

The presence of an *RHD* pseudogene containing a 37 base pair duplication and a nonsense mutation in Africans with the Rh D-negative blood group phenotype

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Antigens of the Rh blood group system are encoded by 2 homologous genes, *RHD* and *RHCE*, that produce 2 red cell membrane proteins. The D-negative phenotype is considered to result, almost invariably, from homozygosity for a complete deletion of *RHD*. The basis of all PCR tests for predicting fetal D phenotype from DNA obtained from amniocytes or maternal plasma is detection of the presence of *RHD*. These tests are used in order to ascertain the risk of hemolytic disease of the newborn. We have identified an *RHD* pseudogene (*RHD*_Δ) in Rh

D-negative Africans. *RHD*_Δ contains a 37 base pair (bp) insert in exon 4, which may introduce a stop codon at position 210. The insert is a sequence duplication across the boundary of intron 3 and exon 4. *RHD*_Δ contains another stop codon in exon 6. The frequency of *RHD*_Δ in black South Africans is approximately 0.0714. Of 82 D-negative black Africans, 66% had *RHD*_Δ, 15% had the *RHD-CE-D* hybrid gene associated with the VS+ V- phenotype, and only 18% completely lacked *RHD*. *RHD*_Δ is present in about 24% of D-negative African Americans and 17% of

D-negative South Africans of mixed race. No *RHD* transcript could be detected in D-negative individuals with *RHD*_Δ, probably as a result of nonsense-mediated mRNA decay. Existing PCR-based methods for predicting D phenotype from DNA are not suitable for testing Africans or any population containing a substantial proportion of people with African ethnicity. Consequently, we have developed a new test that detects the 37 bp insert in exon 4 of *RHD*_Δ. (Blood. 2000; 95:12-18)

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Introduction

Rh is a highly complex red cell blood group system with 52 antigens and numerous phenotypes.^{1,2} The Rh antigens are encoded by 2 closely linked homologous genes, each consisting of 10 coding exons. *RHCE* encodes the Cc and Ee antigens, and *RHD* encodes the D antigen. There are also many uncommon fusion genes, comprising part of *RHCE* and part of *RHD*, which may encode abnormal D and CcEe antigens and 1 or more low-frequency Rh antigens.^{3,4}

The most important Rh polymorphism from the clinical aspect is the D polymorphism. About 82%-85% of Caucasians are D-positive, the remainder lack the D antigen. The D-negative phenotype has a frequency ranging between 3% and 7% in Africans and <1% in the people from the Far East.¹ D-negative red cells lack the D protein, and alloanti-D consists of antibodies to a variety of epitopes on the D polypeptide. Colin et al⁵ found that the D-negative phenotype resulted from homozygosity for a complete deletion of *RHD*. Rare exceptions to this molecular basis of D-negative, in which at least some *RHD* exons are present, have been described. In almost all these exceptions, the aberrant *RHD* belongs to a haplotype producing C antigen. Examples found in Caucasians include *RHD* containing a nonsense mutation⁶ or a 4-nucleotide deletion,⁷ and *RHD-CE-D* fusion genes containing *RHD* exons 1 and 10 or *RHD* exons 1-3 and exons 9 and 10, with the remainder of the exons derived from *RHCE*.^{8,9} An *RHD-CE-D* fusion gene, in which the 3' end of exon 3 plus exons 4-8 are derived from *RHCE*, is sometimes associated with a D-negative

phenotype in people of African origin.¹⁰ In 130 D-negative Japanese, 36 (28%) had an apparently intact *RHD*, 2 had at least exon 10 of *RHD*, and 1 of these also had *RHD* exon 3.¹¹ Despite these variants, however, it is generally accepted that lack of *RHD* is the usual molecular background for the D-negative phenotype.

The D antigen is of clinical importance because anti-D antibodies are capable of causing severe hemolytic disease of the fetus and newborn (HDN), resulting in hydrops fetalis and fetal death. In those severely affected infants who are born alive, jaundice may lead to kernicterus.¹² The prevalence of RhD HDN has been greatly reduced since the 1960s by the introduction of anti-D immunoglobulin prophylaxis, which prevents D-negative mothers from being immunized by fetal D-positive red cells as a result of fetomaternal hemorrhage at parturition. Some D-negative women, however, still produce anti-D for a variety of reasons including undetected antepartum fetomaternal hemorrhage and failure to administer anti-D, and any subsequent D-positive fetus is at risk from HDN. It is now common practice for anti-D immunoglobulin to be administered antenatally as well as postnatally in an attempt to reduce the risk of D immunization.

Discovery of the molecular basis of the D polymorphism created the possibility of predicting fetal D phenotype from fetal DNA derived from amniocytes obtained by amniocentesis. D phenotyping of the fetus of a D-negative woman with anti-D provides valuable information for predicting the risk of HDN and for subsequent management of the pregnancy. The first tests to be

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Submitted June 3, 1999; accepted September 2, 1999.

Supported by a grant from DiaMed AG, Cressier-sur-Morat, Switzerland.

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devised involved a polymerase chain reaction (PCR). The tests either determined the presence or absence of *RHD*-specific sequences in exon 10 or exon 7 or recognized the size differences between introns 4 of *RHD* and *RHCE*.¹³⁻¹⁶ These techniques were found to be unreliable.¹⁶ The absence of certain *RHD* regions in hybrid genes encoding partial D antigens may predict a D-negative phenotype, and the presence of some *RHD* regions in genes encoding no D antigen may predict a D-positive phenotype. In order to avoid these complications, methods where more than 1 region of *RHD* is detected in a single PCR were introduced.^{6,11,17}

Amniocentesis is a highly invasive procedure and not without risk to the fetus. It may also enhance the risk of maternal immunization. Methods have been described for predicting D phenotype from fetal DNA in maternal plasma. It has been proposed that these techniques could be used for testing all D-negative pregnant women to ascertain whether they require administration of antenatal anti-D immunoglobulin.^{18,19} Testing would reduce wastage of this valuable resource by avoiding anti-D immunoglobulin administration to women with a D-negative fetus.

All techniques for predicting D phenotype from DNA or mRNA are based on the dogma that, with only very rare exceptions, the D-negative phenotype results from the absence of all or most of the *RHD* gene. Most tests, however, have been performed on people of European origin, and the molecular basis of D polymorphism has not been adequately investigated in other races, particularly in people of African origin. We have tested for the presence of the *RHD* gene in Africans and African Americans and found that the majority of D-negative Africans and about a quarter of D-negative African Americans have an inactive *RHD* gene with a 37 base pair (bp) insert and a nonsense mutation; the remainder have either the *RHD-CE-D* gene or a complete deletion of the *RHD* gene.

Materials and methods

Blood samples

D-negative blood samples were obtained from black African blood donors from South Africa, Zimbabwe, and Ghana; from South African donors of mixed race, referred to as "Coloured" in South Africa; and from African American donors, selected on the basis of appearance, from Shreveport, Louisiana. Blood samples from D-positive black South Africans were derived from another study.²⁰ All blood samples were collected into a citrate-phosphate-dextrose (CPD) anticoagulant. Blood samples used for screening purposes were the by-product of blood donation and were collected according to the approved protocol of the transfusion center involved. Whole units of blood, for transcription analysis, were taken with informed consent.

Serological studies

All red cell samples were tested for D, C, c, E, e, G, VS, and Rh32 antigens. VS+ samples were also tested for V antigen. Serological tests were performed by the method most suited to the particular reagent: direct hemagglutination, with native or papain-treated cells, or indirect hemagglutination with antihuman IgG (BPL, Elstree, England). All D-negative red cells were tested by an antiglobulin test with 2 potent alloanti-D that are known to react with red cells of all partial D phenotypes. An acid-elution method (Elu-kit II, Gamma Biologicals, Houston, TX) was used for adsorption/elution tests with anti-D.

Molecular analyses on genomic DNA

Genomic DNA was isolated from mononuclear cells by a standard phenol-chloroform-isoamyl alcohol procedure or from whole blood with a DNA purification kit (Promega, Madison, WI). PCR primers are listed in

Table 1. The method used for *RHD* screening, the multiplex PCR described by Avent et al,⁶ detected the presence of *RHD* exon 10 and intron 4 and *RHCE* intron 4, as an internal control.

The method for screening for the 37 bp insert of *RHD* exon 4 was a PCR with a sense primer (RHDIN3F) in intron 3 and an *RHD*-specific antisense primer (RHD4R) in intron 4 (Table 1). Electrophoresis of amplification products in 1.5% agarose gel revealed a 381 bp product from wild type *RHD* and a 418 bp product from *RHD* with the 37 bp insert. As an internal control, primers RHEX2IN1F and RH2R amplified a 509 bp product across exon 2 of *RHD* and *RHCE* for all samples. Screening for the *RHD* exon 6 T807G nonsense mutation involved PCR with RH6F (sense primer) and a sequence-specific antisense primer, RHD ψ E \times 6R (Table 1).

The sequences of *RHD* exons 1, 3-7, and 10 were determined by direct sequencing of PCR products. Many of the primers used for exon amplification were described by Wagner et al²¹ (Table 1). Following agarose-gel electrophoresis, PCR products were cut out of the gel, purified (QIAEX II kit; QIAGEN, Dorking, England), and sequenced (373A DNA sequencer; Applied Biosystems, Warrington, England).

Screening for the presence of *RHD* exons 3, 4, 5, 6, 7, and 9 was performed in separate PCRs, with pairs of primers (Table 1) in which at least 1 had an *RHD*-specific sequence by the conditions employed. The test for an *RHD-CE* hybrid exon 3, characteristic of the D-negative, (*C*)*ce*^s (*r*⁵) complex, utilizes an *RHD*-specific primer to the 5' end of exon 3 and an *RHCE*-specific primer to the 3' end.²⁰ A test for identifying the C733G polymorphism in *RHCE* exon 5, which encodes a Leu245Val substitution in the RhCcEe protein responsible for the absence or presence of VS antigen, involved an *RHCE*-specific sense primer in intron 4 and an allele-specific antisense primer in exon 5 of *RHCE*.²⁰

Transcript analysis

Erythroblasts were cultured from a D-negative black South African with the *RHD* 37 bp insert. Mononuclear cells were separated from whole blood in CPD (Histopaque-1077 gradient; Sigma-Aldrich, Poole, England), and contaminating red cells were lysed in ammonium chloride. CD34⁺ cells were isolated (Mini-MACS columns; Miltenyi Biotech, Bisley, England) by 2 cycles of positive selection with anti-CD34 and immunomagnetic beads. The isolated cells were then cultured in the presence of fetal-calf serum (N.V. HyClone, Belgium), recombinant human (rH) erythropoietin (Boehringer Mannheim, Frankfurt, Germany), rH granulocyte-macrophage colony stimulating factor (R&D Systems, Abingdon, England), and rH interleukin-3 (IL-3) (R&D Systems), as described by Malik et al.²²

After 11 days, cells were harvested and analyzed for the presence of glycophorin A (GPA) and Rh protein. This was accomplished using flow cytometry with monoclonal antibodies BRIC 256 and BRIC 69 (IBGRL Research Products, Bristol, England). BRIC 256 detects an epitope on the extracellular domain of GPA, and BRIC 69 detects an extracellular epitope common to the RhCcEe and RhD polypeptides. About 80% of the cells expressed both Rh protein and GPA.

We isolated mRNA from 10⁶ cells (The PolyATtract System 1000 kit; Promega). First-strand cDNA for PCR amplification was prepared with oligo (dT)₁₆ as the primer and avian myeloblastosis virus reverse transcriptase (Pharmacia, Milton Keynes, England).²³ cDNA was amplified with the following primers: 5'-CTGAGGATGGTCATCAGTAA-3' (nt 457-476, exon 3 of *RHCE* and *RHD*) and 5'-GAGATCAGCCAGCCAC-3' (nt 914-898, exon 6 of *RHCE* and *RHD*). The 458 bp product was isolated by gel purification and sequenced. In addition, an *RHD*-specific PCR was performed with a sense primer in exon 3 (RHDEX3F, Table 1) and an antisense primer in exon 7 (5'-ACCCAGCAAGCTGAAGTTGTAGCC-3') to give a 645 bp product with *RHD* cDNA. As a control, an *RHCE*-specific PCR was carried out with a sense primer in exon 3 (5'-TCGGTGCTGATCT-CAGCGGG-3') and an antisense primer in exons 8 and 7 (5'-CAATCATGCCATTGCCGTTCCA-3') to give a 715 bp product with *RHCE* cDNA.

Reticulocyte RNA was extracted from a D-negative black South African by isolating CD71⁺ cells from 10 mL of phosphate buffered saline-washed

Table 1. Oligonucleotide primers for amplifying whole exons or for identifying the presence of exons

Exon	Primer Name	Direction	Specificity	Primer Position*	Sequence (5' to 3')
1	RH1F (rb13)	S	D + CE	Promoter -675 to -652	CTAGAGCCAAACCACATCTCCTT
	RH1R (re11d)	AS	D + CE	Intron 1 129 to 106	AGAAGATGGGGGAATCTTTTCTCT
	RHD1F (re01)	S	D	Promoter -149 to -132	ATAGAGAGGCCAGCACAA
	RHIN1R	AS	D + CE	Intron 1 84 to 64	TCTGTGCCCTGGAGAACCAC
2	RHEX2IN1F (re 13)	S	D + CE	Intron 1 -72 to -53	ACTCTAATTTTCATACCACCC
	RH2R (re23)	AS	D + CE	Intron 2 250 to 227	AAAGGATGCAGGAGGAATGTAGGC
3	RH3F (ra21)	S	D + CE	Intron 2 2823 to 2842	GTGCCACTTGACTTGGGACT
	RHDIN2F (rb20d)	S	D	Intron 2 -25 to -8	TCCTGGCTCTCCCTCTCT
	RH3R (rb21)	AS	D + CE	Intron 3 28 to 11	AGGTCCCTCCTCCAGCAC
	RHDEX3F	S	D	Exon 3 364 to 383	TCGGTGTCTGATCTCAGTGGGA
	RHDEX3R	AS	D	Exon 3 474 to 455	ACTGATGACCATCTCAGGT
4	RHDIN3F	S	D	Intron 3 -36 to -16	GCCGACTCACTGCTCTTAC
	RHD4R (rb12)	AS	D	Intron 4 197 to 174	TCCTGAACCTGCTCTGTGAAGTGC
	RHDEX4F	S	D	Exon 4 582 to 602	AACGGAGGATAAAGATCAGAC
	RHDIN4R	AS	D	Intron 4 193 to 173	GAACCTGCTCTGTGAAGTGCCT
5	RHD5F (rb11)	S	D	Intron 4 -267 to -243	TACCTTTGAATTAGCACTTCACAG
	RHIN5R	AS	D + CE	Intron 5 73 to 56	GTGGGGAGGGGCATAAAT
	RHDMULTI5F	S	D	Exon 5 648 to 667	GTGGATGTTCTGGCCAAAGT
	RHDMULTI5R	AS	D	Intron 6 16 to exon 5 799	GCACGCCCTGCTCACCTT
6	RH6F (rf51)	S	D + CE	Intron 5 -332 to -314	CAAAAACCCATTCTTCCCG
	RHDIN6R	AS	D	Intron 6 41 to 21	CTTCAGCCAAAGCAGAGGAGG
	RHEX6F	S	D + CE	Exon 6 898 to 916	GTGGCTGGGCTGATCTACG
	RHDEX6R	AS	D	Intron 7 15 to exon 6 932	TGTCTAGTTTCTTACCGCCAAGT
	RHD Ψ EX6R	AS	D Ψ	Exon 6 824 to 807	AACACCGCACTGTGCTCC
7	RHDIN6F (re621)	S	D	Intron 6 -102 to -85	CATCCCCCTTTGGTGGCC
	RHDIN7R (re75)	AS	D	Intron 7 168 to 151	AAGGTAGGGGCTGGACAG
	RHEX7F2	S	D + CE	Exon 7 949 to 967	AACCGAGTGTGGGGATTC
9	RHDEX7R	AS	D	Exon 7 1059 to intron 8 7	ACCCACATGCCATTGCCGGCT
	RHD9F	S	D	Exon 9 1154 to 1170	AACAGGTTTGCTCCTAAATCTT
10	RHEX9R	AS	D	Exon 9 1219 to 1193	AAACTTGGTCATCAAAATATTAACTT
	RH10F (re91)	S	D + CE	Intron 9 -67 to -45	CAAGAGATCAAGCCAAATCAGT
	RHD10R (rr4)	AS	D	3' UTR 1541 to 1522	AGCTTACTGGATGACCACCA

*Indicates that numbers for exon positions represent nucleotide position in cDNA. Numbers for introns are relative to adjacent exons. Primer names in parentheses are as described by Wagner et al.²¹ Introduced mismatch in RHD Ψ EX6R is underlined.

peripheral blood using anti-CD71 magnetic beads (Dynal, Wirral, England). Total RNA was extracted with an RNA extraction kit (SV total RNA, Promega), and first-strand cDNA was then synthesized. *RHD* transcripts were analyzed by the amplification of 2 overlapping cDNA fragments, 1 encompassing exons 1-7 and the other exons 7-10. PCR primers were exons 1-7, 5'-TCCCCATCATAGTCCCTCTG-3' and RHDIN7R (Table 1), and exons 7-10, 5'-TGGTGCTTGATACCGCGGAG-3' and 5'-AGTGCATAATAAATGGTGAG-3'. PCR conditions were as follows: denaturation at 94°C for 1 minute; annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes (Expand™ RT-PCR kit; Boehringer, Lewes, Sussex, England) for 30 cycles with 2.5 mmol/L Mg²⁺ (final concentration) supplied with the kit. A final 30-minute incubation at 72°C was performed to maximize the

d(A) tailing of the PCR products, which were cloned into the pCRII vector (Invitrogen, Groningen, The Netherlands) and sequenced on both strands.

A new multiplex PCR-based screening test for fetal D typing

A 10-primer multiplex PCR-based method has been developed for determining the presence of *RHD* exon 7 and intron 4, the 37 bp insert in *RHD Ψ* exon 4, and the *RHCE* C and c alleles. The primers and their concentrations are shown in Table 2. Thirty cycles of PCR were performed at 94°C for 1 minute, 65°C for 1 minute, and 72°C for 3 minutes 30 seconds.

Table 2. Multiplex PCR test primers for predicting Rh D and C/c phenotype and the presence of the *RHD* pseudogene

Primer Name	Sequence (5' to 3')	Concentration (ng/ μ L)	Product Size (bp)
Exon7/for	AGCTCCATCATGGGCTACAA	1	95
Exon 7/rev	ATTGCCGGCTCCGACGGTATC	1	
Intron 3/for 1	GGGTGGGCTGGTAAAGCTCT	1	498 or 535
Intron 4/rev	GAACCTGCTCTGTGAAGTGTCT	4	
Intron 3/for 2	AACCTGGGAGGCAAATGTT	10	250
Exon 4 insert/rev	AATAAAACCCAGTAAGTTCATGTGG	10	
C/for	CAGGGCCACCACATTGAA	12	320
C/rev	GAACATGCCACTTCACTCCAG	1.5	
c/for	TCGGCCAAGATCTGACCG	7	177
c/rev	TGATGACCACCTTCCCAGG	7	

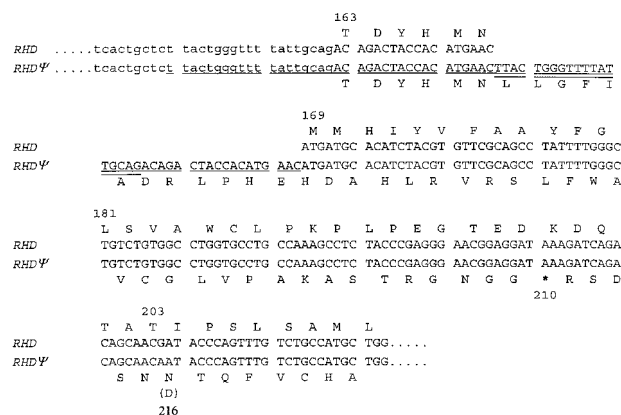


Figure 1. Nucleotide and encoded amino acid sequence for the 3' end of intron 3 and the 5' end of exon 4 of *RHD* and *RHDψ*. The region of the 37 bp duplication is underlined (single and double). The putative exon sequence derived from intron 3 is double-underlined. * Indicates a translation-termination codon.

Results

Identification of an *RHD* pseudogene in D-negative Africans

An *RHD* gene was found in some D-negative Africans. Sequencing of exon 4 of this apparently inactive *RHD* revealed a 37 bp insert (Figure 1). This insert appears to introduce a reading frameshift and a potential translation-termination codon at codon 210. The 37 bp insert is a duplication of a sequence spanning the intron 3–exon 4 boundary (Figure 1). Sequencing of exons 1, 3-7, and 10 of the inactive *RHD* revealed additional changes from the common *RHD* sequence. In exon 4, there is a G to A point mutation at nucleotide 609 (original position). Normally, this would not result in an amino acid change, but the 37 bp insertion and subsequent frameshift gives rise to an aspartic acid to asparagine substitution at codon 203 (original reading frame) or codon 216 (new reading frame) (Figure 1). In exon 5, 3 missense mutations (G654C, T667G, C674T) were

detected, encoding Met218Ile, Phe223Val, and Ser225Phe substitutions. In exon 6, a T807G transversion changes the codon for Tyr269 (TAT) to a translation-termination codon (TAG). PCR with a sequence-specific primer revealed that all individuals with the *RHD* 37 bp insert also had the T807G nonsense mutation. The inactive *RHD* gene will be referred to here as *RHDψ*.

Two samples from D+ C- c+ E- e+ Africans were sequenced as controls. Both had the wild type sequence in exon 4, except 1 had a C602G transversion encoding Thr201Arg. One had the wild type sequence in exon 5, the other had the Phe223Val change. Neither of the African control samples had the T807G nonsense mutation, but 1 had a G819A silent mutation (codon for Ala273). Thr201Arg, Phe223Val, and G819A are associated with the weak D phenotype called weak D type 4 by Wagner et al.²¹

Analysis of Rh mRNA transcripts from Africans with *RHDψ*

mRNA transcripts were isolated from D-negative donors with *RHDψ* using either whole blood or erythroblasts cultured from CD34+ cells. An *RHD*-specific PCR gave a 645 bp product with cDNA prepared from a D-positive control, but the PCR did not give a product with cDNA prepared from a D-negative with *RHDψ*. An *RHCE*-specific PCR gave 715 bp products with all samples. We did not find a clone with an *RHD* insert, and an *RHDψ* sequence was not detected. These results suggest that there is no transcript from *RHDψ*.

Screening D-negative donors for *RHD* exon 10 and intron 4 and for the *RHDψ* 37 bp insert

All D-negative donors were tested by the multiplex PCR method designed to determine the presence of *RHD* exon 10 and intron 4. Intron 4 of *RHCE* was always detected as a control for successful amplification. Three patterns of reaction were apparent: presence of both *RHD* regions; absence of both *RHD* regions; and presence of *RHD* exon 10, but absence of *RHD* intron 4. Frequencies of these

Table 3. Results of testing for presence or absence of *RHD* intron 4 and exon 10 and for the *RHD* exon 4 37 bp insert

D Phenotype	Population	Number	%	Intron 4	Exon 10	Exon 4		
						Normal*	Insert	
Negative	Black South African	29	20	69	+	+	-	+
			6	21	-	+	-	-
			3	10	-	-	-	-
Negative	Zimbabwean	19	13	68	+	+	-	+
			2	11	-	+	-	-
			4	21	-	-	-	-
Negative	Ghanaian	34	21	62	+	+	-	+
			4	12	-	+	-	-
			8	23	-	-	-	-
Negative	African American	54	13	24	+	+	-	+
			12	22	-	+	nt	nt
			29	54	-	-	nt	nt
Negative	Mixed-race South African	41	7	17	+	+	-	+
			1	2	-	+	nt	nt
			33	81	-	-	nt	nt
Positive	Black South African	95	83	87	+	+	+	-
			12	13	+	+	+	+
Positive	Caucasian	13	13	100	+	+	+	-

*Indicates that the 37 bp insert was not detected. The *RHCE* intron 4 was detected in all samples.

patterns are shown in Table 3 for each of the populations tested. Of the 82 D-negative black Africans tested (from South Africa, Zimbabwe, and Ghana), 67% had both regions of *RHD*, 15% had *RHD* exon 10, and only 18% lacked *RHD*. The frequencies of the 3 genotypes were different in African Americans and mixed-race South Africans, with a substantially higher proportion having neither region of *RHD* (Table 3).

Of 75 D-negative samples from people of African origin with *RHD* (including African Americans and mixed-race South Africans), 74 had the exon 4 37 bp insert (Table 3, Figure 2). None of these 74 had normal *RHD* exon 4. These individuals must be homozygous for *RHD* ψ , hemizygous for *RHD* ψ , or heterozygous for *RHD* ψ and the grossly abnormal *RHD-CE-D^s* gene associated with VS+ V- phenotype. Further investigation of the Ghanaian sample with *RHD* exon 4 (without a 37 bp insert), intron 4, and exon 10 has not been possible.

PCR-based tests on selected donors with sequence-specific primers revealed that those donors with *RHD* exon 10 and intron 4 also had *RHD* exons 3, 4, 5, 6, 7, and 9, suggesting the presence of grossly intact *RHD*. Red cells of all donors with *RHD* exon 10, but without *RHD* intron 4, were C+ and VS+; almost all were also V-. In addition to *RHD* exon 10, donors of this type had *RHD* exon 9 and a hybrid exon 3 comprising a 5' end derived from *RHD* and a 3' end derived from *RHCE*. This suggests that these donors have the *RHD-CE-D* gene associated with the (C)*ce^s* (*r³⁵*) complex.¹⁰ This gene will be referred to as *RHD-CE-D^s*. Selected donors with neither exon 10 nor intron 4 of *RHD* also lacked *RHD* exons 3, 4, 5, 6, 7, and 9, suggesting homozygosity for an *RHD* deletion.

Fifty-two of the 74 D-negative red cell samples with *RHD* (and with the 37 bp insert) in the 5 populations were D- C- c+ E- e+ VS- (probable genotype *ce/ce*). Of the remaining 22, 11 were D- C- c+ E- e+ VS+ V+, and 11 were D- C+ c+ E- e+ VS+ V-. These 22 were all heterozygous for C733G in exon 5 of *RHCE* and so had probable genotypes of *ce/ce^s* and *ce/(C)*ce^s**, respectively.²⁰

Screening for *RHD* ψ in random black South African blood donors

Ninety-eight random samples from black South Africans, consisting of 95 D-positive samples and 3 D-negative samples, were tested for *RHD* ψ . Of the 98, 14 had *RHD* ψ . This gives an approximate *RHD* ψ gene frequency of 0.0714. This figure is based on the assumption that 1 of the D-negative donors is hemizygous, not homozygous, for *RHD* ψ . The other randomly selected D-negative donor with *RHD* ψ is VS+ V- with the

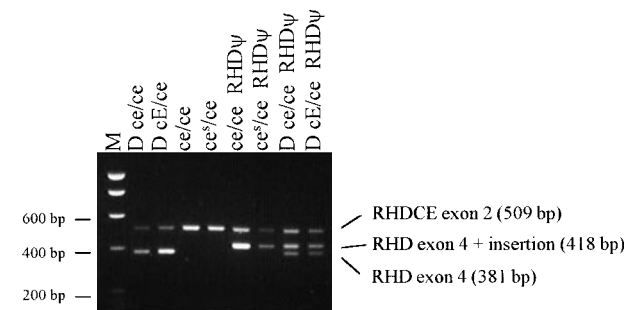


Figure 2. Agarose gel showing results of PCR screening test for the 37 bp *RHD* exon 4 insert with samples from black South Africans.

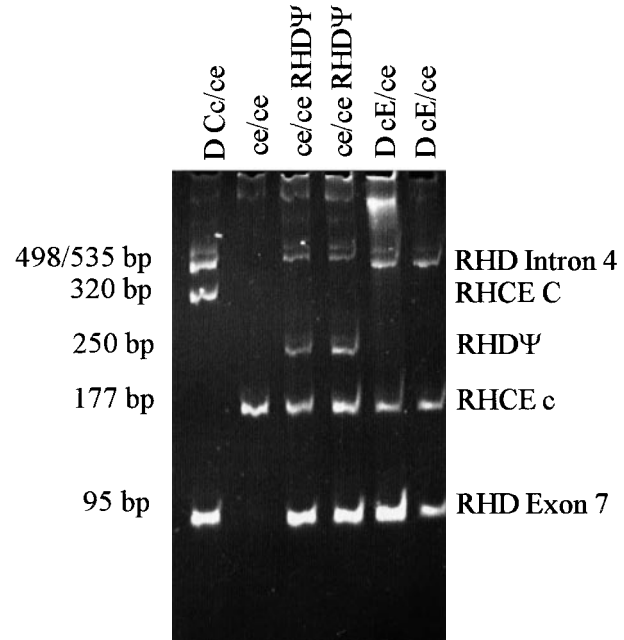


Figure 3. Polyacrylamide gel showing results with multiplex PCR method for predicting D and C/c phenotype and for detecting the presence of *RHD* ψ .

RHD-CE-D^s gene, and the donor is assumed to be heterozygous for *RHD* ψ .

Serological confirmation that the donors tested were D-negative

The donors used in this study were random D-negatives, as determined by the routine methods of the relevant transfusion services. In addition, their red cells gave negative results, by an antiglobulin test, with 2 alloanti-D reagents that react with all known partial D antigens and weak D antigens (with the exception of the extremely weak form of D known as D_{el}). Several red cell samples from donors with *RHD* ψ were tested with alloanti-D by an adsorption and elution technique that would be expected to detect a very weak D antigen. No anti-D could be detected in the eluate.

Of 5 African women who had produced anti-D, 4 had *RHD* ψ , and the other had no *RHD*. This provides further evidence that these Africans with *RHD* ψ are truly D-negative. One of the anti-D antibodies from a D-negative Ghanaian woman with *RHD* ψ caused HDN, which required exchange transfusion.

A pregnant black English woman with anti-D had *RHD* ψ . Testing of DNA derived from amniocytes obtained by amniocentesis showed that her fetus had *RHD* ψ plus *RHD* with a normal exon 4. The fetus is, therefore, almost certainly D-positive and at risk from HDN.

A new PCR-based test for predicting D phenotype from genomic DNA

We have developed a new multiplex PCR-based test for detecting *RHD*, *RHD* ψ , and the C and c alleles of *RHCE*. Examples of the results are shown in Figure 3. *RHD* and *RHD* ψ give rise to 498 bp and 535 bp products, respectively, from *RHD* intron 4 exon 4. Because of the small difference in size between these 2 products, primers specific for the 37 bp insert in *RHD* ψ were incorporated, and they gave rise to a 250 bp product when *RHD* ψ was present.

Both genes produce a 95 bp product from *RHD* exon 7. In addition, primers recognizing a *C*-specific sequence in *RHCE* intron 2 amplify a 320 bp product when a *C* allele is present; primers recognizing a *c*-specific sequence in *RHCE* exon 2 amplify a 177 bp product when a *c* allele is present.

Discussion

The D-negative phenotype of the Rh blood group system is generally considered to result, almost invariably, from homozygosity for a deletion of the D gene, *RHD*. Although this is true in Caucasians, it is certainly not the case in Africans. We have found that about two-thirds of D-negative Africans have an inactive *RHD* gene. This pseudogene (*RHD* ψ) has a 37 bp insert in exon 4, which may introduce a reading frameshift and premature termination of translation and a translation stop codon in exon 6. Of the remaining one-third of African D-negative donors, about half appear to be homozygous for an *RHD* deletion and about half have the *RHD-CE-D^s* hybrid gene characteristic of the (*C*)*ce^s* haplotype that produces c, VS, and abnormal C and e, but no D. In D-negative African Americans and South African people of mixed race (Coloured), the same 3 genetic backgrounds are present, but 24% of African Americans and 17% of South African donors of mixed race have *RHD* ψ , and 54% of African Americans and 81% of South African donors of mixed race have no *RHD*.

The most common D-negative Rh haplotype in Africans is *RHD* ψ with the *ce* allele of *RHCE*, although *RHD* ψ might also be occasionally associated with *ce^s*. The 37 bp insert in exon 4 of *RHD* ψ is a duplication of a sequence spanning the boundary of intron 3 and exon 4. This insert may introduce a reading frame shift and a translation stop codon at position 210. However, the duplication introduces another potential splice site at the 3' end of the inserted intronic sequence in exon 4 (shown double-underlined in Figure 1). If splicing occurred at this second splice site, the sequence of exon 4 would not be changed and could be translated normally. No RhD protein would be expected, however, because another stop codon is present in exon 6 of the gene. *RHD* mRNA was not detected in D-negative individuals with *RHD* ψ , despite the presence of *RHCE* transcripts. mRNA transcripts containing premature termination codons are often rapidly degraded as a result of nonsense-mediated mRNA decay.²⁴

The initial report of the molecular basis for the Rh D-negative phenotype⁵ was followed by the publication of a number of PCR-based tests for predicting D phenotype from genomic DNA.²⁵ Such techniques have proved valuable for predicting the D phenotype of fetuses of women with anti-D antibodies in order to assist in assessing the likelihood of HDN and in managing the pregnancy. Fetal DNA is usually derived from amniocytes obtained by amniocentesis or from chorionic villus samples. Antenatal administration of prophylactic anti-D immunoglobulin at around 28 weeks of gestation to all pregnant D-negative women is an enormous drain on stocks of anti-D immunoglobulin. About 40% of the fetuses of D-negative Caucasian women would be expected to be D-negative, yet these women still receive antenatal immunoglobulin prophylaxis. Less invasive PCR-based tests using maternal plasma are currently being developed, and they may make it possible to test the fetal D phenotype of all D-negative pregnant women.^{18,19,26}

All existing methods for predicting D phenotype from genomic

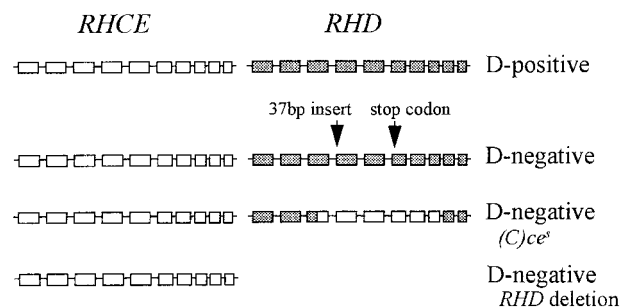


Figure 4. Representation of the genomic organization of the D-positive haplotype and 3 D-negative haplotypes in Africans. □ indicates *RHCE* exon; ■, *RHD* exon.

DNA or mRNA are based on detecting the presence or absence of various regions of *RHD* and are unsuitable for testing African populations or any populations containing a substantial proportion of people with African ethnicity. We have devised a new test that detects the presence of *RHD* ψ . This test incorporates a primer that specifically detects the presence of the 37 bp insert in *RHD* ψ . In addition, the presence or absence of exon 7 and intron 4 of *RHD* and *RHD* ψ are detected. This test would give either an unusual result or a D-positive result with genes encoding partial D antigens. The multiplex PCR test also includes primers that determine the presence of *C* and *c* alleles of *RHCE*. This will be particularly informative because the D-negative phenotype is not usually associated with *C* expression. Most inactive *RHD* genes, with the exception of *RHD* ψ , are in *cis* with a *C* allele of *RHCE*. Typing the fetus for the *C* allele is also valuable because anti-G may be responsible for HDN.²⁷

The Rh proteins exist in the red cell membrane as part of a complex of proteins and glycoproteins. In most people this complex comprises RhCcEe polypeptide, RhD polypeptide, and the Rh-associated glycoprotein. This complex may also be associated, in the membrane, with LW glycoprotein and CD47 and possibly with Duffy glycoprotein and glycophorin B.²⁸ In D-negatives, the RhD polypeptide is not present in the Rh membrane complex. There are 3 common genetic mechanisms responsible for the D-negative phenotype: deletion of *RHD*, a pseudogene *RHD* containing a 37 bp insert and 1 or 2 stop codons, and a hybrid *RHD-CE-D^s* gene that probably produces an abnormal C antigen but does not produce a D antigen (Figure 4). Functions of the Rh proteins are not known, so it is difficult to speculate on the evolutionary significance of the D-negative phenotype and, therefore, why 3 different mechanisms have evolved to create this phenotype. D-negative fetuses have an advantage in D-negative mothers as they are not destroyed by maternal anti-D. Consequently, HDN may provide a selection pressure in favor of inactive *RHD*, which has driven the evolution of the D polymorphism with different genetic backgrounds in different ethnic groups. It is unlikely, however, that HDN alone has provided the selective pressure responsible for the high prevalence of D negativity.

Acknowledgment

We are grateful to the Western Province Blood Transfusion Service for obtaining blood samples from mixed-race donors in South Africa.

References

- Daniels GL. Human Blood Groups. Oxford, England: Blackwell Science; 1995.
- Issitt PD, Anstee DJ. Applied Blood Group Serology. 4th ed. Durham, NC: Montgomery Scientific Publications; 1998.
- Huang CH. Molecular insights into the Rh protein family and associated antigens. *Curr Opin Hematol*. 1997;4:94-103.
- Cartron JP, Bailly P, Le Van Kim C. Insights into the structure and function of membrane polypeptides carrying blood group antigens. *Vox Sang*. 1998;74(suppl 2):29-64.
- Colin Y, Chérif-Zahar B, Le Van Kim C, Raynal V, Van Huffel V, Cartron JP. Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. *Blood*. 1991;78:2747-2752.
- Avent ND, Martin PG, Armstrong-Fisher SS, et al. Evidence of genetic diversity underlying Rh D⁻, weak D (D^u), and partial D phenotypes as determined by multiplex polymerase chain reaction analysis of the *RHD* gene. *Blood*. 1997;89:2568-2577.
- Andrews KT, Wolter LC, Saul A, Hyland CA. The RhD⁻ trait in a white patient with the RhCCee phenotype attributed to a four-nucleotide deletion in the *RHD* gene. *Blood*. 1998;92:1839-1840.
- Huang CH. Alteration of *RH* gene structure and expression in human dCCee and Dc^w- red blood cells: phenotypic homozygosity versus genotypic heterozygosity. *Blood*. 1996;88:2326-2333.
- Faas BH, Beckers EA, Simsek S, et al. Involvement of Ser103 of the Rh polypeptides in G epitope formation. *Transfusion*. 1996;36:506-511.
- Faas BH, Beckers EA, Wildoer P, et al. Molecular background of VS and weak C expression in blacks. *Transfusion*. 1997;37:38-44.
- Okuda H, Kawano M, Iwamoto S, et al. The *RHD* gene is highly detectable in RhD-negative Japanese donors. *J Clin Invest*. 1997;100:373-379.
- Mollison PL, Engelfriet CP, Contreras M. *Blood Transfusion in Clinical Medicine*. 10th ed. Oxford, England: Blackwell Science; 1997.
- Bennett PR, Le Van Kim C, Colin Y, et al. Prenatal determination of fetal RhD type by DNA amplification. *New Engl J Med*. 1993;329:607-610.
- Wolter LC, Hyland CA, Saul A. Rhesus D genotyping using polymerase chain reaction. *Blood*. 1993;82:1682-1683.
- Arce MA, Thompson ES, Wagner S, Coyne KE, Ferdman BA, Lublin DM. Molecular cloning of RhD cDNA derived from a gene present in RhD-positive, but not RhD-negative individuals. *Blood*. 1993;82:651-655.
- Simsek S, Faas BH, Bleeker PM, et al. Rapid Rh D genotyping by polymerase chain reaction-based amplification of DNA. *Blood*. 1995;85:2975-2980.
- Maaskant-van Wijk PA, Faas BH, de Ruijter JA, et al. Genotyping of *RHD* by multiplex polymerase chain reaction analysis of six *RHD*-specific exons. *Transfusion*. 1998;38:1015-1021.
- Faas BH, Beuling EA, Christiaens GC, von dem Borne AE, van der Schoot CE. Detection of fetal *RHD*-specific sequences in maternal plasma. *Lancet*. 1998;352:1196.
- Lo YMD, Hjelm NM, Fidler C, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *New Engl J Med*. 1998;339:1734-1738.
- Daniels GL, Faas BH, Green CA, et al. The Rh VS and V blood group polymorphisms in Africans: a serological and molecular analysis. *Transfusion*. 1998;38:951-958.
- Wagner FF, Gassner C, Müller TH, Schönitzer D, Schunter F, Flegel WA. Molecular basis of weak D phenotypes. *Blood*. 1999;93:385-393.
- Malik P, Fisher TC, Barsky LLW, et al. An in vitro model of human red blood cell production from hematopoietic progenitor cells. *Blood*. 1998;91:2664-2671.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: A laboratory manual*. 2nd ed. Plainview, NY: Cold Spring Harbor Press; 1989.
- Ruiz-Echevarria MJ, Czaplinski K, Peltz SW. Making sense of nonsense in yeast. *Trends Biochem Sci*. 1996;21:433-438.
- Flegel WA, Wagner FF, Müller TH, Gassner C. Rh phenotype prediction by DNA typing and its application to practice. *Transfus Med*. 1998;8:281-302.
- Hamlington J, Cunningham J, Mason G, Mueller R, Miller D. Prenatal detection of rhesus D genotype. *Lancet*. 1997;349:540.
- Hadley AG, Poole GD, Poole J, Anderson NA, Robson M. Haemolytic disease of the newborn due to anti-G. *Vox Sang*. 1996;71:108-112.
- Anstee DJ, Cartron J-P. Towards an understanding of the red cell surface. In: Garratty G, ed. *Applications of Molecular Biology to Blood Transfusion Medicine*. Bethesda, MD: American Association of Blood Banks; 1997:17.