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The most clinically important blood group systems in transfusion medicine, excluding the ABO system, are the RH, Kell, and Kidd systems. Alloantibodies to antigens of these systems may be produced following blood transfusion or during pregnancy and can result in serious hemolytic transfusion reactions and hemolytic disease of the newborn. We developed rapid and robust techniques for RHD, RHCE, KEL, and JK genotyping with the use of a real-time polymerase chain reaction instrument. Two fluorescence-based methods for the detection of amplification products were used: for KEL1/KEL2, JK1/JK2, and RHE/RHe (exon 5) we used the hybridization probes protocol; for RHc/RHe the analysis was done in sequences of exon 1 for RHc and exon 2 for RHe; and for RHD, analysis was done in sequences of intron 4, exon 7, and exon 4 pseudogene using the SYBR Green I protocol. The genotyping tests were validated with samples from 85 Caucasian Portuguese and 15 Black European blood donors. Complete phenotype-genotype correlations were obtained. The potential use of the presented methods can be predicted in clinical transfusion medicine, allowing appropriate monitoring, early intervention, and improved care.

Reliable immunohematology tests play a critical role in the safe blood transfusion practices used today. Certain circumstances, e.g., recently transfused patients or RBCs that are coated with immunoglobulin, make phenotyping by classic hemaglutination methods complex, time-consuming, and difficult to interpret. In these cases, molecular biology techniques can be used to predict the blood group antigen profile of a patient. The genetic mechanisms for the generation of structural diversity of blood groups include nucleotide substitution (the most common, producing amino acid change or modification of splice site or alteration of glycosylation), exon duplication, exon skipping, unequal crossover, and gene conversion.

The K antigen is a strong immunogen and can cause severe reactions to transfusion of incompatible blood. It has a frequency of about 9 percent in Europeans. The K antigen is antithetical to k and it results from a single transition located within exon 6, which gives rise to an amino acid substitution (698 C>T) in the Kell glycoprotein (Met193Thr). Anti-K is the most common immune RBC antibody outside of the ABO and Rh systems.

The Kidd (JK) antigens are located on a RBC urea transporter. The observation that RBCs from Jknull individuals lacking the JK antigens exhibit an increased resistance to lysis in aqueous 2 M urea established the link between these antigens and the urea transport function. This fact was confirmed several years later, by the demonstration that Jknull RBCs have a selective defect in urea transport. The JK gene consists of 11 exons, of which exons 4 to 11 contain all the coding information for the protein. The molecular basis of the JK1/JK2 blood group polymorphism was shown to be
a single transition (G838A), resulting in an Asp280Asn amino acid substitution. These two codominant alleles define three common phenotypes, Jk(a+b−), Jk(a−b+), and Jk(a+b+). The Jk(a−b−) phenotype (JKnull) has been reported mainly in individuals of Asian and Polynesian origin, but has also been found in Caucasians. Two genetic backgrounds may account for this: the homozygous inheritance of a “silent” allele JK at the JK locus, caused by at least four distinct mechanisms that may affect either JK2 (splice site mutations; missense mutation S291P) or JK1 allele (non-sense mutation Tyr194stop and gene deletion removing exons 4 and 5), or the inheritance of a dominant inhibitor gene In(Jk), which is not linked to the JK locus. These individuals do not suffer from a clinical disorder, except for a urine concentrating deficiency.

The RH locus is composed of two homologous genes, RHD and RHCE (96% identity between the proteins), which are closely linked on chromosome 1p34–p36. Both are composed of 10 exons and share a similar exon/intron organization. In RhD-positive Caucasian individuals (85%) both RHD and RHCE genes are present at the RH locus, whereas in the RH-negative individuals (15%) the RHD gene is missing, most likely following a deletion event. However, in other populations (Black, Japanese), where the RH-negative phenotype is rare, the D gene may be either intact or rearranged by partial deletion, by recombination between the D and the CE genes (conversion), by single point mutation, or by other rare mechanisms. This varied genetic basis for D− individuals and partial D phenotypes obliges the laboratory to use more than one molecular biologic approach to have a sufficient degree of certainty in the genotyping result.

The molecular basis of the C/c specificity resulted from four amino acid differences: Cys16Trp (exon 1 of the CE gene) and IleE60Leu, Ser68A, and Ser103Pro (exon 2). However, it seems that only the Ser103Pro substitution is relevant to the C/c genetic polymorphism. The E/e polymorphism resulted from a single amino acid substitution: Pro226Ala (exon 5 of the CE gene).

These blood group systems are clinically important in transfusion medicine, as the antibodies may cause severe immediate or delayed hemolytic transfusion reactions and hemolytic disease of the newborn (HDN). In the case of Kell antibodies, the evolved mechanisms are hemolytic but can also suppress erythropoiesis.

Our purpose was to develop new molecular tools for diagnostic determination of a patient’s blood groups, complementing classic methods used in transfusion services, with results available in a few minutes, allowing them to be used on a routine basis. To achieve that, we used the LightCycler® (Roche Molecular Systems, Somerville, NJ) polymerase chain reaction (PCR) instrument, a microvolume fluorometer integrated with a thermal cycler that combines rapid-cycle PCR with real-time fluorescence monitoring. This assay is based on the fluorescence resonance energy transfer (FRET) principle. Incorporation of labeled hybridization probes with the initial reaction mix and target nucleic acid allows detection and analysis of PCR products with the LightCycler system in a closed reaction vessel. In our protocols we used the two fluorescence-based methods for the detection of amplification products: DNA binding dye SYBR Green I, and hybridization probes.

**Materials and Methods**

Blood samples from 85 Caucasian Portuguese and 15 Black European blood donors, obtained by venipuncture of the antecubital vein after informed consent, were phenotyped by routine hemagglutination with reagents from DiaMed AG (Cressier sur Morat, Switzerland), according to the instructions of the manufacturer. DNA was extracted using the Magna Pure LC System, with reagents from DiaMed AG (Cressier sur Morat, Switzerland), according to the instructions of the manufacturer. DNA that was not used immediately was frozen at –70°C until assayed.

With these assays, a short fragment harboring the particular polymorphic site is amplified. In the hybridization probes protocol, the amplicons are detected by fluorescence using a 3′-fluorescein-labeled probe and a 5′-LightCycler Red 640-labeled probe that in FRET when hybridized to the same strand internal to the unlabeled PCR primers. Homogeneous genotyping is achieved by positioning one of the probes over the polymorphic nucleotide. When fluorescence is monitored as the temperature increases through the melting point (Tm) of the probe/product duplex, a characteristic melting profile is obtained, depending on the presence or absence of a base pair mismatch in the heteroduplex. The fluorescence signal is then plotted in real time against temperature to produce melting curves for each sample, and then converted to derivative melting curves by plotting the negative derivative of the fluorescence with respect to temperature against temperature.
In this study, for KEL1/KEL2, JK1/JK2, and RHE/RHe (exon 5), we used the hybridization probes protocol; for RHC/RHc, the analysis was done in sequences of intron 4, exon 7, and exon 4 pseudogene using the SYBR Green I protocol. We used the primers listed in Table 1; the PCR reaction mixes are described in Table 2 in accordance with the amplification conditions listed in Table 3. In the case of RHE/RHe, the primers were designed to amplify only the RHCE gene, and the probes to genotype the alleles. In any case, less than 1 hour was needed to perform the entire analysis, including amplification and detection.

For confirmation of the KEL1/KEL2 genotypes, PCR restriction fragment length polymorphism (RFLP) was performed, and for JK1/JK2 PCR allele-specific primers (ASP) was done. For confirmation of the results regarding RHC/RHc, RHE/RHe, and RHD genotypes, classical PCR reactions were applied. DNA testing was done without previous knowledge of the phenotype.

Results

KEL1/KEL2

The phenotypes obtained were all confirmed by both genotyping methods (64 heterozygous for KEL1/KEL2, 34 homozygous for KEL2, and 2 homozygous for KEL1), with no discrepant results. The melting point for KEL1 was at 66°C and for KEL2 at 59°C. In different runs, the positions and distances of the melting peaks were identical and differed by less than 1°C for the same allele (see Fig. 1A and Fig. 2).

JK1/JK2

The phenotypes obtained were all confirmed by both genotyping methods (48 heterozygous for JK1/JK2, 26 homozygous for JK1, and 26 homozygous for JK2), with no discrepant results. The melting point

Table 1. Sequences of the primers and probes used to genotype the KEL1/KEL2, JK1/JK2, RHD, RHC/RHc, RHE/RHe alleles, and the length of the PCR product

<table>
<thead>
<tr>
<th>Primers and probes*</th>
<th>Sequences (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEL 5'-GCTTGGAGGCTGGCGCAT-3'</td>
<td>148</td>
</tr>
<tr>
<td>Anchor 5'-CTGGATGACTGGTGTGTGTGGA-3'</td>
<td></td>
</tr>
<tr>
<td>Sensor TTAACCGAACGCTGAGACTTCTGA</td>
<td></td>
</tr>
<tr>
<td>JK 5'-ATTCACCCCTCAAGTTTCCT-3'</td>
<td>165</td>
</tr>
<tr>
<td>Anchor 5'-ATGAACATTCCTCCCATTGC-3'</td>
<td></td>
</tr>
<tr>
<td>Sensor CCCCCCTTCTCTGTGAGATCCTTG</td>
<td></td>
</tr>
<tr>
<td>RHC 5'-GATGCCCTGGTGGTGGAAC-3'</td>
<td>112</td>
</tr>
<tr>
<td>Anchor 5'-GCTGCTTCCAGTGCTTGGC-3'</td>
<td></td>
</tr>
<tr>
<td>Sensor CCCCCCTTCTCTGTGAGATCCTTG</td>
<td></td>
</tr>
<tr>
<td>RHe/RHe 5'-GCAACAGAGCAAGAGTCCATC-3'</td>
<td>392</td>
</tr>
<tr>
<td>Anchor 5'-GAACATGGCATTCTTCCTTTG-3'</td>
<td></td>
</tr>
<tr>
<td>Sensor CGCCCTCTTCTTGTGGATGTTCT</td>
<td></td>
</tr>
<tr>
<td>RHC/RHc 5'-GATGCCTGGTGCTGGTGGAAC-3'</td>
<td>112</td>
</tr>
<tr>
<td>Anchor 5'-GCTGCTTCCAGTGCTTGGC-3'</td>
<td></td>
</tr>
<tr>
<td>Sensor CCCCCCTTCTCTGTGAGATCCTTG</td>
<td></td>
</tr>
<tr>
<td>RHD (intron 4) 5'-GACCTGGTGGTGGTGGTGGTGG-3'</td>
<td>600</td>
</tr>
<tr>
<td>Anchor 5'-AGATGCCCTCGGAGGCAG-3'</td>
<td></td>
</tr>
<tr>
<td>Sensor CCCCCCTTCTCTGTGAGATCCTTG</td>
<td></td>
</tr>
<tr>
<td>RHD (exon 7) 5'-AGCTCCATCATGGGCTACAA-3'</td>
<td>96</td>
</tr>
<tr>
<td>Anchor 5'-ATTGCCGGCTCCGACGGTATC-3'</td>
<td></td>
</tr>
<tr>
<td>Sensor CCCCCCTTCTCTGTGAGATCCTTG</td>
<td></td>
</tr>
<tr>
<td>RHD (exon 4) 5'-GCCGACACTCAGGTCCTTAC-3'</td>
<td>381</td>
</tr>
<tr>
<td>Anchor 5'-TCCTGAACCTGCTCTGTGAAGTGC-3'</td>
<td></td>
</tr>
<tr>
<td>Sensor CCCCCCTTCTCTGTGAGATCCTTG</td>
<td></td>
</tr>
</tbody>
</table>

*Primers and probes were synthesized by TIB MOLBIOL (Berlin, Germany)

Table 2. PCR reaction mixes used in the protocols of the LightCycler

<table>
<thead>
<tr>
<th>H2O (µL)</th>
<th>MgCl2 (µM)</th>
<th>Primer (µM)</th>
<th>Probe (µM)</th>
<th>Master Mix (µL)</th>
<th>DNA (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEL1/KEL2</td>
<td>10.8</td>
<td>1.6</td>
<td>0.8</td>
<td>2†</td>
<td></td>
</tr>
<tr>
<td>JK1/JK2</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RHE/RHe</td>
<td>13.2</td>
<td>0.8</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RHC/RHc</td>
<td>12.8</td>
<td>1.2</td>
<td>2.5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>RHD (intron 4)</td>
<td>12.4</td>
<td>1.6</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RHD (exon 7)</td>
<td>12.4</td>
<td>1.6</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RHD (exon 4)</td>
<td>12.4</td>
<td>1.6</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Each primer and each probe
† DNA-Master Hybridization Probes
‡ FastStart DNA Master SYBR Green I protocol

Table 3. Conditions of amplification used in the LightCycler

<table>
<thead>
<tr>
<th>Amplification</th>
<th>Denaturation (Temperature-Time)</th>
<th>Annealing (Temperature-Time)</th>
<th>Extension (Temperature-Time)</th>
<th>Cycles</th>
<th>Melting curve* (Temperature)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEL1/KEL2</td>
<td>95°C-30s</td>
<td>62°C-10s</td>
<td>72°C-10s</td>
<td>45</td>
<td>50°C-75°C</td>
</tr>
<tr>
<td>JK1/JK2</td>
<td></td>
<td>55°C-10s</td>
<td>72°C-10s</td>
<td>40</td>
<td>45°C-75°C</td>
</tr>
<tr>
<td>RHE/RHe</td>
<td></td>
<td>56°C-60s</td>
<td>72°C-16s</td>
<td>40</td>
<td>53°C-90°C</td>
</tr>
<tr>
<td>RHC/RHc</td>
<td></td>
<td>72°C-3s</td>
<td>72°C-6s</td>
<td>40</td>
<td>86°C-95°C</td>
</tr>
<tr>
<td>RHD (intron 4)</td>
<td>95°C-0s</td>
<td>63°C-4s</td>
<td>72°C-8s</td>
<td>45</td>
<td>78°C-95°C</td>
</tr>
<tr>
<td>RHD (exon 7)</td>
<td></td>
<td>70°C-3s</td>
<td>72°C-72s</td>
<td>45</td>
<td>84°C-99°C</td>
</tr>
<tr>
<td>RHD (exon 4)</td>
<td></td>
<td>71°C-1s</td>
<td>72°C-10s</td>
<td>40</td>
<td>80°C-95°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>66°C-5s</td>
<td>72°C-17s</td>
<td>45</td>
<td>80°C-95°C</td>
</tr>
</tbody>
</table>

* Cooling and Heating Temperatures (0.1°C/s)
† Seconds
for JK1 was at 56°C and for JK2 at 62°C. In different
runs, the positions and distances of the melting peaks
were identical and differed by less than 1°C for the
same allele (see Fig. 1B).

RHD
The phenotypes obtained were all confirmed by
both genotyping methods (68 RHD-positive, 32 RHD-
negative). There were no discrepant cases between the
two real-time PCR methodologies (see Fig. 3 and Fig. 4).
In one Black European who was RHD-negative by
serotyping, the pseudogene was detected.

RHCE
The phenotypes obtained were all confirmed by
classic and real-time methods (20 RHC/RHC, 55
RHC/RHc, 25 RHc/RHc, 6 RHE/RHE, 38 RHE/RHe;
and 56 RHe/RHe). The melting point of RHE was at
62°C and of RHe at 68°C. In different runs, the posi-
tions and distances of the melting peaks were identical
and differed by less than 1°C for the same allele (see
Fig. 1C).

Discussion
Blood group genotyping techniques are necessary
when we must resolve inconsistent, weak, or posttrans-
fusion mixed-field reactions or positive direct antigo-
bulin tests or in HDN. The classic genotyping methods
pose several problems by (1) using separate tubes for
specifically amplifying each allele (PCR-ASP needs
amplification of internal control fragments) or by (2)
using restriction enzymes (used in PCR-RFLP, they pose
the risk of incomplete digestion of amplicons or
of generating falsely positive results if silent mutations occur
near the mismatch, not allowing the
enzyme to cut.) The potential benefits of homogeneous detection
systems, in rela-
tion to the classic
methods, have long
been recognized: simple
processing and rapid
analysis and no
postamplification
processing, eliminat-
ing sampling track-
ing errors and end-
product contamination.

The methods we
describe are reliable
and have few
manual processing
steps, allowing high
throughput and
rapid results in an economical process. Beyond these advantages, they can also detect other mutations under the probes (a characteristic not achieved using PCR-ASP or PCR-RFLP), which can be very useful. In theory, our system for the JK genotyping could also detect one of the causes of the rare Jk(a–b–) phenotype, the T871C missense mutation. However, when we intend to use the molecular genotyping results in clinical practice, we must be alert that in rare situations the genotype may not correlate with the antigen expression on the RBCs. A dominant inhibitor gene that is not linked to the locus analyzed, mutations in a location other than that being studied, mutations in the GATA box, or the problem of hybrid genes could generate false interpretations with clinical significance. In addition, many DNA-based techniques still must be evaluated. In a recent workshop report on the genotyping of RBC alloantigens, a 5.2 percent rate of mistyping in the ABO system was found, demonstrating that further efforts are needed to improve the precision of the genotyping techniques. The real-time fluorescence PCR has proved its real value in this specific area, as was the case of genotyping the FY blood group locus; nevertheless, larger comparative studies are needed before we can rely solely on genotyping information.

Since this paper was accepted for publication, another procedure was added (using the same technology) to genotype the RHC allele by amplifying intron 2, looking for the insertion of a 109 bp length. This was done because some cases involving cde or cDε phenotypes could generate false results. However, in our study, we did not have such a case.

References
2. Reid ME, Rios M, Powell VI, Charles-Pierre D, Malavade V. DNA from blood samples can be used to genotype patients who have recently received a transfusion. Transfusion 2000;40:48-53.
3. Rozman P, Dovc T, Gassner C. Differentiation of autologous ABO, RHD, RHCE, KEL, JK, and FY blood group genotypes by analysis of peripheral blood samples of patients who have recently received multiple transfusions. Transfusion 2000;40:936-42.


Many methods have been used to assess the potential clinical significance of red cell antibodies: serologic tests (e.g., antibody specificity, thermal amplitude, subclass, and concentration), estimates of in vivo survival of red blood cells (RBCs) using \( ^{51} \text{Cr} \)-labeled RBCs, and more recently, in vitro cellular assays.\(^1\)\(^4\) There are two distinct groups of patients for whom it would be particularly helpful to determine whether an antibody should be considered clinically significant: first, transfusion candidates; and second, pregnant women who have IgG blood group antibodies. A discussion of all the factors that affect the pathogenicity of alloantibodies is beyond the scope of this article. Rather, presented here will be a brief review of the more common methods used for predicting clinical significance, followed by a more detailed discussion of the principles and application of the in vitro cellular assays.

### Clinical significance predicted by serologic characteristics

The in vitro serologic characteristics of an antibody, in particular the specificity and temperature of reactivity, provide the first clues to the potential clinical significance of that antibody. For transfusion, experience and \( ^{51} \text{Cr} \) survival studies have provided information about the ability of various antibody specificities to cause immune destruction of incompatible RBCs.\(^1\)\(^4\) Alloantibodies of certain specificities often do not react at 37°C (e.g., anti-A\(_b\), -M, -N, -Le\(_b\), -Le\(_a\), -P\(_1\), and -I\(_1\)). If such antibodies do not react at 37°C, they are considered clinically insignificant; however, if they do react at 37°C, then they have to be considered as having the potential to cause increased RBC destruction. Antibodies of common specificities generally reactive at 37°C (e.g., Rh, Duffy, Kidd, Kell, and SsU specificities) have been known to cause hemolytic transfusion reactions, and antigen-negative units are obtained for transfusion. Antibodies of some specificities, though reactive at 37°C, are rarely clinically significant (e.g., anti-Ch, -Rg, -Sd\(_b\), and -Xg\(_a\)). Another group of antibodies have questionable clinical significance for transfusion in that sometimes they cause immune destruction and other times they cause no destruction of transfused RBCs; these are the Cartwright, Gerbich, Dombrock, Lutheran, Lan, and LW specificities (Table 1). The questionable significance of these latter antibodies does not seem to relate to the strength of the antibody or to the IgG subclass.\(^1\)\(^7\)

### Table 1. Clinical significance of 37°C reactive antibodies for transfusion

<table>
<thead>
<tr>
<th>Usually significant</th>
<th>Not or rarely significant</th>
<th>Sometimes significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABO</td>
<td>Bg</td>
<td>Cartwright (e.g., Yt(a^+))</td>
</tr>
<tr>
<td>Rh</td>
<td>Le(a^-)</td>
<td>Gerbich</td>
</tr>
<tr>
<td>Kell</td>
<td>Ch/Rg</td>
<td>Dombrock</td>
</tr>
<tr>
<td>Kidd</td>
<td>Knops</td>
<td>Lutheran (e.g., Lu(a^-))</td>
</tr>
<tr>
<td>Duffy</td>
<td>JMH</td>
<td>Lan</td>
</tr>
<tr>
<td>Ss,U</td>
<td>Cs(a^-)</td>
<td>LW</td>
</tr>
<tr>
<td>Vel</td>
<td>Sd(a^-)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xg(a^-)</td>
<td></td>
</tr>
</tbody>
</table>

For hemolytic disease of the newborn (HDN), specificity and immunoglobulin class of an antibody provide the first clues of clinical significance, supported by historical evidence of severity of disease or lack thereof. In addition, other characteristics affect the severity of disease: antibody concentration, antigen density, tissue distribution of the antigen, and degree of expression of the antigen on fetal RBCs.\(^4\) IgG molecules of all four subclasses cross the placenta, whereas IgM and IgA do not. Thus, clinically significant IgG antibodies that have the potential to cause HDN are generally those that recognize antigens that are reasonably
developed on fetal RBCs and that are restricted to RBCs (rather than those having a broad tissue distribution). Anti-D is still the most common cause of HDN, followed by anti-c, -k, and -e.4

Determining the subclass of IgG alloantibodies has little practical value in predicting the survival of incompatible RBCs or in predicting the severity of HDN. IgG1, IgG2, and IgG3 can activate complement and interact with macrophage Fc receptors. IgG3 has been shown to react more efficiently with macrophages than IgG1,7,8 but there are not many data on the in vivo destruction of RBCs by IgG1 versus IgG3 antibodies. Multiple studies have been published on the association of IgG1 and IgG3 with the severity of HDN; however, the results are contradictory.4,7 IgG4 antibodies should not cause RBC destruction since they do not activate complement, nor do they interact with macrophages. Unfortunately, most alloantibodies are IgG1 (alone or in combination with other subclasses), some of these are clinically significant while others are not, and IgG4 antibodies rarely occur alone.7,9 Additionally, not all antibodies can be subclassed, i.e., some do not react with any subclassing sera by the commonly used serologic methods. In a retrospective review of 15 years of data, Garratty et al. were unable to subclass 62 (42%) antibodies by a standardized capillary antiglobulin test.9

Antibody concentration studies, e.g., serologic titers and quantitative assays for antibodies detected during pregnancy, though commonly used, are not considered to be reliable predictors of the severity of HDN.4,14 Rather, they serve better as screening methods to determine when invasive techniques, e.g., amniocentesis, are required to more accurately assess the severity of HDN.10,11 A two-fold or greater increase in titer or a titer equal to or greater than a critical level signals a fetus at greater risk. A critical titer can vary from institution to institution, and generally ranges from 8 to 32.10 Anti-D concentrations below 4 IU/mL, as measured on the autoanalyzer, are considered unlikely to cause HDN, however, concentrations above 4 IU/mL do not always cause HDN.10

In vivo survival studies

The gold standard for predicting whether an antibody would be clinically significant is the 1-hour 51Cr RBC survival study recommended by the International Committee for Standardization in Haematology in 1980.13 A 0.5 mL aliquot of incompatible donor RBCs is labeled with 51Cr and a portion of those labeled RBCs are suspended in saline and injected into the patient; the remainder of the labeled RBCs are retained to prepare a standard for measurement. Anticoagulated samples are collected at 3, 10, and 60 minutes. The plasma is separated and the RBCs are lysed; the radioactivity of each plasma and hemolyzed RBC sample is measured. The sample drawn at 3 minutes is taken to represent 100 percent survival, allowing for mixing of the labeled RBCs with the patient’s circulating RBCs. If the radioactivity of the plasma samples at 10 and 60 minutes does not exceed 3 percent of the total radioactivity injected and the RBC survival at 1 hour is at least 70 percent, transfusion of the incompatible donor RBCs carries negligible risk, i.e., the patient will not have an immediate symptomatic reaction; however, the transfused RBCs may not have “normal” survival.1,5 A later sample at 24 hours may provide additional information, e.g., indicating increased destruction, if the 1 hour measurement is ≥ 70 percent.6 Unfortunately, these survival studies are difficult to arrange, even in large metropolitan areas.

An “in vivo” or “biological” crossmatch is performed by transfusing 10 to 50 mL of unlabeled incompatible RBCs and collecting a blood sample 1 hour later to observe for hemoglobinemia, bilirubinemia, or both. Although intravascular hemolysis of 5mL of RBCs will be readily visible in plasma (pink to red), this test only detects acute complement-mediated intravascular hemolysis; it does not predict even severe extravascular destruction. As a result, a “negative” test could lead to a false sense of security, though doing this test, considered not very sensitive, is better than doing no test at all as long as the limitations of the test are understood.1,13

Functional cellular assays

Immunologists used functional cellular assays in the 1960s to study membrane receptors, but starting in the 1980s these assays have been applied to the field of transfusion medicine to predict the clinical significance of RBC alloantibodies. The two primary applications for these tests are for (1) transfusion patients who have alloantibodies to antigens of high frequency with unknown specificity or associated with questionable clinical significance; and (2) prediction of the severity of HDN.

General Principles of Cellular Assays Used in Immunohematology

There are three cellular assays used in immunohematology for the prediction of clinical significance of RBC antibodies: the monocyte monolayer assay (MMA),14,16 the antibody-dependent cell-mediated cytotoxicity (ADCC) assay,17,18 and the chemiluminescence
(CL) assay or test. These assays are based on the interaction of IgG ± C3-sensitized RBCs with mononuclear cells and are an attempt to simulate destruction of sensitized RBCs in vivo. Mononuclear cells (macrophages and monocytes) have specific receptors for IgG1, IgG2, IgG3, and the C3 component of complement, through which they effect destruction of sensitized RBCs.

All three assays start with a sensitization phase. RBCs (lacking and possessing the antigen under investigation) are sensitized with the patient's antibody under conditions similar to those used for serologic testing. The sensitized RBCs are incubated with mononuclear cells. During this time, adherence to the mononuclear cells through the Fc and/or complement receptors can occur. Once adherence takes place, two independent events can occur, as shown in Figure 1.

The in vitro assays are designed to measure those events. One event, illustrated on the lower left side of Figure 1, is cytotoxic lysis: the RBCs adhering to the outside of the mononuclear cell membrane are lysed by an enzyme. This is the basis of the ADCC assay. Since the RBCs are first labeled with $^{51}$Cr for this assay, the release of radioactivity into the supernatant is proportional to the lysis of the RBCs that were damaged in the cytotoxic reaction. The other event, illustrated on the right side of Figure 1, is phagocytosis and the subsequent lysis of RBCs inside the mononuclear cell. This is the basis of the MMA and the CL assay. The MMA measures adherence and/or phagocytosis of RBCs. The CL assay measures the next step, which is the release of oxygen radicals when the RBCs are phagocytized and destroyed; in the presence of the chemical luminol these oxygen radicals can be measured as light.

All three assays have their advantages and disadvantages and, when performed well and for the appropriate applications, can be used to correlate in vitro results with in vivo reactions of alloantibodies. The MMA has been primarily used in the United States for predicting the outcome of transfusing incompatible RBCs; the MMA is less sensitive for predicting the severity of HDN. The ADCC and CL assays have had more use in Europe for predicting the severity of HDN, though the CL assay has also been applied more recently to transfusion.

**Monocyte monolayer assay**

The MMA (Fig. 2) reflects adherence and/or phagocytosis of sensitized RBCs by peripheral monocytes. Macrophages have also been used in this assay, but it is more difficult to obtain and maintain a supply of macrophages than to obtain peripheral monocytes. The test involves making a monolayer of monocytes on a plastic or glass surface before adding sensitized RBCs. The monocytes and RBCs are incubated at 37°C for 1 to 2 hours, then the RBCs that are not bound to the monocytes are removed by washing. After they have been fixed and stained, the monocytes that have RBCs adhering and/or phagocytized are counted, using a standard light microscope. The MMA does not require radioisotopes or specialized equipment (e.g., a luminometer), so the method is more amenable to being performed by more laboratories. However, the MMA is very labor intensive and the reading of the stained slides is subjective.
Antibody-dependent cell-mediated cytotoxicity assay

ADCC assays are carried out either with monocytes (ADCC[M])\textsuperscript{18} or with K lymphocytes (ADCC[L]).\textsuperscript{17} In the ADCC(L), the RBCs are enzyme treated. In the ADCC (Fig. 3), RBCs are labeled with \( ^{51}\text{Cr} \), then sensitized with antibody, washed, and then incubated with the effector monocytes or lymphocytes. After incubation at 37°C, a sample of the supernatant is taken to measure the radioactivity, in a gamma counter. Lysis of the RBCs damaged in the cytotoxic reaction with the effector cells is measured by estimating the \( ^{51}\text{Cr} \) released into the supernatant and the results are compared to a dose-response curve.\textsuperscript{3} RBCs coated with IgG3 appear to be lysed more efficiently than RBCs coated with IgG1. The ADCC is objective and quantitative; however, it does require the use of radioisotopes and a gamma counter to measure the radioactivity.

Chemiluminescence assay

As an alternative to measuring phagocytosis as in the MMA, the CL assay (Fig. 4) uses luminol to measure the oxidative burst that accompanies phagocytosis. Mononuclear cells are incubated at 37°C with sensitized RBCs and the chemical luminol.\textsuperscript{8} The oxygen radicals produced during erythrophagocytosis and following the activation of the respiratory burst react with the luminol to produce light, which is then measured in a luminometer. The chemiluminescence results of the sensitized RBCs are compared with results with control nonsensitized RBCs. The CL assay is a simple and semi-quantitative method. It has an advantage over the MMA in that it produces objective results and is not as labor intensive. However, it requires a luminometer.

Application of the Cellular Assays

Predicting the clinical significance of alloantibodies in transfusion

Only the MMA has been used extensively to predict the outcome of transfusing incompatible RBCs. Studies have shown that the MMA results correlate very well with clinical significance of antibodies as demonstrated by \( ^{51}\text{Cr} \) survival studies or transfusion.\textsuperscript{14–16} In general, a negative MMA result (<3–5%)\textsuperscript{14,22} indicates that incompatible RBCs probably will not be rapidly destroyed, i.e., the patient will not have an acute reaction and incompatible RBCs can be given with minimal risk, but this does not guarantee that the RBCs will have normal survival. And since antibody characteristics can change, it is important to reevaluate the potential clinical significance before each transfusion of incompatible RBCs.\textsuperscript{23} A positive result in the MMA indicates that the incompatible RBCs will not have normal survival, but the patient may or may not have clinical signs of a reaction. The application of the MMA should be restricted to antibodies of questionable clinical significance or when compatible blood is difficult to obtain. Other-wise, the risk of an anamnestic response and the resulting delayed transfusion reaction is high.

In 1997, Garratty et al. presented data on 166 antibodies they tested by the MMA.\textsuperscript{24} Of these, 69 percent gave positive MMA results, including examples of specificities in the questionable significance group: 36/54 (67%) Yta, 11/21 (52%) Gerbich, 13/16 (68%) Dombrock, 19/21 (90%) Lutheran, and 8/9 (89%)
Lan. These data indicate that if MMA or $^{51}$Cr survival studies are not available, most antibodies to antigens to high-frequency antigens should be presumed to be potentially clinically significant.

Recently, the CL assay has been evaluated in comparison to the MMA for predicting outcome of transfusion. The results indicated that the two assays performed similarly, but the MMA is slightly more sensitive.

Predicting the severity of HDN

All three cellular assays are helpful in predicting the severity of HDN; however, they are not fully reliable. The predictive value of a negative result is better than that of a positive result. In other words, it is easier to predict that a baby will be unaffected than how severe the HDN will be, and the assays can be used to indicate when invasive procedures can be avoided. The results of the ADCC(M) have been found to correlate better with severity of HDN than those of the ADCC(L). Results of the CL assay correlate similarly to those of the ADCC with the severity of HDN, but the ADCC yields a better predictive value than the MMA. The MMA was reported to accurately predict the severity of HDN when Rh antibodies were present and when the fetus carried the corresponding antigen to the maternal antibody. However, a later study demonstrated decreased sensitivity (increased falsely positive results) when antibodies other than Rh were evaluated and when the fetus did not carry the corresponding antigen. Both studies did find that an MMA result less than 20 percent is highly predictive of the absence of severe fetal or neonatal disease.

In a recent retrospective review, the Netherlands group, which has been using the ADCC since the 1980s, evaluated its data from mothers with anti-D who gave birth to D+ infants. The authors found, when they correlated anti-D titers and ADCC results with pregnancy outcome, that the ADCC was a better predictor of fetal hemolytic anemia than was the antibody titer. These results confirmed that the ADCC is a useful screening test to select the patients requiring referral to specialized centers for further evaluation and follow-up.

Variables of Published Assays

Performing these assays is best left to specialized laboratories. These are live cell assays, and experienced laboratories work for consistency in the method and have correlated results with other measures of clinical significance. There are many variables that can affect the results of the cellular assays: source of effector cells (monocytes vs. macrophages vs. lymphocytes); variation among donor mononuclear cells; sensitization of the RBCs; effect of complement, incubation times, and atmosphere; reproducibility of the assay; and expression of the results. These should be taken into account when comparing results of one assay with those of another or one laboratory's results with those of another laboratory that is using the same type of assay.

References


Regina M. Leger, MSQA, MT(ASCP)SBB, CQMgr(ASQ), Research Associate II, American Red Cross Blood Services, Southern California Region, 1130 S. Vermont Ave., Los Angeles, CA 90006.
Screening for RBC antibodies—what should we expect from antibody detection RBCs

G. Garratty

In the United States, the Food and Drug Administration mandates that red blood cells (RBCs) for antibody detection possess the following antigens: C, D, E, c, e, M, N, S, s, P1, Le°, K, k, Fya°, Fyb°, Jka°, and Jkb°. Although not required, it is generally agreed that homozygosity for C, D, E, c, e, Fya°, and Jka° is also preferable. There is no requirement for low-frequency antigens to be present. However, manufacturers of antibody detection RBCs receive requests for these RBCs to possess Cw°, Kpa°, and Wra°. There are no data to support the considerable expense and effort involved in providing RBCs that possess low-frequency antigens such as Cw°, Kpa°, and Wra°. The risk of clinically-significant hemolytic transfusion reactions occurring when such antibodies are not detected because antibody detection RBCs lack such antigens is about 1 in 500,000 to 1 in 1 million transfusions. Immunohematology 2002;18:71–77.

Key Words: antibody detection, low-frequency antigens, hemolytic transfusion reactions, hemolytic disease of the newborn, alloantibodies, antibody detection RBCs

Introductory Note

Manufacturers of red blood cells (RBCs) for antibody detection (companies and blood centers) receive many requests for these RBCs to include low-frequency antigens such as Cw°, Kpa°, and Wra°. I was approached by one manufacturer (Ortho-Clinical Diagnostics, Raritan, NJ) aware of my opinions on this matter to write an opinion piece for their customers. I believe that some of these demands come from a generation of immunohematologists who are unfamiliar with the risk/cost/benefit arguments that were made in the 1980s to support the move to abbreviate compatibility testing by deleting the antiglobulin phase of the crossmatch. The antibody screen can sometimes be positive when one or more of the crossmatches are negative. Some reasons for this are:

(1) The antibody detection RBCs express antigens the crossmatched units do not; such antigens are often of moderate frequency (e.g., K, Lu°, Co°, and Yt°).

(2) The antibody detection RBCs are from individuals whose RBCs express a double dose of an antigen, e.g. Jk(a+b-) in contrast to crossmatched RBCs expressing a single dose, e.g., Jk(a+b+).

Background

Originally, RBCs for transfusion were typed for ABO and Rh(D) and a crossmatch was performed using the recipient's serum and donor RBCs (major crossmatch). In most countries only a major crossmatch was performed, but in the United States (until the 1970s), an additional minor crossmatch (donor's serum and recipient's RBCs) was performed. Later, sera were screened for antibodies using a pool of RBCs from two donors, or two donors separately; the antibody detection RBCs had most of the antigens that would react with antibodies of potential clinical importance. The original intent of this screening test was to detect antibodies in patients' sera before the blood was crossmatched or needed for transfusion, thus allowing time to investigate any problems that might occur during crossmatching (e.g., detection and identification of antibodies and selection of donor blood lacking putative antigens). In the United States, compatibility testing eventually included ABO and Rh(D) typing, antibody screening, and crossmatching (i.e., the antibody screen was now performed at the same time as the crossmatch). One reason the antibody screen was performed at the time of the crossmatch was because patients were being admitted closer to the time of transfusion and the original purpose of forecasting and dealing with problems before crossmatching was therefore negated. One could question why the screen was retained; the major argument was to detect antibodies that reacted only, or more strongly, with RBCs from donors who were homozygous for certain genes (e.g., C, E, c, Jke°, and Fya°).

The antibody screen can sometimes be positive when one or more of the crossmatches are negative.
On occasion, one or more of the crossmatches may be positive when the antibody screen is negative. This result may be because:

1. The antibody detection RBCs are group O and the antibodies are anti-A or anti-B.
2. A low- or moderate-frequency antigen (e.g., Wr\(^r\), Kp\(^a\), Js\(^s\), C\(^s\), C\(^u\), D\(^i\), G\(^o\), Sc\(^2\), M\(^i\), Lu\(^i\), Co\(^\circ\), and Yt\(^b\)) is present on the crossmatched RBCs, but not on the antibody detection RBCs.
3. The antibody detection RBCs may not have antigens such as f(ce). That is, R\(_1\)R\(_1\) and R\(_2\)R\(_2\) RBCs will not be f+, but the crossmatched units (e.g., rr RBCs) may have such an antigen.
4. The crossmatched donor RBCs are fresher than the antibody detection RBCs; RBC antigens get weaker on storage and very weak antibodies may react more strongly with the freshest RBCs. This phenomenon is possible but should be rare as Food and Drug Administration (FDA) licensing requires manufacturers to submit data on the effects of storage on RBC antigens. This is a major factor determining the expiration date of the antibody detection RBCs. Having said that, we recently encountered problems with several Xg(a+) RBC samples from one manufacturer that did not react with several anti-Xg\(^a\). This observation may have been due to lability of the Xg\(^a\) antigen, possibly during transit (unpublished observations 2001).

(5) The donor's RBCs express a double dose of an antigen, e.g., Jk(a+b-) and the antibody detection RBCs express a single dose, e.g., Jk(a+b+).\(^3,4\)

Abbreviation of Compatibility Testing

In 1964, Grove-Rasmussen questioned the value of performing an antiglobulin test (AGT) for crossmatches when the antibody screen included an AGT and was negative.\(^5\) He surveyed 20 laboratories where 37,961 antibodies had been detected; 145 (0.4%) antibodies were not detected by antibody screens, but were detected by crossmatches. Only two of 222 hemolytic transfusion reactions (HTRs) were due to such antibodies (1 anti-Kp\(^a\) and 1 anti-Js\(^s\)). Although the 1963 edition of the American Association of Blood Banks (AABB) Standards did not require an antiglobulin test if the antibody screen was negative, the 1970 edition of the AABB Standards required the antiglobulin test for crossmatching.

Approaches to compatibility testing changed dramatically in the 1980s.\(^1,2\) Most of these changes involved abbreviation of the crossmatch. The first change involved deletion of room-temperature testing; this change was stimulated by Dr. Eloise Giblett's remarks in her Karl Landsteiner Award lecture at the 1976 AABB meeting (published in 1977).\(^7\) The second major change was the introduction of the "type and screen" (T/S) approach,\(^7,9\) which was used in association with the maximum surgical blood ordering schedule (MSBOS).\(^8\) The suggested approach was to perform only a blood group (ABO and Rh[D]) and an antibody screen on donors and selected recipients. These selected recipients were surgical patients who rarely used blood during surgery (e.g., cholecystectomy) and patients requiring urgent or massive transfusion. Data were produced to show that ABO and Rh(D)-matched blood could be transfused to such patients with little risk of a hemolytic event if the antibody screen was negative (i.e., crossmatching was not necessary, although most hospitals did perform an "immediate spin" crossmatch [for ABO incompatibility before issuing the blood]). Boral and Henry\(^9,10\) detected 451 antibodies when testing sera from 30,331 patients by screen and crossmatch; 14 (3.1%) antibodies (7 anti-Lu\(^i\); 1 each of anti-Wr\(^a\), Kp\(^a\), V, -Vw, -V\(\text{S}\), -f, and -D\(^i\)) were detected by crossmatch, but not by the antibody screen. Oberman et al.\(^11\) detected 148 antibodies in 13,950 patients (82,647 crossmatches); eight (5.4%) antibodies were not detected by the screen. Thus, combining the data from both studies, about 95 percent of antibodies were detected by the screen, or 24 antibodies were missed in 44,281 patient samples (1 per 1,845 samples).

Table 1. Frequency of antibodies of potential clinical significance detected by crossmatch but not antibody screen

<table>
<thead>
<tr>
<th>References</th>
<th>No. Patients</th>
<th>No. samples</th>
<th>No. Crossmatches</th>
<th>Not detected by screen*</th>
<th>Risk of missing antibody per sample†</th>
<th>Risk of missing antibody per crossmatch‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>9,10,11,13</td>
<td>NA</td>
<td>30,331</td>
<td>NA</td>
<td>15</td>
<td>1 in 2022</td>
<td>NA</td>
</tr>
<tr>
<td>14</td>
<td>25,917</td>
<td>NA</td>
<td>164,083</td>
<td>17</td>
<td>1 in 6470</td>
<td>1 in 9652</td>
</tr>
<tr>
<td>15</td>
<td>40,000</td>
<td>58,227</td>
<td>126,771</td>
<td>9</td>
<td>1 in 4472</td>
<td>1 in 14,086</td>
</tr>
<tr>
<td>16</td>
<td>46,000</td>
<td>116,278</td>
<td>261,136</td>
<td>26</td>
<td>1 in 14,330</td>
<td>1 in 10,044</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>113,839</td>
<td>NA</td>
<td>8</td>
<td>1 in 5494†</td>
<td>1 in 10,615‡</td>
</tr>
<tr>
<td>TOTAL</td>
<td>318,675</td>
<td>551,990</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*My interpretation of the data (e.g., only antibodies of potential clinical significance included)
†My calculations
‡58 antibodies in 318,675 samples; 52 antibodies in 551,990 crossmatches
NA = Data not available
These data stimulated the suggestion that the T/S approach could be used for all patients. At the time this was suggested, it was impossible in the United States because federal regulations required that the antiglobulin test be used for crossmatching. In 1981, data were presented at a public meeting, at the National Institutes of Health, in an effort to have this regulation changed. By 1984, the FDA and AABB Standards allowed the T/S approach (i.e., no antiglobulin test required in the crossmatch if the antibody screen was negative) for all patients. Table 1 shows data from seven studies in five different institutions, on the relative risk of this approach. The risk of missing an antibody of potential clinical significance, if the antiglobulin test phase of the crossmatch was not performed, seemed to be about one in every 5,494 patient samples, or one in every 10,615 crossmatches. Table 2 shows the specificities of the antibodies that were best detected by the crossmatch. It is interesting to note that, even if the antiglobulin test phase of the crossmatch is not performed, and some of the specificities, involving antigens often missed if one of the antibody detection RBCs was R1R1 and R2R2 there were six antibodies to low-frequency antigens (2 anti-J§, 2- V, 1 -C*, and 1 -Wr) giving 1 per 36,666 crossmatches. It is obvious from these data that even using a “3-cell” screen would not have helped detect most of the antibodies that were undetected by the antibody screen.

Clinical significance of antibodies not detected by antibody detection RBCs

The data above and Table 1 show that there is a small added risk of missing antibodies if the antiglobulin test phase of the crossmatch is not performed, and some of the specificities, involving antigens often missed from antibody detection RBCs, are known to have been associated with HTRs (see Table 3). But a major question remained: How many patients were being harmed because these antibodies were not being detected?

Shulman et al. presented data on 3 years’ experience using an abbreviated crossmatch. The antibody screen in this study was composed of an immediate spin, followed by a 20-minute, 37°C incubation phase with 22% albumin, followed by an anti-IgG antiglobulin test; a set of two antibody detection RBCs was used; homozygosity for Jk was required only for the last 6 months of the study. During the study period, 113,839 crossmatches. A “2-cell” screen was used in all three periods. There were 29 antibodies (e.g., anti-K, -Jka, -Jkb, and -Fy) undetected by the screen where lack of homozygosity might have explained the difference, yet 17 of these were Rh antibodies and the two antibody detection RBCs were R,R, and R,R. There were six antibodies to low-frequency antigens (2 anti-J§, 2 -V, 1 -C*, and 1 -Wr) giving 1 per 36,666 crossmatches. It is obvious from these data that even using a “3-cell” screen would not have helped detect most of the antibodies that were undetected by the antibody screen.

### Table 2. Specificities of antibodies of potential clinical significance, detected by crossmatch but not antibody screen

<table>
<thead>
<tr>
<th>References</th>
<th>No. antibodies</th>
<th>Antibody specificities</th>
</tr>
</thead>
<tbody>
<tr>
<td>9,10</td>
<td>15</td>
<td>Lu&lt;sup&gt;7&lt;/sup&gt;, Wr&lt;sup&gt;*&lt;/sup&gt;, Kp&lt;sup&gt;+&lt;/sup&gt;, V&lt;sup&gt;+&lt;/sup&gt;, V&lt;sup&gt;−&lt;/sup&gt;, V&lt;sup&gt;−&lt;/sup&gt;, f&lt;sup&gt;(2)&lt;/sup&gt;, D&lt;sup&gt;+&lt;/sup&gt; (1)</td>
</tr>
<tr>
<td>11,13</td>
<td>17</td>
<td>E&lt;sup&gt;(7)&lt;/sup&gt;, Jk&lt;sup&gt;+&lt;/sup&gt;, J&lt;sup&gt;+&lt;/sup&gt;, K&lt;sup&gt;+&lt;/sup&gt;, C&lt;sup&gt;+&lt;/sup&gt;, C&lt;sup&gt;−&lt;/sup&gt;, c&lt;sup&gt;(1)&lt;/sup&gt;, e&lt;sup&gt;(1)&lt;/sup&gt;, V&lt;sup&gt;(1)&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>9</td>
<td>Wr&lt;sup&gt;(2)&lt;/sup&gt;, Co&lt;sup&gt;+&lt;/sup&gt;, Kp&lt;sup&gt;−&lt;/sup&gt;, Fy&lt;sup&gt;+&lt;/sup&gt;, S&lt;sup&gt;(1)&lt;/sup&gt;, P&lt;sup&gt;(1)&lt;/sup&gt;, S&lt;sup&gt;−&lt;/sup&gt;, V&lt;sup&gt;(1)&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>26</td>
<td>unidentified&lt;sup&gt;(8)&lt;/sup&gt;, Kp&lt;sup&gt;−&lt;/sup&gt;, C&lt;sup&gt;+&lt;/sup&gt;, Wr&lt;sup&gt;(3)&lt;/sup&gt;, J&lt;sup&gt;+&lt;/sup&gt;, M&lt;sup&gt;(2)&lt;/sup&gt;, E&lt;sup&gt;(1)&lt;/sup&gt;, Fy&lt;sup&gt;+&lt;/sup&gt;, Le&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>16</td>
<td>8</td>
<td>Le&lt;sup&gt;(4)&lt;/sup&gt;, E&lt;sup&gt;(1)&lt;/sup&gt;, Jk&lt;sup&gt;+&lt;/sup&gt;, V&lt;sup&gt;(1)&lt;/sup&gt;, A&lt;sup&gt;(1)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
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</tr>
</tbody>
</table>

### Table 3. Antibodies to antigens often not present on antibody detection RBCs that have been reported to cause hemolytic transfusion reactions

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Antigen frequency*</th>
<th>Antibody frequency†</th>
<th>Incompatibility frequency‡</th>
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</thead>
<tbody>
<tr>
<td>f&lt;sup&gt;(ce)‡&lt;/sup&gt;</td>
<td>.650</td>
<td>.0002</td>
<td>.000130</td>
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<tr>
<td>Co&lt;sup&gt;+&lt;/sup&gt;</td>
<td>.100</td>
<td>NA</td>
<td>NA</td>
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<td>Lu&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>.020</td>
<td>.0100</td>
<td>.000100</td>
</tr>
<tr>
<td>Kp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>.020</td>
<td>.0017</td>
<td>.000034</td>
</tr>
<tr>
<td>J&lt;sup&gt;+&lt;/sup&gt;</td>
<td>.100</td>
<td>.0017</td>
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<td>V&lt;sup&gt;(ce)‡&lt;/sup&gt;</td>
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<tr>
<td>VS&lt;sup&gt;(e)‡&lt;/sup&gt;</td>
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<tr>
<td>Go&lt;sup&gt;+&lt;/sup&gt;</td>
<td>&lt;.01</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Based on predominantly Caucasian populations. Marked differences of some antigens in Asians or Blacks (see reference 2)
†Only rough estimate (see reference 19)
‡Not present if only R,R, and R,R, RBCs are used as antibody detection RBCs; will be present if rr RBCs are used
NA = Not available

Low-frequency antigens on antibody detection RBCs
sera from 19,818 patients were screened for antibodies; 3419 (3%) sera contained antibodies. Twenty-eight patients received RBCs that were later found to be incompatible by the crossmatch; only seven of the missed antibodies were of potential clinical significance (Le" [4], Jkα [1], V [1], and passively transfused anti-A [1]). Retrospectively, the anti-Jkα was found to react with RBCs from a Jkα homozygote, which were not used for the antibody screen. None of the patients had any signs of a hemolytic transfusion reaction. In 1990, Shulman18 gathered data from 20 hospitals in different parts of the United States. These hospitals had issued blood for transfusion based on 1.3 million negative antibody screens and negative immediate spin crossmatches. Only five patients experienced an acute overt HTR (a risk of one HTR per 260,000 crossmatches). The antibodies associated with the HTRs, and undetected by the antibody screen, were one example each of an anti-Jkα, -C, -c, -Wra, and -Kpa.

Significance of undetected antibodies to low-frequency antigens

The data in Table 3 emphasize that even if the antibody (e.g., anti-Wrα) is not rare (i.e., about 2% of sera contain anti-Wrα), the incompatibility frequency, reflecting the chance that a crossmatched unit would possess the putative antigen and might be transfused if the AGT crossmatch is not performed, is extremely low (e.g., 1 in 50,000 for Wrα). Some specific studies are of interest. Wallis et al.20 reported that over a 34-month period they detected ten antibodies, of potential clinical significance, that were detected by the crossmatch but not by the antibody screen; seven of these were anti-Wrα and anti-Kpα. They studied the incidence of anti-Wrα and the Wrα antigen in patients and donors. Anti-Wrα was detected, with no other unexpected antibodies, in 8 percent of 1112 patients and 1 percent of 5098 blood donors. Only two of 5253 (1 in 2600) donors were Wr(a+). Wallis et al.20 wisely concluded that it was not practical to include Wr(a+) RBCs in a set of screening RBCs. They suggested either performing an antiglobulin crossmatch, typing all donors for Wrα, and only performing AGT crossmatches on Wr(a+) donors, or assuming the risk. Arriaga et al.21 found 14 of 10,000 (1 in 714) donors to be Wr(a+). They detected anti-Wrα in one of 31 patients, one of 21 pregnant women, and one of 37 blood donors. In the patients, pregnant women, and donors, 60 percent, 93 percent, and 30 percent, respectively, of anti-Wrα were IgG. In a 10-year period where 300,000 units were transfused and 60,000 deliveries occurred, Arriaga et al.21 only observed three HTRs and a positive DAT in one newborn (without HDN) associated with anti-Wrα.

If we consider only antibodies reacting with antigens not expressed on the antibody detection RBCs (i.e., 1 anti-Wrα and 1 anti-Kpα) in the Shulman study,18 then the risk was one HTR per 650,000 crossmatches. As mentioned earlier, Grove-Rasmussen5 reported that two of 222 HTRs (in 20 hospitals) were caused by antibodies (1 anti-Kpα and 1 anti-Jsα) not detected by antibody detection RBCs. Rouger22 reported that in a 30-year period involving 600,000 antibody screens, he had encountered only two HTRs due to antibodies to low-frequency antigens. De la Rubia and et al.23 encountered only one mild HTR, due to anti-Wrα, in a 5-year period (1987–91) when 100,000 antibody screens were performed. Recently, Arriaga et al.21 reported that in a 10-year period where 300,000 units of RBCs were transfused, only three HTRs due to anti-Wrα were encountered.

Hemolytic disease of the fetus/newborn

Although there have been many reports of hemolytic disease of the fetus/newborn (HDFN) due to low-frequency antigens, it occurs rarely and most newborns have mild HDFN (i.e., newborns did not require RBC transfusion). If RBC transfusions are necessary, it is easy to obtain serologically compatible RBCs quickly, as most units will lack the low-frequency antigen. Lubenko and Contreras24 reported the incidence of HDFN attributable to anti-Wrα. In 10 years, they encountered only four cases with a positive DAT due to anti-Wrα. Unfortunately, these authors did not state whether any of these infants had a diagnosis of HDFN. They found that 63 percent of 44 anti-Wrα contained an IgG component; 14 of 28 IgG anti-Wrα had titers of 32 or more. They made the point that 1 in 300,000 pregnancies could potentially result in Wrα-associated HDFN, but the incidence is much rarer, indicating that many IgG anti-Wrα do not cause clinically significant HDFN. As mentioned above, Arriaga et al.21 only encountered one positive DAT in 60,000 deliveries due to anti-Wrα, in a newborn who had no HDFN.

All of the risk data above are from the United States and Europe. As emphasized in a footnote to Table 3, the prevalence of some low-frequency antigens is more common in Caucasian populations and can be very different from that in Black or Asian populations. For instance, the prevalence of Jsα in Blacks and Miα in Chinese are 0.195 and 0.06, respectively, compared with 0.01 and < 0.01, respectively, in Caucasians. As anti-Miα is a common antibody in Chinese and
6 percent of the population are Mi\(\text{a}^+\); it has been argued that, in this population, it is important that one of the antibody screening RBCs be Mi\(\text{a}^+\).\textsuperscript{25} Others have argued that most anti-Mi\(\text{a}\) are not clinically significant and the rarity of HTRs does not warrant the additional expense of using a specially selected antibody detection RBC that is Mi\(\text{a}^+\).\textsuperscript{26}

A lesson to be learned from the above data is that calculating the prevalence of a certain antibody and even adding an incompatibility frequency does not necessarily give an accurate picture of the true clinical significance. This paradox is due mainly to the fact that not all antiglobulin test-reactive antibodies are clinically significant.\textsuperscript{27} The data in Table 4 further emphasize this. During a 19-year period, the Mayo Clinic encountered 559 delayed immune reactions to transfusion associated with antibodies of potential clinical significance.\textsuperscript{28,29}

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Total Number</th>
<th>DHTR</th>
<th>DSTR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>184</td>
<td>47</td>
<td>137</td>
</tr>
<tr>
<td>Jk(\text{a})</td>
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<td>45</td>
<td>50</td>
</tr>
<tr>
<td>Fy(\text{a})</td>
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<td>26</td>
<td>36</td>
</tr>
<tr>
<td>K</td>
<td>62</td>
<td>16</td>
<td>46</td>
</tr>
<tr>
<td>c</td>
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<td>18</td>
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</tr>
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<td>0</td>
</tr>
<tr>
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<tr>
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<tr>
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<tr>
<td>TOTAL</td>
<td>559</td>
<td>197 (35%)</td>
<td>362 (65%)</td>
</tr>
</tbody>
</table>

*Alloimmunization with a posttransfusion positive DAT but no obvious hemolytic anemia or clinical signs of a HTR

Table 4. Delayed hemolytic (DHTR) and serologic (DSTR) transfusion reactions at Mayo Clinic during a 19-year period (1980–1998)\textsuperscript{28,29}

It is also interesting to note that in 19 years at the Mayo Clinic, only five HTRs were due to antibodies to antigens that are not usually present on antibody detection RBCs (3 anti-C\(\text{w}\), 1 -Kp\(\text{a}\), and 1 -Lu\(\text{a}\)).

**Conclusions**

In the United States, the FDA requires that antibody detection RBCs have the following antigens: C, D, E, c, e, M, N, S, s, P\(\text{1}\), Le\(\text{e}\), Le\(\text{e}\), K, k, Fy\(\text{a}\), Fy\(\text{b}\), Jk\(\text{a}\), and Jk\(\text{b}\). There is no requirement for RBCs to express homozygosity (e.g., Jk\(\text{a}^+\text{b}^+\)). A pool of two RBC samples can be used for screening donors, but two or more separate RBC samples must be used for screening recipients. It is obvious from the data above that the risk of not detecting a clinically significant antibody that would be detected by an antiglobulin crossmatch, and that an incompatible unit will be transfused, is very small (about 1 per 250,000 “immediate spin” crossmatches). Most of the risk data relate to a “2-cell” screen. A survey in the United States showed that, since 1996, more than 60 percent of the respondents were using a “3-cell” screen, presumably to increase the possibility of having RBCs that express homozygosity, e.g., Jk\(\text{a}^+\text{b}^+\).\textsuperscript{31} This will decrease the risk somewhat, but others have argued that the increased workload and limited benefit do not justify the added expense.\textsuperscript{17}

There have been approximately 150 million units of RBCs transfused in hospitals in the United States since the onset of using antibody detection RBCs not selected for antigens such as C\(\text{w}\), Kp\(\text{a}\), or Wr\(\text{a}\) for antibody screens, followed by an “immediate spin” crossmatch. There have been no data published to suggest that this is an unsafe approach. As can be seen from the data presented in this review, the risk of a patient’s suffering because of a laboratory missing an antibody to a low-frequency antigen that is potentially clinically significant and additionally of the patient’s also receiving an antigen-positive unit is extraordinarily small (approximately 1 chance per 500,000 to one million transfusions). If readers are so conservative that this concerns them, then they should not be using abbreviated compatibility testing (which is based on a risk/benefit/cost calculation), but should always use an antiglobulin phase in their crossmatches. Even if the latter is used, patients can still have HTRs due to antibodies that are not detectable in the best of laboratories by current tests;\textsuperscript{32} we have lived with this small risk for many decades.
References
22. Rouger P. International Forum. Do you think that the cross match with donor red cells can be omitted when the serum of a patient has been tested for the presence of red cell alloantibodies with a cell panel? Vox Sang 1982;43:151-68.

George Garratty, PhD, FRCP, Scientific Director, American Red Cross Blood Services, Southern California Region, 1130 S Vermont Avenue, Los Angeles, CA 90006.
Sixty-two samples from 62 donors were investigated to determine the significance of warm IgG autoantibodies that were detected using a gel system during compatibility testing. The presence of autoantibodies on the red cells was confirmed by elution studies. Twelve of 23 strongly positive samples, 7 of 19 moderately positive samples, and 6 of 11 weakly positive samples were studied. The remaining nine samples were found positive during crossmatching, then negative when it was repeated. These nine samples were not included in this study. With a tube test, most of the antibodies had titers from 4 to 8. IgG subclass studies showed that 14 of 25 samples with reactive eluates contained IgG1, one contained IgG1+IgG2, one contained IgG1+IgG4, and two contained IgG1+IgG3 weak. The frequency of donors with a positive direct antiglobulin test (DAT) was ~1 in 3000 and males were twice as likely to be DAT positive (8 females vs. 17 males in this study). None of the donors had hemolysis. Two donors showed low-titer anticardiolipin antibodies. We conclude that DAT-positive donors can be a problem during compatibility testing when sensitive methods are used. Immunohematology 2002;18:78–81.

**Key words:** direct antiglobulin test, blood donors, antibodies, elution

A positive direct antiglobulin test (DAT) occurs occasionally in normal blood donors, and often is discovered when the donor's red cells are incompatible in a compatibility test. The frequency of donors with a positive DAT was estimated to be 1 in 9000 to 1 in 14,000.1 In the majority of cases, long-term follow-up revealed no clinical condition that would account for the positive IgG DAT. IgG autoantibodies that are responsible for immune-mediated destruction of red blood cells (RBCs) in patients with warm-antibody autoimmune hemolytic anemia (AIHA) are predominantly IgG, subclass 1,1,2 IgG3 autoantibodies, however, are most effective in producing RBC destruction. The presence of IgG3 on the RBCs of a patient without hemolytic anemia is unusual, and it may indicate a dysfunction of the reticuloendothelial system.3

In the past 3 years, we have investigated the significance of warm type IgG autoantibodies in blood donors discovered during compatibility testing of 69,765 RBC units. Eluates were prepared using samples from the units, and the antibodies were tested to determine their IgG subclass.

**Materials and Methods**

Sixty-two samples from 62 blood donors, with a mean age of 43 ± 9 years, were tested. Of these donors, 42 were males and 20 were females. All of the donors responded to a request for a further sample. A complete blood count (CBC), a reticulocyte count, and liver function tests (bilirubin [total, direct, and indirect] and transaminases [serum glutamic oxalacetic and serum glutamic pyruvic]) were performed to exclude individuals with autoimmune hemolytic anemia, as well as individuals with positive VDRL tests and viral antibodies. The following serologic tests were performed: DATs were performed by the classic tube technique using polyspecific antiglobulin serum (anti-IgG/C3d, Ortho-Clinical Diagnostics, Raritan, NJ). In this technique, RBCs were washed 3 using NaCl 0.9% and resuspended to a 3–5% saline suspension. Two drops of polyspecific antihuman globulin were added to 1 drop of the washed cells. The contents of the tube were mixed, centrifuged for 1 minute at 1500 rpm, and examined macroscopically for agglutination. All positive results were further tested with monospecific anti-IgG (Ortho-Clinical Diagnostics) and anti-IgA, -IgM, -C3, and -C4 (DiaMed AG, Scheitz, Switzerland). The agglutination reactions were considered as strongly positive (4+ and 3+), moderately positive (2+ and 1+), and weakly positive (w+).4

Gel tests, as well as tube tests, were used in parallel. Gel tests were performed according to the manufacturer's instructions for the antiglobulin card sera: IgG, IgA, IgM, and C3. All materials and reagents were obtained from DiaMed AG. For the polyspecific DAT, 50 µL of a 0.8% RBC suspension in LISS (ID-Diluent 2) was added to the top of each microtube in a LISS/Coombs ID card. The cards were centrifuged at
910 rpm for 10 minutes, using the ID-Centrifuge 24S. All positive results (presence of agglutinated RBCs in the gel matrix) were re-examined using rabbit monospecific anti-IgG, -IgA, -IgM, and -C3d cards. Each negative reaction appeared as a discrete cell button at the base of the column (DiaMed AG).

Crossmatches were performed using the LISS antiglobulin technique and ether eluates were prepared from the DAT-positive samples. The eluates were tested with a standard antibody identification panel (DiaMed AG) using polyethylene glycol (PEG 20% in PBS) or albumin 30% techniques. Polyspecific antiglobulin reagent (Ortho-Clinical Diagnostics) was used. The subclass of the IgG antibodies in the eluates was determined by adding anti-IgG1, -IgG2, -IgG3, and -IgG4 subclass antisera to the sensitized RBCs (CLB, Amsterdam, Netherlands).

Titration studies were performed by the tube test as follows: dilutions of the corresponding sera were added to one drop of 2–4% RBCs from the DAT-positive samples. The tubes were centrifuged for 1 minute at 1500 rpm and then examined for agglutination. Additional serologic tests that were performed on the DAT-positive samples included: VDRL tests for syphilis (Latex Pasteur), HIV1+2 (Abbot, Chicago, IL, AxSYM), HBsAg (Abbot, AxSYM), HTLV1/2 and HCV (ELISA, Ortho-Clinical Diagnostics). Tests for antcardiolipin antibodies (ACA) and lupus anticoagulants (LA) were performed by using ELISA (Fresenious GULL Diagnostics, Gull-Meridian Laboratory, Cincinnati, OH; and Stago Laboratory, Asnieres, France, respectively). Screening for LA included the activated partial thromboplastin time, dilute Russel’s viper venom time, and kaolin clotting time. When the initial screening tests demonstrated prolonged coagulation times due to the presence of an inhibitor, confirmation that the inhibitory activity was directed against phospholipid-containing complexes was mandatory. Blood samples were collected into 0.11M citrate (9 parts blood to 1 part citrate), and the plasma was separated after double centrifugation.

**Results**

Fifty-three DAT-positive samples from 62 blood donors (42 males and 20 females) were studied over the last 3 years. The samples were detected during routine compatibility testing (crossmatching) of 69,765 RBC units. Twenty-three of 53 samples reacted strongly in the IgG gel test (43.3%), 19 reacted moderately (35.8%), and 11 reacted weakly (20.7%). Twelve of the samples also reacted in the C3d gel test (22.7%). The tests with anti-IgM and -IgA were negative. Twenty-five of the 53 samples produced positive eluates while 28 produced nonreactive eluates (Table 1).

**Table 1. Reactivity of samples in gel versus tube tests and elution results**

<table>
<thead>
<tr>
<th>Methods</th>
<th>Strongly positive</th>
<th>Moderately positive</th>
<th>Weakly positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel test positive</td>
<td>23</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>Tube test positive</td>
<td>12/23</td>
<td>7/19</td>
<td>6/11</td>
</tr>
<tr>
<td>Eluate positive</td>
<td>12/23</td>
<td>7/19</td>
<td>6/11</td>
</tr>
<tr>
<td>Eluate negative</td>
<td>11/23</td>
<td>12/19</td>
<td>5/11</td>
</tr>
</tbody>
</table>

When the samples that reacted in the gel test were tested by IgG tube test, 12 of 23 reacted strongly (52.1%), 7 of 19 reacted moderately (36.8%), and 6 of 12 reacted weakly (54.5%) (Table 2). Six of 11 samples that reacted strongly in the IgG gel test, but produced nonreactive eluates, also reacted by the IgG tube test. Similarly, 6 of 12 samples that reacted moderately in the IgG gel test, but produced nonreactive eluates, reacted in the IgG tube test. Finally, three of five samples that reacted weakly in the IgG gel test, but produced nonreactive eluates, reacted in the IgG tube test (Table 2).

**Table 2. Reactivity of gel positive samples by IgG tube test and elution results**

| Samples          | Strongly positive | Moderately positive | Weakly positive | Total |
|------------------|-------------------|---------------------|----------------|
| Tube pos Eluate pos | 6                 | 1                   | 3              | 10    |
| Tube pos Eluate neg | 6                 | 6                   | 3              | 15    |
| Tube neg Eluate pos | 6                 | 6                   | 3              | 15    |
| Tube neg Eluate neg | 5                 | 6                   | 2              | 13    |
| Total            | 23                | 19                  | 11             | 53    |

Most of the autoantibodies had titers from 4 to 8 in the tube test, and only two samples had titers of 32. Fourteen out of 25 autoantibodies (56%) produced reactive eluates that contained IgG1, one contained IgG1+IgG2, one contained IgG1+IgG4, and two contained IgG1+IgG3 weak. Four of 25 eluates were strongly reactive, 18 were moderately reactive, and 3 were weakly reactive. No specificity of the autoantibodies was found (broad specificity).

The DAT-positive blood donors who produced reactive eluates, 8 females and 17 males with a mean age of 42 years old (range: 28 to 60 years old), were followed for an average of 38.5 months (median follow-up 42 months). The frequency of DAT-positive samples
found by crossmatching 69,765 samples was 25, or approximately 1/3000.

Two donors with positive DATs, a woman 44 years old and a man 45 years old, were found to have low-titered ACA IgG antibodies (titers: 12 and 15 U/mL, respectively). RBCs from these two donors produced reactive eluates with titers of 8 and 16, respectively. The eluates from both of these donors contained IgG1. They have been tested every 6 months for the past 24 months, and there has been no apparent cause for these findings. None of the donors has shown any signs of hemolysis. Liver function tests, complete blood counts, and reticulocyte counts were within the normal ranges. Autoantibody tests for collagen vascular disease were normal. Viral screening tests were negative.

Discussion

The reported incidence of positive DATs among blood donors has been estimated as 1 in 14,000.1,2 A much higher frequency of 1 in 1000 was reported by Alan and Garratty; however, the discrepancy may be related to the fact that over 90 percent of the reactions were only ≤1+. Twenty of 22 samples were coated with IgG1, and the number of IgG1 molecules per RBC varied from 110 to 950. The remaining two samples were coated with IgG4. In another series of ten subjects, five had IgG1 only, three had IgG4, one had IgG2, and one had IgG1+IgG3. The presence of IgG3 on the RBCs of a patient without hemolytic anemia is unusual, and it may indicate dysfunction of the reticuloendothelial system.3 A strong positive correlation with increasing age was noted in a study of hospitalized patients with positive DATs.2 Only 1 out of 65 went on to develop autoimmune hemolytic anemia. The RBCs may be agglutinated by anti-complement (anti-C3d) or anti-IgG. The frequency of finding both IgG and complement on RBCs varies widely in different series, i.e., 15%, 49%, and 70%.1

Another study demonstrated that HLA-DQ6 has a negative association with a positive DAT result in patients with evidence for hemolysis, and it may be a resistance antigen for clinically relevant RBC autoantibodies.9 The frequency of HLA-DQ6 was higher in asymptomatic DAT-positive blood donors (8.38%) than in DAT-positive hospitalized patients, 96 percent of whom had evidence of clinical hemolysis. Antiphospholipid antibodies may also be a coincidental cause of positive DATs in healthy blood donors with false positive VDRL tests.10

This study found 8 female and 17 male donors with positive DATs, which were confirmed by elution studies. Thus, the frequency of DAT-positive donors was one in 2790 collected blood units, i.e. ~1 in 3000. The 25 DAT-positive donors produced reactive eluates, confirming their studies. The titers of their antibodies were low (from weak to 8), and only two samples showed a titer of 32. Fourteen out of 25 positive samples (56%) demonstrated IgG1 autoantibodies, and four samples demonstrated mixed types (one of IgG1+IgG2, one of IgG1+IgG4, and two of IgG1+IgG3 weak). Two donors with low-titered ACA antibodies have been tested every 6 months. None of the donors in this study has developed autoimmune hemolytic anemia or needed therapy.

We believe that the high incidence of positive DATs among blood donors in this study can be attributed to the high sensitivity of the new methods, especially the gel technique,11 in detecting warm IgG autoantibodies. We recommend confirming positive DAT results by the tube test DAT as well as by performing an elution.12 Positive DATs seem to exist without symptoms for years in some people, but in others they have been associated with viral infections and immune disorders. ACA and LA antibodies have also been reported as coincidental findings in apparently healthy people.13,14 Moreover, units from DAT-positive donors are a real nuisance in compatibility testing, especially in massive transfusions or emergency conditions. Also, units from DAT-positive blood donors are a major problem when the units are of a rare blood group. We recommend informing blood donors who have positive DATs about the laboratory finding, especially when antiphospholipid antibodies are present. In conclusion, we believe the high incidence of positive DATs among blood donors needs further investigation.

References

Blood donors with positive DATs


A Reviewer's Comments:
Because the incidence of DAT-positive blood donations in this study was 25 of 69,763 samples found during crossmatching (~1 in 3000), the magnitude of the problem seems small; however, the authors were concerned that these units posed a problem in providing compatible units in emergency situations. Such units were regularly detected in their laboratory during compatibility testing; however, many laboratories routinely use the immediate-spin crossmatch or the electronic crossmatch, which would not detect donors with a positive DAT. A positive DAT may be age-related and benign, but it may also be symptomatic of autoimmune disease. This study presents a thought-provoking dilemma. Should we be concerned about detecting donors with a positive DAT?

Marianna Bellia, Hematologist-Registrar; Ist Regional Transfusion Center, Hippokrateion Hospital, Athens, Greece; John Georgopoulos, Microbiologist-Senior Registrar, Hippokrateion Hospital, Athens, Greece; Vasilis Tsevrenis, Hematologist-Registrar; Hippokrateion Hospital, Athens, Greece; Efrosini Nomikou, Hematologist-Registrar; Hippokrateion Hospital, Athens, Greece; Niki Vgontza, Hematologist-Senior Registrar, Hippokrateion Hospital, Athens, Greece; and I. Kontogpoulous-Griva, Hematologist, Medical Director, Hippokrateion Hospital, Athens, Greece.
An algorithm to locate hrB– donors for individuals with sickle cell disease

R.R. Gammon and N.D. Velasquez, Jr

Many African Americans with sickle cell disease (SCD) develop alloantibodies to antigens in the Rh blood group system. Others have shown that from D– individuals, those lacking the high-incidence hrB antigen (> 98% prevalence) may be found among r'r African Americans. We describe an algorithm to locate units for African Americans with SCD and anti-hrB and -D. From 46,539 donations, 5136 listed African American as race. Our primary reference laboratory performed Rh phenotyping (D, C, c, E, e) for first-time donors and those not tested previously. Specimens typing r'r were sent to a secondary reference laboratory for hrB phenotyping after each donation. Hemoglobin S screening was performed. Of 24 donors (27 donations) who phenotyped r'r, seven donors, 29.2 percent (nine donations) were hrB–. Two of seven who donated twice consistently tested hrB–. One of 24 donors initially tested hrB–, but hrB+ on repeat donation. The donor tested hrB– by a second reference laboratory. Reagents for phenotyping high-incidence antigens are often not readily available, requiring a specialized reference laboratory that adds cost and turnaround time. Our algorithm selected r'r African American donors most likely to lack hrB for further evaluation by a second reference laboratory. We felt this was the most judicious use of resources and provided the greatest opportunity to find compatible components for individuals with SCD and anti-hrB and -D.

Materials and Methods

During a 10-month period, 46,539 volunteers at our blood collection center were asked to designate their race. The software in use in our center allowed classification of race into one of six categories. Donors did not have the option to designate more than one race. During the study, 5136 donors listed themselves as African American. The primary reference laboratory for our blood collection center performed an Rh phenotypic evaluation (using anti-D, -C, -E, -c, -e) for first-time African American donors or for those for whom no previous phenotype was available. Rh phenotyping was performed by tube testing according to manufacturers' instructions.

Specimens from donors who phenotyped as r'r were sent to a secondary reference laboratory for hrB phenotyping. Specimens from r'r donors were typed for hrB after each donation. Rh phenotyping was repeated only for those Rh antigens that typed negative on the previous donation. At least two different sources of anti-hrB were used for evaluation.

Donors who phenotyped as r'r were screened for the presence of hemoglobin S, using a solubility assay (SickleScreen, Pacific Hemostasis, Huntsville, NC).

If a unit typed hrB–, hemoglobin S negative, it was inventoried and stored frozen for the primary reference laboratory at the discretion of the Technical Director. Information on the donor was entered into a database for future recruitment (Fig. 1).

Due to our patients' need, our study was limited to hrB–, D– donors.
Results

Twenty-four African American donors (from 27 donations) were phenotyped as r'r. Seven donors, 29.2 percent (from nine donations) were found to be hr B–. Two of the seven donors who presented twice consistently tested hr B–. One of the 24 donors initially tested hrB–, but tested hrB+ on repeat donation. Because of discrepant test results, that sample was sent to an independent reference laboratory, which reported the donor to be hrB–. Future units from this donor were not transfused to individuals with anti-hrB. Using the algorithm, the probability that an hrB– donor would be found increased from 2 percent to 29.2 percent (Fig. 2).

Discussion

High-incidence antigens are observed in more than 90 percent of most random populations. Individuals with congenital hemolytic anemias, such as SCD, who require repeated transfusions often develop multiple antibodies, some of which may be to high-incidence antigens. In some situations, because of the rarity of blood that is negative for the antigen, as may be the case with hr, it may be necessary to go beyond local inventories and institute a nationwide search. These actions may result in compatible units not being available to satisfy clinical urgency.

To expedite transfusion when an individual develops an antibody to a high-incidence antigen, reference laboratories often keep inventories of frozen RBCs with uncommon phenotypes.

Fig. 1. Rh phenotyping was performed by the primary reference laboratory on first-time donors and donors not previously phenotyped who listed race as African American. Specimens from donors whose phenotype was r'r and hemoglobin S negative were sent to a secondary reference laboratory for hrB phenotyping. Those units that tested hrB– were placed in frozen inventory and the donors listed in a computer database for future recruitment.

Fig. 2. The pie chart on the top shows that only 2.0 percent of the general population is hrB–. The pie chart on the bottom shows that by using our algorithm of sending samples from a select population of African American r'r donors for hrB evaluation, we were able to increase the probability of finding an hrB– donor approximately 15 times, with 29.2 percent of the evaluable donors testing as hrB–.
Our primary reference laboratory continually screens donors in an attempt to maintain an inventory that would meet the clinical needs of those individuals. The inventory in most cases is an actual unit, frozen at the discretion of the Technical Director of the reference laboratory, but it may also be a virtual unit in that the donor’s name is added to a database. The donor is recruited as the need arises and the unit is captured for inventory.

Using our algorithm, the probability that an hrA-donor would be found increased 15-fold, from 2 percent to 29.2 percent (Fig. 2). We feel that this allowed for the most judicious use of scarce reagents and provided the greatest opportunity for us to find compatible blood components for those individuals with SCD and anti-D and -hrA.

Although we limited our study to those patients with SCD, this algorithm could be applied to other chronically transfused populations at risk for alloantibody formation, such as those with congenital hemolytic anemias and chemotherapy patients.

References
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Phone, Fax, and Internet Information: If you have any questions concerning Immunohematology, Journal of Blood Group Serology and Education, or the Immunohematology Methods and Procedures manual, contact us by e-mail at immuno@usa.redcross.org. 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

Attention: State Blood Bank Meeting Organizers
If you are planning a state meeting and would like copies of Immunohematology for distribution, please contact Mary McGinniss, Managing Editor, 4 months in advance, by phone or fax at (301) 299-7443.
Policy Alternatives in Transfusion Medicine. James P. AuBuchon, MD; Lawrence Petz, MD; and Arlene Fink, PhD, eds. Bethesda, MD: American Association of Blood Banks (AABB) Press, 2001. Softcover, 226 pp. List price: $105, member price: $75. Stock #022005. To order: e-mail: sales@aabb.org or fax: (301) 951-7150.

This reviewer found Policy Alternatives in Transfusion Medicine to be an excellent book and, in fact, so compelling, interesting, and readable I completed it in one day.

In his preface, Dr. AuBuchon states that the “book was written to provoke the reader into considering the dilemmas in which blood banking in transfusion medicine professionals now find themselves.” Indeed, the book does just that. In addition, it offers the reader a substantial amount of current information that otherwise is difficult to access. This includes considerations of ethical principles by Sugarman and Devine, legal principles by Lipton, Parker, and Mullikan, and economic principles and an overview of health-care payment systems by Rice and AuBuchon.

The book also covers policy issues and infectious disease screening, pathogen inactivation for cellular components, bacterial contamination, and under- and mistransfusion in sections written by such noted authorities as Blajchman, Dodd, Busch, Linden, and Kleinman.

Perhaps even more interesting and valuable is a chapter entitled “Desired Directions for Transfusion Medicine,” which includes perspectives from knowledgeable authors, featuring “A Recipient’s Perspective,” “A Health-Care Policy Analyst’s Perspective,” “A Surgeon’s Perspective,” and “A Perspective from Transfusion Medicine.”

In short, Policy Alternatives in Transfusion Medicine is timely, informative, well-written, and unique in its coverage and perspectives. I highly recommend this work for anyone interested in the principles that provide the foundation for much of our current practice in transfusion medicine, as well as for its future.

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1st week in January for the March issue
1st week in April for the June issue
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Fax these items to Mary H. McGinniss, Managing Editor, (301) 299-7443.
BOOK REVIEW

Alloimmune Disorders of Pregnancy: Anaemia, Thrombocytopenia and Neutropenia in the Fetus and Newborn. Andrew Hadley, PhD; and Peter Soothill, eds. Cambridge University Press, 2002. Hardback, 282 pp. List price: $80.00. Stock #0-521-78120-5. To order: e-mail: orders@cup.org or phone: (800) 872-7423 or fax (914) 937-7895.

This reviewer agreed to review a book edited by Andrew Halley and Peter Soothill on alloimmune disorders of pregnancy after preparing a presentation on neonatal alloimmune thrombocytopenia. I wish I had read the book prior to preparing my lecture.

Alloimmune Disorders of Pregnancy, as noted in the book's foreword, attempts to bring together different aspects of the alloimmune cytopenias. The genetics, pathophysiology, diagnosis, and management of the affected fetus and newborn are described for anemia, thrombocytopenia, and neutropenia. The book has 14 chapters, written by 19 authors, from the United Kingdom (UK) and the United States (U.S.).

The first chapter, on pathophysiology of the alloimmune cytopenia, focuses on the maternal alloimmunization response, transfer of IgG to the fetus, and immune destruction of cells in the fetus. Chapters 2 and 3 review blood groups and laboratory methods used in evaluating hemolytic disease of the fetus and newborn (HDFN). Epidemiology and screening for alloimmune thrombocytopenia are covered in chapter 4. Chapters 5 and 6 focus on the use of anti-D immunoglobulin in the prevention of Rh alloimmunization and HDFN. Fetal genotyping and other laboratory assays for determining the severity of HDFN are described in chapters 7 and 8. Chapters 9–11 provide information on the clinical aspects of HDFN, including discussing methods to predict the severity of fetal anemia, as well as antenatal and neonatal therapies. The book concludes with a description of the diagnosis and treatment of alloimmune thrombocytopenia and neutropenia, in chapters 12–14.

As the majority of authors (as well as the editors) are from the UK, one should not be surprised to find that this book has been written from a British medical perspective. For example, chapter 4 considers the case for antenatal thrombocytopenia screening against criteria set by the UK's Department of Health National Screening Committee. This perspective is usually balanced with information about U.S. practice and, even when that is not provided, the text imparts valuable knowledge.

This book is a well-written publication that will serve the interests of transfusion service medical directors, hematologists, obstetricians, medical technologists, and all professionals involved in the care of an alloimmunized pregnant patient and her cytopenic child. I recommend it.

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Manuscripts: The editorial staff of Immunohematology welcomes manuscripts pertaining to blood group serology and education for consideration for publication. We are especially interested in case reports, papers on platelet and white cell serology, scientific articles covering original investigations, and papers on the use of computers in the blood bank. Deadlines for receipt of manuscripts for the March, June, September, and December issues are the first weeks in November, February, May, and August, respectively. Instructions for scientific articles and case reports can be obtained by phoning or faxing a request to Mary H. McGinniss, Managing Editor, Immunohematology, at (301) 299-7443, or see “Instructions for Authors” in every issue of Immunohematology or on the Web. Include fax and phone numbers and e-mail address with your manuscript.
Letter to the Editors

Remembering September 11

In response to the invitation from Delores Mallory and Mary McGinniss to share our September 11 blood-bank-related stories, I have attached a brief statement that I wrote shortly after the event for a Day of Unity recognition that was arranged for our entire hospital personnel to share their stories of preparedness, strength, and unity. As you may know, New York-Presbyterian Hospital/Weill Cornell Medical Center is located just 4½ miles from ground zero, on the upper east side of Manhattan. As it turned out, we used more blood for the World Trade Center victims than any other hospital, even though we were not the closest to the site. That was because we are the main burn center for the city. In the hours after the disaster, we did not transfuse a single unit in the Emergency Department. My blood-bank-related story and dedication to the Blood Bank staff follows.

It was not in our Disaster Plan, but within minutes of our learning of the September 11 tragedy, the entire Blood Bank staff and our Pathology Residents had sprung into action like a team that had done it all before; i.e., to pack coolers full of ice and units of blood and deliver them to the Emergency Department (ED) for the expected flood of victims. Soon after, a small team had set up a dispensing station in the ED, ready to transfuse blood as needed under the safest possible conditions, by more than a hundred physicians who were silently poised for action. Meanwhile, the entire universal donor inventory of about 230 units of group O blood in the Blood Bank was quickly packed and on ice and made ready for immediate transfer to the ED if it was needed. The teamwork and cooperation was incredible; all was quietly accomplished without confusion and within minutes. What a wonderful group of people! What a wonderful response to an incomprehensible disaster. What devotion to the vital task at hand. What overwhelming sadness, when later in the day it became clear there would be no flood of casualties needing our help. But the next day began the blood donor drive. We kept busy, not thinking about what had happened, but thinking about our duty to those who had borne the brunt of the attack, both living and dead, and thinking about all our colleagues and loved ones, and how fortunate we were to still be here and able to love one another as never before.

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Ortho Dedication

Out of the 18 years that Immunohematology has been published, Ortho-Clinical Diagnostics has generously given its support for 12 of those years! Each of the last 12 years, Ortho-Clinical Diagnostics has distributed copies of every issue of the journal to members of their Bankers Club and each September, has contributed to the publication of the September issue—or the “Ortho” issue, as it has become known to the editors and staff. This is no small contribution. Once again, it demonstrates the sincere regard of the company for the customer, the patient, and the field of immunohematology.

If you appreciate this as much as we at Immunohematology do, please tell your Ortho-Clinical Diagnostics sales representatives how much you enjoy their support of this journal.

Delores Mallory
Editor-in-Chief

Mary McGinniss
Managing Editor
Grace Neitzer received a Bachelor of Arts degree from Wayne State University, Detroit, Michigan, in 1947; ASCP certification in Medical Technology in 1944; and ASCP certification in Blood Banking in 1958.

Her career began in 1953: with Kay Beattie she was co-Technical Director of the Michigan Community Blood Center in Detroit, Michigan. She was a career member of the Michigan Association of Blood Banks from 1957 until 2002, and, as the organization’s President, served on many committees: Education, Scientific Programs, Workshops, and Annual Scientific Programs.

In 1971 she moved to Pittsburgh, Pennsylvania, to become the Technical Director of the Central Blood Bank of Pittsburgh.

In 1977 Mrs. Neitzer became Technical Director of Baptist Memorial Hospital Blood Bank in Memphis, Tennessee, a position she held until her retirement. She was also Vice President, President-Elect, and President of the Tennessee Association of Blood Banks.

In 1980, she became President of the Mid-South Blood Bank Organization and in 1982, the Vice President and then President of the Southeastern Area Blood Banks Association.

She was a career member of many prestigious organizations: American Association of Tissue Banks since 1982, Tennessee Department of Public Health Laboratory Advisory Committee since 1978, Tennessee Association of Blood Banks since 1978, American Association of Clinical Pathologists since 1968, and American Society of Allied Health Professionals since 1973.

All of the above activities she joined at the state and local levels. She taught, inspected, mentored, and led. And then she did all this at the national level.

Mrs. Neitzer joined the American Association of Blood Banks in 1957 and remained an active member. She chaired Accreditation, Education, Ethics, and Competency Evaluation committees and was an I & A Inspector. In addition, she served as AABB Secretary, Vice President (twice), President-Elect, President, and Past President. She did it all.

She received many greatly deserved awards in her career. She received three distinguished awards from the American Association of Blood Banks: The Ivor Dunsford Award in 1969, the John Elliott Memorial Award in 1979, and the Distinguished Service Award in 1988.

She also received two awards from the Tennessee Association of Blood Banks: The Lemuel W. Diggs Award in 1985 and the Lyndall Molthan, MD, Memorial Lecture Award in 1989.

Two awards were presented to Mrs. Neitzer by the Michigan Association of Blood Banks: The Merit Award in 1963 and the Facendes Award in 1983. The South Central Association of Blood Banks presented her with the L. Jean Stubbins Award.

For many of us, she was our mentor. She took great pleasure in introducing the new medical technologists and Specialists in Blood Banking to the people on committees and in the office. She encouraged newcomers to join and volunteer, to attend meetings, to give papers, and to think. She set the standard, raised the bar, and seemed to enjoy every minute of her blood bank career.

She loved blood banking. She wanted it to be right and good and she wanted blood bankers to be right and good and to enjoy their work.

She loved her family, her dogs, and blood banking, and each knew that they came first. She will be greatly missed by many grateful people.
ANNOUNCEMENTS

Annual Symposium. On September 26, 2002, the National Institutes of Health (NIH), Department of Transfusion Medicine, will hold its 21st annual symposium: "Immunohematology and Blood Transfusion," at NIH. The symposium is cohosted by the Greater Chesapeake and Potomac Region of the American Red Cross and is free of charge. However, registration is required. Contact: Karen M. Byrne, NIH/CC/DTM, Bldg. 10, Rm. 1C711; 10 Center Drive MSC 1184, Bethesda, MD 20892-1184. Phone: (301) 402-1360, e-mail: kcipolone@dtm.cc.nih.gov, or visit our website: www.cc.nih.gov.dtm.

Monoclonal antibodies available. The New York Blood Center has developed murine monoclonal antibodies that are useful for donor screening and for typing red cells with a positive direct antiglobulin test. Anti-Rh17 is a direct agglutinating monoclonal antibody. Anti-Fy^a, anti-K, anti-Js^a, and anti-Kp^a are indirect agglutinating antibodies that require anti-mouse IgG for detection. These antibodies are available in limited quantities at no charge to anyone who requests them. Contact: Marion Reid, New York Blood Center, 310 E. 67th Street, New York, NY 10021; e-mail: mreid@nybc.org.

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For further details and application forms please contact:
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