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D variants at the RhD vestibule in the weak D type 4 and Eurasian D clusters

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Abstract

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The last two authors are senior authors to this publication.
W.A.F. designed the study, collected samples, interpreted the data, and wrote the paper. I.v.Z. analyzed and interpreted data, and wrote the paper. A.D. and Ma.Pi. collected and analyzed samples critical to the study. F.F.W. collected and analyzed samples and contributed to the interpretation of the phylogeny. K.P.S., C.Ge., and Mi.Pa. collected samples critical to the study. J.P collected and serologically analyzed the *DOL-1/RHDYΨ* sample. H.P. and C.Ga. collected and analyzed the DOL-2 and most DFL samples. N.D.A. performed experiments, analyzed and interpreted data, and wrote the first draft of the paper.

CONFLICT OF INTEREST

W.A.F., I.v.Z., and F.F.W are current or former employees of the DRK Blutspendedienst Baden-Württemberg–Hessen. D.R.K. and W.A.F. are holding patents or have patents pending on nucleotide sequences and their use in molecular genetics of the Rhesus blood group.

N.D.A. is a member of the scientific advisory board of Progenika Biopharma, which developed BloodChip that tests for the known *DOL* alleles.

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BACKGROUND: One branch of the *RHD* phylogenetic tree is represented by the *weak D type 4* cluster of alleles with F223V as the primordial amino acid substitution. F223V as well as a large number of further substitutions causing D variants are located at the extracellular RhD protein vestibule, which represents the entrance to the transmembraneous channel of the RhD protein.

STUDY DESIGN AND METHODS: *RHD* and *RHCE* nucleotide sequences were determined from genomic DNA and cDNA. D epitope patterns were established with commercial monoclonal anti-D panels.

RESULTS: The *RHD* alleles *DOL-1* and *DOL-2* had the two amino acid substitutions M170T (509T>C) and F223V (667T>G) in common. *DOL-2* harbored the additional substitution L378V (1132C>G). Both alleles were observed in Africans and are probably evolutionary related. *DMI* carried M170I (510G>A), which differed from the DOL-typical substitution. DFW and DFL harbored the substitutions H166P (497A>C) and Y165C (494A>G). The antigen densities of DOL-1, DFL, and DFW were only moderately reduced.

CONCLUSION: *DOL-1* and *DOL-2* belong to the *weak D type 4* cluster of *RHD* alleles. Together with *DMI*, *DFL*, and *DFW* they represent D variants with amino acid substitutions located at extracellular loops 3 or 4 lining the RhD protein vestibule. These substitutions were of minor influence on antigen density while adjacent substitutions in the transmembraneous section caused weak D antigen expression. All these D variants were partial D and alloanti-D immunizations have been observed in DOL-1, DMI, and DFL carriers. The substitution at position 170 causes partial D although located deep in the vestibule.

A small fraction of D+ individuals have the potential to produce anti-D after exposure to D+ blood during transfusion or pregnancy. This apparent paradox was explained when the molecular bases underlying partial D were investigated.[1] Findings at the genetic level confirmed the hypothesis of Tippett and coworkers[2] that partial D phenotypes arise where part of the D antigen "mosaic" is lacking and that exposure to the complete D antigen could elicit an immune response to the missing parts of the mosaic. It was found that many partial D are variants of the prevalent RhD protein caused by single point mutations or by gene conversions between the *RHCE* and *RHD* genes. However, several partial D did not fit into this picture, because they harbor multiple point mutations that are dispersed throughout the *RHD* gene. The compilation of these alleles has been instrumental to construct an evolutionary tree of *RHD* alleles.[3]

The present phylogenetic model of *RHD* in humans discerns four allele clusters: the Eurasian D cluster with "normal" *RHD* as the primordial allele from which numerous alleles derive as well as three "African" groups of alleles designated DIVa, DAU, and weak D type 4 clusters.[4–6] Each of these African clusters is characterized by a distinct primordial amino acid substitution relative to the normal *RHD* allele: T379M in the DAU cluster, N152T in the DIVa cluster, and F223V in the weak D type 4 cluster. The existence of the allele *DFV*, corresponding to the single-substitution F223V, has been postulated[4] long before it was actually found in individuals of different ethnic origin. It represents the primordial allele of the weak D type 4 cluster, which comprises many clinically relevant alleles like *weak D type 4.0, 4.1*, and *4.2* (*DAR*) and *RHDΨ*.

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We describe several partial D carrying amino acid substitutions at positions 170 and 223, which are located at the extracellular RhD protein vestibule. The African partial D DOL-1[7–10] and DOL-2 harbor the F223V substitutions in a *cDe* haplotype, which qualifies them as members of the weak D type 4 cluster. Both DOL types also carry the amino acid substitution M170T. These substitutions are located at the RhD vestibule, which represents the extracellular entrance to the transmembraneous protein channel recognized by homology modeling.[11] The presently described *DMI* (M170I) and the known *DFV* (F223V), DCS-1 (F223V A226P), and DFR-1 (M169L, M170R, I172F) harbor substitutions at the amino acid positions 170 or 223. Furthermore, we describe the alleles *DFL*[12,13] and *DFW*[14] with single substitutions at positions 165 and 166, because of their proximity to position 170. We found that individuals carrying amino acid substitutions at the extracellular RhD protein vestibule were prone to making alloanti-D even if their substitution seemed to be located deep in the vestibule.

MATERIALS AND METHODS

Immunohematology

Serologic testing for agglutination was done by tube test with low-ionic-strength saline (LISS) and indirect antiglobulin test or in a gel matrix test (LISS-Coombs 37°C, DiaMed-ID Micro Typing System, DiaMed, Cressier sur Morat, Switzerland).[15] Polyclonal antisera against RH10, RH20, RH23, and RH32 were from the International Blood Group Reference Laboratory (IBGRL, Bristol, UK) and the Australian Red Cross Blood Service (ARCBS, Sydney, Australia) collections. For phenotyping the C antigen, monoclonal anti-C clones MS24 (Ortho, Neckargmünd, Germany) and MS273 (Immucor, Rödermark, Germany) were used. Anti-LW^a was obtained through the SCARF exchange program.

The mean D antigen density was determined by flow cytometry according to the protocol described previously[16] with monoclonal immunoglobulin G (IgG) anti-D BS221, BS227, BS228, BS229, BS231, and H4111B7 (Biotest, Dreieich, Germany). The secondary antibody was goat anti-human IgG, Fab-fragment, fluorescein isothiocyanate conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA).

Molecular analysis of genomic DNA

RHD nucleotide sequencing from genomic DNA for the *RHD* exons 1 through 10 including adjacent flanking intron regions was performed as described previously.[5,12,15,17–19] The presence of the VS typical nucleotide substitution 733C>G was assessed by sequencing of *RHCE* exon 5; *RHCE* exon 1 was sequenced to investigate the concomitant presence of the nucleotide substitution 48G>C.[20] The LW^a molecular polymorphism was determined by sequencing of *ICAM4* exon 1.[21]

Molecular analysis of cDNA

The nucleotide sequence of *DOL-1*, as part of the compound heterozygous *DOL-1/(C)cde*^{s} and *DOL-1/RHDΨ* genotypes, was also determined from cDNA. Total RNA was isolated in one method from whole blood (RiboPure blood kit, Ambion, Austin, TX). RNA was reverse transcribed and cDNA was amplified in one-step reverse transcription buffer (SuperScript,

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Invitrogen, Carlsbad, CA), 2.5 mmol per L $MgSO_4$, 500 nmol per L of each of the primers rh5[22] and rr3,[22] and 1 μL of reverse transcriptase/polymerase (SuperScript II reverse transcriptase, platinum *Taq* DNA polymerase, Invitrogen) in a total volume of 50 μL. Incubation was carried out for 30 minutes at 55°C followed by 2 minutes at 94°C; thereafter 60 cycles were performed with 30 seconds at 92°C, 30 seconds at 62°C, and 3 minutes at 68°C followed by a final incubation for 10 minutes at 72°C. The mixture was then kept at 10°C. Amplified cDNA products were treated with exonuclease and alkaline phosphatase (ExoSAP, USB, Cleveland, OH). Nucleotide sequencing was performed with a cycle sequencing kit and a DNA sequencer (ABI 310, Applied Biosystems, Foster City, CA). Twenty-five cycles were performed, each cycle for 15 seconds at 94°C, 15 seconds at 58°C, and 4 minutes at 60°C; the mixture was finally kept at 10°C.

Primers used

RHD cDNA nucleotide sequencing primers were D150r, 5′-AACTTGATAGGATGCCACGAGCCC-3′ (antisense, cDNA nucleotide position 150-127); DCE91f, 5′-TTTACCCACTATGACGCTTC-3′ (sense, 91-110); D4s, 5′-ACATGATGCACATCTACGTGTTCGC-3′ (sense, 503-527); and D1036f, 5′TTGCTGGTGCTTGATACC-3′ (sense, 1036-1053).

Nomenclature

The designation DOL was derived from the D antigen and the first two letters of the last name of the patient with *DOL-1/RHDΨ*.[7] DOL-2 is molecularly similar to DOL-1. The designations DFW and DFL (RIR-16)[23] were derived from DFR-like and Württemberg or Linköping, respectively, where the original observations were made. DMI represents *RHD*(M170I) and was named after its amino acid substitution M>I.

RESULTS

RHD alleles

In 1994 a D+ young male patient was recognized because he had developed an alloanti-D. His partial D did not fit into the classification of D categories. The underlying allele was *DOL-1* (Table 1),[13] which the patient carried in combination with *(C)cde^s*. Since then, *DOL-1* was discovered in two young female patients. One was hemizygous *DOL-1*, and the other was heterozygous *DOL-1/RHDΨ*. The name DOL was derived from the initials of the *DOL-1/RHDΨ* patient.7 *DOL-2* was detected in a child due to weak D antigen expression. Her allele harbored an additional amino acid substitution compared to *DOL-1*. Another female patient carried the D variant DMI with a single substitution at amino acid position 170 (Table 1). DFW and DFL were two more variants with adjacent amino acid substitutions. *DOL-2* was inherited from the father who carried *DOL*-2 in combination with a normal *RHD* (Fig. 1).

Anti-D immunization

A woman with DFL was reported to the Rhesus Immunization Registry (RIR-16),[23] because of an anti-D acquired by transfusion or pregnancy despite a D+ phenotype (Table 2). The patient with *DOL-1/(C)cde^s* was double heterozygous for sickle trait (hemoglobin S) and

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β-thalassemia. Treatment of sickle thalassemia with two red blood cell (RBC) transfusions was the probable cause of anti-D immunization (RIR-27). An anti-D was found in a woman with DMI 5 months after D+ RBC transfusion (RIR-110). No anti-D was detected in the other 21 DFL and 3 DOL carriers.

Immunohematology

The D epitope (epD) patterns of D variants were determined using panels of monoclonal anti-D (Table 3).[24] Results are shown for the hemizygous DOL-1 sample, for DOL-2, DMI, DFW, and DFL in comparison to the known patterns of DCS-1, DFR-1, and DFV.[21] Weak D type 4.0 was used as a weak D control with *cDe* haplotype. A D+ phenotype was usually assigned to DOL-1, DMI, DFW, and DFL using the routine monoclonal anti-D (Table 3). The antigen densities determined for DOL-1, DFW, and DFL were typical for a D+ phenotype (Table 4).[25] Based on its antigen density, DFR can be typed D+, but routine monoclonal anti-D often miss DFR-1 because it lacks several epitopes, especially parts of epD 6.

All *DOL* alleles observed in this study were associated with the *cDe* haplotype. The samples with hemizygous *DOL-1*, with *DOL-1/RHDΨ* and with *DOL-2* had a ccDee phenotype. The weakened expression of the C antigen in the sample of the patient with *DOL-1/(C)cde*^{s} was explained by the presence of the *(C)cde*^{s} allele in trans: agglutination titers were similar for this sample and controls of known *(C)cde*^{s} using 11 monoclonal anti-C (Table 5).[26]

The *DOL-1/RHDΨ* sample was antigen G positive (RH12) and negative for the antigens V (RH10), VS (RH20), D^w (RH23), RH32 and FPTT (RH50); the *DOL-1/(C)cde*^{s} sample was VS positive. According to molecular analysis the hemizygous *DOL-1* carrier was *VS* negative while the two *DOL-2* carriers were *VS* positive. However, *DOL-2* was inherited independent of *VS* (Fig. 1). We concluded that the *DOL-1* and *DOL-2* alleles were not associated with VS.

Ethnic origin

The *DOL-1/(C)cde*^{s} patient (Table 2) lived in Germany but was born in Togo[27] and his family belonged to the Ewe for at least three generations. The Ewe people are with 22 percent the largest ethnic group of Togo and live in southeast Ghana, southern Togo, and southern Benin. The individual with *DOL-1/RHDΨ* lived in Australia but came from Botswana. The individual with hemizygous *DOL-1* was Czech, but her father originated from Lebanon. The parents of the *DOL-2* baby lived in Austria but were natives of Ghana. The ethnic origin was German for the *DMI* carrier and German and Sri Lankan for the *DFW* carriers. The original DFL carrier was Swedish; 1 German and 20 Austrian carriers have been observed since.

DFL population survey

DFL carriers were identified among blood donors of Upper Austria[12] on the basis of weak reactions with monoclonal anti-D HM10. Between August 15, 2005, and July 31, 2008, we found 11 DFL carriers among 103,251 donors (frequency estimate 1 in 9386; 95% confidence interval, 1 in 5420 to 1 in 19,397, Poisson distribution). Considering this

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frequency in donors, at least 71 to 253 carriers of the DFL phenotype would be predicted to occur in the population of 1,370,000 inhabitants of Upper Austria. Twenty DFL carriers have already been identified at the Linz blood center among donor and patient reference blood samples, including one individual with the compound heterozygous alleles *DFL/weak D type 3*.

DISCUSSION

DOL-1 and *DOL-2* are African alleles. The probands originated from Western and Southern Africa (three cases) or the Middle East (one case), although they were recognized in Germany, Australia, the Czech Republic, and Austria because of their variant D antigens. The three probands from Africa with DOL carried further *RHD* or *RHCE* alleles typical of African populations, such as *(C)cde^s*,[28] *RHDΨ*,[18] and *VS*.[28,29] People of African ancestry are frequently heterozygous for two different variants of *RHD* alleles. The exact molecular basis of such compound heterozygous *RHD* alleles is difficult to define without transcript analysis.

Phylogenetically *DOL* alleles belong to the weak D type 4 cluster, because they share the templated F223V substitution and occur in a *cDe* haplotype (Fig. 2).[4] In a templated mutation, an isolated *RHD*-specific nucleotide is replaced by its *RHCE*-specific counterpart, the mechanism of which may be a short gene conversion or a single point mutation. Characteristic of *DOL-1* is the additional M170T substitution and of *DOL-2* the additional M170T and L378V substitutions, both of which are nontemplated. Hence, *DOL-2* probably evolved from *DOL-1*. Compatible with this assumption were the origins of one DOL-1 carrier and the DOL-2 family, who came from the neighboring West African states of Togo and Ghana.

The templated 667T>G substitution causing F223V represents the primordial event in the evolution of the African weak D type 4 cluster (Fig. 2). As postulated when the phylogenetic tree was initially established,[4] a *DFV* allele was found carrying F223V in isolated form and occurring in a *cDe* haplotype.[25] Recently, two additional *DFV* alleles were observed associated with the haplotypes *CDe*[6] and *cDE*.[21] Based on the differing haplotype associations, these two *DFV* alleles may be grouped to the Eurasian D cluster; they probably evolved independently of the primordial allele of the weak D type 4 cluster (Fig. 2).[21] Hence, a specific *RHD* allele may belong to more than one cluster. F223V is also found in the group of *DV* alleles, which are probably caused by *RHD-CE-D* gene conversions involving DNA stretches of the *RHCE* gene of different lengths. Likewise, the partial D DCS-1 carrying F223V and A226P may have arisen from a gene conversion.

Crossing over events may have caused the *DAU-5* allele (recombination of *DV type 1* with *DAU-0*)[5] as well as the *RHD*-negative alleles of different haplotypes (Fig. 2). There are examples of *RHD* alleles, for which different evolutionary routes are probable. For instance, *DFR-1* is often associated with *Ce*, rarely with *cE*. Hence, *DFR-1* associated with *cE* may have derived from a chromosomal crossing over. Alternatively, *DFR-1* associated with *cE* may have arisen from a gene conversion, where a stretch of amino acids including the three *DFR*-typical positions was transposed from the *RHcE* gene to the *RHD* gene. For some

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alleles relevant data are still missing and their definite position in the phylogeny is pending. *RHCE* associations may need reevaluation once more data will have accumulated, especially since there seems to be a relevant frequency of recombination events; for example, *ceAR* and *ceEK* may also be associated with *RHD* deletions.

The phylogenetic derivation of alleles may provide insight in older nucleotide substitutions that define whole groups of alleles, like D clusters, and are instrumental for devising efficient genotyping strategies. As exemplified by DFV, alleles can be predicted to occur before their actual observation. Systematic characterization will allow simplifying a hitherto confusing assortment of seemingly unrelated alleles.

The partial D DFL, DFW, and DMI with single amino acid substitutions at positions 165, 167, and 170, respectively, were allocated to the Eurasian D cluster, because the underlying nucleotide substitution occurred in the Eurasian *RHD* allele. In addition, their haplotype association was *CDe* and all probands encountered were from Europe or Asia.

As evident from three-dimensional homology modeling, the DOL typical amino acid substitutions F223V and M170T lie at the entrance to the transmembrane channel of the RhD protein, which is known as the extracellular RhD protein vestibule (Fig. 3).[11] Amino acid residue 170, which seems to be located deep in the vestibule,[11] is particularly variable with M170T in DOL-1 and DOL-2, M170I in DMI, and M170R in DFR. All of these alleles are partial D and several anti-D immunizations have been observed (Table 2).[30] Moreover, the *RHce*(R170S) allele was described[31] expressing the D antigen without D-specific amino acids.[20,32] Hence, antigenic defects (Table 2)[30] and neoantigens[31] have been found in this region of the Rh protein. According to the Rh “antigenic vestibule” concept presented by Avent and colleagues,[11,33] most RhC→RhD amino acid substitutions critical for Rh antigenicity lie in exofacial positions (i.e., localized at loops 3, 4, and 6) and line the boundary of the antigenic vestibule.[33] Furthermore, several D variants harbor amino acid substitutions at these loops. So far, 18 D variants have been identified at loops 3 and 4 and the adjoining helices 6 and 7 (Table 4). D variants with substitutions in extracellular parts were partial D with normal or moderately depressed D antigen density. D variants with substitutions in transmembraneous sections were weak D with low D antigen expression. The unexpectedly low D antigen density of the partial D DCS-1 and DCS-2 was previously explained by the presence of proline at position 226.[21]

The antigenic relationship of DOL-1 and DOL-2 with DFV DCS-1, DMI, and DFR was investigated, because these D variants share amino acid substitutions either at positions 223 or 170. F223V seems to have little influence on the protein structure, because DFV was almost undistinguishable from normal D (Table 3).[21] In contrast, DOL and DCS-1 phenotypes showed distinct serologic profiles (Table 3). Therefore, the additional M170T and A226P substitutions, respectively, seem to induce structural changes reflected in serologically recognizable differences in antigenicity. The serologic profile of DOL-1 was similar to that of DOL-2 and DCS-1. Compared to DOL-1, DOL-2 carried an additional conservative substitution located in the transmembraneous helix 12; this substitution was of minor influence on antigenicity. The serologic profiles of DOL, DMI, DFW, and DFL were compatible with partial D. Despite some similarity between these D variants and DFR,

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the overall epD pattern showed considerable differences (Table 3). DFR expresses the low prevalence antigen FPTT (RH50),[30] which was absent in DOL-1.

DOL, DFL, and DMI qualified as partial D also by the presence of alloanti-D after D+ transfusion (Table 2). DFL carriers are quite frequent in Upper Austria with 20 cases detected so far. Nevertheless, only one anti-D immunization in a Swedish individual (RIR-16) was reported to the Rhesus Immunization Registry.[23] Therefore, anti-D immunizations in DFL carriers are probably infrequent. DOL carriers are typed D+ by standard monoclonal anti-D reagents (e.g., BS226 and RUM-1, Table 3) and will be transfused with D+ RBC units and not receive prophylactic anti-D in pregnancy. It will be important to determine the frequency of the *DOL* allele in the African population to assess the risk for anti-D formation after transfusion or pregnancy. With *DOL-1* and *DOL-2, DFL, DFW*, and *DMI*, further alleles are added to the growing list of *RH* variants that are difficult to discern by current serologic methods, but are clearly amenable to specific detection by blood group genotyping.

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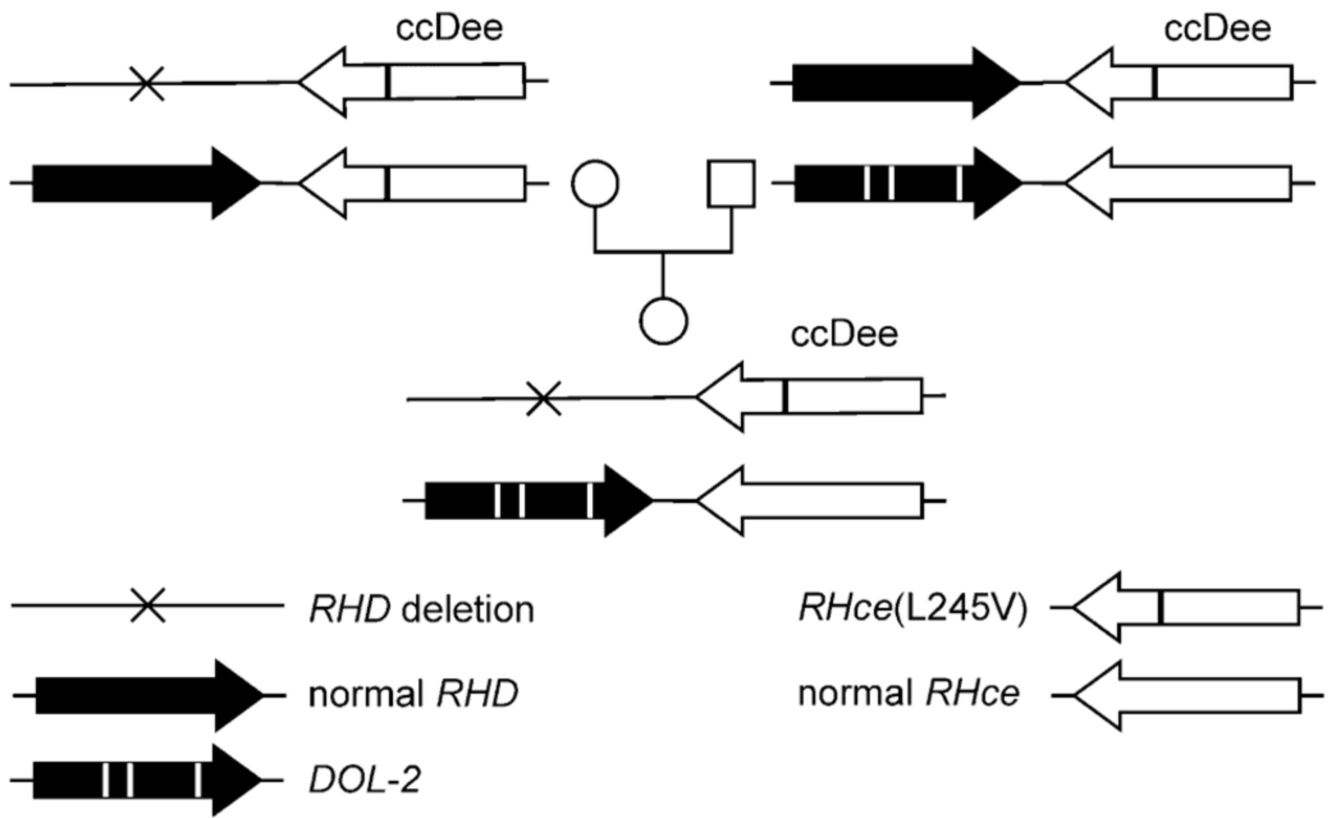

Fig. 1.

Inheritance of the *DOL-2* allele. The Rhesus phenotype of the child with DOL-2 and her parents are shown along with their *RHD* and *RHCE* haplotypes. The normal *RHce* allele represents GenBank Accession Number DQ322275.

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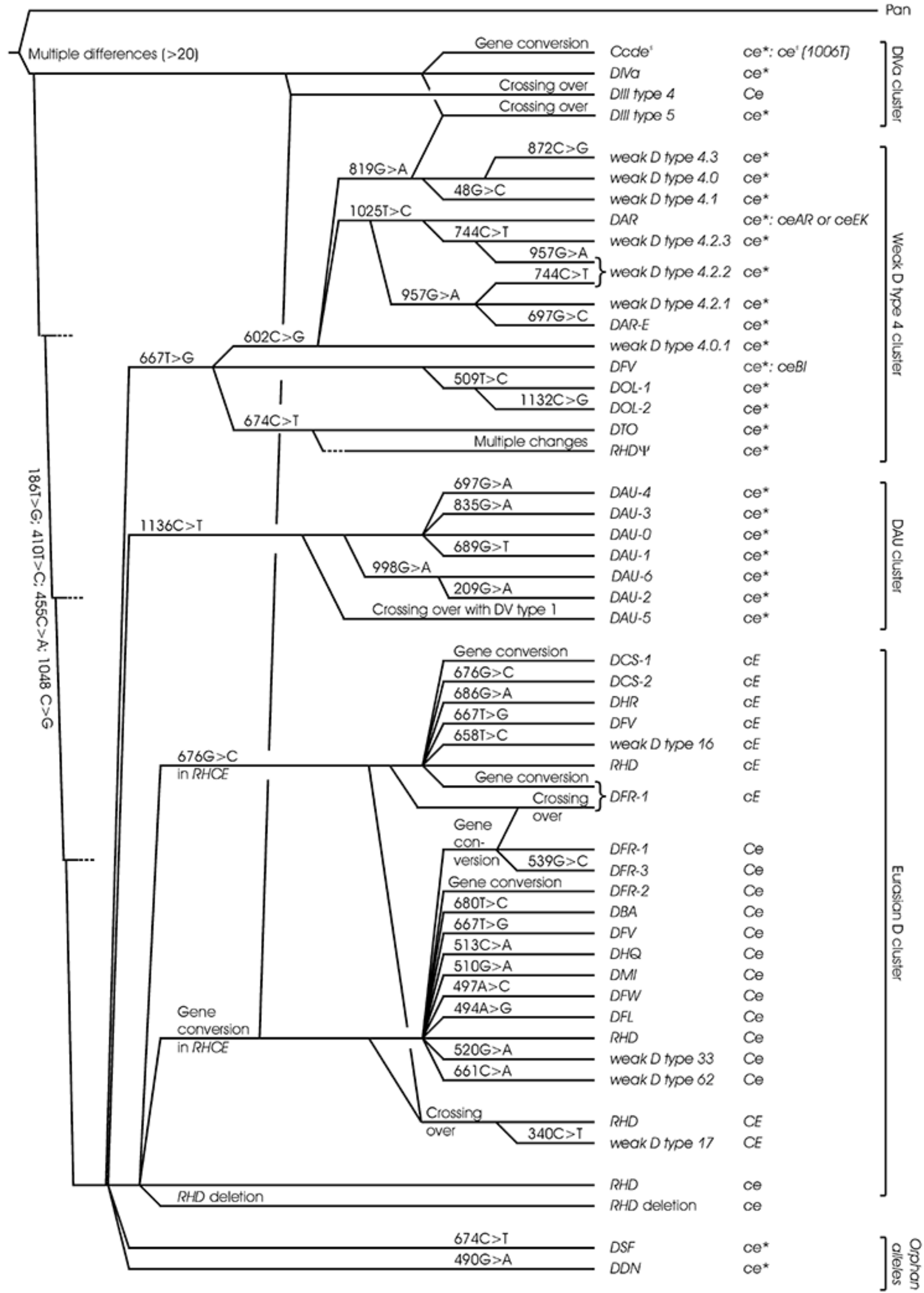

Fig. 2.

Phylogeny of *Rhesus* haplotypes. The phylogenetic tree is based on a previous tree for *RHD*[20] taking into account the available data on *Rhesus* haplotypes. Four main clusters of *RHD* alleles have been described. The *RHD* alleles of the DIVa cluster share the three characteristic ancestral amino acids 62F, 137V, and 152T; those of the weak D type 4 cluster the F223V substitution; and those of the DAU cluster the T379M substitution. These three "African" clusters are segregated from the Eurasian D cluster with "normal" *RHD* as the primordial allele. The *RHD* alleles of the three African clusters generally occur in a *cDe*

haplotype, which indicates that the *cE* and *Ce* alleles of *RHCE* evolved in the Eurasian branch after its divergence from the other branches. For each *RHD* allele, the accompanying *RHCE* allele is indicated. *ce** is a general indicator of a *ce*-like allele which frequently may be a variant; if known, the typically associated *RHCE* alleles are given.

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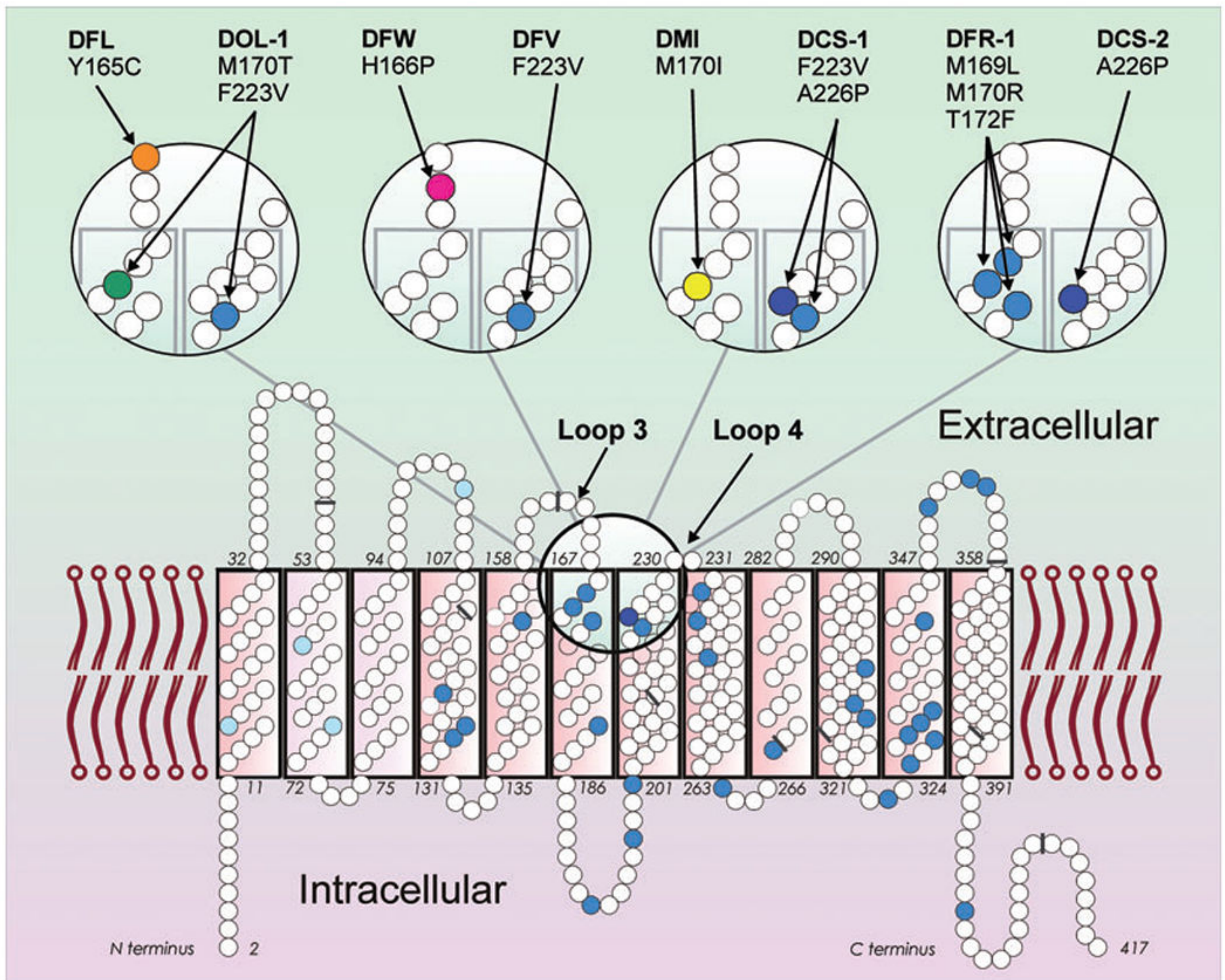

Fig. 3.

Partial D with amino acid substitutions at the extracellular RhD protein vestibule. The two-dimensional model of the RhD protein (bottom) with 417 amino acids (small circles) depicts amino acids that differ in RhCE (blue) with the four C-typical substitutions (light blue) and the one E-typical substitution A226P (dark blue). The vestibule is lined in part by amino acids of loops 3 and 4 (large circle). This region is shown in more detail (top). The amino acid substitutions characteristic of eight partial D are indicated (colored circles and arrows). Four partial D harbor RhCE-like substitutions (blue circles). The nine exon boundaries in the *RHD* cDNA, as reflected in the amino acid sequence, are indicated (gray bars).

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TABLE 1.

Partial D at the extracellular RhD protein vestibule described in this study

Trivial name	Exon involved	Allele*	Nucleotide change	Effect on protein sequence	Membrane localization	Phenotype	Haplotype	Number of probands
DOL-1	4	*RHD*(M170T, F223V)	509T>C	Met to Thr at 170	Rh vestibule	ccDee†	*cDe*	3
	5		667T>G	Phe to Val at 223	Rh vestibule			
DOL-2	4	*RHD*(M170T, F223V, L378V)	509T>C	Met to Thr at 170	Rh vestibule	ccDee	*cDe*	1
	5		667T>G	Phe to Val at 223	Rh vestibule			
	8		1132C>G	Leu to Val at 378	Transmembraneous			
DMI	4	*RHD*(M170I)	510G>A	Met to Ile at 170	Rh vestibule	CcDee	*CDe*	1
DFW	4	*RHD*(H166P)	497A>C	His to Pro at 166	Rh vestibule	CcDee	*CDe*	2
DFL‡	4	*RHD*(Y165C)	494A>G	Tyr to Cys at 165	Rh vestibule	CcDee	*CDe*	22

*The nucleotide sequence data were deposited in EMBL under Accession Numbers AM087651 (cDNA) and FM201788 (genomic DNA) for *DOL-1*, AM072761 (genomic DNA) for *DOL-2*, and AM998551 for *DMI.*

†Weak C antigen expression in one *DOL-1* proband was caused by $Ccde^{s}$ in trans. The other two *DOL-1* probands carried *DOL-1/RHDΨ* and hemizygous *DOL-1*, respectively.

‡The nucleotide sequence data were deposited in EMBL under Accession Numbers AM072758 and AM177314. The phenotype was CcDee in 21 samples and CCDee in 1 heterozygous DFL/weak D type 3 sample. An independent observation was reported from France (Accession Number EF105442).[13]

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TABLE 2.

Probands with alloanti-D

Case*	*RHD* alleles	Phenotype	Anti-D titer	DAT	Possible immunization date	LW blood group	Ethnicity
RIR-16	*DFL*	CcDee	32	Negative	Before 1997	LW(a+)	Swedish
RIR-27	*DOL-1/(C)cde*^{s}	(C)cDee	128	Negative	1992	LW(a+)	Ewe (West Africa)
RIR-110	*DMI*	CcDee	4	Positive	2007	*LW*A/LW*A*	German

*Rhesus Immunization Registry (RIR) entries 16, 27, and 110; data available online.[23]

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TABLE 3.

Serologic reactivity with commercial panels of anti-D

Monoclonal anti-D			Reactivity*								
Clone	Isotype	epD†	DOL-1 ccDee	DOL-2 ccDee	DMI CcDee	DFW CcDee	DFL CcDee	DCS-1 ccDEe	DFR-1 CcDee	DFV ccDEe	Weak D type 4.0 ccDee
BS226‡	IgM	6.4	++	++++	+++	++	+++	++	–	++++	+++
BS232‡	IgM	6.4	++	ND	+++	++	+++	++	–	++++	+++
RUM-1‡	IgM	6.1	+++	ND	+++	+++	+++	++	–	++++	++++
D175-2‡	IgM	6.1	+++	ND	++++	++++	+++	+++	+	++++	+++
HM10§	IgM	6.6	+	(+)	++++	++	+	+	–	++++	+++
HM16§	IgG	6.4	+++	++++	ND	+++	+++	+++	–	++++	+++
P3X61§	IgM	6.1	++	++++	+++	+++	+++	+	–	++++	++++
P3X35§	IgG	5.4	+++	+	ND	–	+	+++	–	++++	+++
P3X212 11 F1§	IgM	8.2	–	–	–	–	–	–	–	++++	++
P3X212 23 B10§	IgM	9.1	++	++++	+++	+++	+++	++	+++	+++	+
P3X241§	IgG	5.4	+++	++++	ND	+++	+++	+++	++	+++	+++
P3X249§	IgG	2.1	++++	++++	ND	+++	++++	++++	++++	++++	++++
P3X290§	IgG	3.1	+++	++	ND	++++	+++	++++	+++	++++	+++
LHM76/58‖	$IgG_{1\lambda}$	ND	++++	ND	ND	+++	++++	+++	+++	++++	++++
LHM76/59‖	IgG_1	ND	++++	ND	ND	+++	++++	++++	+++	++++	+++
LHM174/102‖	$IgG_{3\kappa}$	1.2	–	ND	ND	+++	+++	+	–	+++	+++
LHM50/2B‖	$IgG_{1\lambda}$	6.3	++++	ND	ND	++++	+++	++++	+++	++++	++++
LHM169/81‖	$IgG_{3\kappa}$	1.1	++++	ND	ND	++++	+++	+++	+++	++++	++++
ESD1‖	$IgG_{1\kappa}$	ND	++++	ND	ND	+++	++++	++++	+++	++++	++++
LHM76/55‖¶	$IgG_{1\kappa}$	3.1	+++	++++	ND	+++	+++	+++	+++	+++	++++
LHM77/64‖¶	$IgG_{1\kappa}$	9.1	++++	++++	ND	++++	+++	++++	++++	++++	++++
LHM70/45‖¶	$IgG_{1\lambda}$	1.2	–	–	ND	+++	+++	+	–	+++	+++
LHM59/19‖¶	$IgG_{3\kappa}$	8.1	–	–	ND	(+)	+++	+	+	++++	+++

Monoclonal anti-D			Reactivity*								
Clone	Isotype	epD†	DOL-1 ccDee	DOL-2 ccDee	DMI CcDee	DFW CcDee	DFL CcDee	DCS-1 ccDEe	DFR-1 CcDee	DFV ccDEe	Weak D type 4.0 ccDee
LHM169/80‖¶	$IgG_{3\lambda}$	6.3	+++	++++	ND	+++	+++	+++	+++	+++	+++
LHM57/17‖	$IgG_{1\lambda}$	6.3	++	ND	–	–	–	+	–	++++	+++
LDM-1¶	IgM	ND	+++	++++	+++	+++	+++	+++	(+)	+++	+++
BS221**	IgG	6.3	+++	ND	ND	+++	+++	++++	++++	+++	+++
BS227**	IgG	2.2	+++	ND	ND	+++	++++	++	–	+++	+++
BS228**	IgG	6.3	++++	ND	ND	+++	++++	++++	++++	++++	+++
BS229**	IgG	5.4	++++	ND	ND	++++	+++	+++	–	++++	++++
BS231**	IgG	5.4	++++	ND	ND	+++	++	++++	–	+++	++++
H41**	IgG	3.1	+++	ND	ND	++++	++++	+++	++++	++++	+++

*Tests were performed in gel matrix test with antiglobulin if not stated otherwise. DOL-2 testing was done in tube, except for the six monoclonal anti-D from DiaMed.

†epD patterns as described previously by Scott.[24]

‡Monoclonal anti-D approved for routine use in Germany (BS226 and BS232, Seraclone anti-D (RH1), Biotest; RUM-1, immuClone anti-D rapid and D175-2, immuClone anti-D fast, Immucor).

§D-Screen, Diagast, Loos, France.

‖Advanced partial RhD typing kit, Alba Bioscience, Edinburgh, UK. The results obtained with LHM76/58 and with the monoclonal antibody Number 74, documented as LHM76/58, in the Nantes workshop[24] differed.

¶ID-Partial RhD-Typing Set, DiaMed.

**Monoclonal anti-D panel, Biotest.

ND = not determined.

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TABLE 4.

D variants with amino acid substitutions at loops 3 and 4

		Amino acid position and substitution														
		Extracellular loop 3/transmembraneous helix 6								**Transmembraneous helix 7/extracellular loop 4**						
D variant	**D antigens/RBC***	**164**	**165**	**166**	**169**	**170**	**171**	**172**	**174**	**220**	**221**	**223**	**225**	**226**	**227**	**229**
DDN†	ND	**N**	Y	H	M	M	H	I	V	W	P	F	S	A	L	R
DFL	7,499	D	**C**	H	M	M	H	I	V	W	P	F	S	A	L	R
DFW	10,019	D	Y	**P**	M	M	H	I	V	W	P	F	S	A	L	R
DFR-1	4,895	D	Y	H	**L**	**R**	H	**F**	V	W	P	F	S	A	L	R
DOL-1	7,158	D	Y	H	M	**T**	H	I	V	W	P	**V**	S	A	L	R
DMI	ND	D	Y	H	M	**I**	H	I	V	W	P	F	S	A	L	R
DHQ	ND	D	Y	H	M	M	**Q**	I	V	W	P	F	S	A	L	R
Weak D type 33	ND	D	Y	H	M	M	H	I	M	W	P	F	S	A	L	R
Weak D type 16	184	D	Y	H	M	M	H	I	V	**R**	P	F	S	A	L	R
Weak D type 62	ND	D	Y	H	M	M	H	I	V	W	**T**	F	S	A	L	R
Weak D type 27	409	D	Y	H	M	M	H	I	V	W	**S**	F	S	A	L	R
DFV	9,422	D	Y	H	M	M	H	I	VV	W	P	**V**	S	A	L	R
DTO	ND	D	Y	H	M	M	H	I	V	W	P	**V**	**F**	A	L	R
DSF‡	ND	D	Y	H	M	M	H	I	V	W	P	**F**	**F**	A	L	R
DCS-1	2,958	D	Y	H	M	M	H	I	V	W	P	**V**	S	**P**	L	R
DCS-2	835	D	Y	H	M	M	H	I	V	W	P	F	S	**P**	L	R
DBA	ND	D	Y	H	M	M	H	I	V	W	P	F	S	A	**P**	R
DHR	ND	D	Y	H	M	M	H	I	V	W	P	F	S	A	L	**K**
RhD consensus	NA	D	Y	H	M	M	H	I	V	W	P	F	S	A	L	R
RhCE consensus	NA	D	Y	H	**L**	**R**	H	**F**	V	W	P	**V**	S	**P/A§**	L	R

*Samples were tested by flow cytometry with six monoclonal anti-D (BS221, BS227, BS228, BS229, BS231, and H41; Biotest). Weak D type 4.0 RBC run as a control showed 1689 D antigens/RBC. DFL (RIR-16) shown, 6310 D antigens/RBC measured for DFL of German donor. Hemizygous DOL-1 shown, 6154 D antigens/RBC measured for *DOL-1/Ccde^S^*. The antigen densities of DCS-1 and DCS-2 were previously reported.[21]

†The nucleotide sequence data were deposited in EMBL under Accession Number FM212439.

‡Originally described as D674.[25]

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[§]Amino acids P and A are typical for E and e antigens, respectively.

NA = not applicable; ND = not determined.

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TABLE 5.

Serologic reactivity with research-grade anti-C

Monoclonal anti-C		Agglutination titer in gel matrix test with antiglobulin*		
			Controls	
Clone	**Isotype**	***(C)cdes/cDe***	***(C)cdes/cdE***	***CDe/cde***
C-93/44	IgM	0	0	32
MS253†	IgM	1	4	16
HMR7	IgG_3	4	8	64
MS242	IgG_3	16	32	64
MS23	IgG_1	32	64	128
388F3	IgM	64	128	256
MS24	IgM	64	256	256
MS273	IgG_1	64	256	512
MS257	IgG	128	256	256
P3x255 13G8	IgM	256	128	512
DGC02	IgM	1024	1024	4096

*The phenotypes of the samples were (C)cDee^S (patient expressing a DOL-1 with anti-D, RIR-27), (C)cddEe^S and CcDee (controls); the derived genotypes are given in the table head.

†Reported as anti-C^W.[26]