# Immunohematology

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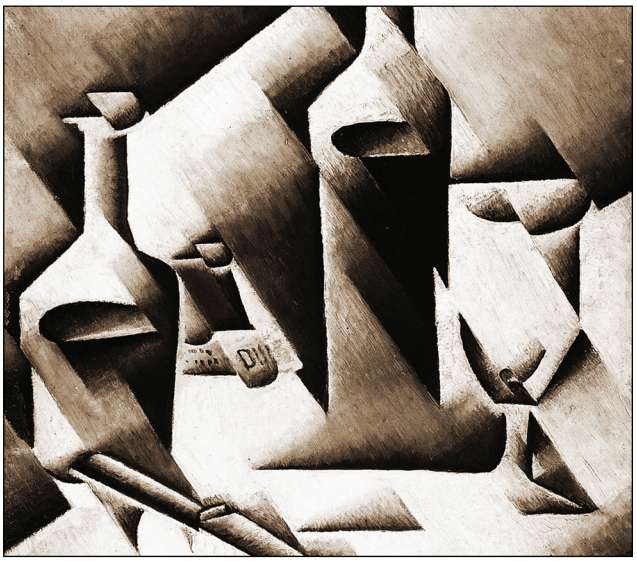

**On Our Cover**
"Bottles and Knife" 1911–1912, Juan Gris

Along with Pablo Picasso, Juan Gris was one of the early innovators of Cubism, an avant garde movement in the visual arts. In a radical break from traditional representation, cubist paintings evinced a radical vision and a remarkable proliferation of perspective in which the surfaces and lines of a painting's subject were distorted, multiplied, and flattened from a cohesive whole into manifold planes. In a similar way, advances in immunohematology elucidating the structure and function of red blood cell surface molecules have led to simultaneous, coexistent models with diverse planes of understanding—biochemical, antigenic, molecular, genetic, genomic, and microbiologic. Like the Gris painting, the Duffy glycoprotein represents a break from the traditional view of the antigen-antibody perspective as it is also a receptor for multiple chemokines and acts as the portal of entry for *Plasmodium vivax*.

——David Moolten, MD

# Consortium for Blood Group Genes (CBGG): 2009 report

G.A. Denomme, C.M Westhoff, L.M. Castilho, M. St-Louis, V. Castro, and M.E. Reid

The Consortium for Blood Group Genes is a worldwide organization whose goal is to have a vehicle to interact, establish guidelines, operate a proficiency program, and provide education for laboratories involved in DNA and RNA testing for the prediction of blood group, platelet, and neutrophil antigens. Currently, the consortium operates with representatives from Brazil, Canada, and the United States. Membership is voluntary with the expectation that members actively contribute to discussions involving blood group genetics. This year witnessed a change in the standing committee membership and the institution of a representative for the human platelet antigens group. Looking forward, the consortium sees challenges for the nomenclature of blood group alleles and user-required specifications for laboratory information systems to store genotype information. ***Immunohematology 2010;26:47–50.***

**Key Words:** blood group alleles, Consortium for Blood Group Genes, proficiency, target alleles

The Consortium for Blood Group Genes (CBGG) is a not-for-profit organization established in 2004 by a group of like-minded people with scientific or industry experience and interest in the field of the genetics of red cell, platelet, and neutrophil antigens, collectively known as blood group genetics. The mission of the consortium is "to establish guidelines, to provide education, and to provide a proficiency exchange for laboratories involved in DNA or RNA testing for the determination of blood group, platelet, and neutrophil antigens." The consortium is coordinated by three country representatives: Lilian Castilho (Hemocentro de Campinas) for Brazil, Maryse St-Louis (Héma-Québec) for Canada, and Connie Westhoff (American Red Cross) for the United States, with Marion Reid (New York Blood Center) as facilitator and Greg Denomme (BloodCenter of Wisconsin) as secretary. Vagner Castro is coordinator for the platelet group. Members are encouraged to refer to the published CBGG articles for background information and progress of the consortium.[1–5] Exchange of information is mainly accomplished through electronic mailings and a yearly meeting, with proficiency evaluation exercises occurring in the spring and fall of each year.

## The CBGG Document

The *CBGG Document* is the sole information document for members, is made available by electronic transfer, and outlines the function of the CBGG. This document contains information on the structure, organizational rules and bylaws, regulatory compliance plan, preferred terminology, and progress on the working parties including a summary of the proficiency exchange program (from 2007 to date), guidelines of practice, DNA repository, funding, forms and disclaimers, and proposed Web site. It is highly recommended that CBGG members refer to this document for the aforementioned duties and activities. A meeting is held annually, in part for members to discuss outstanding issues, to provide input, to accept amendments, and to summarize the proficiency evaluations.

## Regulatory Affairs

CBGG members continue to recognize the importance of appropriately worded reports, including the use of disclaimers, of molecular analyses for both blood donors and transfusion recipients. Data from molecular testing continue to be a source of information for the resolution of complex serologic problems, and presently are not intended as the sole means for patient transfusion management decisions.

As an international consortium, CBGG does not provide guidance on regulatory affairs; members are responsible for knowledge of and compliance with the regulations in their own countries. For US members, we bring to your attention the Code of Federal Regulations (CFR) Part 864—Hematology and Pathology Devices, Subpart E—Specimen Preparation Reagents; Sec. 864.4020 Analyte specific reagents.

## Allele Nomenclature

The list of target alleles was adopted by the CBGG in 2007 and updated in 2009. The preferred current terminology is listed under the heading "Target antigen (target allele)" in Table 1 of this report. However, the final naming of alleles awaits the decision of the International Society for Blood Transfusion. When referring to a particular single nucleotide change, it is important to follow the designated notation, e.g., 125G>A, with intronic nucleotide changes represented in the lower case, e.g., –67t>c. The associated amino acid substitutions flank the designated position number, e.g., Pro103Ser or P103S. Because gene numbering systems vary, and to avoid ambiguity in the location of nucleotide changes, the CBGG has adopted GenBank gene reference sequences (RefSeqGene) and reference SNP numbers (rs#) for blood group genes and nucleotides (Table 1). These numbers refer to a set of common documents used to communicate or report molecular testing results and are linked to other GenBank reference files.

**Table 1.** List of targets and recommended controls for prediction of certain RBC antigens†

| ISBT system name (symbol) number | Target antigen (target allele) | ISBT gene name (RefSeqGene) | Target nucleotide (SNP rs#)‡ | Controls and comments (monitor assay performance with DNA controls known to be homozygous and heterozygote for both alleles unless otherwise noted) |
|---|---|---|---|---|
| ABO (ABO) 001 | A (*ABO*A1*) | *ABO* (NG_006669.1) | consensus | Targets for nondeletional (261ΔG) ABO*O alleles are not listed. Various alleles have been described and multiple targets or sequencing is required for identification. |
| | A2 (*ABO*A2*) | | nt 1061ΔC (rs56392308) | |
| | B (*ABO*B1*) | | nt 526C>G (rs7853989)<br>nt 703G>A (rs8176743)<br>nt 796C>A (rs8176746)<br>nt 803G>C (rs8176747) | |
| | O (*ABO*O1*) | | nt 261G/ΔG (rs8176719) | |
| MNS (MNS) 002 | M (*GYPA*M*)<br>N (*GYPA*N*) | *GYPA* (NG_007470.2) | nt 59C>T;71G>A;72T>G (rs7682260;7687256; 7658293) | Position 72 is the 3rd nt of codon 24 (not for clinical use). |
| | S (*GPB*S*)<br>s (*GPB*s*) | *GYPB* (NG_007483.1) | nt 143T>C (rs7683365) | |
| | S silenced | | nt 230C>T, intron 5+5g>t | 230T/T not required, I5+5t/t not required |
| Rh (RH) 004 | D | *RHD* (NG_007494)<br>*RHDΨ* | Exon 4 and 7<br>Exon 4 37-bp insert | Targets may vary as there are many approaches. |
| | C (*RHCE*C*)<br>c (*RHCE*c*) | *RHCE* (NG_009208) | intron 2 insertion<br>nt 307T>C (rs676785) | |
| | E (*RHCE*E*)<br>e (*RHCE*e*) | | nt 676C>G (rs609320) | |
| | $C^w$ | | nt 122A>G | 122G/G not required |
| | $C^x$ | | nt 106G>A | 106A/A not required |
| | V & VS | | nt 733C>G (rs1053361) | 733G/G not required |
| | V (VS−) | | nt 1006G>T | 1006T/T not required |
| Lutheran (LU) 005 | $Lu^a$ (*LU*01* or *LU*A*)<br>$Lu^b$ (*LU*02* or *LU*B*) | *LU* (NG_007480.1) | nt 230A>G (rs28399653) | |
| Kell (KEL) 006 | K (*KEL*01*)<br>k (*KEL*02*) | *KEL* (NG_007492.1) | nt 578T>C (rs8176058) | |
| | $Kp^a$ (*KEL*03*)<br>$Kp^b$ (*KEL*04*) | | nt 841T>C (rs8176059) | |
| | $Js^a$ (*KEL*06*)<br>$Js^b$ (*KEL*07*) | | nt 1790C>T (rs8176038) | |
| Duffy (FY) 008 | $Fy^a$ (*FY*01* or *FY*A*)<br>$Fy^b$ (*FY*02* or *FY*B*) | *FY* (NG_011626.1) | nt 125G>A (rs12075) | |
| | $Fy^x$ (*FY*265T*) | | nt 265C>T (rs34599082) | 265T/T not required |
| | Fy null (RBC) | | nt -67t>c (rs2814778) | GATA nucleotide change |
| Kidd (JK) 009 | $Jk^a$ (*JK*01* or *JK*A*)<br>$Jk^b$ (*JK*02* or *JK*B*) | JK (NG_011775.1) | nt 838G>A (rs1058396) | Testing for nulls may be appropriate in some situations. |
| Diego (DI) 010 | $Di^a$ (*DI*01* or *DI*A*)<br>$Di^b$ (*DI*02 or DI*B*) | *DI* (NG_007498.1) | nt 2561T>C (rs2285644) | |
| Yt (YT) 011 | $Yt^a$ (*YT*01* or *YT*A*)<br>$Yt^b$ (*YT*02* or *YT*B*) | *YT* (NG_007474.1) | nt 1057C>A (rs1799805) | |
| Scianna (SC) 013 | Sc1 (*SC*01*)<br>Sc2 (*SC*02*) | *SC* (NG_008749.1) | nt 169G>A (rs56025238) | 169A/A not required |
| Dombrock (DO) 014 | $Do^a$ (*DO*01* or *DO*A*)<br>$Do^b$ (*DO*02* or *DO*B*) | *DO* (NG_007477.1) | nt 793A>G (rs11276) | |
| | Hy (*HY*) | | nt 323G>T (rs28362797) | |
| | $Jo^a$ (*JO*) | | nt 350C>T (rs28362798) | |
| Colton (CO) 015 | $Co^a$ (*CO*01* or *CO*A*)<br>$Co^b$ (*CO*02* or *CO*B*) | *CO* (NG_007475.1) | nt 134C>T (rs28362692) | 134T/T not required |
| Landsteiner-Wiener (LW) 016 | $LW^a$ (*LW*05* or *LW*A*)<br>$LW^b$ (*LW*07* or *LW*B*) | *LW* (NG_007728.1) | nt 308A>G | 308G/G not required |
| Cromer (CR) 021 | $Cr^a$ (*CR*01* or *CR*A*) | *CROM* (NG_007465.1) | nt 679G>C (rs60822373) | |
| Knops (KN) 022 | $Kn^a$ (*KN*01* or *KN*A*)<br>$Kn^b$ (*KN*02* or *KN*B*) | *KN* (NG_007481.1) | nt 4681G>A (rs41274768) | |
| | $McC^a$ (*KN*03*)<br>$McC^b$ (*KN*06*) | | nt 4768A>G (rs17047660) | 4768G/G not required |
| | $Sl^a$ (*KN*04*)<br>Vil (*KN*07*) | | nt 4801A>G (rs17047661) | 4801G/G not required |
| Indian (IN) 023 | $In^a$ (*IN*01* or *IN*A*)<br>$In^b$ (*IN*02* or *IN*B*) | *IN* (NG_008937.1) | nt 252C>G | |
| OK (OK) 024 | $Ok^a$ (*OK*01* or *OK*A*) | *OK* (NG_007468.1) | nt 274G>A | Homozygous mutated and heterozygote not required |

†Predicted antigen negativity should be confirmed by hemagglutination with licensed reagents if available, with unlicensed reagents if available, or by a crossmatch performed by the laboratory issuing the product to the patient.
‡Numbering of nucleotide (nt) is based on "A" of AUG

## Guidelines for Molecular Testing

The CBGG published ISO format guidelines for molecular testing for blood groups in 2007. The AABB standards for molecular testing for red cell, platelet, and neutrophil antigens was published in 2008.[6,7] AABB is currently assembling accreditation guidelines and training accreditors to certify laboratories that perform molecular testing for red cell, platelet, and neutrophil antigens. The intent of the CBGG is to remain as an independent forum and voice for molecular testing guidelines in ISO format for use by international laboratories. The members will update, modify, or otherwise amend the CBGG guidelines by process of discussion and consensus. The CBGG guidelines will not become standards as such to reflect the fact that the CBGG is not responsible for laboratory inspection or accreditation.

## Proficiency Program

As of spring 2008, the alleles of RBC antigens for proficiency exchanges are currently restricted to *RHCE*E/RHCE*e, GYPB*S/GYPB*s, KEL*1/KEL*2, FY*A/FY*B, FY*–67C/T GATA*, and *JK*A/JK*B*. The cost of sample preparation and shipping the DNA sample is borne in turn by each submitting laboratory. To participate in the sample exchange, proficiency program members must agree to provide a sample for distribution in a subsequent year through a predetermined rotation. Although participation in the CBGG proficiency program mandates that samples be discarded after the results have been validated, proficiency program members must ensure they comply with the requirements of regulatory bodies. Thus, before joining the proficiency exchange program and committing to supplying a sample for the exchange, new members should address their institutional requirements on informed consent. To prevent communication errors caused by different reporting mechanisms, a report form has been developed specifically for the CBGG proficiency program, a copy of which is provided in Figure 1 with examples. The proficiency evaluations for platelets and neutrophils are distributed among a group of members headed by Vagner Castro (Platelet Immunology Laboratory of Hematology and Hemotherapy Center of the State University of Campinas, UNICAMP, Campinas, São Paulo, Brazil). Serologic confirmation is not mandated by this group because of the lack of regulated antisera.

## Electronic Data Records and Databases

Members of the CBGG have recognized the need to provide manufacturers of electronic laboratory information systems (LIS) with appropriate input as they make decisions on changes to their operating systems. Presently, LIS do not have place-holder fields for genotype results or the capability to compare the DNA test result to the phenotype when available. Neither do algorithms exist to make phenotype predictions from the DNA test results. The CBGG members also discussed that a position paper from the CBGG could be developed to outline what users desire from manufacturers of DNA testing platforms and what nucleotide targets are needed to define a predicted antigen phenotype. It was recommended that a focus group be assembled to address LIS user requirements.

**CBGG Proficiency Exchange Program Result Form**

**NOTE: Report results on this Form and use the nucleotide designation from "List of Targets…. " in the current CBGG Document.**

**Date sent:** _____ **Date due:** _____

| | Originating Laboratory: | Receiving Laboratory: |
|---|---|---|
| Name: | | |
| Address: | | |
| Phone #: | | |
| Fax #: | | |

**DNA Sample ID:** _____ **Molecular Assay Requested:** [*JK*A/B* or *JK*01/02*]

**Receiving (Testing) Laboratory:** **Date sample arrived:** _____

| DNA Method | DNA Result (Genotype) | Predicted Phenotype | Technologist Date | Supervisor Date |
|---|---|---|---|---|
| [AS-PCR] | [*JK*A/JK*B*] | [Jk(a+b+)] | [Signature] | [Signature] |
| | | | [Date] | [Date] |

**Comments/Disclaimers:**

**Originating (Verification) Laboratory:**
**Acceptable response:** _____

| DNA Method | DNA Result (Genotype) | Actual Phenotype | Reviewed by: Date |
|---|---|---|---|
| [PCR-RFLP] | [*JK*A/JK*B*] | [Jk(a+b+)] | [Name] |
| | | | [Date] |

**Comments/Disclaimers:**

**Acknowledgement of verified results:** _____

Originating Laboratory: Complete all information for the receiving laboratory to perform testing.
Testing Laboratory: Fill in testing information; sign and date. Fax form to submitting laboratory.
Submitting Laboratory: Fill in results of submitting laboratory, sign and date. Fax verification to testing lab. NOTE: Discard DNA as soon as verification is received

**Fig. 1.** Example of the CBGG proficiency exchange program result form.

## Conclusions

The CBGG is a self-help, not-for-profit organization designed as an interactive collaborative for members to learn from each other and to strive to achieve excellence in molecular testing of blood group, platelet, and neutrophil antigens. Anyone interested and willing to contribute intellectually is welcome to join. Important information on analyte specific reagents in the context of laboratory-developed tests, decisions on the specifications of information systems, and the appropriate targets for molecular testing are important topics for discussion in 2010.

**Acknowledgments**

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**Manuscripts**

The editorial staff of *Immunohematology* welcomes manuscripts pertaining to blood group serology and education for consideration for publication. We are especially interested in case reports, papers on platelet and white cell serology, scientific articles covering original investigations, and papers on new methods for use in the blood bank. Deadlines for receipt of manuscripts for consideration for the March, June, September, and December issues are the first weeks in November, February, May, and August, respectively. For instructions for scientific articles, case reports and review articles, see Instructions for Authors in every issue of *Immunohematology* or on the Web at www.redcross.org/immunohematology. Include fax and phone numbers and e-mail address with all articles and correspondence. E-mail all manuscripts to immuno@usa.redcross.org.

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# The Duffy blood group system: a review

G.M. Meny

Duffy was the first blood group mapped to an autosome (chromosome 1) using cytogenetic studies. Duffy antigens are located on a glycoprotein that can be found on erythrocytes and other cells throughout the body. $Fy^a$ and $Fy^b$ are products of their respective alleles (*FY*A, FY*B*). $Fy^x$, characterized by weak $Fy^b$ expression, is a result of an additional mutation in *FY*B*. The Fy(a–b–) phenotype, most commonly found in Blacks, occurs primarily as a result of a GATA promoter region mutation upstream of the *FY* allele. This mutation prevents expression of Duffy glycoprotein on erythrocytes only, while permitting expression on nonerythroid cells. Other antigens include Fy3, Fy5, and Fy6. Antibodies to Duffy antigens are usually clinically significant and have been reported to cause hemolytic disease of the fetus and newborn. This review provides a general overview of the Duffy blood group system, including the role of the Duffy glycoprotein as a chemokine receptor (Duffy antigen receptor for chemokines) and in malarial infection. ***Immunohematology 2010;26:51–56.***

**Key Words:** Duffy antigen receptor for chemokines, DARC, *FYA, FYB*

## History

The initial description related to the Duffy blood group system was published in 1950 when anti-$Fy^a$ was observed during an investigation of a hemolytic transfusion reaction.[1,2] The antibody was described in a 43-year-old group O, D– individual with hemophilia who received a 3-unit transfusion for treatment of an episode of spontaneous bruising and bleeding. The transfusions were followed by rigors. Jaundice developed the day after transfusion. Investigation revealed an antibody that was detected only by the IAT and was named anti-Duffy (anti-$Fy^a$) after the patient. An $Fy^a$ phenotype frequency of 64.9 percent was calculated, and gene frequencies for both $Fy^a$ and the hypothetical $Fy^b$ were described.

One year later, anti-$Fy^b$ was discovered by Ikin et al.[3] in a patient 2 days after the birth of her third child. None of the children were noted to show signs of HDN. Antibody investigations demonstrated that stronger reactions were observed when the antibody was tested in the presence of albumin than of saline and at 37°C than at room temperature. Confirmation of a previously calculated $Fy^b$ phenotype frequency was noted. Of interest, the authors speculated on the possibility of a rare third allele, which would react with neither antibody.

Duffy was the first blood group locus to be assigned to an autosome (a nonsex chromosome). Investigators performed linkage analysis between serologic blood typing results and cytogenetic studies on four families, one of which was a three-generation family.[4] The Duffy locus segregated with the uncoiler (*Un)* locus on chromosome 1 in three family studies and an inversion of chromosome 1 in one family study. The *Un* locus causes chromosome 1 to have an unusual lengthy appearance when viewed in metaphase. Describing a family with an inversion break point provided additional evidence for assigning the Duffy locus to chromosome 1.

## Genetics and Inheritance

Both *FY* and *RH* gene loci reside on chromosome 1. However, the *FY* locus is located on the long arm at position 1q22→ q23, whereas *RH* resides on the short arm.[5] $Fy^a$ and $Fy^b$ are antithetical antigens produced by codominant alleles, *FYA* and *FYB*. Four phenotypes are defined by the corresponding antibodies, anti-$Fy^a$ and anti-$Fy^b$ (see Table 1). The Duffy system antigens are listed in Table 2.

**Table 1.** Duffy phenotypes, prevalence, and inherited alleles

| Red cell phenotype | Prevalence % | | Allele |
|---|---|---|---|
| | Caucasians | Blacks | |
| Fy(a+b–) | 20† | 10† | *FY*01/FY*01* or *FY*A/FY*A* |
| Fy(a–b+) | 32 | 20 | *FY*02/FY*02* or *FY*B/FY*B* |
| Fy(a+b+) | 48 | 3 | *FY*A/FY*B* |
| Fy(a–b–) | Very rare | 67 | *FY*/N.01–05, FY*/N.01–02*‡ |

†Present in 70–90% of some Asian populations.
‡Nomenclature pending approval by the ISBT Working Party on Terminology for Red Cell Surface Antigens
Table modified from Daniels.[6,7]

**Table 2.** Duffy antigens[7]

| Antigen | ISBT symbol | ISBT no. |
|---|---|---|
| $Fy^a$ | FY1 | 008001 |
| $Fy^b$ | FY2 | 008002 |
| $Fy^3$ | FY3 | 008003 |
| $Fy^4$ | FY4 | 008004 |
| $Fy^5$ | FY5 | 008005 |
| $Fy^6$ | FY6 | 008006 |

The Fy(a–b–) phenotype is the major phenotype in Blacks, but is very rarely found in Caucasians. The phenotype found in Blacks is characterized by the presence of $Fy^b$ antigen on nonerythroid cells, but an absence of the $Fy^b$ antigen on RBCs.[8] A mutation in the erythroid promoter GATA-1 binding motif explains why Fy(a–b–) individuals do not make anti-$Fy^b$ (see Molecular section). The Fy(a–b–) phenotype found in Caucasians is characterized by a lack of

Duffy antigen expression in both erythroid and nonerythroid tissues. Different mutations are present in either the *FYA* or *FYB* gene, which prevent the Duffy protein from being formed. These individuals, interestingly, tend to form anti-Fy3.[9–11]

Other alleles have been reported at the *FY* locus. The $Fy^x$ phenotype is associated with weak expression of $Fy^b$, Fy3, and Fy5 antigens. Chown et al.[12] first reported the $Fy^x$ gene and estimated the phenotype frequency in a Caucasian population was not more than 2 percent. It is now known that $Fy^x$ is caused by a point mutation in the *FYB* gene.[13,14]

## Antibodies in the System

### Anti-$Fy^a$ and -$Fy^b$

Anti-$Fy^a$ and -$Fy^b$ are found after transfusion or, less frequently, as a result of pregnancy. They are rarely naturally occurring. Duffy antibodies are predominantly of the IgG1 subclass, and 50 percent of anti-$Fy^a$ examples bind complement. Anti-$Fy^b$, identified about 20 times less frequently than anti-$Fy^a$, is usually present in sera with other alloantibodies.[15] Both antibodies cause immediate and delayed hemolytic transfusion reactions.[16] When Fy(a–b–) Black individuals develop Duffy antibodies, they usually produce anti-$Fy^a$, which may be followed by anti-Fy3 or anti-Fy5.[17,18] Anti-$Fy^b$ is not produced.

With regard to hemolytic disease of the fetus and newborn (HDFN), anti-$Fy^a$ was identified in 5.4 percent of atypical alloantibodies in a group of women receiving obstetric care at a tertiary-care center. Of antibodies capable of causing HDFN, Kell blood group antibodies were identified most frequently (22%). In contrast, anti-$Fy^b$ was infrequently identified (0.2%). This compares with 0.5 percent to 3.1 percent of Duffy system antibodies detected in four other series of obstetric patients.[19]

Hughes et al.[20] reviewed the clinical outcome of 18 pregnant women between 1959 and 2004 in whom anti-$Fy^a$ was the only alloantibody identified and the fetus was Fy(a+). Significant HDFN was identified in 2 of 18 (11%) pregnancies, resulting in exchange transfusion or intrauterine transfusion. Maximum serum titers in these cases were 32 and 128. Hydrops fetalis was not identified in any fetus, and no deaths attributable to HDFN were reported. A rare case of HDFN caused by anti-$Fy^b$ has been reported.[21]

### Anti-Fy3, -Fy4, -Fy5, and -Fy6

Anti-Fy3 was first described by Albrey et al.[9] in a Caucasian individual who was pregnant with her third child. The authors noted that this antibody's reactivity "suggests that the Duffy system is more complicated than it seemed before." Anti-Fy3 was unique in that it reacted with enzyme-treated Fy(a+) or Fy(b+) RBCs, but failed to react with Fy(a–b–) RBCs from Black individuals. Clinically, the baby was reported to have mild HDFN (weakly positive DAT), but no treatment was required.

Subsequent reports of anti-Fy3 have also been described in Black individuals during investigation of acute or delayed hemolytic transfusion reactions.[17,22,23] Of interest, Vengelen-Tyler[17] noted that anti-Fy3 developed after anti-$Fy^a$ in individuals receiving multiple RBC transfusions for treatment of sickle cell disease, and Olteanu et al.[22] reported a case of an acute hemolytic transfusion reaction caused solely by anti-Fy3 in an 8-year-old Black individual treated for repair of a femoral neck fracture.

The only example of anti-Fy4 was described by Behzad et al.[24] in a 12-year-old patient with sickle cell disease. This antibody appeared to react with Fy(a–b–), some Fy(a+b–) or Fy(a–b+), but no Fy(a+b+) RBCs. However, the existence of this antibody is in doubt owing to the lack of consistent test results between laboratories and sample instability on storage and shipment.

Colledge et al.[25] reported the first example of anti-Fy5 in an 11-year-old Fy(a–b–) Black individual who died of acute leukemia shortly after the antibody was discovered. Like anti-Fy3, anti-Fy5 reacted with enzyme-treated Fy(a+) or Fy(b+) RBCs. No reactivity was seen with Fy(a–b–) RBCs from Black individuals, or $Rh_{null}$ cells with normal expression of $Fy^a$ and $Fy^b$ antigens. One Fy(a–b–) RBC sample from a Caucasian individual was positive. Anti-Fy5 is reported to cause delayed hemolytic transfusion reactions in patients with sickle cell disease who develop this antibody in conjunction with other blood group antibodies such as anti-$Fy^a$,[17,26] and anti-K, -E, and -C.[27]

No human anti-Fy6 has been identified. Monoclonal antibodies have been raised against Fy6 epitopes, as well as other Duffy blood group epitopes.[28–30]

## Biochemistry

The Duffy protein is composed of 336 amino acids. The numbering of amino acids (and nucleotides) has varied because two kinds of Duffy mRNA have been described: a less abundant form, that was the first to be discovered and cloned, encodes a protein of 338 amino acids whereas the more abundant form encodes a protein of 336 amino acids and is the form that is represented in Figure 1.[31,32] The Duffy protein is likely organized in the RBC membrane as an *N*-glycosylated protein that spans the membrane seven times (Fig. 1). $Fy^a$ and $Fy^b$ differ by a single amino acid change at position 42 on the extracellular domain, with glycine resulting in $Fy^a$ expression and aspartic acid resulting in $Fy^b$ expression.[5,33] Both $Fy^a$ and $Fy^b$ are sensitive to destruction when RBCs are treated with proteolytic enzymes such as papain or ficin. Trypsin treatment of RBCs does not result in destruction of $Fy^a$ or $Fy^b$.[6]

The Fy(a–b+$^w$) phenotype is associated with weak $Fy^b$, Fy3, and Fy6 expression. This phenotype results from a mutation in the *FYB* gene. The $Fy^x$-associated mutation at position 89 in the first cytoplasmic loop (Fig. 1) causes the Duffy protein to be unstable. This intracellular amino acid change causes a quantitative reduction in the amount of Duffy protein and, hence, a decreased amount of $Fy^b$, Fy3, and Fy6 expression. The Arg89Cys change was found in 3.5 percent of Caucasians, but was not found in Blacks. Another mutation in the same area (Ala100Thr) does not alter Duffy expression.[13,34]

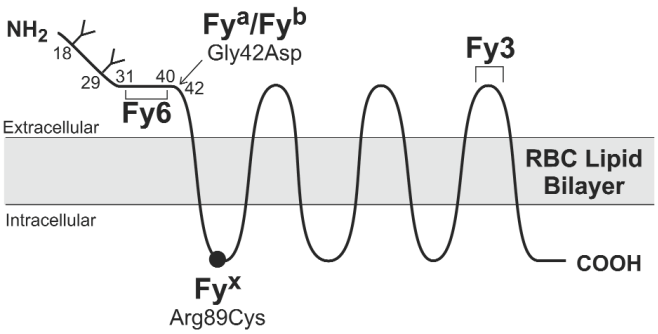


**Fig. 1.** The predicted Duffy glycoprotein seven-transmembrane domain structure. Amino acid changes responsible for the $Fy^a/Fy^b$ polymorphism, the $Fy^x$ mutation, and Fy3 and Fy6 regions are indicated. *N*-glycosylation sites are shown as Y. Reprinted with permission from Westhoff and Reid.[32]

The epitopes identified by monoclonal antibodies to Fy3 and Fy6 have been characterized. The Fy3 epitope is present on the third extracellular loop.[35] Fy3, like Fy5, is resistant to destruction when RBCs are treated with proteolytic enzymes.[6] The Fy6 epitope is located *N*-terminal to the $Fy^a/Fy^b$ site and is composed of multiple amino acids located between positions 19 and 25.[28] Unlike Fy3, Fy6 is destroyed when RBCs are treated with proteolytic enzymes. Like $Fy^a$ and $Fy^b$, trypsin treatment of RBCs does not result in destruction of Fy6.[6]

## Molecular

### *FYA* and *FYB*

The biochemical differences in $Fy^a$ and $Fy^b$ antigens can be explained at a molecular level by a single nucleotide substitution. This substitution (G codes for $Fy^a$ and A codes for $Fy^b$) allowed DNA typing of the main Duffy antigens to be performed as the *FY*A* sequence correlated with a *Ban*I restriction site.[36]

### Fy(a–b–) Phenotype

The Fy(a–b–) phenotype, detected in approximately 70 percent of Black individuals, is identified very rarely in Caucasians. The molecular basis for this disparity is not only interesting from a scientific perspective, but has clinical implications as well (see Clinical Significance). Erythroid-only suppression of Duffy antigen expression occurs because of a point mutation in the GATA-1 binding site in Black individuals who have the Fy(a–b–) phenotype.[8] GATA sequences are plentiful in the genome and function as promoters of many genes, including those involved in hematopoiesis.[37] The mutation present in the GATA promoter region of *FY*B* (–67, T to C) disrupts a binding site for the GATA-1 erythroid transcription factor. A similar mutation has been identified in the GATA-1 promoter region of *FY*A* as well.[38] Duffy antigen expression is prevented on erythrocytes, but not on other cells.[39] Thus, Duffy mRNA can be detected in nonerythroid cells such as lung, spleen, and colon of Black individuals with a mutated GATA box. However, bone marrow cells from the same individuals are negative for Duffy mRNA expression.

The genetic mutations found in Fy(a–b–) Caucasians do not resemble those identified in Black individuals. Three individuals from multiple ethnic backgrounds (Cree Indian, Lebanese Jewish, and Caucasian English) were found to have point mutations that encoded premature stop codons in either *FY*A* or *FY*B*. These mutated genes, if translated into proteins, result in unstable products that are quickly degraded. Thus, the Duffy proteins in Caucasian individuals are absent from all tissues, including RBCs.[11]

## Clinical Significance

### Duffy Glycoproteins and Chemokines

Chemokines are proteins secreted by cells, such as immune cells, which are used as communication signals to guide their interactions.[40] Chemokine messages secreted from one cell are received and decoded by another cell via specific receptors, leading to various responses such as leukocyte chemotaxis and adhesion. Similar to the Duffy glycoprotein, many chemokine receptors have seven transmembrane domains. However, whereas other chemokine receptors specifically bind chemokines of a single class, the Duffy glycoprotein was found to bind a variety of chemokines and is known as the Duffy antigen receptor for chemokines (DARC).[41,42]

The function of DARC is yet to be clearly defined. It has been suggested that DARC may permit the erythrocyte to serve as a chemokine "sink" or scavenger, thus limiting activation of leukocytes in the systemic circulation. However, it is unclear how long chemokines remain bound to the cell surface or what happens to the chemokines at the end of the erythrocyte lifespan. In addition, it is unclear as to the importance of this function in inflammatory or infectious disease as Fy(a–b–) erythrocytes do not bind chemokines, although Fy(a–b+$^w$) erythrocytes bind reduced amounts compared with Fy(a–b+) cells.[34,41–43]

### DARC and Renal Disease

If DARC serves as a scavenger or chemokine sink as part of an effort to limit inflammation, what role could DARC play in modulating an immune response in renal disease and renal transplantation? Using an anti-Fy6 monoclonal antibody, Liu et al.[44] performed immunohistochemical studies of renal biopsies from children with renal disease to examine Duffy antigen expression. Renal cell DARC expression was found to be upregulated in multiple causes of renal cell injury, including HIV nephropathy and hemolytic uremic syndrome. The authors speculate that the increased DARC expression may be the kidney's attempt to bind and neutralize chemokines and control inflammation. They also

speculate that the high incidence of HIV nephropathy in Black individuals may thus be associated with the Fy(a–b–) phenotype.

Other groups have attempted to find a correlation between Duffy antigen expression and renal graft survival, with mixed results. Akalin and Neylan[45] found Duffy-negative graft recipients had lower allograft survival compared with recipients of other phenotypes and speculated that a loss of ability to bind chemokines leads DARC-negative recipients to be more vulnerable to poor graft function. Mange et al.[46] did not confirm an association between a graft recipient with a null DARC phenotype and an increased incidence of acute renal allograft rejection or delayed graft function. One recent paper examined Duffy antigen mismatches between recipient and donor renal transplants and suggests a potential role for Duffy as a minor histocompatibility antigen.[47]

### DARC and Malaria

In addition to serving as a chemokine receptor, the Duffy glycoprotein has been shown to be the erythroid receptor for *Plasmodium vivax* and *Plasmodium knowlesi*. Initially, *P. knowlesi*, a monkey malaria parasite, was used as an in vitro model to study human malaria. However, Miller et al.[48] performed blood typing on 11 volunteers exposed to *P. vivax*–infected mosquitoes and found that those who contracted malaria were Fy(a+) or Fy(b+), whereas those whose erythrocytes were resistant to parasitic invasion were Fy(a–b–). Evidence has since shown that the *P. vivax* Duffy-binding protein (PvDbp) interacts with Duffy antigens on RBCs to permit RBC infection and that PvDbp may be a candidate for vaccine development.[49]

*P. vivax* malaria is the most widely distributed malaria in the world, with approximately 70 to 80 million cases occurring per year.[50] Individuals with the Fy(a–b–) phenotype may have a selective advantage in that their RBCs are resistant to *P. vivax* invasion. This is evident in West Africa, where *P. vivax* malaria is absent and greater than 95 percent of the population is Fy(a–b–). However, a few contradictions to a genetic adaptation hypothesis remain to be explained: the Fy(a–b–) phenotype is not common in Southeast Asia, another endemic area of *P. vivax* malaria, and *P. vivax* malaria infection is not lethal.[5,50]

### The Clinical Value of Duffy Genotyping

The use of Duffy DNA-based genotyping determinations can be an adjunct to traditional phenotyping in clinical situations such as assessing for risk of HDFN and locating matched blood for alloimmunized patients. Goodrick et al.[51] noted that although anti-Fy$^a$ rarely causes significant HDFN, the ability to perform Duffy genotyping of fetal amniocytes can be of benefit when the father is heterozygous for *FY*A*. Regularly transfused patients, such as individuals with sickle cell disease, and any patient who makes one alloantibody are at a higher risk of forming multiple antibodies.[52] Duffy genotyping may be of assistance in providing matched blood by determining, for example, which Fy(a–b–) patients carry the GATA-1 mutation in the promoter region of *FY*B*, as it is theorized that they can receive Fy(b+) blood without risk of forming anti-Fy$^b$ or anti-Fy3.[53] Issues to consider in the use of Duffy genotyping include the need to detect silencing mutations, the potential for contamination of PCR-based assays, and the importance of correlating genotype results with phenotype results.

## Summary

"Kell Kills, Duffy Dies, Lewis Lives"

Medical student mantra related to alloantibody clinical significance

Many of the important discoveries in blood group serology in the first half of the last century were descriptive in nature and focused on identification of RBC antigens and the clinical significance of their corresponding antibodies. The Duffy blood group system illustrates the progress made in elucidating the structure and function of blood group antigens. The Duffy glycoprotein acts as an erythrocyte receptor for certain malarial parasites and as a chemokine receptor (DARC). DARC may play a role in modulating the effects of certain renal diseases, as well as other disease states such as HIV[54] or malignancy.[55] Although significant progress has been made, much research remains to be completed to understand the structure and function of DARC.

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# *RHCE*ceAR* encodes a partial c (RH4) antigen

C. Halter Hipsky, C. Lomas-Francis, A. Fuchisawa, and M.E. Reid

The Rh blood group system is highly complex both in the number of discrete antigens and in the existence of partial antigens, especially D and e. Recently, several partial c antigens have been reported. Here we report findings on an African American man with sickle cell disease whose RBCs typed C+c+ and whose plasma contained anti-c. Hemagglutination tests, DNA extraction, PCR-RFLP, reticulocyte RNA isolation, RT-PCR cDNA analyses, cloning, and sequencing were performed by standard procedures. RBCs from the patient typed C+c+ but his plasma contained alloanti-c. DNA analyses showed the presence of *RHCE*Ce* in *trans* to *RHCE*ceAR* with *RHD*D* and *RHD*Weak D Type 4.2.2*. The amino acid changes on RhceAR are such that a C+c+ patient made alloanti-c. This case shows that RhceAR carries a partial c antigen and illustrates the value of DNA testing as an adjunct to hemagglutination to aid in antibody identification in unusual cases. ***Immunohematology* 2010;26:57–59.**

**Key Words:** blood groups, Rh blood group system, blood transfusion, partial antigen

The Rh blood group system is the most complex of the 30 human blood group systems.[1,2] This is attributable not only to the 50 discrete antigens[3] but also to the fact that some of the antigens, notably D, C, and e, have numerous altered forms, the so-called partial antigens.[4] Partial c antigens have also been described. The first example of alloanti-c in a c+ (presumed phenotype $R_1r$) person was reported in 1982.[5] Anti-Rh26, which can appear as anti-c, has been made by an Rh26–, c– person[6] and also by an RH26–, c+ person.[7] Molecular studies revealed that Rh26 is antithetical to the low-prevalence antigen LOCR, and serologic studies have shown that the LOCR+ phenotype encodes altered (weakened) expression of c.[8] Recently, it has been shown that *RHCE*ce$^S$(340)* and *RHCE*(C)ce$^S$* each encode a partial c antigen.[9,10] Each of these alleles encodes a different haplotype, and the alloanti-c may not be of identical specificity.

One of a growing number of *RHCE*ce* alleles that encode an Rhce protein with an altered c antigen is *RHCE*ceAR*. *RHCE*ceAR* has six nucleotide changes (48G>C, 712A>G, 733C>G, 787A>G, 800T>A, and 916A>G), which predict the amino acid changes of Trp16Cys, Met238Val, Leu245Val, Arg263Gly, Met267Lys, and Ile306Val, respectively.[11] The *RHCE*ceAR* allele encodes an altered e, a weak V, but no Rh18 or hr$^S$.[12] In this article, we describe serologic and DNA testing on blood from a C+c+ African American patient whose plasma contains alloanti-c, thereby revealing that *RHCE*ceAR* encodes a partial c antigen. We published this finding in an abstract,[13] and while this manuscript was in preparation, a report by Peyrard and coworkers[14] appeared and thus there are two such cases.

## Materials and Methods

Blood samples from a 17-year-old multi-transfused African American man with sickle cell disease were analyzed.

### Hemagglutination

Reagents were from our libraries and obtained from numerous colleagues and commercial sources. Hemagglutination was performed in test tubes using the method best suited to the antibody being tested. Eluates were prepared using Gamma Elu-Kit II (Gamma/Immucor, Norcross, GA).

### DNA and RNA Isolation, RT-PCR, Sequencing, and Cloning

Genomic DNA was prepared from 200 µL of the buffy coat layer of peripheral blood using a DNA extraction kit (QIAamp DNA Blood Mini Kit, Qiagen, Valencia, CA). RNA was isolated from the reticulocytes (TriZol and PureLink Micro-to-Midi Total RNA Purification System, Invitrogen, Carlsbad, CA). Reverse transcription was carried out with gene-specific *RHD* and *RHCE* primers listed in Table 1 and Superscript III, according to manufacturer's instructions (Supercript III First Strand Synthesis SuperMix, Invitrogen). PCR amplification was carried out with primers cRHx1F and cRHx5R to amplify exons 1–4; and cRHx4F and cRHx10R to amplify exons 5–10 on *RHD* (RefSeq accession NM_016124) and *RHCE* (RefSeq accession NM_020485) cDNA using HotStarTaq Master Mix Kit (Qiagen). PCR amplicons were checked for purity on agarose gels, cleaned using ExoSAP-IT (USB Corporation, Cleveland, OH) according to manufacturer's instructions and directly sequenced by GeneWiz Inc. (South Plainfield, NJ). Cloning reactions were carried out and sequenced by GeneWiz Inc. Sequences were aligned, and protein sequence comparisons were performed using Sequencher v4.8 (GeneCodes, Ann Arbor, MI). The complete sequences of *RHCE* and *RHD* were analyzed using gene-specific cDNA direct sequencing, and to verify the results and determine which alleles carry which alterations, all RT-PCR products of *RHCE* and *RHD* were also cloned and sequenced. The sequence of *RHD* exon 7 was determined by amplifying and sequencing exon 7 individually using genomic DNA as described previously. To verify the *RHCE*ceAR* nucleotide change 48G>C that does not always sequence well owing to its early position in exon 1, PCR-RFLP was conducted on genomic DNA.

Table 1. Sequence and location of primers

| Primer name | Primer sequence (5′–3′) | Location |
|---|---|---|
| cDx10R | gtattctacagtgcataataaatggtg | Exon 10 |
| cCEx10R | ctgtctctgaccttgtttcattatac | Exon 10 |
| cRHx1F | agctctaagtacccgcggtctgtcc | Exon 1 |
| cRHx5R | tggccagaacatccacaagaagag | Exon 5 |
| cRHx4F | acgatacccagtttgtctgccatg | Exon 4 |
| cRHx10R | tgaacaggccttgtttttcttggatgc | Exon 10 |

## Results

### Hemagglutination

The patient's RBCs typed as group B, D+C+E–c+e+, and his serum contained anti-c. His RBCs were agglutinated by 12 commercial anti-c reagents (reagents included monoclonal and polyclonal antibodies) to the same strength as control RBCs expressing a single dose of c antigen. As this was a surprising result given that he had made anti-c, we performed DNA analyses.

### *RH* cDNA Sequence Analysis

Collectively, the results of DNA analyses showed the presence of the following genes: *RHD*D, RHD*Weak D Type 4.2.2, RHCE*Ce*, and *RHCE*ceAR*. The weak D Type 4.2.2 allele is the same as the *RHD*DAR* allele, except it harbors a 744C>T and a 957G>A nucleotide change.[15] It is likely that the two haplotypes in this patient are *RHD*D/RHCE*Ce* and *RHD*Weak D Type 4.2.2/RHCE*ceAR*.

## Discussion

We report findings on an African American with sickle cell disease who had been transfused on numerous occasions. His RBCs typed C+c+, and his serum contained anti-c reactive by the IAT. This study reveals that the amino acid changes on RhceAR (Trp16Cys, Met238Val, Leu245Val, Arg263Gly, Met267Lys, and Ile306Val) are such that a C+c+ patient can make alloanti-c. Thus, ceAR carries a partial c antigen. The strong reactivity of anti-c reagents with the patient's RBCs that express Ce/ceAR and the absence of a known low-prevalence antigen on RBCs expressing ceAR preclude detection of the altered c antigen associated with ceAR.

This case shows the value of DNA testing as an adjunct to hemagglutination to aid in antibody identification in unusual cases. After we published our findings in a preliminary form[13] and while this manuscript was in preparation, Peyrard and coworkers reported a case of anti-c in a person with the C+/ceAR phenotype.[14] Thus, there are two such published cases. The clinical relevance of the alloanti-c in this latter case is unknown, because after it was identified, the patient received c– RBC components. Interestingly, to date, the majority of partial c antigens have been in persons of African or Hispanic ancestry.

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# The Gerbich blood group system: a review

P.S. Walker and M.E. Reid

Antigens in the Gerbich blood group system are expressed on glycophorin C (GPC) and glycophorin D (GPD), which are both encoded by a single gene, *GYPC*. The *GYPC* gene is located on the long arm of chromosome 2, and Gerbich antigens are inherited as autosomal dominant traits. There are 11 antigens in the Gerbich blood group system, six of high prevalence (Ge2, Ge3, Ge4, GEPL [Ge10*], GEAT [Ge11*], GETI [Ge12*]) and five of low prevalence (Wb [Ge5], Ls$^a$ [Ge6], An$^a$ [Ge7], Dh$^a$ [Ge8], GEIS [Ge9]). GPC and GPD interact with protein 4.1R, contributing stability to the RBC membrane. Reduced levels of GPC and GPD are associated with hereditary elliptocytosis, and Gerbich antigens act as receptors for the malarial parasite *Plasmodium falciparum*. Anti-Ge2 and anti-Ge3 have caused hemolytic transfusion reactions, and anti-Ge3 has produced hemolytic disease of the fetus and newborn (HDFN). ***Immunohematology* 2010;26:60–65.**

**Key Words:** blood group, Gerbich, glycophorin, GPC

## History

In 1960, Rosenfield et al.[1] described the first examples of anti-Gerbich in the sera of three women, including Mrs. Gerbich, after whom the blood group system is named. A year later, Cleghorn[2] and Barnes and Lewis[3] reported on a Turkish Cypriot woman, Mrs. Yus, whose RBCs were compatible with two of the original three sera but were incompatible with serum from Mrs. Gerbich. In 1970, Booth et al.[4] reported on the prevalence of the Gerbich (GE) blood group in Melanesians, and in 1972, Booth[5] reported that certain Ge+ individuals demonstrated an antibody that was compatible with RBCs expressing the Gerbich or the Yus phenotype, but was incompatible with up to 15 percent of Ge+ Melanesians.

After Zelinski et al.[6] demonstrated that Gerbich is genetically discrete from all other existing systems, the Gerbich antigen collection (ISBT Collection 201) was upgraded to the GE blood group system (ISBT system symbol GE and number 020) by the ISBT Working Party on Terminology for Red Cell Surface Antigens.[7]

## Biochemistry

In 1984, Anstee et al.[8] reported that individuals who lack Gerbich blood group antigens have alterations in their erythrocyte membrane sialoglycoproteins. In 1984, these proteins were called β-syaloglycoprotein and γ-syaloglycoprotein; however, the current terminology is glycophorin C (GPC) and glycophorin D (GPD). Gerbich antigens are found on GPC and GPD. These sialic acid–rich glycoproteins are also known as CD236R, and they attach to the RBC membrane through an interaction with protein 4.1R and p55. GPC and GPD contain three domains: an extracellular $NH_2$ domain, a transmembrane domain, and an intracellular or cytoplasmic COOH domain (Figure 1). GPC and GPD are encoded by the same gene, *GYPC*. When the first AUG initiation codon is used, GPC is encoded, whereas when the second AUG is used, GPD is encoded. Thus, GPD is a shorter version of GPC, and the amino acids in GPD are identical to those found in GPC but lacking the first 21 amino acids at the N-terminal of GPC.[9,10]

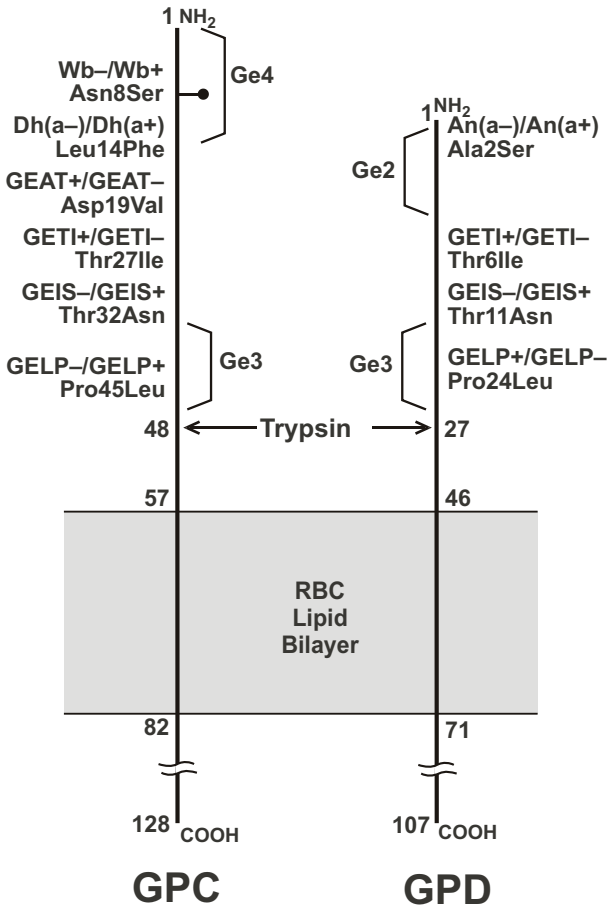


**Fig. 1.** Molecules (glycophorin C [GPC] and glycophorin D [GPD]) showing location of various Gerbich antigens. The stick figures give the amino acid residue numbers defining the extracellular, transmembrane, and intracellular domains of GPC and GPD. Also shown is the trypsin cleavage site and location of all antigens except Ls$^a$, which is the result of amino acids encoded by the duplication of exon 3.

*Nomenclature pending approval by the ISBT Working Party on Terminology for Red Cell Surface Antigens.

Certain Gerbich antigens (Ge4, Wb, Dh[a], GEAT) are only expressed on GPC, two (Ge2, An[a]) are only expressed on GPD, and others (Ge3, Ls[a], GEIS, GEPL, GETI) are expressed on both GPC and GPD. Despite the fact that GPC possesses all of the amino acids that are found on GPD, the likely explanation for why some antibodies only react with GPD is that the antibodies require a conformational epitope that is present at the amino terminus of GPD but absent in the subterminal amino acid sequence of GPC. Some examples of anti-Ge2 do not react with RBCs after the acetylation of membrane proteins with acetic anhydride, suggesting that a free amino group is involved in the epitope detected by these antibodies.[11] A diagram showing the trypsin cleavage site and location of Ge2, Ge3, and Ge4 antigens is given in Figure 1.

In 1990, Reid et al.[12] reported that GPC plays a functionally important role in maintaining erythrocyte shape and regulating the membrane properties through its interaction with protein 4.1R. In 1993, Alloisio et al.[13] showed that p55, a peripheral membrane protein in human erythrocytes, is associated in precise proportions with the protein 4.1R–GPC complex, linking the cytoskeleton and the membrane. The absence of GPC and GPD is associated with hereditary elliptocytosis, which is described later in this discussion.

## Inheritance and Molecular Genetics

In 1986, Colin et al.[14] cloned the gene *GYPC*, and Mattei et al.[15] determined that *GYPC* is located on chromosome 2, in the region of q14–q21. The *GYPC* gene consists of 13.5 kilobase pairs (kbp) of gDNA, comprising four exons. Exons 2 and 3 are homologous, with less than 5 percent nucleotide divergence. This can lead to unequal crossing over during meiosis and loss (outsplicing) of exon 2 or exon 3. In 1987 Le Van Kim et al.[16] reported that a deletion of approximately 3 kb in the *GYPC* gene is associated with the Gerbich blood group deficiency types Yus (GE:–2,3) and Gerbich (GE:–2,–3). In 1989, High et al.[17] reported that the absence of exon 2 results in the Yus phenotype, whereas the absence of exon 3 results in the Gerbich phenotype. The Gerbich phenotype has also been produced by a nucleotide change and deletion of exon 3 of the *GYPC*.[18] The Leach phenotype (GE:–2,–3,–4) may be produced by two different mechanisms. The "PL" type of the Leach phenotype is caused by a deletion of exons 3 and 4, whereas the "LN" type is a consequence of a 131G>T nucleotide change (134delC in exon 3; Trp44Leu) that leads to a frame shift and a premature stop codon. Other Ge antigens are a consequence of nucleotide changes in *GYPC* (Table 1).[19] The products of the Ge alleles are inherited in an autosomal codominant manner.[20] A gene map is shown in Figure 2.

**Table 1.** Summary of nucleotide and amino acid changes in Ge phenotypes

| Phenotype | Traditional name | Nucleotide change exon/ intron | Amino acid change | Ethnicity (occurrence) |
|---|---|---|---|---|
| GE:–2,3,4 | Yus type | del exon 2 | Deletion of amino acids, altered GPC | Hispanic, Israeli, and Mediterranean populations (rare) |
| GE:–2,–3,4 | Gerbich type | del exon 3 | Deletion of amino acids, altered GPC | Melanesians (50%) Others (rare) |
| GE:5 | Wb+ | 23A>G in exon 1 | Asn8Ser in GPC | Wales and Australia (few) Others (rare) |
| GE:6 | Ls(a+) | Duplicated or triplicated exon 3 | Duplication, altered GPC | Blacks (2%) Finns (1.6%) Others (Rare) |
| GE:7 | An(a+) | 67G>T in exon 2 | Ala23Ser in GPC^; Ala2Ser in GPD | Finns (0.2%) Others (rare) |
| GE:8 | Dh(a+) | 40C>T in exon 1 | Leu14Phe in GPC | Original proband was Danish (rare) |
| GE:9 | GEIS+ | 95C>A in exon 2 | Thr32Asn in GPC; Thr11Asn in GPD | Japanese (rare) |
| GE:–10 | GEPL– | 134C>T in exon 3 | Pro45Leu in GPC[19] Pro24Leu in GPD | (rare) |
| GE:–11 | GEAT– | 56A>T in exon 2 | Asp19Val in GPC[19] | (rare) |
| GE:–12 | GETI– | 80C>T in exon 2 | Thr27Ile in GPC[19] Thr6Ile in GPD | (rare) |
| **Null phenotype** | | | | |
| GE:–2, –3,–4 | Leach type (PL) | del exons 3 and 4 | | (rare) |
| GE:–2, –3,–4 | Leach type (LN) | 131G>T; 134delC in exon 3 | Trp44Leu; 45fs; 55Stop | (rare) |

GPC = glycophorin C; GPD = glycophorin D.
^ the altered GPC does not express An[a].

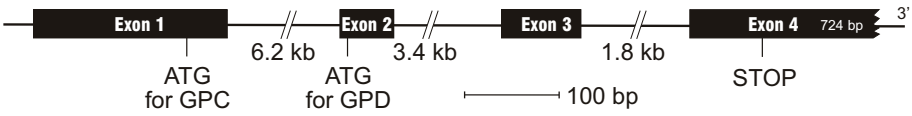


**Fig. 2.** Glycophorin C (*GYPC*) gene map. *GYPC* is composed of four exons and three introns that are distributed over 13.5 kb of genomic DNA. Exons are the regions of the gene sequence that code for the amino acids that constitute the glycoproteins, GPC and GPD. The introns separate the exons, and they are not encoded. Locations of the ATG (start codon) for initiation of GPC and GPD are indicated.

## Gerbich Antigens

There are six high-prevalence antigens and five low-prevalence antigens in the Gerbich blood group system. As examples of anti-Ge1 are no longer available, the Ge1 antigen was declared to be obsolete by the ISBT working party for terminology of red cell surface antigens.

### High-Prevalence Antigens

Ge2 is absent from RBCs with the Yus, Gerbich, or Leach phenotype. Ge2 is located at the $NH_2$ terminal 19 amino acids of GPD and is not expressed on GPC. Ge3 is absent from RBCs with the Gerbich or Leach phenotype. Ge3 is expressed on both GPC and GPD within their extracellular portion close to the lipid bilayer. Ge4 is absent only from RBCs with the Leach phenotype, which is the null phenotype in the Gerbich blood group system. Ge4 is located within the $NH_2$ terminal 19 amino acids of GPC (Figure 1; Table 2).

The Ge-negative phenotypes, which can be difficult to differentiate by hemagglutination with polyclonal antibodies, are readily distinguished by testing trypsin-treated RBCs with monoclonal anti-Ge4. The reaction patterns are shown in Table 3.

Three other high-prevalence Ge antigens, GEPL (Ge10*), GEAT (Ge11*), and GETI (Ge12*), are each a consequence of a nucleotide change in *GYPC* (Table 1).[19]

**Table 2.** Gerbich-negative phenotypes

| Traditional phenotype name | ISBT phenotype name | Antibodies | Compatible with |
|---|---|---|---|
| Yus | GE:–2,3,4 | Anti-Ge2 | GE:–2,3,4, GE:–2–3,4, and GE:–2,–3,–4 |
| Gerbich | GE:–2,–3,4 | Anti-Ge3 or anti-Ge2 | GE:–2,–3,4 and GE:–2,–3,–4 (if anti-Ge2 then compatible with GE:–2,3,4) |
| Leach | GE:–2,–3,–4 | Anti-Ge4, anti-Ge3, or anti-Ge2 | GE:–2,–3,–4 only |

**Table 3.** Differentiation of Ge-negative phenotypes using monoclonal anti-Ge4

| RBCs | Normal | Yus | Gerbich | Leach |
|---|---|---|---|---|
| Untreated | 4+ | 0–2+ | 0–2+ | 0 |
| Trypsin-treated | 0 | 0 | 4+ | 0 |

### Low-Prevalence Antigens

Wb (Ge5)(Webb),[21] An$^a$ (Ge7)(Ahonen),[11] Dh$^a$ (Ge8)(Duch),[22] and GEIS (Ge9)[23] each result from a nucleotide change in *GYPC* (Table 1). The Wb (Ge5) antigen results from the substitution Asn8Ser near the $NH_2$ terminus of GPC. This substitution interrupts the consensus sequence for *N*-glycosylation (Asn-X-Ser/Thr), which results in a loss of the *N*-glycan and the gain of an *O*-glycan.[24] Ls$^a$ (Ge6)(Lewis)[25] results from a novel amino sequence encoded by a duplication or triplication of exon 3 of *GYPC*. The allele with a duplication of exon 3 is the reciprocal product of the altered *GYPC* (*GYPC.Ge*) that lacks exon 3 and encodes the Gerbich phenotype.

### Altered Antigen Expression

In protein 4.1R–deficient RBCs, Gerbich antigens are expressed weakly. As GPC and GPD interact with protein 4.1R, an absence of this protein causes a reduced level of GPC and GPD in the RBC membrane.[12] The weakening of Ge2 and Ge3 antigens can be such that, under certain testing conditions, they can appear to be absent.

Gerbich-negative RBCs may show a weakened expression of certain other blood group antigens, notably Kell and Vel. Nine of 11 GE:–2,–3 samples showed different degrees of weakening of Kell system antigens, whereas none of six GE:–2,3 samples showed Kell depression.[26] Similarly, 3 of 14 examples of anti-Vel failed to react with four GE:–2,–3,4 samples, but they did react with one example each of GE:–2,3,4 and GE:–2,–3,–4 RBC samples.[27]

### Antibodies to Gerbich Antigens

#### Anti-Ge2

Anti-Ge2 may be immune or naturally occurring and reacts with an antigen on GPD. Anti-Ge2 is usually an IgG antibody that reacts by the IAT. Some examples of anti-Ge2 have been complement binding and hemolytic. Treatment of RBCs with papain or ficin results in the loss of reactivity with anti-Ge2; however, when RBCs treated with 200 mM DTT are tested with anti-Ge2, variable results are obtained. Individuals with Yus, Gerbich, or Leach phenotypes can make anti-Ge2 (Table 2).[28] The clinical significance of anti-Ge2 is discussed below.

#### Anti-Ge3

Anti-Ge3 reacts with an antigen on both GPC and GPD. Anti-Ge3 is usually an IgG antibody that reacts by the IAT; however, some IgM forms have been reported. Many examples of anti-Ge3 bind complement and are hemolytic. Anti-Ge3 reacts with RBCs that were treated with papain or ficin and 200 mM DTT. Individuals with Gerbich or Leach phenotypes can make anti-Ge3 (Table 2).[28] The clinical significance of anti-Ge3 is discussed later.

#### Anti-Ge4

Alloanti-Ge4 is very rare; only one human example has been described That antibody was IgG, and it reacted by the IAT.[29] Numerous examples of monoclonal antibodies with Ge4 specificity have been produced.[30,31] Treatment of RBCs with papain or ficin results in the loss of reactivity with anti-Ge4, however, treatment of RBCs with 200 mM DTT does not affect their reactivity with anti-Ge4.[28] Individuals with Leach phenotype can make anti-Ge4 (Table 2).[28] There is no information about the clinical significance of anti-Ge4.

### Antibodies to Low-Prevalence Antigens
#### Wb(Ge5), Ls$^a$(Ge6), An$^a$(Ge7), Dh$^a$(Ge8), GEIS (Ge9)

These antibodies may be IgM or IgG, and they may be naturally occurring. They react at room temperature and by the IAT, and none are complement-binding. Treatment of antigen-positive RBCs with papain or ficin results in the loss of reactivity with these antibodies; however, the antigens are resistant to treatment with 200 mM DTT.[28] There are no reports of clinically significant transfusion reactions or HDFN associated with these antibodies.

## Clinical Significance
### Transfusion Reactions

Some examples of anti-Ge2 and anti-Ge3 have caused moderate transfusion reactions—both immediate and delayed; however, other examples have failed to produce shortened RBC survival when antigen-positive incompatible units were transfused.[32–34] Pearson et al.[35] reported a case of alloanti-Ge in which there were discrepant results between an in vivo chromium-51 ($^{51}$Cr) survival study and an in vitro monocyte assay. In that case, the in vivo $^{51}$Cr survival study yielded zero survival of Gerbich-positive cells after 24 hours; however, a monocyte assay showed less than 1 percent lysis of Gerbich-positive cells. The clinical significance in this case was not determined because only Gerbich-negative blood was transfused during surgery.

### HDFN

Anti-Ge2 has been associated with a positive DAT in infants with GE:2 RBCs; however, no cases of clinical HDFN have been reported. By contrast, anti-Ge3 appears to be capable of causing severe HDFN. An interesting recent publication shows that the mechanism for anemia, and possibly for thrombocytopenia, in HDFN caused by anti-Ge3 may be attributed to interference with the erythropoietin signaling cascade.[36] Similar to the mechanism of erythroid suppression described in HDFN caused by anti-K,[37] anti-Ge3 has been associated with antibody-dependent hemolysis, as well as inhibition of erythroid progenitor cell growth in the infant. In these cases, the affected infants may require initial treatment at delivery, followed by monitoring for signs of anemia for several weeks after birth.[38,39]

### Autoimmune Hemolytic Anemia

Several cases of autoimmune hemolytic anemia (AIHA) with anti-Ge specificity have been reported. In two cases, the course of the AIHA was as expected, i.e., the patients typed Ge+, their serum demonstrated anti-Ge antibodies, their DATs were positive, and eluates from the autologous RBCs demonstrated Ge-like antibodies.[40,41]

In one case, the patient typed Ge+ and the serum was nonreactive, but an eluate from the patient's RBCs demonstrated anti-Ge specificity.[42] This is the first report of IgM-mediated warm AIHA associated with autoanti-Ge. In two other cases, the patients typed Ge+ and their serum demonstrated anti-Ge, but their serum failed to react with the autologous RBCs (DAT-negative).[43,44] However, in both of these cases, eluates from the patients' RBCs demonstrated an antibody with Ge specificity. Without the eluate results, these cases could have been confused with alloanti-Ge. One possible explanation for these findings could be a weakening of the Gerbich antigens during the course of the AIHA. In cases of severe life-threatening hemolysis, it might be advisable to select Ge-negative units for transfusion.

### Hereditary Elliptocytosis

Gerbich antigens interact with protein 4.1R, which contributes to the stability of the RBC membrane.[12,45,46] In 1986, Daniels et al.[31] described a family with hereditary elliptocytosis that was associated with the Leach phenotype. In 1991, Telen et al.[47] further explained the molecular basis for the elliptocytosis as the deficiency of GPC and GPD that is associated with the Leach phenotype. Patients with hereditary elliptocytosis rarely require transfusions. If such a patient requires transfusions for other reasons (e.g., surgery) and the patient demonstrates alloanti-Ge, it might be prudent to select Gerbich-negative units for transfusion, if such rare blood is available.

### Malaria

In northern Papua New Guinea, where malaria is endemic, Serjeantson[48] reported in 1989 that Gerbich-negative Melanesians appear to have a selective advantage for avoiding infections with *Plasmodium falciparum* and *Plasmodium vivax*. Subsequent studies confirmed that *P. falciparum* binds to RBCs through a receptor on wild-type GPC, which is missing on Gerbich-negative cells that express a truncated form of GPC.[49–51]

## Summary

The Gerbich blood group system is composed of six high-prevalence antigens, which are expressed on GPC, GPD, or both. GPC and GPD are encoded by a single gene, *GYPC*, which is located on the long arm of chromosome 2. By interacting with protein 4.1R, GPC and GPD contribute stability to the RBC membrane, and a deficiency in these proteins has been associated with hereditary elliptocytosis. Also, Gerbich antigens apparently act as receptors for *P. falciparum malaria*. Certain Gerbich antibodies are clinically significant, e.g., anti-Ge2 and anti-Ge3 have caused hemolytic transfusion reactions, and anti-Ge3 has produced HDFN.

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# Application of real-time PCR and melting curve analysis in rapid Diego blood group genotyping

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The paucity of appropriate reagents for serologic typing of the Diego blood group antigens has prompted the development of a real-time PCR and melting curve analysis for Diego blood group genotyping. In this study, we phenotyped 4326 donor blood samples for Di$^a$ using semiautomated equipment. All 157 Di(a+) samples were then genotyped by PCR using sequence-specific primers (PCR-SSP) for *DI*02* because of anti-Di$^b$ scarcity. Of the 4326 samples, we simultaneously tested 160 samples for Di$^a$ and Di$^b$ by serology, and for *DI*01* and *DI*02* by PCR-SSP and by real-time PCR. We used the same primers for Diego genotyping by real-time PCR and PCR-SSP. Melting curve profiles obtained using the dissociation software of the real-time PCR apparatus enabled the discrimination of Diego alleles. Of the total samples tested, 4169 blood donors, 96.4 percent (95% confidence interval [CI], 95.8–96.9%), were homozygous for *DI*02* and 157, 3.6 percent (95% CI, 3.1–4.2%), were heterozygous *DI*01/02*. No blood donor was found to be homozygous for *DI*01* in this study. The calculated *DI*01* and *DI*02* allele frequencies were 0.0181 (95% CI, 0.0173–0.0189) and 0.9819 (95% CI, 0.9791–0.9847), respectively, showing a good fit for the Hardy-Weinberg equilibrium. There was full concordance among Diego phenotype results and Diego genotype results by PCR-SSP and real-time PCR. *DI*01* and *DI*02* allele determination with SYBR Green I and thermal cycler technology are useful methods for Diego determination. The real-time PCR with SYBR Green I melting temperature protocol can be used as a rapid screening tool for *DI*01* and *DI*02* blood group genotyping. ***Immunohematology* 2010;26:66–70.**

**Key Words:** blood donors, genotyping, real time, PCR, blood groups, Diego blood group, population, gene frequency

The Diego blood group system was named after identifying a new antibody, anti-Di$^a$, which caused HDN resulting in the death of a Venezuelan newborn in 1955.[1,2] Anti-Di$^b$ was described in 1967 in two Mexicans by Thompson et al.[3] Anti-Di$^a$ and -Di$^b$ are clinically relevant. Both have been implicated in transfusion reactions and in HDN.[4–6]

The Diego blood group system comprises 21 antigens, and Di$^a$ and Di$^b$ are the most clinically significant.[7] Although Di$^b$ is present in virtually all populations, Di$^a$ incidence varies substantially worldwide. It is found in 7 to 54 percent of South American Indians, 5 to 8 percent of Asians (Chinese, Korean, Japanese), and 14.7 percent of Mexican Americans, whereas it is rare in Whites and Blacks (0.01%).[3,8,9]

Di$^a$ and Di$^b$ are carried on band 3 protein. The single *SLC4A1* gene (solute carrier family 4, anion exchanger, member 1) controls band 3 expression. This gene extends on an 18-kilobase (kb) genomic DNA, maps to chromosome 17q12–q21, and consists of 20 exons.[10] The *DI*01* and *DI*02* alleles code for the antithetical Di$^a$ and Di$^b$ antigens, respectively.[11]

*DI*01* and *DI*02* polymorphism is determined by a T>C nucleotide substitution at position +2561 in exon 19 of the *SLC4A1* gene, changing the leucine at amino acid position 854 to a proline in the band 3 protein.[12] No healthy individual with a Diego null phenotype has been reported, reflecting the functional importance of band 3. Although no Di(a–b–) subjects have been recognized by serologic testing, Alloisio et al.[13] described one individual who is homozygous for a band 3 mutation, Va1488Met (band 3 Coimbra), that results in almost complete deficiency of band 3. At the clinical level, Diego blood group antigens are of considerable importance in relation to their role in transfusion reactions and HDN. The Brazilian population is composed of a highly mixed ancestry, with an incidence of 1.3 percent of Di(a+) blood donors.[14] Consequently, multiply transfused individuals can have anti-Di$^a$ or rarely anti-Di$^b$.[14–16] Furthermore, commercial anti-Di$^a$ for serologic testing is scarce, and there is no available commercial anti-Di$^b$ for routine use. The aim of this study was therefore to describe a DNA-based typing method that allows blood samples to be tested for *DI*01* and *DI*02* using a PCR real-time method.

## Material and Methods

This is a prospective study performed at Fundação Pró-Sangue/Hemocentro, São Paulo, Brazil. A total of 4326 venous blood samples from unrelated Brazilian volunteer blood donors were collected in EDTA and tested for Di$^a$ by hemagglutination. Every Di(a+) blood sample was then tested for Di$^b$ by PCR using sequence-specific primers (PCR-SSP) owing to anti-Di$^b$ scarcity. We then performed real-time PCR validation for Diego genotyping. Finally, we performed Diego analysis in 160 of 4326 blood samples for Di$^a$ and Di$^b$ by serologic studies, PCR-SSP, and real-time PCR simultaneously. The results were interpreted blinded from the serologic results.

Di$^a$ phenotyping was performed using 50 µL of anti-Di$^a$ (DiaMed AG, Cressier-sur-Morat, Switzerland) and 25 µL of a 1% RBC suspension dispensed into a microplate using semiautomated equipment (Megaflex-TECAN, TECAN AG, Hombrechtikon, Switzerland). After incubation for 30 minutes at 37°C, the microplates were centrifuged at 468*g* for 15 seconds. The microplates were then washed three times

with saline solution and centrifuged at 468*g* for 15 seconds. Fifty µL of antihuman globulin (AHG) serum (DiaMed Latino America, Lagoa Santa, Brazil) was added to each well; the microplates were centrifuged at 468*g* for 15 seconds, and read immediately. $Di^b$ phenotyping was performed in gel cards (DiaMed Latino America) using anti-$Di^b$ previously identified in a patient. The donor's RBCs were washed three times in 0.9% saline solution and suspended in LISS (ID-Diluent 2, DiaMed Latino America) to a final 0.8% suspension. In a microtube of the LISS/AHG ID card (DiaMed Latino America), 50 µL of 0.8% donor RBCs and 25 µL of patient's serum were dispensed and incubated at 37°C for 15 minutes in an appropriate incubator (ID-Incubator 37SI, DiaMed AG). After incubation, the cards were centrifuged for 10 minutes in an appropriate centrifuge (ID-Centrifuge 24S, DiaMed AG). After centrifugation, the cards were examined for agglutination or hemolysis according to the manufacturer's instructions. Positive and negative known control samples were included in each batch for Diego phenotyping validation results.

### DNA Extraction

Human genomic DNA was isolated from whole blood in duplicate, using a commercial DNA extraction kit (QIAamp DNA Blood Mini Kit, QIAGEN Science, Hilden, Germany). DNA was extracted from 200 µL of blood and eluted in 100 µL of buffer according to the manufacturer's recommendations. For all samples, a mean of 100 ng of DNA was obtained. DNA was stored at –20°C for long-term storage.

### PCR-SSP

All samples were genotyped in duplicate for *DI*01* and *DI*02* alleles by the PCR-SSP method and real-time PCR simultaneously, using primers designed by Wu et al.[17] (Table 1) The F2/AR pair of primers detected the *DI*01* allele, and the BF/R pair detected *DI*02*. Primers amplifying a fragment of the human growth hormone (HGH) gene served as an internal control.[18,19] PCR-SSP was carried out in a final volume of 25 µL containing 100 ng of purified DNA, 1.5 µL of $MgCl_2$ (1.5 mM), 1 µL of dNTP mix (0.2 mM, Invitrogen, Carlsbad, CA), 1.5 U of *Taq* polymerase (Platinum Taq DNA Polymerase, Invitrogen, São Paulo, Brazil), 2.5 µL of Tris-HCl (10 mM, pH 8.3, 50 mM KCl), 0.3 µM of each forward and reverse primer, and 0.08 µM of internal control. Amplifications were programmed on the thermocycler (Mastercycler gradient, Eppendorf AG, Hamburg, Germany), at the following conditions: denaturation at 95°C for 5 minutes, then 30 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 90 seconds at 72°C.[17] The reactions were completed by an elongation step for 5 minutes at 72°C. PCR products were visualized in a 2% agarose gel stained with ethidium bromide under 100 V using photodocumentation equipment (Eagle-eye; Stratagene, La Jolla, CA).

### Real-time PCR

The same two sets of primers used for *DI*01* and *DI*02* polymorphism detection by PCR-SSP were also used for real-time PCR. Real-time PCR was carried out in 0.1-mL strip tubes and Caps (Corbett Research, Mortlake, Australia) using the same primers used for the PCR-SSP method (Table 1). Each reaction contained 7.5 µL of 2x Quantitect SYBR Green PCR Master Mix (QIAGEN Science), 0.3 µM of each forward and reverse primer, 100 ng of genomic DNA, and nuclease-free water in a final volume of 15 µL. PCR amplifications and fluorescence detection were performed using Rotor Gene 3000 equipment (Corbett Research, Sydney, Australia). The PCR amplification profile was a 95°C enzyme activation step (10 minutes), followed by 35 cycles of 95°C denaturation (30 seconds), 60°C annealing (30 seconds), and 72°C extension (90 seconds). Melting curves were generated by monitoring the continuous decrease in fluorescence of the SYBR Green signal from 75° to 95°C at the end of each run. Data acquisition and analysis were handled by the Rotor Gene 6 software (Corbett Research). In each run, we included samples homozygous for *DI*01* and *DI*02* used in the validation process as control samples.

### Statistical Analysis

Allele frequencies were calculated by direct gene counting, and the differences were analyzed by the $\chi^2$ test using a 2 × 2 contingency table. All analyses were performed on Statistica software (SAS Institute, Cary, NC).

## Results

### Real-time PCR Validation

In the validation process of real-time PCR for *DI*01* and *DI*02*, we tested 62 reference samples from the Fundação Pró-Sangue, after which these samples were genotyped for *DI*01* and *DI*02* using PCR-SSP (Figure 1A) and by real-time PCR (Figure 1B) on two occasions. The results were interpreted blinded from the serologic results. We found a genotype-specific melting profile for *DI*01* and *DI*02*, with both amplifications performed in one single run. This step was critical for the optimization of our real-time PCR protocol

**Table 1.** Primers used for *DI* genotyping by PCR SSP and real-time PCR

| Primer | Sequence (5′-3′) | Ref | Pair of primers | Gene detected | PCR Product (bp) |
|---|---|---|---|---|---|
| *F2*† | GTGCTGGGGTGTGATAGGC | (17) | F2/AR | *DI*01* | 139 |
| *AR*† | CAGGGCCAGGGAGGCCA | | | | |
| *BF*† | GGTGGTGAAGTCCACGCC | (17) | BF/R | *DI*02* | 129 |
| *R*† | CCAGGCAGCCACTCACAC | | | | |
| HGH-F | TGCCTTCCCAACCATTCCCTTA | (18) | HGHF/ HGHR | *HGH* | 434 |
| HGH-R | CCACTCACGGATTTCTGTTGTGTTTC | | | | |

†GenBank accession no. AC003043 for *SLC4A1*
F = indicates forward primer; R = reverse primer.

and for determination of the Tm (melting temperature at which double-strand DNA is broken down to single-strand DNA) for *DI*01* (86.94°C) and *DI*02* (88.04°C) (Figure 2). Of 62 samples tested, 49 were found to be homozygous for *DI*02*. Eleven were *DI*01/02*, and two were *DI*01/01*. Our results for *DI*01* and *DI*02* genotyping with real-time PCR were totally concordant with those obtained by PCR-SSP. The two samples with a *DI*01/01* genotype by PCR-SSP allowed us to establish and validate the detection of the *DI*01* homozygous allele by real-time PCR.

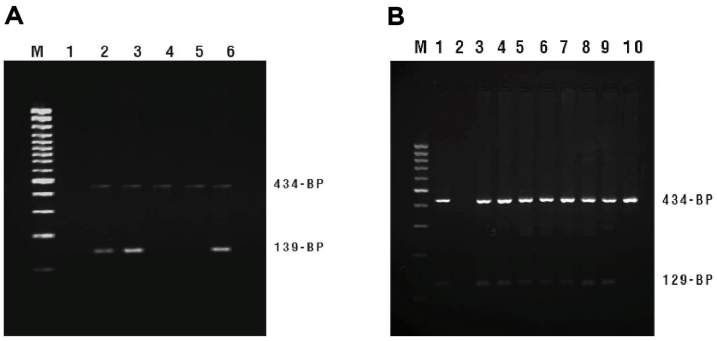


**Fig. 1.** PCR-SSP typing for *DI*01* and *DI*02* alleles. A 434-bp fragment of *HGH* was amplified as an internal control. Lane M shows a 100-bp ladder. (**A**) *DI*01* was determined by the presence of 139-bp fragment. From left to right: Blank (lane 1), *DI*01*-positive samples (lanes 2, 3), *DI*01*-negative (lanes 4, 5), *DI*01*-positive control (lane 6). (**B**) *DI*02* allele was determined by the presence of specific PCR product (129-bp).From left to right: *DI*02*-positive control (lane 1), Blank (lane 2), *DI*02*-positive samples (lanes 3, 4, 5, 6, 7, 8, 9) and negative control for *DI*02* (10).

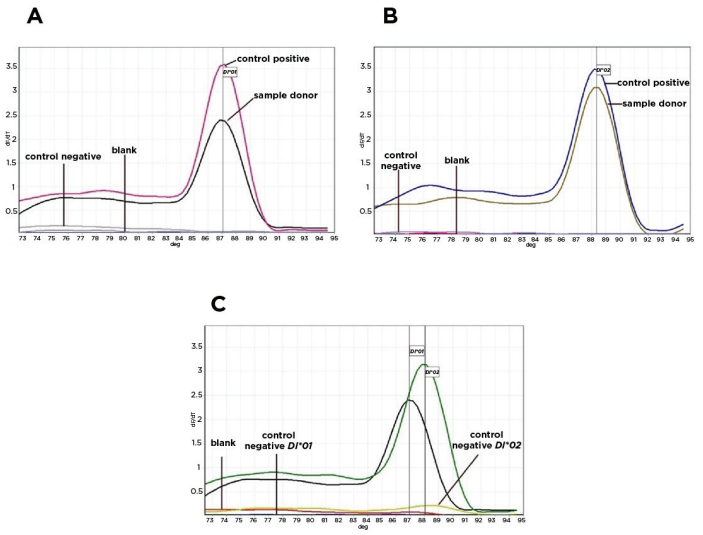


**Fig. 2.** Melting curve analysis of samples genotyped as (**A**) *DI*01/01*, (**B**) *DI*02/02*,and (**C**) *DI*01/02*. The *DI*01* and *DI*02* Tm were determined at 86.94° and 88.04°C, respectively.The arrows indicate controls (positive, negative) for *DI*01* and *DI*02*, blank, and sample donors tested.

### Comparison of Phenotyping with Hemagglutination, PCR-SSP, and Real-time PCR for *DI*01* and *DI*02*

The sensitivity of our real-time PCR assay was verified by correctly identifying 160 samples for *DI*01* and *DI*02* by serology, PCR-SSP, and real-time PCR. Of these, 127 were found to be *DI*02/02* and 33 were *DI*01/02*. No single *DI*01/01* sample was identified in this series. All homozygous and heterozygous alleles have been distinguished by melting curve analyses. Although no *DI*01* homozygous individual was found in this series, we tested two *DI*01/01* samples in the validation step, and both were also identified by real-time PCR. Our results of Diego genotyping using real-time PCR were consistent and totally concordant with those results obtained using PCR-SSP. No discrepant results were observed among the three methodologies evaluated in our study.

### Analysis of Diego Genotype and Allele Frequencies in Brazilian Blood Donors

Phenotype frequencies were estimated in Brazilian blood donors (Table 2). Among 4326 blood donors tested, 4169 (96.4%; 95% confidence interval [CI], 95.8–96.9%) were homozygous for *DI*02* and 157 (3.6%; 95% CI, 3.1–4.2%) were *DI*01/02* heterozygous. No blood donor was found to be homozygous for *DI*01* in this study (Table 2). We assumed that no blood donor presented the very rare band 3–deficient phenotype for Diego genotype and allele frequency calculation. The calculated *DI*01* and *DI*02* allele frequencies were 0.0181 (95% CI, 0.0173–0.0189) and 0.9819 (95% CI, 0.9791–0.9847), respectively, showing a good fit for the Hardy-Weinberg equilibrium (Table 3).

**Table 2.** Diego phenotype incidence in 4326 Brazilian blood donors

| Phenotype | Number | % | 95% CI |
|---|---|---|---|
| Di(a–b+) | 4169 | 96.4 | 95.8–96.9 |
| Di(a+b+) | 157 | 3.6 | 3.1–4.2 |
| Di(a+b–) | 0 | 0 | — |
| Total | 4326 | 100 | |

$\chi^2$ = 1.4776.
CI = confidence interval.

**Table 3.** Allele frequencies of *DI*01* and *DI*02* in 4326 Brazilian blood donors

| Diego alleles | Allele frequency | 95% CI |
|---|---|---|
| *DI*01* | 0.0181 | 0.0173–0.0189 |
| *DI*02* | 0.9819 | 0.9791–0.9847 |

CI = confidence interval.

## Discussion

In the last 10 years, the vigorous globalization process has made international travel and significant immigration extraordinarily common. Rare blood can be necessary in unexpected regions, causing an extra impact on blood services. Consequently, the Diego blood group system is of

clinical importance not only in Latin America but also in many other countries. Different approaches can be taken to deal with this situation.

Diego phenotyping has not been routinely performed in several blood services owing to the scarcity of commercial anti-Di$^a$. The situation is critical for Di$^b$, as there is no available commercial anti-Di$^b$. Therefore, whenever there is a suspicion of anti-Di$^a$ or -Di$^b$, two problems are faced: one is to confirm the antibody specificity against the Diego antigens with appropriate controls (negative and positive), and the other is to find units negative for the antigen involved.

This need led us to search for an alternative approach for Diego blood group antigen testing and for the selection of blood units negative for Di$^a$ or Di$^b$. Diego genotyping can be performed using the PCR-SSP method. However, PCR-SSP is a time-consuming assay as it requires post-PCR handling, and it has been shown to be inadequate for large-scale implementation.[20]

Wu et al.[17] described a PCR-SSP method for *DI*01* and *DI*02* genotyping that we used in this study. They tested different primer combinations and tried to use a single tube for PCR-SSP technique to identify *DI*01* and *DI*02* alleles in the same reaction. However, the results were disappointing, and they had to use two tubes, generating two separate products. Consequently, we used two separate protocols to test for *DI*01* and *DI*02* by PCR-SSP.

Real-time PCR for *DI*01* and *DI*02* allele determination can overcome these drawbacks because it allows specific amplification without post-PCR manipulations. Moreover, real-time PCR decreases the risk of error by the simple fact that it reduces the number of manual steps.[21] Recently, Polin et al.[22] also described the methodology for real-time PCR as an effective tool for blood group genotyping.

As labeled probes are costly when compared to real-time PCR with SYBR Green I, we decided to use the latter for cost reduction.[23] This decision also optimized our strategy for DNA analysis of other blood group systems, as we saw no need to purchase special primers.

The real-time PCR protocol that we developed allowed us to use the same primers we had been using for PCR-SSP in a universal cycling program, performing the same protocol to identify *DI*01* and *DI*02* alleles. The advantages of this real-time PCR method are the rapid performance and the detection of two alleles in the same run. The PCR assay revealed 100 percent specificity as assessed by comparison of genotype data to those generated by serologic typing. The Diego genotyping method described here, based on SYBR Green I by real-time PCR, can be used as a high-throughput discrimination of the *DI*01* and *DI*02* alleles (Figure 2). This approach takes advantage of the fluorescent property of SYBR Green I and of the melting curve analysis for the detection and discrimination of amplicons differing in length and nucleotide content.

One limitation of this study is that we were not able to detect the rare band 3 mutation called Coimbra, because we had not tested all samples by the three methods chosen. However, it would be highly unlikely to find one, as to date band 3 Coimbra mutation has been associated with anemia, and we only included nonanemic individuals (blood donors) in our study.[13]

Finally, we analyzed *DI*01* and *DI*02* genotype and allele frequencies in 4326 Brazilian blood donors. There are significant differences of *DI*01* and *DI*02* allele expression among world populations. The *DI*01* allele is considered rare in Whites,[24,25] but is characteristic in some Asians and in South American Indians with a gene frequency as high as 40 percent.[1,4] We detected Di$^a$ in 3.6 percent of blood donors, with a *DI*01* gene frequency of 0.0181, indicating the complex ancestry miscegenation of the Brazilian population. This study was conducted in São Paulo State, located in southern Brazil, which is characterized by lower levels of African and higher degrees of European contributions when compared with other Brazilian groups.[26] Moreover, our results can be explained in part by the successive migratory waves from 1500 to the 20th century that contributed to the formation of the multiethnic highly admixed Brazilian population. This heterogeneity was documented in several genetic studies, which demonstrated a typical although nonuniform triethnic (European, African, and Amerindian) population gene pool.[27]

In conclusion, we developed a real-time PCR protocol for *DI*01* and *DI*02* genotyping that is feasible and easy to perform on a high-throughput scale. It can improve and facilitate anthropologic and epidemiologic studies on *DI*01* and *DI*02* allele determination.

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# The Dombrock blood group system: a review

C. Lomas-Francis and M.E. Reid

The Dombrock blood group system (Do) consists of two antithetical antigens (Do$^a$ and Do$^b$) and five antigens of high prevalence (Gy$^a$, Hy, Jo$^a$, DOYA, and DOMR). Do antigens are carried on the Dombrock glycoprotein, which is attached to the RBC membrane via a glycosylphosphatidylinositol linkage. The gene (*DO, ART4*) encoding the Do glycoprotein, located on the short arm of chromosome 12, has been cloned and sequenced, allowing the molecular basis of the various Do phenotypes to be determined. Do$^a$ and Do$^b$ have a prevalence that makes them useful as genetic markers; however, the paucity of reliable anti-Do$^a$ and anti-Do$^b$ has prevented this potential from being realized. The ease with which these antigens can be predicted by analysis of DNA opens the door for such studies to be carried out. Anti-Do$^a$ and anti-Do$^b$ are rarely found as a single specificity, but they have been implicated in causing hemolytic transfusion reactions. This review is a synthesis of our current knowledge of the Dombrock blood group system. ***Immunohematology* 2010;26:71–78.**

**Key Words:** ART, blood group system, Dombrock blood group system, mono-ADP-ribosyltransferase, ADP-ribosyltransferase

The Dombrock (Do) blood group system illustrates the value of different methods for the advancement of knowledge. Classical hemagglutination showed certain characteristics of the antigens and antibodies, a relationship of Hy to Gy$^a$, and the fact that the Gy(a–) phenotype was the null of the Dombrock blood group system. Immunoblotting provided a tool to allow further characterization of the Dombrock glycoprotein, including the fact that it is linked to the RBC membrane by a glycosylphosphatidylinositol (GPI) anchor. In silico analysis aided in cloning the *DO* gene and led to PCR-based assays not only to identify the nucleotide changes associated with the antigens but also to screen for antigen-negative donors and identify new alleles. Transfection and hybridoma technology has been used for the production of monoclonal antibodies to Do, and DNA array technology provides a means to do high-throughput testing of donor blood. This review summarizes the understanding of the Dombrock blood group system that has evolved as methods were developed and applied.

## History

Anti-Do$^a$ was identified in 1965,[1] and anti-Do$^b$, which recognizes the antithetical antigen, was described 8 years later.[2] These two antibodies define three phenotypes, the prevalence of which differs in various ethnic groups (Table 1). For populations other than Whites,[2,4] studies have been restricted to testing with anti-Do$^a$, and thus the numbers given for Do(a–b+) in Table 1 are calculated from the prevalence given for Do$^a$.[4–6] Do$^a$ and Do$^b$ were placed in the Dombrock blood group system (DO; 014) by the ISBT Working Party on Terminology for Red Cell Surface Antigens in 1985.[7]

**Table 1.** The Dombrock blood group system

| RBC phenotype | Reactivity with anti- | | | | | Occurrence (%) in | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Do$^a$ | Do$^b$ | Gy$^a$ | Hy | Jo$^a$ | Whites | Blacks | Japanese | Thai | Chinese |
| Do(a+b–) | + | 0 | + | + | + | 18 | 11 | 1.5 | 0.5 | |
| Do(a+b+) | + | + | + | + | + | 49 | 44 | 22 | 13 | |
| Do(a–b+) | 0 | + | + | + | + | 33 | 45 | 76.5 | 86.5 | |
| Gy(a–) | 0 | 0 | 0 | 0 | 0 | Rare | Rare* | Rare | Not found | Rare† |
| Hy– | 0 | wk | wk | 0 | 0/wk | Not found | Rare | Not found | Not found | Not found |
| Jo(a–) | wk | 0/wk‡ | + | wk | 0 | Not found | Rare | Not found | Not found | Not found |

*One Gy(a–) Black proband has been reported.[3]
†One Gy(a–) Chinese proband has been found (unpublished data).
‡RBCs will most often be Do(b–) when the Do glycoprotein is encoded by *JO/JO* but Do(b+$^W$) when encoded by *HY/JO*.

The high-prevalence antigens Gregory (Gy$^a$) and Holley (Hy), described independently in 1967[8,9] were shown to be phenotypically related. RBCs from Caucasians with the Gy(a–) phenotype are Hy–, and RBCs from Black people of African descent with the Hy– phenotype are Gy(a+$^w$).[10] On the basis of this observation, Gy$^a$ and Hy were upgraded from the ISBT Series of High Incidence Antigens to the Gregory Collection (206) (Table 2).[11] Gy$^a$ and Hy were shown to be located on the same glycoprotein by immunoblotting in 1991.[12]

**Table 2.** ISBT terminology for the Dombrock (014) blood group system antigens

| Traditional name | ISBT name | ISBT number | Previous ISBT number |
|---|---|---|---|
| Do$^a$ | DO1 | 014001 | |
| Do$^b$ | DO2 | 014002 | |
| Gy$^a$ | DO3 | 014003 | 206001; 900005 |
| Hy | DO4 | 014004 | 206002; 900011 |
| Jo$^a$ | DO5 | 014005 | 901004; 900010 |
| DOYA | DO6 | 014006 | |
| DOMR | DO7 | 014007 | |

The high-prevalence antigen Jo$^a$ was first described in 1972[13] and later shown to have a phenotypic association with Gy$^a$ and Hy because RBCs with either the Gy(a–) phenotype or the Hy– phenotype are also Jo(a–).[14,15] Jo$^a$ was assigned a number in the ISBT Series of High Incidence Antigens before its association with Gy$^a$ and Hy was realized.[7] When Jo$^a$ was shown to reside on the Gregory glycoprotein by immunoblotting,[16] it was not placed in the Gregory collection but it was promoted directly to the Dombrock blood group system.[17,18] Another high-prevalence antigen, Jc$^a$, was shown to be associated with Gy$^a$ and Hy,[19] and although it was reported to be the same as Jo$^a$,[20] there remained some doubt and an ISBT number was not assigned to Jc$^a$. On the basis of subsequent work, this doubt was justified (see later discussion).[21]

In 1992, Banks and coworkers[22] revealed that in addition to being Hy– and Jo(a–), Gy(a–) RBCs were Do(a–b–). Thus, it was shown that RBCs with the Gy(a–) phenotype are the null phenotype of the Dombrock blood group system.[23] After this discovery, Gy$^a$, Hy, and Jo$^a$ were assigned ISBT numbers in the Dombrock blood group system (Table 2).[17,18]

## Dombrock Glycoprotein

Antigens in the Dombrock blood group system are carried on a GPI-linked glycoprotein.[12,16,24] The Dombrock glycoprotein has an apparent $M_r$ of approximately 47,000 to 58,000 in SDS-PAGE under nonreducing conditions.[12,23] In the membrane-bound form, the Do glycoprotein has five potential *N*-linked glycosylation sites and four or five cysteine residues.[25] The susceptibility of some Do antigens to sulfhydryl compounds suggests that the tertiary conformation of the glycoprotein is dependent on disulfide bonds.

The Do glycoprotein is expressed primarily on erythroid cells in adult bone marrow and in fetal liver. Expression may also occur in the lymph nodes (on lymphocytes), testes, spleen, and fetal heart. Although the Do glycoprotein is a member of the mono-ADP-ribosyltransferase family, no enzyme activity has been demonstrated on the RBC. ADP-ribosyltransferases catalyze the transfer of ADP-ribose from $NAD^+$ to a specific amino acid in a target protein that modulates protein function. ADP-ribosylation can be reversed by ADP-ribosyl hydrolases, which remove the ADP-ribose and restore protein function.[26,27] Thus, Do may be involved in the regulation of cellular protein function.

## Dombrock Antigens

The characteristics of antigens in this system are summarized in Table 3. The susceptibility and resistance of antigens in the Dombrock system to treatment of RBCs with various proteolytic enzymes and DTT, and their absence from paroxysmal nocturnal hemoglobinuria (PNH) type III RBCs,[24] can be used to aid the identification of antibodies to Dombrock antigens.

**Table 3.** Characteristics of antigens in the Dombrock blood group system

| |
|---|
| Resistant to papain or ficin treatment of antigen-positive RBCs. Often the reactivity is enhanced. |
| Sensitive to trypsin treatment of antigen-positive RBCs. |
| Do$^a$, Do$^b$, Gy$^a$, Hy, DOYA, and DOMR are sensitive to DTT (200 mM) treatment of antigen-positive RBCs. Jo$^a$ is variably affected by such treatment. All antigens are resistant to treatment of antigen-positive RBCs with 50 mM DTT. |
| Weakened by α-chymotrypsin or AET treatment of antigen-positive RBCs. |
| Expressed on cord RBCs; although Gy$^a$, Hy, and Jo$^a$ may be weaker than on RBCs from adults. |
| Absent from PNH III RBCs. |
| Some variation in expression on different RBCs and on RBCs from different people. |
| Carried on a mono-ADP-ribosyltransferase (ART-4), a GPI-linked protein. |
| Do$^a$, Do$^b$, Hy, and Jo$^a$ are not highly immunogenic. |

AET = 2-aminoethylisothiouronium bromide; GPI = glycosylphosphatidylinositol; PNH = paroxysmal nocturnal hemoglobinuria.

## Dombrock Antibodies

Studies involving the Dombrock blood group system have been hampered by the paucity of reliable monospecific antisera. Antibodies in the Dombrock blood group system can be difficult to identify. This is especially true for the differentiation of anti-Hy from anti-Jo$^a$. As will be described later, determination of the molecular basis associated with Hy and Jo$^a$ has provided an explanation for this

particular difficulty. Common characteristics of antibodies to Dombrock blood group system antigens are summarized in Table 4.

**Table 4.** Characteristics of antibodies to Dombrock antigens

| |
|---|
| Usually IgG. |
| React optimally by column agglutination technology or by the IAT using papain- or ficin-treated RBCs. |
| Usually weakly reactive. |
| Do not bind complement. |
| Stimulated by pregnancy and by transfusion. |
| Usually present in sera containing other alloantibodies. The exception is anti-Gy$^a$, which often occurs as a single specificity. |
| Often deteriorate in vitro and fall below detectable levels in vivo. |
| Have not caused clinical HDFN (positive DAT only) but have caused transfusion reactions. |

HDFN = hemolytic disease of the fetus and newborn.

## Clinical Importance

Although transfusion reactions caused by anti-Do$^a$ or anti-Do$^b$ have been reported,[28–37] they may be underreported. One reason is that events usually associated with transfusion reactions may not be observed. For example, the DAT is often negative, no antibody is eluted from the patient's RBC samples after transfusion, there is no lag phase in the antibody reactivity, and no increase in titer of the antibody is observed. However, in our experience, the provision of Do(a–) or Do(b–) blood as predicted by DNA analysis has improved RBC survival in patients with the corresponding antibody who receive chronic transfusions. At least one anti-Hy has caused biphasic destruction of Hy+ RBCs[38]; other examples of anti-Hy, and anti-Gy$^a$ and anti-Jo$^a$, have caused moderate transfusion reactions. In the absence of Gy(a–) blood, Hy– blood has been a suitable substitute (personal observations). Some anti-Gy$^a$ appear to be benign: 10 Gy(a+) units were transfused to a man with anti-Gya without adverse consequences.[39] Antibodies in the Dombrock blood group system typically do not cause clinical HDN, although RBCs of some antigen-positive babies were positive in the DAT. One baby of a mother with anti-DOMR was born icteric and required phototherapy.[40]

## The *DO* Gene

For some time, it has been known that *DO* is located on the short arm of chromosome 12 (Fig. 1).[41] In silico analysis aided in identification of a candidate *DO* gene, which has been cloned and sequenced (GenBank accession number; AF290204).[25] *DO* is the first blood group gene to be cloned by an in silico approach.[42]

The *DO* gene is identical to *ART4*, described in 1997 (GenBank accession number X95826),[43] and has been renamed *DO* (GenBank accession number NM_021071, AF290204). The data sets for the chromosomal arms of *ART3* and *ART4* were inadvertently switched,[43] so *ART4* was incorrectly reported to reside on the long arm of chromosome 12. Had it not been for this switch, it is likely that *ART4* would have been recognized as the *DO* gene in 1997! Although the original publication for *ART4* did not include exon 1,[43] the entire sequence of *ART4* is identical to *DO* (F. Koch-Nolte, personal communication). Cloning and sequencing of *DO* allowed the determination of the molecular basis associated with various Do phenotypes (see later discussion) and led to PCR-based assays to screen for antigen-negative donors and identify new alleles.

The *DO* gene consists of three exons distributed over 14 kilobase pairs (kbp) of DNA (Fig. 1). The messenger RNA, which consists of 1.1 kbp, is predicted to encode a protein of 314 amino acids that has both a signal peptide and a GPI-anchor motif (Fig. 1).[25] Both these are cleaved and not present in the membrane-bound form of Do. The *DO*B* allele encodes an Arg-Gly-Asp (263-RGD-265) motif. RGD motifs within adhesive ligands are commonly involved in cell-to-cell interactions involving integrin binding.[44] However, because the *DO*A* allele, and the chimpanzee *DO* (*pDO*) homolog, encode asparagine (N) instead of aspartic acid (D),[25,45] it is unlikely that the RGD motif encoded by the *DO*B* allele is a critical one.[46]

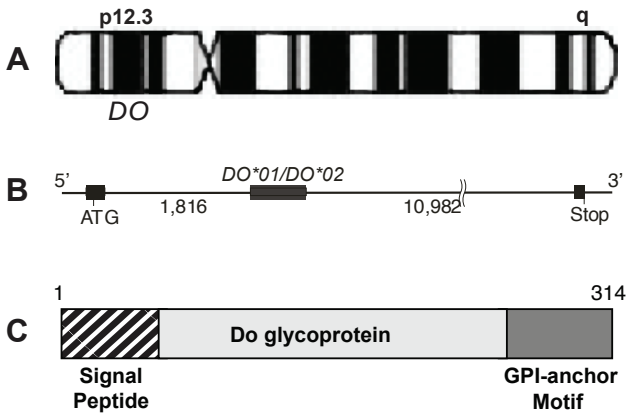


**Fig. 1** Diagram of *DO*, its organization, and the Do glycoprotein. (**A**) Location (12p12.3) of *DO* on the short arm of chromosome 12. (**B**) The organization of the three *DO* exons. (**C**) The Do protein with signal peptide at the amino terminus and glycosylphosphatidylinositol (GPI)-anchor motif at the carboxyl terminus.

## Molecular Basis of Antigens and Phenotypes

### The Polymorphic Do$^a$ (DO1) and Do$^b$ (DO2) Antigens

The common forms of *DO*A* and *DO*B* alleles differ in three nucleotide positions in exon 2. Two are silent nucleotide changes (378C>T, Tyr126Tyr; 624T>C, Leu208Leu); the third is a missense change (793A>G, Asn265Asp), which encodes, respectively, Do$^a$ and Do$^b$ (Table 5).[25] These three nucleotide changes can be readily differentiated by PCR-RFLP, using *Dra*III for 378C>T,[47] *Mnl*I for 624T>C,[47] and BSeRI for 793A>G.[49] Allele-specific PCR also can be used to differentiate *DO*A* from *DO*B*.[50] The ability to distinguish *DO*A* from *DO*B* makes it feasible to predict the Do type of patients and blood donors. This is a tremendous advantage

because, owing to the paucity of reliable reagents, screening for large numbers of Do(a–) or Do(b–) blood donors using classical hemagglutination methods has not been feasible.

**Table 5.** *DO* alleles defining phenotypes†

| Phenotype | ISBT allele name | Nucleotide change | Exon | Amino acid change |
|---|---|---|---|---|
| DO:1 or Do(a+) | *DO*01* or *DO*A* | | | |
| DO:2 or Do(b+) | *DO*02* or *DO*B* | 793A>G | 2 | Asn265Asp[25,47] |
| DO:–4 or Hy– | *DO*02.–04*‡ | 323G>T | 2 | Gly108Val[21] |
| DO:–5 or Jo(a–) | *DO*01.–05*‡ | 350C>T | 2 | Thr117Ile[21] |
| DO:–6 or DOYA– | *DO*01.–06*‡ | 547T>G | 2 | Tyr183Asp[48] |
| DO:–7 or DOMR– | *DO*02.–07*‡ | 431C>T; 432C>A | 2 | Ala144Glu[40] |

†Reference allele *DO*01* (AF290204; shaded) encodes Do$^a$, Gy$^a$, Hy, Jo$^a$, DOYA, and DOMR antigens.See Table 6 for alleles that result in the Gy(a–) phenotype.
‡ISBT proposed allele name pending ratification June 2010.

## The High-Prevalence Gy$^a$ (DO3) Antigen

An absence of Gy$^a$, in addition to an absence of all antigens in the Dombrock blood group system, defines the Do$_{null}$ [Gy(a–)] phenotype. To date, *DO* has been described to be silenced by five molecular bases (Table 6). Two of them, a mutation in the donor splice site[52] and a mutation in the acceptor splice site,[51] lead to outsplicing of exon 2. The third mechanism is a nonsense change in a *DO*A-HA* allele [350C; 378T (*DO*B*); 624T (*DO*A*); 793A (*DO*A*), see later section].[52] A fourth proband has a deletion of eight nucleotides within exon 2 that leads to a frameshift and a premature stop codon,[53] and the fifth is attributable to an amino acid substitution of Phe62 to Ser.[54]

**Table 6.** Molecular basis for Gy(a–) phenotype†

| Phenotype | Allele name | Nucleotide change | Exon | Amino acid change |
|---|---|---|---|---|
| DO:–3 or Gy(a–) | *DO*02N.01*§ | IVS1–2 a>g | 2 | Skips exon 2[51] |
| DO:–3 or Gy(a–) | *DO*02N.02*§ | IVS1 +2 t>c | 2 | Skips exon 2[52] |
| DO:–3 or Gy(a–) | *DO*01N.03*‡§ | 442C>T | 2 | Gln148Stop[52] |
| DO:–3 or Gy(a–) | *DO*01N.04*§ | 343de1343–350 | 2 | Frame-shift; premature; stop codon[53] |
| DO:–3 or Gy(a–) | *DO*01N.05*§ | 185T>C | 2 | Phe62Ser[54] |

†Changes from the reference allele (GenBank accession number AF290204) are given.
‡The background for this allele is actually *DO*A-HA* (378T, 524T, 793A).[55]
§ISBT proposed allele name pending ratification June 2010.

## The High-Prevalence Hy (DO4) Antigen

The nucleotide change associated with Hy+/Hy– phenotypes is 323G>T in exon 2, which is predicted to encode Gly108Val. The change is associated with the absence of Hy and is on an allele carrying 378C (*DO*A*), 624C (*DO*B*), and 793G (*DO*B*) (Table 5). Its association with 793G (265Asp) explains why RBCs with the Hy– phenotype are invariably Do(a–b+). There are two forms of the allele giving rise to the Hy– phenotype, one with 898G (300Val) and the other with 898C (300Leu). Nucleotide 898C (300Leu) is present on the allele encoding the wild-type Hy+. As the 898G allele was present in the sister of the original Hy– proband it was named *HY1*, and the *HY* allele with the wild-type nucleotide 898C, *HY2*.[21] Testing with one potent example of anti-Jo$^a$ showed that some Hy– RBCs express Jo$^a$ very weakly.[56]

## The High-Prevalence Jo$^a$ (DO5) Antigen

A single nucleotide change of 350C>T in exon 2 is predicted to encode isoleucine at amino acid residue 117. Nucleotide 350T is associated with the absence of the Jo$^a$ antigen and is predominantly on an allele carrying 378T (*DO*B*), 624T (*DO*A*), and 793A (*DO*A*) (Table 5). The genotype of people whose RBCs have the Jo(a–) phenotype can be *DO*JO/JO* or *DO*HY/JO*.[21] Its association with 793A (265Asn) explains why most RBCs with the Jo(a–) phenotype are Do(a+b–) (Fig. 2). RBCs from individuals with the *DO*HY/JO* genotype will type Do(a+b+$^w$).

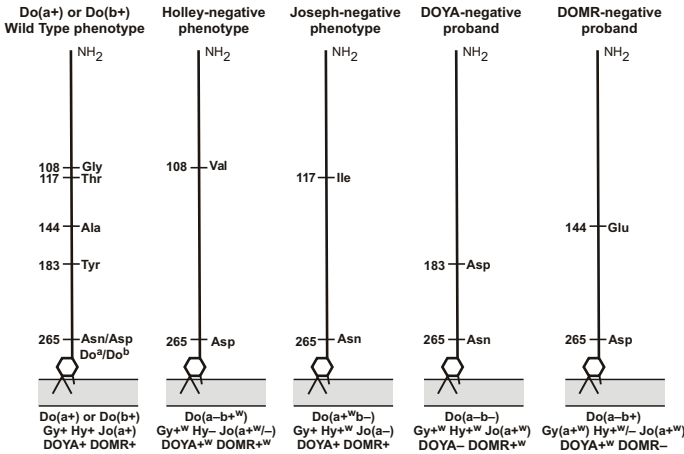


**Fig. 2** Diagram showing amino acids associated with the various Do phenotypes. The effect of the amino acid changes associated with Hy–, Jo(a–), DOYA–, and DOMR– phenotypes on the other Do antigens is given under each stick figure. The figures for the DOYA– and DOMR– phenotypes are each based on the only known proband.

## The Jc$^a$ Antigen

DNA analysis on four samples that had been originally typed as Jc(a–) revealed a combination of *DO*HY* and *DO*JO* alleles (*DO*HY1/HY1; DO*HY1/HY2; DO*HY1/JO; DO*JO/JO*). These results show that Jc$^a$ is not a discrete antigen.[21]

### The High-Prevalence DOYA (DO6) Antigen

The nucleotide change associated with DOYA+/DOYA– phenotypes is *DO*A.547T>G* in exon 2, which is predicted to change Tyr183 to Asp (Table 5). In the DOYA– Turkish Kurd proband, this change silenced the expected Do[a], so her RBCs typed Do(a–b–). They also have a weakened expression of Gy[a], Hy, and Jo[a] (Fig. 2).[57]

### The High-Prevalence DOMR (DO7) Antigen

The novel nucleotide changes associated with DOMR+/DOMR– phenotypes are 431C>A and 432C>A, which are predicted to encode Ala(GCC)144Glu(GAA) (Table 5). The change is associated with the absence of the DOMR antigen and is on a DO*B-WL allele (see later section). Its association with 793G (265Asp) explains why RBCs with the DOMR– phenotype are Do(b+), albeit weakly. RBCs from the DOMR– Brazilian Black proband have a weakened expression of Gy[a], Hy, and Jo[a] (Fig. 2).[40]

### Other *DO* Alleles

Testing of DNA from Blacks from Brazil or the Congo led to the recognition of additional *DO* alleles. These are *DO*A-HA*,[55] *DO*A-SH*,[58] *DO*A-WL*,[59] *DO*B-SH*,[55] *DO*B-WL*,[58] *DO*B-SH-Q149K*,[60] and *DO*B-I175N* (it is possible that this last allele could be on a *DO*B-WL* background) (Table 7).[60] The initials used in these allele names are derived from the initials of investigators who reported them. Information is lacking about the nature of the Do glycoprotein or antigenic expression, if any, encoded by these alleles. These alleles are not known to encode novel antigens.

Chapel-Fernandes and coworkers[60] measured the expression of various Do proteins on transduced K562 cells by flow cytometry using monoclonal anti-Do. They report that the protein encoded by *DO*B-SH, DO*B-WL*, and *DO*B-SH-Q149K* alleles was expressed in lower copy number than the protein encoded by *DO*B* or *DO*B-I175N* alleles. This finding may provide insights into the variable expression of Do[b] on RBCs.

### *DO* and Anthropology

The chimpanzee *DO* allele has the human wild-type nucleotides at positions 323, 350, and 898 and has 378T (*DO*B*), 624C (*DO*B*), and 793A (*DO*A*).[21] This sequence has not been found in humans and does not provide an insight as to the primordial *DO* gene. The sequence of the allele from three unrelated chimpanzees was identical (GenBank accession numbers AF373016, AF373017, and AF374727).

### Transfection of *DO* cDNA

Cells transfected with *DO* cDNA have been used to study expression levels of Do.[25,60] Cells transfected with *DO* cDNA have also been used as an immunogen to produce several monoclonal antibodies (MoAbs). Two MoAbs, MIMA-52 that recognizes a DTT-sensitive epitope and MIMA-53 that recognizes a DTT-resistant epitope, strongly agglutinate RBCs from humans [except Gy(a–)] and other great apes but not from lesser apes, old world monkeys, new world monkeys, prosimians, rabbits, dogs, sheep, or mice.[45] Three other MoAbs (MIMA-64, MIMA-73, and MIMA-98) agglutinated RBCs from humans, chimpanzees, greater apes, and

**Table 7.** *DO* alleles, including some that were described only at the DNA level

| Allele name | | nt (aa) | nt (aa) | Nt | nt | nt (aa) | nt (aa) | nt (aa) |
|---|---|---|---|---|---|---|---|---|
| ISBT | Other | 323 (108) | 350 (117) | 378 | 624 | 793 (265) | 898 (300) | Other |
| *DO*01* | *DO*A* | G (Gly) | C (Thr) | C | T | A (Asn) | C (Leu) | |
| *DO*01.–05* | *DO*JO* | G (Gly) | T (Ile) | T | T | A (Asn) | C (Leu) | |
| *DO*01.–06* | *DOYA* | G (Gly) | C (Thr) | C | T | A (Asn) | C (Leu) | 547T>G (Tyr183Asp) |
| | *DO*A-HA*† | G (Gly) | C (Thr) | T | T | A (Asn) | C (Leu) | |
| | *DO*A-SH*† | G (Gly) | C (Thr) | C | C | A (Asn) | C (Leu) | |
| | *DO*A-WL*† | G (Gly) | C (Thr) | C | T | A (Asn) | G (Val) | |
| *DO*02* | *DO*B* | G (Gly) | C (Thr) | T | C | G (Asp) | C (Leu) | |
| *DO*02.–04* | *DO*HY1* | T (Val) | C (Thr) | C | C | G (Asp) | G (Val) | |
| *DO*02.–04* | *DO*HY2* | T (Val) | C (Thr) | C | C | G (Asp) | C (Leu) | |
| *DO*02.–07* | *DOMR* | G (Gly) | C (Thr) | T | C | G (Asp) | G (Val) | 431C>A and 432C>A (Ala144Glu) |
| | *DO*B-SH*† | G (Gly) | C (Thr) | C | C | G (Asp) | C (Leu) | |
| | *DO*B-SH-Q149K*† | G (Gly) | C (Thr) | C | C | G (Asp) | C (Leu) | 445C>A (Gln149Lys) |
| | *DO*B-WL*† | G (Gly) | C (Thr) | T | C | G (Asp) | G (Val) | |
| | *DO*B-I175N*† | G (Gly) | C (Thr) | T | C | G (Asp) | C (Leu) | 524T>A (Ile175Asn) |

†Alleles defined only through DNA-based assay; not investigated serologically.
aa = amino acid; nt = nucleotide.

lesser apes but not RBCs from the other animals (unpublished observations). A homolog of the DO has been found in mouse,[43] and thus the lack of reaction with mouse RBCs indicates that the specific epitope recognized by the MIMA anti-Do is restricted to apes.[61]

## Expression of Dombrock Antigens

The molecular basis associated with Hy– and Jo(a–) phenotypes was determined only after numerous samples were analyzed. During this analysis, it became clear that RBC samples had been misidentified. The most significant was that of SJ after whom the Joseph phenotype and Jo$^a$ antigen were named. Surprisingly, DNA from SJ typed *DO*HY1/HY2* and DNA from her Hy+ brother was *DOHY2/DO*B*.[21] Thus, any "anti-Jo$^a$" that was identified by using SJ RBCs may actually be anti-Hy. The use of such RBCs and antibodies as reagents has led to the inadvertent incorrect labeling of reagents. To correctly identify anti-Hy and anti-Jo$^a$, RBCs whose type has been predicted by DNA analysis should be used. The various possible combinations and expected antigen expression are given in Table 7. RBCs from people with *DO*JO/JO* or *DO*JO/HY* genotypes will have the same phenotype, although the latter may have a slightly weaker expression of Hy.

The close proximity of amino acids associated with Hy (residue 108) and Jo$^a$ (residue 117) antigen expression may explain why Hy– RBCs lack or have an extremely weak expression of Jo$^a$ and why Jo(a–) RBCs have a weak expression of Hy (Fig. 2). The two critical residues are separated by only eight amino acids, which is within the range of an antigenic determinant.[62,63] The reason for the weak expression of Gy$^a$ on Hy– RBCs, the weak expression of Do$^b$ on RBCs with the Hy– phenotype, and the weak expression of Do$^a$ on RBCs with the Jo(a–) phenotype is still not understood but likely involves conformation or the effect of steric hindrance or charge on the conformation. Interestingly, the amino acid changes involved with an absence of DOYA or DOMR also weaken other Do antigens (Fig. 2).

It is apparent that the immune response in different people with the same Dombrock phenotype varies. One possibility is that Hy– patients may produce anti-Do$^a$ in addition to anti-Hy, and Jo(a–) patients may produce anti-Do$^b$ in addition to anti-Jo$^a$. Furthermore, a patient with a Jo(a–) phenotype and a *DO*HY/JO* genotype should have RBCs that type Do(a+$^w$b+$^w$) Gy(a+) Hy(a+$^w$) Jo(a–) and thus would be expected to make only anti-Jo$^a$. The results of DNA analysis of serologically identified samples with unusual Do phenotypes provide an explanation for the diversity of typing results in antibody producers and for the diversity of the reactivity of their plasma and serum of their immune response.

## Conclusions

Determination of the molecular basis underlying the antigens in the Dombrock blood group system has several advantages and has changed practice in transfusion medicine. The first, as outlined earlier, is the possibility of using RBCs with a bona fide antigen profile in antibody identification studies. The second advantage is to predict the phenotype of patient RBCs to aid in the antibody identification process; the third is the ability to type donors for *DO*A* and *DO*B* and thereby select Do(b–) RBCs for transfusion to a patient who has or has had anti-Do$^b$. Yet another value is to be able to type donors whose RBCs are used in antibody identification panels.[64] This is, perhaps, the first instance in which DNA analysis is more reliable than hemagglutination. This is because the antibodies (especially anti-Do$^a$ and anti-Do$^b$) are rarely available as a single specificity with strength and volume to make accurate typing possible.

There is no known disease entity associated with the Do$^a$ form of the Dombrock glycoprotein (RGD→RGN) nor, indeed, with an absence of the entire glycoprotein [Gy(a–)]. The presence of RGN in the chimpanzee homolog[21] suggests that this, and not RGD, may be the primordial sequence. Expression of Dombrock and other ectoenzymes (Kell and Yt) on RBCs may provide a readily transportable steady-state level of these enzymes for tissues in the vascular space.

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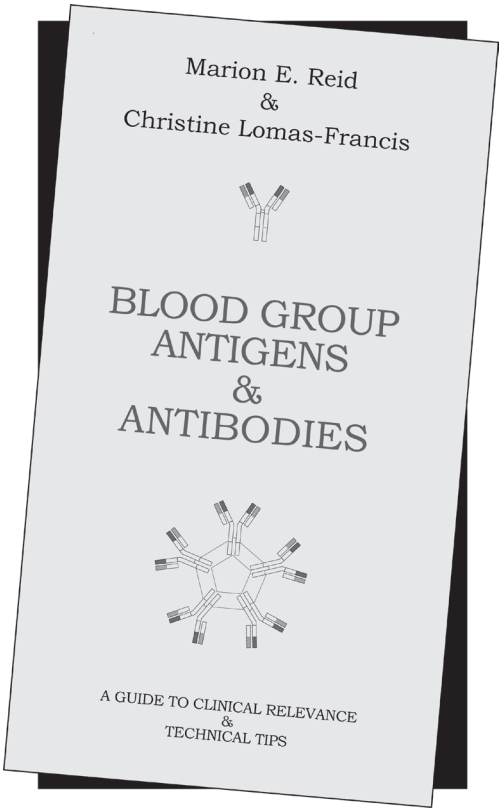


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Revised August 2007

# Immunohematology
Journal of Blood Group Serology and Education


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2. Abstract
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4. Acknowledgments
5. References
6. Author information
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8. Figures

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      Selection and number of subjects, samples, items, etc. studied and description of appropriate controls, procedures, methods, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer's name, city, and state. Do not use patient's names or hospital numbers.
   d. Results
      Presentation of concise and sequential results, referring to pertinent tables and/or figures, if applicable
   e. Discussion
      Implication and limitations of the study, links to other studies; if appropriate, link conclusions to purpose of study as stated in introduction
5. Acknowledgments: *Acknowledge those who have made substantial contributions to the study, including secretarial assistance; list any grants.*
6. References
   a. In text, use superscript, Arabic numbers.
   b. Number references consecutively in the order they occur in the text.
7. Tables
   a. Head each with a brief title; capitalize the first letter of first word (e.g., Table 1. Results of . . .) use no punctuation at the end of the title.
   b. Use short headings for each column needed and capitalize first letter of first word. Omit vertical lines.
   c. Place explanation in footnotes (sequence: *, †, ‡, §, ¶, **, ††).
8. Figures
   a. Figures can be submitted either by e-mail or as photographs (5″ × 7″ glossy).
   b. Place caption for a figure on a separate page (e.g. Fig. 1 Results of…), ending with a period. If figure is submitted as a glossy, place first author's name and figure number on back of each glossy submitted.
   c. When plotting points on a figure, use the following symbols if possible: ○ ● △ ▲ □ ■.
9. Author information
   a. List first name, middle initial, last name, highest degree, position held, institution and department, and **complete** address (including ZIP code) for **all** authors. List country when applicable.

## III. EDUCATIONAL FORUM

**A. All submitted manuscripts should be approximately 2000 to 2500 words with pertinent references. Submissions may include:**

1. An immunohematologic case that illustrates a sound investigative approach with clinical correlation, reflecting appropriate collaboration to sharpen problem solving skills
2. Annotated conference proceedings

**B. Preparation of manuscript**

1. Title page
   a. Capitalize first word of title.
   b. Initials and last name of each author (no degrees; all CAPs)
2. Text
   a. Case should be written as progressive disclosure and may include the following headings, as appropriate
      i. Clinical Case Presentation: *Clinical information and differential diagnosis*
      ii. Immunohematologic Evaluation and Results: *Serology and molecular testing*
      iii. Interpretation: *Include interpretation of laboratory results, correlating with clinical findings*
      iv. Recommended Therapy: *Include both transfusion and nontransfusion-based therapies*
      v. Discussion: *Brief review of literature with unique features of this case*
      vi. Reference: *Limited to those directly pertinent*
      vii. Author information (see II.B.9.)
      viii. Tables (see II.B.7.)

## IV. LETTER TO THE EDITOR

**A. Preparation**

1. Heading (To the Editor)
2. Title (first word capitalized)
3. Text (written in letter [paragraph] format)
4. Author(s) (type flush right; for first author: name, degree, institution, address [including city, state, Zip code and country]; for other authors: name, degree, institution, city and state)
5. References (limited to ten)
6. Table or figure (limited to one)

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