQP1 expression in RBC membrane</td>
<td>No</td>
<td>No</td>
<td>Turkish</td>
<td>Arnaud et al.\textsuperscript{30}</td>
</tr>
</tbody>
</table>

AQP1 = aquaporin 1; HDFN = hemolytic disease of the fetus and newborn. According to the recommendations of the Human Genome Variation Society and National Institutes of Health Single Nucleotide Polymorphism database, “c.” means that the position of the polymorphism refers to the complementary DNA, “p” means that the position of the polymorphism refers to the protein.

**Fig. 2** The AQP1 water channel is shown as an hourglass configuration created by the association of the six transmembrane domains and the crossing of loops B and E with the electrostatic field produced by the NPA motifs forming the water channel.\textsuperscript{21}
vivo survival studies of $^{57}$Cr-labeled Co(b+) RBCs in patients with anti-Co$^a$ showed survival at 94 percent and 85 percent, respectively, at 1 hour, decreasing to 51 percent at 24 hours and 10 percent at 96 hours.\textsuperscript{41,42} Co$^b$ is fully developed at birth, but no significant HDFN has been reported to date.\textsuperscript{34}

Anti-Co$^3$ was reported to cause severe HDFN requiring neonatal transfusion.\textsuperscript{43} Transfusion of incompatible Co(a+b–) RBCs in a patient with anti-Co$^3$ was reported to be responsible for a mild hemolytic transfusion reaction.\textsuperscript{27} There are also reports of autoantibodies mimicking Colton specificities.\textsuperscript{44}

AQP1-deficient individuals were studied for their renal function and capillary permeability before and after water deprivation.\textsuperscript{45} Baseline studies did not reveal any abnormalities; however, after water deprivation this study revealed an inability to concentrate urine. Thus, Colton-null individuals may be subject to serious hydroelectrolytic and metabolic disorders should they become severely dehydrated.

**Acknowledgments**

The authors thank Robert Ratner for preparation of the manuscript and figures, and Hallie Lee-Stroka for her critical review and helpful comments. This work was funded in part by a grant from the MetLife Foundation (GH).

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Alloimmunization to the D antigen by a patient with weak D type 21

H. McGann and R.E. Wenk

Antibodies of apparent D specificity may be found in D+ patients. We report a D+, multi-transfused Caucasian woman with myelodysplasia who exhibited several alloantibodies. One antibody was a moderately strong (2+) anti-D that persisted for 9 months, until the woman died. Molecular analysis of the patient’s RHD gene identified the rare weak D type 21 (938C>T) allele. D alloantibodies do not occur in patients with most weak D types, but some patients with a weak D phenotype, including those with type 21, can produce antibodies to nonself epitopes of the wild-type D antigen. Immunohematology 2010;26:27–29.

Key Words: alloimmunization, anti-D, RHD gene, weak D

Table 1. Evaluation and transfusion of patients with D+ RBCs and serum anti-D

<table>
<thead>
<tr>
<th>Etiology/pathogenesis</th>
<th>Diagnostic evidence</th>
<th>Preferred RBC transfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passive alloantibody</td>
<td>History of transfusion with D– RBC, plasma, IVIG, RhIG, other product with anti-D</td>
<td>D– until anti-D wanes</td>
</tr>
<tr>
<td>Alloantibodies from passenger lymphocytes in donor organ</td>
<td>Organ transfant from D– donor to D+ recipient</td>
<td>D– until anti-D wanes</td>
</tr>
<tr>
<td>Autoantibody to D antigen</td>
<td>Positive direct/indirect antiglobulin test (anti-IgG)</td>
<td>D– over D+</td>
</tr>
<tr>
<td>Autoantibodies or alloantibodies to LW</td>
<td>Antibody fails to react with RBC treated with dithiothreitol</td>
<td>D– over D+</td>
</tr>
<tr>
<td>Autoantibody to epitope absent from normal D protein</td>
<td>Antibody is absorbed by D– RBC</td>
<td>D– only</td>
</tr>
</tbody>
</table>

In this case report, we describe a woman whose RBCs were strongly D+ at the phenotypic level, but had an RHD genotype consistent with weak D, and an alloanti-D.

Case Report

A 72-year-old, gravida 3, Caucasian woman with myelodysplasia and pancytopenia was transferred to another hospital, where in 6 months of treatment, she had received an unknown number of platelet transfusions, but no RBC transfusions.

In our hospital and before any RBC transfusion, the patient’s RBCs serologically typed as D+ (4+) by tube agglutination at immediate spin phase, using a monoclonal-polyclonal blended anti-D (Ortho Clinical Diagnostics Co., Raritan, NJ). During a disease course of 13 months, the patient received 37 apheresis-processed platelet units and 108 leukocyte-reduced units of RBCs, which produced alloimmunization to K, E, and Cw. After 9 months, her serum also demonstrated a 2+ anti-D in a gel-based, antibody detection system (Micro Typing Systems, Inc., Ortho Clinical Diagnostics, Pompano Beach, FL). The initial DAT (tube, anti-IgG) and all but one subsequent DAT were negative as was the auto-control. On the one occasion, the DAT became weakly positive in a mixed-field reaction. Acid eluates prepared from the patient’s RBCs contained only anti-E. There was no history of organ transplantation or administration of a plasma product that might contain anti-D. The anti-D persisted throughout the patient’s course. Blood samples were collected and referred for RHD and RHCE gene analyses.

Although continued transfusions with D– blood were prescribed, the patient refused all further medical treatment. Her myelodysplasia progressed to early acute leukemia before she expired at home.

Molecular Test Results

Molecular tests were performed at the Molecular Red Cell and Platelet Testing Laboratory of the American Red Cross, Penn-Jersey Region, and the University of Pennsylvania DNA Sequencing Facility, both in Philadelphia,
Pennsylvania. The patient was found to be heterozygous for \textit{RHD}. No changes associated with any of the known partial D phenotypes were identified. A change (938C>T) was identified that is associated with weak D type 21 that is predicted to encode an intracellular amino acid change of Pro313Leu. The patient's RH genotype could be interpreted to be \textit{RHD*weak D type 21, RHCE*Ce/RHCE*ce}.

\section*{Discussion}

Initially, the finding of anti-D in a D+ patient was puzzling and the several causes listed in Table 1 were considered. Patients whose anti-D was passively transferred in plasma products (IVIG, RhIG, etc.) or whose serum antibodies were produced by a donor's passenger lymphocytes may require D− RBCs until the acquired anti-D has weakened, usually in a matter of weeks to months.

Passive anti-D, however, was ruled out by history. Before hospital admission at our institution, the patient had been transfused only with apheresis platelets, but she had not been transfused with plasma or cellular products from D− donors, nor had she been infused with IVIG or injected with RhIG. Persistence of the anti-D was further evidence that the anti-D did not involve passive immunization.

The transfer of passenger lymphocytes could be excluded by history as well. The patient had never received a transplant. Passenger lymphocytes are alloantibody-secreting B cells that are carried in a transplanted donor organ to a recipient. An anti-D produced by passenger lymphocytes arises in the donor by alloimmunization to the D antigen before transplantation. Donor lymphocytes continue to manufacture detectable anti-D for about 3 months after transplantation.

Many patients with autoimmune hemolytic anemia and mimicking antibodies to Rh antigens are treated medically and transfusion is avoided altogether. In other patients, transfusion can be delayed until the concentration of antibodies is reduced by medical treatment. Many mimicking warm autoantibodies have apparent Rh specificity, especially anti-e. Only rare D+ patients will possess warm autoantibodies that mimic anti-D. If a patient with autoantibodies requires transfusion, antigen-negative donor RBCs may be preferable because they may survive longer than antigen-positive RBCs.

Antibodies to LW are almost always autoantibodies; those that are less potent appear to have anti-D specificity because the number of D antigen sites per RBC correlates with the number of LW antigen sites. Thus, D− RBCs, which have fewer LW antigen sites than D+ RBCs, may not be agglutinated by an anti-LW. However, anti-LW may be distinguished from anti-D by its failure to agglutinate RBCs treated with DTT. If transfusion is required for a patient with autoanti-LW, D− RBCs are preferable to D+ RBCs providing such a patient is c+.

Patient autoantibodies, including anti-LW, are often transient, but our patient’s anti-D persisted for 4 months at constant serologic strength (2+ by the IgG gel test). Alloantibodies to Rh antigens are often durable (85%). Negative DATs and absence of RBC agglutination in autocontrol tests also indicated the presence of D alloantibody.

LW alloantibodies, which can mimic anti-D, arise only in very rare individuals whose RBCs are LW(a−b+) or LW(a−b−). Neither autoantibodies nor alloantibodies to LW were specifically evaluated in the patient described here.

Alloantibodies to the D antigen occur in people who are known to lack one or more epitopes of D (partial D phenotype). On the other hand, anti-D alloimmune responses in patients with other weak D types are unusual because their RBCs do not appear to lack D epitopes when tested with a standard battery of anti-D monoclonal reagents.

Suspicion that the patient had been alloimmunized to D antigen was raised by the serologic finding of negative DATs and the antibody’s persistence. In addition, people who carry variants of the D antigen that are detectable only by molecular means are capable of producing antibodies to wild-type protein. In the present case, finding a variant \textit{RHID} gene supported the clinical and serologic evidence of D alloimmunization.

Historically, some D+ individuals who made anti-D were initially considered to express a weak D phenotype (first termed “D\\textsuperscript{\textsc{\textit{Weak}}}”) when tested with human polyclonal reagents. Serologic evidence of alloimmunization to D in a D+ person included a negative DAT, a serum antibody, and, for some partial D phenotypes, the patient’s RBCs also expressed a low-prevalence Rh antigen. A D+ patient with alloanti-D was thought to lack part(s) of the D antigen, i.e., a “partial D” type. A person with a partial D phenotype would only produce antibody to those epitopes of the D antigen missing from his or her own RBCs. Investigative use of monoclonal antibody revealed many epitopes of the D antigen and supported the concept that partial D types lacked one or more epitopes of their D antigen. The monoclonal antibodies also extended the identification and classification of the various partial D types initially defined by polyclonal anti-D.

Many individuals, however, were found to have RBCs that agglutinated poorly with common anti-D reagents, but demonstrated the presence of all known epitopes. These D+ RBCs were called “weak D” in preference to D\\textsuperscript{\textsc{\textit{Weak}}}”. Weak D RBCs expressed fewer D molecules per RBC, a finding that accounted for their weak agglutination with reagent antibodies. Theoretically, individuals who carried these weak D types should not be able to develop alloantibodies to D. However, it is virtually impossible to distinguish a person with a weak D phenotype from a person with a weak partial D phenotype by serologic tests; only the production of alloanti-D will reveal someone to have a weak partial D phenotype.

When molecular analysis became available, the \textit{RHD} (and \textit{RHCE}) gene(s) revealed far more extensive and complex polymorphisms than were evident by serologic methods. Weakly agglutinating D serotypes could be related to point mutations, nonsense mutations, deletions, and tandem
rearrangements in the RHD and RHCE DNA. The cause of weak RBC agglutination was confirmed to be an absence of epitopes in partial D types, a decreased number of protein molecules in weak D types, or both. In addition, partial D phenotypes are expressions of genotypes that encode for changes in the amino acids on the outer surface of the RBC where the D protein loops outside the RBC membrane. Patients whose RBCs express partial D types are often immunized to D+ RBCs by transfusion or pregnancy. On the other hand, genotypes that encode weak D phenotypes are predicted to result in intracellular or intramembranous changes in amino acids that, in theory, express all epitopes of D so that patients whose RBCs express weak D types are not expected to produce anti-D after immunization by transfusion or pregnancy. Theory is partly supported by evidence—the most common weak D variants (types 1–3), which constitute 70 to 90 percent of all weak D variants, do not produce alloanti-D.6

Molecular examination of the various partial and weak D types indicated that many serologic phenotypes had more than one basis, but did not suggest that new epitopes could be identified.7 However, serologic evidence suggested otherwise. Anti-D has been found in some weak D patients whose D protein is predicted by their DNA sequences to have altered intramembranous and intracellular amino acids of the D protein. Thus, some weak D molecular alleles contain missense mutations that not only reduce the number of antigens per RBC and cause weak RBC agglutination by reagent antibodies but also cause a loss of D epitopes and produce changes in the three-dimensional antigenic structure of the D protein.

The case presented here indicates that the rare weak D type 21 is an allelic variant whose patient-carriers are susceptible to transfusion alloimmunization by wild-type D antigen. Weak D type 21 is the result of a point mutation that alters an amino acid thought to be in an intracellular region of the D protein.8 The rare allele was first discovered in one individual during investigation of 270 known weak D samples in people from northern Germany,9 and there has been one subsequent independent observation of it in Austria.10 In both cases, the allele has been found on the same chromosome (cis) as a RHCE*Ce allele in the haplotype RHDweak D type 21 RHCE*Ce. The Pro313Leu change in the translated D protein is in its tenth intracellular helix, and all 37 tested epitopes of the D antigen appear to be present.9 As expected, the number of D antigen sites per RBC is reduced (5.2 × 103) when compared with the normal number (104 to 2.5 × 105) of sites.11 This antigen density is the highest among all known weak D alleles. The high density probably accounts for the patient’s 4+ serotype and explains why she was not identified as a weak D carrier.

Summary

We evaluated a patient with a strongly expressed D antigen with a posttransfusion antibody to the D antigen. The antibody had clinical and serologic characteristics of an alloantibody. After RHD and RHCE genotyping, the patient was found to have the RHDweak D type 21 RHCE*Ce/RHCE*ce genotype (Rweak D type 21r phenotype). Although most patients with a weak D phenotype do not produce alloanti-D, patients who carry the rare weak D type 21 in the absence of a normal RHD allele appear susceptible to the production of D alloantibodies.

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References


Heather McGann, MT(ASCP) SBB (corresponding author), Supervisor, Blood Bank, Department of Pathology, and Robert E. Wenk, MD, Consulting Pathologist, Department of Pathology, Sinai Hospital, 2401 West Belvedere Avenue, Baltimore, MD 21215.
A review of the Chido/Rodgers blood group

R. Mougey

... and the blind, wise blood bankers said, “These antibodies are high-titered and have low avidity, they are red cell, white cell antibodies, they are clinically insignificant, are not reactive with enzyme-treated red cells, and can be absorbed with white cells!” Then the chorus of other blind, wise blood bankers said, “NO, THEY ARE NOT!”

Based on memories of discussions about these antibodies back in the 1970s and the fable of the elephant and blind wise men—R. Mougey

It was a delight, an honor, and also a little intimidating to be asked to write a review of the Chido/Rodgers blood group system, especially considering that the last author to pen a review for Immunohematology on this subject was Carolyn M. Giles, the author of so many important publications on Chido and Rodgers. Moreover, the recent discoveries about Chido and Rodgers have moved well beyond the field of blood group serology to an understanding of the functional protein and genetics at a molecular level.

My delight was in the opportunity to revisit my early days in performing antibody identification and think again about the challenges that blood bankers faced then in working with a group of antibodies that no one could agree about. Chido and Rodgers were usually lumped in with this group, which in light of current knowledge is quite understandable. As I recall even the pronunciation for Chido was a matter of some dispute, with adherents claiming that “Cheedo” with a short i vowel, not Chido with a long i sound, was correct. It occurred to me that this review should cover a historical perspective, which might be helpful to those blood bankers whose testing experience is derived from the newer methods of gel column or solid-phase technology and who might be wondering what all the fuss was about. The review will then cover the current understanding of the serology, function, and biochemistry, and then the genetics of C4 and the Chido/Rodgers blood group system. The nomenclature for the complement system is very confusing even for blood bankers who deal routinely with a nomenclature for a blood group that is just as confusing.

Historical Perspective

In the beginning there was confusion and chaos. During the 1960s and '70s, the process for antibody identification was usually a straightforward and consistent exercise with good consensus between individual technical staff and among laboratories, except there was this group of antibodies that “would not cooperate.” In the reference laboratory where I worked, we called them “the grubbies,” but we were too frustrated by working with them to go much further than that. The usual experience was to spend an inordinate amount of time performing every possible test, which led only to excluding what the antibody could not be. Then when additional samples were obtained to continue the investigation, it was not uncommon to find that tests that had appeared to be nonreactive were now reactive with the new antibody sample or with a freshly collected sample of the test cell. It took me a while to recognize that with these antibodies, if you wanted to test for specificity by typing the patient's RBCs or testing known antigen positive or negative RBCs, it was best to use the freshest RBCs. If you wanted to find compatible blood, older donor units were more likely to be nonreactive. That is, it was generally agreed that there were some patients whose antibodies could not be identified as any of the then known antibodies and whose reactivity was so weak there was a lot of disagreement as to which test RBCs were truly nonreactive. This made it very difficult to define and describe the true characteristics of the antigens and the antibodies and show that the antigens were expressed on the RBC as an inherited trait. This variability was partly a result of testing methods, which relied heavily on a multiphasic set of incubation temperatures and the use of a polyclonal antiglobulin reagent. In that period, blood bankers went to great lengths to detect and identify room temperature–reactive antibodies as well as antibodies active at 37°C. The use of fresh serum for testing was usually considered essential, with the end result that blood bankers were pretty good at detecting clinically significant antibodies but even better at finding clinically insignificant antibodies. Blood bankers got lots of practice at identifying these clinically insignificant antibodies, and neutralizing them with human breast milk, avian egg albumin, hydatid cyst fluid, saliva, and human and guinea pig urine. The grubbies defied these efforts, and there was much discussion, some of it acrimonious, on how patients with these antibodies ought to be managed. The term high titer, low avidity was preferred by many if only for its acronym HTLA, even though everyone knew that these antibodies were not always of high titer or of low avidity. When such weak antibodies were shown to be of high titer, it defied logic that the antibody should show such poor avidity. Obviously, the fault could lie with the antigen, either its orientation or its number of sites, or some other property not yet realized. Again, there was a contradiction in the patient making a high-titered antibody if there was so little antigen to stimulate the production of antibodies. The first glimmer of understanding in these contradictory findings was the reports on anti-Chido.
Discovery and Serology of Chido/Rodgers

The Chido antibody was first studied in a patient in 1962, and in a collaborative effort between laboratories in Minneapolis, Minnesota, and London, United Kingdom, it was found to be reactive with nearly all cells tested but was consistently nonreactive with the RBCs of a London panel donor. Six additional examples were found, and the antibody was named Chido after one of the patients and the combined results were published by Harris et al. in 1967.3 Their title is as good a description of the grubbies as any, “A nebulous antibody responsible for crossmatching difficulties.” The authors reported that the antibodies could not be inhibited by saliva, urine, or hydatid cyst fluid.

During this time, there was much speculation about these grubby antibodies being RBC-WBC antibodies, that came from some successful experiments using WBCs to adsorb these antibodies.6 This whole issue of mistaken identity was very understandable because of the not uncommon antibody problem known as anti-Bg. Bg antibodies had been shown to be hemagglutinins with anti-HLA specificity, and the Bg antigens were thought to be remnants of those HLA antigens expressed on the RBC before the loss of its nucleus. Because Bgs were antigens of lower frequency, it would have been so tidy to have them be the antithetical allele to Chido as the variable strength of reactivity seemed so similar. Eventually it was realized that the “grubby” antibodies had nothing to do with WBC antigens. There may have been sufficient residual plasma in the WBC preparations used to adsorb the antibodies, now known to be capable of inhibiting anti-Chido, to lead to this mistaken conclusion. The speculation that the Chido antigen was a WBC antigen did lead to studies for possible linkage with HLA, an important advance as linkage studies had shown no association with any other blood groups.

Many additional examples of anti-Chido were found, and it was thought that these antibodies did not seem to cause obvious destruction of Chido-positive donor RBCs. The work of Middleton and Crookston3 published in 1972 provided the clue that led to recognizing the true nature of the blood group. In an attempt to enhance the reactivity of the anti-Chido they were studying, inert human plasma was added to the tests; a common practice of the time was to add a source of protein to enhance antibody binding for antihuman globulin (AHG) reactions when using polyspecific AHG. The authors recognized that when the antibody reactivity disappeared after the addition of plasma the Chido antigen must also be in plasma. They then found that performing Chido antigen typings using the plasma of the person being typed in a neutralization test was a much more reliable way to type individuals for the Chido antigen than testing their RBCs. They also noted that weak Chido antigen reactivity on an individual’s RBCs did not appear to correlate with the amount of antigen in that person’s plasma. The ability to neutralize anti-Chido was also a useful tool for antibody identification, especially in sera with multiple antibodies. This was the first way in which Chido was distinguished from the other so-called HTLA specificities Knops, Cs, and Yk, although some workers continued to think of anti-Yk as also showing some inhibition with plasma.4

At this point, the Chido antigen was thought to be a plasma antigen that was adsorbed onto the RBC much like the Lewis antigens. Once accurate, reproducible antigen typings were possible, family studies on the inheritance of the Chido gene led to the discovery that Chido was linked to HLA.5 This potential relationship to HLA prolonged the use of the term RBC-WBC antibodies, but the recognition of HLA linkage to Chido led to the studies to see whether other genes known to be linked to HLA would also show linkage to Chido.

The description of anti-Rodgers, a second antibody reactive with an RBC antigen of high prevalence that could be neutralized by plasma, followed in 1976.6 The antibody was formed by a transfused patient whose RBCs were Chido-positive who also made an anti-E. The anti-Rg’ (as anti-Rg was referred to at that time) gave weak to 4+ reactions with E– RBCs (not your typical low-avidity antibody). Using plasma neutralization tests, Longster and Giles found that 3.1 percent of donors could not inhibit the antibody, i.e., were Rodgers-negative, although they found some donors who were only partial inhibitors. Nordhagen et al.7 were also able to demonstrate partial inhibition of anti-Chido by the plasma of Chido-positive subjects, and Giles8 showed that partial inhibition was an inherited trait. These findings would explain conflicting results in tests on Chido/Rodgers antibodies among laboratories if a single source of plasma was used in inhibition tests. The common practice now is to use a pool of inert plasmas when attempting to determine whether an unknown antibody is neutralizable by human plasma.

Rodgers was also shown to be linked to HLA in two studies in 1976 that set the stage for the discovery of the relationship of Chido and Rodgers to the C4 protein.9,10 In 1978, O’Neill et al.11 showed that Chido and Rodgers are antigenic determinants expressed on the C4 complement protein. Then Tilley et al.12 showed that the Chido and Rodgers determinants are on the C4d fragment of the C4 molecule. The small amount of C4d normally found on the RBC membrane is the limiting factor that explains the low avidity of anti-Chido or anti-Rodgers. In fact, if large amounts of C4d are bound to the RBC under low ionic conditions, anti-Ch or anti-Rg can be turned into a strongly reactive, direct agglutinin.13 The puzzle of incomplete or partial inhibition of the antibodies is caused by the extreme polymorphism of the C4 genes with multiple alleles that cause variations in the Ch or Rg determinants.

All this progress made Chido and Rodgers antibodies easier to recognize with the exception of Chido-positive variant individuals who have made anti-Chido to the part of the Chido antigen that they lack.14 The further serologic complexities of Chido/Rodgers specificity will be discussed in the section on genetics.
The inexorable unraveling of the puzzle of the true nature of the blood group Chido/Rodgers is an excellent example of the importance of recognizing anomalous findings in the study of antibodies that most blood bankers wished could just be ignored! The final refutation of the term HTLA came from the revelation that the Knops, McCoy, Swain-Langley, and York blood group sera recognize antigens that are carried on the RBC complement receptor CR1.15,16 However, this finding seemed to have brought a level of kinship to these antibodies with Chido and Rodgers that must have been satisfying to those who wanted to believe that Chido, Knops, etc. must be related. Again the low avidity of most Knops antibodies is probably related to the low copy number of the individual’s CR1 receptor. It also seems likely that these blood groups will be forever linked in the literature as those HTLAs when they are studied by aspiring blood bankers.

Clinical Significance of Chido/Rodgers

It is well documented that many patients with Chido/Rodgers antibodies have been safely transfused with “random” RBC units, but there are also reports of significant reactions to plasma or platelet transfusions in patients with anti-Chido or -Rodgers,17,18 a cautionary incident reminding blood bankers not to ignore the potential significance of an antibody based on specificity alone. Strupp et al.19 also pointed out the danger in assuming that antibodies that appear to be of low avidity may be safely ignored without further investigation. They reported 4 patients with sickle cell disease for whom the provision of compatible blood and benefit of transfusion were delayed because Dombrock antibodies were not initially recognized and identified. However, there is a need to determine the best way to detect and identify these antibodies without incurring unnecessary delays and expense to the patient, but how much testing is enough? It sometimes seems that when we had the time, resources, and people to detect and identify these antibodies, we did not have a clue what they were. Now we know all about them and do not have the time, people, or resources to do much with them. In this area as with so many other blood group issues, perhaps molecular methods will identify good patient practices, and with the use of microarray technology, definitive answers to some of these thorny issues of cost versus benefit will be found. Until that time, when patients with known anti-Chido or anti-Rodgers require RBC transfusions, antibody detection, identification, and crossmatching methods should include plasma-neutralization studies. After all, these patients are known antibody formers. If transfusion of plasma components is required, there should be some consideration given to the possible effects on the patient of receiving a large volume of plasma, such as the formation of precipitating immune complexes or anaphalaxis.20,21 Finally, the presence of antibodies to C4 proteins may indicate a potential disease association such as is seen with the patients who have anti-Ch and anti-Rg and are C4-deficient.

The story of Chido and Rodgers blood groups has moved well beyond the “crossmatching difficulties,” and there is now a wealth of new material that shows the C4 genes and proteins to be a fascinating model for the evolution of the genes for critical biologic processes that can both protect and attack their host organism.

Structure and Function of the C4 Protein

The C4 protein expressing the Chido or Rodgers epitopes occurs in two isoforms, called C4A for acidic (formerly C4F) and C4B for basic (formerly C4S).20 Awdeh and Alper20 proposed the change from F for fast and S for slow to conform with the then new International System for Human Gene nomenclature and because in their experiments sialic acid was removed from plasma proteins before electrophoresis, resulting in distinct but different bands from those in the experiments of O’Neill et al.,21 which were the first to link the Chido/Rodgers antigen to C4 proteins. C4A proteins usually carry the Rodgers antigens and C4B, the Chido antigens, although a number of haplotypes have been discovered in which these associations were switched.21,22

C4A or C4B protein is expressed as a single-chain precursor of 1744 amino acid residues, and the C4A or C4B amino acid sequences are nearly identical (99%) except near residues 1000 to 1200.23 The amino acid substitutions that occur at residues 1101 to 1106 distinguish C4A from C4B. Single-nucleotide polymorphisms (SNP) are also found in the region that distinguishes the alleles of C4A from each other and from C4B and its alleles. The Chido and Rodgers antigens are thought to differ by both amino acid substitution as well as conformational binding sites.24 After translation, the pro-C4 protein is cleaved into alpha, beta, and gamma polypeptide chains that are linked by disulfide bonds.25 However, incomplete processing actually leads to multiple structural forms of the peptide being secreted in the plasma.26 The activated complement component C1 initiates cleavage of 77 amino acids from the N-terminus of the alpha chain, releasing C4a, and the remainder of the molecule C4b changes shape, exposing a thioester bond between the sulfhydryl group of Cys-991 and the carbonyl group of Gln-994. This binding site that is exposed on activation will allow the formation of either a covalent ester or an amide linkage.27,28 Activated C4B is more efficient than C4A at pathogen cell lysis because of its histidine residue at position 1106 and its serine residue at position 1102.29

The liver is the primary site of C4 production, with smaller amounts produced by macrophages in other tissues.30 There is a wide variation in the amount of C4 produced among individuals. The concentration of C4 in the plasma varies from 80 to 1000 µg/mL with variable concentrations of C4 isotypes.31 Moulds et al.32 found variation in patterns of C4 protein levels in individuals that showed either a complete absence of C4A or C4B, more C4A than C4B, more C4B than C4A, or equal amounts of both. There
is some correlation between C4 gene copy number variations and certain polymorphisms with the level of C4 protein produced, but the effect at the haplotype level can be lost in the diploid gene expression.\textsuperscript{33}

C4 can be activated via the classical pathway or the mannann-binding lectin pathway, although this may be a simplistic view as a recent paper demonstrated the potential for localized release of anaphylatoxins C3a and C4a by tryptase under certain conditions such as those found in the airways of asthmatics.\textsuperscript{34} Although it is simpler to consider organized pathways in which complement proteins perform their complex dance with the other parts of the innate and humoral immune response, it is likely that the protection or attack effect is far more chaotic and layered in multiple mechanisms that activate, block, or break down these proteins (see discussion on disease association).

In the classical pathway, C4 is processed by activated C1, which removes C4a anaphylatoxin from the alpha chain. C4a is a mediator of local inflammation and can induce the contraction of smooth muscle, increase vascular permeability, and cause histamine release from mast and basophilic WBCs. C4a levels along with the anaphylatoxins C3a and C5a in plasma were shown to be elevated during normal pregnancy as an indication of the increased activation of the complement system, possibly as a protection against infection.\textsuperscript{35}

The remaining C4b fragment forms the enzymes C3 convertase (C4bC2a) and C5 convertase (C3bC4bC2a). These enzymes catalyze the membrane attack proteins, which open a channel into the cell membrane. C4 binding protein (C4bp) is another plasma protein that regulates C4 activity by blocking the formation of and promoting the decay of the classical pathway C3 convertase. Deficiency of C4bp would be expected to result in increased activation of C3 and complement activity in response to classical pathway or lectin pathway activation by immune complex formation, bacterial infections, apoptosis, and other triggering mechanisms.\textsuperscript{36}

Activated C4b (C4b-B) binds to its target rapidly but has a short half-life of less than 1 second and has greater hemolytic activity than C4A.\textsuperscript{28} Activated C4A (C4b-A) is 10 times slower than C4b, but it forms amide bonds with amino group targets of immune complexes (IC) or protein antigens. C4B more efficiently forms ester bonds with carbohydrate antigens, resulting in better clearance of pathogens.

Besides activation of the complement cascade via the classical or lectin pathways, the covalent binding of C4 to immunoglobulins and to IC enhances the solubilization of immune aggregates and the clearance of IC by binding to complement receptor on RBCs (CR-1). This IC binding on the RBC then acts as a shuttle and delivers the IC for phagocytosis by macrophages. Thus C4 plays multiple roles for both the innate and adaptive immune systems in vertebrate animals.\textsuperscript{37

Part of this multiple role is the normal constant low level of activation of C3 via the classical pathway and the alternate pathway. In the classical pathway, it was thought that specific antigen binding by an antibody capable of activating C1 would initiate the pathway, but Manderson et al.\textsuperscript{38} have shown that C3 is also being activated by C1–C4 in the absence of antibody-antigen complexes. They propose that although circulating constant low levels of IC could account for their findings, the continual circulation of the normal levels of IgG antibodies may form “spontaneous unstable Ig aggregates that would provide for a C3 tickover” as well. In their experiments, vascular stasis would encourage or initiate complement activation and would therefore be augmented at sites of inflammation. This would facilitate pathogen recognition by allowing the deposition of activated C3 fragments onto foreign surfaces (host cell surfaces with complement regulatory proteins would be unaffected).

Thus the various polymorphisms of C4 affect the normal functioning of C4 and can be affected by the individual’s ability to produce suitable levels of C4 proteins. Deficiencies of C4 proteins whether acquired or inherited can increase the risk of severe viral and bacterial infections.\textsuperscript{33} How high levels of C4 may also contribute to the risk of diseases is just beginning to be understood (disease association will be discussed later).

**Genetics of C4 and Chido/Rodgers**

The original notation of Ch\textsuperscript{a} and Rg\textsuperscript{a} implied the expectation that there would be additional antithetical alleles Ch\textsuperscript{b} and Rg\textsuperscript{b}.\textsuperscript{1,6} However, it was soon realized that this blood group did not fit this pattern of inheritance. Chido, or Ch, and Rodgers, or Rg, are used to describe the cluster of determinants that are expressed on the C4A or C4B proteins that can neutralize the antibodies formed by Chido- or Rodgers-negative individuals. Also individuals can type as positive with anti-Chido, but form anti-Chido to the Chido epitopes they lack. Currently nine antigens have been described: six of high prevalence for Chido, two of high prevalence for Rodgers, and one of low prevalence, WH.\textsuperscript{24} Eight phenotypes for the CH/RG system, with their respective occurrences, are shown in Table 1 as the prevalence for the other antigen combinations has not been established (Table 1).\textsuperscript{39} The polymorphisms for the Ch and Rg epitopes as well

<table>
<thead>
<tr>
<th>Chido phenotype</th>
<th>Prevalence (%)</th>
<th>Rodgers phenotype</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch:1,2,3</td>
<td>88.2</td>
<td>Rg:1,2</td>
<td>95.0</td>
</tr>
<tr>
<td>Ch:1,–2,3</td>
<td>4.9</td>
<td>Rg:1,–2</td>
<td>2.5</td>
</tr>
<tr>
<td>Ch:1,2,–3</td>
<td>3.1</td>
<td>Rg:1,–2</td>
<td>2.5</td>
</tr>
<tr>
<td>Ch:–1,–2,3</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ch:–1,–2,–3</td>
<td>Rare</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ch:–1,2,3</td>
<td>(proposed but</td>
<td>Rg:–1,2</td>
<td>(proposed but</td>
</tr>
<tr>
<td>Ch:–1,–2,3</td>
<td>may not exist)</td>
<td></td>
<td>may not exist)</td>
</tr>
</tbody>
</table>
as the polymorphisms for the electrophoretic behavior of the C4 protein confounded the early investigations on the inheritance of the C4 genes. No attempt is going to be made by this author to explain the reported phenotypic frequencies of Ch and Rg antigens with the current understanding of the multiple copy number variation of the C4 gene.

In early studies of the inheritance of Chido/Rodgers, it appeared that the genes must be encoded by two closely linked loci. The majority of persons were Chido-positive and Rodgers-positive. Approximately 1.7 percent of those studied were Chido-negative, Rodgers-positive, and 3 percent were Chido-positive, Rodgers-negative. After the linkage of Chido/Rodgers with HLA was established and the discovery of C4 as the plasma protein carrier was made, family studies detecting both serologic allelic forms and electrophoretic alleles seemed initially to be in agreement with this model. It was proposed that null genes or duplicated genes for C4A or C4B led to lack of Rodgers or Chido.

As methods and antisera improved, numerous C4 alleles were discovered and the results of a workshop to define and standardize nomenclature were published in a joint paper by Mauff et al. They described 14 determinants for the C4A gene and 17 for the C4B gene. Genes that express no C4A or C4B are designated with a Q0 (meaning quantity naught). In patients with complete deficiency of C4 (C4A*Q0/B*Q0), null alleles were thought to be attributable to gene deletions or point mutations that cause a frameshift in the DNA sequence, leading to defective or no expression of the protein. One such example is a 2-bp insertion into the sequence for codon 1213 at exon 29 of the mutant C4A gene seen in both Caucasians and those of African descent, but very rarely in those of Asian descent.

The origin of the complement system C3 and C4 proteins predates the ability to make lymphocytes or antibodies; the latter did not arise on the evolutionary scene until cartilaginous fish and sharks. Homology between C3 and C4 proteins indicates that the C4 gene may have evolved from C3 as a duplicated gene. The C4A gene is thought to have arisen by gene duplication and mutation from C4B because of its efficiency in facilitating the clearance of IC.

The C4A or C4B gene consists of 41 exons that in addition to encoding for an acidic or basic isotype can also exist as a long form of 20.6 kb or a short form of 14.2 kb. The long form has an insertion of a human endogenous retrovirus (HERV-K(C4)) into intron 9. The insertion of this retroviral material is thought to have occurred after duplication of the ancestral mammalian C4A gene but before the divergence of humans from the ancestors of other primates. The reading frames of the retrovirus are all closed, but there is still promoter activity and the HERV-K(C4) insert is thought to be able to modulate the expression of the C4 gene. The long form was originally thought to be a C4A gene, but C4 genes can be C4A (long or short) or C4B (long or short). However, most C4A genes have the HERV-K(C4) insert and a greater proportion of C4B genes do not.

The genes for C4A and C4B were shown to be located between HLA-DR and HLA-B loci on the short arm of chromosome 6 along with C2 and factor B. C4A and C4B proteins are distinguished phenotypically by their migration patterns in electrophoretic gels and genetically by four nucleotide substitutions that encoded changes at residues 1101 to 1106 where C4B encodes for LSPVLD and C4A encodes for PCPVLID at the protein level. C4A and C4B are also distinguished phenotypically by their expression of either the Chido or the Rodgers antigen, which are the result of two amino acid substitutions at residues 1188 to 1191. The Ch1 epitope has ADLR and the Rg1 has YDLR. As data from family studies began to contradict a simple two-gene model, it was realized that a haplotype could have one, two, three, or four C4 genes and that the C4 genes were inherited as part of a complex of genes. The two-gene theory had to give way to the current hypothesis of the inheritance of a haplotype modular gene complex that can vary by C4 copy number and is part of a larger gene complex in the MHC locus. This model explains both the qualitative and quantitative nature of the C4 gene expression as regards epitopes, concentration of the C4 protein in the plasma, the frequency of certain haplotypes, and the association of C4 copy number variation with certain diseases. In fact, the C4 gene as part of the MHC complex can serve as an example of how the genes of an important biologic pathway evolve via the mechanics of meiosis and through the pressure of natural selection to conserve important genes and to maximize the utility of polymorphism potential. Gene duplication, translocation, single-nucleotide polymorphisms, insertions, and deletions all have played a role in the plastic nature of the C4 gene.

Both forms of C4 are highly homologous but either by SNP or by insertions or deletions display great qualitative and quantitative diversity. The copy number variation comes from whether an individual has inherited haplotypes that have one, two, three, or four C4 genes. This gene cluster occurs as a complex in the MHC locus near the centromere with flanking genes in the following order: first an intact RP gene (a serine/threonine kinase gene), an intact C4 gene (either C4A or C4B), then an intact CYP21B gene (cytochrome P450 21-hydroxylase gene), and an intact TNXB (tenascin-X protein). Approximately 17 percent of persons of Northern European ancestry have this haplotype, termed monomodular. In 83 percent of those of Northern European ancestry the C4 gene complex can contain one, two, or three duplicated gene clusters made of fragments or mutations of the above flanking genes and an intact C4 gene. The duplicated gene complex, called RCCX, occurs between the C4 gene and the intact CYP21B gene and consists of a CYP21 (usually a mutated form) fused to a 4.9-kb fragment of TNXA fused to an RP2 0.91-kb fragment and an intact C4 (A or B and long or short). Depending on the number of RCCX modules duplicated, the complete C4 gene may be a monomodular (no RCCX duplication), bimodular, trimodular, or quadrimodular form.
This modular gene duplication maximizes opportunities to conserve the C4 gene, whereas the opportunity for recombination allows greater diversity of polymorphisms. This great diversity should allow for a better response to pathogens but can create opportunities for unequal crossing over during meiosis. Thus low or high copy number variations could be as important a risk factor for disease as the actual C4 polymorphism.

C4 gene copy number studies done on healthy subjects of different ages indicate that those individuals with a low copy number for C4B may be less healthy or have a shorter lifespan than those having more than two copies of the C4B gene. These studies found that in healthy individuals older than 50 years, there was a lower incidence of low copy number for the C4B gene when compared with the low copy number gene frequency in younger healthy individuals.

C4 and Disease Associations

The association of C4 and a number of diseases has been a fruitful area of investigation, and at the genetic level, the frequency of C4 nulls or low copy variation shows a solid association with autoimmune disease, particularly systemic lupus erythematosus (SLE). Although the varying efficiencies of complement function that result from partial or complete deficiency may have a profound effect on autoimmune susceptibility, above-average C4 protein levels may also increase risk. Once a loss of self-tolerance occurs, having high levels of C4 may exacerbate the disease. Moreover, it cannot be overstated that association is not the same as causation. SLE can be thought of as a prototype for the intricacies of the interaction of C4 proteins both in the development of autoimmunity and in disease progression. Inheritance of a C4A*QO null gene shows a greater risk in Caucasian and African populations, but C4B*Q0 nulls are more frequent in SLE patients of Asian descent. SLE is a heterogeneous disease that is characterized by the presence of multiple autoantibody specificities that attack various tissues, resulting in tissue damage, which then presents more antigen to the host’s immune system. It has become increasingly clear that multiple genetic pathways are likely involved and that C4 polymorphism, especially copy number variation, can be a negative or a positive risk factor in the development of autoimmune diseases. This postulates that the function of C4 in the initiation of autoimmunity may be related to its role in presenting antigen to the host immune system, which is separate from the role of complement in the inflammatory process once autoantibodies have been formed. The current understanding of SLE genetics is that there are distinctions in severity, risk, and clinical manifestation that vary by ethnicity, geography, and sex with a greater prevalence in women and some non-Caucasian ethnic groups. Although genetics undoubtedly plays a role, SLE may also be associated with poverty and exposure to infectious diseases.

Progress both in human genetics, particularly by genome-wide association studies, and in the understanding of biologic pathways has led to the discovery of more than 20 different loci that show strong correlation to SLE. Most of the genes identified are involved in three kinds of biologic processes. Minor variations in the genes encoding the proteins of IC processing, production of interferon, or signal transduction of immune cells can greatly increase the susceptibility of an individual to SLE. It is to be expected that more than one kind of trigger, such as pathogens, allergens, or trauma, could then lead to disease.

Inasmuch as a major function of the C4 protein is participation in innate immunity, there is a strong association between deficiencies of C4 and recurrent infections; although this is less frequent than other complement deficiencies. Table 2 lists other reported diseases and their possible association with C4 or complement.

<table>
<thead>
<tr>
<th>Date</th>
<th>Disease</th>
<th>Citation</th>
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<tbody>
<tr>
<td>2009</td>
<td>Schizophrenia</td>
<td>Sh110</td>
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<tr>
<td>2008</td>
<td>Role of complement in schizophrenia</td>
<td>Mayr’s50</td>
</tr>
<tr>
<td>2009</td>
<td>Chronic fatigue syndrome</td>
<td>Sorensen51</td>
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<tr>
<td>2008</td>
<td>Autism and missing or nonfunctional C4B gene</td>
<td>Sweeten71</td>
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<td>2008</td>
<td>Alzheimer’s and increased C4 deposition</td>
<td>Zhou72</td>
</tr>
<tr>
<td>2008</td>
<td>Increased morbidity/mortality associated with low expression of C4B, smokers, and stroke</td>
<td>Szilagyi73</td>
</tr>
<tr>
<td>2007</td>
<td>Cardiovascular disease, smokers, and low expression of C4B</td>
<td>Arason44</td>
</tr>
<tr>
<td>2006</td>
<td>C4 nulls and adult rhinosinusitis</td>
<td>Seppänens55</td>
</tr>
<tr>
<td>2002</td>
<td>Systemic sclerosis</td>
<td>Arason86</td>
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<tr>
<td>1996</td>
<td>Insulin-dependent diabetes and C4 polymorphisms</td>
<td>Lhotta77</td>
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<tr>
<td>1993</td>
<td>Graves’ disease</td>
<td>Ratana-chaiyavong88</td>
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<tr>
<td>1991</td>
<td>C4A deficiency and IgA nephropathy</td>
<td>Wyatt59</td>
</tr>
<tr>
<td>1990</td>
<td>Felty’s syndrome</td>
<td>Hilarby70</td>
</tr>
<tr>
<td>1989</td>
<td>Psoriasis</td>
<td>Wyatt71</td>
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<tr>
<td>1989</td>
<td>Rheumatoid arthritis</td>
<td>Fielder72</td>
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<tr>
<td>1988</td>
<td>C4 allotypes in juvenile dermatomyositis</td>
<td>Robb73</td>
</tr>
<tr>
<td>1987</td>
<td>C4B2 and primary biliary cirrhosis</td>
<td>Briggs74</td>
</tr>
<tr>
<td>1984</td>
<td>Glomerulonephritis, Henoch-Schönlein purpura</td>
<td>McLean75</td>
</tr>
</tbody>
</table>

In their paper, Atkinson and Frank53 also warn that patients with complement C2- or C4-deficient states (either acquired or inherited) have backup complement activation mechanisms that must be considered in defining the role of complement and a disease. For example, the immune response of specific antibody produced to a pathogen (antibody...
excess) may be all that is needed to trigger more ancient pathways to perform cell lysis in the absence of sufficient complement proteins.

Summary

The C4 protein plays an important role in maintaining health and, in some situations complicated by poor expression of the C4 protein, may lead to or exacerbate certain diseases. The blood groups Chido and Rodgers are epitopes on the C4 protein, and polymorphisms associated with these epitopes may lead to the formation of antibodies to the Chido or Rodgers antigens in transfused patients. Identification of anti-Ch or anti-Rg is still based on the antibody neutralization with plasma from Ch-positive or Rg-positive individuals and lack of reactivity with qualified Ch-negative or Rg-negative RBCs. These antibodies may be useful in genetic studies of C4 polymorphisms or, in the case of C4-deficient patients, a signal of the potential for serious illnesses. The recognition of the extreme polymorphism of the C4 gene and the gene complex RCCX should lead to more insights in the understanding of disease risk and potential treatment.

References


Ruth Mougey MT(ASCP)SBB, President, Mougey, Incorporated, 1705 Highland Avenue, Carrollton KY 41008.

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In Memoriam: Marie Cutbush Crookston
1920–2009

D. Mallory

Marie Cutbush Crookston, a dynamic person and exceptional scientist, was born in 1920 in Victoria, Australia. She received a Bachelor of Science degree from the University of Melbourne, and, in 1947, she set sail for England. There, she worked for 10 years with Dr. P. L. Mollison at the Medical Research Council Blood Transfusion Research Unit and contributed to the writing of the first edition of Dr. Mollison’s Blood Transfusion in Clinical Medicine.

In 1957, she married Dr. John Crookston, a hematologist from Toronto, who was in London working for Dr. J. V. Dacie. Giving up her studies for a PhD, she moved to Toronto to raise two sons and to continue to work in the field of immunohematology. In 1964 she joined the University of Toronto, Department of Pathology, where she directed research, and in 1978 she became an associate professor. She also served as immunohematologist in the Department of Hematology at the Toronto General Hospital and acted as a consultant for the Blood Transfusion Laboratory until she retired in 1986.

Marie’s contributions to immunohematology are outstanding and have made a permanent impact on the field. She and her late husband John helped transform the educational level and direction of immunohematology in the 1960s when they returned to Toronto from London. There, they helped to organize the Ontario Antibody Club, which was active for 25 years.

Marie’s studies on conversion of incomplete to complete antibodies by chemical modification were used by commercial companies to make chemically modified Rh antiserum. In addition, she was known for her discoveries of anti-Fy$a$ and anti-Lu$b$ and her early work in exchange transfusion of the newborn, long-term preservation of RBCs by freezing, and linkage of Chido and HLA. She and John Crookston also coined the term HEMPAS to describe an inherited chronic hemolytic anemia with multinucleated cells in the bone marrow associated with a positive acidified serum test (modified Ham’s solution).

Everyone who knew Marie and read any of her 65 published papers remembers her wonderful ability to write in plain intelligible English, or as she would say, “deathless prose,” and how she kept strictly to proper blood group terminology. Her papers were always accepted as submitted. She was on the editorial boards of Immunohematology, Journal of Blood Group Serology and Education, Transfusion, and Vox Sanguinis.

These are some of her awards:
\begin{itemize}
  \item The Karl Landsteiner Memorial Award in 1976 with Dr. Eloise Giblett from the American Association of Blood Banks, awarded “for her discoveries of anti-Fy$a$, anti-Lu$b$, and anti-HEMPAS: for her studies of the relationship between blood-group antibody activity in vitro and in vivo: for her description of red cell uptake of Le$a$ and Le$b$ antigens from plasma; for her investigation of the Chido antigen in plasma; and for the detection of the linkage between Ch and HLA.” (quotation from citation on award)
  \item The Nuffield Foundation Award in 1952 with Dr. Hermann Lehmann for studying the blood groups of tribes in the Nilgiri Hills of South India.
  \item The Buchanan Award in 1980 from the Canadian Society of Transfusion Medicine.
  \item The Canadian Blood Services Award in 2002 for “Lifetime Achievement.”
\end{itemize}

Marie always found the time to help students and scientists who needed directions with their studies or help with their manuscripts. She was a wonderful, caring person who was an enthusiastic, intelligent, and brilliant scientist and one who will be missed by her many friends and colleagues around the world.

Delores Mallory, MT(ASCP)SBB
Emeritus Editorial Board
Eloise “Elo” Giblett was born in Tacoma, Washington, in 1921 and, in 1931, the family moved to Spokane. Elo developed a keen interest in music at an early age, taking piano, dance, and violin, the instrument on which she would focus, becoming concertmaster of her high school orchestra. She put aside music, turned to science, and received a BS in bacteriology from the University of Washington (U of W) in 1942. World War II had started, so Elo joined the WAVES in 1944, became a medical laboratory technician, and served until 1946. She returned to the U of W and received her MS degree in microbiology in 1947 and then, in 1951, graduated first in her class in the second class to graduate from the new medical school at the U of W.

During the medical internship and hematology fellowship at U of W, her interest in human genetics was encouraged. In 1955, she became the first full-time physician at King County Blood Bank (now the Puget Sound Blood Center) as the Associate Director of the Typing and Crossmatch Laboratory and was given a 6-month sabbatical with Dr. Patrick Mollison at the Blood Transfusion Research Unit in London, England. There, she studied the Lewis blood group system, measured the rate of tagged RBC destruction, became acquainted with the clinical potential of the Coombs antiglobulin test, and mastered the tests used at that time in Dr. Mollison’s laboratory. When she returned to Seattle, she began to use the antiglobulin test to find RBC antibodies and discovered anti-V and anti-Js.

Her numerous studies of the polymorphisms of haptoglobin and transferrins and her interest in RBC, plasma, and protein polymorphisms resulted in the renowned 1969 publication of her book, Genetic Markers in Human Blood. In addition, she discovered that two forms of inherited immunodeficiency disease were caused by deficiencies of the enzymes adenosine deaminase and purine nucleoside phosphorylase.

In 1979, Dr. Giblett became the Director of the Puget Sound Blood Center and remained so until she retired in 1987. Upon her retirement, she became Director Emeritus of the Blood Center and Professor Emeritus of the U of W Medical School. After 40 years, she returned to the violin and even helped found a new music school.

Dr. Giblett was president of the American Society of Human Genetics (1973); a member of the Advisory Board of the American Society of Hematology (1980–1986); a member of the Editorial Board of Blood, Transfusion, and The American Journal of Human Genetics (among a few); and chairperson of the Enzyme Nomenclature Committee of the International Workshop on Human Gene Mapping.

She received many honors during her lengthy career, including two from the American Association of Blood Banks:

- 1975—Emily Cooley Lecture Award (American Association of Blood Banks)
- 1976—Karl Landsteiner Memorial Award (American Association of Blood Banks)
- 1978—The Philip S. Levine Award (American Society of Clinical Pathology)
- 1980—Election, National Academy of Science
- 1987—Distinguished Alumna Award (University of Washington)

Dr. Giblett has left a legacy of original scientific discoveries, a book that was groundbreaking for the field of immunohematology, and colleagues who were scientifically mentored and generously helped by her excellent editorial skills. Those who knew her will remember a very interesting and giving human being.

Delores Mallory, MT(ASCP)SBB
Emeritus Editorial Board
Masters (MSc) in Transfusion and Transplantation Sciences at The University of Bristol, England

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 2010 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 1179 595 455, e-mail: p.a.denning-kendall@bristol.ac.uk.
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.

Monoclonal antibodies available at no charge
The New York Blood Center has developed a wide range of monoclonal antibodies (both murine and humanized) that are useful for donor screening and for typing RBCs with a positive DAT. These include anti-A1, -M, -s, -U, -D, -Rh17, -K, -k, -Kp, -Js, -Fy, -Fy3, -Fy6, -Wr, -Xg, -CD99, -Do, -H, -Ge2, -Ge3, -CD55 (both SCR2/3 and SCR4), -Ok, -I, and anti-CD59. Most of the antibodies are murine IgG and require the use of anti-mouse IgG for detection (anti-K, k, and -Kp). Some are directly agglutinating (anti-A1, -M, -Wr and -Rh17) and a few have been humanized into the IgM isoform (anti-Js). The antibodies are available at no charge to anyone who requests them. Please visit our Web site for a complete list of available monoclonal antibodies and the 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 >immunochemistry >current list of monoclonal antibodies available.
Reference and Consultation Services

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

For information, contact Meh dizadeh 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

CLIA licensed, ASHI accredited

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, or e-mail: flickingerc@usa.redcross.org, or write to:

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

CLIA licensed

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, call Kathy Kaherl at: (860)678-2764, e-mail: kaherlk@usa.redcross.org, or write to:

Reference Laboratory
American Red Cross Biomedical Services
Connecticut Region
209 Farmington Ave.
Farmington, CT 06032

CLIA licensed

National Reference Laboratory for Blood Group Serology

Immunohematology Reference Laboratory
AABB, ARC, New York State, and CLIA licensed
(215) 451-4901—24-hr. phone number
(215) 451-2538—Fax

American Rare Donor Program
(215) 451-4900—24-hr. phone number
(215) 451-2538—Fax
ardp@usa.redcross.org

Immunohematology
Journal of Blood Group Serology and Education
(215) 451-4902—Phone, business hours
(215) 451-2538—Fax
immuno@usa.redcross.org

Quality Control of Cryoprecipitated-AHF
(215) 451-4903—Phone, business hours
(215) 451-2538—Fax
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 Immunohematology
  New York Blood Center
  310 East 67th Street
  New York, NY 10065

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

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

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

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

Why be an SBB?
Professional growth  Job placement  Job satisfaction  Career advancement

How does one become an SBB?
• Attend a CAAHEP-accredited Specialist in Blood Bank Technology Program  OR
• Sit for the examination based on criteria established by ASCP for education and experience

However: In recent years, a greater percentage of individuals who graduate from CAAHEP-accredited programs pass the SBB exam.

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

Contact the following programs for more information:

<table>
<thead>
<tr>
<th>Program</th>
<th>Contact Name</th>
<th>Phone Contact</th>
<th>Email Contact</th>
<th>Website</th>
<th>On site or On line</th>
</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@NAAMEDARMD.MIL">William.Turcan@NAAMEDARMD.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 Cooper</td>
<td>909-859-7946</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 Lamence</td>
<td>414-937-6403</td>
<td><a href="mailto:Lynne.Lamence@bcw.edu">Lynne.Lamence@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@cbctcs.org">nlang@cbctcs.org</a></td>
<td><a href="http://www.cbctcs.org/education/sbb.htm">http://www.cbctcs.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:cwong@giveblood.org">cwong@giveblood.org</a></td>
<td><a href="http://www.giveblood.org/education/">www.giveblood.org/education/</a> distance/htm</td>
<td>On line</td>
</tr>
<tr>
<td>Hoxworth Blood Center, Univ. 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>
</tr>
<tr>
<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>
<td><a href="http://www.indianablood.org">www.indianablood.org</a></td>
<td>On line</td>
</tr>
<tr>
<td>Johns Hopkins Hospital</td>
<td>Jan Light</td>
<td>410-955-6580</td>
<td><a href="mailto:jlight5@jhmi.edu">jlight5@jhmi.edu</a></td>
<td><a href="http://pathology2/jhu/department/divisions/tranfusion/sbb.cfm">http://pathology2/jhu/department/divisions/tranfusion/sbb.cfm</a></td>
<td>On site</td>
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<tr>
<td>Medical Center of Louisiana</td>
<td>Karen Kirkley</td>
<td>504-933-3954</td>
<td><a href="mailto:kirkkl@lsuhsc.edu">kirkkl@lsuhsc.edu</a></td>
<td>none</td>
<td>On site</td>
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<tr>
<td>NIH Clinical Center Dept. of Transfusion Medicine</td>
<td>Karen Byrne</td>
<td>301-496-8335</td>
<td><a href="mailto:Kbyrne@mail.nih.gov">Kbyrne@mail.nih.gov</a></td>
<td><a href="http://www.cc.nih.gov/divisions/dtm">www.cc.nih.gov/divisions/dtm</a></td>
<td>On site</td>
</tr>
<tr>
<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">www.rushu.rush.edu/health/dept</a>. html</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 1514</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>
</tr>
<tr>
<td>Univ. of Texas Health Science Center at San Antonio</td>
<td>Linda Myers</td>
<td>210-731-5526</td>
<td><a href="mailto:lmyers@bloodtransfusion.org">lmyers@bloodtransfusion.org</a></td>
<td><a href="http://www.uthscsa.edu">www.uthscsa.edu</a></td>
<td>On site</td>
</tr>
<tr>
<td>Univ. 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>
<td>On line</td>
</tr>
<tr>
<td>Univ. of Texas SW Medical Center</td>
<td>Barbara Laird-Fyer</td>
<td>214-648-1785</td>
<td><a href="mailto:barbaralaird-fyer@UTSouthwestern.edu">barbaralaird-fyer@UTSouthwestern.edu</a></td>
<td><a href="http://telecampus.utsystem.edu">http://telecampus.utsystem.edu</a></td>
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</table>
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
   b. Case Report (if indicated by study)
   c. Materials and Methods
   d. Results
   e. Discussion
   f. Case should be written as progressive disclosure and may include the following headings, as applicable
     i. Clinical Case Presentation
     ii. Immunohematologic Evaluation and Results
     iii. Interpretation
     iv. Recommended Therapy
   g. Discussion

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” x 7” glossy).
   b. Use short headings for each column needed and capitalize first letter of first word. Omit vertical lines.
   c. Place explanation in footnotes (sequence: *, †, ‡, §, ¶, **, ††).

   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
      ii. Immunohematologic Evaluation and Results
      iii. Interpretation
      iv. Recommended Therapy
   b. Discussion: Brief review of literature with unique features of this case
   c. Reference: Limited to those directly pertinent
   d. Author information (see II.B.9.)
   e. 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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