Overview of molecular methods in immunohematology

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BACKGROUND

Blood group antigens are inherited, polymorphic, structural characteristics located on proteins, glycoproteins, or glycolipids on the outside surface of the red blood cell (RBC) membrane. RBCs carrying a particular antigen can, if introduced into the circulation of an individual who lacks that antigen, elicit an immune response. It is the antibody from such an immune response that causes problems in transfusion incompatibility, maternal-fetal incompatibility, and autoimmune hemolytic anemia.

The classical method of testing for blood group antigens and antibodies is hemagglutination. This technique is simple, inexpensive, and when carried out correctly, has a specificity and sensitivity that are appropriate for the clinical care of the vast majority of patients. However, hemagglutination, which is a subjective test, has certain limitations: 1) it does not reliably predict a fetus at risk of hemolytic disease of the newborn (HDN); 2) it is difficult to type RBCs from a patient who recently received a transfusion or those that are coated with IgG; 3) it does not precisely indicate RHD zygosity in D+ people; 4) a relatively small number of donors can be typed for a relatively small number of antigens, thereby limiting antigen-negative inventories; 5) it requires the availability of specific reliable antisera; 6) it is labor-intensive as is the manual data entry; 7) the source material is expensive and diminishing; 8) the cost of commercial reagents (Food and Drug Administration [FDA]-approved) is escalating; 9) many antibodies are not FDA-approved and are characterized (often partially) by the user; and 10) some antibodies are limited in volume, weakly reactive, or not available. The understanding of the molecular bases associated with many blood group antigens and phenotypes enables us to consider the identification of blood group antigens and antibodies using molecular approaches. Screening donors by deoxyribonucleic acid (DNA) testing would conserve antibodies for confirmation by hemagglutination of predicted antigen negativity. The purpose of this overview was to discuss how molecular approaches can be used in transfusion medicine, especially in those areas where hemagglutination is of limited value.

BLOOD TRANSFUSION SUPPORT IN PATIENTS WITH SICKLE CELL DISEASE (SCD)

The Stroke Prevention Trial II corroborated the efficacy of continuous transfusions for preventing strokes in patients with SCD. So clear were the results that the National Heart, Lung, and Blood Institute aborted the 6-year trial after only 2 years. Of the proposed 100 patients, only 79 had been enrolled, 41 of whom were selected to discontinue transfusion. Of these, 14 reverted to high-risk transcranial Doppler ultrasound profiles and resumed transfusion. Of these 14 patients, 2 had suffered a stroke and received a transfusion, and 6 others resumed transfusion for other reasons.1 In contrast, none of the 38 patients who continued to receive transfusions had strokes or reverted to a high-risk state. The National Heart, Lung, and Blood Institute accordingly issued an alert to inform and advise physicians who treat children with SCD that interruption of transfusions for primary stroke prevention is not recommended. Unfortunately, one major risk of chronic transfusion therapy, whether given for stroke prevention or for other indications, is blood group alloimmunization with incidence rates of approximately 20 percent or higher, compared with 5 percent in other transfusion-dependent patient populations.2-6 Patients with SCD often produce many alloantibodies to blood group antigens, which makes the provision of appropriate antigen-negative blood very problematic. Our ability to test a large number of donors for minor antigens is restricted by labor-intensive test procedures and data entry, and limited supplies or unavailability of typing grade antisera. Currently, patients with alloantibodies to multiple blood group

ABBREVIATION: IRB = Institutional Review Board; SCD = sickle cell disease.

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Antigens, or to an antigen of high prevalence, may have to wait for matched RBC components to be obtained through a national or even international Rare Donor Registry, or to undergo surgery with fewer units than the optimal. The unavailability of compatible blood may extend hospital in-days (often in intensive care) and contributes to morbidity and mortality. If we are to transfuse these patients effectively, we must find better ways of reducing the risk of transfusion reactions, hyperhemolysis syndrome, and alloimmunization.

The genes encoding 28 of the 29 blood group systems (only the P system remains to be resolved) have been cloned and sequenced, the molecular bases of blood group antigens and phenotypes have been determined. Analysis of DNA involves amplification of the target sequence of nucleotides, followed by analysis by such techniques as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), allele-specific PCR, real-time PCR, sequencing, and microchip. The results of DNA testing can be used to predict the presence of blood group antigens in various scenarios, and to express antigens in heterologous systems not only to detect and identify blood group antibodies in a single, objective, automated assay but also as immunogens for the production of monoclonal antibodies (Table 1). For the first time in the history of blood transfusion, microchip technology makes it feasible to contemplate precisely matching the antigen-negative status of a donor to that of a patient destined to receive chronic transfusions.

**DNA ASSAYS TO IDENTIFY A FETUS AT RISK FOR ANEMIA OF THE NEONATE**

Hemagglutination, including titters, gives only an indirect indication of HDN risk and severity. Antigen prediction by PCR-based assays can be of value in the prenatal setting in identifying the fetus who is not at risk of HDN (i.e., predicted to be antigen-negative) so that the mother need not be aggressively monitored. Sources of fetal DNA include amniocytes and maternal plasma.

DNA-based typing should be considered when a mother’s serum contains an immunoglobulin G (IgG) alloantibody that has been associated with HDN and the father’s antigen status for the corresponding antigen is heterozygous, indeterminable, or he is not available for testing. For prenatal diagnosis of a fetus at risk of HDN, the approach to DNA typing should err on the side of caution. Thus, the strategy should be to detect a gene even if the product is not expressed on the RBC membrane rather than fail to detect a gene whose product is expressed, because this could result in inadequate monitoring throughout pregnancy. Another potential pitfall is the possibility of contamination by maternal DNA.

As the incidence of genes varies substantially in different populations, it can be helpful to know the ethnicity of the parents. Also, to limit the gene pool, it is helpful to test DNA from the parents. An important practical consideration is to determine whether the mother has undergone medical procedures such as artificial insemination, in vitro fertilization, or whether she is a surrogate mother.

The RHD type is a prime target because anti-D is notoriously clinically significant in terms of HDN (reviewed in Avent and Reid). DNA analysis for the prediction of fetal D phenotype is based on detecting the presence or absence of portions of RHD. In Europeans, the molecular basis of the D− phenotype is usually associated with deletion of the entire RHD, but several other molecular bases have been described. Approximately one-third of D− Japanese have an intact but inactive RHD, and as many as 10 percent of Japanese donors who had RBCs nonreactive with anti-D have the Del phenotype. Approximately one-quarter of D− African Americans have an RHD pseudogene (RHD∗p), which does not encode the D antigen, and many others have a hybrid RHD-CE-D gene (e.g., the r’S phenotype). To predict the RhD antigen type by DNA analysis requires probing for multiple single-nucleotide polymorphisms. Establishing the fetal K genotype is also of great clinical value in determining whether a fetus is at risk for severe anemia, because the strength of the mother’s anti-K does not correlate with the severity of the infant’s anemia.

When performing DNA analysis in the prenatal setting, it is also important to always determine the RHD
status of the fetus, in addition to the test being ordered. In so doing, if the fetus has a normal \textit{RHD}, there is no need to provide Rh– blood for interuterine transfusions. This is especially true if the mother has anti-c and fetal DNA is being typed for \textit{RHCE}\textsuperscript{+}c.

**DNA Typing for Blood Group Antigens for Patients**

When a patient receives a transfusion, the presence of donor RBCs in the patient’s peripheral blood makes RBC phenotyping by conventional hemagglutination techniques complex, time-consuming, and possibly inaccurate. Indeed, the “best guess” of a patient’s antigen type based on the strength of hemagglutination, the number of RBC components transfused, the length of time since transfusion, the estimated blood volume of the patient, and the prevalence of the antigen in question is more often inaccurate than accurate.\textsuperscript{17} To overcome this problem, PCR-based assays using DNA isolated from white blood cells (WBCs), buccal smear, or urine sediment can be used to predict the antigen type of patients.\textsuperscript{18-21}

DNA-based antigen typing of patients with autoimmune hemolytic anemia, whose RBCs are coated with immunoglobulin (have a positive direct antiglobulin test [DAT]), is valuable when 1) direct agglutinating antibodies, or murine monoclonal antibodies, are not available; 2) antisera are weakly reactive; 3) antigen is sensitive to the IgG removal treatment; and 4) diagnostic antibodies require the indirect antiglobulin test and IgG removal techniques are not effective at removing bound immunoglobulin. DNA-based assays are also useful as a tool to distinguish alloantibodies from autoantibodies and to identify the molecular basis of unusual serologic results.

When recommendations for clinical practice are based on molecular analyses, it is important to remember that, in rare situations, a genotype determination will not correlate with antigen expression on the RBC.\textsuperscript{22} If a patient has a grossly normal gene that is not expressed, he or she could produce an antibody following transfusion of antigen-positive blood. When feasible, the appropriate assay to detect a mutation that silences a gene should be part of the DNA-based testing, e.g., GATA box and FY-265 analyses with \textit{FY} typing,\textsuperscript{23} presence of \textit{RHD} pseudogene with \textit{RHD} typing,\textsuperscript{15} and exon 5 analysis with \textit{GYPBS} typing.\textsuperscript{24} In addition, it is important to obtain an accurate medical history of the patient because with certain medical treatments, such as stem cell transplantation, results of DNA typing may differ from results obtained by hemagglutination.

**DNA Testing for Screening Blood Donors**

PCR-based assays can be used to predict the antigen type of the donor blood for transfusion and for antibody identification reagent panels.\textsuperscript{25} This is particularly useful when antibodies are not available or are weakly reactive. Examples are Do\textsuperscript{a}/Do\textsuperscript{b}, Js\textsuperscript{i}, and V/VS where DNA-based assays are being used to type patients and donors to overcome the dearth of reliable reagents. PCR-based assays are also useful for confirming whether an antigen is truly present in a double dose, especially S, D, Fy\textsuperscript{a}, Fy\textsuperscript{b}, Jk\textsuperscript{a}, and Jk\textsuperscript{b}. In our laboratory, DNA-based assays were found to be valuable for differentiating specific Knops antigen negativity from a low copy number of CR1 (CD35).

With donor typing, the presence of a grossly normal gene whose product is not expressed on the RBC surface would lead to the donor being falsely typed as antigen-negative, and although this would mean loss of an antigen-negative donor, it would not jeopardize the safety of blood transfusion. As automated procedures attain higher and faster throughput at lower cost, typing of blood donors by DNA-based assays is likely to become more widespread. Screening for rare donors by analysis of DNA is valuable for typing for “minor” antigens and Rh variants. Those antigens that are predicted to be negative should be confirmed by hemagglutination. In this manner, precious antibodies are conserved for the confirmation of DNA typing predictions.

**Why not determine routine ABO and RHD by testing DNA?**

Like all donors, antigen-negative donors must have their ABO and Rh type determined. However, for several reasons DNA analysis is not the method of choice for routine ABO and D determination of donor blood. They include the following: the naturally occurring anti-A or anti-B in the plasma of most people who lack the corresponding antigens provides a built-in check when performing ABO typing by hemagglutination; potent, well-standardized monoclonal reagents are available for ABO and D typing; hemagglutination is relatively simple and rapid; and systems are in place to test and record, relatively efficiently, the ABO and D type of a donor. In both ABO and Rh systems, there are few antigens and many alleles. In the ABO system, there are four primary phenotypes (A, B, AB, O) but well over 100 alleles. In the Rh system, D is one antigen but there are close to 200 alleles known. In both scenarios, it is highly likely that more alleles exist and await detection. Furthermore, RBCs with a weak expression of the D antigen are almost always C+ or E+. Thus, the fear of transfusing apparently D– RBCs that actually express some serologically nondetectable D antigen can be more easily overcome by transfusing D– C– E– RBCs (which are usually truly D–) than by using DNA assays to detect the multiple alleles involved in the weak D phenotypes. For routine ABO and D determination, DNA testing is more time-consuming, more expensive, prone
to misinterpretation, and not an improvement over hemagglutination.

DNA analysis for ABO and Rh types can be of value in the resolution of ABO and D discrepancies, to show that a discrepancy is due to a genetic variant and not to technologist error or reagent failure and thus, not an FDA reportable error. ABO genotyping can also be useful for distinguishing an acquired phenotype from an inherited one without having to perform laborious family studies. Many Rh phenotypes cannot easily be defined by serologic methods, either because suitable panels of monoclonal antibodies are not readily available or because the antibodies are not available in the needed strength or volume. DNA assays may be useful for defining some and precisely match the D and e antigen status of a donor to a recipient, especially those with SCD.

**Testing for donors lacking Do antigens**

RBC typing for Do\(^a\), Do\(^b\), Hy, and Jo\(^a\) antigens of the Dombrock blood group system is notoriously difficult because the corresponding antibodies, although clinically significant, are often weakly reactive, available only in small volume, and present in sera containing other alloantibodies.\(^{26}\) At the New York Blood Center, PCR-RFLP analysis is used to type donors selected to lack certain combinations of antigens [e.g., C–, E–, K–, Fy(a–), Jk(b–)] for DOA and DOB for patients who have antibodies to such multiple antigens, in addition to anti-Do\(^a\) or anti-Do\(^b\). Donors whose RBCs react weakly or do not react with human polyclonal anti-Gya for DO-HY and DO-JO are also tested. This has provided us with a larger inventory of valuable Hy– and Jo(a–) donors. Because of the dearth of appropriate antisera, testing for polymorphisms in the Dombrock system by DNA analysis surpasses hemagglutination for antigen typing.

**DNA typing for low-prevalence antigens**

Patients in New York often need Js(a–), V–, VS–, Go(a–), or DAK– RBC components. Providing RBC products for these patients is difficult because patients make antibodies to these antigens in addition to several others, e.g., anti-C, -E, -K, -Fy\(^3\), and -Jk\(^b\). While these immunogenic antigens are of low prevalence in Caucasian donors, they are present on up to 20 percent of RBCs from African American donors. As a natural consequence of transfusing Rh- and K-matched RBC components to patients, in the Stroke Prevention Trials, the patients have made antibodies to these “low prevalence” antigens. These antigens are not on antibody screening RBCs; the corresponding antibodies are not available to screen for donors; and the crossmatch is not always reliable for their detection. PCR-based assays provide a tool to mass screen donors, thereby increasing the antigen-negative inventory and improving patient care.

As an illustration, we had a patient whose serum contained anti-U–, -C–, -E–, -K–, plus -VS, and -Js\(^a\). Of 95 eligible U–, C–, E–, K– donors in the center’s inventory, four had been typed as VS–, Js(a–), 27 were VS+ or Js(a+), and 64 had not been typed for either antigen. Anti-VS and anti-Js\(^a\) were not available in sufficient volume for typing. PCR-based methods, which do not require special reagents, can be used to screen for antigen-negative donors. Current manual methods are time-consuming and labor intensive; however, the prospect of using microchips to screen large numbers of donors is appealing. This case is not unique, and many such examples throughout the United States exist.

**DNA typing for high-prevalence antigens**

As anti-Lu\(^b\), -Yta, -Sc\(^1\), -I\(^W\), and -Co\(^a\) are inconsistently available, testing DNA is a desirable alternative. The ready availability of anti-k–, -Kp\(^2\), -Js\(^a\), -Fy\(^3\), and -Jk\(^3\) often makes hemagglutination the method of choice for these antigens. On the other hand, if the appropriate single-nucleotide polymorphisms can be added to a microchip at little incremental cost, all of the above antigens could be predicted. Detection of Vel–, Lan–, At(a–), or Ir(a–) donors is restricted to hemagglutination because the molecular bases of these antigens are not yet known. Detection of null phenotypes such as Rhnull, Knull, Gy\(^a\), Ge–, or McLeod is complex because of multiple molecular bases associated with these phenotypes.\(^8\)

**LIMITATIONS OF DNA ANALYSIS**

Testing by DNA analyses does have technical, medical, and genetic pitfalls.\(^27\) Medical pitfalls include recent transfusions, stem cell transplantation, and natural chimerism. In these scenarios, results of testing DNA may not agree with hemagglutination results. In addition, stem cell transplantation and natural chimerism may cause the results of testing DNA from somatic cells to differ from the results of testing DNA from WBCs. Thus, when embracing DNA testing, it is important to obtain an accurate medical history. There are many genetic events that cause apparent discrepant results between hemagglutination and DNA test results;\(^28\) the genotype is not the phenotype.

The majority of DNA-based assays will detect a grossly normal gene that is not expressed and this can lead to a donor being falsely identified as antigen-positive. This would mean that a valuable antigen-negative (e.g., null) donor would be lost to the inventory, but would not jeopardize the safety of a patient receiving blood transfusion. Confirmation by hemagglutination of predicted antigen negativity is recommended using a
reagent antibody, if available, or by crossmatching using a method optimal for the detection of the antibody or antigen in question.

Not all blood group polymorphisms can be easily analyzed, for example, if a large number of alleles encode one phenotype (e.g., ABO, Rh, and null phenotypes in many blood group systems); or alleles with a large deletion (e.g., Ge−) or alleles encoded by a hybrid gene (e.g., in the Rh and MNS systems); or when the molecular basis is not yet known (e.g., Vel, Lan, Jr⁰). Additionally, there is a high probability that not all alleles in all ethnic populations are known.

As discussed earlier, the molecular bases associated with a large number of antigens have been reported. However, in many cases, the analysis has been restricted to a relatively small number of people with known antigen profiles. This information is being applied to DNA typing with the assumption that such analysis will correlate with RBC antigen typing in all populations. However, we are still in our infancy of understanding gene polymorphisms in different ethnic groups and their significance to the expression of blood group antigens. A much larger number of people from a variety of ethnic backgrounds needs to be analyzed to establish more firmly the correlation between genotype and the blood group phenotype. Until such data are available, caution should be exercised when recommending clinical practice based on DNA typing for blood group antigens.

**MICROCHIP SCREENING FOR ANTIGEN-NEGATIVE DONORS**

Microchip technology simultaneously performs multiple assays on one sample; thereby a large number of antigens can be predicted on a large number of donor samples. The results are analyzed and interpreted by computer, and can be directly downloaded to a donor database, which will reduce data entry errors inherent in manual systems. The cost of the approach will depend on how much the manufacturers charge for kits they develop. An added cost of doing DNA testing could be the expense of investigating any discrepancies. High-throughput technologies have the potential to dramatically increase inventories of antigen-negative blood.

**POSSIBLE USES OF PHENotypically MATCHED BLOOD**

If antigen-negative inventories were sufficient, the following uses of antigen-matched blood could be contemplated:

- to match antigen profiles in chronically transfused patients who are immune responders, especially those with SCD disease;
- to match unusual Rh phenotypes, especially in African Americans (hrB−, hrS−, etc.);
- to predict the type of antigens for which there is no antibody (e.g., V/VS, Go⁰, DAK, Js⁰, Do⁰, Do⁰);
- to match for Jk⁰ and Jk⁰ if the patient has been exposed, to prevent transfusion reactions and deaths due to anti-Jk⁰ or anti-Jk⁰. Reports by the FDA in the United States and the Serious Hazards of Transfusion study in the UK have revealed that a handful of patients die annually after receiving transfusion of antigen-positive blood;
- to transfuse RBC components with weak expression of the RhD antigen to patients with similarly weak RhD expression (e.g., match for D₃D and D₃weak);
- to transfuse patients with antibodies to high prevalence antigens; and
- to transfuse patients with autoimmune hemolytic anemia to eliminate labor-intensive procedures that are required to ensure that there are no underlying clinically significant antibodies.

**OTHER CONSIDERATIONS**

To apply molecular approaches to clinical situations, several areas of knowledge are needed, for example, a knowledge of molecular techniques, of gene structure and processing, of the molecular bases of blood groups, of hemagglutination techniques, of the expression of blood group antigens, of factors that may affect the interpretation of genotype (e.g., chimeras), and of regulatory com-
pliance (Good Laboratory Practices, IRB, FDA), as well as an ability to correlate DNA and serologic results to the clinical problems being addressed.

Numerous studies have analyzed blood samples from people with known antigen profiles and identified the molecular bases associated with many antigens. The available wealth of stockpiled serologically defined variants has contributed to the rapid rate with which the genetic diversity of blood group genes has been revealed. Initially, molecular information associated with each variant was obtained from only a small number of samples. This information was applied to DNA analyses with the hopeful assumption that the molecular analysis would correlate with RBC antigen typing. With the gathering of more information it became obvious that many molecular events result in the genotype and phenotype being apparently discrepant and that more than one genetic event can give rise to the same phenotype. This is especially true for null phenotypes, e.g., for Rh, Kell, and Kidd systems, and the p phenotype.

Other considerations include establishing the extent of testing alleles for each antigen, e.g., GATA and nt FY-265 with FY typing, and whether to use the results without confirmation by hemagglutination if it is unlikely to harm the patient. If we had a simple, inexpensive way of positively identifying a donor at subsequent donations, DNA typing of a donor could be performed only once. Of value would be a system of automated DNA preparation and positive sample identification from the beginning of the process to the end.

Hemagglutination is the gold standard technique to type RBCs for the presence or absence of blood group antigens. PCR-based assays, used as an adjunct to hemagglutination, will be a powerful tool that could radically change approaches used to support patients in their transfusion needs.

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