



DEL RBC transfusion should be avoided in particular blood recipient in East Asia due to allosensitization and ineffectiveness*

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Abstract: Previously, both primary and secondary anti-D alloimmunizations induced by "Asian type" DEL (*RHD1227A* allele) were observed in two incidents. We investigated how often these alloimmunization events occur. The transfusions of any D-negative patients were investigated in the First Affiliated Hospital of Xi'an Jiaotong University Medical College, China, during the entire 2009. The antigens of D, C, c, E, and e were routinely serotyped. The "Asian type" DEL variant was genotyped and the *RHD* heterozygote was determined through two published methods. The changes in anti-D levels were monitored by the indirect antiglobulin test (IAT) and flow cytometry. Thirty D-negative transfused patients were included in the study. We focused on 11 recipients who were transfused with packed red blood cells (RBCs) from DEL donors at least one time. Of those 11 recipients, seven were anti-D negative before transfusion and four were anti-D positive (one patient with an autoantibody). One of the seven pre-transfusion anti-D negative patients produced a primary-response anti-D after being transfused with 400 ml of DEL blood twice. All four pre-transfusion antibody positive patients were not observed hemoglobin (Hb) levels increased, as expected after transfusions. Two patients had an increase in anti-D from 1:8 to 1:64 by IAT, which was also shown by flow cytometry. None of the patients experienced an acute hemolytic episode. Our data indicated that the primary anti-D induced by DEL transfusion or the secondary anti-D elevated by DEL in a truly D-negative patient might not be unusual. We suggest that a truly D-negative childbearing-aged woman should avoid DEL transfusion to protect her from primary anti-D allosensitization. In addition, anti-D positive recipients should also avoid DEL red cell transfusion due to the delayed hemolytic transfusion reaction (DHTR).

Key words: Rh blood group, DEL, Allo-anti-D, Transfusion, Pregnancy, Delayed hemolytic transfusion reaction

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1 Introduction

The Rhesus (Rh) blood group is of clinical importance because the D antigen presents a strong immunogenicity in transfusion and mother-fetus al-

loimmunization. Commonly D, encoded by either *RH* genes or *RHD*, is divided into D-positive and D-negative phenotypes. It has been observed that D is weakly expressed in many D variants, such as DEL (or D_{el}) (Okubo *et al.*, 1984). The D antigen on a DEL red blood cell (RBC) is so weak that it is serotyped as D-negative by a conventional indirect antiglobulin test (IAT), and needs to be confirmed by an absorption-elution test. Currently neither patients in clinics nor donors in blood banks are screened for

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DEL, which means we deem DEL as a D-negative donor and a D-negative recipient in transfusion.

DEL alleles were first described by Chang *et al.* (1998). Although most of them are observed in individuals of European descent, the DEL variant is quite rare in these populations (Wagner *et al.*, 2001), and is absent in individuals of African descent. In East Asia, however, the DEL variant is presented at a rate of 28% in Japanese (Fukumori *et al.*, 1997) and 17% in Koreans among apparent D-negative persons (Kim *et al.*, 2005). We reviewed a number of published papers on DEL that were conducted in Hong Kong, Taiwan, and mainland China (including Chinese papers that weren't shown in the references) (Mak *et al.*, 1993; Sun *et al.*, 1998; Shao *et al.*, 2002; Chen *et al.*, 2004; Wang *et al.*, 2005; Xu *et al.*, 2005; Sun *et al.*, 2008; Li *et al.*, 2009). There were 1869 (approximately 27.1%) DELs found by elution or DNA detection among 6906 apparent D-negative cases. The DELs were genotyped, and it was found that more than 98% (667/678) were carrying the *RHD1227A* allele, which predominates in East Asians, and is thus called the "Asian type" (Körmöczsi *et al.*, 2005; Shao, 2010; Shao *et al.*, 2010).

Although DEL is nearly D antigen negative, some truly D-negative patients were still observed being induced allo-anti-D by DEL red cells in transfusions in Europeans and Asians (Körmöczsi *et al.*, 2005; Wagner *et al.*, 2005; Yasuda *et al.*, 2005; Kim *et al.*, 2009). In a case report, Yasuda *et al.* (2005) reported a secondary anti-D alloimmunization in a woman who received a *RHD1227A* DEL blood transfusion in Japan, and more recently, Kim *et al.* (2009) observed a primary allo-anti-D immunization in a 68-year-old Korean man who was transfused with "Asian type" DEL red cells. These studies, along with the high rate of DEL in apparent D-negative individuals, suggest that further clinical investigation is needed to determine whether DEL should be identified in clinics in East Asian countries.

2 Materials and methods

2.1 Design

This study started in January 2009 and lasted throughout the whole year. We routinely observed every D-negative transfusion in the First Affiliated

Hospital of Xi'an Jiaotong University Medical College, Shanxi Province, central China. The D antigen was detected for blood recipients in saline, then D-negative donor blood was applied for transfusion. The weak D and partial D phenotypes are rare in Chinese populations. The DEL variant is not currently tested and distinguished from apparent D-negative blood (determined by IAT) within the blood supply institutions of China. For this study, after pre-transfusion tests, both recipient and donor samples of any D-negative transfusions were collected and sent to the Blood Group Reference Laboratory, Shaanxi Blood Center in Xi'an for further serological and genetic analyses. The following information was recorded: recipients, alloimmunization histories, blood transfusions, transfusion reactions, and the results of anti-D, hemoglobin (Hb), indirect bilirubin and haptoglobin tests.

2.2 Serological tests

The routine blood tests for Hb and hematocrit (HCT) or packed cell volume were performed by using a blood cell counter (Hematology Analyzer MEK 6400, Nihon Kohde, Tokyo, Japan) up to 24 h prior to transfusion and then again 24 h post-transfusion. When necessary, the tests were repeated at other points in time. Serum haptoglobin and bilirubin levels were also tested. The pre-transfusion tests, including irregular serum antibody screening and cross-matching tests, were performed using a commercial gel card method (Diana, Branchville, NJ, USA). When irregular antibodies were detected, the serum sample would be re-screened and identified through conventional IAT using erythrocyte screening cells (Immucor Inc., GA, USA, or Sanquin Reagents, Amsterdam, the Netherlands, or Shanghai Blood Center, China), and an erythrocyte panel for antibody specificity identification (Immucor Inc., GA, USA, or Sanquin Reagents, Amsterdam, the Netherlands). Autoantibody was tested too, but the procedure was not completed systematically. The post-transfusion anti-D was tested during the 4th week after the first transfusion. Occasionally, patients were unavailable for additional testing. For Rh blood group typing, the C, c, E, and e antigens were tested in saline (anti-C: MS24; anti-E: MS12; anti-c: MS33; and anti-e: MS16. Immucor Diagnostik GmbH, Roedermark, Germany), and the D was typed by IAT (anti-D,

IgM+IgG, Clone 175-2 and Clone 415 1E4. Dominion Biological Limited, Nova Scotia, Canada, or Immucor Inc., Norcross, GA, USA).

2.3 Genotyping

Genomic DNA was isolated from patient and donor whole blood samples (Promega wizard genomic DNA extraction kit, Promega Corporation, Madison, WI, USA). DNA was also extracted from the patient's husband if her serum anti-D detected positive. The *RHD* was first genotyped through a sequence-specific polymerase chain reaction method (PCR-SSP) by using a commercial kit for partial D detection (BAG Health Care GmbH, Lich, Germany). This method detects all ten exons of *RHD* and typed C, c, E, and e of the *RHCE* gene according to the manufacture's procedures. If a sample was *RHD* positive for any of the exons, it would be sent to Shenzhen Blood Center for detection of the *RHD1227A* allele ("Asian type" DEL) and the *RHD* zygosity using methods detailed elsewhere (Shao et al., 2002; Perco et al., 2003).

2.4 Flow cytometry

To remove the ambiguities inherent to the IAT method, the changes of serum anti-D titer were also confirmed by flow cytometry. The serum samples were mixed 4:1 with 4% RBC suspension in phosphate buffer saline (PBS). The D-positive red cells were pooled from three previously typed DCCee (DcE/DCe) donors (Shao et al., 2002). The D-negative control cells were pooled from three ddcee samples (Shao, 2010). The negative control serum sample came from a D-positive, AB donor. The tubes were incubated at 37 °C for 45 min, and shaken every 15 min. The RBCs were washed with 0.9% (9 g/L) saline solution 5 times, and the supernatant was removed. A total of 15 µl of goat anti-human IgG antigen-binding fragments (Fab) antibody labeled with fluorescein isothiocyanate (FITC) was added (Jackson Immuno Research, AP, USA), and the mixture was kept in a light-proof container at room temperature for 24 min, and shaken by hand every 5 min. The RBCs were then washed five times with normal saline solution. On the last wash, the supernatant was removed and 2 ml of normal saline was added and mixed for cytometry (FACSCanto II, BD Biosciences, MA, USA). A total of 30000 to 50000 events were

collected. The median fluorescence intensities were compared across the samples.

3 Results

In 2009 at the Department of Transfusion, Hospital of Xi'an Jiaotong University Medical College, there were 30 D-negative (in IAT) transfusion inpatients. Among them, 11 were transfused with packed red blood cells (PRBCs) or washed red blood cells (WRBCs) from "Asian type" DEL donors at least once. Two patients were found to be heterozygous for the *RHD1227A* allele (D_{el}/dCcee, 18.2%) and nine patients were dd (81.8%, Table 1, patient No. 15 has nonfunctional *RHD* allele, *RHD-RHCE(2-9)-D*). We found that four of the 11 patients had anti-D antibodies before transfusion (one had autoantibody). After two units of PRBCs transfused (one unit is derived from 200 ml of whole blood), two patients tested anti-D titers jumped from 1:8 to 1:64 (50.0%). In the seven anti-D negative patients (two are DEL variants), one of them developed anti-D (1:2) after being transfused twice with two units of DEL PRBCs (14.3%). It was not surprising that the "Asian type" DEL elicits or enhances anti-D to a truly D-negative blood recipient, although primary anti-D is difficult to test. There was no clinical evidence of acute hemolysis or rise in serum haptoglobin in any of the patients.

Patient No. 3 (Table 1) was a 33-year-old woman, who was diagnosed with dysfunctional uterine bleeding (DUB). She had two gestations and one parturition (G2P1). She denied any history of transfusion, and denied ever receiving Rh immune globulin prophylaxis. Her husband was a D-positive phenotype with D/D genotype. The day before transfusion, a routine blood test showed whole blood Hb of 60 g/L, RBC $1.80 \times 10^{12} \text{ L}^{-1}$, platelet count (PC) $80 \times 10^9 \text{ L}^{-1}$, and HCT of 0.22. In pre-transfusion tests, serum antibody screening and identification showed only anti-D positive at a titer of 8-fold. Two units of cross-match-compatible group B and D-negative PRBCs were transfused simultaneously. No acute transfusion reactions were observed; however, the routine blood test on the third day showed Hb 55 g/L, RBC $1.68 \times 10^{12} \text{ L}^{-1}$, PC $70 \times 10^9 \text{ L}^{-1}$, and HCT 0.17, and her serum indirect bilirubin rose to 18.5 µmol/L, which indicated a delayed hemolytic transfusion

reaction (DHTR) had occurred. No further transfusions were prescribed. Upon recover, the patient was discharged from the hospital two weeks later. Twenty-five days post transfusion, a blood sample was drawn and an IAT detected anti-D at a titer of 1:64. The titers of pre-transfusion anti-D and post-transfusion anti-D were also compared by flow cytometry (Fig. 1).

Patient No. 7 was a 45-year-old man with colon carcinoma. His family members declared that he had a transfusion of whole blood 20 years earlier during an appendicitis operation in a county hospital. The

number of blood units transfused was unknown; however, Rh(D) blood group testing was not required in China at that time. After being transfused with two units of PRBCs from a DEL donor, his anemia had not improved significantly (Hb 55 to 57 g/L). A second bag of red cells (two units) was applied on the third day, and his Hb increased to 60 g/L. The patient's serum indirect bilirubin remained in the normal range. Four weeks after transfusion, IAT detected a 4-fold increase in his anti-D (1:8 to 1:64), which was confirmed by flow cytometry (Fig. 1).

Patient No. 11 was a woman at our center who

Table 1 Truly D-negative patients transfused with DEL RBCs

Patient No.	Sex	Age (year)	Diagnosis	Rh phenotype	Immune history	Pre-transfusion Abs	Transfusion (Rh/Vol (ml))			Post-transfusion anti-D	Reaction
							1	2	3		
2	M	52	Fracture	ddccee	None	None	DEL/400			None	None
3	F	33	DUB	ddccee	G2P1 with D ⁺ child	Anti-D 1:8	DEL/400			1:64	Hb: 60→55 g/L
7	M	45	Colon cancer	ddCcee	Probably D ⁺ transfusion	Anti-D 1:8	DEL/400	dd/400		1:64	Hb: 55→57→60 g/L
8	M	42	Hemolytic anemia	ddccEE	Apparent D ⁻ transfusion	Auto-Ab ⁺	dd/400	DEL/400	dd/200	Auto-Ab ⁺	Hb: 38→40→40→46 g/L
11	F	28	Caesarean birth	ddccee	G3P1 with D ⁺ child, probably D ⁺ transfusion	Anti-D 1:512	DEL/400			1:512	Hb: 46→48 g/L
15	M	68	Gastric carcinoma	ddCcee	None	None	DEL/400	DEL/400		1:2	Hb: 57→62→68 g/L
17	F	31	Trauma	ddccEE	G1P1 with D ⁺ child	None	DEL/400			None	None
20	M	27	MDS	ddCcee	None	None	DEL/400			None	Rash
25	F	12	AML	ddCcee	None	None	DEL/200			None	None

DUB: dysfunctional uterine bleeding; MDS: myelodysplastic syndromes; AML: acute myeloid leukaemia; Vol: volume; Ab: antibody

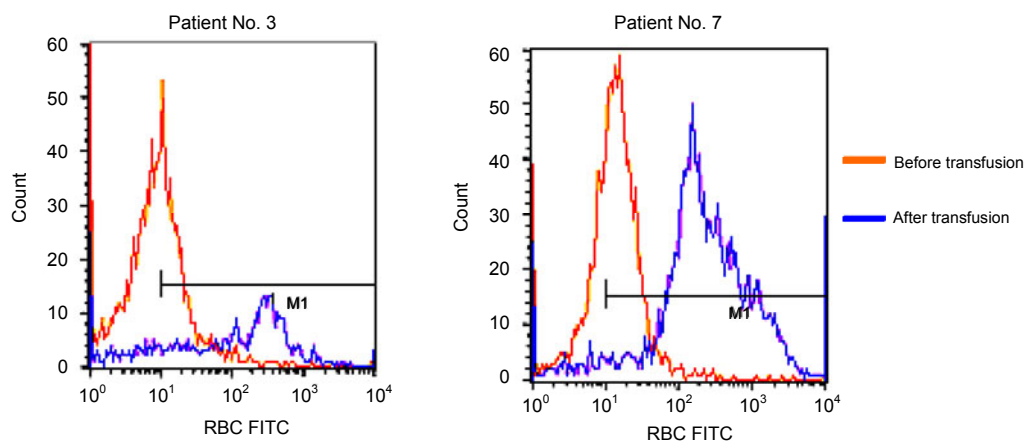


Fig. 1 Flow cytometry analysis of anti-D before and after transfusion

The fluorescein isothiocyanate (FITC) intensities of pre-transfusion and post-transfusion anti-D to D-positive red cells were compared. In patient No. 3, the mean fluorescence intensity and the peak fluorescence intensity before transfusion were 10.22 and 14.28, respectively. Both values after transfusion were 224.74 and 317.54. In patient No. 7, the values were 11.56 and 19.38 pre-transfusion, and 315.35 and 233.26 post-transfusion, respectively

needed a caesarean section due to hemolytic disease of the fetus and the newborn (HDFN). Her anti-D titer was 1:512. She had a history of transfusion due to a childhood surgery and had two miscarriages. She received two units of DEL PRBCs after her operation. Her child recovered with a treatment of exchanged blood. Her anti-D titer remained unchanged by the 4th week of testing, but her Hb dropped and her serum indirect bilirubin rose by the fifth day (26.1 $\mu\text{mol/L}$) indicating a DHTR.

Seven other patients, who tested negative for anti-D at admission, had no history of D-positive blood transfusions. Patient No. 4 was transfused with apparent D-negative red cells many times. Patient No. 17 was recorded G1P1 (her husband was tested D/D). After DEL transfusions, six of them had no detectable anti-D (two patients, Nos. 4 and 13, were typed as DEL and would not be expected to develop anti-D from DEL transfusions). However, one male patient (No. 15), with no transfusion history, received DEL PRBCs twice, and 22 d after his first transfusion he was found to have a 1:2 titer of anti-D. This appears to be characteristic of primary anti-D alloimmunization.

4 Discussion

Although the D-negative phenotype is rare in East Asian populations (0.3%–0.5%) compared to Caucasians (about 15%), the DEL variant is common among the apparent D-negative individuals. The highest rate reported is approximately one-third (Mak *et al.*, 1993). In this study, among all 30 apparent D-negative patients, six were genotyped DEL (20%), and among the donors to those patients, 12 of 51 are DEL (29%) (data not shown). This indicates that a truly D-negative blood recipient frequently receives DEL blood. It is, therefore, important to explore the effects of the DEL phenotype in clinical blood transfusions or for mother-fetus alloimmunization prophylaxis in East Asia countries.

Commonly we see a donor with a weak D phenotype as a D-positive in transfusion. Although D antigen can be detected on DEL red cells, we deem a DEL as a D-negative donor as there are fewer than 22 RhD antigens per “Asian type” DEL red cell (Körmöcz *et al.*, 2005), and a DEL is routinely typed as

D-negative by IAT. According to this study, we should reconsider our screening policy for DEL in donors and recipients. Although the cases reporting primary and secondary anti-D immunizations by DEL (Yasuda *et al.*, 2005; Kim *et al.*, 2009) were once considered a rare event, our primary data show that 50% of truly D-negative patients with anti-D developed DHTR after DEL RBCs transfusion, and 14.3% of apparent D-negative patients with no detected anti-D developed primary allosensitization. If we excluded two DEL recipients (Shao, 2010; Shao *et al.*, 2010), the latter number would be 20.0%. This indicates that the ineffective transfusions elicited by the “Asian type” DELs are a frequent occurrence. Therefore, a truly D-negative woman of childbearing age, should be protected from primary anti-D allosensitization by transfusing her with DEL red cells. In addition, for an anti-D positive recipient, the use of DEL blood, for an effective red cell transfusion, should be avoided. The first step, however, should include screening all donors and recipients for the DEL phenotype.

Another important consideration to the Rh(D) blood group is prophylaxis of mother-fetus alloimmunization, the hemolytic disease of the fetus and the newborn. When a mother is a DEL variant, it is safe to give birth to a D-positive child (Shao, 2010; Shao *et al.*, 2010). However, when a mother is truly D-negative and the fetus or newborn is a DEL variant, according to our study it is important to consider the possible occurrence of allosensitization or a secondary alloimmunization. To our knowledge, an HDFN case due to a DEL variant newborn as never been reported. Maybe a mild DEL HDFN is easily ignored as physiological jaundice. Further research is needed in order to implement a prophylaxis policy in East Asian countries.

It is well-known that the DEL variant occurs in approximately 1/4–1/3 of apparent D-negative East Asian individuals. Although more investigation is needed, our primary data indicates that it is not unusual that the “Asian type” DEL RBCs induce a DHTR in a truly D-negative blood recipient. Therefore it is suggested that blood centers and blood banks need to identify the presence of the DEL variant in apparent D-negative donors and recipients. A truly D-negative blood recipient should not receive DEL transfusion in order to protect him or her from primary anti-D allosensitization or severe DHTR.

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