Review: Cell Typing the Sensitized Transfusion-Dependent Patient

Maria Rios, Kim Hue-Roye, Jill R. Storry, and Robert F. Reiss
New York Blood Center, New York, New York

Abstract. Extended red cell typing is required for the management of transfusion-dependent patients to confirm the identity of suspected alloantibodies or determine the specificity of potential additional antibodies that may be formed in the future. Typing may be complicated by the presence of circulating allogeneic cells or a positive direct antiglobulin test. Phenotyping such individuals by hemagglutination is dependent on the separation of a reticulocyte-enriched fraction by differential centrifugation. Flow cytometric typing of reticulocytes is also possible. The effectiveness of these techniques is limited in those who are heavily transfused or have low reticulocyte counts. Heavily transfused patients with sickle cell anemia may be typed, however, following hypotonic lysis of allogeneic cells. In patients with a positive direct antiglobulin test, sensitized cells are usually typed with either direct agglutinating antisera and/or IgG antisera following elution of the autoantibody. Inactivation of some antigens during the elution process or the lack of some antisera specificities limit such typing. By designing appropriate oligonucleotide primers, polymerase chain reaction (PCR) amplification of target gene sequences for most blood group systems and the identification of a large number of their allelic specificities is now possible. Peripheral blood leukocytes can be used as the DNA source. Restriction fragment length polymorphism determination is widely adopted for the identification of allelic specificity of the amplified target sequence. Alternate strategies, including allele-specific PCR, are often employed if the genetic basis of the polymorphism is more complex than a single nucleotide substitution, or if it does not create or ablate a restriction endonuclease cleavage site. These techniques may permit genotyping of sensitized transfusion-dependent patients, and can improve transfusion safety and efficacy. (received 27 April 2000, accepted 20 June 2000).

Keywords: Red cell antigens, phenotyping, genotyping, polymerase chain reaction, transfusion

Introduction

The classical method of testing for blood group antigens and antibodies is hemagglutination. Hemagglutination is conceptually simple and easy to perform, does not require highly qualified technical skills or equipment, is inexpensive and, when done correctly, has a specificity and sensitivity that is appropriate for the clinical care of the vast majority of patients. In most cases, if antisera specificities are available, cell typing by hemagglutination is straightforward. On the other hand, performance of extended cell typing for transfusion-dependent patients who have developed allo- or autoantibodies remains a difficult laboratory problem for the transfusion medicine specialist. Transfusion-dependent patients are defined as those who require transfusion on a consistent basis. Patients included in this category are those with thalassemia, selected patients with sickle cell anemia who are managed with chronic transfusion therapy, some patients with aplastic anemia and myelodysplasia who are ineligible for stem cell transplantation, and patients with autoimmune hemolytic anemia.

Red cell typing is essential in sensitized patients to confirm the identity of suspected alloantibodies and to facilitate the identification of antibodies that may be formed in the future. Typing cells from patients who have been heavily transfused is complicated because it is difficult to distinguish between donor and patient cells. While it would be optimal to perform...
extended red cell typing on these patients prior to initiating transfusion, one is often faced with patients whose typing has never been performed or is unknown. Typing cells from patients with a positive direct Coombs test who have been sensitized with IgG is also complicated because the presence of this immunoglobulin on the patient cells makes it difficult to use IgG reagents requiring the performance of the antoglobulin test.

The development of molecular DNA typing technologies has permitted the elucidation of blood group antigen polymorphisms at the genomic level. These techniques have great potential value in clinical problems that are difficult to address with current hemagglutination methodology. Problems include the identification of red cell antigens for which antisera are not available or are weakly reactive; identification of fetuses at risk for hemolytic disease of the newborn; and typing of patients who have been recently transfused or have a positive direct Coombs test.

The goal of this paper is to review some techniques that permit cell typing of transfusion-dependent patients who have been recently transfused with large amounts of allogeneic red cells or have positive direct Coombs tests. Specifically, the paper will review some of the methods that facilitate the use of classical immunologic techniques and will describe the molecular basis of DNA genotyping and discuss its application in addressing these clinical challenges.

Cell Typing by Immunological Techniques

**Typing recently or heavily transfused patients.** Typing patients who have received recent transfusions or have been transfused with massive amounts of red cells depends on the separation of the patient's reticulocytes and young red cells from older transfused cells by differential centrifugation. These techniques are based on the fact that younger red cells are less dense than older cells. In the classic method, washed red cells drawn from the patient are centrifuged in microhematocrit tubes over mixtures of dibutyl and dimethyl phthalates with differing specific gravities. When the mixture of phthalate esters that gives good separation between lighter and heavier cells has been identified, larger amounts of red cells are drawn from the patient and centrifuged over the mixture possessing the specific gravity that gave optimal separation. The cells are then harvested from the microhematocrit tubes, washed with saline, and typed with routine hemagglutinating reagents [1]. Subsequently, a simplified method of differential centrifugation was found to suffice in most situations. In this method, the centrifugation is performed without the use of the phthalate ester mixture [2].

More complex methods utilizing separation of red cells over a Percoll-Renografin density gradient [3] do not lend themselves to routine serological work in most hospital transfusion service laboratories. Use of these separation techniques is precluded if the patient has been heavily transfused or has a low reticulocyte count. Furthermore, it has been reported that some red cell antigens in the Rh, Duffy, and Kidd systems are more weakly expressed on reticulocytes [4]. Whether this is of significance when using current generation antisera is unknown.

A direct method to phenotype reticulocytes for red cell antigens by two-color flow cytometric analysis has been described [5]. In this method, reticulocytes are stained with a thiazole orange fluorescent stain; these cells are phenotyped by a modified indirect Coombs reaction utilizing commercially available IgG antibodies, biotinylated anti-IgG as the secondary label, and avidin-phycoerthrin as the fluorescent stain. If validated for the range of clinically significant antigens, this technique offers a simple method for reliably typing the red cells of heavily transfused patients, but it is limited to those institutions in which flow cytometric analysis is readily available.

Importantly, phenotyping patients with sickle cell disorders who have been heavily transfused may be easily accomplished by hemagglutination, since cells that are SS or SC are resistant to lysis, while allogeneic cells containing hemoglobin A are lysed with hypotonic solutions. In the most commonly used procedure, the red cells drawn from the transfused patient are washed repeatedly in 0.3% saline until the supernatent is free of visible hemoglobin. The remaining patient cells are then suspended in normal saline and typed with routine procedures [6].

**Typing patients with positive direct Coombs tests.** Direct phenotyping of patients with autoimmune hemolytic anemia is limited to those antigens for which
direct agglutinating monoclonal antibodies are commercially available. Monoclonal antibodies to antigens of the Rh system are widely used today. Recently, monoclonal antibodies to some antigens of other blood group systems (e.g., Kell, Kidd) are becoming available. While the variety of directly agglutinating reagents is expected to grow in the future, most non-Rh antigens continue to be typed with IgG reagents that are incapable of causing direct agglutination of antigen positive red cells.

Patients with positive direct Coombs tests due to sensitization with IgG can only be typed with IgG antisera following successful elution of the autoantibody from the cell surface. Many such elution procedures have been described. Gentle heating of red cells to 45 °C will sometimes remove sufficient immunoglobulin to phenotype them reliably with these reagents, but heavily sensitized cells cannot be typed. Elution procedures utilizing enzyme-dithiothreitol reagents [7], which are widely used to prepare red cells for absorption procedures, denature and destroy antigens of the Duffy, MNSs, and Kell systems.

Among the more successful techniques in current use are those utilizing citric acid [8], glycine-HCl-EDTA [9], or chloroquine diphosphate [10] solutions to elute IgG from red cell surfaces. Unfortunately, the acid elution techniques inactivate Kell antigens, while treatment of red cells with chloroquine may weaken Rh antigens. Care must therefore be taken in order to insure the correct interpretation of the patient's apparent phenotype, by the use of appropriate positive and negative controls.

**Cell Typing by Molecular DNA Technology**

**Genetic basis for blood group polymorphisms.** The most frequent genetic mechanisms responsible for red blood cell antigen polymorphisms are point mutations, deletions, insertions, and altered splicing events. In addition, chromosomal translocations, intragenic and intergenic crossovers, gene conversions, and other gene rearrangements may cause such polymorphisms. These mechanisms have been reviewed recently [11-13].

Point mutations consist of the substitution of a single nucleotide in the coding region of a DNA strand. The consequences of the substitution are varied and can lead to the following consequences: no alteration in the amino acid specified by the codon (silent mutation); change in the identity of the encoded amino acid (missense mutation); and conversion of a codon specifying an amino acid into a stop codon coding for termination of translation (nonsense mutation). The majority of the blood group antithetical antigens arise from missense mutations (see Table 1). Nonsense mutations are associated with null phenotypes of the Duffy and Colton systems. Deletions may involve the loss of a single nucleotide or a segment of DNA.

A deletion of nucleotides in multiples of three, if in-frame, will result in absence of one or more amino acid(s) and, most likely, a protein with different characteristics. A deletion of three nucleotides out of frame or in multiples of anything other than three will induce a shift in the correct reading frame, resulting in an inactive mutant protein. Some blood group null phenotypes arise as a consequence of such deletions.

<table>
<thead>
<tr>
<th>Blood group system</th>
<th>Antigen</th>
<th>Amino acid change</th>
<th>Nucleotide change</th>
<th>Restriction enzyme</th>
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</thead>
<tbody>
<tr>
<td>Rh (RHCE) Cl</td>
<td>Ser103^Fle200</td>
<td>T307^C</td>
<td>Ser</td>
<td>A^Cl</td>
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<tr>
<td></td>
<td>Leu</td>
<td>A178^C</td>
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<td>Ser</td>
<td>G203^A</td>
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<td>Pro</td>
<td>G676^G</td>
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<td></td>
<td>Glu^Arg</td>
<td>A122^G</td>
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<td>Kell Kl</td>
<td>Met193</td>
<td>Thr</td>
<td>T598</td>
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<td>Trp^Arg</td>
<td>T651</td>
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<td>C1910</td>
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<td></td>
<td>G2019</td>
<td>T</td>
<td>DdE</td>
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<td>Duffy Fa</td>
<td>Gly^Asp</td>
<td>G306^A</td>
<td>BanI</td>
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<tr>
<td>Kidd Jk</td>
<td>Asp^Asn</td>
<td>G838^A</td>
<td>MnII</td>
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<tr>
<td>Diego Di</td>
<td>Leu</td>
<td>Ser^Leu</td>
<td>T2560^C</td>
<td>Nael</td>
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<tr>
<td></td>
<td>Lys^Glu</td>
<td>A1972^G</td>
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<td>MNS M</td>
<td>Ser^Leu</td>
<td>C2^T</td>
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<tr>
<td>S</td>
<td>Gly^Glu</td>
<td>G14^A</td>
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<tr>
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<td>A229^G</td>
<td>AdA</td>
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<tr>
<td>Colton Co</td>
<td>Ala^Val</td>
<td>C13^T</td>
<td>PybM1</td>
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</table>
(eg, Jk (a-b-), U-, D--). Insertions may involve the gain of a single nucleotide or a segment of DNA. As with deletions, insertions can be in-frame or can cause a frame shift. The Co(a-b-) null phenotype may arise by this mechanism.

Altered splicing events are consequences of nucleotide substitutions in the 5' intronic donor splice site or the 3' intronic acceptor sequence upon which orderly outsplicing of introns is dependent. A change in a single nucleotide in these motifs can alter the splicing to induce such events as exon skipping. This mechanism can give rise to the Jk(a-b-) phenotype and the S-s-U- phenotype.

**DNA typing methodology.** DNA typing technologies are based upon variations of techniques for polymerase chain reaction (PCR) amplification of target sequences of the genes of interest and identification of the gene alleles. DNA can be obtained from several sources including buccal epithelial cells, urinary tract epithelial cells and leukocytes [14], or any other body fluid containing nucleated cells. Importantly, blood samples collected from heavily transfused patients can give reliable genotyping, as the circulating leukocytes in such patients are predominantly from recipients [15]. In a study performed in over 60 such patients, no trace of microchimerism was found [16].

Several kit methods for the isolation of DNA are available, including DNAzoI (Gibco BRL, Grand Island, NY); Easy DNA Kit (Invitrogen, Carlsbad, CA); Puregene (Gentra Systems, Minneapolis, MN); and QIAamp (Qiagen, Santa Clara, CA). The first three kits involve similar methods that include the use of a kit-specific detergent lysis solution, followed by precipitation of proteins using organic solvents, and finally DNA precipitation with ethanol. The Qiagen method entails the attachment of nucleic acid to silica columns following cell lysis and its subsequent elution.

Extracted DNA in Tris/EDTA can be stored at 4°C indefinitely. Prior to analysis the DNA should be tested for quantity and quality, since extremely high or low concentrations of DNA or degraded DNA can lead to poor or absent amplification. Between 50 and 200 ng of DNA is ideal for amplification of blood group genes. The estimation of concentration of DNA can be made spectrophotometrically by comparison against DNA standards of known concentrations. Poor or absent amplification may also be observed if the quality is poor (degraded DNA), particularly if the fragment to be amplified is larger than 400 base pairs. The DNA may be tested for degradation by electrophoresis to assure that an excessive number of bands is not present.

PCR uses sequences of oligonucleotides complementary to those that flank a specific DNA sequence of interest, termed primers, to initiate DNA replication. These specific sequences are extended by Taq DNA polymerase in the presence of excess deoxynucleotides. The amplification process occurs in cycles of three steps: denaturation by heating the reaction mixture to 94°C in order to melt the double-stranded DNA into separate strands; annealing (hybridization) of primers to their complementary sequences by cooling to 62°C; and finally, elongation (extension) of the hybridized primers by the DNA polymerase after bringing the reaction mixture to 72°C. After each cycle of denaturation, annealing, and elongation, the newly formed strand serves as template for the next cycle. It is estimated that a single DNA template will generate over a billion copies at the end of 30 cycles of amplification. Since the quantity and quality of DNA in clinical samples is often less than ideal, it is recommended that primers be designed to generate relatively short PCR amplified products (smaller than 400 base pairs) to maximize amplification efficiency [14,16].

There are several approaches to identify the allelic specificity of the amplified product. The most common are post-amplification analysis by restriction fragment length polymorphism (PCR-RFLP) and PCR amplification with allele-specific primers (AS-PCR). The use of allele-specific oligonucleotide probes has not been widely adopted for red cell genotyping. In the PCR-RFLP assay, the amplified products are subjected to enzyme digestion with specific restriction endonucleases, and the digestion fragments are then subjected to electrophoresis. The size of the resultant fragments is dependent on the presence of restriction enzyme sensitive sites specific to the allele. On the other hand, in the AS-PCR assay, the amplification product is already specific for the presence of the allele. It is recommended that known homozygotes and heterozygotes be tested in parallel with the unknown samples and that genotyping be performed without knowledge of phenotyping results.
Typing red cell antigens in sensitized patients

PCR-RFLP analysis can be used for the identification of alleles when they differ from each other by a nucleotide(s) substitution(s) that is (are) associated with the generation or ablation of restriction enzyme sites. Fortunately, this situation occurs with a large number of antigen systems [11]. Table 1 lists some blood groups systems and their associated polymorphisms, as well as examples of the respective restriction endonucleases used for identification. The primers are selected to flank the sequences containing the enzyme restriction sites characteristic of the polymorphism. Subsequently, amplified products are subjected to enzyme treatment followed by electrophoresis in agarose or acrylamide gel. Fig. 1 illustrates the application of PCR-RFLP in genotyping Duffy polymorphism, including primers, enzyme restriction sites, and resolution of polymorphic fragments by electrophoresis in acrylamide gel.

AS-PCR is conceptually simpler than PCR-RFLP. In this technique, the 3'-end of the primers that are selected to amplify the target sequence is specific for only one of the alternate alleles, so that the presence or absence of the allele is determined by the presence or absence of the PCR product. Although this procedure is rapid and simple to perform, it is difficult to achieve specificity if the alleles differ by only one nucleotide. AS-PCR requires the concurrent amplification of an internal control to assure assay performance. AS-PCR is used primarily with complex gene differences or when single point mutations do not result in generation

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**Fig. 1.** Left panel: Cartoon depicting the nucleotide sequences of the two Duffy alleles, *FYB* (top) and *FYA* (bottom). The fragment sizes created by digestion with the BanI restriction enzyme are indicated above the bars corresponding to each allele. Also indicated are the BanI cleavage site common to both alleles, and the new site specific for the *FYA* allele. Right panel: Photograph of an acrylamide gel after electrophoresis of the PCR products. Lane 1: 100 base pair molecular ladder. Lane 2: PCR product not treated with the BanI enzyme (uncut product). Lane 3: Products from a sample homozygous for *FYA* (*FYA*/*FYA*). Lane 4: Products from a sample homozygous for *FYB* (*FYB*/*FYB*). Lane 5: Products from a heterozygous *FYA, FYB* (*FYA/FYB*) sample.
Fig. 2. Primers specific for GYPBS were run with known positive and negative samples together with two unknown samples on the right, while the same controls and samples were run with primers specific for GYPBs on the left. The top sample is a homozygous GYPBS control, the second is a heterozygous control, and the third is a homozygous GYPBs control. Both unknowns are homozygous GYPBs.

or ablation of a restriction endonuclease site. Fig. 2 illustrates the application of AS-PCR for typing for GYPBS and GYPBs. In this case, two separate AS-PCR assays are performed. One assay is performed with primers specific for GYPBS, and the other with primers specific for GYPBs.

The use of allele-specific probes to identify PCR amplification products is also conceptually simple and lends itself to automation. However, this technique has not yet been widely adopted for the molecular identification of blood group alleles.

Discussion

Successful typing of transfusion-dependent patients who have been recently or massively transfused is often possible with classical hemagglutination techniques, following the separation of the patients' reticulocytes and young red cells from the older transfused cells. Recently, a method has been described that permits laboratories with access to flow cytometry instrumentation to phenotype the patients' reticulocytes directly. The increasing availability of a wide variety of direct hemagglutinating monoclonal antibodies promises the capability of phenotyping patients who have positive direct Coombs tests due to sensitization with IgG antibodies. Unfortunately, at present, comprehensive phenotyping for all clinically significant antigens requires the elution of antibody from the red cell surface. Commercial kits enable the laboratory to perform elution procedures with relative ease. While these techniques are effective in many cases, their utility is limited in those patients with decreased numbers of reticulocytes or red cells that have been heavily coated with IgG immunoglobulin.

Establishment of DNA genotyping capabilities in major medical centers and reference laboratories permits these facilities to perform accurate red cell
antigen typing when immunological methods cannot be employed. While establishment of such testing requires considerable investment, referral of difficult typing cases to such reference laboratories can reduce testing time, as well as increase the accuracy of results. Unfortunately, genotyping remains limited by the fact that, to date, gene sequences of some clinically significant blood group systems (eg, Dombrock) have not yet been reported. Expertise is required to avoid false positive or false negative results and to understand the pitfalls in genotype interpretation described below.

An accurate medical history must be obtained from the patient before interpreting genotyping results. As previously noted, the apparent phenotype of circulating red cells in heavily transfused patients may not reflect the true recipient type as determined by genotyping. Furthermore, results can differ with DNA obtained from different cell or tissue sources. In patients who have received a transplant of allogeneic progenitor cells from cord blood, bone marrow, or peripheral blood, the results obtained on DNA from white blood cells will differ from the results obtained on DNA from other somatic cells.

It is important to understand that discordance between genotype and phenotype can also be due to molecular events. In these cases, PCR-based assays would predict a certain antigen profile, but the gene product would not be expressed in the erythrocyte membrane. In some cases molecular events, such as crossovers and other gene rearrangements, can lead to situations where the genotype and phenotype do not agree. In these situations, the primers might not anneal to the hybrid gene and thereby could give false genotyping results. Gene expression may be lacking if transcription is absent. For example, expression of the Duffy protein in red blood cells is dependent upon expression of the GATA transcription factor [11]. A single missense mutation in the GATA binding motif is responsible for the absence of Duffy on the red cells of 68% of African-Americans.

In addition, the expression of some blood group antigens is dependent upon "modifying" genes. For instance, to be fully expressed, Kell system antigens require that the Xk protein be present [17]; Rh antigens (D, C, c, E, e) require the presence of the Rh-associated glycoprotein (RhAG) for expression [18]. Finally, antigens in the Kidd and Lutheran blood group systems are weakened by the presumed presence of the unidentified modifying genes, In(JK) [19] and In(LU) [20], respectively.

**Conclusions**

Transfusion-dependent patients who are sensitized and have been recently transfused with large amounts of blood may often be accurately phenotyped using classical hemagglutination techniques, following separation of their younger red cells and reticulocytes from the older transfused red cells by differential centrifugation or methods that employ centrifugation over density gradients. Patients with sickle cell anemia may be typed following hypotonic lysis of transfused cells. Patients who are found to have positive direct Coombs test reactions, due to sensitization of their red cells with IgG, may often be typed with monoclonal direct agglutinating antisera for Rh antigens, or with IgG reagents following elution of immunoglobulin from their cells.

In transfused patients who have few circulating reticulocytes or patients whose own red cells are sensitized with large amounts of IgG, accurate genotyping for most red cell antigens by variations of PCR technology is usually possible. A few blood group system genes have not been cloned and sequenced, so that genotyping for these antigens is not currently feasible. Correct interpretation of genotyping results is dependent upon a knowledge of the patient's history and an understanding of the reasons that the expected gene product (red cell antigen) may not be expressed. Current techniques permit genotyping of many sensitized transfusion-dependent patients and contribute to transfusion safety and efficacy.

**References**

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