Variant *RH* alleles and Rh immunisation in patients with sickle cell disease

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Background. Alloimmunisation is a major complication in patients with sickle cell disease (SCD) receiving red blood cell (RBC) transfusions and despite provision of Rh phenotyped RBC units, Rh antibodies still occur. These antibodies in patients positive for the corresponding Rh antigen are considered autoantibodies in many cases but variant *RH* alleles found in SCD patients can also contribute to Rh alloimmunisation. In this study, we characterised variant *RH* alleles in 31 SCD patients who made antibodies to Rh antigens despite antigen-positive status and evaluated the clinical significance of the antibodies produced.

Materials and methods. RHD and RHCE BeadChip™ from BioArray Solutions and/or amplification and sequencing of exons were used to identify the *RH* variants. The serological features of all Rh antibodies in antigen-positive patients were analysed and the clinical significance of the antibodies was evaluated by retrospective analysis of the haemoglobin (Hb) levels before and after transfusion; the change from baseline pre-transfusion Hb and the percentage of HbS were also determined.

Results. We identified variant *RH* alleles in 31/48 (65%) of SCD patients with Rh antibodies. Molecular analyses revealed the presence of partial *RHD* alleles and variant *RHCE* alleles associated with altered C and e antigens. Five patients were compound heterozygotes for *RHD* and *RHCE* variants. Retrospective analysis showed that 42% of antibodies produced by the patients with *RH* variants were involved in delayed haemolytic transfusion reactions or decreased survival of transfused RBC.

Discussion. In this study, we found that Rh antibodies in SCD patients with *RH* variants can be clinically significant and, therefore, matching patients based on *RH* variants should be considered.

Keywords: sickle cell disease, *RH* alleles, Rh alloimmunisation, *RHD* and *RHCE* variants.

Introduction

Alloimmunisation remains a major problem in transfused patients with sickle cell disease (SCD). In an effort to reduce alloimmunisation to red blood cell (RBC) antigens some programmes have been implemented to provide RBC transfusions matched for at least Rh and K to patients with SCD who are in need of chronic transfusion support[1-3]. However, it has been observed that despite provision of Rh phenotyped matched RBC to patients with SCD, these patients still produce Rh antibodies[4,5] and in many cases the antibodies are considered autoantibodies because the patient's own RBC type serologically positive for the corresponding antigen.

The Rh blood group system is one of the most important and complex blood group systems with a large number of antigens and is involved in RBC alloimmunisation, haemolytic transfusion reactions and haemolytic disease of the foetus and newborn[6]. It comprises 54 antigens defined serologically and recognised by the International Society of Blood Transfusion (ISBT)[7]. The most common antigens are D, C, c, E and e, defined by commercial reagent antibodies. The RH locus on chromosome 1 consists of two homologous genes: RHD encoding the D protein and RHCE encoding the Ce, ce, cE and CE proteins[6,8]. As a consequence of the homology and opposite orientation of the two RH genes, many rearrangements occur between these two genes and result in hybrid genes. These arrangements are thought to allow "hairpin" formation and exchange between the genes (the so-called gene conversion mechanism). A large number of RH variants and low incidence antigens arise from this mechanism[8,9]. Over of 200 *RHD* alleles and 80 *RHCE* alleles have been reported, and new alleles are still being discovered (ISBT[10] and Rhesus Site[11]). Patients with partial antigens and/or lacking high prevalence antigens, such as hr^B and hr^S, may develop Rh alloantibodies[12]. The high prevalence of altered *RH* alleles in Africans contributes to the high rate of Rh alloimmunisation in

SCD patients[5,13]. Alloimmunisation to Rh antigens in populations of African origin is complicated by the genetic diversity of the *RH* locus and the limitations of serological methods to distinguish the variant antigens[5]. *RH* genotyping has revealed that many patients with SCD carry alleles encoding partial Rh antigens but little clinical or biological evidence related to alloimmunisation and haemolytic transfusion reactions is available for all the *RH* variant alleles. The identification of alloantibodies in patients with SCD carrying *RH* variants is important and a complete characterisation of the variants producing alloantibodies is required, because alloantibodies in those patients may be confused with autoantibodies. In this study, we characterised variant *RH* alleles in patients with SCD who made antibodies to Rh antigens despite their antigen-positive status and evaluated the clinical significance of the antibodies produced. We demonstrated that 65% of the patients with SCD alloimmunised to Rh antigens carried *RH* variant alleles and that 42% of the antibodies encountered had clinical significance.

Material and methods

Patients' samples

We studied DNA samples from 48 African Brazilian patients with SCD (homozygous for haemoglobin S) who had been multiply transfused and were receiving RBC units antigen-matched for RhD, C, E, c, e and K, and made one or more antibodies with Rh specificities. All patients agreed to participate in this study by signing an Institutional Review Board-approved informed consent form.

Serology

RBC from of all alloimmunised patients were typed for D, C, c, E and e by haemagglutination in gel cards (Bio-Rad, Cressier sur Morat, Switzerland) using two different commercial sources of anti-sera (Immucor, Norcross, GA, USA; Bio-Rad). An indirect antiglobulin test was used to screen for and identify antibodies. A direct antiglobulin test and autologous control were performed in gel for all samples antigen-positive for the corresponding Rh antibody specificity. Eluate studies were performed on all samples with a positive direct antiglobulin test using an acid elution method (DiaCidel, Bio-Rad). Adsorption onto autologous RBC was also performed to aid the differentiation of autoantibodies and alloantibodies.

DNA preparation

Genomic DNA was extracted from whole blood by a manual spin column separation technique (QIAmp, Qiagen, Valencia, California, USA), according to the manufacturer's instructions. The concentration of the DNA was measured with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA).

DNA array analysis

DNA array analysis was performed using wRHCE and wRHD BeadChips from BioArray Solutions (Immucor, Warren, NJ, USA) containing 34 markers associated with altered RhD expression and 25 polymorphisms associated with altered *RHCE* alleles. It should be noted that wRHD and wRHCE BeadChips devices are for "research use only" to date. The two assays were performed with 8 mL of each DNA sample, containing ~10 to 80 ng/μL of genomic DNA for wRHD and ~10 ng/μL for wRHCE, according to the manufacturer's recommendations. The polymerase chain reactions (PCR) were performed in a Veriti thermal cycler (Life Technologies) and BeadChips carriers containing single-stranded amplicons and an elongation mixture were incubated at 53 °C in a high humidity hybridisation oven ([Boekel InSlide-Out, model 241000], Boekel, Feasterville, PA, USA). The fluorescence of each bead was analysed on the Bioarray Solutions® Array Imaging System to determine positive and negative reactions. BioArray Solutions Information System (BASIS) software was used to calculate the adjusted intensity of every reaction to assign a genotype and predicted phenotype.

DNA sequence analysis

DNA sequence analysis was performed on PCR products amplified from genomic DNA in all samples that were not characterized by the RHD and RHCE BeadChips in order to determine the specific allele present using *RHD* and *RHCE* specific primers as previously reported[13,14]. PCR products were purified by elution from 1% agarose gels using a Qiaex II gel extraction kit (Qiagen, Valencia, CA, USA), and sequenced directly, without subcloning, on an ABI 373XL Perkin Elmer Biosystems sequencer, with the Perkin Elmer Biosystems Big Dye reagent BD Half-term (GenPak, Perkin Elmer Biosystems, Foster City, CA, USA).

In order to determine *RH* allelic combinations on samples identified with *RHCE* variants, we performed Rh-cDNA cloning and sequencing. RNA was isolated from reticulocytes with TriZol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out with Superscript First Strand Synthesis (Invitrogen, Carlsbad, CA, USA) using gene-specific primers. PCR products amplified from cDNA were purified with ExoSAP-IT (USB, Cleveland, OH, USA), cloned into a TA vector (Invitrogen, Carlsbad, CA, USA) and sequenced using previously reported primers[15].

Determination of *RHD* zygosity

For specific detection of the *RHD* gene deletion we used a PCR-restriction fragment length polymorphism (amplification of the downstream and hybrid Rhesus

box as well as digestion of the PCR products with the restriction enzyme Pst I) as previously reported[16]. We also used a quantitative PCR approach[17] complemented by the specific detection of *RHDΨ*[18].

Analysis of Rh immunisation

Records of transfusions, serological and molecular features in all antigen-positive patients with corresponding antibodies were analysed. The clinical significance of the antibodies was evaluated by retrospective analysis of the haemoglobin (Hb) levels before and after transfusion and determined by changes from the patient's baseline values calculated as the mean pre-transfusion percentage of HbS and Hb levels at the time of antibody detection.

Results

Serology

Among the 48 SCD patients with Rh antibodies studied, nine were antigen-negative for the corresponding antibody specificity (3 anti-D in D-negative patients and 6 anti-C in C-negative patients) and 39 were antigen-positive with 4+ or weak agglutination reactions (15 anti-D whose RBC typed D-positive; 16 anti-e whose RBC typed e-positive; 4 anti-C whose RBC typed C-positive; 2 anti-D,-e whose RBC typed positive for both D and e; and 2 anti-D,-C whose RBC typed positive for both D and C). Autologous controls, the direct antiglobulin test and eluate were positive in 8/39 (20.5%) patients whose RBC typed serologically positive for the corresponding antibody. Adsorption

Table I - RH genotypes and predicted phenotypes in 39 SCD patients with Rh antibodies whose RBC typed positive for the corresponding antigen.

N.	RH Genotypes and Predicted Phenotypes	
	D+ patients with anti-D considered to be alloantibodies	
	Genotypes*	**Predicted phenotypes**
3	*DIIIa-ceS/DIIIa-ceS*	partial D, C−E−, partial c, partial e
3	*DAR-ce/DAR-ceAR*	partial D, C−E−c+e+
1	*DAR-ce/ceEK*	partial D, C−E−c+e+
2	*DAU0-ce/D–ce*	partial D, C−E−c+e+
1	*DAU3-cE/DAU5-ce*	partial D, C−E+c+e+
1	*DAU4-ce48C/DAU4-ce48C*	partial D, C−E−c+e+^{weak}
3	*Weak partial 4.0-ce733G/weak partial 4.0-ce733G*	partial D, C−E−, partial c, partial e
1	*Weak partial 4.0-ce/DIIIa-CE(4-7)-D-ceS*	partial D, partial C, E−c+e+
	e+ patients with anti-e considered to be autoantibodies	
	Genotypes	**Predicted phenotypes**
5	*D-ce/D-ce*	D+C−E−c+e+
1	*D-Ce/ce*	D+C+E−c+e+
2	*D-cE/ce*	D+C−E+c+e+
	e+ patients with anti-e considered to be alloantibodies	
	Genotypes	**Predicted phenotypes**
2	*D-Ce/D-ce48C,733G*	D+C+E−, partial c, partial e
3	*ceS/ce733G*	D−C−E−, partial c, partial e
2	*D-ce48C/D-ceS*	D+C−E−c+, partial e
1	*ce48C,733G/ce733G*	D−C−E−, partial c, partial e
	D+ and e+ patients with anti-D and anti-e considered to be alloantibodies	
	Genotypes	**Predicted phenotypes**
1	*DAR-ceS/DAR-ceAR*	partial D, C−E−, partial c, partial e
1	*DAU5-ce48C/DAU0-ceMO*	partial D, C−E−, partial e
	C+ patients with anti-C considered to be alloantibodies	
	Genotypes	**Predicted phenotypes**
1	*DIIIa-CE(4-7)-D-ceS/DAU0-ce*	partial D, partial C, E−c+e+
3	*DIIIa-CE(4-7)-D-ceS/ DIVa-2-ceTI*	partial D, partial C, E−c+, partial e
	D+ and C+patients with anti-D and anti-C considered to be alloantibodies	
	Genotypes	**Predicted phenotypes**
2	*DIIIa-CE(4-7)-D-ceS/ DIVa-2-ceTI*	partial D, partial C, E−c+, partial e

* The order of the two alleles within the RH genotypes is arbitrary and does not correspond to the presumed haplotype.

onto autologous RBs showed that these eight patients had autoantibodies.

Molecular analyses

Molecular analyses revealed *RHD* and *RHCE* alleles associated with variant D, C and e antigens in 31/48 (65%) of SCD patients with Rh antibodies (Table I). Except for *RHD*DAU0*, found in three samples by sequencing, the other *RH* variants were identified by wRHD and wRHCE BeadChips. Homozygous, heterozygous and compound heterozygous alleles were found by cDNA analysis. Allelic associations showed that five patients (2 with anti-D, -e; 1 with anti-C and 2 with anti-D, -C) were compound heterozygotes for *RHD* and *RHCE* variants (Table I).

Rh immunisation

All 39 patients with "unexplained" Rh antibodies had received ten or more units of RBC cells within the preceding 6 months. Autologous adsorption results associated with molecular findings of *RH* variants in 31 patients showed that eight patients had autoantibodies and 31 had alloantibodies. Retrospective analysis showed that 13/31 alloantibodies (4 anti-D, 5 anti-e and 4 anti-C) were involved in delayed haemolytic transfusion reactions or decreased survival of transfused RBC as verified by the change from the patients' baseline pre-transfusion Hb and percentage of HbS at the time of antibody detection. Those antibodies were associated with worsened anaemia and/or an increase in HbS (Table II).

Discussion

In our institution matching in patients with SCD includes, in addition to ABO, phenotyping for Rh, K1, Fy^a, Jk^a, S and Di^a prior to RBC transfusion, with the aim of preventing alloimmunisation to these RBC antigens and as part of the antibody identification process. In this study, we performed *RH* genotyping in 48 patients with SCD immunised to Rh antigens to determine *RH* variants and evaluated the clinical significance of the antibodies by change in Hb levels at the time of antibody detection.

We report here variant *RH* alleles associated with Rh alloimmunisation in 31/48 of African Brazilian patients with SCD. *RHD* variants were found in 23/31 patients, *RHCE* variants in 28/31 and *RHD* and *RHCE* allele combinations in 20/31 patients. The most frequent *RHD* alleles found in our group of patients were *RHD*DAR, RHD*weak* partial 4.0, *RHD*DIIIa* and *RHD*DAU0*. With regards to *RHCE* alleles carried by these SCD patients, the most frequent alleles were *RHCE*ceS*, *RHCE*ce48*, *RHCE*ce733G*, *RHCE*ceAR* and *RHCE*ceTI*. Variant *RHD* alleles were present in the homozygous and in the heterozygous state in 44% of cases and in the hemizygous state in 12% of patients. Variant *RHCE* alleles were found in the homozygous state in 25% of cases, in compound heterozygosity in 43% and in heterozygosity with a conventional allele in 29% of patients.

The *RH* variants characterised herein have already been associated with Rh alloimmunisation in SCD patients[5,12,13,19]. The main difference in this study is the inclusion of patients with Rh antibodies only. The majority of patients had single Rh antibodies although four (13%) had more than one Rh antibody. In 31 cases, antibodies occurred in patients whose RBC were positive for the antigen and were associated with Rh variants by *RH* genotyping. Thirteen of the Rh antibodies associated

Table II - RH genotypes found in 13 patients with SCD associated with clinically significant antibodies involved in decreased survival of transfused RBC.

ID	Antibody specificity*	*RH* genotype	RBC transfusions	Haemoglobin S (%) at antibody detection		Haemoglobin (g/dL) at antibody detection	
			N.	Pre-transfusion	Post-transfusion	Pre-transfusion	Post-transfusion
375	Anti-e	*ceS/ce733G*	64	23.8	45.8	7.8	6.9
509	Anti-e	*ceS/ce733G*	38	24.3	58.1	8.5	6.4
510	Anti-e	*D-ce48C/D-ceS*	142	29.6	42.1	9.8	8.1
516	Anti-e	*ceS/ce733G*	94	25.3	40.8	8.9	7.5
517	Anti-e	*ce48C,733G/ce733G*	18	28.9	46.1	9.5	8.2
534	Anti-D	*DIIIa-ceS/DIIIa-ceS*	103	19.6	44.6	8.4	7.2
536	Anti-D	*DIIIa-ceS/DIIIa-ceS*	32	29.3	47.2	9.0	7.8
538	Anti-D	*DAR-ce/DAR-ceAR*	14	26.1	34.6	7.9	6.8
562	Anti-D	*DAU4-ce48C/DAU4-ce48C*	16	23.1	43.6	8.6	8.0
711	Anti-C	*DIIIa-CE(4-7)-D-ceS/ DIVa-2-ceTI*	84	24.1	45.3	7.9	6.8
722	Anti-C	*DIIIa-CE(4-7)-D-ceS/ DIVa-2-ceTI*	114	44.3	54.8	6.9	5.9
738	Anti-C	*DIIIa-CE(4-7)-D-ceS/ DIVa-2-ceTI*	62	27.6	58.1	8.6	7.6
752	Anti-C	*DIIIa-CE(4-7)-D-ceS/DAU0-ce*	321	26.4	50.7	7.3	6.5

* Rh antibodies considered to be alloantibodies. RBC: red blood cells.

with altered D, C and e antigens due to homozygous or compound heterozygous *RHD* and *RHCE* variant alleles were found to be clinically significant, as shown by a worsened anaemia and/or increase in HbS when the patients were transfused with the corresponding antigens. Patients who are homozygous, hemizygous or compound heterozygous for RH alleles therefore require a different approach to transfusion management. For example, two patients who developed clinically significant anti-C had the hybrid *DIIIa-CE(4-7)-D* that encodes a partial C antigen associated with variant *RHD* alleles encoding partial D: for these patients strategies to provide D−, C− should be implemented. Although, antibodies against high-prevalence antigens, such as Hr^s (RH18) and Hr^B (RH34) were not identified in our SCD cohort, they have been documented in SCD patients with variant *RHCE* alleles in a homozygous state[19]. It has also been demonstrated by other authors that the Rh antibodies produced by patients with some of the variants found among our patients or antibodies against high-prevalence antigens can be involved in the occurrence of delayed haemolytic transfusion reactions or deaths[5,13].

Furthermore, in patients with SCD there is a strong association between autoantibody development and alloimmunisation[5,12]. In this study, the *RHCE* gene analysis together with serological analysis led to the prediction of autoantibodies against e antigen in 8/48 (17%) of the patients, whereas alloantibodies against D, C and e antigens were detected in 31/48 (65%). Knowledge of the prevalence of *RH* variants and reports such as this one on the clinical significance of antibodies produced in individuals carrying such variants support the development of strategies to match *RH* to avoid Rh alloimmunisation and the risk of haemolytic transfusion reactions and/or poor transfusion outcomes.

Molecular Rh typing has been used to identify altered RH alleles and to predict whether an antibody is an autoantibody or alloantibody and is playing an important role in expanding matching of the RH system in SCD patients and donors[5,8,12]. Transfusion therapy could be improved in such patients if donor centres performed molecular screening in large numbers of donors to identify donors with *RH* variants for matching[20]. As blood group genotyping is becoming part of laboratory routine and platforms targeting a large number of *RH* polymorphisms are becoming available we believe that molecular analysis of RH variants in patients and donors would be feasible.

In conclusion, we characterised a cohort of SCD patients prone to alloimmunisation and delayed haemolytic transfusion reactions and demonstrated that some variant RH alleles were frequent in these patients. With this information, a strategy to prevent Rh alloimmunisation can be implemented. Our finding reinforces the importance of recognising SCD patients with *RH* variants in order to provide them with Rh genetically-matched RBC units.

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