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Transfusion. Author manuscript; available in PMC 2014 November 01.

Published in final edited form as:
Transfusion. 2013 November ; 53(11 0 2): . doi:10.1111/trf.12145.

D category IV: a group of clinically relevant and phylogenetically diverse partial D

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Abstract

Background—The D typing strategies in several European countries protect carriers of D category VI (DVI) from anti-D immunization but not carriers of other partial D. Besides DVI, one of the clinically most important partial D is D category IV (DIV). A detailed description and direct comparison of the different DIV types was missing.

Study design and methods—*RHD* nucleotide sequences were determined from genomic DNA. D epitope patterns were established with commercial monoclonal anti-D panels.

Results—DIV comprises several variants of the D antigen with distinct serology, molecular structures, evolutionary origins and ethnic prevalences. The DIV phenotype is determined by 350H shared by all, but not limited to, DIV variants which are further divided into DIVa and DIVb. The DIVa phenotype is expressed by *DIV type 1.0* harboring 350H and the dispersed amino acids 62F, 137V and 152T. The DIVb phenotype is expressed by *DIV type 3* to *type 5* representing *RHD-CE-D* hybrids. 4 of the 6 postulated DIV variants were encountered among 23 DIV samples analyzed. Of 12 DIV carriers with anti-D, 10 were female and 7 likely immunized by pregnancy. 2 *DIV* related alleles are newly described: DWN which differs from DIV type 4 by 350D and epitope pattern. DNT carries 152T, known to cause a large D antigen density.

Conclusion—*DIV* alleles arose from at least 2 independent evolutionary events. *DIV type 1.0* with DIVa phenotype belongs to the oldest extant human *RHD* alleles. *DIV type 2* to *type 5* with DIVb phenotype arose from more recent gene conversions. Anti-D immunization, especially

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Conflict of Interest: FFW and WAF receive royalties for *RHD* genotyping patents. WAF holds intellectual property rights for *RHD* genotyping and serves on the Scientific Advisory Board of Immucor (non-remunerated). JMM serves as principal investigator for an investigator agreement with BioArray Solutions, Immucor. IvZ declares no conflict of interest.

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dreaded in pregnancies, will be avoided not only in carriers of DVI but also in carriers of other D variants like DIV, if our proposed D typing strategy is adopted.

Introduction

D is the clinically most important protein antigen on red blood cells (RBC) and the leading cause of alloimmunization in D negative individuals. In 1953, allo-anti-D was also detected in D positive individuals.[1] This ostensible contradiction was explained by partial defects of the D antigen. Individuals lacking part of the D antigen may develop an antibody against the missing part following transfusion, transplant or pregnancy. The term “partial D” for these D variants was introduced in 1984;[2,3] however, D variants missing different epitopes had been recognized much earlier.

In 1959, the D antigen was divided into Rh^A, Rh^B, Rh^C [4,5] and Rh^D [6,7] and summarized as the 4 “blood factors”.[8] Independent of this earlier work,[4-7,9] D categories I to VI were defined in 1962,[10] of which D category I was retracted[11,12] while D category VII was added later.[12,13] The D category I samples of 1977 represented a heterogeneous set of weak D phenotypes from Caucasians,[11] difficult to characterize by serology because of the weak expression of the D antigen,[11] while the heterogeneity of the weak D types likely involved was unknown at the time. The original nomenclature comprised of the “blood factors”[4,5] was abandoned for the D category classification,[10] which is still in use.[14]

The current terminology for D categories comprises DII to DVII.[15] Several subtypes of D categories[14] have since been detected as well as many partial D, whose serological appearance and molecular basis did not match any defined D category. Today, D categories represent only a fraction of all partial D alleles. At present, 85 partial D are listed in the internet-based registry of *RHD* alleles (The RhesusBase),[16] of which only 26 belong to D categories and their subtypes.

DII is very rare; only 3 individuals expressing DII are known in 2 pedigrees.[14,15] DVII is the most prevalent D category in Caucasians with a phenotype frequency of 1 in 900 in Germany.[17] DVI, not to be confused with DIV, is much less frequent with 1 in 6214,[18] but is the clinically most relevant partial D in Europeans with respect to immunization by normal D.[19] Therefore, in several European countries a D typing strategy involving the use of two monoclonal anti-D antibodies that do not recognize DVI is mandatory for recipients.[20] While this strategy prevents anti-D formation in DVI individuals, there are other partial D that are typed as D positive, like DIV, R_0^{Har}, DNB, DVII, DIII, weak D type 4.2 and DV.[21] These partial D may remain undetected and anti-D immunizations may occur with the consequence of transfusion incompatibility and complications during pregnancy.[22,23]

The RhD protein has 12 transmembraneous segments and forms 6 extracellular loops. DII, DHK (identical to DV type 5) and DVII type 1 are caused by single amino acid substitutions in the extracellular loops 6, 4 and 2, respectively. These substitutions are not related to the RhCE amino acid sequence and are, hence, not caused by gene conversions.[24,25] In contrast, DIII, DIV, DV and DVI harbor amino acid substitutions which may be explained by gene conversions because they are found in the RhCE protein.

DIV has been divided serologically into DIVa and DIVb using polyclonal anti-Go^a, an antibody defining the low-prevalence RhD antigen RH30 (Go^a). All DIVa are Go^a positive and DIVb are considered Go^a negative.[11] Similarly, DIVa but not DIVb carry the high-prevalence D epitope 4 (epD4) detectable with monoclonal antibodies (9 epitope model).[26] Molecular characterization suggested different phylogenetic origins for DIVa and

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DIVb.[27-29] Several molecular subtypes have been reported for DIVb,[30-35] while DIVa is less diverse at the molecular level.

A complete record of the DIV types and their phylogeny was missing. We provide a detailed and systematic description of DIV alleles and propose an easy and comprehensive nomenclature. A large case collection of DIV carriers with anti-D is presented. We also propose an improved routine typing strategy, which would avoid anti-D immunization in pregnancies and transfusions of individuals carrying the DIV phenotype or some other partial D.

Materials and Methods

Immunohematology

Serologic testing of IgG and IgM monoclonal antibodies for agglutination was done by a gel matrix test (LISS-Coombs 37°C with polyspecific rabbit anti-IgG and monoclonal anti-C3d, clone C139-9, ID Micro Typing System; DiaMed; Cressier sur Morat, Switzerland).[36] Monoclonal IgM anti-D approved for routine use in Germany were BS226 and BS232 (Seraclone Anti-D (RH1); Biotest, Dreieich, Germany), RUM-1 (immuClone Anti-D rapid; Immucor, Rödermark, Germany) and D175-2 (immuClone Anti-D fast; Immucor). Several monoclonal anti-D from RhD typing kits were used as detailed in the Results (D Screen; Diagast, Loos, France; and Advanced Partial RhD Typing Kit; Alba Bioscience, Edinburgh, UK). Two monoclonal IgG anti-D LOR17-6C7 and LHM76/58 came from the International Workshop on Monoclonal Antibodies against Human Red Blood Cells and Related Antigens in Nantes 1996.[37] The sources of further antisera are detailed in Table 3.

Commercial monoclonal IgM anti-C were MS24 (Ortho BioClone; Ortho, Neckargemünd, Germany and Gamma-clone; Immucor, Norcross, USA) and MS273 (immuClone 2; Immucor, Rödermark, Germany). The anti-Go^a sera A1149 and DL34678B were kindly provided by M.K. Moulds and showed identical results with all partial D and weak D types except weak D type 4.0. The 3 weak D type 4.0 samples tested did not react with the A1149 serum, were noted as Go^a negative in the figure, but reacted with the DL34678B serum possibly due to an additional specificity in the DL34678B serum.

The mean antigen density was determined by flow cytometry according to the protocol described previously[38] with monoclonal IgG anti-D BS221, BS227, BS228, BS229, BS231 and H4111B7 (Biotest). The secondary antibody was goat anti-human IgG, Fab-fragment, FITC-conjugated (Jackson ImmunoResearch Laboratories, West Grove, USA).

Molecular methods

RHD and *RHCE* nucleotide sequencing from genomic DNA for exons 1 through 10 including adjacent flanking intron regions and *RHD* exon specific PCR was performed as described.[31,39-41] All *DIV type 1.0* samples were sequenced in full length as well as *DWN*, *DNT* and at least one example of each reported *DIV* allele; further samples were identified by *RHD* exon specific PCR and nucleotide sequencing of relevant *RHD* exons. The presence of the *ceTI* typical nucleotide substitution 1025C>T was determined by sequencing of *RHCE* exon 7.[42]

Serological population screen

From February until September 1995, we examined 78,156 blood donations in South-Western Germany, of whom 60,965 were D positive. An autoanalyzer (Olympus PK7100; Olympus, Hamburg, Germany) was used to test for reactivity with 7 IgM anti-D including BS226 and BS232 (Biotest), P3X212 11 F1 and P3X212 23 B10 (Diagast) and RUM-1

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(Immucor).[17,43] P3X212 23 B10 binds to epD9,[15] also published as epD23,[26,44] which is absent from DIVa and DIVb. P3X212 11 F1 binds to epD8,[15] also published as epD22,[26,44] which is absent e.g. from DVII, DVI, R_0^{Har} and DHMI. The antibodies were used in a dilution of 1:1,000 in phosphate buffered 0.9% NaCl (Immusol; Dade Behring, Liederbach, Germany) with 0.22% bovine serum albumin (Ortho, Neckargemünd, Germany) as described previously.[43] 25 μl of an 1.6% RBC suspension prepared in Immusol with 0.1% bromeline (Roth, Karlsruhe, Germany) was mixed with 25 μl antibody solution. After 1 h at 25 °C the incubation mixture was read with the autoanalyzer. D positive donors typing negative with P3X212 23 B10 or P3X212 11 F1 were further tested with panels of monoclonal anti-D. All DIV were also characterized by molecular methods. Several publications have covered samples from this 9 months' blood donor survey in 1995,[17,19,24,29,31,32,38,45-48] which commenced the molecular D variant analysis that took place in Ulm since.

Rhesus Immunization Registry

Samples from D positive patients with suspected anti-D were collated in the Rhesus Immunization Registry (RIR) from 1998 to 2009.[21] A possible immunization event, such as a transfusion or pregnancy, had been documented in all patients.

Nomenclature

We followed the common practice to abbreviate the names of the D categories; for example, we used the abbreviation DIV for D category IV.

All names for alleles and other genetic structures are italicized; all names for phenotypes and protein structures are in regular font.

DIII type 4 and DIII type 5 are related to DIV type 1.0 and presented for comparison. DIII type 5 was first characterized in 2002;[28] according to recent findings the nucleotide sequence of DIIIa is in fact identical to DIII type 5,[49,50] while the sequence originally assigned to DIIIa in 1997 was incomplete.[51] These findings imply that the phenotypes previously described by DIII type 5 [28] and DIIIa [51] are identical. We are aware of the internet-based allele terminology that had been drafted by an ISBT subcommittee and proposed at the time of manuscript submission.[52] Because 15 years of literature refers to a putative nucleotide structure dubbed *DIIIa*,[51] now known to be incorrect,[50] we propose discontinuing the use of *DIIIa* as an allele designation. We advocate reserving the designation DIIIa for the description of the serologic phenotype,[12] which represents its original definition, and using *DIII type 5* for the allele and DIII type 5 for the protein,[28] which makes the *DIIIa* allele and DIIIa protein designations[51] obsolete.

The allele *DWN* was named after the two characteristic amino acid substitutions tryptophane (W) and asparagine (N) and has been observed before without attribution of a name.[53] The designation *DNT* was derived from the amino acid substitution asparagine (N) to threonine (T).

Results

RHD alleles

We collected and characterized 23 samples with DIV phenotype (Table 1), [16,27,28,31-35,54-58] among which 4 molecular structures were encountered. The 3 DIV type 1.0 samples differed from the DIV type 1.1 described in 1995[30] by the presence of the additional amino acid substitution A137V[28] (Table 1). The partial D DNT carried the amino acid substitution N152T in isolated form; which is one of the four amino acid substitutions characteristic of

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DIV type 1.0. The partial D DWN is similar to DIV type 4 except for amino acid position 350, which is variant in all DIV types but normal in DWN.

Anti-D immunization

In an internet-based survey from 1998 to 2009 (Rhesus Immunization Registry), we collated cases of anti-D immunizations occurring in D positive individuals.[21] 124 samples were submitted and analyzed at the molecular level. 12 were DIV, among which *DIV type 4* and *type 3* were the most frequent (Tables 1 and 2). Strong anti-D antibodies were observed in *DIV type 1.0* and *type 4* carriers. Also *DWN* and *DNT* were recognized because their carriers had produced anti-D.

Ethnicity

At least 2 of the 3 DIV type 1.0 carriers were of African origin; the ethnicity of the 3rd carrier is unknown. All 20 DIV type 3 to DIV type 5 carriers were Caucasians; 17 were donors or patients from Germany and 1 patient each was of Turkish, Yugoslavian and Swiss origin. The DNT carrier was a Caucasian from Germany while the DWN carrier was an African American.

Immunohematology

DIV types and related D variants were tested with monoclonal anti-D to establish the epitope patterns (Table 3).[26,37] Samples of DIIIc,[32] DIII type 4,[32] DNU,[59] DNB[59] and DWI[60] were tested for comparison. Characteristic of DIV were the lack of reactivity with anti-D clones specific for epD 1.1, 1.2, 2.1, 2.2, 3.1 and 9.1. An antibody directed against epD4.1 (clone no. LOR17-6C7) recognized DIV type 1.0 but not DIV type 3 to type 5, DWN and DNB. Only DIV type 1.0 samples were Go^a positive (Fig. 1).

Antigen densities

The number of D antigens per RBC were large in DIV type 1.0, DIII type 4 and type 5 as well as DNT, while moderately reduced in DIV type 3 to type 4, DWN, DNU and DNB (Fig.1).

Population frequencies

Among 78,156 blood donations,[17] we detected 8 samples with DIV phenotype by applying a high throughput serologic screening procedure followed by further serological and molecular characterization. 4 DIV type 3, 3 DIV type 4 and 1 DIV type 5 donors were identified (Table 4), who are among the 23 DIV samples listed in Table 1. Furthermore, we detected 80 samples with DVII phenotype and 1 sample each of DNU and DFW.

DIV and *ceTI*

Our 3 DIV type 1.0 samples were serologically C negative with 3 monoclonal IgM anti-C including Gamma-clone, which is known to detect weak C expression in rare samples with the DIVa(C) phenotype.[42,61] They all carried the *RHCE* allele *ceTI*[42] as evident from the presence of a heterozygous T/C at nucleotide position 1,025. Samples of DIV type 3 (n = 3), DIV type 4 (n = 2), DIV type 5 (n = 3), DWN (n = 1), DIII type 4 (n = 1), DIII type 5 (n = 1) and DNT (n = 1) did not carry the *ceTI* allele.

Discussion

A comprehensive characterization is available for most of the clinically important partial D but was missing for the group of *DIV* alleles. The current D typing strategy applied in Germany and several European countries protects individuals with DVI from immunization

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by normal D. However, patients with DIV and other partial D are typed D positive and hence can get immunized by transfusion of D positive blood. An improved typing strategy will be particularly relevant in women in childbearing age and girls. To avoid adverse effects of anti-D immunization, detailed knowledge is required for the involved alleles and the susceptibility to become immunized by normal D. This objective prompted us to address the molecular basis and to determine the clinical importance of the various *DIV* alleles.

What was initially defined as DIV, was later recognized to comprise a group of different phenotypes and alleles. DIV and its subgroups DIVa and DIVb are serologically defined; DIV lacks epD 1, 2, 3 and 9 or parts thereof;[24,26,60,62,63] DIVa carries epD4 and the low prevalence antigen Go^a (RH30), which are absent from DIVb (Table 5).[11,26,64] The differences between DIVa and DIVb at the serological level are mirrored by distinct molecular structures, evolutionary origins and ethnic prevalences (Fig. 1 and Table 5). 350H is the only variant amino acid residue that is shared by all DIV types.

The DIVa phenotype is expressed by DIV type 1.0. The original publication of DIV type 1.1[30] has been incorrect;[56] if DIV type 1.1 is ever found, the DIVa phenotype will need to be tested in that sample. We observed DIV type 1.0 only (Table 1), which is the primordial allele of the DIVa cluster (Fig. 2).[28] The DIVa cluster is one of the 3 African clusters and encompasses also the *DIII type 4* and *DIII type 5* alleles as well as the *(C)cde^s* haplotype. All these DIVa-related variants (Fig. 1A) share the amino acids 62F, 137V and 152T, which are likely remnants of evolutionary old alleles.[28,32] 62F and 137V are highly conserved in the animal kingdom and occur in the Rh-related proteins RhAG, RhBG and RhCG. 152T is an RhCE typical amino acid present in several mammal species and is regarded as the specific amino acid substitution of the DIVa cluster relative to the normal Eurasian *RHD* allele.[28] We observed the corresponding D variant with N152T as the sole difference to the normal RhD protein. This allele, dubbed DNT, was evolutionary unrelated to the DIVa cluster and may belong to the Eurasian D cluster because it differed in haplotype. Furthermore, the DNT sample was found in a Caucasian, while DIV type 1.0 typically occurs in Africans.

The DIVb phenotype is expressed by DIV type 2 to type 5, which are structurally distinct from DIV type 1 because they are encoded by *RHD-CE-D* hybrid alleles of the *RHD* gene (Table 1, Fig. 1B and Table 5). They arose as members of the Eurasian D cluster (Fig. 2). While *DIV type 1* typically occurs in Africans, all our samples of *DIV type 3* to *type 5* were from Caucasians (Table 5). *DIV type 4* represents the *RHD-CE-D* gene conversion with the shortest *RHCE* segment encoding only 3 amino acids (Table 1). All DIVb-related variants share the structural motif of DIV type 4 but harbor further stretches of RhCE-like amino acids which are located in the transmembraneous or intracellular section of the protein (Fig. 1B).

D350H, the sole amino acid substitution shared by all DIV variants, is part of extracellular loop 6 of the RhD protein (Fig. 1 and Fig. 3).[65] This RhCE typical substitution causes a change in charge and hydrophobicity likely affecting anti-D binding. The associated antigenic loss defines the DIV phenotype. The D variant DWN was similar to DIV type 4 but lacked the critical D350H substitution and was instrumental in determining the influence of 350D for D antigen expression. The comparison of epitopes expressed by several D variants with substitutions at loop 6 (Table 3) corroborated previous reports[24,60,62,63] on the allocation of epD 3, 4 and 9 to distinct amino acids of loop 6 (Fig. 3).

In 1977, DIV could be divided into DIVa and DIVb by serologic criteria.[11,26] DIVa is Go^a positive and occurs in African populations, while DIVb lacks Go^a and occurs in Caucasians. The first 2 alleles expressing these 2 DIV phenotypes were characterized on a molecular

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level in 1995 and named *DIVa* and *DIVb*.[30] Neither allele was observed among our 24 DIV samples or has been confirmed since the original description. 4 additional *DIV* alleles have been described since 1995 and confirmed in this study.

We propose to reserve the designations DIVa and DIVb for the description of the serologic phenotypes and to use the designations *DIV type 1* to *type 5* for the alleles characterized molecularly (Table 1). The 2 initially described alleles [30] were named *DIV type 1.1* and *DIV type 2* (Table 1).[55] *DIV type 1.0*[28] has been confirmed independently,[56,66] while *DIV type 2* has not been confirmed and may attract further scrutiny. Alleles encoding the DIVb phenotype are labeled *DIV type 2* to *type 5* (Table 1). *DIV type 2* cannot be frequent,[30,55] if extant at all, and *DIV types 3* and *4* kept their original names.[32-34] The allele *DIVb(J)*, first described in Japan,[35] was designated *DIV type 5.*

A small fraction of DIVa samples express C antigen very weakly.[61] The initial presumption[42] that this C expression may be caused by the *RHCE* variant *ceTI* has not been confirmed;[67] recently, this weak C expression in DIVa(C) was attributed to a hybrid allele at the *RHCE* locus.[64] Our 3 DIV type 1.0 samples, lacking C expression, occurred in a genotype heterozygous for *ceTI*, which is in accordance with independent observations.[42,67] The *ceTI* allele has not been found associated with the DIVb phenotype, at least not in our sample collection.

All DIV variants appear D positive by routine serology, because their densities are larger than 4,500 D antigens per RBC. DIVa-related D variants, which carry the amino acid substitution N152T, had a density of larger than 20,000 D antigens per RBC (Fig. 1A), while DIVb-related D variants had a moderately reduced density of lower than 8,000 D antigens per RBC (Fig. 1B). Typical antigen densities[68] are 22,750 for *CDe/CDe* (R_1R_1)[32] and 24,500 for *CDe/cDE* (R_1R_2).[32] N152T may facilitate the integration of the RhD protein into the RBC membrane.[31] Accordingly, the D variant DNT with the single amino acid substitution N152T had an unusual large density of 28,000 D antigens per RBC.[57] Similarly, increased antigen densities were observed in other D variants harboring the N152T substitution such as DIV type 1, DIII type 4 and DVI type III.[19,31] The relatively large antigen density of DIII type 5 as compared to weak D type 4.0 may be due to the presence of 152T as well. DIII type 5 arose from a recombination between alleles of the DIVa and the weak D type 4 clusters (Fig. 2).[28,69] The substitution T201R likely caused the low antigen density of weak D type 4.0 (Fig. 1A).

During the years 1998 - 2009, cases of D positive patients with anti-D were collated in the Rhesus Immunization Registry.[21] Among 124 cases was 1 patient with anti-LW^a, the others had an allo- or an auto-anti-D. Allo-anti-D was confirmed in 70 cases comprising 12 DIV, 12 DVI, 11 R_0^{Har}, 9 DNB, 6 DVII, 5 DIII, 2 DV, 2 weak D type 4.2/DAR; and 1 each of DAU-3, DAU-4, DDE, DFL, DHMI, DMI, DNT, DOL, DWN, weak D type 11 and weak D type 15. The number of registered cases did not correlate with the population frequency of the involved D variants: the prevalence of D variants in the population of Southern Germany are 1 in 977 for DVII, 1 in 6214 for DVI,[18] and 1 in 9770 for DIV (Table 4). Individuals with DVII are, therefore, much less prone to anti-D immunization than individuals with DVI and DIV. Among the 12 cases with DIV and anti-D were only 2 males. 7 immunizations were suspected or confirmed to be caused by pregnancy (Table 2). 79% of all patients with partial D and anti-D were females and pregnancy was the most frequent immunization cause. Therefore, women of childbearing age and girls would especially benefit from measures to prevent further anti-D immunization. In a study conducted in the USA, among 21 cases of anti-D immunizations in D-positive individuals one third were cases with DIV[70] including 6 DIVa and 1 DIVb; 6 cases involved weak D type 4.2.

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The present typing strategy for the D antigen in recipients, introduced 1996 in Germany, aims at preventing anti-D immunization in patients with DVI (1:6,214),[18] the clinically most relevant partial D in Caucasians.[19,71] DNB (1:292 to 1:1644)[59] and DVII are the most frequent known partial D in Caucasians but of lesser clinical relevance. Currently 2 monoclonal anti-D that react with epD6 [26] and do not recognize DVI are used for typing of recipients. Consequently, recipients with DVI are typed and transfused D negative. However, recipients with DIV are not protected by this strategy developed in Europe[18,71] and adapted to Caucasians. Electing reagents for typing of the D antigen is posing an ongoing challenge.[72]

It would be valuable to also protect carriers of other clinically relevant partial D, including DIV, from becoming immunized by the D antigen. To this end, an adaptation of the current serological D typing strategy for recipients would be necessary and can be implemented at the routine level. A set of 2 monoclonal anti-D antibodies could be used with one not recognizing DVI, and another not recognizing other clinically important D variants. For instance, an antibody binding epD9.1, like P3X212 23 B10 and LHM77/64 (Table 3), would neither recognize any DIV type (Table 3) nor R_0^{Har} [73] or give only weakly positive results with several other partial D. Other useful target epitope and monoclonal anti-D may be epD3.1 and P3X290 or ESD1 (Table 3). For clinical purposes, appropriate monoclonal anti-D might be selected according to the prevalence of partial D in the population served.[74] Discrepant results with the 2 monoclonal antibodies should prompt identification of the allele by molecular methods or evaluation by panels of monoclonal anti-D. Pregnant women and their children would especially benefit from this modified typing strategy.

Acknowledgments

The authors dedicate this paper to the memory of John James Moulds, MT(ASCP)SBB, who died on June 13, 2011 at the age of 67. With his lifetime contributions to blood group serology, he was a key person for our field and contributed profoundly to our understanding of blood group antigens and to establishing the foundation, on which all molecular immunohematology is built.

We thank all contributors of the Rhesus Imunization Registry framework[21] for procuring blood samples and clinical data of D positive patients with anti-D carrying the D variants DIV, DIII, or DNB. Such samples were provided by J. Burkhart, München, W. Endres, Stuttgart, M. Heuberger, Kassel, B. Just, Hagen, A. Mildner, Reutlingen, E. Piro-Biehler, Weiden, P. Pirzer, Amberg, and A. Seltsam, Springe, all from Germany; S. Kilga-Nogler, Innsbruck, Austria; the late N. Eicher, Bern, Switzerland; J. Jorgensen, Aarhus, Denmark; M.R. Combs, Durham, NC, S. Frandson, San Diego, CA, and M.K. Moulds, Shreveport, LA, United States. DWI was a gift from G. F. Körmöczi, Vienna, Austria. One additional sample of *DWN/RHDpsi* came from S. Kuhn, Würzburg, Germany. The authors acknowledge the serology advice by Marylin K. Moulds and the technical assistance by Donna M. Baker and David Hanna in Shreveport, LA, and by Marianne Lotsch, Anita Link and Hedwig Erne in Ulm. This work was supported by the Intramural Research Programs of the DRK-Blutspendedienst Baden-Württemberg – Hessen, Mannheim and the NIH Clinical Center.

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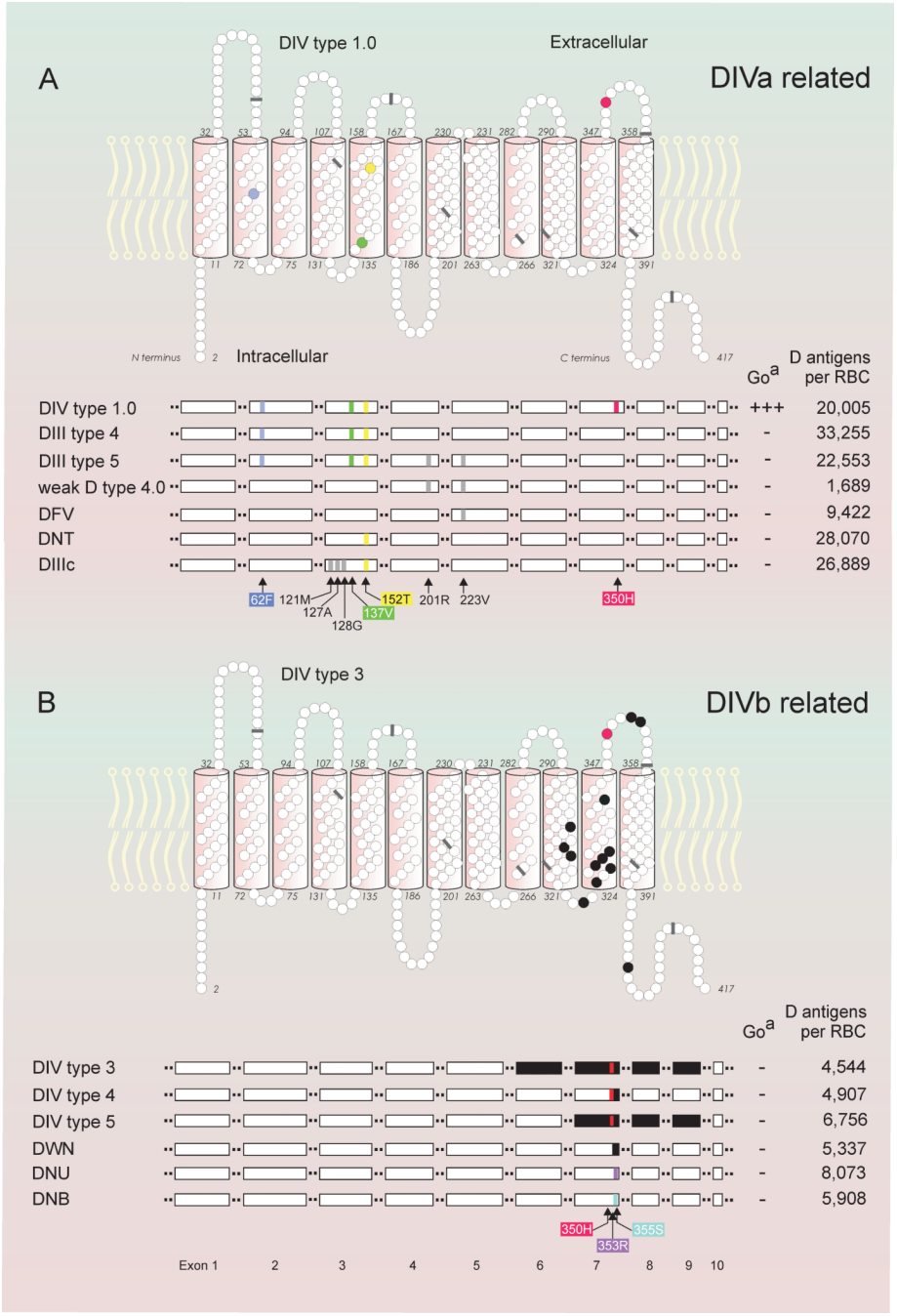

Figure 1.

Molecular structure, antigen density and anti-Go[a] reactivity of DIV variants. DIVa-related variants (panels A) and DIVb-related variants (panels B) are shown with models of the 2-dimensional structure (upper panels of A and B) and the linear exon structure (lower panels of A and B). In the 2-dimensional models of the RhD protein, amino acid substitutions are indicated (colored circles) together with the positions of the 9 exon boundaries (bars) as reflected in the *RHD* cDNA. DIV type 1.0 exemplifies the typical structure of DIVa-related variants with dispersed amino acid substitutions and DIV type 3 represents a typical structure of DIVb variants resulting from gene conversions (black circles). The linear exon structures of several related D variants are compared (white horizontal bars, lower panels of

A and B); typical amino acid substitutions corresponding to nucleotide substitutions (colored and gray vertical bars) as well as *RHCE*-like gene conversions (black bars) are indicated. Antigen densities of DIII type 4 and DIIIc,[32] of weak D type 4.0 and DFV[29] and of DIV type III, DNU and DNB[59] were published previously.

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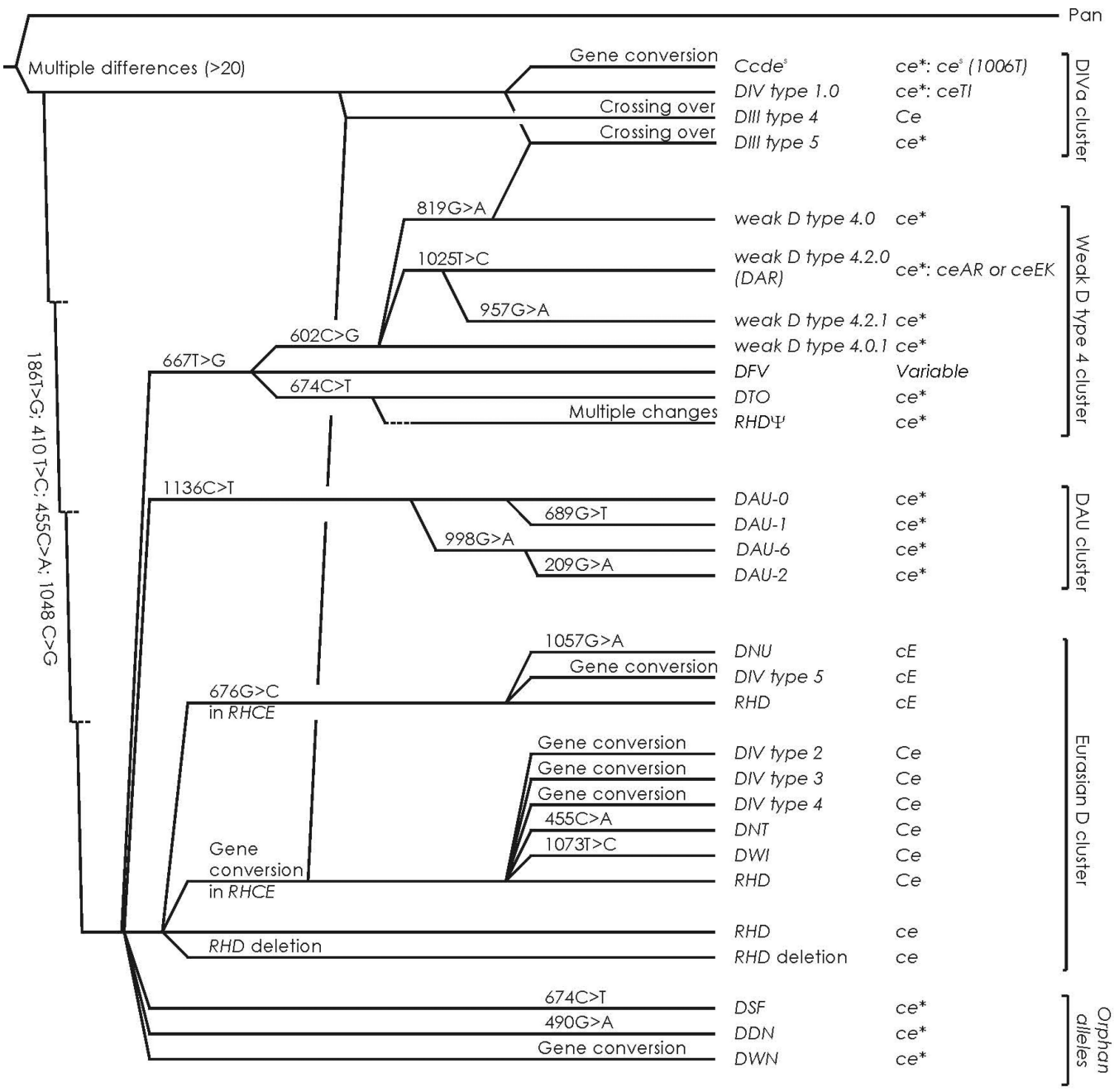

Figure 2.

Phylogeny of DIV. The phylogenetic tree is based on a previous tree for *RHD*[29,39] with 4 main branches representing allele clusters. The three African D clusters have characteristic primordial amino acids and typically occur in a *cDe* haplotype while the Eurasian D cluster has normal *RHD* as the primordial structure in which the *Ce* and *cE* alleles of *RHCE* evolved. The DIVa cluster is one of the 3 African D clusters; D variants of this cluster share the ancestral amino acid substitutions 62F, 137V and152T relative to the consensus RhD. In contrast, DIV variants with DIVb phenotype evolved in the Eurasian D cluster by gene conversions. The typically associated *RHCE* alleles are shown.

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	Amino acid position and substitution									D epitope (epD)		
D variant	350	351	352	353	354	355	356	357	358	3.1	9.1	4.1
Normal RhD	D	T	V	G	A	G	N	G	M	+	+	+
DIV type 1.0	H	T	V	G	A	G	N	G	M	-	-	+
DIV type 3	H	T	V	W	N	G	N	G	M	-	-	-
DIV type 4	H	T	V	W	N	G	N	G	M	-	-	-
DIV type 5	H	T	V	W	N	G	N	G	M	-	-	-
DWN	D	T	V	W	N	G	N	G	M	+	-	-
DNU	D	T	V	R	A	G	N	G	M	+	-	+
DNB	D	T	V	G	A	S	N	G	M	+	+	-
DWI	D	T	V	G	A	G	N	G	T	+	+	+
Normal RhCE	H	T	V	W	N	G	N	G	M	-	-	-

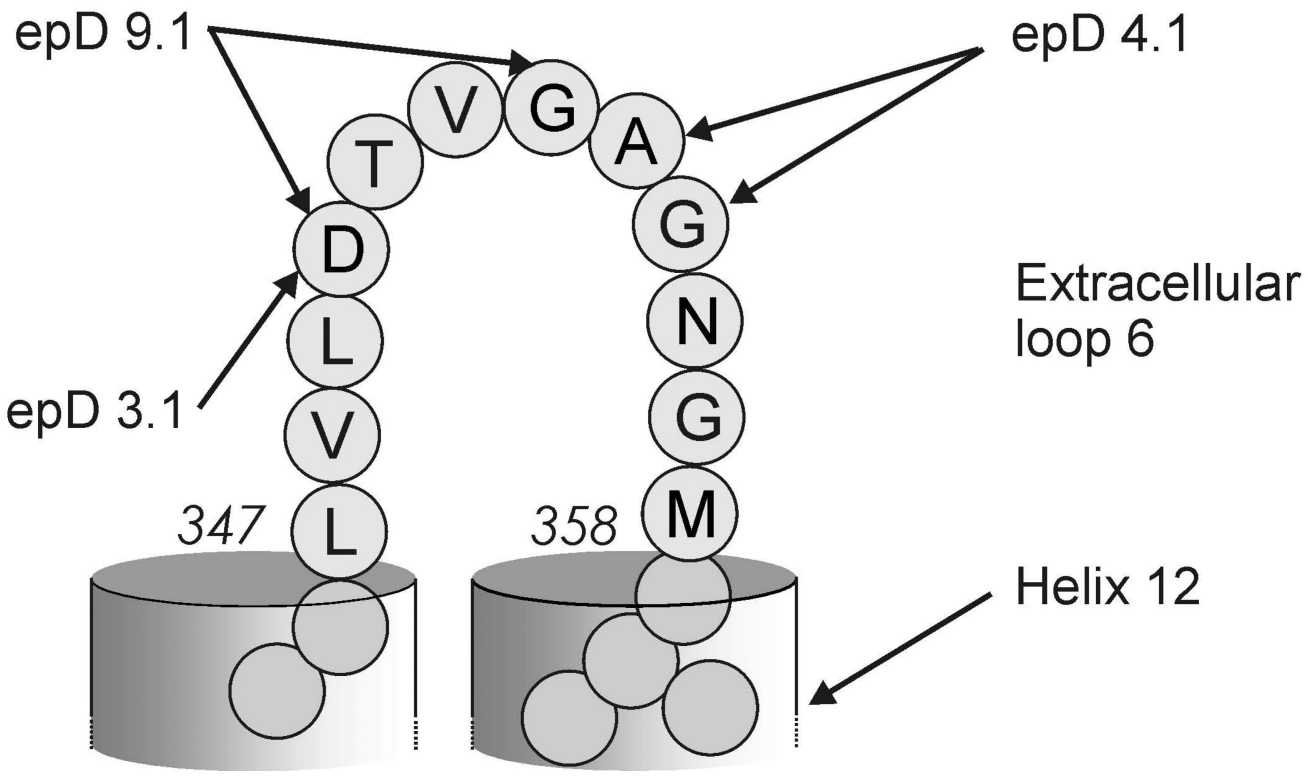

Figure 3.

Amino acids at extracellular loop 6 critical for D epitope (epD) expression. epD were allocated on the basis of reactivities with monoclonal anti-D P3X290 and LHM56/55 (epD 3.1), with LOR17-6C7 (epD 4.1) as well as P3X212 23 B10 and LHM77/64 (epD 9.1). Further splits of these epD were previously described.[24,60] DNAK is another partial D[65] with a single amino acid substitution in loop 6 (G357D, not shown).

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Table 1

Molecular bases of DIV types and the 2 partial D DWN and DNT

RHD allele						Probands of this study			
Proposed name*	**Previous designation[16, 54, 55]**	**Nucleotide substitution in *RHD* gene†**	**Effect on protein sequence**	**Exon involved**	**Predicted membrane localization†**	**probable Haplotype**	**Phenotype**	**n**	**References**
DIV type 1.0‡	*DIVa-2*	dispersed 186G>T 410C>T 455A>C 1048G>C	dispersed L62F A137V N152T D350H	2 3 3 7	TM TM TM EF	*cDe*	ccDee	3	[28]
DIV type 1.1	*DIVa type 1*[54]	dispersed 186G>T 455A>C 1048G>C	dispersed L62F N152T D350H	2 3 7	TM TM EF	NA§	NA	0	[30, 55]; original decription[30] shown to be incorrect[56]
DIV type 2	*DIVb type 2*	gene conversion 1048C to 1193T	gene conversion 350H to 398V	part of 7 to 9	EF, TM, IC	NA	NA	0	[30, 55]
DIV type 3‡‖	*DIVb type 3*	gene conversion 916A to 1193T	gene conversion 306I to 398V	6 to 9	TM, IC, EF	*CDe*	CcDee CCDee CcDEe	5 3 1	[31, 32, 55]
DIV type 4‡	*DIVb type 4*	gene conversion 1048C to 1061A	gene conversion 350H to 354N	part of 7	EF	*CDe*	CcDee	8	[33, 34, 55]
DIV type 5‡	*DIVb(J)*	gene conversion 941T to 1193T	gene conversion 314V to 398V	7 to 9	TM, IC, EF	*cDE*	ccDEe	3	[54]
DWN‡	NA	gene conversion 1053T to 1061A	gene conversion 353W to 354N	part of 7	EF	*cDe*	ccDee	1	this study
DNT‡	NA	455A>C	N152T	3	TM	*CDe*	CcDee	1	this study[57, 58]

*This name is also proposed for the D variant protein, for which it should be used in regular font (no italics).

†Numbering refers to cDNA; all gene conversions from *RHCE*. TM = transmembraneous; IC = intracellular; EF = exofacial.

‡The nucleic acid sequence data were deposited in EMBL under accession numbers HF549086 (*DIV type 1.0*), HF549087 (*DIV type 3*), HF549085 (*DIV type 4*), HF549083 (*DWN*), HF549084 (*DNT*). Hyodo et al.[35] have deposited AB037270 (cDNA of *DIV type 5*).

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§NA = not available.

//The 3' breakpoint was confirmed (n = 6) as described previously.[31]

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Table 2

Allo-anti-D in patients with DIV, DWN and DNT between 1998 and 2008

***RHD* allele**	**Case***	**Sex**	**Phenotype**	**Anti-D titer†**	**Possible immunization events**			**Ethnicity**
					RBC unit transfusion	**Pregnancies**	**Date**	
DIV type 1.0	RIR-24‡	female	ccDee	256	none	2//	before 1997	Mulatto (Dominican Republic)
	RIR-118	female	ccDee	16	none	2 live births 1 miscarriage//	2006	African (Togo)
DIV type 3	RIR-23	female	CcDee	1	none	2//	before 1997	Swiss
	RIR-26	female	CcDee	2	unknown	at least 1	before 2000	German
	RIR-94	female	CcDee	8	2 D positive//	unknown	1996	German
DIV type 4	RIR-18	female	CcDee	4	none	4//	before 1990	German
	RIR-29	male	CcDee	256	1 D positive//	NA§	2000	German
	RIR-80	female	CcDee	1	unknown	7//	before 1997	Yugoslavian
	RIR-85	female	CcDee	128	none	2live births	before 1997	German
	RIR-105	male	CcDee	8	10 D positive//	3miscarriages//	2006	Turkish
						NA		
DIV type 5	RIR-87	female	ccDEe	2	unknown	9//	before 1997	German
	RIR-119	female	ccDEe	8	several//	1	1988	German
DWN¶	RIR-112	female	ccDee	16	several	at least 1//	before 2000	African (USA)
DNT	RIR-84	female	CcDee	16	unknown	2//	2001	German

*Rhesus Immunization Registry (RIR) entries, data available online.[21]

†Auto-anti-D were excluded by negative direct antiglobulin tests in 8 of 14 samples. RIR-80, RIR-85, RIR-94, RIR-105, RIR-118 and RIR-119 samples had a positive direct antiglobulin test but antibody elution was negative; autologous absorptions were generally not performed due to lack of an appropriate amount of RBC. Titers were done in gel matrix test.[36] Additional antibodies were anti-E (RIR-23); anti-E (RIR-29); anti-Fy^a (RIR-84); anti-Fy^b, anti-S and anti-M (RIR-85); anti-E and anti-K (RIR-112); and anti-C (RIR-119).

‡RIR-24 compound heterozygous for *DIV type 1.0/RHDΨ*.

§NA = not applicable.

//Likely immunization cause.

¶There was one additional *DWN* sample with anti-D titer 32 and anti-E, carrying the *DWN/RHDΨ* genotype.

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Table 3

Serologic reactivity with panels of anti-D

Monoclonal anti-D			Reactivity*									
Clone	**Isotype**	**epD†**	**DIV type 1 ccDee**	**DIV type 3 CcDee**	**DIV type 4 CcDee**	**DIV type 5 ccDEe**	**DWN ccDee**	**DNU ccDEe**	**DNB ccDEe**	**DIIIc CCDee**	**DIII type 4 CcD.ee**	**DNT CcDee**
BS226‡	IgM	6.4	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++
BS232‡	IgM	6.4	++++	++++	++++	++++	++++	++++	+++	++++	ND	++++
RUM-1‡	IgM	6.1	++++	++++	++++	++++	++++	++++	+++	++++	ND	++++
D175-2‡	IgM	6.1	++++	++++	++++	++++	++++	++++	+++	++++	+++	++++
HM10‡	IgM	6.6	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++
HM16§	IgG	6.4	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++
P3X61§	IgM	6.1	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++
P3X35§	IgG	5.4	++++	++++	++++	++++	++++	+++	+++	++++	++++	++++
P3X212 11 F1§	IgM	8.2	++++	+++	+++	++++	+++	+++	+++	++++	++++	+++
P3X212 23 B10§	IgM	9.1	-	-	-	-	-	-	++	++++	++++	++++
P3X241§	IgG	5.4	++++	++++	++++	+++	+++	+++	+++	++++	++++	++++
P3X249§	IgG	2.1	-	-	-	-	++++	++++	+++	++++	++++	++++
P3X290§	IgG	3.1	-	-	-	-	++++	+++	+++	++++	++++	++++
LHM76/58‖	$IgG_{1\lambda}$	ND	++++	-	-	-	++++	+++	+++	++++	++++	++++
LHM76/59‖	IgG_1	ND	-	-	-	-	++++	+++	+++	++++	+++	++++
LHM174/102‖	$IgG_{3\kappa}$	1.2	-	-	-	-	+++	+++	+++	++++	++++	+++
LHM50/2B‖	$IgG_{1\lambda}$	6.3	++++	-	++	(+)	++++	++++	+++	++++	++++	++++
LHM169/81‖	$IgG_{3\kappa}$	1.1	-	-	-	-	+++	+++	+++	++++	++++	++++
ESD1‖	$IgG_{1\kappa}$	ND	-	-	-	-	++++	+++	++	++++	ND	++++
LHM76/55‖¶	$IgG_{1\kappa}$	3.1	-	-	-	-	++++	+++	+++	++++	++++	++++

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Monoclonal anti-D			Reactivity*								
Clone	**Isotype**	**epD†**	**DIV type 1 ccDee**	**DIV type 3 CcDee**	**DIV type 4 CcDee**	**DIV type 5 ccDEe**	**DWN ccDee**	**DNU ccDEe**	**DNB ccDEe**	**DIIIc CCDee**	**DIII type 4 CcD.ee**	**DNT CcDee**
LHM77/64‖¶	$IgG_{1\kappa}$	9.1	-	-	-	-	-	-	+++	++++	+++	++++
LHM70/45‖¶	$IgG_{1\lambda}$	1.2	-	-	-	-	+	+++	+++	++++	++++	+++
LHM59/19‖¶	$IgG_{3\kappa}$	8.1	+++	+++	+++	++++	+++	+++	+++	++++	+++	++++
LHM169/80‖¶	$IgG_{3\lambda}$	6.3	++++	++++	+++	++++	++++	+++	+++	+++	++++	++++
LHM57/17‖	$IgG_{1\lambda}$	6.3	++	++	+	+++	+++	++	+	+++	ND	+++
LDM-1¶	IgM	ND	+++	+	+	+++	++	++	+	+++	ND	+++
BS221**	IgG	6.3	+++	++	+++	++	+++	+++	+++	++++	ND	++++
BS227**	IgG	2.2	-	-	-	-	+	+++	+++	++++	++++	++++
BS228**	IgG	6.3	++++	++++	++++	+++	++++	++++	+++	++++	ND	++++
BS229**	IgG	5.4	++++	++++	++++	++++	++++	++++	+++	++++	ND	+++
BS231**	IgG	5.4	++++	++++	++++	+++	++++	++++	+++	++++	ND	++++
H41**	IgG	3.1	-	-	-	-	+++	++	+++	++++	++++	+++
LOR17-6C7	IgG	4.1	++++	-	-	-	-	++++	-	++++	++++	++++

*Gel matrix tests with antiglobulin.

†epD patterns as described previously by Scott.[26] ND - not determined. NA – not applicable.

‡Monoclonal anti-D approved for routine use in Germany.

§D-Screen; Diagast.

‖Advanced Partial RhD Typing Kit; Alba Bioscience. The results obtained with LHM76/58 and with monoclonal antibody no. 74 documented as LHM76/58 in the Nantes workshop[37] differed. The reactivity of ESD1 differed from that of “ESD1M?” (monoclonal anti-D no. 1-56) documented in the Paris workshop where specificities for epD4.1 and epD9.1 were listed.[26]

¶ID-Partial Rh D-Typing Set; DiaMed.

**Monoclonal anti-D Panel; Biotest.

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Table 4

Phenotype frequencies of D variants in the Southwest German population

	Phenotype frequency		
partial D	**Estimate**	**95% Confidence interval***	**Donations† (n)**
DVII	1:977	1:788 - 1:1,264	80
all DIV	1:9,770	1:5,238 - 1:23,792	8
DIV type 1.0	NA	1:26,087 - NA	0
DIV type 3	1:19,539	1:8,143 - 1:57,215	4
DIV type 4	1:26,052	1:9,647 - 1:95,545	3
DIV type 5	1:78,156	1:14,683 - 1:1,532,470	1
DNU	1:78,156	1:14,683 - 1:1,532,470	1
DFW	1:78,156	1:14,683 - 1:1,532,470	1

*95% confidence interval of the frequency estimate according to Poisson distribution.

†D variants among 78,156 blood donations, of which 60,965 were D positive. All DIV samples came from different donors. The 80 donations with DVII were from 71 donors.

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Table 5

Differences between DIVa and DIVb

	DIVa[*] ***DIV type 1.0***[†]	**DIVb**[*] ***DIV type 2 to type 5***[†]
Epitopes		
Go[a]	positive	negative
epD4	positive	negative
D antigen expression	high	moderately reduced
RHD nucleotide substitutions	dispersed	*RHD-CE-D* gene conversions
RHCE	associatied with *ceTI*	regular exon 7
Haplotype	cDe or (C)De[≠]	CDe or cDE
Phylogeny	old extant allele, primordial allele of the DIVa cluster in the African cluster	gene conversions in the Eurasian cluster
Typical ethnic origin	African	Caucasian

[*] Defined serologically

[†] Defined at the molecular level

[≠] A minor fraction of DIVa samples expresses a weak C antigen.[64]