Three Unrelated Rh D Gene Polymorphisms Identified Among Blood Donors With Rhesus CCee (r’r’) Phenotypes

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Human red blood cells are traditionally typed as Rhesus (Rh)-positive or -negative depending on the presence or absence of the Rh D antigen. A recent report demonstrated that the Rh D gene is completely absent in Rh D-negative individuals. In this study, Rh D-negative blood donors with ccee (n = 25) and CCee (n = 3) phenotypes were examined for the presence or absence of the D gene. Polymerase chain reaction (PCR) probes that hybridize to the 5' and 3' regions of the Rh CcEe gene and the closely related D gene were used in a Southern analysis. The D gene was absent in all ccee phenotypes examined. The CCee phenotypes showed three Rh D polymorphisms: one donor lacked the D gene, one donor had a partial deletion on one D gene at the 3' region, and the remaining donor appeared to have one normal D gene within the intron/exon regions examined. We conclude that, while the D gene may be absent in the majority of Rh D-negative phenotypes, rarer polymorphisms also occur that prevent expression of the D antigen resulting in the Rh D-negative phenotype.

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The Human Rhesus (Rh) Blood Group is of Clinical Importance Because of Its Association With Hemolytic Disease of the Newborn, Incompatible Transfusion Reactions, and Autoimmune Hemolytic Anemias. Historically, the Rh antigens, particularly D, have served as informative markers for studying the evolution of human populations. The Rh D is the most important clinically because of its high immunogenicity. Red blood cells are traditionally classified as Rh-positive or -negative depending on the presence or absence of the D antigen. The existence of an alternative allele to D, designated d, was postulated to exist in a manner analogous to the Rh Cc and Ee alleles, but a hypothetical d antigen has never been identified. A Southern analysis by Colin et al. in 1991 showed that the D gene is absent in D-negative individuals, thereby providing an explanation for the inability to identify the postulated d antigen.

The Rh D antigen is associated with an integral membrane protein, M, 28,000 to 32,000, for which the corresponding gene has been cloned. This gene shows a high degree of homology with the cloned Rh CcEe gene, with only a 3.5% and 8.4% divergence at the nucleotide and amino acid level, respectively, and both genes encode a 417-amino acid protein. The Rh CcEe gene organization contains 10 exons, and differential splicing at intron/exon boundaries can produce different mRNA isoforms, which may give rise to different peptides carrying the C/c and E/e polymorphisms. It has been well established from family studies that the D and CcEe genes are closely linked, and the cloned Rh d cDNA has been used to confirm the location of the Rh locus on chromosome 1. The two genes were possibly derived by duplication of an ancestral gene.

We have previously reported the use of two Rh probes to the 5' and 3' regions of the CcEe gene in a Southern analysis to identify restriction fragment length polymorphisms (RFLPs) associated with the Cc and Ee alleles, respectively. These probes also hybridized to the closely related D gene and allowed a D gene dosage measurement by comparison of the relative intensity of the D gene fragments to the CcEe fragments. In this present study, the two probes were used to screen DNA from Rh ccee and CCee phenotypes to determine whether the D gene is invariably absent in Rh D-negative individuals.

Materials and Methods

DNA isolation. Genomic DNA was isolated from peripheral blood leukocytes of citrate-phosphate-dextrose (CPD)-anticoagulated blood as described by Miller et al.

Sample selection. Rh D-negative volunteer blood donors were selected from Red Cross Blood Transfusion Service Rh typing panels. The Rh ccee phenotypes (n = 25) were selected at random from the data base at the Brisbane Red Cross Blood Bank. One Rh CCee phenotype was available from the Brisbane Red Cross Blood Bank panel and two from the Sydney Red Cross Blood Bank panel. They are designated as B1, S1, and S2. These have been regular blood donors for up to 17 years. Their blood donations are used routinely for a Rh CCee phenotype D-negative patient who suffers from sickle cell anemia.

Preparation of Rh DNA probes. The 5' and 3' regions of the Rh CcEe gene were amplified from human genomic DNA and cDNA, respectively, using polymerase chain reaction (PCR) techniques described elsewhere. Briefly, using an inverted PCR technique, the 5' region was amplified from genomic DNA that had been digested with Sau3AI and ligated. The PCR reactions for inverted PCR use primers in reverse orientation to standard PCR. Antisense primer was ggagaattcaGGCAGGCCGCAGAGA and corresponds to position 37 to 22 on the published cDNA sequence counting from the initiator codon sequence. Sense primer was ggagaattcaGGCCCTAAACATGGA at position 47 to 62. The PCR product extended 373 nucleotides upstream of the initiation ATG codon and 117 nucleotides downstream into the first exon (GenBank accession no. M84983). The sequence was identical to published sequences for the Rh CcEe and D gene within the coding region and the first 44 and 30 bases upstream from the ATG codon, respectively. It is not yet known whether the remaining upstream region is common to both the CcEe and D. The 3' probe was prepared by extracting total RNA from bone marrow tissue and transcribing mRNA to cDNA using antisense primer gattgagtccccggttttgcccTAAAAATCACCC, which spans bases 1266 to 1243 at the 3' termini.

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nal of the cloned Rh gene. The PCR on the cDNA was performed with the same antisense primer and a sense primer, gattcgaactTGG-
CCATCGTATAGCTCTCACG, spanning bases 1115 to 1137. This was followed by a heminested PCR reaction containing the same sense primer, but a new antisense primer, gggaattctgAACAG-
CCATGGAGGA spanning bases 1245 to 1229. The final PCR product from the 3' region lies between nucleotides 1115 and 1245 counting from the initiation ATG codon on the Rh CcEe gene. The sequence for the 3' probe is identical to that for the published CcEe gene and differs by two bases only from the D gene. The locations of the 5' and 3' probes on the gene are shown in Fig 1. These PCR products (100 ng) were labeled with (α-32P)CTP in 10 PCR cycles and used on the same day in a single Southern analysis that incorporated both probes.

Southern analysis. Human genomic DNA (3 μg) was digested with the restriction enzyme Msp I (10 U/μg), precipitated with ethanol, washed, dried, and resuspended in water. DNA fragments were separated by electrophoresis on a 1.0% agarose gel (Seakern; FMC Bioproducts, Rockland, ME) at 30 V for 20 hours. HindIII-digested lambda-phage and HaeIII-digested products served as size markers. DNA was transferred onto Zeta probe GT membrane (Bio
dynamics, Sunnyvale, CA) at 30 V for 20 hours. HindIII-digested lambda-phage and HaeIII-digested DNA was transferred onto Zeta probe GT membrane (Bio
dynamics, Sunnyvale, CA). Images were optimised, density profiles plotted, and relative intensities of peaks within each profile determined by volume of area integration using ImageQuant software (Molecular Dynamics).

RESULTS

We have previously shown that the 5' and 3' probes can be used on a Southern analysis to identify DNA fragments that associate with the Rh C, c, D, E and e antigens. Figure 2, lanes 1 and 5, indicates the position of Rh gene fragments for genomic DNA that were prepared from two standard CcDee and CCDeEe genotypes, designated C1 and C3, and which were cut with the restriction enzyme Msp I. From the top, the 3' probe hybridizes to a constant CcEe gene fragment at 2.8 kb; the 5' probe hybridizes to a C band of 2.6 kb and/or a c band of 2.5 kb and to two D bands of 2.45 and 2.2 kb; the 3' probe hybridizes to the lower D band of 2.0 kb, and the e band of 1.8 kb; the 5' probe hybridizes to the bottom 1.1 kb constant CcEe band. The Rh D gene, if present, exists in one or two copies in contrast to the Rh CcEe gene, which will, almost invariably, be present in two copies. As the two genes are closely related, the latter gene can be used as a control to measure the D gene dosage. For example, for any one sample, the intensity of the 2.2-kb D band, hybridizing to the 5' probe, is compared with that for the constant 1.1-kb band, which also hybridizes to the 5' probe. As a double check, from among bands identified by the 3' probe, the 2.0-kb D band is compared with the 1.8-kb e band.

All Rh D-negative ccee phenotypes (n = 25) tested by this Southern analysis lacked the designated D gene fragments. One example of a ccee sample, designated C2, is shown in Fig 2, lane 4, which shows that the three D gene fragments and the gene fragment for the C allele are absent.

When the three Rh D-negative CCDeEe phenotypes were analyzed, only one (S1) gave the expected pattern, since it lacked all D fragments and showed the expected C band at 2.6 kb (Fig 2, lane 2). The second Rh D-negative CCDeE sample (S2) is shown in Fig 2, lane 3. The C gene fragment was again present, but, the 5' probe also hybridized to the 2.45- and 2.2-kb D gene fragments. In contrast to the 5' probe, the 3' probe did not hybridize to any D gene fragment. The lack of the 2.0-kb D gene fragment from the 3' region is readily seen by comparing lane 3 with the control CcDeEe (C1) phenotype in Fig 2, lane 1.

When both the c and D genes were present, the 2.5-kb c fragment merged with the top 2.45-kb D fragment. However, for S2 the relative intensity of the C band to this top D fragment indicated that two C genes were present, and c was therefore absent as expected for a CCDeE phenotype (compare with control CcDeEe phenotype in lane 1). The D gene dosage for S2 was determined by comparing the intensity of the middle 2.2-kb D band with the constant 1.1-kb fragment at the bottom of the gel. These comparisons gave similar values to the CcDeEe control (lane 1) and showed that the 3' probe hybridizes to one D gene.

![Diagram](https://www.bloodjournal.org)

Fig 1. Genomic organization of the Rh CcEe gene and location of Rh probes prepared by PCR amplification of 5' and 3' regions. Position of exon/intron boundaries taken from published data. The 5' probe had a length of 490 bp. The 3' probe had a length of 131 bp. Boxed regions represent exons and the lines represent introns. Numbers represent nucleotide positions. There is an Msp I recognition site at nucleotide 27, which results in the 5' probe hybridizing to two gene fragments from both the D and CcEe genes in Southern analysis using Msp I-restricted DNA.
THREE RFLP PATTERNS AMONG THREE ρ′′ DONORS

Fig 2. RFLP analysis of Rh D-negative phenotypes by Southern analysis. A 3-μg sample of human genomic DNA was cut with restriction enzyme Msp I, the DNA electrophoresed on a 1% agarose gel at 30 V for 20 hours, and transferred onto Zeta probe GT membrane using 0.4 mol/L NaOH as transfer buffer. Membranes were washed in 2× SSPE and prehybridized for 1 hour in Church and Gilbert hybridization solution. The radiolabeled [α-32P]dCTP probes to the 5' and 3' regions of the Rh CcEe and D genes were then added to the membrane and hybridized for 16 hours at 65°C. Membranes were washed twice with SSPE for 5 minutes and exposed for 2 days on a phosphor screen. HindIII-digested lambda-phage and HindIII-digested φX174 DNA served as size markers.

Rh phenotypes in lanes 1 to 6 are, respectively: C1, CcDee; S1, CCee; S2, CCee; S2, ccee; C2, ccee; C3, CcDee; and B1, CCee. For C3, lane 5, a double D gene dose was predicted from phenotype and observed on genotype. Samples C3 and B1 were run on a separate gel from the other samples. The shift in mobility observed for the 1.1-kb band is a technical artifact.

The third Rh D-negative CCee sample, B1, showed a different pattern again. For this sample, both the 5' and 3' probes identified all three designated D gene fragments (Fig 2, lane 6). The intensity ratio between the 2.2-kb D and 1.1-kb CcEe gene fragments hybridizing to the 5' probe was 0.6, which compared with 1.1 for the control CCDee genotype, C3, lane 5. Similarly, the ratio of 2.0-kb D and 1.8-kb CcEe fragments hybridizing to the 3' probe was 0.4 for B1 compared with 0.8 for C3. These measurements show that the 5' and 3' probe are each identifying one D gene for B1, and therefore the apparent genotype is CCDee.

DISCUSSION

This study clearly identifies three distinct polymorphisms on the Rh D gene from individuals with Rh CCcee phenotypes who lacked the D antigen. Donor S1 had no detectable D gene in the regions probed. Donor S2 had a single copy of the D gene, but this had a deletion at the 3' end. The extent of this deletion has not been precisely determined, but attempts to amplify exon 7 by PCR also failed, indicating the deletion probably covered exons 7 to 10. Donor B1 apparently had a single copy of a normal D gene, although no antigen was expressed. Further studies are required to determine if there are underlying structural defects (eg, the presence of two D genes, one with a 5' deletion and one with a 3' deletion). In addition to these variants, in an earlier study we observed a sample with a deduced genotype of CCDDee in which one of the D genes also contained a 3' deletion. In this case, the deleted gene still contained exon 7 as judged by PCR (data not shown), which implies that a further Rh D-negative CCcee type is possible.

The presence of several variants of the relatively rare Cc genotype suggests it has arisen on several occasions by independent deletions of part or all of the D gene, presumably from the relatively common CDe or R1 genotype. Although our sample size is small, the results presented here suggest that a significant number of Ce genotypes may contain at least part of the D gene.

These results contrast with data from the ccee donors in whom no trace of a D gene was found. Therefore, the event resulting in the D-negative trait of these ce genotypes must have been different from at least some and possibly all of the Ce genotypes.

The ce and Ce genotypes occur at greatly different frequencies (0.38 and 0.0098, respectively, for white populations). As a result, the findings reported here do not materially alter the conclusion that D-negative subjects have both D genes fully deleted: the expected frequency is approximately 99% of Rh-negative individuals. Nevertheless, it is interesting to note that a small percentage of the population will contain an inactivated or partially deleted D gene.

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