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Original article

Rh antibodies as a result of altered Rh epitopes on transfused red cells: a case series of seven Brazilian patients

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Background - Rh antibodies produced by patients receiving Rh-matched RBC units may be associated with inheritance of altered *RH* alleles or a result of altered Rh epitopes on donor red blood cells (RBC). On this background, our aim was to evaluate unexpected Rh antibodies in Brazilian patients receiving regular transfusions and determine the clinical significance of the alloantibody produced.

Material and methods - We investigated seven patients (5 with sickle cell disease, 1 with myelodysplastic syndrome and 1 with β-thalassaemia) with unexplained Rh antibodies. All patients had complete serological and molecular analyses. A lookback at the donor units transfused to these patients was performed and donors suspected of having Rh variants were recruited for further analysis. Laboratory and clinical findings were used to evaluate the clinical significance of the alloantibodies produced.

Results - The unexpected Rh antibodies found in the patients were not linked to the expression of partial Rh phenotypes according to serological and molecular analyses. Anti-D was found in two patients, anti-C was found in one patient, anti-c was found in one patient and anti-e was found in three patients carrying conventional D, C, c and e antigens respectively. Serological and molecular analyses of donors' samples revealed that six donors whose RBC were transfused to these patients carried partial Rh antigens. Only one anti-e in a patient with β-thalassaemia was autoreactive and could not be explained by *RH* diversity in his donors. Three of the seven Rh antibodies were associated with laboratory and clinical evidence of a delayed haemolytic transfusion reaction or decreased survival of transfused RBC at first detection.

Discussion - Our study provides evidence that patients exposed to RBC units from donors with Rh variants may develop antibodies and some of these may be of clinical significance.

Keywords: *Rh antibodies, Rh epitopes, transfused patients, Rh variants, RBC alloimmunisation.*

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INTRODUCTION

One of the main complications of red blood cell (RBC) transfusion is alloimmunisation, which has become a major concern in transfusion medicine, especially in transfusion-dependent patients such as those with sickle cell disease (SCD), thalassaemia or myelodysplastic

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syndrome (MDS)[1-6]. The antibodies that develop after transfusions remain in the patients' plasma and may be implicated in delayed haemolytic transfusion reactions (DHTR) and in the reduction of the number of compatible blood units for future transfusions, resulting in the inability to give safe transfusions and delays in finding compatible RBC units[7].

In order to prevent the formation of alloantibodies against RBC antigens and the negative consequences subsequent to DHTR, many services have adopted a prospective transfusion protocol of phenotype matching for ABO, Rh (D, C, c, E, e) and Kell (K) antigens, while others have implemented a more extended matching protocol including Fy^a, Fy^b, Jk^a, Jk^b and Ss antigens[8-11]. Transfusion protocols based on the genotypic profile of patients have proven to be even more efficient in preventing alloimmunisation and haemolytic transfusion reactions in patients undergoing chronic transfusion[12-15]. Although these protocols have contributed significantly to the reduction of red cell alloimmunisation and to the improvement of RBC transfusion therapy, it has been observed that some patients still produce antibodies directed to Rh antigens despite receiving transfusions of Rh-matched RBC units. In many cases, these antibodies are considered autoantibodies, because the patient has the corresponding antigen[16,17]. However, molecular analysis has revealed the presence of several altered *RH* alleles predicting expression of partial Rh antigens in these individuals, demonstrating that these antibodies can be classified as alloantibodies and can be clinically significant[18-20]. Conversely, a patient exposed to donor red cells with variant Rh antigens may also recognise these as foreign and form alloantibodies, as suggested in previous studies performed in SCD patients with conventional *RH* alleles and unexplained Rh antibodies[20,21].

The high frequency of altered *RH* alleles in patients and donors, due to the great genetic diversity of the *RH* locus, and the limitations of serological methods to distinguish variant antigens, contribute to the high rate of Rh alloimmunisation in chronically transfused patients[16]. Even though some observations suggest that not all Rh antibodies developed by these patients are associated with inheritance of altered *RH* alleles and may also be a result of altered Rh epitopes on donor RBC[20,21], evidence to prove this is still lacking. Furthermore, the distinction between auto- and allo-antibodies in these patients is difficult and often inconclusive.

Based on this and the fact that donor RBC units with partial antigens are being transfused to Brazilian patients with conventional antigens, our aim was to evaluate Rh alloimmunisation in transfused patients carrying conventional *RH* alleles exposed to partial antigens to provide evidence that Rh antibodies may result from altered Rh epitopes on donor RBC. We also determined the clinical significance of the alloantibodies produced.

MATERIALS AND METHODS

Patients

Seven patients (5 with SCD, 1 with MDS and 1 with β-thalassaemia) on chronic RBC transfusion therapy at the Haematology and Haemotherapy Centre of the State University of Campinas (UNICAMP; Campinas, Brazil) who developed unexplained Rh antibodies in the last 3 years in our institution were evaluated in this study under an institutional review board-approval protocol. These patients had been given Rh and K or extended (Rh, K, Fy^a, Fy^b, Jk^a, Jk^b, S) phenotype/genotype-matched RBC units The transfusion requests and alloimmunisation history from January 2017 to December 2019 were reviewed. The RBC antigen phenotypes of each patient and their history of RBC antibodies were obtained from medical records, the Transfusion Service's computerised database and interviews with the patients. All patients were genotyped for *RH, KEL, FY, JK, MNS, DI, DO, LW, SC* and *RH* variants.

Donors

Donors with weak expression or discrepant results on Rh typing whose RBC were transfused to these seven patients with Rh antibodies were identified in a look-back period of 3 years and recruited for further serological and molecular analyses. From 854 donors evaluated, 11 (1.3%) had weak expression or discrepant results in Rh typing and were recruited: all were repeat donors, had given at least one donation per year in our centre with regular collection and agreed to participate in this study by signing informed consent. Sixteen of these donors were also genotyped for *RH* and *KEL* and for *RH* variants. The study was conducted in accordance with our institutional review board-approval protocol.

Serological analyses

RBC samples collected into EDTA from the seven patients

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with Rh antibodies and from the 11 donors recruited for this study were re-typed for D, C, c, E, e by manual haemagglutination in gel cards (Bio-Rad, Lagoa Santa, MG, Brazil) using two different sources of licensed monoclonal antibodies (Immucor, Norcross, GA, USA and Bio-Rad). Antibody screening and a direct antiglobulin test were performed by gel testing on all patients' samples. Eluate was obtained using an acid elution method (Diacidel, Biorad, Cressier, Switzerland). As most of the patients had been recently transfused, antibodies were tested with autologous cells after performing high-speed microhaematocrit centrifugation to separate autologous RBC from donor RBC[22] and with allogeneic partial Rh antigens when available in our frozen RBC inventory. Adsorption onto autologous RBC was also performed to aid the differentiation of autoantibodies from alloantibodies.

Molecular analyses

Genomic DNA was extracted from whole blood from patients and donors using a QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. RBC genotyping was performed by HEA BeadChip and *RH* variants were genotyped with RHD and RHCE BeadChip arrays (Bioarray, Warren, NJ, USA) contained 35 markers associated with *RHD* and 25 polymorphisms associated with *RHCE* alleles. The assays were performed with 8 µL of each DNA sample, containing approximately 10 to 80 ng/mL of genomic DNA (gDNA), in accordance with the manufacturer's recommendations, and analysed with the web-based software BASIS that automatically generates genotype and predicted phenotype reports. DNA sequence analysis was performed on polymerase chain reaction (PCR) products amplified from gDNA for all samples from patients and donors 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[23,24]. 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 (PEB) sequencer,with the PEB Big Dye reagent BD Half-term (GenPak, Perkin Elmer Biosystems, Foster City, CA, USA).

For specific detection of the *RHD* gene deletion, we used 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[25]. We also used a quantitative PCR approach[26], complemented by the specific detection of *RHDψ*[27].

Analysis of Rh alloimmunisation and clinical significance of the Rh antibodies

Records of transfusions, serological and molecular results were evaluated for all seven patients with unexpected antibodies. The clinical significance of the antibody was determined by comparison of the haemoglobin levels recorded before and after transfusion at the time of antibody detection. A DHTR was defined by a decrease of more than 1 g/dL in haemoglobin levels after transfusion and the transfusion need of the patient.

RESULTS

Subjects

All patients and donors enrolled in this study were from Southeast Brazil and shared African, European and Amerindian ancestries. The patients with SCD had a higher proportion of African ancestry while the patients with thalassemia and MDS had a higher proportion of European ancestry. During this 3-year study the patients received two RBC units every 2-4 weeks for simple transfusion with Rh and K or extended phenotype/genotype-matched units in our Institution.

Unexplained Rh antibodies identified in the patients

Antibodies were classified as auto- or allo-antibodies based on the results of serological testing. Despite transfusions with *RH*-matched RBC, anti-D was found in one patient with SCD and in one patient with MDS whose RBC were D^+. Anti-C was found in one patient with SCD who was typed C^+ and anti-c was found in one patient with SCD typed c^+. Anti-e was found in two patients with SCD and in one patient with thalassaemia who typed e^+. Serological features of the identified antibodies were compatible with the presence of alloantibodies in six patients as these patients' plasma was non-reactive with their own RBC or different kinds of allogeneic partial Rh antigens tested. Only the anti-e detected in a patient with thalassaemia was autoreactive and positive in the eluate.

Rh typing

Patients' RBC showed normal expression of D, C, c, E and e antigens, compatible with conventional antigens. Donors'

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RBC showed weak expression or discordant results with the monoclonal reagents used in Rh typing. Seven donors showed weak or discrepant results on D typing, one donor showed weak expression of C antigen, one donor showed weak and discordant c typing and two donors showed weak or discrepant results on e typing (**Table I**).

Molecular analyses

No altered *RHD* and *RHCE* alleles were found in the DNA samples of the seven patients according to RHD/RHCE BeadChip and sequencing results although these molecular methods may miss clinically relevant *RH* alleles. Among the 11 donors with suspected Rh variant phenotypes who were studied, two had altered *RHD* alleles (*RHD*DAR* and *RHD*DVI*) encoding partial D phenotypes, one had the hybrid allele *RHD*DIIIa-CE(4-7)-D* encoding partial C, one had a partial c encoded by *RHCE*ceJAL* and two had partial e encoded by *RHCE*ceMO* and *RHCE*ce733G*. Discordant results in five donors were due to weak D encoded by *RHD*weak D type 1*, *RHD*weak D type 4.0* and *RHD*weak D type 38* (**Table I**).

Analysis of Rh alloimmunisation

The seven patients with unexplained Rh antibodies received RBC units antigen-positive to the antibody identified. Serological and molecular analyses on donors' samples revealed that six donors whose RBC were transfused into six of the seven patients with unexplained Rh antibodies in the last 3 years carried partial Rh antigens. One SCD patient and one patient with MDS presenting anti-D received RBC units from donors with partial D. SCD patients with anti-C, anti-c and anti-e had been transfused with RBC units carrying partial C, partial c and partial e, respectively. The patient with thalassaemia who developed the autoreactive anti-e was not, apparently, transfused with RBC units from donors with partial e. **Table II** shows the transfusion protocol with the Rh

Table I - *Donors with discrepant results in Rh typing, types of discrepancies and RH genotypes*

Number of donors	Antigen	Type of discrepancy in Rh typing	*RH* genotypes
1	D	Weak expression and discordant results with different clones	*RHD*DVI* type 1/ *RHD*deleted*
1	D	Weak expression and discordant results with different clones	*RHD*DAR/RHD*DAR-RHCE*ceAR/RHCE*ceAR*
1	C	Weak expression	*RHD*DIIIa-CE(4-7)-D*
1	E	Weak expression and discordant results with different clones	*RHCE*ce733G/RHCE*ce733G*
1	E	Weak expression and discordant results with different clones	*RHCE*ce*ceMO/RHCE*cE*
1	C	Weak expression and discordant results with different clones	*RHCE*ceJAL/RHCE*ceJAL*
2	D	Weak expression	*RHD*weak D type 1/RHD*weak D type 1*
1	D	Weak expression	*RHD*weak D type 38/RHD*deleted*
2	D	Weak expression	*RHD*weak D type 4.0/RHD*weak D type 4.0*

Table II - *Patients with unexplained Rh antibodies, transfusion protocol with the Rh phenotypes recommended, red blood cell units with Rh variants received and the patients' transfusion needs*

Pt	Diagnosis	Transfusion protocol (Rh phenotypes)	Rh antibodies	Annual average of RBC transfusions	Genotypes of RBC units with Rh variants transfused to the patients
1	SCD	R_0r	Anti-D	30	*RHD*DAR/RHD*DAR-RHCE*ceAR/RHCE*ceAR* *RHD*weak D type 4.0/ RHD*weak D type 4.0*
2	SCD	R_1r	Anti-C	36	*RHD*DIIIa-CE(4-7)-D ceS/RHCE*ce* *RHD*weak D type 38/RHD*deleted*
3	SCD	R_0r	Anti-e	40	*RHCE*ce733G/RHCE*ce733G* *RHD*weak D type 4.0/ RHD*weak D type 4.0*
4	SCD	R_0r	Anti-c	48	*RHCE*ceJAL/RHCE*ceJAL*
5	SCD	R_2r	Anti-e	26	*RHCE*ce*ceMO/RHCE*cE*
6	MDS	R_1r	Anti-D	42	*RHD*DVI/RHD*deleted* *RHD*weak D type 1/RHD*weak D type 1*
7	Thalassaemia	R_1r	Anti-e	36	*RHCE*Ce/RHCE*ce* RHD*weak D type 1/RHD*weak D type 1

Pt: patient; RBC: red blood cells; SCD: sickle cell disease; MDS: myelodysplastic syndrome.

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phenotypes recommended for each patient, the antibodies developed, the annual average RBC transfusions and the units with Rh variants transfused to the patients.

Clinical and laboratory evidence of delayed haemolytic transfusion reactions

Three of seven patients with unexplained Rh antibodies (1 SCD patient with anti-D, 1 MDS patient with anti-D and 1 SCD patient with anti-C) developed worsening anaemia and/or a decrease in haemoglobin levels greater than 1 g/dL after transfusion with D^+ and C^+ RBC units compared to the pre-transfusion values, compatible with a DHTR at the time of antibody detection, which was also indicated by the increased frequency of transfusions that these patients required.

DISCUSSION

Unexpected Rh antibodies, defined as those produced by patients who have the corresponding antigens, have been the target of many investigations in patients receiving chronic transfusions, especially SCD patients[21]. Individuals with altered *RH* alleles encoding partial antigens lack common Rh epitopes and are at risk of developing clinically significant antibodies[16-20]. Likewise, exposure to altered epitopes on donor RBC can stimulate the formation of antibodies in the patient but the clinical significance of transfusion of a donor unit with partial antigens to patients with conventional antigens remains unknown[28].

We report a 3-year retrospective study in which we investigated the origin of unexplained antibodies in patients undergoing chronic transfusion. We evaluated seven patients with unexpected Rh antibodies and no altered *RHD* and *RHCE* alleles, according to the methods of molecular analysis used. The patients were receiving Rh and K matched RBC units in our institution. All patients had undergone extended genotyping for *RH, KEL, FY, JK, MNS, DI, DO, LW, SC* and *RH* variants and six of them had Rh antibodies with serological features of alloantibodies according to the autoadsorption tests performed and absence of reactivity with their own RBC or with various allogeneic partial Rh antigens tested. A look-back in the records of 854 donors whose RBC units were transfused to these patients identified 11 (1.3%) with weak expression or discrepant results in Rh typing. These donors were repeat donors, had undergone extended antigen typing (Rh, K, Fy^a, Fy^b, Jk^a, Jk^b, Ss) and were re-typed at three donations. Although very mixed, around 40% of donors had a higher proportion of African ancestry. They were recruited for this study and their samples were evaluated by serology and molecular analyses to confirm the presence of Rh variants that could potentially stimulate the production of alloantibodies in the recipients. Six of these 11 donors had altered *RH* alleles encoding partial antigens and five had *RH* variant alleles encoding weak D antigens.

- Patient 1 has SCD and anti-D: phenotyped and genotyped as D^+, this patient received extended phenotype-matched units with partial and weak D antigens encoded by the variant *RHD* alleles *RHD*DAR* and *RHD*weak D type 4.0*. As the patient had a conventional *RHD* gene we suppose that the unexplained antibody had been stimulated by exposure to donor RBC with altered epitopes. An interesting fact is that a blood unit transfused to this patient also had the variant *RHCE*ceAR* and the patient did not develop anti-c or anti-e. Perhaps, this may be explained by the D antigen immunogenicity compared to that of the c and e antigens.
- Patient 2, with SCD, was typed as C^+ and was being given extended phenotype-matched units but developed anti-C after transfusion with a RBC unit containing a partial C encoded by the hybrid *RHD*DIIIa-CE(4-7)-D* allele. This patient was also transfused with one unit of RBC typed as weak D type 38 but did not develop anti-D, suggesting that weak D types with normal epitopes and low antigenic density may not induce alloimmunisation.
- Patient 3 with SCD, typed as e^+ with anti-e and receiving extended phenotype-matched RBC units, was transfused with one RBC unit containing a partial e encoded by the *RHCE*ce733G* allele. This patient also received one RBC unit homozygous for *RHD*weak D type 4.0* alleles but did not develop anti-D. There is still controversy as to whether *RHD*weak D type 4.0* should be considered partial or weak although recently Flegel *et al.* included this type in the list of weak D antigens that do not cause alloimmunisation in transfusion recipients[29]. According to these authors, weak D types 1, 2, 3, 4.0 and 4.1 may be managed safely as D^+ in the context of blood transfusions.
- Patients 4 and 5 both have SCD. Patient 4, receiving extended phenotype-matched units, was typed as c^+ with anti-c. This patient was transfused with a donor unit

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with a partial c encoded by a homozygous *RHCE*ceJAL* allele that probably stimulated the production of the antibody. Patient 5, also receiving extended phenotype-matched units, was typed as e^+ with anti-e and had been transfused with one RBC unit with a partial e encoded by *RHCE*ceMO*, suggesting that this altered e was the cause of production of the patient's unexplained antibody.

- Patient 6, who has MDS and was typed as D^+, was receiving Rh and K-matched RBC units and made anti-D after receiving one RBC unit with a partial D encoded by a hemizygous *RHD*DVI* allele. This patient also received one RBC unit with weak D encoded by a homozygous *RHD*weak D type 1* but we believe that this transfusion was not related to his alloimmunisation as his anti-D was first detected after the transfusion with the partial D category VI unit.
- Patient 7, a patient with β-thalassemia receiving Rh and K-matched RBC units, typed as e^+ with an autoreactive anti-e. All RBC donor units analysed for this patient had a conventional e antigen and we, therefore, consider this antibody as an autoantibody. The only point of note from the look back was that the patient had been transfused with a weak D type 1 RBC unit, reinforcing the theory that weak D types may not induce alloimmunisation.

As only patients 1, 2 and 6 had evidence of DHTR after receiving antigen-positive RBC units we suppose that the clinical significance of the antibodies detected may be related to the immunogenicity of and exposure to the antigen, as suggested by Coleman *et al*[19]. A follow up of the antibodies identified in the patients' serum would be interesting in order to better analyse the clinical significance and evanescence of the antibodies.

The limitations of our study include the molecular methods used that may miss clinically relevant *RH* alleles, the small number of patients, the occurrence of transfusions outside our institution that were not under control and the evaluation period of 3 years. Since these patients are chronically transfused, there is a likelihood that patients 1, 2 and 6 have been exposed to a greater number of red blood cells from donors with Rh variants in other years. The Brazilian population is of mixed ethnicity and therefore our patients, even the SCD patients, receive blood transfusion from donors with an admixture of Caucasian, African and Amerindian ancestry, as reflected by the types of variants found in this study. Thus, patients who receive chronic transfusions are often transfused with RBC units from donors with different types of Rh variants.

Further studies involving all patients who received blood units with altered Rh epitopes are important to confirm our findings.

CONCLUSIONS

In this study, we showed that many Rh epitopes were involved in a small number of patients who developed unexplained Rh antibodies, providing evidence that unexplained Rh antibodies in transfused patients carrying conventional Rh antigens can be alloantibodies as a result of RBC transfusions from donors with altered Rh epitopes.

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AUTHORSHIP CONTRIBUTIONS

MDdM recruited the participants, carried out the serological and molecular genetic studies, analysed data and wrote the manuscript, MRM, TDdS and IL carried out molecular analyses. LC designed and coordinated the study, analysed data and reviewed the manuscript. All authors read and approved the final manuscript.

The Authors declare no conflict of interests.

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