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Three Molecular Structures Cause Rhesus D Category VI Phenotypes With Distinct Immunohematologic Features

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Rhesus D category VI (D^{VI}) is the clinically most important partial D. D^{VI} red blood cells were assumed to possess very low RhD antigen density and to be caused by two *RHD-CE-D* hybrid alleles. Because there was no population-based work-up, we screened three populations in central Europe for D^{VI}. Twenty-six D^{VI} samples were detected and examined by exon-specific *RHD* polymerase chain reaction with sequence-specific primers (PCR-SSP). A new genotype, hereby designated *D category VI type III,* was characterized as a *RHD-Ce(3-6)-D* hybrid allele by sequencing of the cDNA, parts of intron 1, and by PCR-restriction fragment length polymorphism (PCR-RFLP) of intron 2. *Rhesus* introns 5 and 6 were sequenced and the 3′ breakpoints of all known *D^{VI} types* shown to be distinct. We differentiated the 5′ breakpoints of *D^{VI} type I* and *D^{VI} type II* by a newly devised *RHD*-PCR. Thus, the D^{VI} phenotype originated in at least three independent molecular events. Each D^{VI} type showed distinct immunohematologic features in flow cytometry. The number of RhD proteins accessible on the red blood cells' surface of *D^{VI} type III* was normal (about 12,000 antigens/cell; *D^{VI} type I,* 500; *D^{VI} type II,* 2,400) based on the determination of an RhD epitope density profile. *D^{VI} type II* and *D^{VI} type III* occurred as CDe haplotypes, and *D^{VI} type I* as a cDE haplotype. The distribution of the *D^{VI} types* varied significantly in three German-speaking populations. Genotyping strategies should take account of allelic variations in partial RhD. The reconsideration of previous serologic and clinical data for partial D in view of the underlying molecular structures may be worthwhile.

THE RHESUS BLOOD GROUP system is of great importance for transfusion medicine because of the high immunogenicity of its antigens. Rhesus antigens are carried by two highly homologous proteins, the RhD and RhCE proteins.[1-6] The D antigen (ISBT 004.001; RH1) determined by the RhD protein is the most important Rhesus antigen and the leading cause for hemolytic disease of the newborn.[7] About 17% of Caucasians lack the expression of the D antigen.[8] The transfusion of a single unit of D positive red blood cells to a D negative patient is associated with an immunization rate of greater than 80%.[9]

The D antigen comprises several different antigenic epitopes. Rare individuals carry a partial D antigen[10] and may produce alloantibodies directed against D epitopes that are lacking in their RhD protein. Initially, these individuals have been classified into six distinct categories (D^{II} to D^{VII}, D^{I} being obsolete) based on the mutual reactivity with polyclonal anti-D sera from immunized partial D carriers.[11] Today, characterization of partial D is performed by differential reactivity with monoclonal anti-D antibodies.[12,13] D category VI (D^{VI}) is the clinically most important partial D. Severe cases of hemolytic disease of the newborn have occurred in RhD positive babies born to D^{VI} mothers with anti-D.[14] D^{VI} is the most abundant serologically defined partial D occurring among weak D samples. D^{VI} is reported to comprise about 6% to 10% of weak D samples[8,15] and has a phenotype frequency of 1:6,200 in Germany (range, 0.02% to 0.05% in Caucasians).[8,15,16] The majority of RhD positive individuals with allo-anti–D were D^{VI}.[17]

D^{VI} occurs in CDe, cDE, and cDe haplotypes.[17] The $CD^{VI}e$ haplotype is due to an *RHD-RHCE* hybrid molecule in which exons 4 to 6 of *RHD* were substituted by the respective exons of *RHCE*.[18] Samples with a $cD^{VI}E$ haplotype were initially assumed to carry a deletion of exons 4 to 6,[18] but in fact, are due to an exon 4 to 5 hybrid.[19,20] Most $CD^{VI}e$ haplotypes carry the low frequency Rhesus antigen BARC (ISBT 004.052; RH52).[21] No other consistent serologic differences in D^{VI} have been described.[21]

We recently completed a serologic random survey for partial D in southwestern Germany.[8,22] Here we report the results of the molecular and immunohematologic work-up of D^{VI} samples. We describe a novel molecular event that caused a D^{VI} phenotype carrying a normal number of RhD proteins accessible on the red blood cells' surface. We show that the three *D^{VI} types* may be readily discriminated by flow cytometry based on distinct immunohematologic features. We demonstrate considerable differences in the distribution of D^{VI} types within German-speaking populations showing the importance of a full molecular description for *Rhesus* genotyping purposes, eg, in prenatal testing.

MATERIALS AND METHODS

Random Survey and Blood Samples

EDTA- or citrate-anticoagulated blood samples came from southwestern Germany (DRK-Blutspendedienst Baden-Württemberg, Ulm, Germany), northern Germany (DRK-Blutspendedienst Niedersachsen, Oldenburg) and Tyrol, Austria (Zentralinstitut für Bluttransfusion und Immunologische Abteilung Innsbruck, Innsbruck, Austria). As described previously,[8] blood samples in Ulm screened for differential reactivity with a monoclonal IgM anti-D (BS226; Biotest, Dreieich,

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The nucleic acid and amino acid sequence data were deposited in EMBL/GenBank/DDBJ under the accession numbers Z97026; Z97030; Z97031; Z97333; Z97334; Z97362 and Z97363.

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Germany; not reactive with D^{VI}) and with polyclonal anti-D in antiglobulin technique were checked for the D^{VI} phenotype by a panel of monoclonal anti-D (D-Screen; Diagast, Lille, France). The D^{VI} phenotype was further confirmed by reactivity with monoclonal anti-D BS221, H41 (Biotest), and BRAD-2 (International Blood Group Reference Laboratory, Bristol, UK), as well as absence of reactivity with BS227, BS229, BS231, BS232 (Biotest) and RUM-1 (Bio Products Laboratory, Elstree, UK). Similarly, blood donors in Oldenburg and Innsbruck with weak D phenotype were screened by the *RHD* exon-specific polymerase chain reaction with sequence-specific primers (PCR-SSP; see below).

Molecular Biology

DNA was prepared using a modified salting out procedure[23] or QIAAmp Blood DNA isolation kit (Qiagen, Hilden, Germany). RNA was isolated using RNeasy kit (Qiagen). Reverse transcription was performed with oligo-dT-priming and Moloney murine leukemia virus (MMLV) reverse transcriptase (Sigma, Deisenhofen, Germany). *RHD* exon-specific PCR-SSP was performed as previously described.[24] cDNA was amplified in a nested PCR-reaction (High Fidelity PCR system, Boehringer Mannheim, Mannheim, Germany) with external primers RR1 and RR3 and internal primers Rh5 and Rh7. The 5′ part of intron 1 was amplified with primers RB13 and RB45. Restriction fragment length polymorphism-PCR (RFLP-PCR) of intron 2 was performed using the primers of Poulter et al.[25] The 3′ region of intron 3 was amplified with primers RB46 and RB5, RB12 and RI4R2. The intron 3 length polymorphism data were based on seven RhD-negative and 20 RhD-positive samples. Intron 5 was amplified using primers RA9B and Rh2 and intron 6 using primers RB25, RB7, and RB27.

Nucleotide sequencing was performed with a DNA sequencing unit (Prism dye terminator cycle-sequencing kit with AmpliTaq FS DNA polymerase; ABI 373A, Applied Biosystems, Weiterstadt, Germany). We subcloned the PCR product into pMos-T-vectors (pMos-T-kit, United States Biochemicals, Cleveland, OH). Three independent cDNA clones were sequenced using T7 promoter primers, U19 reverse plasmid primers, and internal *Rhesus* primers. Genomic sequences were established from cloned PCR fragments using both primer walking and nested deletion strategies (Nested deletion kit, Pharmacia, Freiburg, Germany) and verified by sequencing of PCR products using internal *Rhesus* primers. Intron 1 sequences were based on six RhD-negative (three ccee, two ccEE, one CCee), eleven RhD-positive samples and one sample of each D^{VI} type, intron 3 sequences on six *RHCE* (two ce, Ce, cE each) and four *RHD* alleles, intron 5 and 6 sequences on at least two *RHCE* (ce) and *RHD* alleles. Intron DNA sequences were analyzed for the presence of repetitive sequences by the CENSOR program[26] (censor@charon.lpi.org).

Primer Sequences

RB13, ctagagccaaacccacatctcctt (promoter,[27] position -675 to -653 relative to the A of the start codon of the cDNA); RR1, tgttggagagaggggtgatg (5′ untranslated, -60 to -41); Rh5,[28] gcacagagacggacacag (5′ untranslated, -19 to -2); Rh1,[29] tatctagagacggacacaggATGAGC (5′ untranslated to exon 1, -17 to 6); RB45, acactgttgrctgaatttcggtgc (intron 1, antisense); RA21,[25] gtgccacttgacttgggact (intron 2, sense); RA22,[25] gtggacccaatgcctctg (intron 2, antisense); RB46, tggcaagaacctggaccttgacttt (intron 3, sense); RA9B, GGTGCCTGCCAAAGCCTCTACCC (exon 4, 554 to 576); RB5, GGCAGACAAACTGGGTATCGTTGC (exon 4, 627 to 604); RB12, tcctgaacctgctctgtgaagtgc (intron 4, antisense, *RHD*-specific); RI4R2, ttggctcactgcaacctccaccac (intron 4, antisense, *RHCE*-specific); RB25, agcagggaggatgttacag (intron 4, sense); Rh2,[29] AGAAGGGATCAGGTGACACG (exon 5, 900 to 881); RB7, ATCTCTCCAAGCAGACCCAGCAAGC (exon 7, 1022 − 998); RB27, AGCCCAgtgacccacatg (exon7/intron 7); Rh7, acgtacaaatgcaggcaa (3′ untranslated, 1330 − 1313); RR3, cagtctgttgtttaccagatg (3′ untranslated, 1512 − 1431, *RHD*-specific).

Immunohematology

Monoclonal anti-D were provided by the Workshop on Monoclonal Antibodies against Human Red Blood Cells and Related Antigens.[30] All monoclonal anti-D were tested for agglutination in a gel matrix test (LISS-Coombs 37°C, DiaMed-ID Micro Typing System, DiaMed, Cressier sur Morat, Switzerland). As detailed in the Results, positive reactivities were obtained with BIRMA-D6; BTSN6; BTSN10; HIRO-3; HIRO-4; HIRO-7; HIRO-8; H41; LHM76/55; LHM76/59; LHM-77/64; LOR11-2D9; LOR17-6C7; LOR29-F7; MCAD-6; MS26; NAU3-2E8; NAU6-4D5; P3G6; P3x21223B10; P3x290; 822; negative with AUB-2F7/Fiss; BIRMA-D56; BRAD-3; BRAD-5; BS229; BS231; BS232; B9A4B2; CAZ7-4C5; CLAS1-126; C205-29; D6D02; D10; D89/47; D90/12; D90/17; F5S; HeM-92; HG/92; HIRO-1; HIRO-2; HIRO-6; HM10; HS114; H2D5D2F5; ID6-H8; LHM50/2B; LHM50/3.5; LHM59/19; LHM59/20; LHM59/25; LHM70/45; LHM-76/58; LHM169/80; LHM 169/81; LHM174/102; LORA; LOR12-E2; LOR17-8D3; LOR28-7E6; LOR28-21D3; L87.1G7; MAR-1F8; MS201; NaTH28-3C11; NaTH53-2A7; NaTH87-4A5; NAU6-1G6; NOI; NOU; P3AF6; P3F17; P3F20; P3x35; P3x61; P3187; RAB.B15; RUM-1; SALSA-12; SAL17-4E8; SAL20-12D5; T3D2F7; VOL-3F6; ZIG-189; 17010C9; 175-2; 819; and weak positive or variable with BIRMA-DG3; BTSN4; D90/7; LORE. Furthermore, reactivity with two polyclonal anti-D produced by carriers of the D^{VI} phenotype (CcD^{VI}ee and ccD^{VI}Ee), as well as with anti-BARC (ISBT 004.052; RH52) serum and eluate (kindly provided by Drs Geoff Daniels and Carole A. Green, Bristol, UK) was checked.

Determination of RhD antigen density was performed by indirect immune fluorescence as described previously.[31,32] All 22 IgG monoclonal anti-D reactive to RhD epitopes present in D^{VI} [30] were used (BIRMA-D6, BTSN6, BTSN10, HIRO-3, HIRO-4, HIRO-7, HIRO-8, H41, H41.11B7, LHM76/55, LHM76/59, LHM 77/64, LOR11-2D9, LOR17-6C7, LOR29-F7, MCAD-6, MS26, NAU3-2E8, NAU6-4D5, P3G6, P3x290, and 822). The secondary antibody was goat antihuman immunoglobulins, fluorescein isothiocyanate (FITC)-conjugated (Sigma).

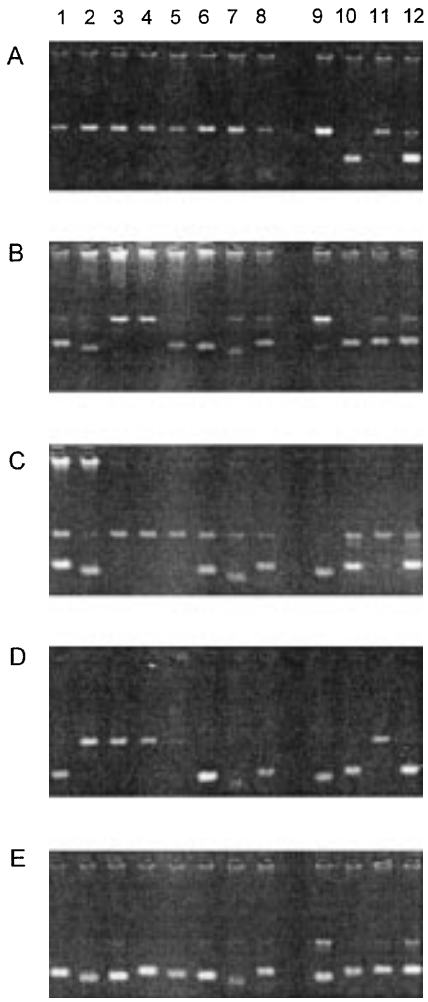
All blood samples were stored on fluid nitrogen. The fluorescence was compared with that of a standard CDe/cde red blood cell (13,000 RhD antigens per cell). Background fluorescence was determined with RhD-negative samples. The number of RhD epitopes detected on the sample cells was calculated as [median fluorescence of sample—background fluorescence] ÷ [median fluorescence of standard cell—background fluorescence] × RhD antigen density of standard cell.

Markers were set to count all red blood cells, even if a fraction of red blood cells appeared unstained. To account for a log-normal distribution, we based the parametric statistical analysis on the logarithms of the RhD antigen densities.

RESULTS

Detection of Three Independent Molecular Events Causing D Category VI

Twenty-six D^{VI} samples were examined using *RHD*-specific PCR-SSP for exons 2, 3, 4, 5, 6, 7, 9, and 10 (3′ untranslated).[24] All D^{VI} samples differed in their PCR-SSP pattern from the wild-type *RHD* allele and showed one of three PCR-SSP patterns (Fig 1). Two PCR-SSP patterns were compatible with the previously described genomic rearrangements associated with *D^{VI} type I* (lack of *RHD* exons 4 and 5) and *D^{VI} type II* (lack of *RHD* exons 4 to 6).[18,19,33] A third pattern could not be explained by any known *RHD/RHCE* variation and is hereby called *D^{VI} type III*.



Reaction (lane)	Specificity	Exon	Nucleotide position(s)	Product (bp)
1	D/C	2	201 307	148
2	D	3	383	113
3	D	4	602	122
4	D	5	676 787	157
5	D	6	916	132
6	D	7	1048	122
7	D	9	1193	83
8	D	10	1255	147
9	C/c[2]	1	48	112
10	c/c[2]	2	201 307	149
11	E	5	787	158
12	e	5	787	158

Fig 1. PCR-SSP of D^{VI} samples. Agarose gels of representative D^{VI} samples are shown along with negative and positive controls. The specificities of the 12 reactions for the *RHD*/*RHCE* exons are shown on the right side (box). The nucleotide position(s) detected by the PCR-SSP are given along with the expected sizes of the specific products. The control band represents a 434-bp product of the growth hormone gene. For D^{VI} samples, three different reaction patterns are observed: pattern I (B) lacks specific signals for *RHD* exons 4 and 5 and is compatible with *D^{VI} type I*.[19] Pattern II (C) lacks those for *RHD* exons 4 to 6 being compatible with *D^{VI} type II*.[18] Pattern III (D) lacking specific products for *RHD* exons 3 to 6 is novel. c^2 indicates the c(cyt^{48}) allele.

Molecular Characterization of D^{VI} Type III

Coding sequence. Because the PCR-SSP pattern of *D^{VI} type III* was novel, we determined the full-length coding sequence of its cDNA (EMBL/GenBank/DDBJ nucleotide sequence database accession number Z97026). The *D^{VI} type III* cDNA comprising all 10 *Rhesus* gene exons represented a *RHD-CE-D* cDNA, in which the complete exons 3, 4, 5, and 6 of the *RHD* gene were replaced by the corresponding exons of the *RHCE* gene.

The exons 3 to 6 are derived from the RHCe allele. We applied a PCR-RFLP method for the characterization of the *Rhesus* genes' intron 2.[25] A length polymorphism discriminates between the *RHC* and *RHc/RHD* alleles of the two *Rhesus* genes (Fig 2A). An RFLP allows the further separation of the *RHC*, *RHc* and *RHD* alleles (Fig 2B). We excluded the presence of *RHD*-specific sequences in *D^{VI} type III* at the position of this polymorphism in intron 2 (Fig 2B). The discrimination between an *RHC*- or *RHc*-origin of the *D^{VI} type III* intron 2 was achieved by the length polymorphism. The *D^{VI} type III* sample showed an enhanced band of 1,177 bp size (*RHC*) compared with that of 1,068 bp size (*RHc*) (Fig 2A). This indicated that two copies of *RHC*-like intron 2 sequences were present in the *CD^{VI}e/ce* genotype, one from the *D^{VI} type III* allele, the other from the *Ce* allele of the *CD^{VI}e* haplotype. We concluded that the *RHCE*-derived genomic sequences of the *D^{VI} type III* allele originated from the *RHCe* allele and extended 5′ of this polymorphism, which is located in the middle of intron 2.

Exon 1 is of RHD origin. The guanosine at nucleotide position 48 relative to the A of the translation start codon in the *D^{VI} type III* cDNA was compatible with both an *RHD* or an *RHc* origin.[3,28] To prove the *RHD* derivation of exon 1, we characterized the 5′ portion of intron 1 for both *Rhesus* genes (EMBL/GenBank/DDBJ nucleotide sequence database accession number Z97362 and Z97363). *D^{VI} type III* presented all three nucleotide substitutions and the insertion characteristic for the *RHD* allele (Fig 3). This observation indicated that the genomic sequences of the *D^{VI} type III* allele 5′ of this part of intron 1 were derived from the *RHD* gene. The molecular characteristics of *D^{VI} type III* were summarized and compared with other published alleles (Fig 4).

Demonstration of Distinct Breakpoints in the Three D^{VI} Types

The 3′ breakpoints of D^{VI} type II and D^{VI} type III are different. To define the 3′ limits of the conversion regions of the three *D^{VI} types*, we established the complete nucleotide sequence ranging from exon 5 to exon 7 including both introns 5 and 6. We found

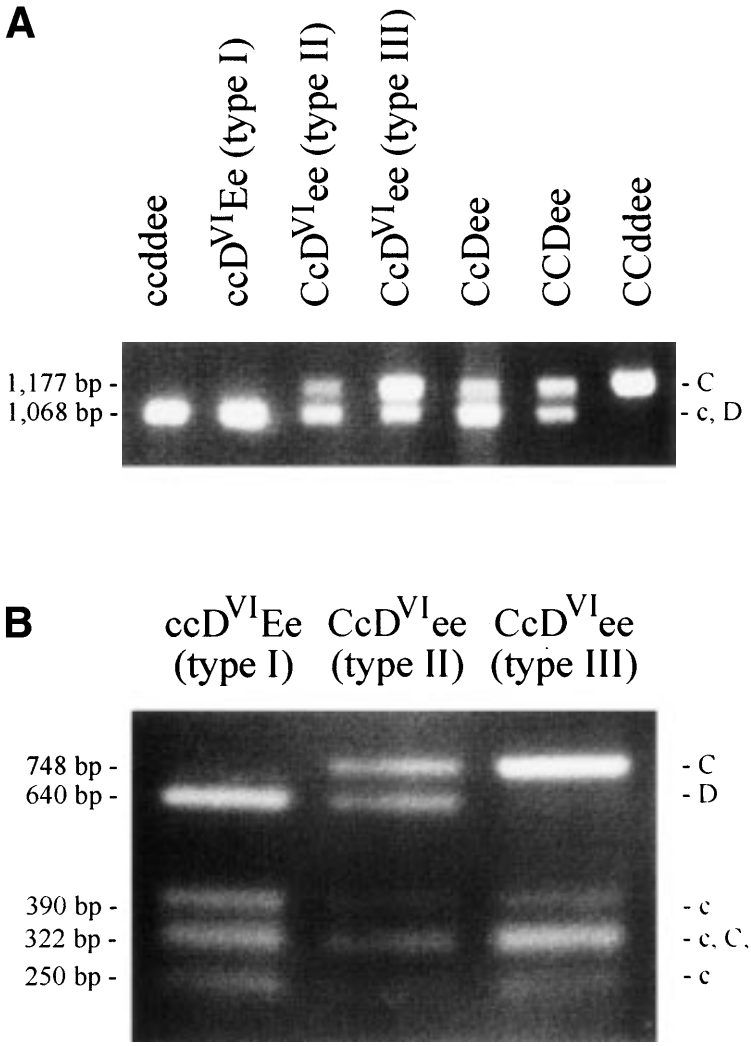


Fig 2. PCR-RFLP of intron 2 of the *Rhesus* genes. An intron 2 polymorphism was analyzed by PCR amplification and digestion by PstI as previously described.[25] Agarose gels are shown with fragment lengths[25] and fragment specificities indicated. (A) The 1,177-bp product is specific for *RHC* alleles, the 1,068-bp product is representative for *RHD* or *RHc* or both. The CcD^{VI}ee type III sample shows a strong band at the *RHC* position and a weaker band at the *RHD*/*RHc* position. In contrast, the CcD^{VI}ee type II and the CcDee samples show a weak band at *RHC* position and a strong band at the *RHD*/*RHc* position. (B) The PCR products shown in (A) were digested with PstI to separate *RHD* from *RHc*-specific products. The D^{VI} type III sample lacks the *RHD*-specific fragment (640 bp), whereas all other RhD positive samples show this fragment.

the breakpoint of *D^{VI} type I* to be located at the border of intron 5 and exon 6 in a nucleotide range of 215 bp between -100 bp and +115 bp relative to the first nucleotide of exon 6 (Fig 5). The breakpoint of *D^{VI} type III* was located in intron 6 between +360 bp and +963 bp (range of 603 bp) relative to the first nucleotide of intron 6. Finally, the breakpoint of *D^{VI} type II*[34] was also located in intron 6 between +1,781 and +1,821 bp (range of 40 bp) relative to the first nucleotide of intron 6.

```
                Exon 1---> Intron 1-->
RHCE            TCCTATCAAG gtgagagttc attggaacag tggtcacagg agcaaatagc aggggcaggg gcgggggagg
D^VI type III   TCCTATCAAG gtgagagttc attggaaaag tggtcacagg agcaaatagc aggggcaggg gcgggggagg
RHD             TCCTATCAAG gtgagagttc attggaaaag tggtcacagg agcaaatagc aggggcaggg gcgggggagg
                          1        10         20         30         40         50         60

RHCE            cctatggttc tccaggggca cagatgttcc tttctacaaa atcccgagga aaa-gattcc cccatcttct
D^VI type III   cctgtggttc tccaggggca cagatgttcc tttctacaaa atcccaagga aaaagattcc cccatcttct
RHD             cctgtggttc tccaggggca cagatgttcc tttctacaaa atcccaagga aaaagattcc cccatcttct
                       70         80         90        100        110        120        130
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Fig 3. 5′ portion of the *Rhesus* genes' intron 1. One hundred thirty nucleotides of intron 1 adjacent to exon 1 are shown for the *D^{VI} type III* allele along with the common *RHCE* and *RHD* genes. The *RHCE* and *RHD* genes differ by three nucleotide substitutions and one insertion (boxed). The *D^{VI} type III* allele is identical to *RHD* at these positions. As expected, *D^{VI} type I* and *D^{VI} type II* alleles are also identical to *RHD* (not shown). Nucleic acid sequence accession numbers were Z97362 (*RHCE*) and Z97363 (*RHD*).

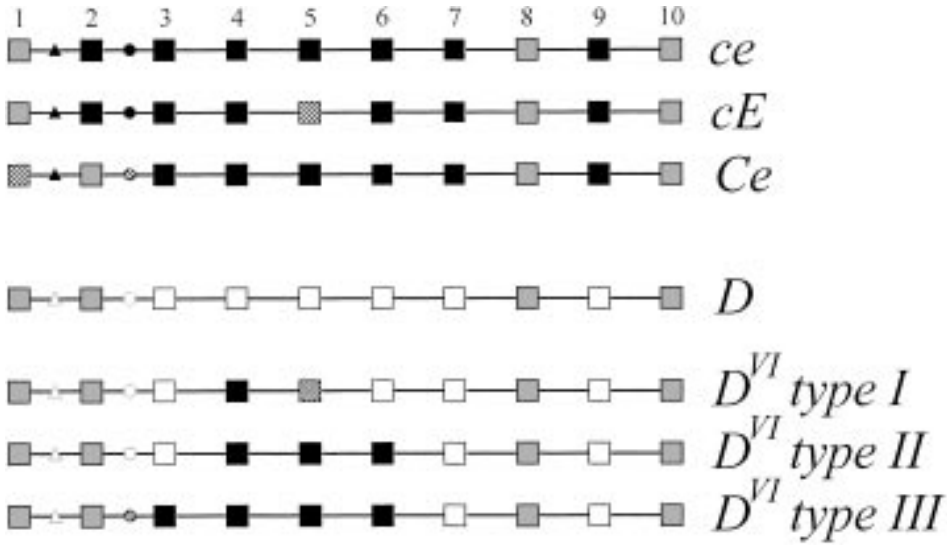

Fig 4. Schematic representation of the genomic structure of *D^VI type III* compared with other alleles of the *RHD* and *RHCE* genes. The 10 exons of the *Rhesus* genes are symbolized by squares and numbered 1 to 10, the introns are represented by lines. The triangles denote the base substitutions and insertion occurring in intron 1 distinguishing *RHD* and *RHCE*. The circles denote the intron 2 polymorphism distinguishing *RHD*, *RHC* and *RHc*. Black symbols represent *RHCE*-specific sequences, open symbols *RHD*-specific sequences. Sequences shared by at least one *RHCE* and the wild-type *RHD* allele are indicated by gray symbols, sequences specific for *RHC* or for *RHE* are hatched. The nucleic acid and amino acid sequence accession number of *D category VI type III* was Z97026.

An intron 3 length polymorphism differentiates the 5′ breakpoints of D^VI type I and D^VI type II. We found a 288-bp deletion in intron 3 of the *RHD* gene, when compared with the *RHCE* gene. Based on this deletion, a PCR typing method for *RHD* was devised. In this intron 3 PCR, *D^VI type I* and *D^VI type III* samples reacted like *RHD* negative controls, while *D^VI type II* samples displayed the shorter, *RHD*-specific band (Fig 6A). This indicated that the conversion point of *D^VI type I* had to be 5′ of the intron 3 deletion. We confirmed the conversion point of *D^VI type II* adjacent to an Alu repeat[34] (data not shown, see Z97030 and Z97031) and 5′ of the conversion point were two additional Alu repeats with inverse orientation, one of which was partly deleted in *RHD* (Fig 6B).

Linkage of the D^VI types to different Rhesus haplotypes. We observed the three *D^VI types* associated with specific *Rhesus* haplotypes: all *D^VI type I* samples (n = 14) were found in the *cD^VIE* haplotype, all *D^VI type II* (n = 9), and *D^VI type III* (n = 3) in the *CD^VIe* haplotype. Because the genomic structure of *D^VI type III* is *D-Ce(3-6)-D*, a conversion event among the two *Rhesus* genes in cis-position may be the cause of this hybrid allele.

Regional frequency variation of the D^VI types. The distribution of the different *D^VI types* varied depending on the regional origin of the samples (Table 1). In Tyrol (Austria), all samples were *D^VI type I*, while in southwestern Germany, *D^VI type I* and *D^VI type II* were observed about equally frequently. In northern Germany, the only D^VI samples that we found so far were *D^VI type II*.

Serology of D^VI Samples

Polyclonal antibodies. One sample of each *D^VI type* was tested with two polyclonal anti-D and anti-BARC (Table 2). *D^VI type III* qualified as a D category VI, because it was nonreactive with anti-D produced by probands of *D^VI type I* and *D^VI type II*. Further, *D^VI type III* carried the BARC antigen (ISBT 004.052; RH52). Anti-BARC did not differentiate *D^VI type II* and *D^VI type III*.

Monoclonal anti-D. One sample of each *D^VI type* was tested with the full panel of monoclonal anti-D provided in the recent Workshop on Monoclonal Antibodies against Human Red Blood Cells and Related Antigens.[35] The three *D^VI types* did not differ in their reaction pattern (Table 3, upper panel). All positive and most negative reactivities reported by the Workshop coordinator[30] were confirmed. Four anti-D (BIRMA-DG3; BTSN4; D90/7; LORE), reported to be nonreactive,[30] showed discrepant results and were tested with additional D^VI samples (Table 3, lower panel). We found variable, ie, negative or weak positive, reactivity. This reactivity would have been considered negative by the Workshop criteria[30] and thus our observations were in full agreement with the Workshop results.

Flow Cytometric Analysis of the D^VI Types

Epitope density profiles. Fifteen D^VI samples and three control samples were tested with the 22 IgG monoclonal anti-D of the Workshop[30] that bind the RhD epitopes of D category VI. In contrast to the control samples, the number of RhD epitopes per cell detected on the *D^VI* samples varied considerably depending on the monoclonal antibody used (Fig 7). This variation in the number of epitopes detected did not correlate with the epitope specificity[30] of the anti-D (data not shown, $P = .23$ in the analysis of variance). *D^VI type I* and *D^VI type II* presented consistently low numbers of RhD epitopes per cell with all anti-D. Interestingly, many monoclonal anti-D detected normal, if not enhanced, numbers of RhD epitopes per cell in *D^VI type III*.

RhD antigen density (antigens/cell). Using the results of all 22 anti-D, we calculated the RhD antigen densities as correlates of the number of RhD proteins accessible on the red blood cells' surface (Table 4). The RhD antigen density of *D^VI type III* was similar to the CcDee control and several fold higher than that of

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     1        10        20        30        40        50        60        70        80        90       100
RHCE ccttagaggcagtagtgagctggcccaccgtgtccactgatgaaggacacgtagccccaacacaggggagaggtggtttcaggatcagcaaagcagggag  1700
RHD  ---------------------------t-a-----------------------------------------a----------------------------
                                                                           |****************************

RHCE gatgttacagggttgccttgttcccagcgtgctggtcacttgcagcaagatggtgttctctctaccttgcttcctttacccacacgctatttctttgc  1800
RHD  ----------------------------------------------------------------------------------------------------
     **** D category VI type I **************************************************************************

RHCE agACTTATGTGCACAGTGCGGTGTTGGCAGGAGGCGTGGCTGTGGGTACCTCGTGTCACCTGATCCCTTCTCCGTGGCTTGCCATGGTGCTGGGTCTTGT  1900
RHD  ----------------------------------------------------------------------------------------------------
     **** D category VI type I **************************************************************************

RHCE GGCTGGGCTGATCTCCATCGGAGGAGCCAAGTGCCTGCCGgtaagaaactagacaactaatgctctctgctttggctgaaggccagcaggacgctgggac  2000
RHD  ----------------G----G----------A--------------------------cctc-------------------------------------
     ****************|

RHCE ctgatgggccactgtgcagtgcacagctgcattaggcaggtgttggtgcattctcttattggcttcaacgcctagcgagggatccatcctggctcggtgg  2100
RHD  -------------------------------------------c--c-----------------------------t------------------------

RHCE cacatttgttaagatgctggggagcaggtggcagaacccatttgagcttgcttgggcactggggagaatttgttaccaggctacaggggtgtcacagaac  2200
RHD  -g----------------c---------------------------------------t----------------t--------t---------------

RHCE tcaaggacagggactggagtgttgtggggagcccagaagcccctgttttacttctttctttgcttttcctgaatatctgctttattcttactctatagac  2300
RHD  ----------------------------------c-----------------------------------------------------------------

RHCE ctgcttcctcctctttcaccccacattgtggggtgtagtcttttgcttcaagaaagcagcctggtggatggaatctcttggccccaatcccaaattctct  2400
RHD  a---------------------------------------------------------------------------------------------------
     |*** D category VI type III ************************************************************************

RHCE ggagaaggggctctttggtttaacttggataatgttgtcttcagctgggggtgggcacatcgtgcatatgtggctgctgccggggaaccacgtggatgat  2500
RHD  ----------------------------------------------------------------------------------------------------
     **** D category VI type III ************************************************************************

RHCE gtgagaggagcagcacccagaagagggagtgctgggctgatggtccaggtcgtgtccacttctgattgtttaattcttcttctaagtggatggatctttc  2600
RHD  ----------------------------------------------------------------------------------------------------
     **** D category VI type III ************************************************************************

RHCE tccaatactcagcaaatcctgatcgttccagaatacttcattatagccaattggttataatgtgcttctctaagagaaatatttagggacaacaaatctt  2700
RHD  ----------------------------------------------------------------------------------------------------
     **** D category VI type III ************************************************************************

RHCE catgggtttgaagacttgatggaggaaaaaggagtagattttcgaaggctggatttggatgaacaggggctattcagggagtgcattccaacctaaaatt  2800
RHD  ----------------------------------------------------------------------------------------------------
     **** D category VI type III ************************************************************************

RHCE aggaaaaactggctgggcgcagtggctcacgcgctttgggaggccgaggcgggcagatggcctgaggtcaggagttcaagaccagcctggccaacatggt  2900
RHD  ----------------------------------------------------------------------------------------------------
     **** D category VI type III ************************************************************************
               |--->Alu-Sx^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

RHCE gaaacc.atctctactaaaagtacaaaaattagccaggcgtggtggcgggcacctgtcatcttagctactcaggaggctgagatgcgagaatcacttgaac 3000
RHD  ------c-------------------------------a--------------------------g----------------ca----------------
     ******|
     ^^^^Alu-Sx^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

RHCE ctgggagacagagcttgcagtgagccgaaattgcgccactgcactccagcctgggcgacagaacaagactctgtcttaaaaaaaaaaaa.gtgttttatat 3100
RHD  -------------------------t-----c-t----tg-------------------------------------------------a---g-------
     ^^^^Alu-Sx^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^<---||--->^^^^^^

RHCE acagagtggaatattatttagccataaaaagaatgaaatcctgtcatttgcagcaacatggatggaactggaggtcattaaaaaataaaataaaataaat  3200
RHD  ---------------------------------------------------------------------------a---------------t------..
     ^^^^L1MA2^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^<---|

RHCE aaggaaaaacgtatcaatacttcgattgaccaaaaccagggcaaatctgattttcatctttgcaaggggaacaaatttcttttatctcctctggctttga  3300
RHD  ..--------------------------a-----------------------------------------------------------------------

RHCE aaccctgaaatgaaaggaggaagggcagaaaaaagaacacatagcaagttaccatcaggctcagcgcccatcgcattccctgagcttgtttccttgactt  3400
RHD  ---------------------------------------------------t------t-----------------------------------------

RHCE catcactggcaggactattcaaaaatgattccctcattcattcatatattcattcattcatcattccttcattcaacacatacgttttaacactcatctt  3500
RHD  -------------------------------g--------------------------------------------------------------------
                                 |--->TTCA tandem repeats^^^^^^^^^^^^^^^^^^^<---|

RHCE gcttttcaagctatagtttagtgagcgaaatggatacacagaatacagtgtgagaacagctacagggcacatctgagctagcctgggatgggtccggaaa  3600
RHD  ----------------------------------------c-------------------a-g-------------------------------t-----

RHCE tgcttcctggagcagaggaaacggttgacagccaagtgttgacagagaagtagtattagccaggcagagacatggggaatgtattccaggcagaaggcac  3700
RHD  ----------------------------------------------t-----------------------------------------------------

RHCE agtgtgtatgaaagcttattggtaagaagagtgtgtggcccaaccaggaaacagacattctgaaggcatagggtccacccaggagcatggtgaacccaga  3800
RHD  ---------------------t---------------------------------------a------------------------------g-------
                          |*** D category VI type II *************|
```

Fig 5. Exon 6 of the *Rhesus* genes and parts of the adjacent introns. The sequence of the *RHCE* gene extending 202 bp 5′ of exon 6 to 1860 bp 3′ of exon 6 is shown. Numbers indicate the position relative to the first base of exon 5 in the *RHCE* gene. Exon 6 (bases 1902 to 2040) is demarcated by uppercase letters. Dashes denote nucleotides in the *RHD* gene that are identical, dots denote deletions. The breakpoint regions for D^{VI} *type I*, D^{VI} *type II* and D^{VI} *type III* are indicated by asterisks. Repetitive DNA elements are marked by carets. The full intron 5 and intron 6 sequences of *RHCE* and *RHD* were deposited in EMBL/GenBank/DDBJ under accession numbers Z97333 (*RHCE;* 5,134 bp) and Z97364 (*RHD;* 5,146 bp).

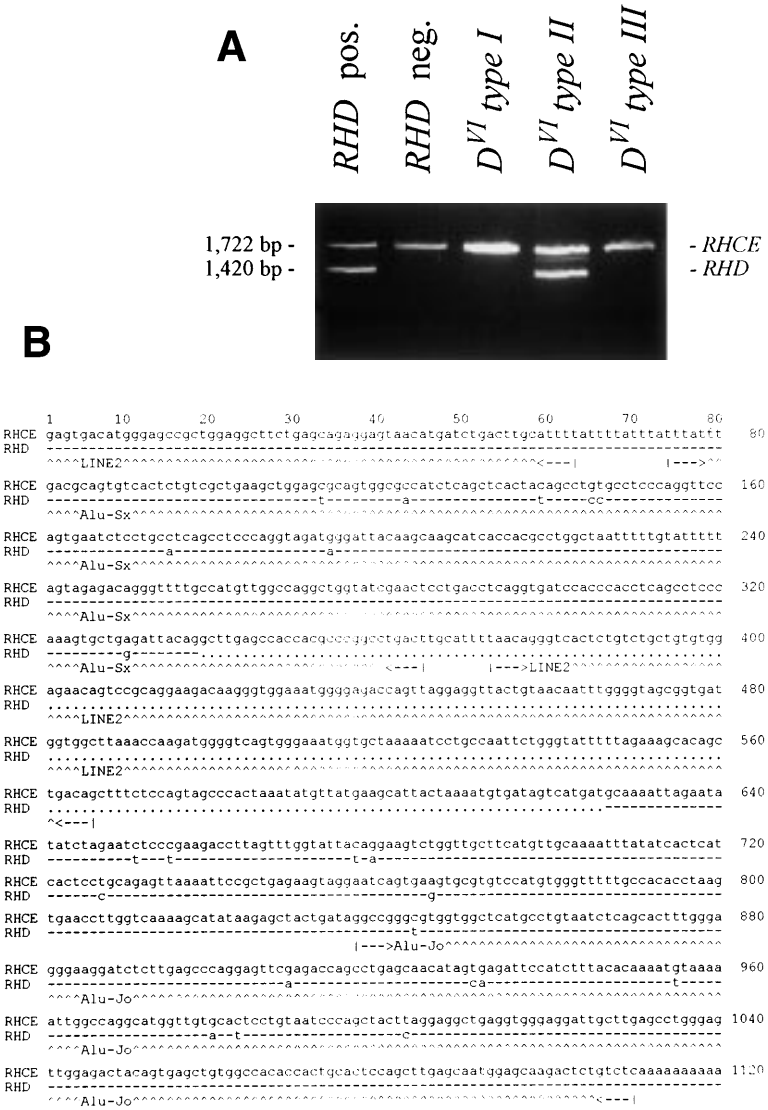

Fig 6. Intron 3 length polymorphism of the *Rhesus* genes. The 3′ region of intron 3 was amplified by PCR using primers RB46 and RB5. (A) The agarose gel shows a 1,722-bp product for the *RHCE* gene. The 1,420-bp product is representative of the *RHD* gene. In the *D^VI type II* sample, a *RHD*-specific product is found. *D^VI type I* and *D^VI type III* samples show no *RHD*-specific product. (B) The nucleotide sequence of the 3′ part of intron 3 of the *RHCE* gene starting 1,556 nucleotides 5′ from exon 4 and of the corresponding parts of the *RHD* gene comprising the diagnostic 288 bp deletion are shown. Dashes denote nucleotides in the RHD gene that are identical, dots denote deletions. Nucleic acid sequence accession numbers were Z97030 (*RHCE;* 1,580 bp) and Z97031 (*RHD*; 1,278 bp).

D^VI type I and *D^VI type II*. Still, the RhD antigen densities of *D^VI type I* and *D^VI type II* differed significantly.

Distinct immunohematologic features of the D^VI types. Two of four monoclonal anti-D that are binding to epD37[30] detected fair numbers of RhD epitopes per cell for *D^VI type I*, but rather low numbers for *D^VI type II* and *D^VI type III*. This deviation from the actual RhD antigen densities (Table 4) was neither observed with the two other monoclonal anti-D binding to epD37 nor any other anti-D binding to the remaining RhD epitopes present in D^VI samples. This heterogeneity of anti-D's binding to epD37 may represent a flow cytometric split: epD37a (BTSN10 and HIRO-3) was detected equally well in all D^VI types, epD37b (MCAD-6 and 822) was reduced in D^VI type II and D^VI type III. The binding characteristic of MCAD-6 was used to discriminate the three *D^VI types* by immunohematologic methods, which also allowed separation from normal controls (Fig 8).

DISCUSSION

The population-based study showed that the variability of the D^VI phenotype is greater than previously reported for the underlying molecular structures[18-20] and the RhD antigen densities.[15,36-39] We characterized a *D-Ce(3-6)-D* hybrid allele of the *RHD* gene. In accordance with the previous nomenclature,[18] this new allele was dubbed *D^VI type III*. Its D^VI type III phenotype is associated with an almost normal number of RhD proteins accessible on the red blood cells' surface. All three D^VI types and RhD controls showed distinct immunohematologic features in flow cytometry. The distribution of the *D^VI types* varied significantly even within German-speaking populations.

The observation of a *D-Ce(3-6)-D* hybrid allele, which represented a D^VI phenotype, contributed considerably to the understanding of the immunoreactivity in partial D. The D^VI phenotype is caused by several different genotypes that are strictly associated with specific *Rhesus* haplotypes. As a common feature, all known *D^VI* genotypes shared *RHCE* exons 4 and 5 and *RHD* exon 7. Substitutions of *RHD* exon 4 or exon 5 alone by the corresponding exon of *RHCE* result in different partial D (exon 4: DFR, exon 5: D^Va),[40] the additional substitution of exon 7 results in the loss of most[41] or all[42] RhD immunoreactivity. Our report of a *D-Ce(3-6)-D* allele proved

Table 1. Distribution of D^{VI} Types in German-Speaking Populations

Regional Origin	D Category VI Samples Observed (n) Type I	Type II	Type III	Total*
Tyrol (Austria)†	9	0	0	9
Southwestern Germany†	5	7	3	15
Northern Germany	0	2	0	2
Total	14	9	3	26

*The D^{VI} samples were found by a serologic survey of RhD-positive samples including weak D (southwestern Germany) and by molecular screenings of weak D samples (northern Germany and Tyrol). All D^{VI} samples of the serologic survey were found in weak D as previously published.[8]

†The observed distributions of the various D^{VI} types in Tyrol and Southwestern Germany were statistically significantly different ($P < .01$, Brandt-Snedecor-χ^2-test for 2×3 contingency tables with correction for multiple testing (n = 3) according to Bonferroni-Holm).

that in contrast to exon 7, *RHD* exon 3 is not necessary for a D^{VI} phenotype. This observation supported the current RhD loop model.[43,44] All polymorphic sites of exon 3 and exon 6 are believed to occur in the transmembrane and intracellular portions, and hence, may not be expected to influence RhD immunoreactivity very much. In contrast, the polymorphic amino acids of the extracellular loops 3, 4, and 6 are determined by exons 4, 5, and 7, respectively. In concordance with several recent reports,[19,20,45] we were unable to find the ''deletion type''[18] that has been proposed for the cDE haplotype of D^{VI}. However, the $ccD^{VI}ee$ phenotype observed in one individual[37] likely represented a fourth *D category VI* genotype (proband lost to follow-up; J.W. Jones, personal communication, 1996). Interestingly, the D-Ce(3-6)-D hybrid protein (*D^{VI} type III)* is complementary to the Ce-D(2-6)-Ce hybrid protein. The latter hybrid protein causes some Evans (D··) phenotypes,[46,47] encodes several RhD epitopes, and lacks all CcEe antigens.

An unexpected feature of D^{VI} type III was its almost normal number of RhD proteins per cell. The determination of epitope density profiles in D^{VI} samples gave unequivocal evidence that the lack of certain RhD epitopes need not correlate with the loss of RhD proteins per cell. The observation of the D^{VI} type III phenotype provided a formal proof that the limited RhD immunoreactivity detected with polyclonal anti-Ds in D^{VI} type I and D^{VI} type II[37] cannot be explained by the lack of these RhD epitopes only, but must be due to a reduced number of RhD proteins accessible on the red blood cells' surface. The Rhesus protein conformation is likely to influence its red blood cell membrane integration. However, there is currently no conclusive model to predict the effect of any substitution on RhD protein expression. This is exemplified by the different *D^{VI} types* showing a paradoxical, inverse correlation between the size of the substituted protein segments and the RhD antigen density. Substitution of exon 3[48] increases RhD antigen density[37] in D^{IIIc}, also. Furthermore, single residue substitutions as occurring in D^{VII}[49,50] and DNU[51] may have considerable effects on RhD antigen density.[32,37]

It is intriguing to note that all three *D^{VI} types* may be explained by gene conversion events occurring among both *Rhesus* genes in cis position. The molecular structures of most *Rhesus* hybrids (D^{IIIb},[52] D^{Va},[40] hybrid-VS,[42] DBT[53]) are also compatible with this proposed mechanism. Only one *RHD* hybrid characterized so far (Rh D-E variant ISBT49[54]) seemed to be caused by a gene conversion in trans position. The impression that conversions in trans position were predominant in *RHCE* hybrids (R^N,[55] R_0^{Har},[56] and r^{G}[57]) is likely due to an observation bias because *RHCE* hybrids will almost exclusively be detected in RhD negative samples.

Table 2. Reactivity of DVI Types With Polyclonal Anti-D and Anti-BARC

Proband's Antiserum	Proband's Genotype	D Category VI Samples Type I (n)	Type II (n)	Type III (n)	RhD Positive Controls (n)
Anti-D	D^{VI} type I	− (1)	− (1)	− (1)	++++ (2)
Anti-D	D^{VI} type II	− (1)	− (1)	− (1)	++++ (2)
Anti-BARC*	—	ND	+++ (6)	++++ (2)	− (3)

Abbreviation: ND, not determined.

*Anti-BARC (ISBT 004.052; RH52) eluate kindly provided by Drs G. Daniels and C. A. Green.

Table 3. RhD Epitopes Expressed by the D^{VI} Types as Detected by Monoclonal Anti-D

RhD Epitopes	D Category VI Type I (n = 1)	Type II (n = 1)	Type III (n = 1)	Monoclonal Anti-D* Tested (n)
epD1	Negative	Negative	Negative	2
epD2	Negative	Negative	Negative	2
epD3	Negative	Negative	Negative	1
epD4	Negative	Negative	Negative	1
epD7	Negative	Negative	Negative	2
epD10	Negative	Negative	Negative	7
epD11	Negative	Negative	Negative	1
epD12	Negative	Negative	Negative	6
epD13	Negative	Negative	Negative	6
epD15	Negative	Negative	Negative	9
epD17	Negative	Negative	Negative	9
epD18	Negative	Negative	Negative	8
epD21	Negative	Negative	Negative	5
epD22	Negative	Negative	Negative	2
epD31	Negative	Negative	Negative	2
epD32	Negative	Negative	Negative	1
epD33	Negative	Negative	Negative	1
epD34	Negative	Negative	Negative	1
epD35	Negative	Negative	Negative	1
epD5	Positive	Positive	Positive	5
epD6	Positive	Positive	Positive	1
epD23	Positive	Positive	Positive	8
epD36	Positive	Positive	Positive	4
epD37	Positive	Positive	Positive	4
	Reactivity With D Category VI Samples			
	Type I (n = 4)	Type II (n = 5)	Type III (n = 3)	Monoclonal Anti-D
epD3	Negative	Variable†	Variable	LORE
epD15	Negative	Variable	Variable	BIRMA-DG3
epD15	Negative	Variable	Variable	BTSN4
epD15	Variable	Variable	Variable	D90/7

*Clone identifications were listed in Materials and Methods as provided by Nantes Workshop.[35] All monoclonal anti-D were tested with an identical random sample of each D^{VI} type.

†Reactivity with the monoclonal anti-Ds indicated on the left side was either weak positive or negative.

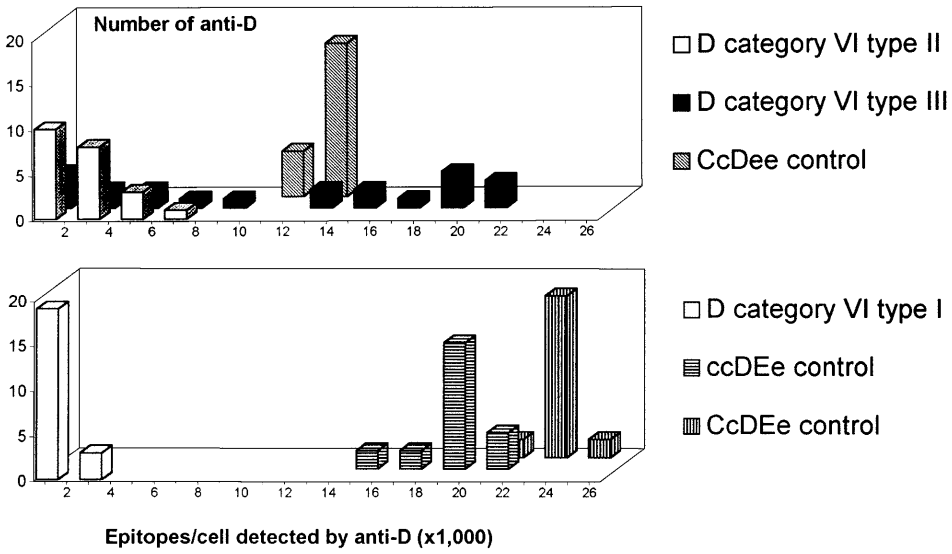

Fig 7. Epitope density profiles of samples with the three D^{VI} types and with normal RhD. On the abscissa, ranges of epitope densities (sites/cell) as detected by various anti-D are given. On the ordinate, the number of anti-D representing the particular ranges of sites/cell are shown. One representative sample is shown for each D^{VI} type. Epitope density profiles obtained with four additional D^{VI} type I, six additional D^{VI} type II, and two additional D^{VI} type III samples were similar.

We referred to *D^{VI} type III* as a *D-Ce(3-6)-D* hybrid, but a *D-Ce(2-6)-D* hybrid could not formally be excluded. The approximately 4,500-bp region encompassing exon 2 was reported to be identical between the *RHD* and *RHCe* alleles and to contain many repetitive elements.[58] The 5′ conversion point of *D^{VI} type III* resided in the region between the polymorphisms in intron 1 and intron 2 (Figs 2 and 3). A further characterization did not seem worthwhile because of the long stretch of identical sequences and repetitive elements in that region. The 5′ conversion points of several independent gene conversion events with substitutions in the *RHCe* allele by *RHD* sequences in D−− probands were shown by Kemp et al[58] to occur also in this stretch of identical sequences. It is tempting to speculate that the sequence identity over more than 4,000 bp including many repetitive elements facilitated conversion events. A similar accumulation of repetitive Alu and LINE elements (Fig 3) occurred adjacent to the breakpoint region of *D^{VI} type II* in intron 3, which hosted the conversion points of four *RHD*/*RHCE* hybrids.[34]

Characterization of the 3′ breakpoint regions of the three *D^{VI} types* (Fig 5) showed that their breakpoints were not clustered. The breakpoint of *D^{VI} type I* occurred in a stretch of 195 bp covering parts of intron 5 and exon 6, that of *D^{VI} type III* in a stretch of identity between *RHD* and *RHCE* over 605 bp including an Alu repeat. The extent of the whole gene conversion sequence thus varied between about 4,800 bp (*D^{VI} type II*) and > 19,500 bp (*D^{VI} type III*). The breakpoint region of *D^{VI} type II* in intron 6 was identical to that recently described by Matassi et al.[34] These findings are compatible with a common origin (identity by descent) of all *D^{VI} type II* samples described so far in France, the Netherlands, and Germany.

Our quantitative RhD epitope analysis of molecularly characterized samples clarified several previously controversial issues of D^{VI} immunohematology. First, the use of epitope density profiles addressed the problem of variable antibody affinities in partial D. Studies based on single or few monoclonal antibodies[15,36] were likely to underestimate the true number of RhD proteins accessible on the red blood cells' surface, in particular,

Table 4. RhD Antigen Density of D^{VI} Types

D Category VI	RhD Antigen Densities (antigens per cell)*			% of Reference‡	D^{VI} Samples Tested (n)§
	Median	Mean†	Range		
Type I	502	489	204-1,169	3	5
Type II	2,458	2,049	634-3,941	20	7
Type III	13,294	12,699	11,018-13,981	106	3

*The RhD antigen density of a sample was calculated as median of the epitopes per cell detected by the 22 IgG monoclonal anti-D.

†Geometric mean of the RhD antigen densities.

‡Median RhD antigen density as percentage of control cells with comparable Rhesus phenotypes (CcDee 12,532 RhD antigens/cell; ccDEe 19,062).

§The RhD antigen densities of all three *D^{VI}* types were significantly different from one another ($P < .007$, *t*-test with correction for multiple testing (n = 3) according to Bonferroni-Holm).

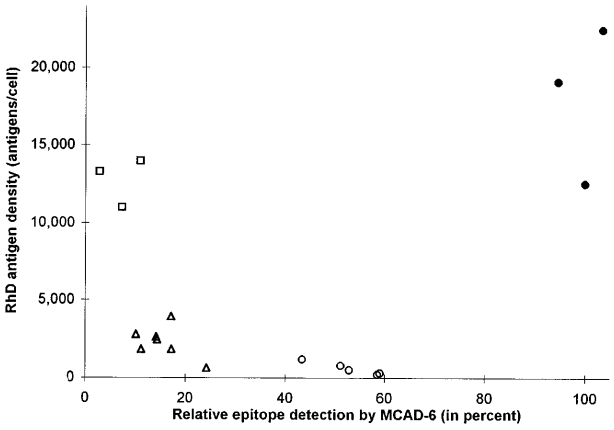

Fig 8. Distinct immunohematologic features of the three D^{VI} types. The RhD antigen density is plotted on the ordinate. On the abscissa, the relative epitope detection by MCAD-6 is shown. This parameter was calculated as follows: [epitopes per cell detected by MCAD–6] ÷ [RhD antigen density] × 100%. Data of 15 D^{VI} samples and three controls are shown. ○, D^{VI} type I, n = 5; △, D^{VI} type II, n = 7; □, D^{VI} type III, n = 3; ●, controls, n = 3.

if the anti-D used[36] happened to lack affinity for D^{VI}.[12] Even with conditions believed to be saturating, variable epitope densities were obtained with different anti-D.[37] We established epitope density profiles using a panel of monoclonal IgG directed to different RhD epitopes present in the partial D tested.[32] Such epitope density profiles in D^{VI} showed the variability of anti-D affinities. In difference to other partial D like D^{VII} and DNU,[32] there was no narrow antigen density peak (Fig 7), and therefore, the median of the results of all antibodies was used. This robust approach may slightly underestimate the RhD antigen density as correlate of the true number of RhD proteins accessible on the red blood cells' surface because antibodies of marginal affinities to D^{VI} were not excluded. However, our results for *D^{VI} type I* and *D^{VI} type II* were in good agreement with previous reports.[37]

Second, the immunohematologic features were correlated with molecular structures instead of serologic haplotypes. Previously, the influence of the molecular structures were not checked. Controversial results indicating low[36-38] or variable[15,39] RhD antigen densities may simply reflect the absence or presence of *D^{VI} type III* samples in the CcD^{VI}ee group tested. The close linkage of *Rhesus* haplotype and molecular structure also explains the observation[37] that the presence of C suppresses RhD antigen density in normal RhD samples,[59,60] but not in D^{VI} samples.[37] In D^{VI}, the slight suppressive effect of antigen C was overwhelmed by the effects of the molecular structures as the principal determinants of RhD protein expression.

Third, the quantitative analysis by flow cytometry separated overall RhD antigen density caused by variations in the number of RhD proteins accessible on the red blood cells' surface from variable expression of certain RhD epitopes. Flow cytometry allowed differentiation of the *D^{VI} types* from one another and from normal RhD. Furthermore, we could demonstrate a flow cytometric split of RhD epitope epD37. However, the only qualitative serologic difference that we could correlate with the molecular structures was a paucity, but not lack, of epD37b on *D^{VI} type II* and *D^{VI} type III*. We suspect that some previously reported serologic splits[8,16,18,21,61,62] that were mainly observed with weak overall antibody reactivity[8,16,18,62] may be due to quantitative differences in RhD epitope expression rather than lack of certain RhD epitopes. We propose that a meaningful report of a serologic split in partial D should exclude the confounding effect of low antigen densities. This exclusion may be achieved by inverse reaction patterns of different monoclonal antibodies,[12] by quantitative methods like flow cytometry,[31,32] and enzyme-linked immunosorbent assay (ELISA)[37] or by the demonstration of different underlying molecular structures.

Our findings have several practical implications for RhD phenotyping and *RHD* genotyping. Comprehensive *RHD* genotyping is a complex task[24] because of many *Rhesus* hybrid genes[18,19,33,40,42,52,53,55-57,63] and of RhD-negative phenotypes still harboring *RHD*-specific sequences.[6,42,64,65] *D^{VI} type III* adds to this complexity. It would type *RHD* negative in a standard intron 2-based PCR method[25] previously believed to type D^{VI} samples reliably as *RHD* positive. The population study (Table 1) provided further evidence for the allelic variation between closely related populations, which influences the specificity and sensitivity of *Rhesus* genotyping. An absolute match of phenotype and genotype is unlikely to be achieved by current technology because sporadic nonfunctional alleles occur rather frequently in genes[66] including *Rhesus*.[67] Hence, the expense of a genotyping system must be weighed against its residual failure rate in phenotype prediction. *D^{VI}* is the clinically most important *RHD* variant and it might be advantageous to dissociate this variant both from *RHD* positive and *RHD* negative. To this end, a simple system testing intron 4 and exon 7 may suffice because any *D category VI* genotype is likely to lack both *RHD* exon 4 and 5 and to retain *RHD* exon 7.

D^{VI} recipients should be transfused with RhD negative blood to limit anti-D immunization,[17] a rationale that prompted RhD negative transfusion in patients carrying weak D. This essentially RhD antigen density-based transfusion strategy is today considered wasteful, as it became apparent that most weak D patients may be safely transfused RhD positive. The wastage might be reduced by lowering of the weak D threshold for RhD negative transfusion. However, this measure would trigger RhD positive transfusion in partial D like *D^{VI} type III*, while still many RhD negative units would be transfused to weak D patients not requiring RhD negative transfusion. In this context, a strategy based on two monoclonal anti-D that do not react with D^{VI} is advantageous.[8,17] This RhD epitope-based transfusion strategy abandons RhD antigen density as the trigger for RhD negative transfusions and became mandatory in Germany in 1996.[68] It should be advocated in all regions where D^{VI} is the single clinically important partial D. For donor typing, weak D is considered Rhesus positive.[69] *D^{VI} type III* proved that D^{VI} erythrocytes may carry rather high RhD antigen densities. The threshold of RhD antigen density and the RhD epitopes that most likely cause anti-D immunization are not fully established. We think the transfusion of D^{VI} red blood cells should be restricted to RhD positive individuals, until further evidence for lack of immunogenicity is established.

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