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

By Franz F. Wagner, Christoph Gassner, Thomas H. Müller, Diether Schönitzer, Friedrich Schunter, and Willy A. Flegel

Rhesus D category VI (DVI) is the clinically most important partial D. DVI 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 DVI. Twenty-six DVI 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 DVI types shown to be distinct. We differentiated the 5' breakpoints of DVI type I and DVI type II by a newly devised RHD-PCR. Thus, the DVI phenotype originated in at least three independent molecular events. Each DVI type showed distinct immunohematologic features in flow cytometry. The number of RhD proteins accessible on the red blood cells' surface of DVI type III was normal (about 12,000 antigens/cell; DVI type I, 500; DVI type II, 2,400) based on the determination of an RhD epitope density profile. DVI type II and DVI type III occurred as CD4 haplotypes, and DVI type I as a CE4 haplotype. The distribution of the DVI 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.

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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,4 blood samples in Ulm screened for differential reactivity with a monoclonal IgM anti-D (BS226; Biotest, Dreieich, Germany). We describe a novel molecular event that caused a DVI phenotype carrying a normal number of RhD proteins accessible on the red blood cells' surface. We show that the three DVI types may be readily discriminated by flow cytometry based on distinct immunohematologic features. We demonstrate considerable differences in the distribution of DVI types within German-speaking populations showing the importance of a full molecular description for Rhesus genotyping purposes, eg, in prenatal testing.

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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\textsuperscript{VI} and with polyclonal anti-D in antiguillin technique were checked for the D\textsuperscript{VI} phenotype by a panel of monoclonal anti-D (D-Screen; Diagast, Lille, France). The D\textsuperscript{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\textsuperscript{25} 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.\textsuperscript{26,27} 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 Poulet et al.\textsuperscript{25} The 3’ region of intron 3 was amplified with primers RB46 and RB5, RB12 and R14R2. 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 (Prism dye terminator cycle-sequencing kit with AmpliTaq FS DNA polymerase; ABI 373A, Applied Biosystems, Weiterstadt, Germany). 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).

**Results**

**Detection of Three Independent Molecular Events Causing D\textsuperscript{VI} Category IV**

Twenty-six D\textsuperscript{VI} samples were examined using RHD-specific PCR-SSP for exons 2, 3, 4, 5, 6, 7, 9, and 10 (3’ untranslated). All D\textsuperscript{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\textsuperscript{VI} type I (lack of RHD exons 4 and 5) and D\textsuperscript{VI} type II (lack of RHD exons 4 to 6). A third pattern could not be explained by any known RHD/RHCE variation and is hereby called D\textsuperscript{VI} type III.
Molecular Characterization of DVI Type III

Coding sequence. Because the PCR-SSP pattern of DVI 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. 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 DVI type III at the position of this polymorphism in intron 2 (Fig 2B). The discrimination between an RHCe- or RHc-origin of the DVI 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 CDVb/ce genotype, one from the D VI type III allele, the other from the Ce allele of the CDVb 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 DVI type III cDNA was compatible with both an RHD or an RHc origin. 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. 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 DVI Types

The 3’ breakpoints of DVI type II and DVI type III are different. To define the 3’ limits of the conversion regions of the three DVI types, we established the complete nucleotide sequence ranging from exon 5 to exon 7 including both introns 5 and 6. We found
the breakpoint of $D^I$ 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 $D^I$ 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 $D^I$ type II 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.

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.\textsuperscript{25} Agarose gels are shown with fragment lengths\textsuperscript{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\textsuperscript{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\textsuperscript{VI}ee type II and the CcDe 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 RH-specific products. The D\textsuperscript{V}I type III sample lacks the RHD-specific fragment (640 bp), whereas all other RhD positive samples show this fragment.

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 $D^I$ 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 $D^I$ type III allele is identical to RHD at these positions. As expected, $D^I$ type I and $D^I$ type II alleles are also identical to RHD (not shown). Nucleic acid sequence accession numbers were Z97362 (RHCE) and Z97363 (RHD).
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 (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^{VI}E haplotype, all D^{VI} type II (n = 9), and D^{VI} type III (n = 3) in the CD^{VI}e 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. 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 were confirmed. Four anti-D (BIRMA-DG3; BTSN4; D90/7; LORE), reported to be nonreactive, 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 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 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 of the anti-D (data not shown, P = 0.23 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
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 DVI type I, DVI type II and DVI 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).
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DVI type I and DVI type II. Still, the RhD antigen densities of DVI type I and DVI type II differed significantly.

Distinct immunohematologic features of the DVI types. Two of four monoclonal anti-D that are binding to epD37 detected fair numbers of RhD epitopes per cell for DVI type I, but rather low numbers for DVI type II and DVI 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 DVI 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 DVI 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 DVI types by immunohematologic methods, which also allowed separation from normal controls (Fig 8).

DISCUSSION

The population-based study showed that the variability of the DVI 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 DVI types and RhD controls showed distinct immunohematologic features in flow cytometry. The distribution of the DVI types varied significantly even within German-speaking populations. The observation of a D-Ce(3-6)-D 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 DVI 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
that in contrast to exon 7, RHD exon 3 is not necessary for a DVI phenotype. This observation supported the current RhD loop model. All polymorphic sites of exon 3 and exon 6 are likely represented a fourth genetic model to predict the effect of any substitution on RhD expression. Only one hybrid characterized so far (Rh D-E variant ISBT49) seemed to be caused by a gene conversion in trans position. The D-VI type III variant is likely 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 DVI types showing a paradoxical, inverse correlation between the size of the substituted protein segments and the RhD antigen density. Substitution of exon 3 (DHI) increases RhD antigen density in DVI, also. Furthermore, single residue substitutions as occurring in DVI (49,50) and DNU (51) may have considerable effects on RhD antigen density.

Table 1. Distribution of DVI Types in German-Speaking Populations

<table>
<thead>
<tr>
<th>Regional Origin</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrol (Austria)†</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Southwestern Germany</td>
<td>5</td>
<td>7</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Northern Germany</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>9</td>
<td>3</td>
<td>26</td>
</tr>
</tbody>
</table>

*The DVI 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 DVI samples of the serologic survey were found in weak D as previously published.†The observed distributions of the various DVI types in Tyrol and Southwestern Germany were statistically significantly different (P < .01, Brandt-Snedecor-χ²-test for 2 × 3 contingency tables with correction for multiple testing (n = 3) according to Bonferroni-Holm).

Table 2. Reactivity With DVI Types With Polyclonal Anti-D

<table>
<thead>
<tr>
<th>Proband's Antiserum</th>
<th>Proband's Genotype</th>
<th>DVI Samples</th>
<th>RhD Positive Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-D</td>
<td>DⅢⅢ type I</td>
<td>3 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Anti-D</td>
<td>DⅢⅢ type II</td>
<td>3 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Anti-BARC*</td>
<td></td>
<td>ND</td>
<td>2 (1)</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

Table 3. RhD Epitopes Expressed by the DVI Types as Detected by Monoclonal Anti-D

<table>
<thead>
<tr>
<th>RhD Epitopes</th>
<th>Type I (n = 1)</th>
<th>Type II (n = 1)</th>
<th>Type III (n = 1)</th>
<th>Monoclonal Anti-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>epD1</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>2</td>
</tr>
<tr>
<td>epD2</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>2</td>
</tr>
<tr>
<td>epD3</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>1</td>
</tr>
<tr>
<td>epD4</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>1</td>
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<tr>
<td>epD5</td>
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<td>Negative</td>
<td>Negative</td>
<td>9</td>
</tr>
<tr>
<td>epD6</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>9</td>
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<td>epD7</td>
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<td>epD8</td>
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<td>epD9</td>
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<td>epD13</td>
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<td>Positive</td>
<td>4</td>
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<tr>
<td>epD14</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>4</td>
</tr>
</tbody>
</table>

*Clone identifications were listed in Materials and Methods as provided by Nantes Workshop. All monoclonal anti-D were tested with an identical random sample of each DVI type.
We referred to D\textsuperscript{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\textsuperscript{58}. The 5\textsuperscript{th} conversion point of D\textsuperscript{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\textsuperscript{th} conversion points of several independent gene conversion events with substitutions in the RHCE allele by RHD sequences in D\textsuperscript{22} probands were shown by Kemp et al\textsuperscript{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\textsuperscript{VI} type II in intron 3, which hosted the conversion points of four RHD/ RHCE hybrids\textsuperscript{34}.

Characterization of the 3\textsuperscript{rd} breakpoint regions of the three D\textsuperscript{VI} types (Fig 5) showed that their breakpoints were not clustered. The breakpoint of D\textsuperscript{VI} type I occurred in a stretch of 195 bp covering parts of intron 5 and exon 6, that of D\textsuperscript{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\textsuperscript{VI} type II) and > 19,500 bp (D\textsuperscript{VI} type III). The breakpoint region of D\textsuperscript{VI} type II in intron 6 was identical to that recently described by Matassi et al\textsuperscript{34}. These findings are compatible with a common origin (identity by descent) of all D\textsuperscript{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\textsuperscript{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\textsuperscript{15,36} were likely to underestimate the true number of RhD proteins accessible on the red blood cells’ surface, in particular,

![Fig 7. Epitope density profiles of samples with the three D\textsuperscript{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\textsuperscript{VI} type. Epitope density profiles obtained with four additional D\textsuperscript{VI} type I, six additional D\textsuperscript{VI} type II, and two additional D\textsuperscript{VI} type III samples were similar.](image)

![Fig 8. Distinct immunohematologic features of the three D\textsuperscript{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\textsuperscript{VI} samples and three controls are shown. ○, D\textsuperscript{VI} type I, n = 5; △, D\textsuperscript{VI} type II, n = 7; □, D\textsuperscript{VI} type III, n = 3; ●, controls, n = 3.](image)

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

<table>
<thead>
<tr>
<th>D Category</th>
<th>RhD Antigen Densities (antigens per cell)*</th>
<th>% of Reference†</th>
<th>D\textsuperscript{VI} Samples Tested (n)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>Median: 502; Mean: 489; Range: 204-1,169</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Type II</td>
<td>Median: 2,458; Mean: 2,049; Range: 634-3,941</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>Type III</td>
<td>Median: 13,294; Mean: 12,699; Range: 11,018-13,981</td>
<td>106</td>
<td>3</td>
</tr>
</tbody>
</table>

*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\textsuperscript{VI} types were significantly different from one another (P < .007, t-test with correction for multiple testing (n = 3) according to Bonferroni-Holm).
if the anti-D used\textsuperscript{16} happened to lack affinity for $D^{VI}$,\textsuperscript{12} Even with conditions believed to be saturating, variable epitope densities were obtained with different anti-D.\textsuperscript{37} We established epitope density profiles using a panel of monoclonal IgG directed to different RhD epitopes present in the partial D tested.\textsuperscript{32} Such epitope density profiles in $D^{VI}$ showed the variability of anti-D affinities. In difference to other partial D like $D^{V}$ and DNU,\textsuperscript{31} 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.\textsuperscript{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\textsuperscript{36-38} or variable\textsuperscript{5,39} RhD antigen densities may simply reflect the absence or presence of $D^{VI}$ type III samples in the CdDV\textsubscript{ee} group tested. The close linkage of Rhesus haplotype and molecular structure also explains the observation\textsuperscript{37} that the presence of C suppresses RhD antigen density in normal RhD samples.\textsuperscript{59,60} but not in $D^{VI}$ samples.\textsuperscript{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 split\textsuperscript{8,16,18,21,61,62} that were mainly observed with weak overall antibody reactivity\textsuperscript{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,\textsuperscript{12} by quantitative methods like flow cytometry,\textsuperscript{31,32} and enzyme-linked immunosorbent assay (ELISA)\textsuperscript{37} or by the demonstration of different underlying molecular structures.

Our findings have several practical implications for RhD genotyping and RhD genotyping. Comprehensive RhD genotyping is a complex task\textsuperscript{24} because of many Rhesus hybrid genes\textsuperscript{18,19,33,40,42,52,53,55-57,63} and of RhD-negative phenotypes still harboring RhD-specific sequences.\textsuperscript{6,42,64} $D^{VI}$ type III adds to this complexity. It would type RhD negative in a standard intron 2-based PCR method\textsuperscript{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\textsuperscript{66} including Rhesus.\textsuperscript{54} 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,\textsuperscript{22} 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.\textsuperscript{8,37} This RhD epitope-based transfusion strategy abandons RhD antigen density as the trigger for RhD negative transfusions and became mandatory in Germany in 1996.\textsuperscript{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.\textsuperscript{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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