Molecular and serologic characterization of DWI, a novel "high-grade" partial D

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BACKGROUND: Accurate D antigen identification is essential for pretransfusion and prenatal evaluation to prevent anti-D alloimmunization. Quantitative and qualitative D variants may pose typing problems and require particular consideration because of differing potential for anti-D induction.

STUDY DESIGN AND METHODS: A novel partial D, DWI, was discovered in an anti-D-alloimmunized D+ Austrian woman. This D variant was investigated by *RHD* genotyping and nucleotide sequencing, as well as characterization of its serologic properties.

RESULTS: The proposita exhibited a single-nucleotide exchange in *RHD* Exon 7 (1073T>C) predicting a Met358Thr substitution in the sixth extracellular loop of the RhD polypeptide. All DWI individuals identified (the proposita and two relatives) were genotyped *DWIdCcee*, which, together with the family tree, was highly suggestive of a *DWICe* haplotype association. Epitope mapping studies revealed only minor D antigen modification with weakening but not loss of epitopes D1.1, D9.1, and D16.1. Antigen density varied individually between 8000 and 8600 D sites per erythrocyte. No known low-frequency Rh antigen was detected. Despite the highly retained D epitope composition, the DWI proposita's serum sample contained alloanti-D from an immunization event many years earlier.

CONCLUSION: The findings of this investigation emphasize the possible clinical significance of "high-grade" partial D variants that are likely to be missed by routine serology.

The highly immunogenic D antigen (RH1) is a complex mosaic of different epitopes (Eps). Partial D individuals genetically devoid of parts of the antigen are able to mount an antibody response against those Eps that are not expressed upon challenge with normal D+ RBCs.[1] A number of such cases have elicited severe HDN.[2-4] Partial D types emerge from *RHD*/*RHCE* gene hybridization events or point mutations in *RHD* stretches encoding extracellular RhD domains, with characteristic loss of D Eps.[5] Weak D types, on the other hand, exhibit amino acid substitutions in transmembraneous or cytosolic RhD segments,[6] leading to diminished expression of, in most instances, grossly normal D antigen.[7] Because most prevalent weak D types as well as the reduced D density brought about by a *C in trans* haplotype[8] are not associated with loss of D Eps,[7] such individuals are not prone to anti-D alloimmunization.

ABBREVIATIONS: Ep(s) = epitope(s); SSP = sequence-specific priming.

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D Eps may be either continuous, represented by a particular amino acid sequence, or highly conformation-dependent, involving more than one of the six extracellular loops of the RhD polypeptide.[9-11] Carefully selected panels of anti-D MoAbs allow for evaluation of antigen integrity and definition of distinct Ep patterns of partial D types.[12,13] This aids in identification of D variants, which has a decisive impact on transfusion strategy and prenatal investigation.[14] We report on a novel partial D termed DWI with grossly conserved Ep composition that was discovered in a patient with alloanti-D. The designation DWI is derived from partial D from Wien (Vienna, Austria).

MATERIALS AND METHODS

DWI proposita

On initial testing the DWI proposita, a Caucasian 74-year-old woman of Viennese (Eastern Austrian) ancestry was typed O, D+. Her serum sample contained apparent alloanti-D suggestive of partial D, which necessitated further evaluation. The patient had delivered two healthy daughters after uneventful pregnancies from her D+ husband. About 25 years ago, she had received several transfusions during and after gynecologic surgery.

Immunohematology

Routine serologic investigations were carried out by tube and gel matrix testing (Micro Typing System DiaMed-ID, Cressier, Switzerland). Blended monoclonal anti-D reagents (DiaClon anti-D, DiaMed; Seraclone anti-D Blend, Biotest, Dreieich, Germany; and anti-D Totem, Diagast, Loos, France) as well as polyclonal anti-D antisera (from Biotest; Gamma Biologicals, Inc., Houston, TX; and Ortho Diagnostic Systems, Neckarsgmünd, Germany) were used for direct agglutination. Additionally, blended monoclonal and polyclonal anti-D (DiaMed) were employed in gel matrix tests. D variant identification was attempted by use of two commercially available anti-D MoAb panels (D-Screen, Diagast; and ID-Partial Rh D-typing set, DiaMed). The DAT was performed with monospecific anti-human globulin reagents (anti-IgG, -IgA, -IgM, -C3c, and -C3d) in gel matrix (DiaMed). Antibody titration was carried out in tubes with polyspecific anti-human globulin (Ortho). Chloroform elution was performed as described.[15] In cross-match experiments with blood group O, partial D, and normal D+ and D– control RBCs, the serum sample of the DWI proposita containing anti-D was applied neat. In case of cross-match with A cells, the proposita's serum sample was used after exhaustive adsorption of anti-A with pooled normal blood group A, ddccee RBCs. D Ep mapping was achieved with 79 anti-D MoAbs from two Rh serology workshops.[12,13]

Molecular biology

Genomic DNA was isolated from EDTA-anticoagulated blood with Nucleon BACC2/3 reagents (Amersham, Buckinghamshire, UK). For *RHD* and *RHCE* genotyping a PCR with sequence-specific priming (SSP) was performed with a commercially available kit (CDE-SSP, Inno-Train, Kronberg, Germany). *RHD* zygosity was determined by PCR-SSP and PCR-RFLP.[16] Nucleotide sequencing of *RHD* Exons 1 to 10 from genomic DNA of the proposita was carried out as described.[17] A specific PCR-SSP was developed to detect DWI with 0.5 µmol per L detection primer *RHD*Ex7+968F (GTAACCGAGTGCTGGGGATGCC) and *RHD*Ex7-DWI+1073R (GGGTAAGCCCAGTGACCCCCG), 0.1 µmol per L control primer, and 1.5 mmol per L $MgCl_2$. All other parameters were exactly as described previously.[18]

Antigen density determination

Antigen density was determined by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany), with minor modifications of a published protocol.[19] Fifty microliters of 2 percent suspensions of washed RBCs in PBS was incubated at 37°C for 1 hour with 50 µL of human anti-D. Nine human IgG anti-D MoAbs were used: P3x290, P3x35, P3x241, P3x249, and HM16 (Diagast); MS26 and ESD1 (DiaMed); and BRAD-3 and BRAD-5 (International Blood Group Reference Laboratory/IBGRL, Bristol, UK). The human clone AEVZ5.3 (IBGRL) served as a negative control. In addition, additional incubations were performed with human polyclonal anti-D (Ost, MIC and PER) and anti-c (She), as well as mouse monoclonal anti-glycophorin A (BRIC256). The human polyclonal reagents had been prepared from alloimmune sera by adsorption and subsequent acid elution from antigen-positive RBCs with an elution method (Elu-Kit II, Gamma Biologicals Inc.). After incubation with primary antibody, the cells were washed and bound antibody was detected by the addition of PE-labeled $F(ab')_2$ fragment rabbit anti-mouse IgG (Dako, Ely, UK) or FITC-conjugated $F(ab')_2$ fragment rabbit anti-human IgG (Dako). After incubation with secondary antibody (30 min at 4°C) and washing, 30,000 events per sample were acquired.

Absolute D antigen densities (D antigens per RBC) were assessed by use of a standard RBC sample with 18,332 D antigens per cell (kindly provided by M. Urbajs, Blood Transfusion Center of Slovenia, Ljubljana, Slovenia) deduced from the standard of the Fourth International Workshop on Monoclonal Antibodies against Human Red Blood Cells. For antigen density calculations, the recommended algorithm was applied.[19]

Population screen for DWI

One human monoclonal IgM anti-D antibody (P3x212 23B10, Diagast) did not agglutinate RBCs from DWI indi-

viduals. Therefore, EDTA-anticoagulated blood samples from 1377 blood donors with a high probability for *RHD* heterozygosity (1008 of D.Ccee phenotype, 311 D.ccEe, and 58 D.ccee) from Eastern Austria (Vienna, Lower Austria, and Burgenland) were screened for reactivity with this MoAb in neutral gel cards (DiaMed) at room temperature. In case of weak or absent reactivity, blood samples were further tested with the anti-D of the DWI proposita, and DNA was extracted for subsequent individual *DWI*-specific PCR-SSP.

In addition, a total of 1152 anonymized DNA samples of PLT donors (911 of which were D+) from Western Austria (Tyrol) were screened for the *DWI* allele by use of *DWI*-specific PCR-SSP. Pools of eight DNA samples each were investigated, whereby each single DNA sample was screened in two different pools.

RESULTS

Antigen and antibody identification by routine serology

Serologic testing of the DWI proposita's RBCs (DWI RBCs) yielded blood group O; D+C+c+E–e+, C^w–, G+ NSs; P1+; Lu(a–b+); K–k+ Kp(a–b+); Le(a–b+); Fy(a+b+); Jk(a+b+). All routine D typing reagents, a commercial panel of anti-D MoAbs for partial D detection, and a polyclonal anti-D from a DIIIa person were uniformly reactive, without evidence for variant D. Nevertheless, results obtained with another commercial MoAb panel indicated altered EpD9.1 based on negative reactivity with one MoAb (clone P3x212 23B10).[13]

The serum sample of the proposita contained anti-D, reacting with 65 different D+ but not with 52 D– RBCs. Bromelain treatment of test RBCs slightly enhanced positive reactions, whereas DTT (0.2 mol/L) had no attenuating effect excluding anti-LW specificity.[20] The anti-D titer in the IAT was 4 as tested against two different D+C–c+E+e– RBC samples. The DAT was negative, and a chloroform eluate prepared from DWI RBCs did not react, indicating that the anti-D was of alloimmune nature.

DWI results from a single-point mutation in *RHD* Exon 7

According to Rh genotyping and *RHD* zygosity determination the DWI proposita was *DdCcee*. *RHD*-specific sequencing of genomic DNA was carried out to elucidate the molecular basis of anti-D formation in this obviously D+ patient. We found a single exchange of the last nucleotide of *RHD* Exon 7, T to C at position 1073, accounting for a Met358Thr substitution in the predicted sixth extracellular loop of the RhD polypeptide (GenBank Accession Number AY170011). All relatives investigated (two daughters, a sister, and a daughter of the sister) and her husband were genotyped *DdCcee*. Only her sister and the niece displayed the DWI polymorphism as demonstrated by the newly developed PCR-SSP directed at the 1073T>C exchange. Both genotyping results and the family tree strongly argued for a *DWICe* haplotype association.

No irregular RBC antibodies were found in the serum sample of the sister or the niece of the DWI proposita. Neither had received any transfusions. The sister had had four pregnancies (one daughter with DWI phenotype and three children whose Rh status could not be evaluated), whereas the niece had one daughter of unknown Rh phenotype.

The cross-match of the RBCs of both the sister and the niece with the anti-D of the proposita was found to be negative in the IAT, further corroborating their DWI status. In addition, cross-matches with RBCs from DIV Type IV and DNB individuals were negative, but positive with DFR Type I and DVII RBCs.

DWI RBCs exhibit only minor D antigen alteration

Because single RhD amino acid exchanges may provide particularly valuable insights into the molecular basis of the D antigen,[10] additional investigations were undertaken for phenotypic characterization of DWI RBCs. The D antigen appeared highly conserved because RBCs of the DWI proposita reacted with 78 of 79 anti-D MoAbs of known Ep specificity (Table 1). Only one of the seven MoAbs to EpD9.1 did not react, indicating that DWI RBCs split this Ep. One MoAb each to EpD1.1 and EpD16.1 reacted only weakly. To our knowledge this pattern of reactions is unique.

Flow cytometric analysis of DWI RBCs labeled with five different anti-D MoAbs demonstrated only moderately reduced expression of D Eps compared to control D+C+c+E–e+ RBCs (Fig. 1). Of note, results with the anti-EpD9.1 MoAb (MS26) were not different from those with MoAbs to other Eps. The application of an additional four monoclonal and three polyclonal anti-D yielded very similar results (data not shown). The absolute D antigen densities of all DWI+ individuals and normal control RBCs calculated from fluorescence intensities of indirect staining with all nine anti-D MoAbs are shown in Table 2. The observed interindividual differences in the D antigen density of DWI RBCs were confirmed by repeat testing of different blood samples. Levels of expression of c and glycophorin A by DWI RBCs were normal (data not shown).

DWI RBCs did not express low-frequency antigens C^x (RH9), V (RH10), VS (RH11), D^w (RH23), Go^a (RH30), Rh32, Rh33, Evans (RH37), Tar (RH40), Riv (RH45), FPTT (RH50), BARC (RH52), JAL (RH48), or JAHK (RH53), all known to be associated with distinct D or CcEe variants.[1,5]

TABLE 1. Reactivity pattern of RBCs of the DWI proposita with 79 monoclonal anti-D from two Rh serology workshops[12,13]

EpD*	MoAbs Positive	MoAbs Negative
1.1	BRAD-7 (weak)	
1.2	LHM70/45	
2.1	NaTH98-10D9, NaTH98-11C2, P3x249	
2.2	5C8	
3.1	LOR-15C9, H1121G6, HIRO-11, HIRO-16, H1121g6, P3x290, LHM76/55, H4111B7	
4.1	ESD1M	
5.1	HIRO-15	
5.2	NaTH109-1G2, OSK3	
5.3	P3Brou7	
5.4	P3x35, P3x241, BS229, BS231	
6.1	P3AF6, OSK3-2	
6.2	822, HMR49, 6D10, SM3H4, OSK3-1, FC3, BRAD-3	
6.3	AB5, F64, Los1, D7, Lorifa, LHM169/80	
6.4	NaTH119-3E5, HM16, P3 × 61, HS114, D1-4, BS223, BS225, P3.187, P3F20, D7B8	
6.5	OSK3-4	
6.6	NaTH118-3C3, HM10, Flos3, HIRO-101, HIRO-102, P3F17	
6.7	FOG-1, N2B8, HIRO-94, HIRO-95	
6.8	2B6, BRAD-5, BRAD-6, FEF3	
8.1	NaTH53-2G10, LHM59/19	
8.2	P3x212-11F1	
9.1	LHM77/64, HIRO-4, HIRO-7, HIRO-8, BRAD-2, MS26	P3x212-23B10
12.1	NaTH87-4A5	
15.1	BTSN6, OSK3-3	
16.1	HIRO-3, MCAD-6, BS87 (weak)	

* Not tested: EpD5.5, EpD8.3, EpD10.1, EpD11.1, EpD13.1, and EpD14.1.

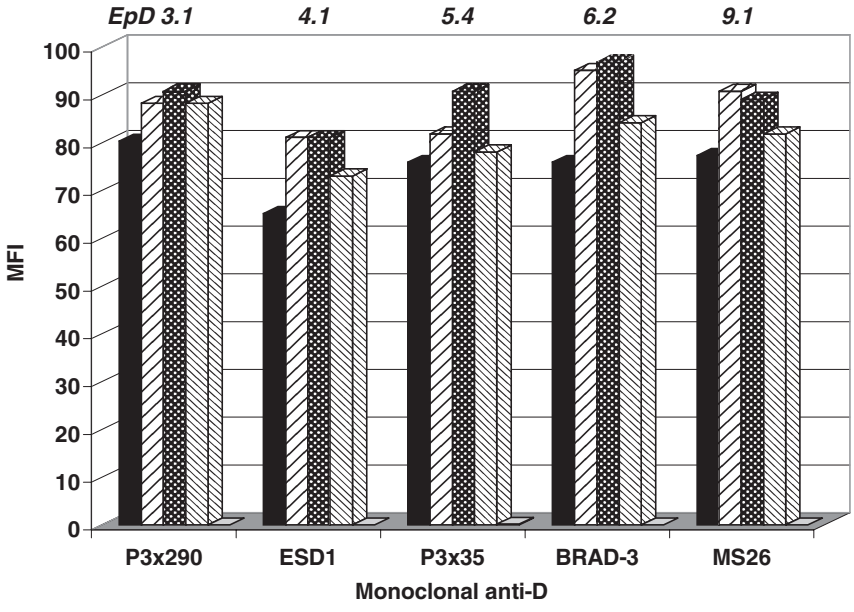

Fig. 1. D Ep expression of RBCs of the DWI proposita as determined by flow cytometry. For comparison, control RBC samples (three of DCcee and one of ddccee phenotype) are included. Human monoclonal anti-D were used in combination with FITC-conjugated F(ab′)$_2$ fragment rabbit anti-human globulin for indirect staining. Data are median fluorescence scores after subtraction of the score of the negative control. (■) DWICcee; (▨) DCcee (Sample 1); (▩) DCcee (Sample 2); (▧) DCcee (Sample 3); (□) ddccee.

Population screen for DWI

Both serologic and DNA-based techniques were employed in an attempt to establish the *DWI* allele frequency in the Austrian population. Nevertheless, no additional DWI individuals were identified among 1377 and 911 D+ blood donors from Eastern and Western Austria, respectively.

DISCUSSION

In this study a novel partial D, DWI, derived from a *RHD* single-point mutation, was identified that was found to be "high grade" in respect to D antigen integrity as well as D antigen density. The predicted RhD Met358Thr substitution appears to directly determine the DWI-specific Ep alterations. EpD9.1, which is probably continuous, was assigned to the sixth extracellular loop of RhD with inferred critical amino acids Asp350, Gly353, and Ala354.[10,21] Our results suggest that Met358 may also be involved, because the DWI reaction pattern indicated splitting of EpD9.1. Similarly, all other known partial D types with single amino acid substitutions in this stretch of the RhD polypeptide, such as DII (Ala354Asp), DNU (Gly353Arg),[10] and DNB (Gly355Ser)[22] exhibit at least partial loss of EpD9.1.[13,22] Also the DIV Type IV variant derived from a *RHD-CE-D* hybrid in which the sixth extracellular RhD loop is affected (Asp350His, Gly353Trp, and Ala354Asn substitutions) features loss of EpD9.1. This possibly accounted for the lack of reactivity of the anti-D of the DWI proposita with DNB and DIV Type IV RBCs, because all these three D variants have EpD9.1 alterations in common. In contrast, RBCs of DVII and DFR phenotype with alterations in the second (Leu110Pro) and third (Met169Leu, Met170Arg, Ile172Phe) extracellular RhD loops,[23,24] respectively, clearly differ from DWI in their antigenic properties, which are recognized by the proposita's anti-D.

Like DNU,[13] DWI displayed some EpD1.1 alteration as evidenced by only weak binding of MoAb BRAD-7. This confirms earlier observations[21] that

TABLE 2. D antigen densities of DWI and normal control RBCs

Rh phenotype	Number of D sites per cell
DWICcee	
Proposita	8,471
Sister	8,642
Niece	7,966
DCcee	
Sample 1	9,748
Sample 2	9,952
Sample 3	9,409
DCCee	19,770
DCcEe	20,127

parts of the sixth extracellular RhD loop contribute to EpD1.1 expression. DII, DNU, DNB, and DWI RBCs are all reactive with LOR-15C9,[13] a MoAb to EpD3.1, previously reported to recognize a non-conformation-dependent antigenic determinant derived from *RHD* Exon 7.[9] Nevertheless, in contrast to DNB, which is known to split EpD3.1,[13] DWI RBCs reacted with all eight EpD3.1-specific MoAbs tested, including HIRO-11 and HIRO-16. Additional differences were positive and negative reactions of DWI and DNB RBCs, respectively, with P3F20 (EpD6.4) as well as with HIRO-7 and HIRO-8 (EpD9.1).[22] Taken together, of all mentioned partial D variants DWI seems to have the most conserved D Ep composition.

The *DWI* polymorphism is localized to the very end of *RHD* Exon 7 and could theoretically lead to aberrant RNA splicing, which would lead to major antigen modification or total loss of D antigen expression. Alternatively, the Met358Thr exchange in DWI renders Asn356 a potential N-glycosylation site.[25] N-glycosylation of the DWI polypeptide could affect its intracellular trafficking, RBC surface expression, folding, and antigenicity. Nevertheless, the only marginally altered Ep composition, the degree of antigenic similarity to related partial D variants, and the near-normal strength of antigen expression strongly argue against both aberrant splicing and N-glycosylation of Asn 356 in DWI.

Remarkably, despite the highly retained D antigen integrity of DWI, alloanti-D had been produced against missing antigenic determinants, which was still readily detectable 25 years after the last possible immunizing event. This emphasizes the potential clinical significance of even high-grade partial D variants. In the vast majority of partial D types, including DII and DNB, alloanti-D formation has been reported.[1,22] Because alloanti-D is generally considered clinically significant with respect to hemolytic transfusion reactions and HDN,[26,27] anti-D alloimmunization should be avoided in persons with partial D phenotypes. Consequently, individuals identified to exhibit the DWI phenotype should preferably receive D– transfusions and, in case of pregnancy, be monitored for the induction of anti-D and administered prophylactic anti-D immunoglobulin after delivery of a D+ baby. One obstacle to this, however, is the fact that DWI and a number of other partial D variants are likely to be missed by routine serology. For this reason, to increase the accuracy of Rh typing, the development of DNA-based methods for fast and economic large-scale routine use may be a promising future perspective.

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