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Transfusion in the age of molecular diagnostics

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Abstract

DNA-based tests are increasingly being used to predict a blood group phenotype to improve transfusion medicine. This is possible because genes encoding 29 of the 30 blood group systems have been cloned and sequenced, and the molecular bases associated with most antigens have been determined. RBCs carrying a particular antigen, if introduced into the circulation of an individual who lacks that antigen (through transfusion or pregnancy), can elicit an immune response. It is the antibody from such an immune response that causes problems in clinical practice and the reason why antigen-negative blood is required for safe transfusion. The classical method of testing for blood group antigens and antibodies is hemagglutination; however, it has certain limitations, some of which can be overcome by testing DNA. Such testing allows conservation of antibodies for confirmation by hemagglutination of predicted antigen-negativity. High-throughput platforms provide a means to test relatively large numbers of donors, thereby opening the door to change the way antigen-negative blood is provided to patients and to prevent immunization. This review summarizes how molecular approaches, in conjunction with conventional hemagglutination, can be applied in transfusion medicine.

During the 20th century, knowledge of blood groups grew from three antigens [A, B, and O (later shown to be H)] to nearly 300 discrete antigens. Most blood group antigens are accommodated in 30 blood group systems,[1-3] and the gene (or a small gene family for MNS, Rh, and Ch/Rg systems) encoding each blood group system (the gene encoding P1 remains to be published) has been cloned and sequenced,[4] and the molecular bases associated with the vast majority of blood group antigens have been determined.[5] 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 clinical practice, such as in patient/donor blood transfusion incompatibility, maternal-fetal incompatibility, and autoimmune hemolytic anemia and the reason why antigen-negative blood is required for safe transfusion in these patients.

The classical method of testing for blood group antigens and antibodies is hemagglutination. This technique has served the transfusion community well for decades, it is simple, requires little equipment, and when done correctly, has a specificity and sensitivity that is appropriate for the clinical care of the vast majority of patients requiring blood transfusion. However, hemagglutination, which is a subjective test, has certain limitations (Table 1). Collectively, the limitations have resulted in a relatively small number of donors being typed for a relatively small number of antigens, thereby limiting antigen-negative inventories.

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The knowledge of the molecular bases associated with many blood group antigens and phenotypes enables us to predict the presence or absence of blood group antigens, thereby overcoming some longstanding limitations of hemagglutination. High-throughput DNA arrays provide a means to test a relatively large number of donors, thereby opening the door to change the way antigen-negative blood is provided to patients.They provide the possibility of preventing immunization, and, for patients who require chronic transfusions, providing RBC components based on matching by DNA testing; this is particularly valuable for Rh and Do blood group systems. Screening donor samples by DNA-based tests allows us to conserve precious antibodies for confirmation by hemagglutination of predicted antigen-negativity. The purpose of this review is to discuss how molecular approaches can be applied in transfusion medicine, especially in those areas where hemagglutination is of limited value, and how DNA testing can be a powerful adjunct to hemagglutination.

Prediction of Blood Groups by DNA Analysis and Clinical Applications

Prediction of a blood group antigen by testing DNA is easy and reliable if the antigen is encoded by a common or “normal” functional allele. However, the many uncommon alleles make this approach imperfect. There are far more alleles than phenotypes. For the 30 blood group systems, there are 34 associated gene loci and 270 antigens, but over 1000 alleles.

The value, reagents, and limitations of hemagglutination, PCR-based assays, and DNA array testing for the prediction of blood groups, are summarized in Table 1. Some applications of PCR-based tests are listed in Table 2. In molecular diagnostic tests, the source of DNA is most frequently extracted from cells that do not express the antigen (eg, WBCs, buccal epithelial cells) and the resulting “genotype” is used to predict the antigen expression on RBCs. Furthermore, if the genotype is not fully understood, inaccurate predictions can ensue.

An increasing number of DNA array platforms are available: BioArray Solutions (BAS) HEA Beadchip™,[6,7] BioTrove (OpenArray), Progenika (BloodGen bloodchip),[8] GTI (Red Cell EZ Type), and SNaPshot™ (ABI, Foster City, CA).[9] Desirable characteristics for a DNA array include that it uses robust chemistry, allows easy addition of probes to detect multiple nucleotide changes and automation with minimal hands-on time, and is reliable, consistent, flexible, specific, sensitive, and of feasible cost.

DNA Typing to Identify a Fetus at Risk for Anemia of the Neonate

Hemagglutination, including antibody titers, gives only an indirect indication of hemolytic disease of the fetus and newborn (HDFN) risk and severity. Antigen prediction by DNA assays can be of value in the prenatal setting to identify the fetus who is NOT at risk of HDFN (ie, predicted to be antigen-negative) so that the mother need not be aggressively monitored. Another value is to predict the D type of a fetus to aid in the evaluation of whether Rh-immune globulin need, or need not, be administered. Testing DNA should be considered when a mother's serum contains an IgG alloantibody that has been associated with HDFN and the father's antigen status for the corresponding antigen is heterozygous, indeterminable, or he is not available for testing. Sources of fetal DNA include amniocytes and maternal plasma.

For prenatal diagnosis of a fetus at risk of HDFN, 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. To reduce the likelihood of error in interpretation, it is helpful to test DNA from the parents. If this is not possible, and because the prevalence of genes can vary substantially

in different populations, it can be helpful to know the ethnicity of the parents. A potential pitfall is the possibility of contamination of the fetal sample with maternal DNA.

The *RHD* type is a prime target because anti-D is notoriously clinically significant in terms of HDFN (reviewed in Avent and Reid[10]), and even today a high proportion of HDFN cases are caused by anti-D. PCR analyses for predicting the D type of a fetus are based on detecting the presence or absence of specific portions of *RHD*. Establishing the fetal *KEL* 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 antibody does not correlate with the severity of the infant's anemia.[11] The same is true for anti-Ge3.[12]

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 *RHD* there is no need to provide Rh-negative blood for intrauterine transfusions. For example, if a mother has anti-c and fetal DNA is being typed for *RHCE*c*, in the absence of a predicted D type of the fetus, r'r' RBCs would be ordered for an intrauterine transfusion, whereas if the fetus is predicted to be D-positive then R_1R_1 RBCs are appropriate.

DNA Typing to Predict Blood Group Antigens in Transfusion Candidates

Knowledge of the RBC phenotype of a patient is an invaluable part of the antibody identification process. When a patient receives transfusions, the presence of donor RBCs in the patient's peripheral blood makes RBC phenotyping by hemagglutination complex, time-consuming, and possibly inaccurate.[13] To overcome this problem, PCR-based assays using DNA isolated from WBCs, buccal smear, or urine sediment can be used to predict the antigen type of patients. In the way the PCR-based assays are performed for this purpose, any donor WBCs in a transfused patient's circulation do not interfere with the interpretation.[13-16]

In the author's laboratory, PCR-based assays are used to aid in identification of more uncommon antibodies, for example, to differentiate specific Knops antigen-negativity from a low copy number of CR1 (CD35) and to identify antibodies to Cromer blood group antigens.[17] If the initial findings of an investigation indicate an antibody to a Cromer system antigen, serological testing is halted and DNA analyses are performed on the sample. Any findings are then confirmed serologically, providing suitable serum and cell samples are available. The scarce quantities of rare red cells and sera for certain blood groups are conserved so as to be available for other investigations and confirmatory testing.

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.[18] If a patient has a grossly normal gene that is not expressed, he/she could produce an antibody if transfused with antigen-positive blood. When feasible, the appropriate assay to detect a nucleotide change that silences a gene should be included as part of the DNA-based testing (see Limitations). Also, it is important to obtain an accurate medical history for 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 Antigen-negative Blood Donors

PCR-based assays are particularly valuable to predict the antigen profile of a blood donor when the antibody reacts weakly or is not readily available, eg, anti-Do^a, -Do^b, -Hy, -Jo^a, -Js^a, -Js^b; -C^W, -V, and -VS. Testing for these antigens by DNA analyses is accomplished

without the need for special reagents (antibodies) and is an example of where PCR-based assays surpass hemagglutination for antigen typing. DNA arrays, with their high-throughput capability, are particularly suited for mass screening donors. This technology provides a tool to enable us to increase the antigen-negative inventory of combinations of the minor antigens and of high-prevalence antigens and, thus, to consider the possibility of providing donor RBC components that are DNA matched to the patient's type. DNA-based assays also can be useful to detect genes that are predicted to encode weakly expressed antigens and thereby prevent immunization or possible transfusion reactions.

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-positive, 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 PCR-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 interpretations.

RBC typing for Do^a, Do^b, Hy and Jo^a antigens is notoriously difficult because the corresponding antibodies, although potentially of clinical significance, are often weakly reactive, available only in small volume, and present in sera containing other alloantibodies.[19] At the New York Blood Center, for over a decade we have used PCR-RFLP LDTs to type donors selected to lack certain combinations of antigens [eg, C–, E–, K–, Fy(a–), Jk(b–)] for *DO*A, DO*B, DO*HY* and *DO*JO* for patients who have antibodies to multiple antigens, in addition to the Dombrock system antibody. Due to the dearth of appropriate antisera, testing for polymorphisms in the Dombrock system by DNA analysis surpasses hemagglutination for antigen typing.

The value of access to larger numbers of antigen-negative RBC components is multifaceted. If antigen-negative inventories were sufficient, several uses of antigen-matched blood could be contemplated to improve patient care (Table 3). Obviously, as with hemagglutination, the rare antigen-negative donor will likely only be found by screening thousands of donors.

DNA Typing for Low-prevalence Antigens

Some antigens that are of low prevalence in Caucasian donors, such as Js^a, V, VS, Go^a, or DAK, are present on RBCs of up to 20% of African-American donors. As a natural consequence of transfusing Rh- and K-matched RBC components, several patients in the area serviced by New York Blood Center have made antibodies to these “low-prevalence” antigens. Thus, providing antigen-negative RBC products can be difficult because patients typically have several other antibodies, for example, anti-C, -E, -K, -Fy^a, and -Jk^b, in their serum/plasma. The “low-prevalence” antigens are not on antibody screening RBCs, the corresponding antibodies are not available to screen for suitable 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.

DNA Typing for High-prevalence Antigens

As anti-Lu^b, -Di^b, -Yt^a, -Sc1, and -Co^a are inconsistently available, testing DNA is a desirable alternative. The ready availability of anti-k, -Kp^b, -Js^b, and -Fy3 often makes hemagglutination the method of choice for these antigens. On the other hand, as probes for various nucleotide changes can be added to a microchip at little incremental cost, all of the above antigens could be assayed simultaneously. Detection of Vel–, Lan–, At(a–), or Jr(a–)

donors is restricted to hemagglutination because the molecular bases of these antigens are not yet known. Detection of null phenotypes such as Rh_{null}, K_0, Gy(a–), Ge–, or McLeod is complex due to multiple molecular bases associated with each of these phenotypes.[5]

DNA Testing for the Prediction of ABO and RHD Alleles

In the author's opinion, DNA analysis is not suited for routine ABO and D determination of donor blood. The reasons include the following: the naturally occurring anti-A and/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 antibodies 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 about 200 known alleles. In the Rh system, D is one antigen but there are over 150 *RHD* known alleles. In both scenarios, it is highly likely that more alleles exist and await discovery. 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 non-detectable D antigen can be overcome by transfusing D- C- E- RBCs (which are usually truly D-) rather than by using PCR-based assays to detect the multitude of alleles associated with weak D phenotypes. There is some debate on this approach, for example, Flegel et al advocate performing DNA analysis of all D- donors.[20] For routine ABO and D determination, DNA testing is more time-consuming, more expensive, prone to misinterpretation, and thus, not an improvement over hemagglutination.

DNA analysis for ABO and Rh types can be of value in the resolution of ABO and D anomalies, by showing that a discrepancy is due to a genetic variant (without having to perform laborious family studies) and not to technologist error or reagent failure and, thus, not a regulatory reportable error. ABO genotyping also can be useful for distinguishing an acquired phenotype from an inherited one. Many Rh phenotypes cannot easily be defined by hemagglutination, either because suitable panels of monoclonal antibodies are not readily available or the antibodies are not available in needed strength or volume. PCR-based assays may be useful to define some Rh variants and to precisely match D and e antigen status of a donor to a recipient, especially in the case of patients with sickle cell disease (SCD). Of particular note are hr^S and hr^B antigens, the absence of which is known to be encoded by several distinct alleles.[21]

Limitations of DNA Analysis

Testing by DNA analyses has technical, medical, and genetic pitfalls,[22;23] and DNA testing methods and reagents licensed by the US Food and Drug Administration (FDA) are not available. As with all methodologies, there are numerous sources of technical errors, but description of these is beyond the scope of this review. Medical pitfalls include recent transfusions, stem cell transplantation, and natural chimerism. Thus, when embracing DNA testing, it is important to obtain an accurate medical history. Many genetic events cause apparent discrepant results between hemagglutination and DNA test results;[23] the genotype is not the phenotype (Table 1). Confirmation by hemagglutination of predicted antigen-negativity is recommended using a reagent antibody if available, and/or by cross-matching using a method optimal for detection of the antibody/antigen in question.

From a practical perspective, not all blood group polymorphisms can be analyzed: for example, in situations when a large number of alleles encode one phenotype (eg, ABO, Rh, and null phenotypes in many blood group systems); alleles with a large deletion (eg, GE:–2,3 and GE:–2–3–4); or a hybrid alleles (eg, in the Rh and MNS systems); or when the

molecular basis is not yet known (eg, Vel, Lan, Jr^a). Detection of null phenotypes, eg, Rh_{null},[10;24] K_0,[25] McLeod,[26] Kidd,[27] and the p phenotype[28] is complex by DNA analysis because of multiple molecular bases, but is easy by hemagglutination. For example, to detect D- test RBCs with anti-Rh17 (or -C & -c), to detect Fy(a-b-) test RBCs with anti-Fy3, and to detect Jk(a-b-) test RBCs with anti-Jk3 or test for the resistance of such RBCs to lysis by 2M urea.[29]

Although the molecular bases associated with most antigens have been reported, 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. A much larger number of people, from a variety of ethnic backgrounds, need 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.

When appropriate and feasible, an assay to detect a nucleotide change that silences a gene should be part of the DNA-based testing; eg, GATA box and *FY*265* (for Fy^x) analyses with *FYA/FYB* typing,[30] presence of *RHD* pseudogene with *RHD* typing,[31] and exon 5/intron 5 analysis with *GYPB*S* typing.[32] While a nucleotide change (t>c) in the GATA-1 binding motif is the basis of the Fy(a-b-) phenotype in blacks, the Fy(a-b-) phenotype in Caucasians is very rare and associated with silencing of *FY*A* or *FY*B* by at least 4 different molecular bases.[30] Given the large number of alleles that silence various blood groups, the question is how much testing is practical or feasible. The limited DNA testing used for clinical utility will not allow accurate interpretation of all alleles; thus, in this scenario, the ”genotype” may not be the phenotype and confirmation of predicted antigen-negativity by hemagglutination or a crossmatch is recommended. Although DNA-based methods have limitations, there are clearly instances where they can have clinical benefit. These and other considerations are presented in an International Forum[33] and in review articles.[8,34-36]

Concluding Remarks

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 interpretation of genotype (eg, chimeras), and of regulatory compliance (cGLP, IRB, FDA). In addition, it is important to be able to correlate DNA and serological results to the clinical problems being addressed.

Other considerations include establishing the extent of testing alleles for each antigen. The number of markers that can be assayed depends on such things as the ethnic population being tested, the reason for the testing, the number of probes that can be placed on one platform, and the cost. Another consideration is whether to use the results without confirmation by hemagglutination if it is unlikely to harm the patient. Furthermore, if there was a simple, inexpensive way to positively identify a donor at subsequent donations, should DNA typing of a donor be performed only once? Of value would be a fully automated system and positive sample identification from the beginning of the process (DNA preparation) to the end (result interpretation entered into data base). 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.

DNA testing for routine ABO and Rh cannot be justified over the simplicity of hemagglutination methods. If a patient has RBCs that express a variant ABO, precise

matching is not necessary because group O blood is a safe alterative. While routine Rh typing by DNA methods is not indicated, in a small cohort of patients with alloantibodies to certain Rh antigens (eg, SCD), matching at the DNA level can provide an added level of safety and efficacy. Matching for the so-called “minor” antigens in the relatively small population of patients whose plasma contains alloantibodies (less than 10% of transfusion recipients make alloantibodies) to different combinations of these antigens or for patients who are known to be responders has value.

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Table 1

Features of hemagglutination and PCR-based assays for blood groups antigens.

Hemagglutination

- Value
  - The "Gold Standard" method to detect the presence or absence of blood group antigens on RBCs that has served the transfusion community well
  - Simple and quick to perform, requires little in the way of equipment, and, when done correctly, has a specificity and sensitivity that is appropriate for the vast majority of transfusions
  - Detects mixed populations of RBCs
- Reagents
  - Specialized and obtained from immunized patients/donors (polyclonal and monoclonal antibodies) or from immunized mice (monoclonal antibodies)
  - Source material is a biohazard and is diminishing
  - Cost of FDA-approved, commercially licensed reagents is escalating
  - Many antibodies are not commercially available and are characterized (often only partially) by the user and some are limited in volume, weakly reactive, or not available
- Limitations
  - Is a subjective test
  - Requires use of reliable antisera
  - Labor-intensive testing so a relatively small number of donors can be typed for a relatively small number of antigens, which has limited the size of antigen-negative inventories
  - Indirect indication of a fetus at risk of hemolytic disease of the fetus/newborn
  - Difficult to phenotype a recently transfused patient
  - Difficult to phenotype RBCs coated with IgG
  - Can be difficult to distinguish an alloantibody from an autoantibody in antigen-positive people
  - Restricted ability to determine zygosity, especially RHD zygosity in D-positive individuals

DNA testing, including DNA arrays

- Value
  - Can be automated
  - High throughput because multiple markers are tested simultaneously on one sample
  - Computerized interpretation and data entry into a patient/donor data base
  - Potential to precisely geno-match donor blood to the patient's type
- Reagents
  - Does not require special reagents, which can be readily purchased
- Limitations
  - Predict an antigen type; it is recommended that the prediction be confirmed by hemagglutination, particularly if negative for the antigen
  - Takes hours
  - DNA and hemagglutination test results may not agree
  - DNA results from somatic cells and from WBCs may not agree
  - More than one genotype can give rise to the same phenotype, especially with the null phenotypes
  - There is a high probability that not all alleles in all ethnic populations are known
  - For research use only; has not been approved by the FDA as the sole test on which to base decisions regarding patient care

Table 2

Applications of PCR-based assays to predict a blood group antigen.

Antigen Typing a Patient
■ To identify a fetus at risk or not for hemolytic disease of the fetus and newborn (HDFN)
■ When antibody is weak or not available (eg, anti-Do^{a}, -Do^{b}, -Js^{a}, -V/VS)
■ Who has been recently transfused to aid in antibody identification and selection of RBCs for adsorption
■ To distinguish an alloantibody from an autoantibody (eg, anti-e, anti-Kp^{b})
■ To help identify alloantibody when a patient's RBCs type antigen-positive and a variant phenotype is suspected (eg, anti-D in a D-positive, anti-e in a e-positive)
■ Whose RBCs are coated with immunoglobulin (+DAT)
■ Who has received an allogeneic stem cell transplant
■ To detect weakly expressed antigens where the patient is unlikely to make antibodies to transfused antigen-positive RBCs
■ Identify molecular basis of unusual serological results, especially Rh variants
■ To determine zygosity
Antigen Typing for Donors
■ Mass screening to increase antigen-negative inventory
■ To find donors whose RBCs lack a high-prevalence antigen
■ To resolve blood group A, B, D, C, and e discrepancies
■ To detect genes that encode weak antigens
■ To type donors for reagent RBCs for antibody screening cells and antibody identification panels

Table 3

Possible uses of antigen-negative blood to improve patient care.

If inventories of antigen-negative donor blood were large enough, it could be feasible to contemplate the following:
■ Prevent morbidity and mortality due to transfusion reactions. The cost to screen for antigen-negative donors is offset by the cost to investigate and treat a patient with a transfusion reaction.
■ Reduce the need to withhold RBC components to alloimmunized patients, especially those with multiple alloantibodies where providing antigen-negative blood has been a challenge. This could ameliorate the clinical risks of withholding blood transfusion.
■ Prevent immunization and transfusion reactions by matching antigen profiles in chronically transfused patients who are immune responders, especially those with sickle cell disease.
■ Provide RBC components lacking antigens for which there is no antibody for screening purposes.
■ Match, based on DNA testing, unusual Rh phenotypes especially in African Americans.
■ Match for Jk^a and Jk^b if a patient has been exposed to either antigen, to prevent transfusion reactions and deaths due to anti-Jk^a or anti-Jk^b. Reports by the FDA in the USA and the Serious Hazards of Transfusion (SHOT) study in the UK have revealed that a handful of patients die annually after being transfused with antigen-positive blood.[27]
■ Transfuse a patient with an alloantibody to a high-prevalence antigen with antigen-matched RBC components, and thereby reduce the need for maintaining a large library of rare reagents.
■ For patients with autoimmune hemolytic anemia (AIHA), reduce need for labor-intensive procedures that are required to detect underlying clinically significant antibodies each time the patient requires blood transfusion.
■ Transfuse RBC components matched for phenotypes such as week D to conserve true D-negative blood for true D-negative patients.