Revisão / Review

Blood group genotyping
Genotipagem de grupos sangüíneos

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*Accurate phenotyping of red blood cells (RBCs) can be difficult in transfusion-dependent patients such as those with thalassemia and sickle cells anemia because of the presence of previously transfused RBCs in the patient's circulation. Recently, the molecular basis associated with the expression of many blood group antigens was established. This allowed the development of a plethora of polymerase chain reaction-based tests for identification of the blood group antigens by testing DNA. The determination of blood group polymorphism at the genomic level facilitates the resolution of clinical problems that cannot be addressed by hemagglutination. They are useful to (a) determine antigen types for which currently available antibodies are weakly reactive; (b) type patients who have been recently transfused; (c) identify fetuses at risk for hemolytic disease of the newborn; and (d) to increase the reliability of repositories of antigen negative RBCs for transfusion. It is important to note that PCR based assays are prone to different types of errors that those observed with hemagglutination assays. For instance, contamination with amplified products may lead to false positive test results. In addition, the identification of a particular genotype does not necessarily mean that the antigen will be expressed on the RBC membrane. Rev. bras. hematol. hemoter. 2004;**26**(2):135-140.*

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Introduction

Blood group antigens are polymorphisms of proteins and carbohydrates on the outside surface of the red blood cell (RBC) and are defined by serum alloantibodies produced in response to an immunizing event such as transfusion or pregnancy. It is the antibody that causes clinical problems in transfusion incompatibility, maternal-fetal incompatibility, and autoimmune hemolytic anemia.

The major risks of transfusions are unexpected incompatibility reactions[1] and the transmission of infectious agents. Iron overload and alloimmunization are also frequently observed among some categories of chronically transfused patients. Alloimmunization leads to an increased risk of transfusion reactions, reducing the available pool of compatible blood for transfusion in subsequent crises. Alloimmunization is the source of a variety of problems during long-term medical and transfusion management, with the main problem being the identification of appropriate antigen-negative RBCs for transfusion.[2]

Thus, in transfusion medicine, much time and effort are expended in detecting and identifying blood group antibodies. Next to ABO, the most clinically significant antibodies are those in the Rh, Kell, Duffy, and Kidd blood group systems.[3]

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Red blood cell phenotyping is essential to confirm the identity of suspected alloantibodies and to facilitate the identification of antibodies that may be formed in the future. Accurate antigen typing of transfused patients is often a difficult task due to the presence of donor RBCs in the patients' circulation. Thus, in these patients phenotyping can be time consuming and difficult to interpret. It is also complicated to type cells when a patient's RBCs have a positive direct antiglobulin test and no direct agglutinating antibody is available.

DNA technology led to the understanding of the molecular basis of many blood group antigens. The genes encoding 28 of the 29 blood group systems (only P remains to be resolved) have been cloned and sequenced,[4-7] which has permitted the elucidation of the molecular basis of many common blood group antigens. There are many molecular events that give rise to blood group antigens and phenotypes (Table 1), however, the majority of genetically defined blood group antigens are the consequence of a single-nucleotide polymorphism (SNP). (For current information regarding blood group antigen SNPs, the following website is recommended: www.bioc.aecom.yu.edu/bgmut/index.htm).This knowledge allows the use of DNA based-assays to detect specific blood group SNPs and that can be used to overcome the limitations of hemagglutination assays.[8,9]

Several assays for blood group genotyping of patients have recently been developed to predict the blood group antigen profile of an individual, with the goal of reducing risk or helping in the assessment of the risk of hemolytic disease of the newborn (HDN).[10-16] They include PCR-RFLP, allele-specific PCR, sequence-specific PCR as single or multiplex assays, real-time quantitative PCR.

These assays can be applied to blood group antigens to type patients who have recently received transfusions; to type patients whose RBCs are coated with immunoglobulin; to identify a fetus at risk for HDN; to determine which phenotipically antigen-negative patients can receive antigen-positive RBCs; to type donors for antibody identification panels; to type patients who have an antigen that is expressed weakly on RBCs; to determine *RHD* zygosity; to mass screen for antigen-negative donors; to resolve A, B, and D discrepancies.

Applications to transfusion medicine

1. Multiply-transfused patients

The ability to determine a patient antigen profile by DNA analysis when hemagglutination tests cannot be used is a useful adjunct to a serologic investigation. Blood group genotyping in the transfusion setting is recommended for multiply transfused patients, as part of antibody identification process.

Table 1
Molecular events that give rise to blood group antigens and phenotypes

- Gene conversion or recombination events (MNs, Rh, Ch/Rg blood group systems)
- Duplication of an exon (Gerbich)
- Deletion of a gene, exon, or nucleotide (s) (ABO, Rh, MNS, Kel,Duffy, Dombrock)
- Insertion of a nucleotide (s) (Rh, Colton)
- Single nucleotide substitutions (Most blood group systems)

The dilemma of using DNA obtained from transfused patients white blood cells (WBC) are that the donor leucocytes contained in the transfused units could, at least theoretically, interfere with genotyping results.[17-19] However, studies performed with patient WBC samples have shown that these cells can be used reliably to determine a blood group polymorphism by polymerase chain reaction (PCR)-based assays even when blood samples from recently transfused patients are used as source of DNA.[13-16] These studies demonstrate that post-transfusion blood samples can be safely used for genotyping blood groups.

Determination of a patient's blood type by analysis of DNA is particularly useful when a patient, who is transfusion-dependent, has produced alloantibodies.[3] This is because identification of the patient's probable phenotype allows the laboratory to determine to which antigens the patient can and cannot respond to make alloantibodies.

We have demonstrated the relevance of genotype determination of blood groups for the management of multiply transfused patients with diseases such as Sickle Cell Disease (SCD) and b-Thalassemia[15,16] by allowing the determination of the true blood group genotype, and by assisting in the identification of suspected alloantibodies and the selection of antigen-negative RBCs for transfusion. Furthermore, we have observed that taking the genotype into account enables a better selection of compatible units for patients with discrepancies between genotype and phenotype, leading to increased cell survival and a reduction of the transfusion frequency.

In addition to its contribution to the general accuracy of identification of red blood cell antigens, genotyping of transfusion-dependent SCD patients allows assessment of the risk of alloimmunization against antigens of the Duffy system due to regulation of antigen expression determined by the GATA-1 box. In the presence of the normal GATA-1 binding motif, phenotype and genotype agree, but when the GATA-1 motif is mutated, a pseudo-discrepancy is observed due to the absence of the *FY* gene expression in the erythroid lineage.[20,21] Thus, patients phenotyped as Fy(b–) who carry the mutated GATA box can be transfused

with Fy(b+) blood units with minimum risk of alloimmunization because the Duffy protein is expressed in other body tissues.[20,21]

2. Patients whose RBCs are coated with IgG

Patients with autoimmune hemolytic anemia (AIHA), whose RBCs are coated with IgG cannot be accurately typed for RBC antigens, particularly when directly agglutinating antibodies are not available, or IgG removal by chemical treatment of RBCs is insufficient. We have shown that blood group genotyping, is very important for determination of the true blood group antigens of these patients.[22] After genotyping, the patients received antigen-matched RBCs that had better in vivo survival, as assessed by rises in hemoglobin levels and diminished frequency of transfusions.

As a word of caution, we should emphasize that the interpretation of genotyping results must take into account the potential of contamination of PCR-based amplification assays and the observation that the presence of a particular genotype does not guarantee expression of this antigen on the RBC membrane.

The latter is illustrated by genes with a silencing mutation in a location other than that being analyzed (e.g., point mutation in the GATA box), a gene that is silenced by an alteration of a gene encoding protein with a modifying effect (e.g. Rh_{mod}, Rh_{null}), or the detection or failure to detect hybrid genes.[23-26]

The possibility to have an alternative to hemagglutination tests to determine the patient's antigen profile should be considered for multiply transfused patients and for patients with AIHA by allowing the determination of the true blood group genotype and by assisting in the identification of suspected alloantibodies and in selection of antigen-negative RBCs for transfusion. This ensures a more accurate selection of compatible donor units and is likely to prevent alloimmunization and reduce the potential for hemolytic reactions.

3. Blood donors

DNA-based typing can also be used to antigen-type blood donors both for transfusion and for antibody identification reagent panels. This is particularly useful when antibodies are not available or are weakly reactive. A good example is the Dombrock blood group polymorphism where DNA-based assays are used to type patients and donors for Do^a and Do^b to overcome the dearth of reliable typing reagents.[27,28] Furthermore, the newer technologies have the potential to screening pools of DNA for rare blood types. The molecular analysis of a variant gene can also assist in resolving a serologic investigation.[29]

As automated procedures attain higher and faster throughput at lower cost, blood group genotyping is likely to become more widespread. We believe that the PCR technology may be used in a transfusion service in the next few years to overcome the limitations of hemagglutination.

4. Resolution of weak A, B, and D typing discrepancies

A proportion of blood donors and patients who historically have been typed as group O are now being recognized as group A or group B with the use of monoclonal antibodies capable of detecting small amounts of the immuno-dominant carbohydrate responsible for A or B specificity. A typing result that differs from the historical record often results in time-consuming analyses. Since the bases of many of the weak subgroups of A and B are associated with altered transferase genes, PCR-based assays can be used to define the transferase gene and thus the ABO group.[30]

Similarly with the D antigen of the Rh blood group system, a proportion of blood donors that historically have been typed as D-negative are now reclassified as D-positive, due to monoclonal reagents that detect small and specific parts of the D antigen. The molecular basis of numerous D variants can be used to identify the genes encoding altered RhD protein in these individuals.[31-33]

Applications to maternal-fetal medicine

Alloimmunization against the RhD antigen during pregnancy is the most frequent cause of hemolytic disease of the newborn (HDN). Immunization occurs when fetal cells, carrying antigens inherited from the father, enter the mother's circulation following fetal-maternal bleeding. The mother, when not expressing the same antigen(s), may produce IgG antibodies towards the fetal antigen and these antibodies can pass through the placenta causing a diversity of symptoms, ranging from mild anemia to death of the fetus. Apart from antibodies to the RhD blood group antigen, other specificities within the Rh system and several other blood group antigens can give rise to HDN, but RhD is by far the most immunogenic.[34]

Prenatal determination of fetal RhD status is desirable in pregnancies to prevent sensitization and possible hydrops foetalis in fetuses of RhD negative mothers with RhD positive fathers. Fetal DNA has been detected in amniotic cells, chorionic villus samples, and as recently reported, in maternal plasma.[35] It is now well accepted that a minute number of copies (as low as 35 copies/mL) of cell-free fetal *RHD* DNA in the maternal plasma[36] can be utilized as a target for non-invasive genotyping of the fetus. Unlike fetal DNA isolated from the cellular fraction of maternal blood samples (where micro-chimerism has been shown to persist for decades),[37] free fetal DNA isolated from maternal plasma has been shown to be

specific for the current fetus and is completely cleared from the mother's circulation by post partum.[38]

It has been reported that fetal *RHD* can be determined by PCR in DNA extracted from maternal plasma of pregnant women with RhD positive fetuses, in a non-invasive procedure.[39-44] PCR amplification of *RHD* in maternal plasma may be useful for the management of RhD negative mothers of RhD positive fetuses and for the study of fetus-maternal cell trafficking.

There are, however, several challenges in fetal *RHD* genotyping due to the high complexity of the Rh system. This complexity with more than 50 registered variants[45] is partly a result of the presence of two genes (*RHD* and *RHCE*) that share 96.8% homology at the nucleotide level, and molecular elucidation of the Rh phenotypic variants indicates that a substantial proportion of the variants are caused by homologous recombination between the two genes.[45] Additional point mutations add to the complexity. Gene conversion between two homologous flanking regions of the *RHD*-gene is the proposed mechanism behind the most common RhD-negative phenotype where the *RHD* gene is deleted.[45] A major problem for *RHD* genotyping is the presence of an *RHD* pseudogene (*RHD*Y) in Africans with an RhD-negative phenotype.[46] *RHD*Y needs to be considered in PCR-based genotyping to avoid a false-positive result.

These complexities highlight the importance of examining more than one exon of the *RHD* gene when attempting antenatal genotyping.[46,47] Adding to the difficulty of being certain whether the detected genotype is representative for the phenotype is the need for a fetal-specific DNA control to confirm the presence of fetal DNA when the fetus is *RHD* negative. The amount of free DNA is obviously a limiting factor for fetal *RHD* genotyping in maternal blood. For the fetus, the consequence of a false-negative *RHD* determination may be more severe than a false positive result, as the latter would only result in unnecessary prophylaxis. Fetal DNA obtained from maternal plasma is unstable (even at 4 ºC) and samples to be analyzed should be frozen.[48] Large-scale clinical trials and standardization of protocols are still required; however, it is likely that determination of fetal *RHD* type using this noninvasive procedure, will become routine clinical practice.

Before interpreting the results of DNA analysis, it is important to obtain an accurate medical history and to establish if the study subject is a surrogate mother, if she has been impregnated with non-spousal sperm.

The discovery of fetal DNA in maternal plasma has opened up new and exciting possibilities for the non-invasive prenatal determination of fetal blood group status. However, a number of technical issues still need to be addressed and large scale multicenter clinical trials need to be carried out. When these issues are resolved, it is likely that the prenatal testing of fetal blood group type will be carried out routinely and safely.

Conclusions

The determination of blood group polymorphism at the genomic level facilitates the resolution of clinical problems that cannot be addressed by hemagglutination. They are useful to (a) determine antigen types for which currently available antibodies are weakly reactive; (b) type patients who have been recently transfused; (c) identify fetuses at risk for hemolytic disease of the newborn; and (d) to increase the reliability of repositories of antigen negative RBCs for transfusion.

It is important to note that PCR based assays are prone to different types of errors than those observed with hemagglutination assays. For instance, contamination with amplified products may lead to false positive test results. In addition, the identification of a particular genotype does not necessarily mean that the antigen will be expressed on the RBC membrane.

Analysis of *RHD* gene has shown that there are several mechanisms that silence the expression of D antigen.[45,47,49] In D-negative people of European descent, the most common mechanism is deletion of the entire *RHD* gene that is thought to have arisen by recombination of the two flanking Rheus boxes to form a hybrid box with subsequent deletion of the intervening sequence.[47] However, the D-negative phenotypes of people of African and Asian ancestry result from a number of different molecular backgrounds of which gene deletion is just one. These include the insertion of a 37 bp nucleotide sequence at the introns 3/exon 4 boundary which alters the open read frame and encodes a premature stop codon,[45] the presence of a *D-CE-D* hybrid that encodes a variant C antigen, VS, c and e antigens but no D antigens.[45,50,51]

The available wealth of serologically defined variants has contributed to the rapid rate with which the genetic diversity of blood group genes is revealed. Initially, molecular information associated with each variant was obtained from only a small number of samples and applied to DNA analysis 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 RBC phenotype being apparently discrepant.[3]

A large number of people from a variety of ethnic backgrounds need to be studied to determine the occurrence of particular genotypes and to establish more firmly the correlation between blood group genotypes and phenotypes.

Hemagglutination has identified many phenotypic variants encoded by *RHD*, *RHCE*, or hybrids of the two and molecular analysis has revealed remarkable variation

within the variants. Numerous partial D and weak D phenotypes have been defined at the molecular level[46] and this information, together with clinical and serologic data, will be used to guide transfusion and testing policy for patients and donors. Many new ABO alleles have been defined[52] and ABO genotyping is a useful clinical tool for resolution of typing discrepancies.

Advances are ongoing in the automation of SNP and DNA sequence analysis and the success of sequencing the human genome has shown the potential for phenotyping large numbers of samples.[53] It should soon be possible to analyze major and many minor blood group alleles on a single synthetic chip. Indeed, it will be possible to test for many genetically defined conditions.

The identification of the molecular basis of blood group antigens provides an insight into the generation of gene diversity in humans. A molecular understanding of blood groups has enabled the design of simple assays that may be used to facilitate the provision of blood to patients who require antigen-matched red cells, both by phenotyping the patient to determine his/her requirements and by phenotyping red cell units.

Transfusion-dependent patients who have sickle cell disease, thalassemias and aplastic anemias frequently become alloimmunized. Blood group genotyping contributes substantially to the safety of blood transfusion in these recipients. Although it is unlikely that molecular genotyping will replace hemagglutination any time in the near future, together these techniques have substantial value in the resolution of clinical laboratory problems, and consequently in the quality of patient care.

Resumo

*Os antígenos eritrocitários são herdados geneticamente e definidos por seqüências de aminoácidos específicos constituindo uma proteína ou por carboidratos ligados a estas proteínas ou à lipídios. A diversidade dos antígenos de grupos sangüíneos, como para qualquer outro traço biológico, encontra-se ao nível do gene. Existem atualmente mais de 250 antígenos eritrocitários que se encontram distribuídos em 29 sistemas de grupos sangüíneos, de acordo com a Nomenclatura da Sociedade Internacional de Transfusão Sangüínea (ISBT). Os genes que codificam 28 dos 29 sistemas de grupos sangüíneos já foram clonados e seqüenciados. Assim, os problemas que ainda não são resolvidos por testes sorológicos podem ser agora solucionados por técnicas moleculares. O objetivo desta revisão é apresentar os mecanismos moleculares responsáveis pelo aparecimento dos antígenos associados aos fenótipos de grupos sangüíneos; mostrar a aplicação da técnica de PCR na medicina transfusional e materno-fetal, e discutir os problemas clínicos que potencialmente podem ser resolvidos pelas técnicas moleculares. A possibilidade de realizar genotipagem de grupos sanguíneos em conjunto com hemaglutinação muda a gama de possibilidades nos procedimentos transfusionais aumentando assim a segurança dos pacientes transfundidos. No entanto, é importante lembrar que a detecção de um gene através das técnicas de genotipagem molecular não significa necessariamente que a proteína carreando o antígeno será expressa. Assim, resultados falso-negativos e falso-positivos podem ocorrer. Embora seja pouco provável que a genotipagem molecular venha a substituir a hemaglutinação nos próximos anos, estas técnicas utilizadas em conjunto têm um valor potencial importante na segurança transfusional e materno-fetal. Rev. bras. hematol. hemoter. 2004;**26**(2):135-140.*

Palavras-chave: *Antígenos de grupos sangüíneos; DNA; hemaglutinação; medicina transfusional; medicina materno-fetal.*

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