TRANSFUSION MEDICINE

High prevalence of red blood cell alloimmunization in sickle cell disease despite transfusion from Rh-matched minority donors

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Key Points

- Rh serologic phenotype–matched transfusions from minority donors do not prevent all Rh alloimmunization in patients with SCD.
- Variant *RH* genes are common in patients with SCD and contribute to Rh alloimmunization and transfusion reactions.

Red blood cell (RBC) transfusion is a key treatment of patients with sickle cell disease (SCD) but remains complicated by RBC immunization. In the present study, we evaluated the effects of antigen matching for Rh D, C, and E, and K and transfusion from African American donors in 182 patients with SCD. Overall, 71 (58%) chronic and 9 (15%) episodically transfused patients were alloimmunized. Fifty-five (45%) chronic and 7 (12%) episodically transfused patients were Rh immunized. Of 146 antibodies identified, 91 were unexplained Rh antibodies, one-third of which were associated with laboratory evidence of delayed transfusion reactions. Fifty-six antibodies occurred in patients whose RBCs were phenotypically positive for the corresponding Rh antigen and 35 in patients whose RBCs lacked the antigen and were transfused with Rh-matched RBCs. High-resolution *RH* genotyping revealed variant alleles in 87% of individuals. These data describe the prevalence of Rh alloimmunization in patients with SCD transfused with phenotypic Rh-matched African American RBCs. Our results suggest that altered *RH* alleles in both the patients and in the donors contributed to Rh alloimmunization in this study. Whether *RH* genotyping of patients and minority donors will reduce Rh alloimmunization in SCD needs to be examined. (*Blood*. 2013;122 (6):1062-1071)

Introduction

The use of transfusion therapy for sickle cell disease (SCD) is increasing due to expanded indications, increased availability of erythrocytapheresis, and oral chelators to treat transfusional iron overload.[1] However, alloimmunization to red blood cell (RBC) blood group antigens remains a major complication for patients with SCD and often presents significant challenges in their medical management.[2,3] The incidence of alloimmunization in patients with SCD ranges from 7% to 47%, dependent on age, RBC exposures, and extent of antigen matching for blood groups other than ABO and RhD.[4-12] An estimated 4% to 11% of patients with SCD who receive transfusions develop overt delayed hemolytic transfusion reactions (DHTRs),[13-15] but mild DHTRs may be unrecognized.

Sensitization to Rh antigens (D, C, c, E, and e) and to K comprise the majority of the RBC antibodies encountered in SCD.[4,5,9] One major explanation of the high rates of alloimmunization is the disparate distribution of RBC antigens between donors primarily of European ancestry and patients with SCD primarily of African ancestry.[9] One strategy to decrease alloimmunization in SCD is provision of phenotype matched RBCs for C, E, and K antigens.[8,10,12,16] Transfusion with units from African American donors has also been suggested,[9,10] although an increase in production of antibodies to low incidence antigens present primarily in minority groups is predicted.[17] Phenotype matching for additional minor antigens in the Kidd, Duffy, and MNS systems reveal that more stringent matching results in lower alloimmunization rates,[6,18] but there is no standard of practice.[19]

The Rh system is a complex blood group system and includes >50 different serologic specificities.[20] The *RH* locus is comprised of 2 homologous genes, *RHD* and *RHCE*, which encode the D antigen and the CE antigens in various combinations (ce, cE, Ce, or CE), respectively. *RHD* and *RHCE* are inherited as haplotypes, and expression of the proteins is exclusive to erythrocytes. Genetic diversity of the *RH* locus has been revealed in the last decade, with >200 *RHD* and 80 *RHCE* alleles reported. These potentially encode variant or altered antigens due to amino acid changes in the Rh proteins.[3,21,22] The RBCs may lack common Rh antigenic epitopes or carry novel epitopes. Standard RBC antigen typing does not distinguish the presence of Rh variants, which are more prevalent in individuals of African ancestry. For example, *RHD* encodes the D antigen ("Rh positive"), but individuals with altered *RHD* encoding partial D, defined as missing some D epitopes, may form anti-D when exposed to conventional D antigen.[23-26] In contrast, individuals with altered *RHD* encoding weak D, defined as expressing a reduced amount of D antigen but not lacking epitopes, are not typically at risk for anti-D.[20,27] Variant *RHCE* alleles are also prevalent in individuals of African descent, with alleles encoding partial e antigen most often encountered.[24,28-32]

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A frequent variant allele in this ethnic group is the hybrid *DIIIa-CE(4-7)-D* gene, in which several *RHCE* exons have replaced the corresponding region of *RHD*.[33] This allele encodes a partial C antigen and does not encode D antigen. RBCs are phenotypically C+, but individuals with this allele are at risk for anti-C when exposed to C antigen encoded by a conventional allele.[29]

This study was undertaken to assess the effects of antigen matching for D, C, E, and K and transfusion primarily from African American donors on alloimmunization rates, antibody specificity, and clinical significance in pediatric and young adult patients with SCD. The association of inherited *RH* genotypes with antibody production in patients on chronic and episodic transfusion protocols was investigated.

Methods

Study design

This study is a 15-year retrospective analysis of patients with SCD at the Comprehensive Sickle Cell Center (CSCC) at the Children's Hospital of Philadelphia (CHOP), transfused with RBCs that were prospectively matched for D, C, E, and K and were primarily from African American donors. Molecular analyses were performed between 2009 and 2012.

Transfusion protocol

Since 1994, the institutional policy for transfusion of patients with SCD has been to antigen match prospectively for D, C, E, and K antigens; patients whose RBCs lack the antigen are transfused with antigen-negative donor units. Efforts to provide ethnically matched RBC units began in 1997 through the "Blue Tag" Program consisting of donors who self-identify as African American and designate their donation to support children with SCD, although patients are not assigned to specific individual donor(s). Transfusions were mostly from African American donors, with some exceptions related to patient-specific antibodies rather than supply. These included patients with anti-D, for whom antigen-negative donors are primarily found in whites, and occasional patients alloimmunized to multiple antigens needing urgent transfusion. Patients received hemoglobin S-negative, leukocyte-reduced, and irradiated RBCs <21 days old. Patients on a chronic transfusion program had a target pretransfusion percent hemoglobin S of 30% or 50%, depending on the indication.

Patient population

In accordance with the Declaration of Helsinki, patients were enrolled after informed consent under a protocol approved by the Institutional Review Board at the Children's Hospital of Philadelphia to review medical records and retain patient samples to study alloimmunization. Clinical records were reviewed from patient birth to June 1, 2012. By parental history, all patients were of African descent. Subjects were interviewed at enrollment to determine whether they received all their transfusion support at our facility. Additionally, because >99% of RBC units in this cohort were transfused on an outpatient basis every 3 to 5 weeks for chronic transfusion therapy, the patient history was reviewed for missed appointments or laboratory values reflecting possible off-program transfusion. With the exception of 1 patient, no alloimmunized patients were transfused outside our institution for the 12 months preceding new antibody detection.

Twenty-five of the 123 chronic and 7 of 59 episodically transfused patients received ≥1 transfusion prior to initiation of C, E, and K matching with African American donors. Twelve antibodies occurred in 9 patients before these 2 strategies were implemented: 3 anti-C, 2 anti-K, 3 anti-M, 1 anti-C^W, 1 anti-Co^b, 1 anti-Kn^a, 1 anti-Le^a, and 3 anti-M (supplemental Table 1). We excluded these antibodies, but not the patients, because 23 were not alloimmunized, and thus were still at risk; the 9 with antibodies were at risk for additional antibodies. Of the 9 alloimmunized prior to 1997, 7 formed additional antibodies during the study period: 3 anti-D, 2 anti-C, 1 anti-E, 2 anti-Kp^a, and 1 anti-S (supplemental Table 1).

Laboratory testing

Patient RBCs were serologically phenotyped before transfusion for ABO; Rh (D, C, c, E, e); Kell (K); Duffy (Fy^a, Fy^b); Kidd (Jk^a, Jk^b); Lewis (Le^a, Le^b); MNS (M, N, S, s); and P1. An antibody screen, complete blood count, and hemoglobin quantitation were performed prior to each transfusion. Antibody testing was performed with the low ionic strength saline tube technique until 2000 and with a gel-based method (Ortho Diagnostics) thereafter. We defined a warm autoantibody as the presence of a positive direct antiglobulin test with a panagglutinin in the serum or eluate with similar strength of reactivity to all cells tested with no apparent specificity.

RH high-resolution genotyping

Genomic DNA was extracted from peripheral blood (QIAamp; Qiagen, Valencia, CA), polymerase chain reaction (PCR) amplified, and analyzed by a combination of PCR-restriction fragment length polymorphism assays, as described previously.[24] Samples were also tested with prototype RHD and RHCE BeadChip arrays (BioArray, Warren, NJ). For high-resolution genotyping, PCR was performed with *RHD*- and *RHCE* exon-specific primers in the flanking introns, directly sequenced, and compared with conventional *RHD* (GenBank accession no. L08429) or *RHCE* (GenBank accession no. DQ322275). To establish allelic associations, Rh-cDNA cloning and sequencing were performed. RNA was isolated from reticulocytes with TriZol (Invitrogen, Carlsbad, CA). Reverse transcription was performed with gene-specific primers (Superscript First Strand Synthesis; Invitrogen). PCR products were purified (ExoSAP-IT; USB, Cleveland, OH) and sequenced.

Determination of transfusion outcome and statistical analysis

Antibodies were recorded with detection date and RBC exposure number at the time of antibody development. We compared the percent hemoglobin S and hemoglobin levels at the time of new antibody detection to the patient's baseline values. The baseline was calculated as the mean pretransfusion percent hemoglobin S and hemoglobin levels on each visit in the 6 to 12 months preceding the appearance of a specific antibody. The standard deviation (SD) from the mean was calculated, and antibodies associated with a difference in values >2 × SD of their individual mean (Z-score > 2.0, corresponding to two-tailed $P < .05$) were considered a clinically significant delayed transfusion reaction (DTR). The Student *t* test was used to compare parametric data between groups, and the Mann-Whitney test was used for nonparametric data. The χ^2 test was used for categorical data. A two-tailed $P < .05$ was considered statistically significant.

Results

Subjects

The majority of subjects (91.2%) were homozygous HbSS, 5.0% were HbSC, 3.3% were HbSβ-thalassemia, and 0.5% were HbSO-Arab. A review of the transfusion history was performed for 182 patients with ≥1 RBC exposure (Table 1). Fifty-nine individuals (32.4%) had received transfusions episodically for acute complications of SCD or preoperative preparation. The mean number of exposures per patient was 4.6, the median was 3, and the range was 1 to 15 units. A total of 272 units were transfused to this group, primarily by simple transfusion. One hundred twenty-three patients (67.6%) had been or were currently managed with chronic transfusions and received 44 210 units by simple transfusion or erythrocytapheresis. Within this cohort, the mean number of exposures per patient was 354, the median was 230, and the range was 10 to 1460 units. The most common indications for chronic

Table 1. Transfused patient characteristics

	Overall demographics	Episodic category	Chronic category	Episodic category			Chronic category		
				Alloimmunized	Nonalloimmunized	*P* value	Alloimmunized	Nonalloimmunized	*P* value
No. of patients	182								
Male/female	112/70 (61.5% male)								
Median age (years)	15.7 (range 0.5-41)								
Transfusion category									
No. of patients (%)		59 (32.4)	123 (67.6)						
Donor RBC exposures (units)									
Mean		4.6	354						
Median		3	230						
Range		1-15	10-1460						
Total for group		272	44 210						
Alloimmunization status									
No. of patients (%)				9 (15.2)	50 (84.8)	NA	71 (57.7)	52 (42.3)	NA
No. of male patients (%)				3 (33.3)	28 (56.0)	.317	48 (67.6)	30 (57.7)	**.003**
Mean age (years)				14.5	11.6	.200	19.1	15.7	**.007**
Mean age at first transfusion (years)				3.9	5.3	.327	6.5	6.8	.755
Donor RBC exposures (units)									
Mean				8.9	3.8	**<.001**	408.6	292.3	**.045**
Median				9	2.5	**<.001**	309	192.5	**.008**
Range				3-15	1-12	NA	12-1263	10-1460	NA
Total for group				80	192	NA	29 009	15 201	NA

NA, not applicable; **bold** indicates statistically significant ($P < .05$).

transfusion were primary and secondary stroke prevention (30.1% and 32.5%, respectively).

RBC immunization in patients with SCD

Among 182 transfused patients, 9 of 59 episodic (15%) and 71 of 123 chronically transfused (58%) subjects were alloimmunized (Table 1). For episodically transfused patients, gender, age at data analysis, and age at first transfusion were not significantly different for alloimmunized compared with nonalloimmunized patients. However, alloimmunized patients received a greater number of RBC units compared with nonalloimmunized patients (8.9 vs 3.8 units, $P < .001$). In the chronically transfused cohort, male gender ($P = .003$), older age

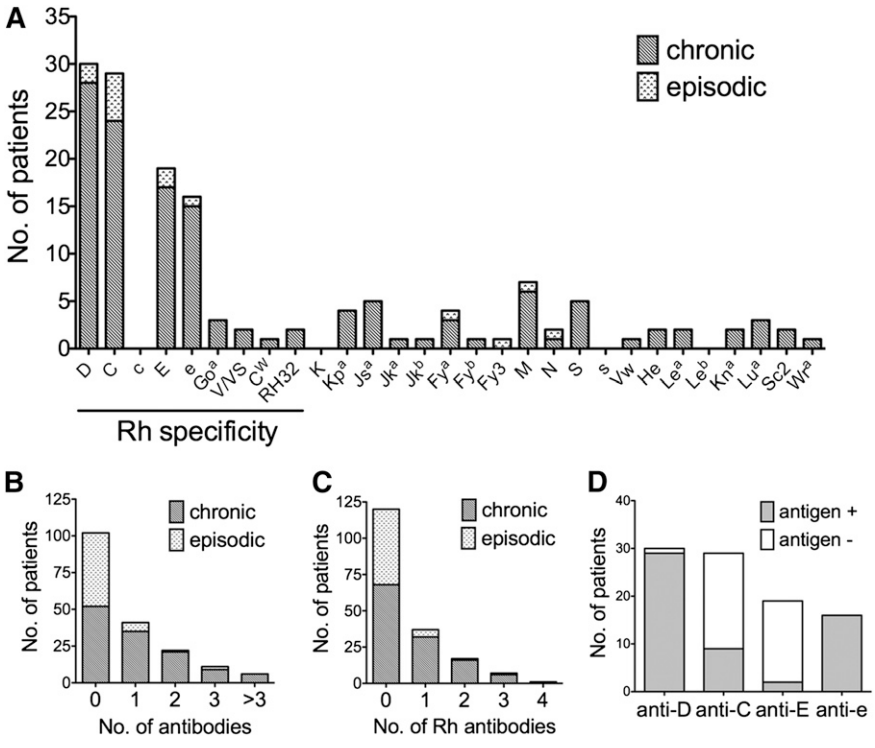

Figure 1. RBC immunization in patients with SCD transfused with Rh D, C, and E and K matched RBCs from minority donors. (A) One hundred forty-six specific antibodies in 123 chronically and 59 episodically transfused patients. (B) Number of antibodies per patient. (C) Number of Rh antibodies per patient. (D) Number of patients with anti-D, -C, -E, or -e in their serum and whose RBCs type positive or negative for the antigen.

Table 2. Twenty-nine Rh antibodies in 25 patients associated with a significant change in hematologic parameters

Antibody specificity	Concurrent Antibodies	ID	Hemoglobin S (%)			Hemoglobin (g/dL)		
			Baseline	At antibody detection	Z-score*	Baseline	At antibody detection	Z-score*
Patients whose RBCs type positive for the corresponding antigen								
D (n = 12)		292†	—	81.7	—	6.5	4.2	**−5.7**
	E	**95**	27.1	68.0	**10.4**	9.1	7.5	**−3.2**
		41	25.9	38.2	**5.9**	9.4	8.9	**−2.4**
		78	28.8	45.1	**5.4**	10.2	9.7	−0.5
	C	**138**	19.3	61.6	**4.3**	10.4	8.2	**−4.0**
	C	**77**	28.8	45.3	**3.5**	8.0	8.1	0.2
		110	27.6	—	—	10.6	7.9	**−2.7**
		50	44.3	58.8	1.6	8.5	6.4	**−2.8**
		99	29.6	45.7	**2.5**	8.9	8.2	−1.0
		34	18.2	23.3	**2.2**	8.8	8.7	−0.2
		145	26.0	32.7	**2.1**	9.5	9.5	−0.1
		100	24.8	40.6	**2.0**	8.9	7.5	−1.8
C (n = 3)		95	25.3	31.2	1.0	9.3	5.0	**−6.9**
	D	**138**	19.3	61.6	**4.3**	10.4	8.2	**−4.0**
		85	28.9	44.0	**2.7**	9.8	8.8	**−2.3**
e (n = 5)		65	29.0	43.6	**4.9**	8.7	8.2	−0.7
		108	23.1	50.7	**4.4**	7.6	6.3	**−4.1**
	C	**97†**	—	76.3	—	9.0	6.3	**−7.6**
		100	26.4	40.6	**2.1**	8.6	8.1	**−2.7**
		86	—	89.5	—	7.7	6.9	**−2.1**
Patients whose RBCs type negative for the corresponding antigen and received antigen negative RBCs								
C (n = 4)		28	25.1	45.8	**8.0**	9.4	8.7	−1.3
	D	**77**	28.8	45.3	**3.5**	8.0	8.1	0.2
	e	**97†**	—	76.3	—	9.0	6.3	**−7.6**
		130	37.5	34.9	−0.8	8.9	8.2	**−5.0**
E (n = 4)	**D**	**95**	27.1	68.0	**10.4**	9.1	7.5	**−3.2**
		103	24.3	66.1	**8.9**	10.0	7.6	**−6.9**
		159	50	—	—	9.2	5.1	**−6.8**
	Jkb	31	43.1	91.5	**2.0**	7.9	6.8	−1.1
Patients whose RBCs type negative for the corresponding antigen and received antigen positive RBCs								
E (n = 1)		63‡	32.3	50.7	**4.4**	9.5	8.9	**−2.1**

For each antibody, the percent hemoglobin S and hemoglobin level at the time of first detection is indicated and compared with the patient's individual baseline value (see Methods). For antibody specificity, the second entry indicates additional antibodies detected concurrently. —, data not available.

*Z-score >2.0 for hemoglobin S level or <−2.0 for hemoglobin level correlates with $P < .05$, indicated in **bold**. ID in bold indicates patients with >1 Rh antibody detected concurrently.

†Episodically transfused.

‡E− transfused with E+ RBCs for management of anti-e.

at data analysis ($P = .007$), and a greater mean number of RBC units ($P = .045$) were associated with alloimmunization (Table 1).

There were 146 specific antibodies identified in the serum of 80 individuals (Figure 1A). Among the 123 chronically transfused patients, 35 had 1 antibody, 21 had 2 antibodies, 9 had 3 antibodies, and 6 had >3 antibodies (Figure 1B). In the 59 episodically transfused patients, 6 had 1 antibody, 1 had 2 antibodies, and 2 had 3 antibodies (Figure 1B). Antibodies to low incidence antigens found primarily on RBCs of African American donors and predicted to be increased in patients receiving transfusion with minority donor units included 3 examples of anti-Go^a, 2 anti-V/VS, and 5 anti-Js^a. Commonly encountered antibodies included 7 anti-M, 5 anti-S, and 4 anti-Fy^a. Antibodies to other low prevalence antigens included 4 anti-Kp^a, 1 anti-C^W, 2 anti-Sc2, and in the MNS system, 1 anti-Vw and 2 anti-He. Fifty percent of chronically transfused patients had a warm autoantibody compared with 5% of episodically transfused individuals (data not shown).

Notably, 94 of the 146 antibodies had specificity for common Rh antigens (D, C, E, e), and comprised nearly two-thirds (64.4%) of all antibodies (Figure 1A). Fifty-five (45%) chronically and 7 (12%) episodically transfused patients were Rh alloimmunized despite prophylactic Rh antigen matching, and 25 (40%) had >1 Rh antibody (Figure 1C). Only three cases of anti-E could be explained by transfusion of E+ RBCs to E− patients because they had made anti-e. Patient interviews excluded transfusion not matched for C, E, K antigens outside our institution (except 1 case), and look-back of donor center records and repeat antigen typing of donors when possible excluded labeling and antigen typing errors. Anti-D was identified in the serum of 30 patients (29 whose RBCs typed D+), anti-C in 29 (9 whose RBCs typed C+), anti-E in 19 (2 whose RBCs typed E+) and anti-e in 16 (all whose RBCs typed e+; Figure 1D). In summary, there were 56 unexplained Rh specificities identified in 45 patients whose RBCs typed positive for the corresponding antigen and 35 unexplained Rh specificities in 33 patients whose RBCs

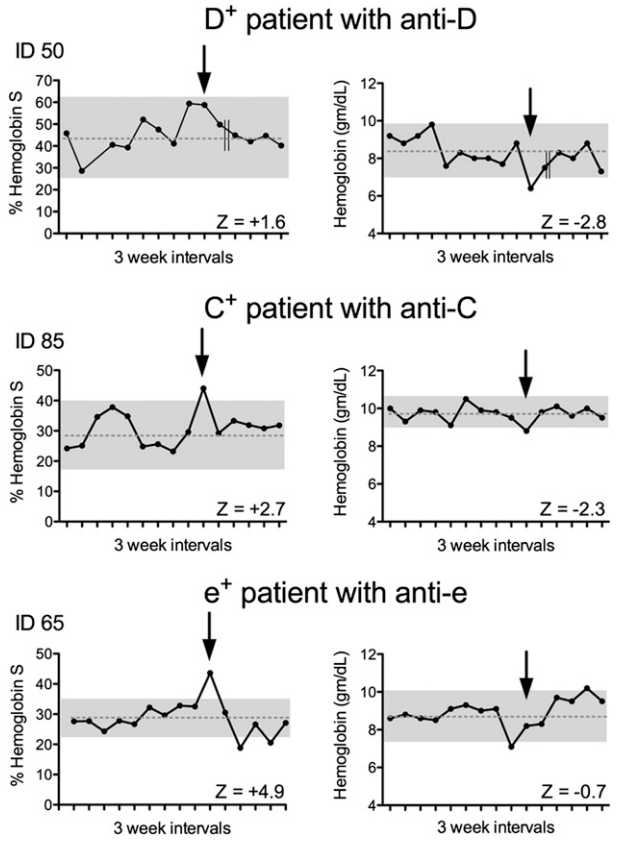

Figure 2. DTRs in patients with unexplained Rh antibodies. Percent hemoglobin S and hemoglobin levels in 3 representative cases of chronically transfused patients with anti-D, -C, and -e (see Table 2 for values of all DTRs associated with Rh antibodies). Each point represents a pretransfusion value coinciding with transfusions occurring at 3-week intervals. Arrows indicate time of antibody detection. The dotted line represents the mean percent hemoglobin S or hemoglobin level for that individual determined pretransfusion for 9 visits preceding antibody detection. The SD for means was calculated. The difference between the baseline and the value at time of antibody formation is expressed as a multiple of this SD, or Z-score. The gray shaded area indicates values that would have a Z-score <2. Z-score >2.0 for hemoglobin S level or <-2.0 for hemoglobin level correlates with $P < .05$. The double lines on the top charts indicate a several-month period when transfusions were discontinued.

typed negative for the antigen and had received antigen-negative units. The Rh specificities had clear preference for antigen-positive cells and were distinguishable from warm autoantibodies.

Clinical significance of unexplained Rh antibodies

One overt hemolytic transfusion reaction requiring hospitalization was associated with anti-D in a D+ patient (Table 2, ID 292). DTRs are underreported in this patient group but are typically accompanied by an increased percent hemoglobin S level and/or a decrease in the hemoglobin/hematocrit.[1] We evaluated whether patients experienced a DTR at the time of Rh antibody production by comparing the percent hemoglobin S and hemoglobin level with the patient's baseline values (n = 82 evaluable occurrences in 56 patients). Figure 2 depicts hematologic data consistent with DTRs for 3 representative patients receiving chronic transfusions who made unexplained anti-D, -C, or -e. Twelve of 29 anti-D, 3 of 9 anti-C, and 5 of 16 anti-e occurring in D+, C+, and e+ individuals, respectively, were associated with a DTR (Table 2). Overall, 20 of 50 (40%) Rh antibodies evaluated in individuals positive for the corresponding antigen and 8 of 29 (28%) in antigen-negative individuals who received antigen-negative blood (Table 2) were associated with a DTR. One of 3 anti-E in E− patients who received E+ RBCs for management of anti-e also had a DTR (Table 2).

RH genetic diversity in patients with SCD

High-resolution analysis of *RHD* and *RHCE* was performed to determine *RH* diversity and allele prevalence in 226 patients with SCD and whether Rh immunization in the 182 transfused patients was associated with specific *RH* genotypes. Thirteen different *RHD*, 14 *RHCE*ce*, and 1 *RHCE*Ce* alleles encoding amino acid changes were present (Figure 3). Patients were homozygous, heterozygous, or compound heterozygous for variant *RH* alleles. At the *RHD* locus, there were 85 alleles encoding the recessive D negative ("Rh negative") phenotype: 52 with a gene deletion, 12 with a 37-bp inactivating insertion (*RHDψ*), and 21 with a *RHD-CE-D* hybrid locus [*DIIIa-CE(4-7)-D*]. Of 367 *RHD* alleles encoding D antigen ("Rh positive"), 235 alleles were conventional sequence (64%) and 132 encoded RhD proteins with amino acid changes (36%). *RHD*DAU0* was common (16% of alleles) and *RHD*weak partial D 4.0* was relatively frequent (5%).

At the *RHCE* locus, the majority of alleles in African Americans are *RHCE*ce* encoding a C−c+ and E−e+ RBC phenotype.[20] Among 358 *RHCE*ce* alleles, 100 were conventional (28%) and 258 were variant (72%). Most frequent variant *RHCE*s were *RHCE*ce(48C)* (19%), *RHCE*ce(733G)* (14%), *RHCE*ce(48C,733G)* (6%), *RHCE*ce(254G)* (6%), and *RHCE*ceS* (6%) (Figure 3). Among 52 *RHCE*Ce*, 1 variant designated *RHCE*CeRN* was present. Forty-two alleles were *RHCE*cE*. No *RHCE*CE* was identified.

More than one-third of *RHD* and more than one-half of *RHCE* alleles differed from the conventional sequence. In total, ⩾1 nonconventional *RH* allele was identified in 86.7% (196/226), and 47.3% of individuals had ⩾1 variant *RHD* and 1 variant *RHCE* allele.

Examination of Rh antibodies with *RH* genotypes for antigen-positive patients

We examined each Rh antibody and the patient's *RH* genotype, clinical outcome, exposure number, and duration of antibody reactivity (Tables 3 and 4). Anti-D was identified in the serum of 29 D+ patients (Table 3). Seven individuals had only variant *RHD* alleles encoding amino acid changes. Anti-D was associated with an overt hemolytic reaction in 1 patient homozygous for *DAU4* (ID 292), and DTRs occurred in 1 patient with *DAU0/DAU5* (ID 50) and 1 with *DAU0* only (ID 34) (DTR details, Table 2). The remaining 22 patients with anti-D had 1 or even 2 conventional *RHD* alleles; 9 cases were associated with a DTR. Eight patients had other antibodies concurrent with anti-D (7 unexplained anti-C or anti-E and 1 anti-S). There was no significant difference in DTR incidence between D+ patients lacking conventional *RHD* and those with ⩾1 conventional *RHD* allele ($P = .690$). The mean RBC exposures at time of anti-D detection was also not significantly different between those 2 groups ($P = .108$) and ranged from 2 to 733 units. After anti-D detection, all patients received D− RBCs. Anti-D duration was not significantly different based on the presence of ⩾1 conventional *RHD* ($P = .946$) or DTR occurrence ($P = .473$), and most were undetectable after 1 to 3 months. In contrast, anti-D in the onlyD− patient persists 9 years after initial detection (Table 3).

Anti-C was identified in the serum of 9 C+ patients (Table 4). Five patients had partial C antigen encoded by a hybrid *DIIIa-CE(4-7)-D* (at the *RHD* locus), and 1 had a DTR. Of 4 patients with 1 conventional *RHCE*Ce* (3 were siblings), 2 had DTRs, and anti-D was also present in 1. DTR occurrence was not significantly different for patients with the hybrid *DIIIa-CE(4-7)-D* compared with those with 1 conventional *RHCE*Ce* ($P = .270$), but patient numbers were small. The mean number of exposures at time of anti-C detection was also not significantly different between the 2 groups ($P = .229$).

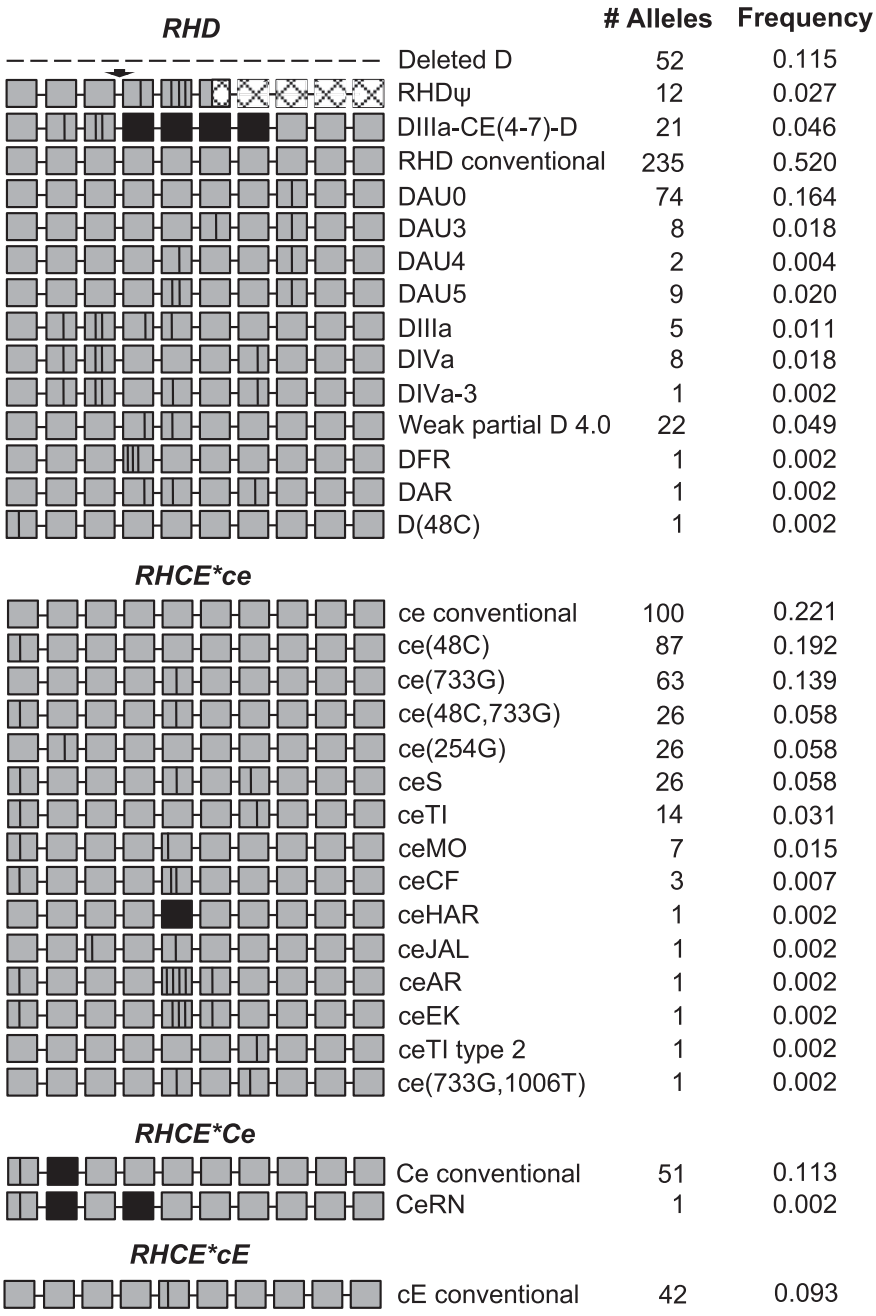

Figure 3. *RHD* and *RHCE* diversity in 226 patients with SCD. *RH* alleles identified in patients with SCD. Each gray box represents 1 of 10 exons in the *RH* genes. Black boxes represent exon exchange between *RHD* and *RHCE*. Vertical black lines indicate position in the exon encoding amino acid substitutions in the protein. Dashed lines indicate gene deletion. Arrowhead indicates 37-bp duplication. Hatched boxes represent exons encoding a frameshift and untranslated region of the inactive *RHD* pseudogene.

After detection, patients received C− RBCs, and anti-C duration was not different based on the presence of 1 conventional *RHCE*Ce* ($P = .400$) or DTR occurrence ($P = .200$).

Anti-e was identified in the serum of 16 e+ patients (Table 4). Fourteen lacked a conventional *RHCE*ce*: 8 were homozygous for variant *RHCE*ce*, 2 had *RHCE*Ce in trans,* and 4 had *RHCE*cE in trans*. Anti-e was associated with a DTR in 5 of these 14 patients, and 3 also had anti-C. Two e+ patients with anti-e had a conventional *RHCE*ce in trans* to an altered *RHCE*ce* allele. The mean exposures at time of anti-e detection were 89 units for e+ patients lacking conventional *RHCE*ce* and 339 for those with 1 conventional *RHCE*ce*, which was significantly different despite the small number in the latter group ($P = .002$). The duration of anti-e demonstration was not significantly different from those with 1 conventional *RHCE*ce* allele ($P = .690$) or DTR occurrence ($P = .942$). After anti-e detection, patients received e− (E+) RBCs, and antibody demonstration ranged between 1 and 49 months. Three E−e+ individuals subsequently developed anti-E; only 1 was associated with a DTR (Table 2, ID 63).

Anti-E was identified in the serum of two E+ patients (Table 4). Both had conventional *RHCE*cE, in trans* to *RHCE*ce* or *RHCE*ce(48C),* respectively. The latter also had anti-D.

Incidence of Rh alloimmunization and related *RH* genotypes

We determined the incidence of Rh alloimmunization in 123 chronically transfused patients with only variant *RH* alleles, variant and conventional alleles, or only conventional alleles (supplemental Table 2). Of 117 D+ patients, anti-D was detected in 5 of 29 (17%) who inherited only variant *RHD*, 8 of 31 (26%) with 1 conventional

Table 3. *RHD* genotype and production of anti-D: clinical significance, donor exposures, and antibody duration

Antibody specificity	Concurrent Antibodies	ID	*RH* genotype				DTR	RBC exposures	Antibody demon-stration (months)
			RHD		*RHCE*				
D+ patients lacking conventional *RHD*									
D (n = 7)		292*	DAU4	DAU4	ce(48C)	ce(48C)	Yes	2	—
		50	DAU0	DAU5	ce(48C)	ce(48C)	Yes	91	1
		34	DAU0	Deleted D	ce(733G)	ce	Yes	269	3
	E	104	DAU3	DAU5	ce(48C)	cE	No	39	19
	C	214*	DAU0	Weak partial 4.0	ce(48C)	ce(48C,733G)	SS	4	—
		117	Weak partial 4.0	Weak partial 4.0	ce(254G)	ce(48C,733G)	No	32	2
		14	DIVa-2	DIIIa-CE(4-7)-D	ce	ceS	No	181	1
D+ patients with one or more conventional *RHD*									
D (n = 22)		100	RHD	DIIIa-CE(4-7)-D	ce(733G)	ceS	Yes	98	14
		141*	RHD	DIIIa-CE(4-7)-D	ce(733G)	ceS	No	5	36
	S	44	RHD	DIIIa-CE(4-7)-D	ce	ceS	No	116	1
		94	RHD	DIIIa-CE(4-7)-D	ce	ceS	No	82	16
	C	138	RHD	DIIIa-CE(4-7)-D	Ce	ceS	Yes	103	5+
		110	RHD	Weak partial 4.0	Ce	ceS	Yes	28	3
		72	RHD	DIVa-2	ceTI	ceTI	No	111	1
	E	28	RHD	DAU0	ceHAR	ce(48C)	No	212	1
		30	RHD	DAU0	Ce	ce(48C)	No	177	1
		99	RHD	Deleted D	Ce	cE	Yes	28	7
		17	RHD	Deleted D	cE	ce	No	331	1
		95	RHD	Deleted D	Ce	ceTI	No	155	4
	E	*95*	*RHD*	*Deleted D*	*Ce*	*ceTI*	*Yes*	*369*	*1*
		21	RHD	Deleted D	Ce	ce(733G)	No	426	1
		78	RHD	Deleted D	ce(733G)	ce(254G)	Yes	199	1
		19	RHD	RHD	ce	ce	No	733	1
		18	RHD	RHD	cE	ce	No	64	54
		41	RHD	RHD	ce	ce(733G)	Yes	429	6
		145	RHD	RHD	Ce	ce(733G)	Yes	48	1
	C	20	RHD	RHD	ce	ce(733G)	No	224	1
		69	RHD	RHD	ce(733G)	ce(733G)	No	230	1
	C	77	RHD	RHD	ce	ce(254G)	Yes	12	5
		103	RHD	RHD	ce(48C)	ceS	—	—	—
D− patient									
D		10	Deleted D	Inactive RHD ψ	ce(254G)	ce(48C)	No	314	112+

DTR, DTR from Table 2; RBC exposures, cumulative number of RBC units transfused prior to antibody detection; antibody demonstration, number of months anti-D was detected. Additional antibody detected concurrently is indicated in the second column. SS, confounded by splenic sequestration; —, data not available; +, remains detectable; italicized patient demonstrates anti-D recurrence.

*Episodically transfused.

and 1 variant *RHD*, and 13 of 57 (23%) with conventional *RHD* only ($P = .720$). Of 36 C+ patients, anti-C was detected in 4 of 10 (40%) individuals with only the hybrid *RHD*DIIIa-CE(4-7)-D* encoding partial C, 1 of 1 with conventional *RHCE*Ce* and hybrid *RHD*DIIIa-CE(4-7)-D*, and 3 of 25 (12%) with conventional *RHCE*Ce* only ($P = .033$). All 123 chronic transfused patients were e+; anti-e was found in 13 of 69 (19%) with only variant *RHCE*ce*, 2 of 36 (6%) with 1 conventional and 1 altered *RHCE*ce*, and 0 of 18 patients with only conventional *RHCE*ce* ($P = .033$). Of 19 E+ patients, anti-E was detected in 2 of 19 (11%) patients with conventional *RHCE*cE*.

One of 6 D− patients made anti-D (17%), 17 of 87 C− patients had anti-C (20%), and 13 of 104 E− patients had anti-E (13%), despite D-, C-, and E-negative transfusions, respectively.

Discussion

We report here the results of a 15-year experience of transfusing patients with SCD with donor units that were antigen matched for Rh D, C, and E, and K, and selected primarily from African American donors. A number of studies demonstrate that antigen matching for C, E, and K is associated with a decrease in antibodies,[10,12,16] and extended phenotype matching to also include Jk^b, Fy^a, and S can further minimize alloimmunization.[5,6] The rationale for providing blood from ethnically similar donors is based on differences in RBC antigen frequency in white and black ethnic groups. For instance, the C−, E−, K−, Fy^a−, and Jk^b− phenotype occurs in 26% of African Americans and 2% of whites. Based on antigen prevalence differences, it was hypothesized that alloimmunization would be reduced in patients with SCD by transfusion with blood selected from ethnically similar donors.[34]

Indeed, antibodies to FY, JK, and S were rare, with a rate of 0.027/100 units. However, the overall incidence of alloimmunization was higher than expected, primarily due to a large number of unexpected Rh antibodies. The alloimmunization rate for patients on chronic transfusions was 0.30/100 units transfused compared with 0.055/100 units in 45 patients reported by Wahl et al[11] and 0.11/100 units in 32 patients by Godfrey et al,[35] all receiving chronic transfusions with C-, E-, and K-matched RBCs. These studies were also in pediatric and young adult patient populations,

Table 4. *RHCE* genotype and production of anti-C, -e, or -E: clinical significance, donor exposures, and antibody duration

Antibody specificity	Concurrent Antibodies	ID	*RH* genotype				DTR	RBC exposures	Antibody demon-stration (months)
			RHCE		*RHD*				
C+ patients lacking conventional *RHCE*Ce*									
C (n = 5)		100	ceS	ce(733G)	DIIIa-CE(4-7)-D	RHD	No	52	39
		94	ceS	ce	DIIIa-CE(4-7)-D	RHD	No	115	4
		105*	ceS	cE	DIIIa-CE(4-7)-D	RHD	No	12	—
		85	ceS	ceTI	DIIIa-CE(4-7)-D	DIVa-2	Yes	65	13
		118	ceS	ceTI	DIIIa-CE(4-7)-D	DIVa-3	—	—	—
C+ patients with one conventional *RHCE*Ce*									
C (n = 4)	D	138	Ce	ceS	RHD	DIIIa-CE(4-7)-D	Yes	103	1
		95	Ce	ceTI	RHD	Deleted D	Yes	365	2
		96	Ce	ce(733G)	RHD	Deleted D	No	82	32
		21	Ce	ce(733G)	RHD	Deleted D	—	—	—
e+ patients lacking conventional *RHCE*ce*									
e (n = 14)	C	97*	ce(48C)	ce(48C)	RHD	RHD	Yes	1	—
		75	ce(48C)	ce(48C)	DAU0	DAU5	No	91	1
		108	ce(48C)	ce(733G)	DAU0	Inactive RHD ψ	Yes	107	1
		102	ce(48C)	ce(733G)	DAU0	RHD	No	295	24
		148	ce(48C)	ce(733G)	Weak partial 4.0	Weak partial 4.0	No	6	4
		65	ce(48C)	ceS	DAU3	DIIIa	Yes	174	19
		63	ce(48C)	ceS	DAU0	DIIIa-CE(4-7)-D	No	195	1
		100	ce(733G)	ceS	RHD	DIIIa-CE(4-7)-D	Yes	62	36
		96	ce(733G)	Ce	Deleted D	RHD	No	64	10
		93	ce(733G)	Ce	RHD	RHD	No	24	1
		45	ce(733G)	cE	RHD	RHD	—	—	—
		86	ce(48C,733G)	cE	Weak partial 4.0	RHD	Yes	8	1
	C	98	ce(254G)	cE	RHD	Deleted D	No	92	11
	C	60	ce(254G)	cE	DAU0	RHD	No	40	22
e+ patients with one conventional *RHCE*ce*									
e (n = 2)		27	ce	ce(48C)	RHD	DAU0	No	396	49
		94	ce	ceS	RHD	DIIIa-CE(4-7)-D	No	281	2
E+ patients with one conventional *RHCE*cE*									
E (n = 2)		17	cE	ce	RHD	Deleted D	—	279	1
	D	104	cE	ce(48C)	DAU3	DAU5	No	39	19

DTR, DTR from Table 2; RBC exposures, cumulative number of units transfused prior to antibody detection; antibody demonstration, the number of months antibody was detected. Additional antibody detected concurrently is indicated in the second column. —, data not available.

*Episodically transfused patient.

and the mean number of units transfused per patient was 243 and 137, respectively. A significant distinction in our study is the large number of unexpected Rh antibodies identified: 45% of chronic transfused patients and 12% of episodically transfused patients had antibodies to D, C, E, or e, and 40% had >1 Rh antibody, with a rate of 0.21/100 units for unexpected Rh antibodies and 0.09/100 for other blood groups. The main differences in the present study are the larger number of patients, the greater mean number of units transfused (354 units), and RBC transfusions primarily from African American donors.

In this study, 91 Rh antibodies were unexpected. Thirty-five occurred in antigen-negative patients receiving corresponding D−, C−, or E− RBCs who should not have made the antibody in the absence of exposure to antigen-positive RBCs. Fifty-six antibodies occurred in patients whose RBCs were positive for the antigen and should not have recognized the antigen as foreign and formed the antibody. Unexplained Rh antibodies in patients with SCD were occasionally observed in previous studies, including one anti-D in a D+ patient, one anti-C in a C− patient, and three anti-E in both E+ and E− patients, despite Rh matching.[10,11,35] A recent study reported 5 patients with Rh variants discovered by *RH* genotyping after immunization to Rh antigens for which their RBCs were positive by serologic phenotype.[12]

These Rh antibodies can be clinically significant, as one-third were associated with delayed transfusion reactions, defined by hematologic laboratory changes at antibody detection. Only a few patients presented with clinical symptoms of increased hemolysis or worsening anemia, but the majority had mild or no symptoms and did not seek medical attention. More data are needed to determine the true incidence of DTR and the clinical significance of Rh antibodies in this patient population. Routine laboratory evaluations that may provide evidence of DTRs should be monitored closely and specifically reviewed when patients develop new antibodies. Clinically significant Rh antibodies occurred after 1 RBC exposure to as many as several hundred units, suggesting that heavily transfused patients remain at risk, in contrast to a prior report that all clinically significant alloantibodies were detected within 6 months of initiating chronic transfusion therapy.[10] Nearly all unexplained Rh antibodies were evanescent and not detected in the patient's serum after several months, consistent with observations that many alloantibodies

disappear within 6 months.[36] However, anti-D in D− patients typically persist, lasting for many years,[36] but >80% of anti-D in D+ patients in this study were no longer demonstrable after 1 to 6 months. In contrast, the one anti-D in a D− patient reported here was likely due to transfusion with an RBC unit that expressed a weak D antigen not detected by standard donor typing,[20] and the anti-D remains detectable after 9 years.

High-resolution *RH* genotyping revealed that 87% of patients inherited ⩾1 variant *RH* allele, demonstrating the tremendous *RH* diversity in this population. These variant alleles potentially encode altered or partial Rh antigens not distinguished from common antigens with routine serologic typing. Twenty-six Rh antibodies occurred in patients homozygous for variant *RH* alleles. Conversely, we observed an absence of anti-e in chronically transfused patients with only conventional *RHCE*ce* (supplemental Table 2). Correlating specific *RH* alleles or haplotypes with alloimmunization or DTR was not possible due to the small numbers of individuals with identical *RHD* and *RHCE* genotypes. Comparison of antibody production, DTR occurrence, exposure number, and antibody duration was performed broadly between patients with and without variant alleles, but a large multi-institutional study is necessary to address the risk of alloimmunization and DTR with specific *RH* haplotypes.

RH diversity in patients contributes to Rh alloimmunization, but 65 unexpected Rh antibodies were not explained by homozygosity for altered alleles at the patient's corresponding *RH* loci. Thus, the role of minority donor RBCs requires study. We hypothesize that African American donors have the same degree of *RH* heterogeneity. Rh specificities in the serum of patients who typed negative for D, C, or E antigens (n = 35 cases) or who tested positive and carry conventional alleles (n = 30 cases) likely reflect an immune response to a foreign Rh complex on African American donor units. Rh epitopes and Rh antigen specificities are complex, may be cross-reactive with other Rh antigens, and are not always straightforward. RhD-like epitopes, reactive with anti-D, can be expressed on altered Rhce proteins,[31,37] C-like epitopes on variant RhD and Rhce proteins,[32,38] and E-like antigens on RhCe proteins.[39] Thus, future studies will address whether RBCs from African American donors stimulate complex Rh specificities.

These findings suggest that *RH* diversity in patients with SCD necessitates an alternative approach to improve RBC matching. As blood group genotyping technology continues to expand beyond reference laboratories to donor centers and hospitals, and as costs further decline, molecular analysis to refine RBC matching for African American patients and donors should be feasible. Although *RH* genotype–matched RBCs may be supply or cost prohibitive for all patients, transfusion management can be tailored for specific patient genotypes. For example, our transfusion policy for C+ patients with the hybrid *DIIIa-CE(4-7)-D* that encodes a partial C antigen (and who lack conventional *RHCE*Ce*) is to provide C− RBCs prospectively.[29] Another potential strategy is to provide D− RBCs to D+ patients whose *RHD* genotypes predict partial D expression only. However, because D− RBCs are much less frequent among African-American donors, such a policy may result in increased alloantibodies to other RBC antigens with disparate distribution between Europeans and Africans (Jk^b, Fy^a, S).[9] More extended matching to include those antigens might be considered to minimize alloimmunization risk[5,6] but may be challenging to supply for patients requiring chronic erythrocytapheresis. Future studies to determine the clinical significance of specific *RH* genotypes are needed, as well as prospective trials to assess RBC matching strategies based on patient and/or donor blood group genotypes.

The major findings of this study are (1) transfusion of patients with SCD using African American donor units antigen matched for D, C, E, and K did not reduce Rh alloimmunization; (2) antibodies to low incidence antigens found primarily on RBCs of African American donors were not significantly increased, but rather a large number of unexplained Rh antibodies with apparent common specificities were found; (3) ~30% of unexpected Rh antibodies were associated with laboratory evidence of delayed transfusion reactions; and (4) altered *RH* alleles were present in 87%, and some, but not all Rh antibodies, were explained by inheritance of altered *RH*. This study suggests that the presence of 1 conventional *RH* allele is not protective against alloimmunization and that *RH* diversity in African American patients and donors contributes to the high rate of Rh alloimmunization seen here. Future studies will aim to determine whether *RH* genotyping of patients and donors can guide RBC selection to prevent Rh alloimmunization.

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Authorship

Contribution: S.T.C., D.F.F., and C.M.W. designed the study, analyzed results, and wrote the manuscript; T.J. and S.V. conducted research, analyzed results, and provided helpful discussions; and K.S.-W. analyzed results, formulated discussions, and assisted with the manuscript.

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