Transfusion Medicine and Hemotherapy

Original Article

Transfus Med Hemother 2015;42:15–21
DOI: 10.1159/000370217

Received: November 15, 2013
Accepted: May 14, 2014
Published online: December 22, 2014

Prospective Evaluation of a Transfusion Policy of RhD-Positive Red Blood Cells into DEL Patients in China

Wei Xu[a,b] Mei Zhu[c] Bao-Long Wang[a] Hong Su[d] Min Wang[a]

[a] Department of Laboratory Medicine, Affiliated Provincial Hospital of Anhui Medical University, Hefei, Anhui, China;
[b] Department of Blood Transfusion, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China;
[c] Department of Nuclear Medicine, The Third Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China;
[d] Department of Epidemiology & Biostatistics, School of Public Health, Anhui Medical University, Hefei, Anhui, China

Keywords
DEL variant · Pregnant women · Complete DEL · Partial DEL · Alloimmunization

Summary
Background: The D antigen is highly immunogenic, requiring only a small quantity of transfused red blood cells (RBCs) to cause alloimmunization in D– immunocompetent recipients. DEL was reported arousing alloimmunization to true Rh– patients. Molecular studies of the *RHD* gene have revealed that DEL individuals retain a grossly intact *RHD* gene or have a portion of *RHD* in their genomes. Avoiding immunization with clinically important antibodies is a primary objective in transfusion medicine. **Methods:** In order to determine whether pregnant DEL women carrying an RhD+ fetus are at risk of anti-D alloimmunization, 808 Rh– pregnant women with a history of gestations or parturitions who regularly visited hospitals for their prenatal anti-D screening and postpartum care from January 2011 to December 2012 were investigated. Samples were analyzed for DEL by PCR with specific primers, PCR-sequence-specific primers (PCR-SSP), reverse transcription-PCR (RT-PCR), PCR-restriction fragment length polymorphism (PCR-RFLP), and by gene sequencing to characterize different alleles. **Results:** Among the 808 Rh– pregnant women of our sample, 178 (22.0%) were typed as DEL; 168 DEL samples were confirmed to have the *RHD* (1,227 G>A) allele, 8 DEL samples were characterized by one base mutation of the *RHD* (3G >A) allele, and the remaining two DEL samples were determined to carry *RHD-CE(4–9)-D* or RHD-CE(2–5)-D. The observation of allo-anti-D in two prominent D epitope loss cases confirmed the partial nature of these DEL phenotypes. **Conclusions:** In conclusion, evidence is provided that different *DEL* genotypes code either for partial or complete D antigen expression. It is suggested that the use of RhD+ RBCs in complete D antigen DEL patients does not induce adverse reaction.

Introduction

The risk of anti-D alloimmunization in D– healthy volunteers who received D+ red blood cells (RBCs) is higher than 80% [1–6]. According to national transfusion guidelines from many countries, D– patients should receive RBCs from D– donors. Unfortunately, in some situations, such as massive transfusion or a shortage of D– RBCs, because Rh– persons represent about 0.3% in China, the transfusion of D+ RBCs to D– patients may be required. However, after transfusion of D+ RBCs to D– patients the anti-D alloimmunization is frequent [1]. Accordingly, a new strategy should be developed in order to optimize their use in China.

Routine serologic typing does not discriminate DEL from the D– phenotype, DEL phenotypes were determined on the basis of no agglutination in the indirect antiglobulin test (IAT) procedure and positive results with adsorption-elution techniques [7, 8]. The DEL phenotype arises from a mutation of the *RHD* gene, and more than 20 different *DEL* alleles have been described [4]. Molecular studies have indicated that DEL individuals retain a grossly intact *D* gene or a partial DEL with detectable D epitope (epD) loss [9–13]. D and CE poly-

Wei Xu and Mei Zhu contributed equally to this publication as first authors.

KARGER

Fax +49 761 4 52 07 14
Information@Karger.com
www.karger.com

1660–3796/15/0421–0015$39.50/0

Accessible online at:
www.karger.com/tmh

Bao-Long Wang
Department of Laboratory Medicine
Affiliated Provincial Hospital of Anhui Medical University
17 Lu Jiang Road, 230001 Hefei, Anhui, China
wbl196555@163.com

peptides are coded for by the highly homologous *RHD* and *RHCE* genes. Partial D variants exhibit some degree of antigenic alteration derived from *RHD*/*RHCE* gene hybridization events, in most instances accompanied by a reduction in antigen density [14]. Amino acid substitutions in extracellular stretches of the D polypeptide lead to loss of one or more epDs, rendering also partial D individuals prone to anti-D alloimmunization upon contact with normal D+ RBCs expressing the complete set of epDs [14, 15]. These reports support the possibility that a partial DEL phenotype could theoretically induce anti-D alloimmunization [9, 11, 14]. The antibody is also capable of causing severe hemolytic disease of the fetus and newborn (HDFN) in sensitized pregnant DEL women carrying an RhD+ fetus [9]. A partial D-like epitope loss has been reported in DEL phenotypes to be associated with the *RHD* (IVS3 + 1G>A) allele, and individuals with this phenotype can make anti-D [11]. Although these anti-D antibodies may indicate a secondary immune response, a rapid primary anti-D immunization attributed to this DEL phenotype was still possible. Our study explored whether pregnant DEL women carrying an RhD+ fetus are at risk of anti-D alloimmunization.

Material and Methods

Study Population

The present study was conducted between January 2011 and December 2012 at the Department of Blood Transfusion of Affiliated Province Hospital of Anhui Medical University, the Department of Blood Transfusion of The First Affiliated Hospital of Anhui Medical University, and the Department of Blood Transfusion of The Third Affiliated Hospital of Anhui Medical University. The three hospitals are located in the capital cities of Anhui province and are the largest tertiary hospitals in Anhui, China. Medical services provided by the three hospitals laboratories include antenatal blood group and antibody screening on more than 150,000 pregnant women per year.

Inclusion Criteria

Only pregnant DEL women carrying Rh+ fetus who declared a history of gestations or parturitions were included. The disease can become progressively more severe in subsequent pregnancies where the fetus is D+. Pregnant women with autoantibodies, a history of blood transfusion, and receiving Rh immune globulin prophylaxis were excluded from the study groups, as were women who were alloimmunized to other blood group antigens. Our study was approved by the Scientific and Ethics Committee of Anhui Medical University. Informed consent was obtained from all participants.

Rh Phenotyping

During a 2-year study period, a total of 313,250 EDTA-anticoagulated blood samples from pregnant women were collected in the three hospitals. The D antigen was determined by direct agglutination with the monoclonal anti-D in saline according to the manufacturers' instructions. Samples that were negative to anti-D in the direc t agglutination were retested by using the indirect antiglobulin test (IAT). Subsequently, all samples typed as D– with the IAT were retested for the DEL phenotype through an adsorption and elution test in tubes. Briefly, RBCs were incubated with an equal volume of polyclonal anti-D at 37 °C for 1 h. The cells were then washed thoroughly. An eluate was prepared by using a heat elution technique. The eluates and final supernatants were used for the IATs. If the result was positive (≥1+), the sample was marked as DEL. RhC/c and E/e were determined by using monoclonal anti-C,c,E,e reagents (Gamma, Houston, TX, USA) according to the manufacturer's instructions.

Molecular Studies

Genomic DNA Extraction

Genomic DNA of DEL samples was extracted from 200 µl of EDTA-anticoagulated peripheral blood samples according to the manufacturer's recommendations (QIAamp DNA Blood Mini Kit, Qiagen GmbH, Hilden, Germany). To minimize the risk of contamination, DNA was isolated under laminar airflow, and aerosol-resistant tips were used. The optical density (OD) of the purified DNA was measured using an ultraviolet spectrophotometer. The DNA was extracted once from each sample and then stored at –20 °C until further processing within 48 h.

RHD Genotyping

Amplification of RHD Exons

We devised *RHD*-specific primers based on intron sequences to amplify genomic DNA for 10 *RHD* exons 1–10. PCR was performed using a commercially available system (Expand High Fidelity Polymerase; Roche Applied Science, Basel, Switzerland). Cycling conditions for *RHD* exon 1 and 10 were denaturation at 95 °C for 40 s, annealing for 40 s at 64 °C (exons 1–4, 6, 8–10), at 60 °C (exons 5, 7) and at 57 °C (β-actin), and extension at 72 °C for 1 min. The final extension was done at 72 °C for 10 min. Amplified DNA products were visualized by electrophoresis in a 1.5% agarose gel with ethidium bromide staining and photographed under UV light. *β-actin* gene was used as an internal control.

DNA Sequencing

The complete *RHD* exons 1–10 including adjacent intron regions were sequenced from PCR products using the respective PCR primers with an ABI PRISM 3730 automated sequencer (Applied Biosystems®; Life Technologies Carlsbad, CA, USA). The nucleotide and deduced amino acid sequences were analyzed and compared with the published sequences. D specificities of primers used for exon amplification and sequencing were identical and are listed in table 1.

PCR-SSP and cDNA Sequencing to Determine RHD-CE-D Hybrid Alleles

For *RHD* and *RHCE* genotyping, testing for different partial and weak D variants and determination of the *RHD* zygosity of investigated and control blood samples, a PCR-SSP was performed with commercially available typing kits (CDE, weak D, RHd, Inno-Train). Kit 'CDE' is capable of properly identifying *RHD-CE-D* hybrid alleles by detecting *RHD*-specific DNA sequences in the 5′-untranslated region and exons 2, 3, 4, 5, 6, 7, 9, and 10 of *RHD* and *RHC*, *RHc* (intron1 and exon 2) and *RHE*, *RHe* (exon 5) of *RHCE* as described previously [16].

Total RNA was isolated with reagent (Trizol; Invitrogen™, Life Technologies). RNA integrity and quantity was assessed by measuring the OD at 260 and 280 nm with the NANO DROP 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Reverse transcription with random hexamer primers was performed with the PrimeScript™ RT Master Mix kit (Takala, Dalian, China) following manufacturer's instructions. Amplification of cDNA was performed on a thermocycler T Gradient (Biometra, Göttingen, Germany) with Primer a 5′-CACAGGATGAGCTCTAAGTAC-3′ located at the 5′ end of *RHD/RHCE* and Primer b 5′-TAAATGGTGAGATTCTCCTC-3′ located in *RHD* 3′ noncoding sequence or with Primer c 5′-CAAATCTGTCTCTGACCTTGTTTC-3′ located in *RHCE* 3′ noncoding sequence. Samples were submitted to an initial denaturation step (5 min at 94 °C), followed by 35 amplification cycles (45 s at 94 °C; 45 s at 50 °C; 1 min at 72 °C) and a final elongation step of 10 min at 72 °C. PCR products were sequenced by a genetic analyzer. Results were compared with the published sequences.

PCR-RFLP for RHD Zygosity Determination

According to the method published by Wagner and Flegel [17], a PCR amplification was performed by using the expand high-fidelity PCR system with primers rez7 (consensus, 5′ of the *Rh box* identity region) and rnb31 (specific

Table 1. Primers used for PCR and DNA sequencing

Primer name*	Sequence 5′ to 3′	Specificity	Genomic region	Position§	Product size (bp)
E1-s (=E1-seq)	TCCATAGAGAGGCCAGCACAA	D	promoter	−152 to −132	340
E1-a	GCTATTTGCTCCTGTGACCACTT	D	intron 1	40–18	
E2-s	TGACGAGTGAAACTCTATCTCGAT	D	intron 1	−1,064 to −1,041	1,606
E2-a (=E2-seq)	GGCATGTCTATTTCTCTCTGTCTAAC	D/CE	intron 2	355–330	
E3-s	GTCGTCCTGGCTCTCCCTCTCT	D	intron 2	−29 to −8	219
E3-a (=E3-seq)	CTTTTCTCCCAGGTCCCTCCT	D/CE	intron 3	39–19	
E4-s	GCCGACACTCACTGCTCTTAC	D/CE	intron 3	−36 to −16	378
E4-a (=E4-seq)	TGAACCTGCTCTGTGAAGTGC	D	intron 4	194–174	
E5-s	CTGCCAAAGCCTCTACCCG	D	intron 4	−502 to −484	984
E5-a (=E5-seq)	GCTGACTCTCGCTCATGGT	D/CE	intron 5	315–297	
E6-s (=E6-seq)	CAGGGTTGCCTTGTTCCCA	D/CE	intron 5	−95 to −77	274
E6-a	CTTCAGCCAAAGCAGAGGAGG	D	intron 6	41–21	
E7-s (=E7-seq)	CTACTCATAGTGTGGTCCGTAGACC	D	intron 6	−280 to −256	543
E7-a	CAAATATTCACCGAAGCCTACTG	D/CE	intron 7	129–107	
E8-s	GGTCAGGAGTTCGAGATCAC	D	intron 7	−594 to −575	771
E8-a (=E8-seq)	GATGGGGCACATAGACATCC	D/CE	intron 8	97–78	
E9-s (=E9-seq)	GGTCCAGGAATGACAGGGCT	D	intron 8	−162 to −143	530
E9-a	CGCTGAGGACTGCAGATAGG	D	intron 9	294–275	
E10-s (=E10-seq)	CAAGAGATCAAGCCAAAATCAGT	D/CE	intron 9	−67 to −45	382
E10-a	AGCTTACTGGATGACCACCA	D	3UTR	290–271	
β-actin-s	GGAAATCGTGCGTGACATT	–	–	–	473
β-actin-a	CGTCATACTCCTGCTTGCTG	–	–	–	

s = Sense primer; a = antisense primer; seq = sequencing primer.
§The positions of the synthetic oligonucleotides are indicated relative to their distances from the first nucleotide position of the start codon ATG for all primers in the promoter and in the exons or relative to their adjacent exon-intron boundaries for all other primers.

for downstream of the *Rh box*, 3′ of the *Rh box* identity region). PCR products were digested with *Pst*I for 3 h at 37 °C, and the fragments were resolved by electrophoresis on a 1% agarose gel.

Lookback

The antibody screening in the pregnant DEL women was performed using fully automated Johnson gel technology using erythrocyte screen cells (Shanghai Blood Center, Shanghai, China). Erythrocyte panels were performed to confirm allo-anti-D specificity (Immucor Inc, Norcross, GA, USA, or Sanquin Reagents, Amsterdam, the Netherlands). After confirmation of the maternal allo-anti-D, routine serological typing of her husband and the newborn was made. The newborn's total bilirubin concentration and reticulocyte count was measured, and an anti-D elution test and a direct antiglobulin test of cord blood were done.

Results

Serologic Studies

In this prospective study, the 808 blood samples of D– pregnant women reacted negatively with the IgM monoclonal anti-D in saline. Using the IAT and the adsorption and elution test, a total of 178 DEL samples was found. Their phenotypes of RhC/c and E/e were displayed in table 2.

Table 2. The phenotypes of RhC/c and E/e in the 808 pregnant women samples

Rh phenotypes	RhC/c and E/e phenotype							
	Ccee	CCee	CCEe	CcEe	ccEe	ccEE	ccee	total
Apparent D–*	280	31	7	21	32	9	428	808
DEL	157	11	6	2	2	0	0	178

*The apparent D negative phenotypes were determined by a microplate test.

Molecular Characterization of DELs

A total of 168 samples were determined by sequencing to carry the *RHD* 1227G>A allele, and the variant shows a silent nucleotide change at the exon boundary. Eight DEL samples were characterized by one base mutation of the *RHD* (3G>A) allele, codon 1–3 were changed from ATG to ATA. The remaining two DEL samples seemed to have *RHD-CE-D* hybrid alleles. According to *RHD/CE* PCR-SSP, one *RHD-CE(4–9)-D* and one *RHD-CE(2–5)-D* for *RHD-CE-D* hybrid alleles were identified as judged by testing for the *RHD* 5′-untranslated region and exons 2, 3, 4, 5, 6, 7, 9, and 10.

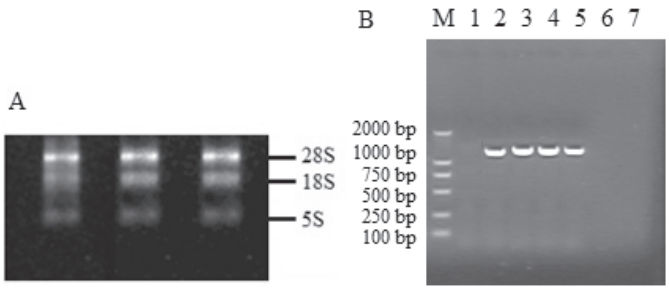

Fig. 1. A The result of RNA electrophoresis. **B** The results of RT-PCR for the *RHD-CE-D* hybrid alleles. M: molecular marker; Lane 1: water control; Lane 2: amplicon of *RHCE*+ control with primer a and *RHCE*-specific reverse primer c (band of 1399 bp); Lane 3: amplicon of *RHD*+ control with primer a and *RHD*-specific reverse primer b (band of 1,446 bp); Lane 4–5: amplicons for *RHD-CE(4–9)-D* and *RHD-CE(2–5)-D* with primer a and *RHD*-specific reverse primer b (band of 1,446 bp); Lane 6–7: amplicons were lacking for *RHD-CE(4–9)-D* and *RHD-CE(2–5)-D* with primer a and *RHCE*-specific reverse primer c.

The 260/280 nm ratios of the samples were >1.8. Sample purity was confirmed by electrophoresis on an agarose gel. All samples contained 18S and 28S ribosomal RNA peaks with no visible degradation products (fig. 1A). The results of RT-PCR are shown in figure 1B. Nucleotide sequencing from their cDNA was performed to show that the two *RHD-CE-D* hybrid alleles did not harbor any additional variation. No novel allele was found in the retrospective study. Frequencies and respective 95% confidence intervals (95% CIs) for the alleles encountered in this study are given in table 3.

RHD Zygosity Determination

All 178 DEL samples presented with Rh box zygosity. 19 samples, including 16 samples with *RHD* (1,227G>A) allele and 3 samples carrying *RHD* (3G>A), were found to be *RHD*+/*RHD*+ homozygotes by PCR-RFLP analysis. The rest of the 159 samples including 2 samples with the *RHD-CE-D* hybrid allele, 5 samples with *RHD* (3G>A), 152 samples carrying *RHD* (1,227G>A) allele were *RHD*+/*RHD*– hemizygous. The associated serological data are described in table 4.

Complete DEL Is Not at Risk of Anti-D

There are 56 ethnic groups in China. More than 90% of the population is Han ethnicity; the rest of the population is composed of 55 minority groups. Of all 178 pregnant DEL women in this prospective study, 70 women were excluded from the study group (table 5) because they did not meet the above mentioned inclusion criteria. All 108 pregnant women were Han Chinese aged 21–41 years at the time of testing. In this cohort, 101 samples (93.5%) showed a *RHD* (1,227G>A) mutation, 5 samples (4.6%) were determined to carry *RHD* (3G>A) allele in *RHD* exon 1, and the remaining 2 samples (1.9%) showed the characteristics of the *RHD-CE(4–9)-D* or *RHD-CE(2–5)-D* hybrid gene. The 2 women carrying the *RHD-CE-D* hybrid developed allo-anti-D, and their children developed mild hemolytic disease of the newborn (table 6).

Table 3. Estimated DEL allele frequencies and 95% CIs in 808 D– pregnant women

Alleles observed	n	Estimate	95% CI	
			lower	upper
RHD (1,227G>A)	168	1:5	1:6	1:4
RHD (3G>A)	8	1:101	1:233	1:52
RHD-CE(4–9)-*D*	1	1:808	1:31,949	1:145
RHD-CE(2–5)-*D*	1	1:808	1:31,949	1:145

Table 4. Results of PCR–RFLP analyses of the 178 DEL population

DEL allele	*RHD* zygosity	*RHCE* haplotypes	Number of subjects
RHD (1227G>A)	16 *RHD*+/*RHD*+	Ccee	12
		CCee	2
		CCEe	1
		CcEe	1
	152 *RHD*+/*RHD*–	Ccee	138
		CCee	8
		CCEe	3
		ccEe	2
		CcEe	1
RHD (3G>A)	3 *RHD*+/*RHD*+	Ccee	2
		CCEe	1
	5 *RHD*+/*RHD*–	Ccee	3
		CCee	1
		CCEe	1
RHD-CE(4-9)-D	1 *RHD*+/*RHD*–	Ccee	1
RHD-CE(2-5)-D	1 *RHD*+/*RHD*–	Ccee	1
Total			178

Prospective Transfusion Policy of DEL Patients in Chinese Han

Indications for mandatory use of D– RBCs: DEL patients with anti-D antibody or carrying partial DEL allele. Indications for recommended use of D– RBCs: women of childbearing age carrying a complete DEL allele, under-age children (<18 years). Indications for acceptable use of D+ RBCs: massive transfusion patients, adult men (>18 years) and women of non-childbearing age with no detectable anti-D antibody in case of insufficient D– RBCs in stock.

Discussion

If RBC units of D+ were transfused to DEL recipients, the clinical consequences are of current interest in China. Molecular studies

Table 5. 70 pregnant women were excluded from this study

Exclusion criteria	Han	Tibetan	Uigur	Hui	Mongol	Sum
A history of blood transfusion	6	1			1	8
Mother receiving RhIG prophylaxis	21				1	22
If mother carries apparent D– fetus	2		1			3
Mother with autoantibodies	2			1		3
Mother with other blood group antibodies	4					4
No a history of gestations or parturitions	27					27
Non-Chinese Han population		1		1	1	3
Total	62	2	1	2	3	70

Table 6. Anti-D alloimmunization among two *RHD-CE-D* hybrid allele pregnant women

Pregnant women					Newborn				
DEL allele	n	ABO	zygosity*	anti-D titer	TBIL#, μmol/l	Ret count$^{\Delta, \%}$	ABO	DAT	anti-A/B
RHD-CE(4-9)-D	1[a]	A	*RHD+/RHD–*	256	185	8.88	AB	+	–
RHD-CE(2-5)-D	1	O	*RHD+/RHD–*	128	190	8.04	O	+	–

*Presence (+) or absence (–) of the *RHD* gene.
#TBIL concentration 24 h after delivery.
$^{\Delta}$Ret count 7 h after delivery.
[a]A 26-year-old Chinese Han woman carrying *RHD-CE(4–9)-D* allele. Routine serotyping demonstrated an A phenotype and genotyping showed hemizygous. Her husband was group B. She was found to be sensitized to the D antigen, apparently after a previous first-trimester miscarriage, with an anti-D titer 256 using an IAT. More detailed maternal anti-D did not react with self RBCs, excluding an autoantibody. A live female infant was delivered at 39 weeks of gestation with a birth weight of 3500 g, the newborn was not anemic. However, the newborn TBIL concentration 24 h after delivery was 185 μmol/l. The reticulocyte count was 8.88% at 7 h after delivery and decreased to 2.44% by 120 h. The infant's phenotype was AB, D+C+c+E–e+. The D antigen was detected with immunoglobulin M anti-D in an immediate-spin tube test indicating normal levels of expression. Cord blood serology showed a positive direct antiglobulin test, and anti-D was eluted from the cord RBCs. It was noted that the infant was AB while the mother was group A; however, anti-B was excluded as the cause of HDFN because anti-B was not detected in the eluate from the cord RBCs. The newborn's hyperbilirubinemia was attributed to maternal anti-D reacting against the infant's D+ RBCs.

have shown that a heterogeneous array of variant *RHD* alleles can result in the DEL phenotype [10, 18–23]. DEL derives from several mechanisms, including splice-site mutation, missense mutation, *RHD-CE-D* hybrid, frame shift mutation, and a long deletion of the *RHD* gene [14, 24–28]. *RHD* (IVS3 + 1G>A) is a DEL allele that has only been observed in Caucasians. *RHD* (1,227G>A) and *RHD* (IVS3 + 1G>A) belong to splice-site mutation type [11, 12]. *RHD* (M295I, 885G>T) is a missense mutation, *RHD* (X418 L) is frame shift mutation [12, 29]. *RHD* (del Ex8) and *RHD* (del Ex9) alleles are classified as long deletions of the *RHD* gene [26, 28], and *RHD* (del Ex9) is now determined to be nonexistent but resulted from a misinterpretation of data on *RHD* (1,227G>A) [12, 27]. *RHD-CE(4–9)-D* belongs to *RHD-CE-D* hybrid gene [14, 30–32]. Körmöczi and colleagues [9] proposed that DEL phenotypes should be divided into two subtypes: complete types where the majority of epDs are conserved such as *RHD* (1,227G>A) and partial D-like variants with characteristic epD loss caused by either *RHD-CE-D* hybrid genes or *RHD* point mutations *RHD* (IVS3 + 1G>A) affecting extracellular RhD loops. The distinction between complete and partial D is of clinical importance, because only partial D individuals exhibit a tendency to anti-D alloimmunization [9, 11, 14].

So far, no report was available that pregnant women with *RHD-CE-D* hybrid *DEL* genes carrying D+ fetus developed allo-anti-D. In this prospective study, the two pregnant women who were typed as DEL carrying *RHD-CE(4–9)-D* and *RHD-CE(2–5)-D* had anti-D alloimmunization. One might speculate that a limited variety of antibody epitope specificity in the two variants is associated with a lower propensity for hemolytic activity because the HDFN was rather mild in relation to the anti-D titer in the two cases reported here. Anti-D alloimmunization in DEL individuals may in fact not be a very rare case; a predisposition to allo-anti-D formation requires epD loss and may therefore likely be partial DEL type-specific [9]. However, this speculation requires confirmation by more case reports because the correlation between anti-D titer and severity of HDFN is weak. Anti-D often persists for a long time and may even be found several decades after initial formation. Whether the two pregnant DEL women elicited primary immunization or secondary immune response is not yet proven as an unnoticed and uncompleted D+ pregnancy could not be excluded as the primary immunization event. In particular, the RHD+/RHD– hemizygote has the potential of stimulating anti-D at least in secondary immunizations. A few instances of primary immunization have also been reported. In the two anti-D cases, a positive direct antiglobulin test was detected in the newborns. Of these one received small-volume RBC transfusions and phototherapy, the other one was treated only with phototherapy. The *RHD* (1,227G>A) DEL phenotypes type may not be prone to anti-D alloimmunization after exposure to 'regular' D+ RBCs [10, 33]. Shao et al. [10] reported no anti-D isoimmunization among 44 women with *RHD* (1,227G>A) who had not received prophylactic anti-D, providing supportive evidence

that the epDs are conserved. So we propose to identify patients carrying *RHD* (1,227G>A) and *RHD* (3G>A) DEL alleles as these patients can receive Rh+ units if necessary. Likewise, pregnant women carrying complete DEL variants *RHD* (3G>A) and *RHD* (1,227G>A) might not be stimulated to make allo-anti-D in response to an Rh+ fetus. Wagner et al. [15] found a new *RHD* allele in a lookback initiated after an unexplained anti-D immunization event. The DEL phenotype expressed by this *RHD* (IVS5–38del4) allele elicited a rapid anti-D immune response. However, Von Zabern et al. [34] reported four samples carrying *RHD* (IVS5–38del4) that does not cause a DEL phenotype; the deletion has no influence on D antigen expression at all, and an association of this 4 bp deletion with the DEL phenotype was excluded.

Our prior policy concerned the use of D– RBCs in DEL recipients and insufficient D– RBCs in stock. The implementation of the new policy will result in a significant change in the use of D– RBCs. The new policy will reduce the consumption of D– RBCs by about 1,560 units/year in the three hospitals. The number of units saved is significant given the demand to obtain Rh– blood for transfusion recipients who are truly at risk of making allo-anti-D. The DEL variant is linked to the C antigen, which permits the rapid exclusion of the DEL variant during recipient pre-transfusion testing. In those apparent Rh–/C+ individuals, the *RHD* (1,227 G>A) and *RHD* (3G>A) alleles, which were the two most frequent DEL alleles in Chinese Han ethnicity, could be detected by PCR-SSP simply and precisely.

Anti-D in pregnant women with the partial DEL phenotype has caused HDFN, but there are no universally accepted guidelines for Rh immunoprophylaxis in DEL variant women. The clinical management of patients carrying DEL phenotypes is based on the predicted clinical relevance: it is recommended that patients carrying complete DEL types could be transfused with D+ blood. Likewise, pregnant women should not receive anti-D prophylaxis (RhIg) if they carry one of these complete DEL types. In contrast, patients and pregnant women who carry one of the partial DEL types should receive transfusion of D– blood or receive RhIg or both.

To the best of our knowledge this is the first paper in which a transfusion policy concerning the use of D+ RBCs in DEL patients is prospectively evaluated. In conclusion, our study shows that the use of D+ RBCs in selected DEL patients carrying complete epDs is justified and may be highly beneficial for institutions.

Acknowledgment

We thank Dr. B.-L. Wang for his excellent technical and financial assistance and valuable advice during the preparation of this manuscript. This work was supported by the National Natural Science Foundation of the People's Republic of China (no. 30972815).

Disclosure Statement

The authors declare that they have no conflict of interest in the subject matter of the manuscript. The authors did not receive any financial support in addition to the project grant mentioned.

References

1 Gonzalez-Porras JR, Graciani IF, Perez-Simon JA, Martin-Sanchez J, Encinas C, Conde MP, Nieto MJ, Corral M: Prospective evaluation of a transfusion policy of D+ red blood cells into D– patients. Transfusion 2008;48:1318–1324.
2 Cook K, Rush B: Rh(D) immunization after massive transfusion of Rh(D)-positive blood. Med J Aust 1974;1:166–168.
3 Gunson HH, Stratton F, Phillips PK: The use of modified cells to induce an anti-Rh response. Br J Haematol 1971;21:683–694.
4 Urbaniak SJ, Robertson AE: A successful program of immunizing Rh-negative male volunteers for anti-D production using frozen/thawed blood. Transfusion 1981;21:64–69.
5 Keith L, Berger GS, Pollack W: The transfusion of Rh-positive blood into Rh-negative women. Am J Obstet Gynecol 1976;125:502–506.
6 Pollack W, Ascari WQ, Crispen JF, O'Connor RR, Ho TY: Studies on Rh prophylaxis. II. Rh immune prophylaxis after transfusion with Rh-positive blood. Transfusion 1971;11:340–344.
7 Krog GR, Clausen FB, Berkowicz A, Jørgensen L, Rieneck K, Nielsen LK, Dziegiel MH: Is current serologic RhD typing of blood donors sufficient for avoiding immunization of recipients? Transfusion 2011;51:2278–2285.
8 Flegel WA, von Zabern I, Wagner FF: Six years' experience performing *RHD* genotyping to confirm D– red blood cell units in Germany for preventing anti-D immunizations. Transfusion 2009;49:465–471.
9 Körmöczi GF, Gassner C, Shao CP, Uchikawa M, Legler TJ: A comprehensive analysis of DEL types: partial DEL individuals are prone to anti-D alloimmunization. Transfusion 2005;45:1561–1567.
10 Shao CP, Xu H, Xu Q, Sun GD, Li JP, Zhang BW, Liang XH, Liu Z, Zhou Y, Li D, Zhuang NB: Antenatal Rh prophylaxis is unnecessary for 'Asia type' DEL women. Transfus Clin Biol 2010;17:260–264.
11 Gardener GJ, Legler TJ, Hyett JA, Liew YW, Flower RL, Hyland CA: Anti-D in pregnant women with the *RHD* (IVS3 + 1G>A) associated DEL phenotype. Transfusion 2012;52:2016–2019.
12 Li Q, Hou L, Guo ZH, Ye LY, Yue DQ, Zhu ZY: Molecular basis of the RHD gene in blood donors with DEL phenotypes in Shanghai. Vox Sang 2009;97:139–146.
13 Li Q, Ye LY, Guo ZH, Zhang YX, Wang LL, Zhu ZY: Molecular basis of D variants between Uigur and Han blood donors in Xinjiang. Transfus Med 2008;18:199–203.
14 Ouchari M, Jemni Yacoub S, Houissa B, Abdelkefi S, Chakroun T, Bouslama M, Jerray I, Belhedi S, Hmida S: System RH: screening of partials D with RHD/RHCE hybrid gene. Transfus Clin Biol 2013;20:35–39.
15 Wagner T, Körmöczi GF, Buchta C, Vadon M, Lanzer G, Mayr WR, Legler TJ: Anti-D immunization by DEL red blood cells. Transfusion 2005;45:520–526.
16 Körmöczi GF, Förstemann E, Gabriel C, Mayr WR, Schönitzer D, Gassner C: Novel weak D types 31 and 32: adsorption-elution-supported D antigen analysis and comparison to prevalent weak D types. Transfusion 2005;45:1574–1580.
17 Wagner FF, Flegel WA: *RHD* gene deletion occurred in the Rhesus box. Blood 2000;95:3662–3668.
18 Shao CP, Xiong W, Zhou YY: Multiple isoforms excluding normal RhD mRNA detected in Rh blood group Del phenotype with RHD 1227A allele. Transfus Apher Sci 2006;34:145–152.
19 Ye L, Yue D, Wo D, Ding X, Guo S, Li Q, Guo Z, Zhu Z: Molecular bases of unexpressed *RHD* alleles in Chinese D– persons. Transfusion 2009;49:1655–1660.
20 Ye LY, Guo ZH, Li Q, Zhu ZY: Molecular and family analyses revealed two novel RHD alleles in a survey of a Chinese RhD-negative population. Vox Sang 2007;92:242–246.
21 Ye SH, Wu DZ, Wang MN, Wu XY, Xu HG, Xu H, Shao CP: A comprehensive investigation of RHD polymorphisms in the Chinese Han population in Xi'an. Blood Transfus 2013;11:1–9.
22 Silvy M, Simon S, Gouvitsos J, Di Cristofaro J, Ferrera V, Chiaroni J, Bailly P: Weak D and DEL alleles detected by routine SNaPshot genotyping: identification of four novel RHDalleles. Transfusion 2011;51:401–411.
23 Sun CF, Chou CS, Lai NC, Wang WT: RHD gene polymorphisms among RhD-negative Chinese in Taiwan. Vox Sang 1998;75:52–57.
24 Shao CP, Maas JH, Su YQ, Köhler M, Legler TJ: Molecular background of Rh D-positive, D-negative, D(el) and weak D phenotypes in Chinese. Vox Sang 2002;83:156–161.
25 Yan L, Wu J, Zhu F, Hong X, Xu X: Molecular basis of D variants in Chinese persons. Transfusion 2007;47:471–477.

26 Richard M, Perreault J, Constanzo-Yanez J, Khalifé S, St-Louis M: A new DEL variant caused by exon 8 deletion. Transfusion 2007;47:852–857.
27 Luettringhaus TA, Cho D, Ryang DW, Flegel WA: An easy RHD genotyping strategy for D- East Asian persons applied to Korean blood donors. Transfusion 2006;46:2128–2137.
28 Chang JG, Wang JC, Yang TY, Tsan KW, Shih MC, Peng CT, Tsai CH: Human Rh Del is caused by a deletion of 1013 bp between introns 8 and 9 including exon 9 of *RHD* gene. Blood 1998;92:2602–2604.
29 Gassner C, Doescher A, Drnovsek TD, Rozman P, Eicher NI, Legler TJ, Lukin S, Garritsen H, Kleinrath T, Egger B, Ehling R, Körmöczi GF, Kilga-Nogler S, Schoenitzer D, Petershofen EK: Presence of *RHD* in serologically D–, C/E+ individuals: a European multicenter study. Transfusion 2005;45:527–538.
30 Szulman A, Nardozza LM, Barreto JA, Araujo Júnior E, Moron AF: Investigation of pseudogenes RHDΨ and RHD-CE-D hybrid gene in D-negative blood donors by the real time PCR method. Transfus Apher Sci 2012;47:289–293.
31 Gunel T, Kalelioglu I, Gedikbasi A, Ermis H, Aydinli K: Detection of fetal RHD pseudogene (RHDΨ) and hybrid RHD-CE-Ds from RHD-negative pregnant women with a free DNA fetal kit. Genet Mol Res 2011;10:2653–2657.
32 Martin FO, de Menezes SS, Chiba AK, Langhi DM Jr, Nardozza LM, Chiattone CS, Bordin JO: *RHD* gene polymorphisms in alloimmunized RhD-negative individuals with high rate of racial admixture. Transfus Apher Sci 2013;48:113–116.
33 Shao CP: Transfusion of RhD-positive blood in 'Asia type' DEL recipients. N Engl J Med 2010;362:472–473.
34 von Zabern I, Flegel WA: IVS5–38del4 deletion in the *RHD* gene does not cause a DEL phenotype: relevance for *RHD* alleles including DFR-3. Transfusion 2007;47:1552–1555.