Analysis of density and epitopes of D antigen on the surface of erythrocytes from DEL phenotypic individuals carrying the *RHD*1227A allele

Juan Gu[1], An-Yuan Sun[2], Xue-Dong Wang[1], Chao-Peng Shao[3], Zheng Li[4], Li-Hua Huang[1], Zhao-Lin Pan[1], Qing-Ping Wang[1,5], Guang-Ming Sun[6]

[1]Department of Clinical Laboratory, The Fifth People's Hospital of Wuxi, Affiliated Hospital of Nanjing Medical University, Wuxi, Jiangsu; [2]Department of Clinical Laboratory, The Affiliated Provincial Hospital of Anhui Medical University, Hefei, Anhui; [3]Shenzhen Blood Center and Institute of Blood Transfusion Medicine, Shenzhen, Guangdong; [4]Department of Clinical Laboratory, The Affiliated Hospital of Guilin Medical College, Guilin, Guangxi; [5]Department of Clinical Laboratory, the Shaoxing Hospital of China Medical University and Central Hospital of Shaoxing County, Shaoxing, Zhejiang; [6]Zhenjiang Maternal & Children Health Care Hospital, The Fourth Affiliated Hospital of Jiangsu University, Zhenjiang, Jiangsu, People's Republic of China

Background. The characteristics of the D antigen are important as they influence the immunogenicity of D variant cells. Several studies on antigenic sites have been reported in normal D positive, weak D and partial D cases, including a comprehensive analysis of DEL types in Caucasians. The aim of this study was to assess D antigen density and epitopes on the erythrocyte surface of Asian type DEL phenotypic individuals carrying the *RHD*1227A allele in the Chinese population.

Materials and methods. A total of 154 DEL phenotypic individuals carrying the *RHD*1227A allele were identified through adsorption and elution tests and polymerase chain reaction analysis with sequence-specific primers in the Chinese population. D antigen density on the erythrocyte surface of these individuals was detected using a flow cytometric method. An erythrocyte sample with known D antigen density was used as a standard. Blood samples from D-negative and D-positive individuals were used as controls. In addition, D antigen epitopes on the erythrocyte surface of DEL individuals carrying the *RHD*1227A allele were investigated with 18 monoclonal anti-D antibodies specific for different D antigen epitopes.

Results. The means of the median fluorescence intensity of D antigen on the erythrocyte membrane surface of D-negative, D-positive and DEL individuals were 2.14±0.25, 193.61±11.43 and 2.45±0.82, respectively. The DEL samples were estimated to have approximately 22 D antigens per cell. The samples from all 154 DEL individuals reacted positively with 18 monoclonal anti-D antibodies specific for different D antigen epitopes.

Discussion. In this study, D antigen density on the erythrocyte surface of DEL individuals carrying the *RHD*1227A allele was extremely low, there being only very few antigenic molecules per cell, but the D antigen epitopes were grossly complete.

Keywords: Rh blood type, D antigen, DEL individual, antigen density, antigen epitope.

Introduction

The Rhesus (Rh) blood group system is the most complicated system among human erythrocyte blood group systems. It is determined by the highly homologous *RHD* and *RHCE* genes encoding the D, C, c, and E, e antigens in common Rh-positive phenotypes, of which the D antigen has the strongest immunogenicity. The *RHD* gene is highly polymorphic and the existence of a large number of alleles results in RhD variant phenotypes. The D antigen carried by the RhD polypeptide is of particular clinical importance with respect to haemolytic disease of the newborn, haemolytic transfusion reactions and autoimmune haemolytic anaemia[1]. According to the quantity and quality of D antigen and differences in antigenicity, the RhD phenotype can be divided into normal D, weak D, partial D, partial weak D and elevated D. Weak D is a variant phenotype in which the expression of D antigen is greatly reduced. The surface of a normal RhD-positive erythrocyte expresses about 10,000-30,000 D antigens per cell while weak D erythrocytes have type-specific antigen densities of between 70 and 4,000 D antigens per cell[2]. Whether the phenotype of weak D erythrocyte surface antigen is completely expressed is still controversial and the specific molecular mechanism

Juan Gu and An-Yuan Sun contributed equally to this work.

is not clear. Beckers *et al*.[2] held that the *RHD* gene in individuals with a weak D phenotype does not show any abnormalities at either the genomic or the transcriptional level when compared to the *RHD* gene in those with a normal D phenotype. Weak D phenotype individuals carry complete D polypeptides and the phenotype reflects a quantitative rather than a qualitative variation of D antigen. Wagner *et al*.[3], however, consider that weak D subjects have qualitative alterations in D antigen. It has been proposed that there is a critical value of RhD antigen density (which can be determined by the IgM anti-D antibodies present) and that transfusion of RhD-positive blood to white individuals with a weak D phenotype whose RhD antigen density is larger than this critical value (400/cell) is safe. In 1984, Okubo *et al*.[4] reported a unique D variant that could be detected among individuals determined to be Rh-negative by the indirect antiglobulin test (IAT), using a more sensitive technique called the adsorption-elution test. This D variant was initially named D^{el} and more recently DEL. It belongs to the weak D phenotype with extremely weak antigenicity. Unlike in white populations, the frequency of the DEL phenotype is very high among the RhD-negative population in Asia. In a large investigation, Wagner *et al*.[5] found that the DEL phenotype occurred in only 0.16% of Caucasian or whites and it has not been reported in Africans. In large population studies of apparently Rh-negative Chinese Han (the major ethnic group, accounting for more than 91% of the population in China), DEL rates were reported to be about 30% in mainland China and Taiwan[6,7]. The rates were 28% and 17% in Japan and Korea, respectively[8,9]. In this study, we explored D antigen density and epitopes on the surface of erythrocytes from DEL phenotypic individuals carrying the *RHD*1227A allele in the Chinese population. So far, there are few reports describing D antigen expression on DEL phenotypic individuals carrying the *RHD*1227A allele. Only three such individuals were studied by Körmöczi *et al.*[10] and all three came from China because of the scarcity of such cases among Caucasians. In summary, the DEL blood group accounts for about one-third of RhD-negative cases among the Chinese population of Han ethnicity. Studies on the DEL blood group in Chinese Han have the advantage of superiority of resources (in terms of number of cases), but also increase research data on the Rh blood group in the world.

Materials and methods

Blood samples

Peripheral blood samples (5.0 mL) were collected from outpatients, inpatients and blood donors at the Anhui Provincial Hospital, the Affiliated Hospital of Anhui Medical University, the Clinical College of Nanjing Medical University, the Affiliated Hospital to Guilin Medical College, the Shaoxing People's Hospital of Zhejiang Province, the 4th Affiliated Hospital of Jiangsu University, Blood Centre of Anhui Province, Shenzhen Blood Centre of Guangdong Province and Wuxi Blood Centre of Jiangsu Province in south-east China. A total of 720 RhD-negative individuals were identified by a microplate technique[11] with monoclonal IgM anti-D (Shanghai Pharmaceutical & Biological Inc., Shanghai, People's Republic of China). All samples typed as D-negative with the IAT were retested. 154 samples of DEL individuals carrying *RHD*1227A were finally confirmed through an adsorption and elution test and polymerase chain reaction analysis with sequence-specific primers (PCR-SSP) was used to screen for *RHD*1227A[12]. All the subjects were Chinese Han, aged 5-65 years old: 70 were male and 84 were female (including 36 pregnant women). C, c, E and e antigens were routinely detected by the tube technique with commercially available monoclonal IgM antibodies (Shanghai Pharmaceutical & Biological Inc., People's Republic of China). In addition, we collected 30 samples from RhD negative and RhD positive donors as negative and positive control groups, respectively.

Serological studies

All samples typed as D-negative with the IAT were retested for the DEL phenotype through an adsorption and elution test. Briefly, 200 μL of packed red blood cells (RBC) were washed with isotonic saline and centrifuged at 3,000 rpm for 1 min. The supernatant was then removed and 200 μL mixed IgG anti-D reagents (clone HM16, P3×35, P3×241 and two different human polyclonal IgG anti-D sera; volume of each reagent, 40 μL) were added to the RBC. The mixture was incubated at 37 °C for 30 minutes then washed three times with 0.9% sodium chloride. Ether was used to elute the RBC. The result of the IAT was displayed. If the result was positive (≥1+), the sample was marked as DEL.

Eighteen monoclonal antibodies are specifically aimed at D antigen epitopes 1-9 (epD1-6 and epD8-9, since the International Transfusion Association cancelled epitope 7). The following monoclonal reagents were used: LHM169/81, LHM70/45, LHM174/102, LHM76/55, LHM59/19, ESD-1, LHM169/80, LHM76/58 and LHM77/64 (Biotest, Dreieich, Germany); P3×35, P3×61, P3×249, P3×290, P3×241, P3×21211F1, P3×21223B10, HM10 and HM16 (Diagast, Loos, France).

Flow cytometry analysis

A FACS Caliber Flow Cytometer (Beckton Dickinson, San Jose, CA, USA) was used to analyse D antigen intensity on the RBC membrane of 30 D-negative cases, 30 D-positive cases and 154 DEL cases. In brief, 2×10^6

washed RBC from subjects with D-negative, D-positive or DEL phenotype were incubated with 50 μL of the monoclonal anti-D for 25 minutes at 37 °C. The cells were then washed three times in 0.1% bovine serum albumin, and 50 μL of 1:500 diluted (in phosphate-buffered saline) secondary antibodies (fluorescein isothiocyanate-labelled polyclonal anti-human IgG antibodies) were added to the tubes and kept for 30 minutes at 37 °C. The cells were again washed three times in 0.1% bovine serum albumin and re-suspended in phosphate-buffered saline. To disaggregate RBC agglutinates, if any, we aspirated the suspensions twice through a 23 gauge needle. Those samples were re-suspended by vortexing just before flow cytometric analysis, and 20,000 RBC were analysed on the flow cytometer. Absolute D antigen densities (D antigens per RBC) were assessed using a standard CcDdee RBCs sample with 9,748 D sites per cell deduced from the standard of the Fourth International Workshop on Monoclonal Antibodies against Human Red Blood Cells. In detail, one sample of CcDdee phenotype that was shown to be *RHD* homozygous by molecular methods[13] was provided as the standard RBC. The antigen density of this CcDdee standard RBC sample was determined to be 9,748 D antigens per RBC by comparison with the previously described standard RBC (13,000 RhD antigens per cell)[14]. These standard RBC were independently prepared twice and were to be used in all tests as internal standards. The threshold of the quantitative test was the median fluorescent intensity (MFI) value of 30 ccddee negative control erythrocytes, and the positive control was Rh-positive individual erythrocytes of CcDee phenotype.

Background fluorescence was determined with RhD-negative samples. The MFI of positive samples or DEL samples was compared with that of the standard CcDee red blood cells (9,748 RhD antigens per cell). The RhD antigen density detected on the sample cells was calculated as:

$$\frac{[\text{median fluorescence of samples} - \text{background fluorescence}]}{[\text{median fluorescence of standard cells} - \text{background fluorescence}]} \times \text{RhD antigen density of standard cells}$$

The MFI of standard RBC, negative controls, positive controls and the test samples were recorded in a Microsoft Excel spread sheet. The relative antigen density was computed from the median fluorescence ratios in the spread sheet. Using the relative antigen density and known antigen density of control RBC, the absolute number of antigen density was calculated[15].

Statistical analysis

The SPSS13.0 software package, version 11.0 (SPSS Inc., Cary, NC, USA) was used for all statistical computations. Quantitative data are expressed as mean values±standard deviation. Differences are considered statistically significant when the p value is <0.05.

Results

Rh phenotype and *RHD*1227A allele

A total of 720 D-negative samples were found through the microplate and IAT determination. OF these, 160 cases were typed as DEL through the adsorption and elution test, of which 156 cases (97.5%) had a RhC positive phenotype (Table I). Thus, in the Chinese population examined, approximately 22.22% (160/720) D-negative individuals had DEL phenotypes. A total of 154 samples were determined to have the *RHD*1227A allele by PCR-SSP and sequencing. Thus, 96.25% (154/160) of the Chinese DEL individuals had the *RHD*1227A allele.

Table I - RhCE phenotype distribution of 160 cases with DEL phenotype.

	Ccee	CCee	CcEe	ccEe	CCEe	CcEE	ccee	total
cases	130	18	6	3	1	1	1	160
%	81.250	11.25	3.750	1.875	0.625	0.625	0.625	100

D antigen density of DEL individuals carrying *RHD*1227A

To quantify D expression of DEL phenotype individuals carrying *RHD*1227A, the absolute numbers of D antigens per RBC were assessed by flow cytometry. First, MFI values of standard RBC were determined twice during each flow cytometer session (at the beginning and at the end of each session). The results were 140.74 and 138.35, respectively. The mean value of the MFI was 139.54. Second, MFI values of D-negative controls, D-positive controls and DEL samples were determined. The mean MFI values were 2.14±0.25, 193.61±11.43 and 2.45±0.82 respectively. In the DEL phenotype individuals carrying *RHD*1227A, specific MFI values with indirect anti-D staining were found to be above the defined detection threshold (i.e., the mean of the MFI obtained withD-negative control RBC plus two times the respective standard deviation, corresponding to 2.14±0.25). These flow cytometric results paralleled the graded reactivity of eluates prepared from DEL erythrocytes adsorbed with routine anti-D reagents, as detailed earlier. According to the related calculation method[15], the absolute number of D antigens per cell of the D-positive controls was approximately 13,584, while that of the DEL individuals was approximately 22 D antigens per RBC (Table II).

D antigen epitopes of DEL individuals carrying *RHD*1227A

Thirty D-positive and 30 D-negative controls were tested with 18 kinds of monoclonal anti-D involving

Table II - RhD antigen density of DEL individuals carrying *RHD*1227A.

RhD phenotype	Samples	Mean MFI	RhD antigens per cell
D(-)	30	2.14±0.25	0
D(+)	30	193.61±11.43	13,584
DEL	154	2.45±0.82*	22

*Compared with D(−)and D(+), p<0.01.

different epitopes of the D antigen through the IAT assay. All the D-positive controls displayed positive reactivity whereas D-negative controls were all negative. Adsorption-elution-based epitope mapping with different anti-D monoclonal antibodies revealed that the 154 samples of DEL from individuals carrying *RHD*1227A expressed all the tested D epitopes, suggesting the expression of a possible complete D antigen on the surface of erythrocytes from such individuals (Table III).

Discussion

The *RHD*1227A allele was first described in European blood donors[5], although it is prevalent in East Asia. The prevalent allele of DEL phenotypes in Caucasians is *RHD* (M295I)[5,18]. In China, approximately 20% to 30% of RhD-negative individuals carry a D variant, termed "Asia type" DEL[6,7,19]. Although DEL is the weakest D-positive phenotype, the potential danger that DEL RBC might cause a clinical transfusion reaction cannot be completely excluded. It was reported that some recipients with a truly D-negative phenotype developed anti-D after transfusion with DEL RBC[20-22]. Moreover, Richard and colleagues found a patient with a DEL phenotype who developed anti-D[23]. Some research results[10] demonstrated that DEL individuals carrying *RHD*(M295I), *RHD*(K409K), *RHD*(X418L) and *RHD*IVS5-38DEL4 have a complete range of D epitopes, while DEL individuals carrying *RHD*IVS3+1G>A lack most D epitopes, expressing only epitopes 2 and 5. All of these findings suggest that the different results obtained might be associated with the different variants of DEL. Analogous cases have not yet been reported in the Chinese population. Körmöczi *et al*.[10] proposed a novel concept of partial DEL *vs* complete DEL phenotype. Such segregation is of clinical importance, as indicated by the fact that two investigated partial DEL individuals were found to be anti-D alloimmunised. We wondered whether the DEL individuals carrying *RHD*1227A found in China were complete DEL, as no DEL sample was found to have anti-D in serum[24]. The molecular basis of DEL variants in different races needs to be explored.

The proportion of DEL individuals carrying the *RHD*1227A allele is relatively high among the Rh-negative Han population in China, but it is rare in Caucasians, and the molecular mechanism of D antigen expression of DEL phenotype of different races is different. So far, at least 14 DEL phenotype alleles have been found, namely *RHD*1227A, *RHD*(M295I), *RHD*IVS5-38del4, *RHD*IVS3+1G>A, *RHD*(delE×9), *RHD*(Y401X) *RHD*(X418L), *RHD*(L18P), *RHD*3G>A, *RHD*(L84P), *RHD*(R10W), *RHD*(A137E), *RHD* (W408R) and *RHD*IVS2-2A>G[5,9,18,20,25-27]. So far, the four DEL alleles existing in Caucasians, *RHD*(M295I), *RHD*IVS3+1G>A, *RHD*(X418L) and *RHD*IVS5-38del4, have not been found in the Chinese population. Among the 160 DEL individuals in our study, 97.5%

Table III - Reactivity pattern of DEL individuals carrying *RHD*1227A with anti-D antibodies.

D epitope		Monoclonal anti-D			Number of positive		
1-9	1-16	IgG		IgM	RhD+	RhD–	DEL
1	1.2	LHM169/81			30	0	154
1	1.2	LHM70/45	LHM174/102		30	0	154
2	2.1	P3×249			30	0	154
3	3.1	P3×290	LHM76/55		30	0	154
4	4.1	ESD1			30	0	154
5	5.4	P3×35			30	0	154
5	5.4	P3×241			30	0	154
6	6.3	LHM169/80			30	0	154
6	6.4			P3×61	30	0	154
6	6.4	HM16			30	0	154
6	6.6			HM10	30	0	154
8	8.1	LHM76/58	LHM59/19		30	0	154
8	8.2			P3×21211F1	30	0	154
9	9.1	LHM77/64		P3×21223B10	30	0	154

Note: Lomas *et al*.[16] divided D antigen epitopes into 1-9 (epitope 7 has been cancelled); Scott M.[17] divided D antigen epitopes into 1-16, the number after the decimal point being sub-epitopes.

were positive for the C antigen (Table I), which demonstrates a possible correlation between C antigen and DEL phenotype. We used 18 kinds of monoclonal antibodies aiming at different D antigen epitopes to test D antigen expression on the surface membrane of DEL erythrocytes: epitopes 1-9 of the 154 DEL individuals carrying *RHD*1227A all gave positive reactions, showing that these individuals may have complete D antigen epitopes (Table II). However, this conclusion theoretically contradicts the molecular mechanism of D antigen expression. Several studies[25,28] have shown that *RHD* of DEL may be mis-spliced, which enables the mature mRNA segments to lose the corresponding sequence of exon 9; truncated isoforms of the RhD cDNA seem to be generated by the splicing of exon 9. We speculated that a possible explanation of the contradiction between serological results and molecular biological findings is that since the amino acid sequence of exon 9 corresponding to RhD protein is located in the transmembrane region, D antigenic determinants of the DEL erythrocyte surface or epitopes were not fully affected. According to the basic principles of immunology, if the DEL erythrocyte membrane expresses complete D antigen, DEL phenotype individuals could not develop isoimmunisation by D antigen, that is to say, Rh positive erythrocytes can be transfused to patients with DEL phenotype and Rh-positive foetuses of DEL phenotype pregnant women would not have the risk of haemolytic disease of the newborn. About 30% of Rh-negative patients (possibly DEL type) in Asia would not have to wait for Rh-negative blood, which would relieve the shortage of the Rh-negative blood supply and reduce delays in treatment of patients who are waiting for rare Rh-negative blood. Of course, the results and conclusion of the present study need to be further confirmed by more and comprehensive monoclonal antibodies with different epitopes of the D antigen, although it is very difficult to obtain such monoclonal antibodies. In addition, large scale clinical observation is required, such as tracing isoimmunisation of Rh-positive foetuses delivered by DEL group pregnant women. In the future, we will further study the characteristics of the DEL allele in the Chinese population. Worldwide, including China, the DEL blood group is currently not routinely identified in patients, pregnant women, or donors, who are regarded as Rh-negative individuals. However, further investigation on the expression of DEL erythrocyte membrane antigens has far-reaching significance.

Our results showed that the MFI of D antigen on DEL erythrocytes is slightly higher than that of D negative erythrocytes, and far less than that of D-positive erythrocytes (Table III), suggesting that D antigen intensity of the DEL blood group is very low and that there are very few D antigens. Körmöczi *et al*.[10] reported that different DEL types exhibited not only qualitative but also quantitative differences in the D antigen. It was observed that RBC from individuals with the DEL phenotype associated with *RHD*(M295I) carried a maximum of 36 D antigens, whereas the number of D antigens on most DEL erythrocyte membranes is <22 (below the threshold of flow cytometry detection). Epitope mapping with adsorption-elution revealed a prominent D epitope loss in the *RHD*IVS3+1G>A associated DEL phenotype, whereas in the *RHD*(K409K), *RHD*(M295I), *RHD*IVS5-38del4 *RHD*(X418L) DEL types no signs of qualitative D antigen alteration were detected. Krog *et al*.[27] found approximately 24-28 D antigens per RBC in the analysis of individuals with the *RHD*IVS2-2A>G DEL phenotype.

So far, there are few reports of anti-D clinical immune incidents caused by DEL. Taking our previous studies into consideration, we infer that the reason why no anti-D isoimmunisation occurs in RhD-negative patients receiving transfusions of DEL erythrocytes may be related to the low density of D antigen on the surface of DEL erythrocytes, with this density being lower than the D antigen threshold necessary to generate anti-D. The safety of RhD-negative patients receiving DEL erythrocyte transfusions should be further confirmed by clinical data from large-scale samples. Whether the DEL group expresses normal RhD protein and whether its raises an immune response to D antigen are keys issues in determining the policy for DEL transfusions. Because the DEL phenotype is common in the Rh-negative population of Chinese Han and studies have shown that almost all individuals with the DEL phenotype among the Chinese Han carry the *RHD*1227A allele, we had a unique superiority in resources for studying these issues and formulating a clinical transfusion strategy for the DEL phenotype in the context of preventing Rh isoimmunisation in the world.

Financial disclosure

This research was supported by the Medical Research Foundation of Wuxi Municipal Health Bureau (Grant n. ML201203) and supported by the Scientific Technology Development Foundation of Nanjing Medical University (Grant n. 2012NJMU071). The funders had no role in the study design, data collection or analysis, decision to publish, or preparation of the manuscript.

The Authors declare no conflicts of interest.

References

1) Daniels G. *Human Blood Groups*. 2nd ed. Oxford: Blackwell Science Ltd; 2002: 232-67.
2) Beckers EA, Faas BH, Ligthart P, et al. Lower antigen site density and weak D immunogenicity cannot be explained by structural genomic abnormalities or regulatory defects of the *RHD* gene. Transfusion 1997; **37**: 616-23.

3) Wagner FF, Frohmajer A, Ladewig B, et al. Weak D alleles express distinct phenotypes. Blood 2000; **95**: 2699-708.
4) Okubo Y, Yamaguchi H, Tomita T, et al. A D variant, DEL? Transfusion 1984; **24**: 542.
5) Wagner FF, Frohmajer A, Flegel WA. *RHD* positive haplotypes in D negative Europeans. BMC Genet 2001; **2**: 10.
6) Xu Q, Grootkerk-Tax MG, Maaskant-van Wijk PA, et al. Systemic analysis and zygosity determination of the *RHD* gene in a D-negative Chinese Han population reveals a novel D-negative *RHD* gene. Vox Sang 2005; **88**: 35-40.
7) Chen JC, Lin TM, Chen YL, et al. *RHD*1227A is an important genetic marker for RhD(el) individuals. Am J Clin Pathol 2004; **122**: 193-8.
8) Fukumori Y, Hori Y, Ohnoki S, et al. Further analysis of DEL (D-elute) using polymerase chain reaction (PCR) with *RHD* gene-specific primers. Transfus Med 1997; **7**: 227-31.
9) Kim JY, Kim SY, Kim CA, et al. Molecular characterization of D-Korean persons: development of a diagnostic strategy. Transfusion 2005; **45**: 345-52.
10) Körmöczi GF, Gassner C, Shao CP, et al. A comprehensive analysis of DEL types: partial DEL individuals are prone to anti-D alloimmunization. Transfusion 2005; **45**: 1561-7.
11) Brecher ME. Red cell typing methods. In: Brecher M editors. Technical Manual 14th edition. Bethesda, MD: American Association of Blood Banks; 2002. p. 677-9.
12) Shao CP, Maas JH, Su YQ, et al. Molecular background of Rh D-positive, D-negative, D (el) and weak D phenotypes in Chinese. Vox Sang 2002; **83**: 156-61.
13) Wagner FF, Flegel WA. *RHD* deletion occurred in the Rhesus box. Blood 2000; **95**: 3662-8.
14) Körmöczi GF, Legler TJ, Daniels GL, et al. Molecular and serologic characterization of DWI, a novel "high-grade" partial D. Transfusion 2004; **44**: 575-80.
15) Wagner FF, Gassner C, Muller TH, et al. Three molecular structures cause Rhesus D category VI phenotypes with distinct immunohaematologic features. Blood 1998; **91**: 2157-68.
16) Lomas C, Tippett P, Thompson KM, et al. Demonstration of seven epitopes on the Rh antigen D using human monoclonal anti-D antibodies and red cells from D categories. Vox Sang 1989; **57**: 261-4.
17) Scott M. Section 1A: Rh serology coordinator's report. Transfus Clin Biol 2002; **9**: 23-9.
18) Gassner C, Doescher A, Drnovsek TD, et al. Presence of *RHD* in serologically D-, C/E+ individuals: a European multicenter study. Transfusion 2005; **45**: 527-38.
19) Yang YF, Wang YH, Chen JC, et al. Prevalence of *RHD*1227A and hybrid Rhesus box in the general Chinese population. Transl Res 2007; **149**: 31-6.
20) Wagner T, Körmöczi GF, Buchta C, et al. Anti-D immunization by DEL red blood cells. Transfusion 2005; **45**: 520-6.
21) Yasuda H, Ohto H, Sakuma S, et al. Secondary anti-D immunization by DEL red blood cells. Transfusion 2005; **45**: 1581-4.
22) Kim KH, Kim KE, Woo KS, et al. Primary anti-D immunization by DEL red blood cells. Korean J Lab Med 2009; **29**: 361-5.
23) Richard M, Perreault J, Constanzo-Yanez J, et al. A new DEL variant caused by exon 8 deletion. Transfusion 2007; **47**: 852-7.
24) Wang QP, Dong GT, Wang XD, et al. An investigation of secondary anti-D immunisation among phenotypically RhD negative individuals in the Chinese population. Blood Transfus 2014; **12**: 238-49.
25) Chang JG, Wang JC, Yang TY, et al. Human RhDel is caused by a deletion of 1,013 bp between introns 8 and 9 including exon 9 of *RHD* gene. Blood 1998; **92**: 2602-4.
26) Li Q, Hou L, Guo ZH, et al. Molecular basis of the *RHD* gene in blood donors with DEL phenotypes in Shanghai. Vox Sang 2009; **97**: 139-46.
27) Krog GR, Clausen FB, Berkowicz A, et al. Is current serologic RhD typing of blood donors sufficient for avoiding immunization of recipients? Transfusion 2011; **51**: 2278-85.
28) 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**: 145-52.

Arrived: 14 March 2013 - Revision accepted: 28 August 2013
Correspondence: Xue-dong Wang
Department of Clinical Laboratory
The Fifth People's Hospital of Wuxi
Affiliated Hospital of Nanjing Medical University
88# xingyuan Road
Wuxi, Jiangsu, People's Republic of China
e-mail: wxd729@126.com