

An investigation of secondary anti-D immunisation among phenotypically RhD-negative individuals in the Chinese population

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Background. Despite the introduction of anti-D prophylaxis into clinical practice, RhD alloimmunisation remains a problem, particularly in the context of transfusions and pregnancy-induced alloimmunisation. The incidence of RhD alloimmunisation among phenotypically RhD-negative individuals is unknown in most countries. We investigated RhD alloimmunisation in RhD-negative pregnant women and transfusion recipients in south-east China in order to optimise the prevention of this phenomenon.

Methods. We analysed the RhD alloimmunisation status of RhD-negative pregnant women and transfusion recipients in south-east China. The RhD blood types of the study population were identified by standard serological methods. The D antigen was further tested with the indirect antiglobulin test to exclude or confirm weak D or partial D types. RhC, c, E and e antigens were typed in all subjects. If anti-D antibody screening was positive, the specificity and titre of the antibody were determined. The Del phenotype was investigated by the polymerase chain reaction sequence-specific primer method.

Results. An anti-D antibody was found in 61 of 416 RhD-negative pregnant women (14.66%), and in 11 of 227 RhD-negative transfusion recipients (4.85%). None of the 72 RhD-negative pregnant women or transfusion recipients with anti-D had the Del phenotype. Anti-D antibodies were not detected among Del phenotype individuals and Del phenotypes were not found in anti-D antibody producing individuals.

Discussion. Our study suggests that the risk of alloimmunity-induced neonatal haemolysis increases in true RhD-negative multipara. Perinatal protection would be necessary in these patients, while antenatal anti-D testing and Rh immune globulin prophylaxis would be unnecessary for RhDel pregnant women. Pregnant women and transfusion recipients with the Del type seldom produce anti-D antibody. RhD-negative recipients are not at risk of alloimmunisation after transfusion with Del red blood cells.

Keywords: RhD negative, pregnancy, transfusion recipient, alloimmunisation, Del phenotype.

Introduction

The Rhesus (Rh) blood group system is clinically important because antibodies against Rh antigens are involved in haemolytic disease of the foetus and newborn and haemolytic transfusion reactions¹. The Rh blood group system is one of the most diverse antigen systems presently known in humans and the D antigen is its most important member because D-negative individuals can be easily anti-D immunised. Indeed, each person who lacks a red blood cell antigen and is exposed to it is at risk of creating an antibody to that antigen. If

RhD-positive foetal erythrocytes enter the circulation of a RhD-negative mother, the maternal immune system may be stimulated and trigger the creation of antibodies by alloimmunisation. The same immune reaction may also occur during transfusion of antigen-incompatible red blood cells.

Studies of the blood group system have shown that racial differences exist not only in the genetic background of the RhD antigen but also in the frequencies of the Rhesus D genotype (*RHD*). About 15% of Caucasoid people are D-negative, most of whom have deletion

of *RHD* between the upstream and the downstream Rhesus boxes². In contrast, the majority of D-negative black Africans have a *RHD* gene, with one study showing that 66% had an inactive *RHD* pseudogene (*RHD* ψ) with a 37 base pair (bp) insert and a nonsense mutation³. In Asian populations, *RHD* ψ is rare, although a certain percentage of RhD-negative individuals have a *RHD-CE-DS* hybrid gene and *RHD1227A* allele. In contrast to Caucasian population, only 0.3-0.5% of Chinese populations have a RhD-negative blood phenotype⁴; however, nearly 30% of the RhD-negative individuals have the RhDel allele, which is a rare variant of the Rh system with a grossly intact *RHD* gene, of which one is the 1227G >A mutation that probably disrupts normal intron splicing. In European populations, the reported frequency of RhDel is 1:3030, and that of the *RHD1227A* allele is 1:9091⁵. There are currently no data on the frequency of Del in Africans. The most frequent *RHD* allele among Asian individuals is the RhDel variant *RHD1227A*, which serves as an important genetic marker in RhDel Asian people⁶⁻⁸. Although Del is the weakest D-positive phenotype, the potential danger that Del red blood cells might cause a clinical transfusion reaction cannot be completely excluded and indeed there are reports of transfusion recipients with a true D-negative phenotype having developed anti-D after transfusion with Del red blood cells^{9,10}. Analogous cases have not as yet been reported in Chinese populations.

In the present study, we analysed the RhD alloimmunisation status of RhD-negative pregnant women and transfusion recipients in a south-eastern region of China. There were no Del phenotype individuals among the RhD-negative pregnant women and transfusion recipients who produced anti-D antibody. Among Chinese populations, RhD-negative pregnant women and transfusion recipients with the Del blood type seldom produce the anti-D antibody. On the basis of our analysis, we advocate that a programme to prevent Rh immunisation and a transfusion strategy be implemented to avoid anti-D alloimmunisation among RhD-negative Chinese populations.

Materials and methods

Blood samples and DNA isolation

Peripheral blood samples (5.0 mL) were collected from 227 transfusion recipients and RhD-negative pregnant women during routine antenatal care visits at the Anhui Provincial Hospital, the Jiangsu Provincial Maternal & Children Health Care Hospital, the Shaoxing Hospital of China Medical University and the Affiliated Hospital of Guilin Medical College. Subjects were excluded from participation in this study if they: were RhD-positive, were RhD-negative

but already immunised against the RhD antigen, had a history of anaphylactic or other severe systemic reaction to immunoglobulins, had been administered anti-D immunoglobulin, had IgA deficiency, had been transfused with RhD-positive blood or been given any other blood-derived product within the 6 months prior to enrolment, when the biological father of the child was RhD-negative, had clinically relevant abnormal laboratory parameters (haematology, biochemistry, coagulation, urinalysis) which, in the opinion of the investigator, were not acceptable.

Plasma was separated by centrifugation at 2,000×g for 10 minutes. Genomic DNA was extracted with commercially supplied kits following the manufacturer's instructions (QIAamp blood mini kit, Qiagen, Hilden, Germany), transferred to new propylene tubes and stored at -20 °C until use. The buffy coat from each blood sample was also collected and stored at -20 °C.

This study was approved by the institutional Ethics Review Board of Nanjing Medical University and all subjects provided written informed consent to their participation in it.

RhD phenotyping

Blood samples were collected into Vacutainer blood tubes containing EDTA. The RhD blood phenotype status was determined by classical serological techniques with direct agglutination. Two commercial anti-D were used (Immucor, Norcross, Georgia, USA; Gamma Biologicals, Houston, Texas, USA). Anti-C (Novaclone, Dominion Biologicals, Dartmouth, Canada), anti-E (Gamma Biologicals), anti-c (Immucor), and anti-e (Immucor) were also used. Samples that were negative with anti-D in the direct agglutination test were re-examined using the indirect antiglobulin test. An adsorption elution test was also performed on all the samples (including those from all RhD-negative donors), which were negative with the indirect antiglobulin test in order to determine the presence of RhDel.

Polymerase chain reaction - sequence-specific primer assay for the *RHD1227A* allele

A polymerase chain reaction -sequence-specific primer (PCR-SSP) assay was used to screen for the *RHD1227A* allele, which is the most common Del allele reported in the Chinese population. Del-U and D-int9-L, designed by Shao *et al.*¹¹, were used to specifically genotype *RHD1227A*. Multiplex PCR amplifications were performed with 1.0 μ L of genomic DNA and 0.5 U DNA polymerase (Applied Biosystems, Foster City, California, USA), 200 μ M dNTPs, *RH* primers, primers for the internal control (IC), 2.5 mM MgCl₂ in a buffer supplied by the manufacturer in a 12.5 μ L

reaction volume. The PCR consisted of denaturation at 95 °C for 5 minutes and then 35 cycles of 10 seconds at 94 °C, 40 seconds at 62 °C, and 30 seconds at 72 °C were carried out with a thermocycler (PE 9700 GeneAmp PCR system, Applied Biosystems). PCR products were separated and visualized in 5% acrylamide gels.

Statistical analysis

The SPSS software package, version 11.0 (SPSS Inc., Cary, North Carolina, USA) was used for all statistical tests.

Results

RhD phenotyping

Four hundred and sixteen blood samples from pregnant women and 227 blood samples from transfusion recipients were collected, which were determined to be RhD-negative by routine serological testing. An indirect antiglobulin test was performed on all samples typed as D-negative, showing that there were no cases of weak D or partial D. About 98% of the individuals were of Chinese Han ethnicity and lived in the south-eastern area of China, in Jiangsu Province, Zhejiang Province, or Anhui Province.

Of all 643 D-negative samples received, 379 samples were typed as ccee. The remaining 264 samples had the C antigen or E antigen, with the majority exhibiting the Ccee or CCee phenotype (237/643; 36.9%). Of the 643 samples determined to be D-negative by the indirect antiglobulin test, 155 cases were actually Del as shown by adsorption/elution testing (Tables I and II).

Anti-D immunisation analysis

Among the 416 RhD-negative pregnant women, 61 produced an anti-D antibody, for a production

rate of 14.66% (61/416) (Table I). Among the 227 RhD-negative transfusion recipients, 11 produced an anti-D antibody, for a production rate of 4.85% (11/227) (Table II).

To investigate the immunogenicity of Del red blood cells in RhD-negative recipients, we investigated the alloimmunisation status of 19 RhD-negative recipients transfused with Del red blood cells. Of these 19 Rh-negative recipients transfused with Del variants, ten were males and the other nine were females with a history of pregnancy. The results of adsorption-elution tests for these 19 subjects showed that five cases were Del and 14 were true Rh negative. Three men and two women identified as Del individuals showed no reactions after transfusion, and irregular antibodies were not detected in their serum. Among the remaining 14 true Rh-negative samples, all subjects gave the same results as the Del samples (Table III).

In the present study, PCR-SSP and sequencing showed that none of the 72 RhD-negative pregnant women or transfusion recipients with anti-D antibodies carried the *RHD1227A* allele. Anti-D antibodies were not detected among Del phenotype individuals (Tables I and II). Further investigations found no cases of anti-D alloimmunisation in D-negative recipients after the transfusion of Del variants (Table III).

Samples with the *RHD1227A* allele

In 151 of the 155 Del phenotyped cases, *RHD1227A* alleles were detected by PCR-SSP (Figure 1). *RHD1227A* alleles were identified in 85 of the D-negative Ccee samples and 66 of the CCee samples. For confirmation, all 151 samples were determined to have the *RHD1227A* alleles by sequencing analysis (Figure 2).

Table I - Anti-D immunisation analysis in 416 RhD-negative pregnant women.

Pregnancy history	Number of cases	Saline test	IAT	Adsorption and elution test		IgG-D (%)	<i>RHD1227A</i>		Foetus or newborn RhD phenotype
				-	+		-	+	
G2P1	153	-	-	121	32	12* (7.8)	122	31	148(+), 4(Del), 1(-)
G2P2	57	-	-	45	12	6* (10.5)	45	12	+
G3P1	98	-	-	78	20	9* (9.2)	79	19	97(+), 1(-)
G3P2	22	-	-	18	4	6* (27.3)	18	4	21(+), 1(Del)
G3P3	9	-	-	6	3	3* (33.3)	6	3	+
GnP2	47	-	-	35	12	13* (27.7)	35	12	46(+), 1(Del)
GnP3	30	-	-	25	5	12* (40.0)	25	5	+

G: gestation; P: parturition; n: number of time (n ≥ 4); IAT: indirect antiglobulin test; *: pregnant woman with non-RhDel phenotype; "+": positive; "-": negative.

Table II - Anti-D immunisation analysis in 227 RhD-negative transfusion recipients.

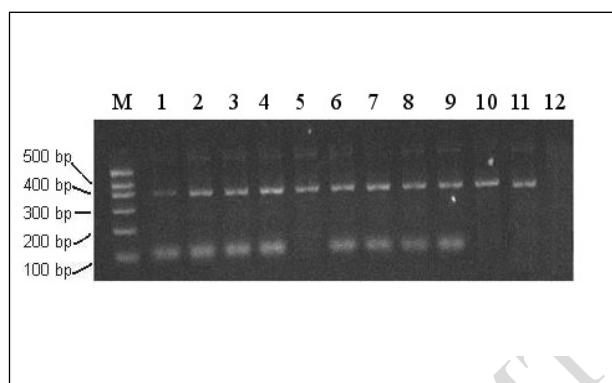
Transfusion history	Number of cases	Saline test	IAT	Adsorption and elution test		IgG-D (%)	<i>RHD1227A</i>		Donors RhD phenotype
				-	+		-	+	
Second times	156	-	-	108	48	4* (2.6)	108	48	142(-), 14(Del)
Multiple	71	-	-	52	19	7* (9.9)	54	17	63(-), 8(Del)

*: recipient with non-RhDel phenotype; "+": positive; "-": negative; Multiple: at least three times; IAT: indirect antiglobulin test.

Table III - Anti-D immunisation analysis in 19 RhD-negative transfusion recipients of RhDel red blood cells.

SN	Sex	Age	Saline test	IAT	Adsorption and elution test	Amount of Del (U)	Phenotype	IgG-D
11005	M	31	-	-	-	2	Ccee	-
11012	M	22	-	-	+	4	CCee	-
11032	M	31	-	-	-	1	ccee	-
11053	F	28	-	-	-	3	ccEe	-
11060	M	69	-	-	-	6	ccee	-
11070	F	35	-	-	-	2	ccee	-
11075	F	57	-	-	-	2	ccee	-
11079	M	53	-	-	-	2	ccEe	-
11082	M	48	-	-	+	2	Ccee	-
11089	F	58	-	-	-	2	ccee	-
11099	F	27	-	-	+	1	Ccee	-
11103	F	69	-	-	-	3	ccee	-
11117	M	31	-	-	-	4	ccee	-
11134	M	32	-	-	-	2	Ccee	-
11171	F	30	-	-	-	4	ccee	-
12204	M	34	-	-	+	2	Ccee	-
12220	M	46	-	-	-	4	ccee	-
12225	F	41	-	-	+	4	CCee	-
12227	F	27	-	-	-	2	ccee	-

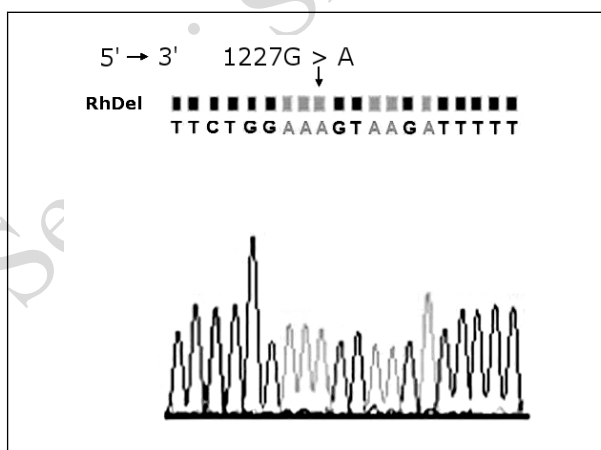
SN: specimen number; F: Female; M: Male; "+": positive; "-": negative; IAT: indirect antiglobulin test; 1 U: red blood cells prepared from 200 mL whole product.

**Figure 1** - PCR-SSP for *RHD1227A* (109 bp band).

Lanes 1-4, 6-9, *RHD1227A* allele detected in Del phenotype samples; Lane 5, non-*RHD1227A* allele gene detected in Del phenotype samples; lane 10, Rh-negative control sample; lane 11, sample with a normal *RHD* gene; lane 12, H₂O control. A representative example of 155 Del phenotyped cases is shown.

Discussion

In this study, we investigated the RhD alloimmunisation rate among RhD-negative pregnant women and transfusion recipients in the south-eastern region of China. Despite the fact that the prevalence of Rh-negative phenotypes is significantly lower among Asians than among Caucasians, China is a populous country, and spouses of RhD-negative women are almost all RhD-positive individuals. We have previously reported the non-invasive determination of foetal RhD blood type using free foetal DNA from Chinese RhD-negative maternal plasma¹². If the foetus is RhD negative, it is unnecessary to administer IgG anti-D at gestation or institute prophylaxis of RhD alloimmunisation in the case of potentially sensitising events, but its application is still very limited. More

**Figure 2** - Sequencing analysis of the *RHD1227A* allele.

The arrow indicates the position of the nucleotide mutation between *RHD* exon 9 and intron 9. A representative example of 151 *RHD1227A* genotyped cases is shown.

than 98% of RhD-negative pregnant Chinese women give birth to RhD-positive infants (Table I).

Most RhD alloimmunisation status studies have been conducted in Caucasian and African populations and their results are not entirely applicable to the Chinese population as there are significant differences in the frequencies and molecular characteristics of RhD variants in different populations. Our previous studies had shown that East Asian populations who carry the Del can safely receive transfusions from RhD-positive donors¹³. However, alloimmunisation associated with the RhD-negative phenotype has been poorly studied among the Chinese population. Many questions remain unanswered and there is an urgent need for the development of a management protocol for this condition among the Chinese population. This

study was conducted to evaluate the state of secondary anti-D immunisation among Chinese individuals with the RhD-negative phenotype.

Several studies have revealed that RhD-negative individuals may have a partial or intact *RHD* gene, and this genotype occurs at different frequencies depending on the ethnic population^{14,15}. The immunogenic impact of D antigen depends mainly on the quantity of D antigen sites on the surface of red blood cells. Red blood cells with weak D express less D and are less immunogenic than red blood cells with normal expression of D antigen¹⁶. However, previous studies have demonstrated that red blood cells from subjects with the weak D type 1 phenotype may cause primary alloimmunisation of anti-D¹⁷, as may those of the weak D type 2 phenotype with a D antigen density of approximately 500 per cell¹⁸, although Del is defined by expression of trace amounts of the antigen that can be detected only by an absorption-elution study. Shao and associates¹¹ deduced that Del is a type of weak D, and different Del individuals might have different amounts of D antigen sites on their red blood cells. Although the Del phenotype is very rare in Caucasians, it is very common in the Rh-negative Chinese population as shown by indirect antiglobulin testing. The reported rates in Chinese Hans (the major ethnic group, accounting for more than 91% of the Chinese population) are from 26% to 30%^{11,19,20}. Moreover through genomic DNA analysis, the studies by Shao *et al.* and Chen *et al.* in Shenzhen, Southern China, and in Taiwan⁶, showed that the *RHD1227A* allele is a dominant genetic marker for Chinese and Taiwanese individuals with Del. These individuals possessed one or two of these alleles with Ccee or CCee phenotypes¹¹. The Del allele, *RHD1227A* has a synonymous mutation at the end of exon 9 compared to the consensus *RHD*. This single nucleotide polymorphism causes complete exon 9 mis-splicing, which has been confirmed through sequence analysis of mRNA transcripts. The exon 9 corresponding region in mRNA codes for transmembranous amino acids^{21,22}. Although Del is the weakest D-positive phenotype, the potential danger that Del red blood cells might cause a clinical transfusion reaction cannot be completely excluded. Indeed, recipients with a true D-negative phenotype have developed anti-D after transfusion with Del red blood cells^{9,10}, although no such cases have yet been reported in the Chinese population. Previous studies demonstrated that "Asian type" Del displays the complete repertoire of RhD antigen epitopes²³. However, carriers of Del can still be RhD-negative donors, since there are fewer than 22 membrane RhD antigens per Del red cell, as compared with thousands of RhD antigens on a normal RhD-positive red cell²³.

In North America, approximately 9% to 10% of all pregnancies involve a Rh-negative woman carrying a Rh-positive infant. Rh alloimmunisation occurs antenatally in 0.4% to 2.0% of first pregnancies involving Rh incompatibility. The risk of alloimmunisation is approximately 3% to 5% after abortion, with the risk increasing with increasing gestational age, and 2.1% to 3.4% after amniocentesis. A cross-sectional retrospective study to determine the prevalence of anti-D immunoglobulin among Cameroonian women of reproductive age indicated an anti-D prevalence of 4% among Rh-negative African women²⁴. In our study of Chinese women, we observed that 18.60% (61/328) of the true RhD-negative pregnant women carrying a RhD-positive foetus produced anti-D IgG antibody. These pregnant women had a history of pregnancy or childbirth. D-antigen may cause primary alloimmunisation that cannot be detected and may not cause a haemolytic immune response, but is responsible for an immune recall response. Abortion and other invasive procedures can lead to maternal RhD primary immunisation. Considering our overall cohort of phenotypically RhD-negative pregnant women, the anti-D detection rate was 14.66% (61/416). Further analysis of the women who had been twice or more, showed an anti-D detection rate of 31.48% (34/108). Among 227 RhD-negative transfusion recipients, 11 produced the anti-D antibody for a production rate of 4.85%. To further investigate the immunogenicity of Del red blood cells in Rh negative recipients, we investigated the alloimmunisation status of 19 RhD-negative recipients transfused with Del red blood cells. No anti-D was detected in any of these 19 RhD-negative recipients. All 72 of the RhD-negative pregnant women and transfusion recipients with anti-D were excluded from carrying the Del phenotype through PCR-SSP analysis and sequencing. Anti-D antibodies were not found among Del phenotype individuals and Del phenotypes were not found in anti-D antibody-producing individuals. Körmöczi *et al.* suggested that the Del phenotypes might be subdivided into two groups, partial Del and complete Del²³. We wondered whether all of the Del found in our Chinese subjects were complete Del, as no Del sample was found to have anti-D in the serum.

Our study supports the biochemical observations that Del variants express normal RhD and pose virtually no risk of inducing anti-RhD antibodies. We suggest that people in East Asian populations who carry Del variants can safely receive transfusions from RhD-positive donors. Our findings would apply to East Asian transfusion recipients in Europe, North America, and elsewhere and could be implemented easily when genetic cross-matching becomes a reality²⁵.

Contribution

Qing-ping Wang and Guang-tao Dong contributed equally to this work.

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