The *RHD* Gene Is Highly Detectable in RhD-negative Japanese Donors

Hiroshi Okuda,* Masaki Kawano,* Sadahiko Iwamoto,* Mitsunobu Tanaka,† Taiko Seno,‡ Yasuto Okubo,‡ and Eiji Kajii*

*Department of Legal Medicine and Human Genetics, Jichi Medical School, Minamikawachi-machi, Kawachi-gun, Tochigi 329-04; and

†Osaka Red Cross Blood Center, Morinomiya, Jyotou-ku, Osaka-shi, Osaka 2-4-43, Japan

Abstract

Recent molecular studies on the Rh blood group system have shown that the Rh locus of each haploid RhD-positive chromosome is composed of two structural genes: *RHD* and *RHCE*, whereas the locus is made of a single gene (*RHCE*) on each haploid RhD-negative chromosome. We analyzed the presence or absence of the *RHD* gene in 130 Japanese RhD-negative donors using the PCR method. The RhD-negative phenotypes consisted of 34 ccEe, 27 ccee, 17 ccEE, 26 Ccee, 19 CcEe, 1 CcEE, and 6 CCee. Among them, 36 (27.7%) donors demonstrated the presence of the *RHD* gene. Others showed gross or partial deletions of the *RHD* gene. These results were confirmed by Southern blot analysis. Additionally, the *RHD* gene detected in the RhD-negative donors seemed to be intact through sequencing of the RhD polypeptide cDNA and the promoter region of *RHD* gene. The phenotypes of these donors with the *RHD* gene were CC or Cc, but not cc. It suggested that there is some relationship between the *RHD* gene and the RhC phenotypes in RhD-negative individuals. In Caucasian RhD-negative individuals, the *RHD* gene has not been found outside of the report of Hyland et al. (Hyland, C.A., L.C. Wolter, and A. Saul. 1994. *Blood*. 84:321–324). The discrepant data on the *RHD* gene in RhD-negative donors between Japanese and Caucasians appear to be derived from the difference of the frequency of RhD-negative and RhC-positive phenotypes. Careful attention is necessary for clinicians in applying RhD genotyping to clinical medicine. (*J. Clin. Invest.* 1997. 100:373–379.) Key words: Rh blood group • RhD genotyping • RhD serotyping • genotype • racial difference

Introduction

Recent studies of the Rh blood group system have shown that *RH* genes are composed of *RHD* and *RHCE* genes (1–5). It is inferred that the *RHD* gene encodes the RhD antigen, and that the *RHCE* gene encodes the Rh C/c and RhE/e antigens (1–5). The Rh system is one of the clinically important blood groups because it induces Rh-incompatible blood transfusion and hemolytic disease of the newborn (HDN)¹ (6). To make a

rapid diagnosis and achieve a good prognosis, especially in HDN, it is necessary to investigate fetal RhD type before birth. In the case of patients who have been recently transfused, and who harbor a large quantity of donor blood cells, it is sometimes difficult to determine the Rh phenotypes with accuracy. Therefore, various trials were carried out to determine the genotypes of the Rh blood group system.

Several groups applied RFLP patterns on Southern blots for Rh genotyping (1, 7, 8). Recently, PCR experiments have been used to examine the Rh genotypes (9–12). Some of these methods have been put in practice. Among them, RhD genotyping has spread widely.

The method for RhD genotyping is based on the presence or absence of the *RHD* gene (9, 11). The *RHD* gene structure is not yet known completely, but preliminary investigations indicate that it resembles closely the *RHCE* gene that comprises 10 exons ranging in size from 72 to 247 bp distributed over 75 kb (13). A deletion of 600 bp in intron 4 of the *RHD* gene, however, and sequence divergences between the *RHD* and *RHCE* genes in the 3' noncoding region, have been identified (2, 3). Substitutions of 41 bp between RhD and RhCE cDNA were indicated. On the basis of features of the *RHD* gene, several RhD genotypings have been performed by PCR, and have demonstrated some cases with discrepancies between RhD serotyping and genotyping (11). Hyland et al. (14) investigated three RhD-negative samples with the CCee phenotype in the Australian population, and found that one appeared to have a normal *RHD* gene. We also detected the *RHD* gene in RhD-negative Japanese individuals (15, 16). Simsek et al. (11) have reported no detection of the *RHD* gene in 56 RhD-negative Caucasians. These results suggest that racial differences exist in the genetic background of the RhD antigen, and call our attention to RhD genotyping. The exact frequency of RhD-negative Japanese individuals with the *RHD* gene has not been clarified. For the purpose of determining the gene frequency and examining its disparity between Japanese and Caucasians, we analyzed the *RHD* gene in 130 unrelated RhD-negative Japanese donors. The gene's incidence is higher than we expected. We would like to report the result, and to discuss the racial difference.

Methods

Blood sample. We have analyzed genomic DNAs from 200 RhD-positive and 130 RhD-negative unrelated Japanese donors. Anticoagulated whole blood was drawn from the cubital vein after informed consent. Serotyping for the RhD, RhC/c, and RhE/e antigens was carried out by polyclonal and monoclonal anti-RhD (Ortho Diagnostic Systems Inc., Raritan, NJ, and Osaka Red Cross Center, Osaka, Japan), and polyclonal anti-C, anti-c, anti-E, and anti-e antibodies (Ortho Diagnostic Systems Inc.).

RNA and DNA isolation. Total RNAs of reticulocytes were isolated according to Tse et al. (17). Genomic DNA was extracted from peripheral blood leukocytes according to a previously reported method (18).

PCR for *RHCE* and *RHD* genes. Table I shows the PCR primers for detection of *RH* gene. To investigate the promoter regions in the

Address correspondence to Hiroshi Okuda, Department of Legal Medicine and Human Genetics, Jichi Medical School, Minamikawachi-machi, Kawachi-gun, Tochigi 329-04, Japan. Phone: 0285-44-2111; FAX: 0285-44-4902.

Received for publication 22 October 1996 and accepted in revised form 21 April 1997.

1. Abbreviations used in this paper: HDN, hemolytic disease of the newborn; RT-PCR, reverse transcription-PCR.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/97/07/0373/07 \$2.00

Volume 100, Number 2, July 1997, 373–379

Table I. The PCR Primers for Detection of the *RHD* and *RHCE* Genes

Primer	Nucleotide sequence	Position	Detection of
F1	5'-GGCCAAGATCTGACCGTGATG-3'	nt 151 to 171 (exon 2)	<i>RHD</i> , <i>RHCE</i> gene
R1	5'-TGAACAGTGTGATGACCACCTT-3'	nt 334 to 313 (exon 2)	<i>RHD</i> , <i>RHCE</i> gene
F2	5'-GCTGGCCACCATGAGTGCTT-3'	nt 342 to 361 (exon 3)	<i>RHD</i> gene
R2	5'-TTACTGATGACCATCCTCAGGT-3'	nt 476 to 455 (exon 3)	<i>RHD</i> gene
F3	5'-TGTTTCGCAGCCTATTTTGGG-3'	nt 521 to 540 (exon 4)	<i>RHD</i> , <i>RHCE</i> gene
R3	5'-TGACACTTGGCCAGAACATC-3'	nt 670 to 651 (exon 5)	<i>RHD</i> , <i>RHCE</i> gene
F4	5'-TTGTGGATGTTCTGGCCAAGTT-3'	nt 646 to 667 (exon 5)	<i>RHD</i> gene
R4	5'-CTGAGATGGCTGTCCACACG-3'	nt 763 to 744 (exon 5)	<i>RHD</i> gene
F5	5'-CAGCTCCATCATGGGCTACAA-3'	nt 972 to 992 (exon 7)	<i>RHD</i> gene
R5	5'-TGCCGGCTCCGACGGTATC-3'	nt 1066 to 1048 (exon 7)	<i>RHD</i> gene
F6	5'-GGATTTTAAGCAAAAGCATCCAA-3'	nt 1246 to 1268 (exon 10)	<i>RHD</i> gene
R6	5'-CGATAAATGGTGAGATTCTCCT-3'	nt 1443 to 1422 (exon 10)	<i>RHD</i> gene

RHD and *RHCE* genes, PCR amplification was performed by a set of primers: Ps (CCAGGTTTTTACTAGAGCCA) and Pa (AGAGCTGCTTCCAGTGTTAG). Primer F3 for a sequence located in exon 4, and primer R3 for a sequence located in exon 5, were used to distinguish the *RHCE* and *RHD* genes (Fig. 1, Table I). The antisense primer R6 recognizes a sequence in the 3' noncoding region of the *RHD* gene (2), and therefore yields a product of 198 bp (Fig. 1, Table I). In the presence of all four primers (F3/R3 and F6/R6), dual PCR I was performed. By making use of the differences in RhD cDNA as compared with RhCE cDNA, six primers (F2/R2, F4/R4, and F5/R5) specific to the *RHD* gene were produced (Fig. 1, Table I). Two primers (F1/R1) to exon 2 were common to both *RHD* and *RHCE* genes, and were used as an internal control to validate the PCR reaction (Fig. 1, Table I). As shown in Fig. 1, eight primers (F1/R1, F2/R2, F4/R4, and F5/R5) were applied to allele-specific amplification for detecting the

RHD gene. Dual PCR II was used in the presence of four primers (F1/R1 and F5/R5) to detect exon 7 of the *RHD* gene and triple PCR was carried out with six primers (F1/R1, F2/R2, and F4/R4) to identify exons 3 and 5 of the *RHD* gene.

Reverse transcription-PCR (RT-PCR). Poly(A)⁺ RNA was reverse transcribed into the first strand of cDNA with oligo(dT)₁₆ as a primer using cloned Moloney murine leukemia virus reverse transcriptase. PCR was performed in a reaction tube (Gene Amp RNA PCR kit; Perkin Elmer Corp., Norwalk, CT). The two primers prepared for PCR and sequencing on the basis of the published cDNA sequence of Rh polypeptides (19, 20) were as follows: 1s (nt -30 to -11); 5'-GTGGAACCCCTGCACAGAGA-3', and 1a (nt 1283 to 1264); 5'-CAGGCCTTGTTTTTCTTGGA-3'.

PCR condition. Three kinds of PCR were performed with the genomic DNA in the capacity of the template, as follows. Method I was

Figure 1. Dual PCR I, II, and triple PCR strategy for the *RHCE* and *RHD* genes. In dual PCR I, primers F3/R3 amplified a 1,225-bp fragment of the *RHCE* gene, and a 576-bp fragment of the *RHD* gene in intron 4, and primers F6/R6 amplified a 198-bp fragment of exon 10 only in the *RHD* gene. Dual PCR II and triple PCR are the methods for detecting exon 7 of the *RHD* gene, and exons 3 and 5 of the *RHD* gene, respectively. The PCR products generated from exons 3, 5, and 7 of the *RHD* gene were 125, 117, and 95 bp, respectively. The PCR fragment obtained with two primers (F1/R1) to exon 2, and that was common to both the *RHD* and *RHCE* genes, was 180 bp, and was used as an internal control.

used in analyzing the promoter region (primers Ps/Pa), for carrying out dual PCR (primers F3/R3 and F6/R6) and RT-PCR (primers 1s/1a). Final concentration was 1× PCR buffer (10× PCR buffer = 15 mM MgCl₂, 100 mM Tris-HCl pH 8.3, 500 mM KCl buffer), 0.24 μM of both 5' and 3' primer, and 2.5 U *Taq* DNA polymerase in a total volume of 100 μl. The first cycle of the PCR was done at 95°C for 5 min to denature DNA, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 3 min at 72°C, and finally, 1 cycle of 10 min at 72°C. Method II was applied to dual PCR (primers F1/R1 and F5/R5) for detecting exon 7 of the *RHD* gene. PCR mixture was of the same composition as that in method I. After an initial cycle of denaturation at 95°C for 5 min, 40 cycles were performed, consisting of 1 min of denaturation at 95°C, 1.5 min of primer annealing at 55°C, 3 min of extension at 72°C, followed by a final cycle of 10 min at 72°C. Method III was fulfilled for identifying exons 3 and 5 of the *RHD* gene. Final concentration of PCR mixture was 1× PCR buffer (same as above), 1.5 μM of both 5' and 3' primers, and 5.0 U *Taq* DNA polymerase in a total volume of 100 μl. The first cycle of PCR was done at 95°C for 5 min to denature DNA, followed by 35 cycles of 30 s at 95°C (denatur-

ation), 30 s at 67°C (annealing), 30 s at 72°C (extension), and finally, 1 cycle of 10 min at 72°C. To evaluate all results of amplification by methods I, II, and III, 5 μl of the reaction mixture was withdrawn and electrophoresed in 3% agarose gel.

Subcloning. PCR products of intron 4 were ligated into pCRTM^{II} (TA cloning kit; Invitrogen Corp., San Diego, CA) according to the supplier's instructions. After transformation, the clones with the insert were screened by color selection with X-Gal. Each white colony was transferred into Luria-Bewtani medium containing ampicillin at 100 μg/ml, and the culture was incubated overnight at 37°C. Plasmid DNAs were isolated by the alkaline lysis method, and were prepared for sequencing by the alkaline-denaturation method.

DNA sequencing and analysis. The ssDNA template was sequenced by the dideoxy chain termination of Sanger et al. (21) using a Sequenase manufacturer. The primers used were either sequence-specific oligonucleotides, or the universal primer. The reaction products were electrophoresed on 6% acrylamide gels containing 8 M urea.

Southern analysis. Genomic DNAs obtained were digested with

Figure 2. The nucleotide sequences of intron 4 of the *RHCE* (upper) and *RHD* (lower) genes. The *RHCE* and *RHD* genes consist of 1,075 and 426 nucleotides, respectively. The black letters demonstrate the common region of the *RHCE* and *RHD* genes. The white letters show the specific region of the *RHCE* gene. The letters surrounded with an oblong frame indicate the nucleotide substitutions between the *RHCE* and *RHD* genes. The broken line indicates the deleted region of the *RHD* gene.

restriction endonuclease BamHI and analyzed by Southern blot hybridization as described (8). The *RHD*-cDNA in the coding region was used as a probe.

Results

Determination of Rh phenotypes. RhD phenotyping of unrelated Japanese donors was performed by polyclonal and monoclonal anti-RhD antibodies. In the case of detecting no agglutination, antiglobulin and adsorption/elution tests were performed to exclude weak D, D^u, or D_{el} antigen (22). The RhD-negative phenotypes were determined through such a process. As regards partial D, the screening test was performed according to the strategy of Okubo et al. (23). Additionally, the phenotypes of RhC/c and RhE/e were observed. In this study, we analyzed 200 RhD-positive donors and 130 RhD-negative donors. The RhD-negative phenotypes consisted of 34 ccEe, 27 ccee, 17 ccEE, 26 Ccee, 19 CcEe, 1 CcEE, and 6 CCee.

Isolation and sequencing of intron 4 of the *RHD* and *RHCE* genes. The *RHCE* gene is composed of 10 exons (13). The *RHD* gene structure has not been established, but preliminary investigations indicated that it is similarly organized (13). Recently, a deletion of 600 bp in intron 4 of the *RHD* gene was identified. Huang showed all sequences of intron 4 in the *RHD* gene, and indicated the existence of the human *Alu* repeat in intron 4 of the *RHCE* gene (24). Westhoff et al. demonstrated the *Alu* element similar to Huang's report in addition to all sequences of intron 4 in both the *RHD* and *RHCE* genes (25). To identify intron 4 of the *RHD* gene and the *RHCE* genes, we carried out the PCR amplification with primers F3/R3. As shown in Fig. 2, intron 4 of the *RHCE* and *RHD* genes consisted of 1,075 and 426 nucleotides, respectively. The size of deletion in intron 4 of the *RHD* gene was 649 bp. It was confirmed that the difference in size between intron 4 fragments of the *RHCE* and *RHD* genes derives from a deletion, and that the nondeleted sequence of the *RHD* gene is identical to the *RHCE* gene except for three nucleotides. The nucleotide sequence of intron 4 in the *RHD* gene was entirely consistent with that reported by Huang. Comparing our data with the report of Westhoff et al. (25), however, there are many nucleotide differences: two deletions (G at nucleotide 1, A at nucleotide 316), a single insertion (G next to nucleotide 426) in intron 4 of the *RHD* gene, four deletions (G at nucleotide 1, G at nucleotide 545, C at nucleotide 953, A at nucleotide 965), four insertions (G at nucleotide 308, A at nucleotide 409, G at nucleotide 414, G next to nucleotide 1075), and three substitutions (C→T at nucleotide 308, A→G at nucleotide 685, G→A at nucleotide 799) in intron 4 of the *RHCE* gene (Fig. 2).

Detection of the *RHD* gene by PCR. For detecting the presence or absence of the *RHD* gene, the PCR amplification for intron 4 of the *RHD* and *RHCE* genes was performed by primers F3/R3. In addition to the primer set for intron 4, another primer set (F6/R6) was prepared that is specific for exon 10 of the *RHD* gene only. The dual PCRI was done by these two primer sets (F3/R3 and F6/R6) (Fig. 1). All 200 RhD-positive donors showed three PCR products (Fig. 3): 1,225 bp from intron 4 of the *RHCE* gene, 576 bp from intron 4 of the *RHD* gene, and 198 bp from exon 10 of the *RHD* gene. On the other hand, the patterns of PCR products from 130 RhD-negative donors could be classified into three categories of A, B, and C (Fig. 3 a, Table II). Intron 4 of the *RHCE* gene was amplified

Figure 3. The results of dual PCRI, II, and triple PCR in donors with various Rh phenotypes. Genomic DNAs from one RhD-positive and five RhD-negative individuals were amplified with dual PCRI, II, and triple PCR. Each lane in *b* and *c* was lined with the same donor as in *a*. Lane 1 (RhD-positive case) belongs to the *RHD* gene nondeletion type (category C). Regardless of RhD-negative cases, lanes 3 and 4 are category C. Lanes 2 and 5 (RhD-negative cases) are gross *RHD* gene deletion type (category A). Lane 6 belongs to partial *RHD* gene deletion type (category B). The PCR products were separated on 3% agarose gel stained with ethidium bromide. (*a*) The products generated from intron 4 in the *RHD* and *RHCE* genes were 576 and 1,225 bp, respectively. Only in the *RHD* gene was the 198-bp fragment of exon 10 amplified. (*b* and *c*) The fragment generated from exon 2 in both the *RHD* and *RHCE* genes was 180 bp. The bands produced from exons 3, 5, and 7 (only in the *RHD* gene) were 125, 117, and 95 bp, respectively. Lane 6 donor belonged to category B, possessed the PCR product from only exon 3 of the *RHD* gene, and lacked the other PCR products from exons 5 and 7 of the *RHD* gene.

in all RhD-negative donors. In category A, both PCR products from intron 4 and exon 10 of the *RHD* gene were absent (gross *RHD* gene deletion type). In category B, the PCR product was present in exon 10 of the *RHD* gene, but not in intron 4 of the *RHD* gene (partial *RHD* gene deletion type). In category C, all three PCR products were detectable (*RHD* gene nondeletion type).

Table II. PCR Amplification for Intron 4 and Exon 10 of the *RHD* Gene in 130 RhD-negative Japanese Individuals

Category	<i>RHD</i> gene		RhD (–) RhC (+) Rhc (+/–)					RhD (–) RhC (–) Rhc (+)			
	Intron 4	Exon 10	CCdee	CcdEE	CcdEe	Ccdee	Total	ccdEE	ccdEe	ccdee	Total
A	–	–	2	1	6	5	14	17	34	27	78
B	–	+	0	0	0	2	2	0	0	0	0
C	+	+	4	0	13	19	36	0	0	0	0
Total			6	1	19	26	52	17	34	27	78

Among 130 RhD-negative donors, 92 (70.8%), 2 (1.5%), and 36 (27.7%) were grouped into categories A, B, and C, respectively (Table II). All 78 RhD-negative donors with RhC-negative and Rhc-positive phenotypes showed category A. 52 RhD-negative donors with RhC-positive phenotypes were divided into 14 (26.9%) of category A, 2 (3.9%) of category B, and 36 (69.2%) of category C. The presence of the *RHD* gene in RhD-negative donors seems to relate strictly to the RhC phenotypes, but not to the Rhc, RhE, or Rhe. As shown in Fig. 1, the dual PCR by two sets of F1/R1 and F5/R5, and the triple PCR by three primer sets of F1/R1, F2/R2, and F4/R4 were performed for 200 RhD-positive donors. In all donors, two bands and three bands were detected in the products of the dual PCR and triple PCR, respectively (Fig. 3, *b* and *c*). Two bands in the dual PCR consisted of 180 bp from exon 2 of both the *RHD* and *RHCE* genes, and 95 bp from exon 7 of the *RHD* gene. The dual PCR and triple PCR were applied to 130 RhD-negative donors. Both of these PCR supported the result of the dual PCR and the classification of categories A, B, and C. One of two donors belonging to category B lacked all PCR products from exons 3, 5, and 7 of the *RHD* gene (not shown), and the other possessed only that of exon 3 (Fig. 3). There appeared to be various size deletions of the *RHD* gene in the partial *RHD* deletion type.

Southern blot analysis. Southern analysis was performed to analyze further the structure of the *RH* locus. BamHI was used as a restriction endonuclease. Fig. 4 shows the RFLP patterns produced by BamHI. The donors with RhD-positive phenotypes exhibited four bands of 1.4, 2.2, 4.3, and 5.2 kb fragments. The RFLP of RhD-negative donors in categories A, B, and C demonstrated three bands (1.4, 2.2, and 5.2 kb) and four bands (1.4, 2.2, 4.3, and 5.2), respectively. The 4.3-kb band appears to be derived from the *RHD* gene, and this result is the same as previous reports (1, 26).

Isolation and sequencing of cDNA-encoding Rh polypeptides. Detection of Rh polypeptide mRNA has been accomplished using the RT-PCR method followed by direct sequencing. The RT-PCR using *Taq* DNA polymerase was performed for the Rh polypeptide cDNAs from 12 RhD-negative donors with the *RHD* gene, and 6 RhD-negative donors without the *RHD* gene using a set of 1s/1a primers. After 35 cycles of amplification, the reaction products were separated by agarose gel electrophoresis, and the fractionated bands were detected in all samples (around 1,313 bp expected). Direct sequencing suggested the presence of RhD-cDNA in PCR products from all the RhD-negative donors with the *RHD* gene, but not in ones from the RhD-negative donors without the *RHD* gene. For the purpose of purification of the Rh-related cDNAs, the RT-PCR products were subcloned with the plasmid pCRTM^{II}

vector. The sequence analyses revealed the intact *RHD*-cDNA, which was correspondent with the RhD cDNA in the report of Le Van Kim et al. (2) except for two substitutions (C→G at nucleotide 654 and G→T at nucleotide 941), in all the RhD-negative donors with the *RHD* gene, but no *RHD*-cDNA in the RhD-negative donors without the *RHD* gene. This clone was identified in RhD-positive Japanese individuals.

Isolation and sequencing of the promoter region of *RH* genes. The PCR amplification was performed for the 5' flanking regions containing the promoters of the *RHD* and *RHCE* genes from three RhD-positive donors and three RhD-negative donors with both genes. The sequences of these PCR products clarified no substitution between the flanking regions of the *RHD* and *RHCE* genes, not only from the RhD-positive donors, but also from the RhD-negative donors.

Discussion

Several theories were put forward on *RH* genetic models until the present. At first, the three-loci theory was proposed by

Figure 4. Southern blot analysis. Genomic DNAs from various Rh phenotypes digested by BamHI were hybridized with the *RHD*-cDNA in coding region. Each category that the donors belonged to was shown in the lower position. All cases possess the 1.4, 2.2, and 5.2 kb band, whereas the 4.3 kb band was not detectable in categories A and B.

Fisher and Race (27). This theory has contributed greatly to understanding the hereditary manner in the Rh blood group system. Furthermore, Fisher (28, 29), by analyzing the frequency ratio of each genotype, postulated that the *RHC/c* gene lies between the *RHD* and *RHE/e* genes. Wiener (30), on the other hand, suggested the single locus theory, in which the inheritance of Rh antigens is determined by a single gene with multiple alleles. Thereafter, the two-loci theory was advocated by Tippett (31), based on serological data. According to this model, *RH* genes consist of two structural genes: one encoding the RhD antigen, and the other encoding both the RhC/c and RhE/e antigens. Actually, two different *RH*-cDNAs (*RHD* and *RHCE* cDNAs) were cloned (2, 3, 15, 19, 20). By Southern analysis of genomic DNA, Colin et al. (1) indicated that two highly homologous genes were present in RhD-positive individuals, but that only one was present in RhD-negative individuals. Absence of the *RHD* gene in RhD-negative individuals was confirmed after cloning of the *RHD*-cDNA (2, 3). These reports are compatible with the two-loci theory.

The present data, however, demonstrated the normal *RHD* gene in 36 (27.7%) of 130 RhD-negative Japanese donors. We used every discretion in determining the RhD-negative phenotypes for excluding partial D, D^u, or D_{el} antigen. All of these RhD-negative donors with the *RHD* gene showed the CC or Cc phenotypes, but not the cc phenotype. These results suggest that there is some relationship between the *RHD* gene and the RhC phenotypes in RhD-negative individuals. As an exception, the *RHD* gene had been isolated from a Japanese sibling with the phenotype of ccdee (16). It was suggested that another mechanism preventing the expression of RhD antigen might participate in the previously reported case. In RhD-negative Caucasians, the *RHD* gene has not been found outside of the report of Hyland et al. (14). By performing careful research, it is possible to detect the *RHD* gene in RhD-negative Caucasians. The frequency of RhD-negative phenotype varies widely in different parts of the world, presenting itself in 17% of English people (22), but only in 0.5% of the Japanese (32). The RhC-positive phenotypes accounted for 18% of Japanese RhD-negatives, but only for a very low percentage of English RhD-negatives. The diverse data on the *RHD* gene of RhD-negative individuals seem to be due to the difference of RhD-negative and RhC-positive frequency between both races. In the present study, the number of RhD-negative and RhC-positive donors was biased because these donors were analyzed actively with the intention of comparing with RhD-negative and RhC-negative donors. With due regard to the percentage (69.2%) of the presence of *RHD* gene in the RhD-negative and RhC-positive individuals, the positive ratio of the *RHD* gene in Japanese RhD negatives is estimated to be 12.5%.

The blood group antigenicity of the Rh-related polypeptides is thought to require one or more comolecules (33, 34). The candidates for the Rh-related comolecules included the Rh50 glycoprotein, LW glycoprotein, glycophorin B, CD47 glycoprotein, and Duffy glycoprotein (33, 34). Among them, Cherif-Zahar et al. (35) have proposed the Rh50 glycoprotein as a comolecule common to the RhD and RhCE polypeptides through investigating five Rhnull individuals whose red cells lacked all antigens (D, C/c, and E/e) of the Rh system. In these Rhnull cases, the Rh50 glycoproteins have shown genomic alterations of frameshift, nucleotide mutations, and failure of amplification. The Rh50 glycoproteins that were immunoprecipitated by Moore and Green (36) are heterogeneously glyco-

sylated membrane proteins of 50 and 45 kD, coprecipitated with the Rh30 protein on immunoprecipitation with anti-Rh-specific antibodies (37). It is thought that the Rh30 polypeptide, which is a nonglycosylated 30-kD erythrocyte membrane protein, is associated with Rh antigen (38). Confirmation of the RhD-negative phenotype with the intact *RHD* gene, and of the -D- phenotype with the intact *RHCE* gene, predicts RhD-specific and RhCE-specific comolecules. In several RhD-negative cases with the *RHD* gene, it was shown in this time that the promoter regions of both *RH* genes were intact, and that each *RHD*-cDNA obtained from them was also intact. In several RhD-negative donors without the *RHD* gene, *RHD*-cDNA was entirely absent. We have also indicated normal *Rh50* transcripts in RhD-negative donor reticulocyte (not shown). By carrying out the coexpression study of *Rh50* and *RHD* or *RHCE* gene in 293 nonerythroid cells, we confirmed that excessive transcript was shown with Northern blotting study, in spite of the lack of RhD and RhC antigens on transduced cells (manuscript submitted for publication). We intend to detect RhD-specific comolecules, and to analyze the mechanism of the expression in RhD-negative cases with the *RHD* gene. The RhD-specific and RhCE-specific comolecules have not been isolated. The present data suggested that the gene of the RhD-specific comolecule that was associated with the expression of RhD-polypeptide correlated with the *RHCE* or *RHCE* gene. The genetic change of the RhD-specific comolecule might induce the disappearance of RhD antigen on red cell membranes.

According to the above-mentioned data, it is probable that the *RHD* gene is highly detected in other ethnic RhD-negative groups, especially with RhC-positive phenotype, as well as Japanese. It is dangerous to perform RhD genotyping by judging the absence or the presence of the *RHD* gene. In applying RhD genotyping to clinical medicine, careful attention is necessary for clinicians. As RhD genotyping must inevitably be carried out to investigate the fetal RhD type before birth for making a rapid diagnosis and achieving a good prognosis, for instance, HDN case, the clinicians have to recognize its limitation and take notice of the interpretation on its result. It is thought that further research in the Rh blood system is necessary to apply the RhD genotyping to clinical medicine in a safer manner.

Acknowledgments

The authors thank Mr. T. Oyamada for technical assistance.

This work was supported by a Grant-in-Aid (No. 07671220) for scientific research from the Ministry of Education, Science, and Culture, Japan.

References

- Colin, Y., B. Cherif-Zahar, C. Le Van Kim, V. Raynal, V. Van Huffel, and J.-P. Cartron. 1991. Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. *Blood*. 78: 2747-2752.
- Le Van Kim, C., I. Mouro, B. Cherif-Zahar, V. Raynal, C. Cherrier, J.-P. Cartron, and Y. Colin. 1992. Molecular cloning and primary structure of the human blood group RhD polypeptide. *Proc. Natl. Acad. Sci. USA*. 89:10925-10929.
- Arce, M.A., E.S. Thompson, S. Wagner, K.E. Coyne, B.A. Ferdman, and D.M. Lubin. 1993. Molecular cloning of RhD cDNA derived from a gene present in RhD-positive, but not RhD-negative individuals. *Blood*. 82:651-655.
- Mouro, I., Y. Colin, B. Cherif-Zahar, J.-P. Cartron, and C. Le Van Kim. 1993. Molecular genetic basis of the human Rhesus blood group system. *Nat. Genet.* 5:62-65.

5. Hermand, P., M.M. Huet, C. Bloy, K. Suyama, J.P. Goldstein, J.-P. Cartron, and P. Bailly. 1993. Immunochemical characterization of Rhesus proteins with antibodies raised against synthetic peptides. *Blood*. 82:669–676.
6. Mollison, P.L., C.P. Engelfriet, and M. Contreras. 1993. Blood Transfusion in Clinical Medicine. 9th edition. Blackwell, Oxford, London, Edinburgh, Boston and Melbourne.
7. Hyland, C.A., L.C. Wolter, Y.W. Liew, and A. Saul. 1994. A Southern analysis of Rh blood group genes: association between restriction fragment length polymorphism patterns and Rh serotypes. *Blood*. 83:566–572.
8. Umenishi, F., E. Kajii, and S. Ikemoto. 1994. A new genetic polymorphism of Rh polypeptide genes. *Biochem. J.* 299:203–206.
9. Bennett, P.R., C. Le Van Kim, Y. Colin, R.M. Warwick, R. Path, B. Cherif Zahar, N.M. Fisk, and J.-P. Cartron. 1993. Prenatal determination of fetal RhD type by DNA amplification. *N. Engl. J. Med.* 329:607–610.
10. Le Van Kim, C., I. Mouro, Y. Brossaet, J. Chavinie, J.-P. Cartron, and C. Colin. 1994. PCR-based determination of Rhc and Rhd status of fetuses at risk of Rhc and Rhd haemolytic disease. *Br. J. Haematol.* 88:193–195.
11. Simsek, S., B.H.W. Faas, P.M.M. Bleeker, M.A.M. Overbeeke, H.M. Cuijpers, C.E. van der Schoot, and A.E.G.Kr. von dem Borne. 1995. Rapid RhD genotyping by polymerase chain reaction-based amplification of DNA. *Blood*. 85:2975–2980.
12. Faas, B.H.W., S. Simsek, P.M.M. Bleeker, N.A.M. Overbeeke, H.M. Cuijpers, A.E.G.Kr. von dem Borne, and C.E. van der Schoot. 1995. Rh E/e genotyping by allele-specific primer amplification. *Blood*. 85:829–832.
13. Cherif-Zahar, B., C. Le Van Kim, C. Rouillac, V. Raynal, J.-P. Cartron, and Y. Colin. 1994. Organization of the gene (*RHCE*) encoding the human blood group RhCcEe antigens and characterization of the promoter region. *Genomics*. 19:68–74.
14. Hyland, C.A., L.C. Wolter, and A. Saul. 1994. Three unrelated RhD gene polymorphisms identified among blood donors with Rhesus CCee (r'r') phenotypes. *Blood*. 84:321–324.
15. Kajii, E., F. Umenishi, S. Iwamoto, and S. Ikemoto. 1993. Isolation of a new cDNA clone encoding a Rh polypeptide associated with the Rh blood group system. *Hum. Genet.* 91:157–162.
16. Umenishi, F., E. Kajii, and S. Ikemoto. 1994. Molecular analysis of Rh polypeptides in a family with RhD-positive and RhD-negative phenotypes. *Biochem. J.* 299:207–211.
17. Tse, W.T., P.G. Gallagher, B. Pothier, F. Costa, A. Scarpa, J. Delaunay, and B.G. Forget. 1991. An increased frameshift mutation of the b-spectrin gene associated with elliptocytosis in spectrin Nice (b220/216). *Blood*. 78:517–523.
18. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
19. Cherif-Zahar, B., C. Bloy, C. Le Van Kim, D. Blanchard, P. Bailly, P. Hermand, C. Salmon, J.-P. Cartron, and Y. Colin. 1990. Molecular cloning and protein structure of a human blood group Rh polypeptide. *Proc. Natl. Acad. Sci. USA*. 87:6243–6247.
20. Avent, N., K. Ridgwell, M.J. Tanner, and D.J. Anstee. 1990. cDNA cloning of a 30kDa erythrocyte membrane protein associated with Rh (Rhesus)-blood-group-antigen expression. *Biochem. J.* 271:821–825.
21. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain terminating. *Proc. Natl. Acad. Sci. USA*. 74:5463–5467.
22. Daniels, G. 1995. Rh blood group system. In Human Blood Groups. 1st edition. Blackwell Science Ltd., Oxford.
23. Okubo, Y., T. Seno, H. Yamano, H. Yamaguchi. 1991. Partial D antigens disclosed by a monoclonal anti-D in Japanese blood donors. *Transfusion*. 31:782.
24. Huang, C.-H. 1996. Alteration of *RH* gene structure and expression in human dCCee and DC^W-red blood cells: phenotypic homozygosity versus genotypic heterozygosity. *Blood*. 88:2326–2333.
25. Westhoff, C.M., and D.E. Wylie. 1996. Investigation of the *RH* locus in gorillas and chimpanzees. *J. Mol. Evol.* 42:658–668.
26. Rouillac, C., Y. Colin, N.C. Hughes-Jones, M. Beolet, A.-M. D'Ambrosio, J.-P. Cartron, and C. Le van Kim. 1995. Transcript analysis of D category phenotypes predicts hybrid Rh D-CE-D protein associated with alteration of D epitopes. *Blood*. 85:2937–2944.
27. Fisher, R.A., cited by R.R. Race. 1944. An incomplete antibody in human serum. *Nature (Lond.)*. 153:771–772.
28. Fisher, R.A., and R.R. Race. 1946. Rh gene frequencies in Britain. *Nature (Lond.)*. 157:48–49.
29. Fisher, R.A. 1947. The rhesus factor. A study in scientific method. *Am. Sci.* 35:95–103.
30. Wiener, A.S., and I.B. Wexler. 1963. An Rh-Hr Syllabus. 2nd edition. Grune & Stratton Inc., New York.
31. Tippett, P.A. 1986. A speculative model for the Rh blood groups. *Ann. Hum. Genet.* 50:241–247.
32. Okubo, Y. 1991. Pretransfusion Testing with Special Reference to Blood Groups in Japanese. Ishiyakushupan Corporation, Japan.
33. Anstee, D.J., and M.J.A. Tanner. 1993. Biochemical aspects of the blood group Rh (rhesus) antigens. In Baillere's Clinical Haematology, Red Cell Membrane and Red Cell Antigens. Vol. 6. Baillere Tindall, London.
34. Cartron, J.-P. 1994. Defining the Rh blood group antigen. *Blood Rev.* 8: 199–212.
35. Cherif-Zahar, B., V. Raynal, P. Gane, M.-G. Mattei, P. Bailly, G. Gibbs, Y. Colin, and J.-P. Cartron. 1996. Candidate gene acting as a suppressor of the *RH* locus in most cases of Rh-deficiency. *Nat. Genet.* 12:168–173.
36. Moore, S., and C. Green. 1987. The identification of specific Rhesus-polypeptide-blood-group-ABH-active-glycoprotein complexes in the human red-cell membrane. *Biochem. J.* 244:735–741.
37. Ridgwell, K., N.K. Spurr, B. Laguda, C. Macgeoch, N.D. Avent, and M.J.A. Tanner. 1992. Isolation of cDNA clones for a 50kDa glycoprotein of the human erythrocyte membrane associated with Rh (Rhesus) blood-group antigen expression. *Biochem. J.* 287:223–228.
38. Ridgwell, K., S.A.C. Evers, W.J. Mawby, D.J. Anstee, and M.J.A. Tanner. 1994. Studies on the glycoprotein associated with Rh (Rhesus) blood group antigen expression in the human red blood cell membrane. *J. Biol. Chem.* 269: 6410–6416.