Molecular characterization of *GYPB* and *RH* in donors in the American Rare Donor Program

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Transfusion of patients with sickle cell disease (SCD) has been a challenge in clinical transfusion medicine, especially when the required donor RBCs must be U– and negative for high-prevalence Rh phenotypes (hr^B, hr^S). It is now possible to genotype donors to identify or confirm U^{var} and U– phenotypes, as well as Rh hr^B– and hr^S– phenotypes, and to characterize the different *RH* backgrounds found in these donors. In a preliminary study of donors registered in the American Rare Donor Program, twelve different *RH* backgrounds were identified in eighteen hr^B– or hr^S– donors. These results, summarized in the current report, confirm the heterogeneous nature of these phenotypes and are relevant for selection of donor units for patients with antibodies to high-prevalence Rh antigens. Not all phenotypically similar units will be compatible, and matching the Rh genotype of the donor to the patient is important to prevent further Rh sensitization. Most donors referred were hr^B– and carry at least one hybrid *RHD-CE(3-7)-D* gene that encodes a variant C antigen linked to *RHCE*ce*^{S} that encodes the VS+V– phenotype. Surprisingly, the majority of donors were heterozygous, some even carrying conventional alleles, suggesting that the loss of expression of the hr^B epitopes on RBCs is a dominant phenotype. Although antigen-matching of patients with SCD with donors for C, E, and K antigens has decreased the incidence of alloimmunization, some patients still become immunized to Rh antigens, indicating the units were not truly matched. RH genotyping can identify those patients with SCD who carry RH alleles that encode altered C, e, or D who are at risk for production of "apparent auto" and alloantibodies to Rh antigens. RH genotyping of alloimmunized patients with SCD, partnered with genotyping of donors, can identify compatible units that would also eliminate the risk of further Rh alloimmunization. ***Immunohematology* 2006;22:143–147.**

The American Rare Donor Program (ARDP) consists of more than 35,000 rare donors from the United States, Puerto Rico, and Milan, Italy.[1] The program has been effective in finding RBC units for alloimmunized patients with sickle cell disease (SCD), but supporting requests for units that are U– or negative for high-prevalence Rh phenotypes such as hr^B and hr^S is a challenge. When units with these phenotypes cannot be located in the United States, blood has been supplied from the rare donor program located in Durban, South Africa. This requires extensive resources for coordination and shipment of units and entails waiver of any infectious disease screening that does not parallel that performed in the United States. Because these phenotypes are more common in Blacks, the search for potential donors in the United States targets African American groups. However, the lack of well-characterized serologic reagents hampers screening efforts.

Genotyping is an important new tool, both for screening donors and for typing patients, to provide appropriate units for transfusion when antibodies to high-prevalence antigens are present. Within the Rh system, the clinically significant anti-hr^B and -hr^S have caused transfusion reactions and fatalities.[2] Transfusion in these situations is not straightforward because not all antibodies that are called anti-hr^B or -hr^S have identical specificities. Neither hr^B- nor hr^S- is associated with one specific genetic polymorphism. These phenotypes encompass multiple different Rh protein polymorphisms encoded by numerous *RHCE*ce* genes, and the structural determinants that define these specificities are not yet known.

An important consideration for transfusion in patients with antibodies to these high-prevalence antigens is that not all donors with these phenotypes will be compatible. Additionally, because they occur on very diverse Rh backgrounds,[3] the complete Rh genotype of the donor and patient should be considered to prevent further Rh sensitization.

With recent advances in genotyping techniques and knowledge of the molecular basis of expression of variant antigens, it is now feasible to genotype donors at the *GYPB* and *RH* loci to identify U– and Rh variant RBC donors. Here we propose an approach to genotype ARDP donors to identify or confirm U^{var} and U– phenotypes, and we illustrate the power of genotyping to confirm hr^B– or hr^S– RBC phenotypes and to characterize the different RH gene backgrounds found in these donors.

GYPB Testing to Characterize U– and U+^{var}

In Black ethnic groups, the S–s– phenotype is usually characterized by complete deletion of the *GYPB* gene and absence of the U antigen, but the S–s– phenotype is also associated with weak expression of U (U+^{var}). The antisera used for phenotyping are not always well characterized and S–s–U+^{var} donors may be misidentified as U–. This is problematic when searching for U– units for *GYPB*-deleted patients with anti-U. The *GYPB* gene is not deleted when the RBCs have a U+^{var} phenotype. The molecular basis of U+^{var} involves changes in or around exon 5 of the *GYPB* gene.[4] The *GYPB(P2)* allele has a splice site mutation in intron 5 (+5 g>t) that causes complete skipping of exon 5, while *GYPB(NY)* results from a point mutation (230C>T) in exon 5 that causes partial exon skipping. Testing for the presence of these mutations discriminates S–s–U– donors from U+^{var} donors. Molecular genotyping is encouraged to confirm the U status of donors entered into the ARDP registry as U– or as S–s–U+^{var}.

RH Testing to Characterize hr^{B}– and hr^{S}–

The two highly homologous genes *RHD* and *RHCE* encode proteins that carry D, and c or C, and E or e antigens, respectively. However, more than 50 Rh serologic specificities are known; these are the result of point mutations or hybrid *RHD* and *RHCE.*[5,6] More than 120 different *RHD* variants, and approximately 50 different *RHCE* variants, have been reported to date.[7,8] The numerous genetic polymorphisms potentially encode different antigenic forms of the Rh proteins. Altered C, e, or D antigens are not uncommon in patients with SCD. The altered C antigen is encoded by a hybrid *RHD-CE(3-7)-D* gene that does not encode D epitopes but encodes a C antigen that differs from that found in Europeans. Altered e antigen expression is encoded by many different genes more commonly found in Blacks,[2,6,9] and altered D antigen is associated with numerous gene mutations found in many different ethnic groups.[6,10] Altered Rh antigens are associated with the absence of the high-prevalence antigens hr^{B} and hr^{S} on RBCs.

To investigate the *RH* genes in potential hr^{B}– or hr^{S}– donors referred to the ARDP, DNA was isolated from WBCs. Fresh RBCs, if available, were tested with the anti-hr^{B}-like monoclonal antibody FOR-2E3.[3] Characterization of the RH genes was performed with a combination of PCR and RFLP, or allele specific (AS)-PCR techniques in addition to amplification and sequencing of RH-specific exons.[3,9,11,12] *RHD* zygosity testing was determined by direct detection of the hybrid Rhesus box.[13]

Summary

Table 1 summarizes the hr^{B} or hr^{S} status of the donor RBCs determined by the referring laboratory, the results of testing with the anti-hr^{B}-like FOR-2E3 monoclonal antibody by the American Red Cross National Reference Laboratory for Blood Group Serology (NRLBGS), and the Rh phenotype of the eighteen donors studied.

Fifteen of the donors were referred to the ARDP as apparent hr^{B}–, two as hr^{S}– (donors 6 and 18), and one was both hr^{B}– and hr^{S}– (donor 9). Fresh RBCs were available from 12 of the 18 donors for testing with anti-hr^{B}-like FOR-2E3 monoclonal antibody. RBCs from only two of the ten hr^{B}– donors tested did not react (donors 7 and 12). The RBCs from one apparent hr^{S}– donor (donor 6) and the hr^{B}–/hr^{S}– (donor 9) also did not react. In total, the monoclonal FOR-2E3 antibody gave negative reactions with only four of twelve donor RBCs that lack high-prevalence Rh antigens.

Six donors were D– and twelve were D+. RBCs from all donors phenotyped as C+, c+, E–, e+ with the exception of one, donor 9, whose RBCs were C– and also c+, E–, and e+.

Table 1. Results of anti-hr^{B} or -hr^{S} and FOR-2E3 monoclonal testing; the Rh phenotype for each donor is indicated

Donor	hr^{B}–/hr^{S}–	FOR-2E3	Rh phenotype
1	hr^{B}–	–	D– C+c+E–e+
2	hr^{B}–	–	D– C+c+E–e+
3	hr^{B}–	1+mf	D– C+c+E–e+
4	hr^{B}–	+w	D– C+c+E–e+
5	hr^{B}–	–	D– C+c+E–e+
6	hr^{S}–	0	D– C+c+E–e+
7	hr^{B}–	0	D+ C+c+E–e+
8	hr^{B}–	–	D+ C+c+E–e+
9	hr^{B}–/hr^{S}–	0	D+ C–c+E–e+
10	*hr^{B}–	2+	D+ C+c+E–e+
11	hr^{B}–	–	D+ C+c+E–e+
12	hr^{B}–	0	D+ C+c+E–e+
13	hr^{B}–	1+s	D+ C+c+E–e+
14	hr^{B}–	1+	D+ C+c+E–e+
15	hr^{B}–	–	D+ C+c+E–e+
16	hr^{B}–	1+s	D+ C+c+E–e+
17	*hr^{B}–	2+	D+ C+c+E–e+
18	hr^{S}–	2+	D+ C+c+E–e+

*sample was positive with one source and negative with another

Table 2. Summary of twelve different RH backgrounds identified in eighteen donors

	Donor	Rh phenotype	Genes		Total # of donors
			RHD	RHCE	
hr^B–	1, 2	D– C+c+E–e+	D-CE(3-7)-D D-CE(3-7)-D	*ce^S ce^S	2
	3, 4	D– C+c+E–e+	D-CE(3-7)-D D-CE(3-7)-D or Deleted D	ce^S ce	2
	5	D– C+c+E–e+	D-CE(3-7)-D Deleted D	ce^S ce (48C)	1
	6	D– C+c+E–e+	D-CE(3-7)-D Deleted D	ce^S ce	1
hr^B–	7	D+ C+c+E–e+	D-CE(3-7)-D DIII type 5	ce^S ce^S	1
	8	D+ C+c+E–e+	D-CE(3-7)-D D	ce^S ce^S	1
	10, 11, 12, 14	D+ C+c+E–e+	D-CE(3-7)-D DAU	ce^S ce	4
	13	D+ C+c+E–e+	D-CE(3-7)-D DAU	ce^S ce (48C)	1
	15	D+ C+c+E–e+	D-CE(3-7)-D DIVa type 1	ce^S ce	1
	16, 17	D+ C+c+E–e+	D-CE(3-7)-D D	ce^S ce	2
hr^B–/hr^S–	9	D+ C–c+E–e+	DAU-0 DAU-0	ceMO ceMO	1
hr^S–	18	D+ C+c+E–e+	DAR D	ceAR Ce	1

*ceS = 48C, 733G, 1006T

Table 2 shows the twelve different RH backgrounds identified in the eighteen donors. The hr^B– phenotype is associated with *RHCE*ce^S,* which carries 48C (16Cys), 733G (245Val), and 1006T (336Cys).[2] Four donors (1, 2, 7, and 8) were homozygous for the ce^S allele but the majority of the hr^B– donors (12) were heterozygous. A hybrid *RHD-CE(3-7)-D* gene encoding altered C expression is linked to the ce^S allele.[14] Indeed, the majority of the ce^S alleles were found with the hybrid *RHD-CE(3-7)-D* gene. However, in donors 7 and 8, *RHD*DIII* type 5 or conventional *RHD* was also found with the ce^S allele.

One of the predicted hr^S– donors (donor 6) had an RH genotype more consistent with a hr^B– phenotype, i.e., a hybrid *RHD-CE(3-7)-D* gene with ce^S. In addition, the RBCs did not react with the anti-hr^B-like FOR-2E3 monoclonal antibody. The hr^B– and hr^S– donor (donor 9) was homozygous 48C (16Cys) and 667T (223Phe), characteristic of *ceMO* alleles.[2] Donor 18 was heterozygous for *RHCE*ceAR* (16Cys, 238Val, 245Val, 263Gly, 267Lys, 306Val)15 and for a conventional *RHCE*Ce.*

All of the D– donors and ten D+, hr^B– donors had at least one *RHD-CE(3-7)-D* hybrid. The RBCs of all phenotyped as C+ but the samples genotyped as RHC– by multiplex PCR assay. RHC/c genotyping results are discordant in samples that carry the hybrid *RHD-CE(3-7)-D* gene that encodes an altered C antigen.

Only three of the donors had a conventional *RHD* (donors 8, 16, and 17). Seven had partial D, which included five DAU, one DIII type 5, and one DIVa type 1. Partial D was also present in the hr^S–/hr^B– donor 9, who was homozygous for DAU-0, and in the hr^S– donor 18 who had a DAR allele and a conventional *RHD.* DAR is known to be linked to the ceAR allele.[15]

Conclusion

This report summarizes the preliminary results of genotyping eighteen ARDP donors identified as negative for the high-prevalence Rh antigens hr^B or hr^S. Twelve different *RH* backgrounds were found in the eighteen donors phenotyped as hr^B– or hr^S–, confirming the heterogeneous nature of these phenotypes.[3]

The majority of donors referred were confirmed to be hr^B–. They all had at least one hybrid *RHD-CE(3-7)-D* gene encoding altered C linked to a ce^S allele that encodes the VS+ V– phenotype. These samples phenotyped as C+ but genotyped as C– by multiplex PCR in intron 2 because of the presence of the hybrid *RHD-CE(3-7)-D* gene. Only four of the sixteen hr^B– donors were homozygous for ce^S alleles associated with the hr^B– phenotype. The majority were heterozygous; some even carrying conventional alleles, suggesting that the loss of expression of the hr^B epitopes on RBCs is a dominant phenotype. The amino acid changes Leu245Val and Gly336Cys may exert a dominant influence on expression of Rh proteins in the cell membrane, despite the presence of conventional *RHCE*ce* (donors 3, 4, 6, 10, 11, 12, 14, 15, 16, and 17).

RBCs from the donor homozygous for $D^{DAU\text{-}0}ce^{MO}$/$D^{DAU\text{-}0}ce^{MO}$ were subsequently confirmed to be both hr^S– and hr^B– with six different anti-hr^S and six different examples of anti-hr^B. This is the first sample with this interesting phenotype to be characterized at the RH locus and the investigation of additional samples will give important insights into the structure of the high-prevalence Rh antigens.

Transfusion of patients with SCD represents a significant challenge in clinical transfusion medicine. SCD may be the single disease for which transfusion therapy may increase in the next decade as a result of the stroke prevention trial in sickle cell anemia.[16,17] Complications of chronic transfusion include iron overload and alloimmunization. The recent availability of oral iron chelation agents is predicted to make transfusion a more acceptable treatment option. To address the problem of alloimmunization, many programs transfuse patients with RBCs that are phenotype-matched for D, C, E, and K, and some programs attempt to supply RBCs from African American donors to SCD patients whenever possible. Although transfusion of antigen-matched units reduces the incidence of alloantibody production, some patients with SCD will still become sensitized to Rh antigens, indicating units were not truly Rh antigen matched. The prevalence in the sickle cell population of RH alleles that encode altered e, C, or D explains why these patients become immunized despite conventional antigen-matching.

The hr^B- and hr^S- donors are an important resource for the management of alloimmunized patients with SCD. With the use of RH genotyping, patients with SCD who are homozygous for variant alleles and who are at risk for production of "apparent auto" and alloantibodies to high-prevalence Rh antigens can now be identified. RH genotyping of these patients, partnered with RH genotyping of donors, would have a positive impact on patient care because it allows the selection of both compatible units and units that eliminate the risk of further Rh alloimmunization. This approach would also optimize the use of donations from members of minority groups.

Our goal is to characterize the *RH* genes in these rare donor units. Patients with SCD who make antibodies to high-prevalence Rh antigens will then be RH genotyped and blood for transfusion will be based on an RH "genetic" match. It is anticipated that the implementation of molecular genetic methods for transfusion in SCD will move transfusion practice into the age of molecular medicine.

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