Complexities of the Dombrock blood group system revealed

Marion E. Reid

The Doa antigen was discovered after I began my career in immunohematology and I have been fortunate to be involved in several fascinating discoveries in the Dombrock blood group system. The Doa antigen and its antithetical antigen, Do, have a prevalence that makes them useful as genetic markers. The paucity of reliable anti-Doa and anti-Do has prevented this potential from being realized; however, our ability to type for DO alleles at the DNA level has made it possible to test cohorts from different populations. In 1992, the Dombrock blood group system was expanded to include three phenotypically related antigens, Gy, Hy, and Jo, when it was discovered that the Gy(a−) phenotype was the null of the Dombrock system. Based on the knowledge that the Dombrock glycoprotein is attached to the RBC membrane via a glycosylphosphatidylinositol linkage and subsequent to the assignment of the corresponding gene to the short arm of chromosome 12, expressed sequence tags from terminally differentiating human erythroid cells were analyzed in silico to identify the DO gene. This allowed determination of the molecular basis of the various Do phenotypes and the realization that DO is identical to the gene encoding a mono-ADP-ribosyltransferase, ART4. No enzymatic activity in RBCs has been demonstrated and the function of this glycoprotein, on the outside surface of RBCs, has yet to be determined. This review is a synthesis of our current knowledge of the Dombrock blood group system.

ABBREVIATIONS: GPI = glycosylphosphatidylinositol; NBF = National Blood Foundation; SNP(s) = single-nucleotide polymorphism(s).

From the New York Blood Center, New York, New York.

Address reprint requests to: Marion E. Reid, PhD, Director, Immunohematology Laboratory, New York Blood Center, 310 East 67th Street, New York, NY 10021; e-mail: mreid@nybloodcenter.org.

This work was funded in part by a NIH Specialized Center of Research (SCOR) grant in transfusion medicine and biology (HL54459).


TRANSFUSION 2005;45:92S-99S.

The application for a National Blood Foundation (NBF) grant was my first experience in writing a grant in the National Institute of Health (NIH) format. Going through the process of thinking, writing, submitting, receiving, and reporting a NBF grant (NBF 93-11) provided me with the basis, confidence, and some preliminary data with which to apply for NIH funding. Through the NIH-Specialized Center of Research (SCOR) grant mechanism, my research has been funded since the beginning of 1995. The focus of my research has been, and still is, to understand blood group antigens and their interactions with the corresponding antibody to improve antibody identification and provision of antigen-negative RBC components to patients with alloantibodies to blood group antigens.

One blood group system that has been of special interest throughout my career, which also illustrates the value of different techniques for the advancement of knowledge, is the Dombrock system. Numerous colleagues have contributed to revealing the complexity of Dombrock. This review will summarize the understanding that has evolved as techniques were applied and includes classical hemagglutination (which showed certain characteristics of the antigens and antibodies, a relationship of Hy to Gy, and the fact that the Gy(a−) phenotype was the null of the Dombrock blood group system); immunoblotting (which 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 (which led to cloning of the gene); manual polymerase chain reaction (PCR)-based assay (which identified the single-nucleotide polymorphisms [SNPs] associated with the five antigens and provided a means to screen for antigen-negative donors); transfection and hybridoma technology (for production of monoclonal antibodies [MoAbs]); and microarray technology (which provides a means to perform high-throughput testing of donor blood and the identification of two new alleles).

CHARACTERISTICS OF DOMBROCK AS REVEALED BY HEMAGGLUTINATION

Some 20 years after the indirect antiglobulin test (IAT) was introduced for pretransfusion testing,1 anti-Doa was iden-
The antibody that recognizes the antithetical antigen, anti-Do\(^b\), was described 8 years later. These two antibodies define three phenotypes, the prevalence of which differs in various ethnicities (see Table 1). For populations other than white persons, 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 the Do\(^a\) antigen. Do\(^a\) and Do\(^b\) were placed in the Dombrock blood group system (DO; 014) by the International Society of Blood Transfusion (ISBT) Working Party on Terminology for Red Cell Surface Antigens in 1985.

The high-prevalence antigens Gregory (Gya) and Holley (Hy), which were described independently in 1967, were shown 8 years later to be phenotypically related. Red blood cells (RBCs) from Caucasian persons with the Gy(a–) phenotype are Hy–, and RBCs from black people of African descent with the Hy– phenotype are Gy(a\(^+\)w). Based on this observation, Gya, Hy, and Jo\(^a\) antigens were assigned ISBT numbers in the Dombrock blood group system (Table 2).

The high-prevalence antigen Jo\(^a\) was shown to have a phenotypic association with Gy\(^a\) and Hy antigens because RBCs with either the Gy(a–) phenotype or the Hy– phenotype are also Jo(a–). Another high-incidence antigen, Jc\(^a\), was shown to be associated with Gy\(^a\) and Hy but later shown not to be a discrete antigen.

It was not until 1992 that a remarkable discovery was made. In addition to being Hy– and Jo(a–), Gy(a–) RBCs were shown to be Do(a–b–). Thus, RBCs with the Gy(a–) phenotype are the null phenotype of the Dombrock blood group system. Upon this discovery, Gya, Hy, and Jo\(^a\) antigens were assigned ISBT numbers in the Dombrock blood group system (Table 2).

**SEROLOGIC CHARACTERIZATION OF DOMBROCK ANTIGENS**

The characteristics of Do antigens are summarized in Table 3. The susceptibility and resistance of antigens in the Dombrock system to various proteolytic enzymes, their sensitivity to treatment of RBCs with dithiothreitol (DTT), and their absence from paroxysmal nocturnal hemoglobinuria Type III RBCs can be used to aid the identification of corresponding antibodies.

**SEROLOGIC CHARACTERIZATION AND CLINICAL SIGNIFICANCE OF DOMBROCK ANTIBODIES**

Characteristics of antibodies to antigens in the Dombrock blood group system, which are summarized in Table 4, explain why they can be difficult to identify. The paucity
of reliable monospecific antisera hampered studies involving the Dombrock blood group system.

Although transfusion reactions attributed to anti-Do\textsuperscript{a} or anti-Do\textsuperscript{b} have been reported,\textsuperscript{23-32} they may be underreported. One reason is that events usually associated with transfusion reactions may not be observed. For example, the direct antiglobulin test (DAT) is often negative, antibody may not be eluted from the patient’s RBC samples after transfusion, the lag phase may be absent, and there may be no increase in titer of the antibody.

At least one anti-Hy has caused biphase destruction of Hy+ RBCs;\textsuperscript{33} other examples of anti-Hy (and also anti-Gy\textsuperscript{a} and anti-Jo\textsuperscript{a}) have caused moderate transfusion reactions. Antibodies in the Dombrock blood group system have not been reported to cause clinical hemolytic disease of the newborn (HDN) although RBCs of some antigen-positive babies were positive in the DAT.

**PROPERTIES OF THE DOMBROCK GLYCOPROTEIN REVEALED BY IMMUNOBLOTTING**

Immunoblotting showed that Gy\textsuperscript{a}, Hy, and Jo\textsuperscript{a} antigens are located on the same glycoprotein.\textsuperscript{34,35} Jo\textsuperscript{a} was assigned a number in the ISBT Series of High Incidence antigens before its association with Gy\textsuperscript{a} and Hy was realized.\textsuperscript{7} The antigen bypassed joining the Gregory collection to be assigned a number in the Dombrock system. When it was recognized that the Gy(a–) phenotype was the null of the Dombrock system,\textsuperscript{19} the Gregory glycoprotein was renamed the Dombrock (Do) glycoprotein.

The Do glycoprotein has an apparent M\textsubscript{r} of approximately 47,000 to 58,000 in sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions\textsuperscript{19,34} and is attached to the RBC membrane by a GPI linkage.\textsuperscript{22,34,35} It has several potential N-linked glycosylation sites, and four or five cysteine residues in the membrane-bound protein.\textsuperscript{36} The susceptibility of Do antigens to sulfydryl compounds suggests that the tertiary conformation of the glycoprotein is dependent on disulfide bonds.

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

- Usually IgG
- React optimally by column agglutination technology or by the IAT with 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, with the exception of anti-Gy\textsuperscript{a}, which can occur as a single specificity
- Often deteriorate in vitro and fall below detectable levels in vivo
- Have not caused clinical HDN (positive DAT only) but have caused transfusion reactions

**IN SilICO ANALYSIS AND CLONING THE GENE ENCODING THE DOMBROCK GLYCOPROTEIN**

Based on the knowledge that the Do glycoprotein is attached to the RBC membrane via a GPI linkage, and on the assignment of the DO to the short arm of chromosome 12,\textsuperscript{37} expressed sequence tags from terminally differentiating human erythroid cells were analyzed in silico. A candidate gene, DOK1, was detected within a BAC clone (GenBank Accession Number AC007655) and, by a series of experiments, was proven to be the gene that encodes the Do glycoprotein\textsuperscript{36} (GenBank Accession Number AF290204). The DOK1 gene is identical to the ART4 gene (GenBank Accession Number X95826)\textsuperscript{38} and has been renamed DO (GenBank Accession Number XM_017877). The Do glycoprotein (ART4) is a member of the mono-ADP-riboseyltransferase family, but has no demonstrable enzyme activity on the RBC.\textsuperscript{39,40}

The DO gene consists of three exons distributed over 14 kb of DNA. The messenger RNA is predicted to encode a protein of 314 amino acids that has both a signal sequence and a GPI-anchor motif.\textsuperscript{36} Computer prediction programs suggested that Do glycoprotein has a signal peptide of 44 amino acids,\textsuperscript{36} which would be unusually long. The possibility exists that initiation occurs at the second AUG and that Met22 to Thr44 is the signal peptide (G. Cross, personal communication). Indeed, Met1 is not conserved in the mouse, whereas Met22 is conserved.\textsuperscript{10,41} In either case, the N-terminal amino acid in the membrane-bound protein is predicted to be Glu45. The predicted GPI-anchor motif of 17 amino acids (residues 298-314)\textsuperscript{36} is less than the minimum length found in other GPI-anchored proteins in mammalian cells.\textsuperscript{42-45} and it is possible that the C-terminal 30 amino acids (residues 285-314) forms the GPI-anchor motif (G. Cross, personal communication). Thus, the membrane-bound Dombrock glycoprotein is likely to consist of 240 amino acids (residues 45-284) rather than 253 amino acids (residues 45-297) (Fig. 1).

In this review, the numbering used by Gubin and coworkers\textsuperscript{26} is used, that is, nucleotide 1 is the A of the first AUG initiation codon and amino acid 1 is the first methionine.

**MANUAL PCR-BASED ASSAYS REVEAL SNPs AND PROVIDE A MEANS TO FIND ANTIGEN-NEGATIVE BLOOD**

The antithetical antigens, Do\textsuperscript{a} and Do\textsuperscript{b}

The DOA and DOB alleles differ at three nucleotide positions. Two are silent mutations (378C>T; 1261yr and 624T>C; 208Leu) and one is a missense mutation (793A>G;
Asn265Asp), which encodes, respectively, Do\textsuperscript{a} and Do\textsuperscript{b} (Table 5 and Fig. 2). These three mutations each can be readily differentiated by PCR-restriction fragment length polymorphisms (RFLP), with \textit{Dra}\textsubscript{III} for 378C>T, \textit{Mnl}I for 624T>C,\textsuperscript{46} and \textit{BSe} RI\textsuperscript{47} for 793A>G, or by allele-specific PCR.\textsuperscript{48}

The ability to distinguish \textit{DOA} from \textit{DOB} makes it feasible to type patients and blood donors. This is a tremendous advantage, because owing to the paucity of reliable reagents, screening for large numbers of Do(a\textsuperscript{–}) or Do(b\textsuperscript{–}) blood donors by hemagglutination has not been possible. PCR-RFLP analysis has shown that previously typed Do(a+b\textsuperscript{–}) donors have both \textit{DOA} and \textit{DOB} alleles. Although RBCs from some of these donors have been shown to have Do(a+b\textsuperscript{+}) RBCs, others do not express Do\textsuperscript{b}. Based on these results, the Do\textsuperscript{b} antigen may have a slightly higher prevalence than reported. It has been observed by this author that provision of Do(b\textsuperscript{–}) blood as determined by DNA analysis has improved RBC survival in patients with anti-Do\textsuperscript{b}.

**The high-prevalence Hy antigen**

The nucleotide change associated with Hy+/Hy– is 323G>T, which is predicted to encode Gly108Val. The change is associated with the absence of the Hy antigen and is on an allele carrying 378C (\textit{DOA}), 624C (\textit{DOB}), and 793G (\textit{DOB}). Its association with 793G (265Asp) explains why RBCs with the Hy– phenotype are invariably Do(a–b\textsuperscript{+}) (Fig. 2). There are two forms of the allele encoding the Hy– phenotype, one with 898G (named \textit{HY1} because it was found in the sister of the original Hy– proband) encoding 300Val and the other with 898C (named \textit{HY2}) encoding 300Leu (which is present on Hy+ wild type).\textsuperscript{17} Table 5).

**The high-prevalence Jo\textsuperscript{a} antigen**

A single-nucleotide change of 350C>T is predicted to encode isoleucine at amino acid residue 117. Nucleotide 350T is associated with the absence of the Jo\textsuperscript{a} antigen and is on an allele carrying 378T (\textit{DOB}), 624T (\textit{DOA}), and 793A (\textit{DOA}) (Table 5; Fig. 2). The genotype of people whose RBCs have the Jo(a\textsuperscript{–}) phenotype can be \textit{JO/JO} or \textit{HY/JO}.\textsuperscript{17}

**Gy(a–) or Do\textsubscript{null} phenotype**

To date, four molecular bases causing silencing of the \textit{DO} have been described. Two of them, a mutation in the donor splice site\textsuperscript{49} and a mutation in the acceptor splice site,\textsuperscript{50} lead to outsplicing of exon 2. A third proband has a deletion of eight nucleotides within exon 2 that leads to a frameshift and a premature stop codon\textsuperscript{51} and the fourth mechanism is a nonsense mutation in a novel allele [350C; 378T (\textit{DOB}); 624T (\textit{DOA}); 793A (\textit{DOA})] that has been named \textit{GY5}\textsuperscript{52} (Table 6). There is no known pathology associated with the Do\textsuperscript{a} form of the Dombrock glycoprotein, which has RGN in place of RGD (a motif within adhesion ligands that is commonly involved in cell-to-cell interactions involving integrin binding). Furthermore, no pathology has been observed with an absence of the entire Do glycoprotein [Gy(a–)].
TRANSFECTION AND HYBRIDOMA TECHNOLOGY

The ability to transfect cells with DO cDNA provided a tool to immunize mice as the first step to production of MoAbs to Dombrock antigens.\textsuperscript{52,53} Although MoAb anti-Do\textsuperscript{b} is useful to screen for Do(b–) blood donors, it has the same limitations as human anti-Do\textsuperscript{b}, and it is recommended to confirm the type by DNA-based analyses. MoAb anti-Do recognizes epitopes on RBCs from humans and other apes but not from monkeys, rabbits, dogs, sheep, or mice.\textsuperscript{53} These MoAbs anti-Do have also made it possible for the Do glycoprotein to be assigned the cluster of differentiation number CD297.\textsuperscript{54}

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

<table>
<thead>
<tr>
<th>Molecular basis</th>
<th>Associated allele</th>
<th>Proband</th>
</tr>
</thead>
<tbody>
<tr>
<td>a→g in an acceptor splice site in intron 2, leading to skipping of exon 2</td>
<td>DOB</td>
<td>GY1, GY2, GY3\textsuperscript{a}</td>
</tr>
<tr>
<td>t→c in donor splice site in intron 2, leading to skipping of exon 2</td>
<td>DOB</td>
<td>GY4\textsuperscript{c}</td>
</tr>
<tr>
<td>Deletion of nucleotides 343-350 in exon 2, frameshift and premature stop codon</td>
<td>DOA</td>
<td>H\textsuperscript{f1}</td>
</tr>
<tr>
<td>442C&gt;T in exon 2, Gln148Stop</td>
<td>GY5</td>
<td>GY5\textsuperscript{b}</td>
</tr>
</tbody>
</table>

MICROARRAY TECHNOLOGY

The ability to detect SNPs on microarrays or bead arrays provides a means to revolutionize the way we provide antigen-negative blood in transfusion medicine. This approach not only provides high-throughput analysis of multiple SNPs on one chip but also electronically downloads the massive amount of data generated directly to a database.\textsuperscript{55-58} This eliminates two labor-intensive steps that are currently being used for the identification of antigen-negative blood donors. In testing DNA samples from distinct ethnic groups (69 Ashkenazi Jew, 58 Chinese, 188 Israeli, 100 Thai, and 218 predominantly black African American New York donors) with a BeadChip array that included probes for five DO SNPs, two new DO alleles were found. One, DOB-SH, is the same as HY at nucleotides 378, 624, and 793 but has wild-type nucleotide 323 and is predicted to express Do\textsuperscript{b} antigen (the appropriate alleles, i.e., DOA/DOB-SH or DOB-SH/DOB-SH, have not yet been found and, thus, testing has not been possible). The other, DOA-HA, is the same as JO at nucleotides 378, 624, and 793 but has wild-type nucleotide 350 (Table 5; Fig. 1) and has been shown to express Do\textsuperscript{a} antigen.\textsuperscript{59} DOA-HA was found in all five groups, whereas DOB-SH was only found in African American persons. Even though the number of samples in each ethnic group was relatively small, the prevalences of the alleles found are given in Table 7 (the numbers for the New York donors is not given because they were selected and not random).\textsuperscript{59,60}

PERSPECTIVES

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\textsuperscript{a} antigen were named. Surprisingly, DNA from SJ is homozygous for the HY allele (HY1/HY2).\textsuperscript{17} Thus, any “anti-Jo\textsuperscript{a}” identified by use of SJ RBCs is more likely to be anti-Hy. The subsequent use of such RBCs and antibodies led to the inadvertent incorrect labeling of reagents. Some samples have two different rare alleles, which also leads to confusing serologic test results. For instance, whereas RBCs from people with the JO/JO or the JO/HY genotype
are Jo(a–), the latter would be expected to have a slightly weaker expression of the Hy antigen and to be Do(b+), albeit weakly. The various combinations of alleles and expected corresponding antigen expression are given in Table 8. It is becoming apparent that further complexities exist because people with, for example, HYIDOB have made anti-Hy and those with JOIDOB have made anti-Joa. To correctly identify anti-Hy and anti-Joa, as well as other Dombrock antibodies, it is of value to use reagent RBCs that have been typed by DNA analysis.

It is apparent that the immune response in different people with the same Dombrock phenotype varies. One explanation is that Hy– patients can produce anti-Doa in addition to anti-Hy, and Jo(a–) patients can produce anti-Do in addition to anti-Jo. In contrast, a patient with a HY/JO genotype, who would be expected to have RBCs that type Do(a+b+) Gy(a+) Hy(a+) Jo(a–) and would only be able to make anti-Joa. This helps to explain some of the observed variations in reaction strength when testing plasma and/or serum from people with the same apparent phenotype (see Table 7). The results of DNA analysis of unusual Do phenotypes, as defined by hemagglutination, has provided an explanation for the diversity of RBC typing results in antibody producers and for the diversity of the reactivity of antibodies in their plasma and/or serum.

The close proximity of amino acids associated with Hy (residue 108) and Jo (residue 117) antigen expression may explain why Hy– RBCs appear to lack the Joa antigen (or express it extremely weakly) 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.

In contrast, given the distance of Hy and Jo from, respectively, Do or Do, the reason for weak expression of Do on RBCs with the Hy– phenotype and the weak expression of Do on RBCs with the Jo(a–) phenotype is still not understood but presumably is due to conformation. The reason for the weak expression of Gy antigen on Hy– RBCs is also not understood.

Determination of the molecular basis underlying the antigens in the Dombrock blood group system has several advantages and can potentially change practice in transfusion medicine. The first is the possibility of use of RBCs with a bona fide antigen profile in antibody identification panels. The second advantage is to type patients to aid in antibody identification. Third is the ability to type donors for DOA and DOB and thereby, perhaps for the first time, to reliably select Do(b–) RBCs for transfusion to a patient who has or has had anti-Do. Because antibodies to antigens in the Do blood group system (especially anti-Do)
are rarely available as a single specificity with strength and volume to make accurate typing possible, this is, perhaps, the first instance in which DNA-based analyses are more reliable than hemagglutination.

ACKNOWLEDGMENTS

I am indebted to the numerous colleagues who have allowed me to be included in their studies that made it possible for me to be involved in unraveling the complexities of the Dombrock blood group system. They made it possible for me to be a part of the investigations with different technologies for more than 30 years (1974-2005). I am also grateful to the NBF for providing funding that helped me along the road to grant-funded research. I thank Christine Lomas-Francis for helpful comments and Robert Ratner for assistance in preparation of the manuscript and figures.

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