Novel weak D types 31 and 32: adsorption-elution–supported D antigen analysis and comparison to prevalent weak D types

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BACKGROUND: Weak D types are thought to express rather quantitative than qualitative D antigen variants. Distinct type-specific phenotypes and weak D cases with anti-D alloimmunization, however, suggest a variable degree of D antigen alteration.

STUDY DESIGN AND METHODS: Variant D types were investigated by use of molecular typing, *RHD* sequencing, extended serologic D antigen investigations, and flow cytometric D antigen density determination.

RESULTS: Two novel weak D types were discovered, termed weak D type 31 and 32 with single *RHD* nucleotide substitutions coding for amino acid exchanges in predicted intracellular RhD polypeptide stretches, with antigen densities of approximately 130 and 50 D sites per red blood cell, respectively. Adsorption-elution technique–supported D epitope mapping of these two weak D types, the recently described weak D type 26, and of the most common Central European weak D types (weak D types 1, 2, 3, 4.0, and 4.1) demonstrated the expression of all tested D epitopes. In contrast, a distinct D epitope loss was detected in weak D type 15 and partial D control samples.

CONCLUSION: All novel and prevalent weak D types expressed all tested D epitopes. Our results indicate that adsorption-elution techniques may be of advantage whenever D epitope loss is suspected in extremely weak D variants.

Owing to the high immunogenicity the Rh blood group antigen D (RH1), a high proportion of D– individuals challenged with D+ red blood cells (RBCs) by transfusion or pregnancy develop anti-D. The clinical significance of alloanti-D lies in its potential to cause hemolytic transfusion reactions and severe hemolytic disease of the newborn (HDN).[1] Anti-D alloimmunization can mostly be prevented by adequate transfusion strategies and the timely administration of immunoglobulin (IgG) anti-D prophylaxis. For these reasons, reliable routine D typing methods must be performed, with particular attention to donors with weakly expressed D variants.[2]

The D antigen is composed of a considerable number of distinct D epitopes located on six extracellular loops of the RhD polypeptide.[2] The RhD polypeptide is encoded by the *RHD* gene, whereas the RhCcEe polypeptides carrying the C (RH2) or c (RH4) and E (RH3) or e (RH5) antigens are encoded by the highly homologous *RHCE* gene. The

ABBREVIATION: SSP = sequence-specific priming.

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genetic bases of RhD variants are either *RHD-CE-D* gene hybrids or *RHD* point mutations.[3] Partial D variants are derived from *RHD* alterations affecting partly or exclusively extracellular RhD sections, leading to type-specific D epitope loss and a frequently diminished D antigen density.[4] Such individuals may develop alloanti-D against those D epitopes they are devoid of, sometimes causing severe to fatal HDN.[5-9]

Another group of D variants are weak D types emerging from *RHD* point mutations coding for transmembraneous or cytosolic RhD amino acid substitutions.[10] Most known weak D type RBCs are thought to express quantitatively but not qualitatively altered D antigens. Therefore, such individuals appear not to have a tendency toward alloanti-D induction upon contact with normal D+ RBCs. Nevertheless, RBCs of different weak D types were reported to exhibit characteristic phenotypes not only with regard to a type-specific range of D antigen densities but also to the reactivity with monoclonal antibodies (MoAbs) with known D epitope specificity.[11] This suggests a variable degree of D antigen alteration also in weak D types. Moreover, cases of weak D type 4.2 and weak D type 15 individuals with anti-D alloimmunization indicated that there may be no clear-cut distinction between weak and partial D types but rather a continuous spectrum from absent D antigen alteration to major D epitope loss in RhD variants.[11]

In this study, the molecular background of two novel weak D types, weak D type 31 and 32, and their associated immunohematologic features are presented. In addition, a qualitative analysis of the D antigen expressed by weak D types 31 and 32, the recently discovered weak D type 26, as well as a comparison with the four most common weak D types in Central Europe, weak D types 1 to 4,[12] was performed.

MATERIALS AND METHODS

Investigated blood samples

Weak D type 31 was discovered in Dresden, Germany. Manual routine control of a female blood donor sample following fully automated blood group determination (PK 7200, Olympus, Hamburg, Germany) yielded incongruent results. By means of two different monoclonal anti-D reagents (anti-D Mono-Type, anti-D clone MS201, Medion Diagnostics, Düdingen, Switzerland; ImmuClone anti-D rapid, anti-D clone RUM-1, Immucor, Rödermark, Germany), this sample had been typed D–. However, a weak D+ result was obtained with two different anti-D reagents (Seraclone anti-D blend, clones BS221, H4111B7, BS232, Biotest, Dreieich, Germany; and ImmuClone anti-D duo, anti-D clones MS26 and TH28, Immucor) in tube technique indirect antiglobulin test (IAT).

Weak D type 32 was identified in Linz, Austria. Molecular screening of blood samples typed D– by automated serology (Olympus PK 7200, with anti-D Totem, clones P3×61, P3×21223B10, P3×290, P3×35, Diagast, Loos, France; and microplate technique, Anthos photometer, Krefeld, Germany, with anti-D microscreen, clone HM10, and anti-D Totem phenolic, Diagast) led to recognition of a male donor sample positive for *RHC* along with a previously unidentified *RHD* gene. On serologic reevaluation of RBCs from this sample, a faintly positive result was observed with two different anti-D reagents in tube technique IAT (Seraclone anti-D blend, Biotest; anti-D for weak D, clone ESD1, DiaMed, Cressier, Switzerland).

Weak D type 26 had also been identified by a molecular screening approach of seemingly D–, C, or E+ samples in Innsbruck, Austria. Weak D type 26 showed a *RHD* T26A nucleotide exchange predicting a valine-to-aspartate substitution at intracellular position 9 of the RhD polypeptide. Further details of weak D type 26 are given elsewhere.[13]

Reference samples of weak D types 1 (n = 3), 2 (n = 2), 3 (n = 2), 4 (n = 3), and 15 (n = 2) and *RHD* category VI type I (n = 3) were taken from respective cohorts from Innsbruck, Austria, identified earlier.[12] Weak D type 4 samples were sequenced in *RHD* exons 1, 3, 4, 5, 6, and 7 for discrimination of weak D type 4.0 and 4.1 from weak D type 4.2.[13] The reference samples used in this study were of weak D type 4.0 (n = 1) and 4.1 (n = 2). The *RHD* category IV type IV sample was from Vienna, Austria. Molecular characterization of the respective *RHD* gene was achieved with commercially available *RH* polymerase chain reaction (PCR) typing kits (CDE, weak D and RHd, Innotrain, Kronberg, Germany; details are given below) with sequence-specific priming (SSP) following the manufacturer's instructions.

Molecular biology

Genomic DNA was isolated from ethylenediaminetetraacetate-anticoagulated blood with Nucleon BACC2 reagents (Amersham, Buckinghamshire, UK). Molecular screening of D– samples from Linz was performed in pools of 20 with LightCycler technology as recommended by the manufacturer (Roche, Vienna, Austria), employing *RHD* specific primers for exons 4, 7, and 10 as published previously.[14] For *RHD* and *RHCE* genotyping, testing for different partial and weak D variants and determination of the *RHD* zygosity of investigated and control blood samples, a PCR-SSP was performed with commercially available typing kits (CDE, weak D, RHd, Innotrain). Kit "CDE" is capable of properly identifying *RHD-CE-D* hybrid alleles by detecting *RHD*-specific DNA sequences in the 5′-untranslated region and exons 2, 3, 4, 5, 6, 7, 9, and 10 of *RHD* and *RHC*, *RHc* (intron1 and exon 2) and *RHE*, *RHe* (exon 5) of *RHCE* as described previously.[13,14] Weak D types 1 to 5, 11, and 15 can be typed with kit "weak D," also allowing the discrimination of weak D type 4.0 and 4.1 from 4.2. Detec-

tion of weak D types 1 to 5 without weak D 4 subtype discrimination was described by Müller and coworkers.[12] Kit "Rhd" includes detection for the Rhesus hybrid and upstream *Rhesus* box indicative for the *RHD* zygosity status of an investigated sample, as well as specificity for the common *RHD* DEL alleles M295I, IVS3+1g>a, K409K, the unexpressed *RHD* allele *RHDψ*, and the phenotypic D– *RHD* allele *RHDCe*^{s}. The respective molecular backgrounds are given elsewhere.[15-18] Nucleotide sequencing of *RHD* exons 1 to 10 from genomic DNA of the weak D type 31 and 32 propositi was performed as described earlier.[13]

Immunohematology

Blended monoclonal anti-D reagents (DiaClon anti-D, anti-D clones MS26 and TH28, DiaMed; Seraclone Anti-D blend, Biotest; and Anti-D Totem, Diagast; BioClone, Ortho Diagnostic Systems, Neckargemünd, Germany) were used for direct plate test agglutination as well as gel matrix IAT (low-ionic-strength saline direct antiglobulin test [DAT] cards containing anti-IgG and anti-C3d, DiaMed). Additionally, monoclonal and polyclonal anti-D, anti-C, anti-c, anti-E, and anti-e reagents contained within gel matrix (ABO/Rh for patients; DiaClon Rh-subgroups+K; ABO/Rh; Rh-subgroups+C^{w}+K, DiaMed) were employed. RBC antibody screening with three test cell preparations was performed by IAT in gel matrix (DiaMed), as was the DAT with monospecific anti-human globulin (anti-IgG, -IgA, -IgM, -C3c, -C3d). For D epitope mapping, human IgG anti-D MoAbs (clones P3×249, P3×290, P3×35, HM16 (Diagast, in part donated by Klaus Göttfert); MS26, ESD1, LHM70/45, LHM76/55, LHM59/19, and LHM169/80 (DiaMed, partly provided by Armin Köchli); Brad-2, Brad-3, and Brad-5 (provided by G. Daniels, Bristol Institute for Transfusion Sciences, Bristol, UK); Birma D6 (International Blood Group Reference Laboratory/IBGRL, Bristol, UK); H4111B7, BS221, BS227, BS228, BS229, and BS231 (a gift from M. Ernst, Biotest); and human IgM anti-D MoAbs (P3×61, HM10, P3×21223B10, and P3×21211F1, from Diagast) with known D epitope specificity[19] were used in gel matrix. In case of nonreactivity, IgM MoAbs were adsorbed onto equal volumes of washed packed variant D as well as negative and positive control RBCs (*ccddee* and weak D type 3 *CcDdee*) at room temperature for 10 minutes, followed by 10 washing steps with PBS.[20] To exclude unspecific reactions due to MoAb adsorption onto glass, adsorbed RBCs were transferred to clean tubes after each washing step. After the last wash, RBCs were resuspended in an equal-volume remainder of washing solution, subject to heat elution (10 min at 56°C) in a water bath with occasional agitation, and finally centrifuged to collect the supernatant (eluate). IgG anti-D MoAbs not reactive with weak D type 15 or DVI type I RBC samples in IAT were adsorbed onto equal volumes of RBCs at 37°C for 1 hour and after eight washing steps subjected to acid elution (DiaCidel, DiaMed). The same procedure was performed for DEL phenotype detection with routine anti-D reagents (DiaClon anti-D and Seraclone anti-D blend). Heat and acid eluates and the respective last washing solutions were tested against one D– and two D+ RBC samples in gel matrix IAT.

Flow cytometry

D antigen density of variant D and control RBCs was determined by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany) as described.[21] Eleven human IgG anti-D MoAbs were used: P3×35, P3×290, P3×241, P3×249, HM16, BS221, BS227, MS26, ESD1, Brad-3, and Brad-5. The human IgG clone AEVZ5.3 (IBGRL) was used as negative control. Fluorescein isothiocyanate–conjugated Fab fragment goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) served as secondary reagent. Absolute D antigen densities (D antigens per RBC) were assessed by the combined use of *ccddee*– control and *CcDdee* standard RBC samples with known D antigen density.[22] For D antigen density calculation, the recommended algorithm was applied.[21]

RESULTS

Weak D types 31 and 32 are both caused by single *RHD* point mutations

Initial molecular typing approaches of both weak D type 31 and 32 propositi yielded identical results: According to *RHDCE* PCR-SSP, both were of *CcDee* genotype, without evidence for *RHD-CE-D* hybrid alleles as judged by testing for the *RHD* 5′-untranslated region and *RHD* exons 2, 3, 4, 5, 6, 7, 9, and 10. Typing for weak D excluded weak D types 1 to 5, 11, and 15, and typing for *RHD* zygosity revealed both a hybrid and upstream *Rhesus box* indicative of *RHD* heterozygosity.

To define the molecular basis of the confirmed weak D antigen expression in these two cases, *RHD* DNA sequencing was performed. The sample from the female donor showed a single-nucleotide exchange in *RHD* exon 1, C to T at position 17, predicting a Pro6Leu substitution at the aminoterminal end of the RhD polypeptide. Because of the presumed intracellular localization of this RhD amino acid substitution, this RhD variant was assigned the designation weak D type 31. The DNA sequence of exon 1 of the observed allele (*RHD*(P6L), C>T at 17, including the adjacent 50 bp) of the promoter and intron 1 was deposited under EMBL Accession Number AJ557803.

The sample of the male donor showed an A at coding nucleotide position number 1121 in *RHD* exon 8 instead of T, accounting for a predicted Ile374Asn amino acid exchange in the last transmembraneous spanning part of

the RhD polypeptide. This RhD variant was dubbed weak D type 32. The DNA sequence of exon 8 of this novel allele (*RHD*(I374N), T>A at 1121) including the adjacent 50 bp of intron 7 and 150 bp of intron 8 was deposited under EMBL Accession Number AJ580942 (exons 1-7, and exons 9 and 10 of the same allele were deposited under EMBL Accession Numbers AJ580935 to AJ580941 and AJ580943 and AJ580944, respectively).

A family analysis of the weak D type 31 proposita revealed a daughter typed *ccddee* and another daughter typed *CcDdee* with the weak D type 31 allele. This clearly demonstrated the association of weak D type 31 with *Ce*. No family study of the weak D type 32 propositus could be performed owing to unavailability of family members. During the preparation of this article, however, another unrelated weak D type 32 individual from Linz was discovered by molecular screening and genotyped *CCDdee*, indicating an association of weak D type 32 with *Ce*.

Routine Rh typing of weak D type 31 and 32 RBCs

The weak D type 31 and 32 RBC samples did not react with all routine monoclonal anti-D reagents (DiaClon, Seraclon, and Totem anti-D) in direct agglutination plate tests. By use of these reagents in gel matrix IAT, the two weak D type 31 RBC samples and the sample of the weak D type 32 propositus yielded 2+ and 1 to 2+ positive reactions, respectively, whereas negative reactions were observed with the second weak D type 32 sample. The latter exhibited a DEL phenotype as evidenced by adsorption and subsequent acid elution of routine anti-D reagents. The DEL phenotype in this *CCDdee* individual was probably caused by the suppressive effect of *C* in trans.[11] With polyclonal and monoclonal anti-D contained within gel matrix, only weak D type 31 RBCs displayed extremely faint positive reactions. Both weak D type 31 RBC samples and the first weak D type 32 sample were typed C+ c + E– e+ with polyclonal and monoclonal routine reagents in gel matrix, whereas the DEL sample was C+ c– E– e+.

Weak D type 31 and 32 RBCs express very low D antigen numbers

These results suggested that the two novel weak D alleles were associated with a particularly low D antigen expression. D density quantification by flow cytometry corroborated the routine serologic results. As shown in Table 1, all *CcDdee* weak D type 31 and 32 RBCs exhibited lower-scale absolute D antigen densities compared to other common weak D types. Weak D type 32 RBCs expressed substantially lower numbers of D antigens than weak D type 31 RBCs, paralleling the reactivity of anti-D reagents in gel matrix. The D densities obtained with RBC samples of the most frequent weak D types 1, 2, 3, 4.0, and 4.1 as well as with weak D type 15 and partial D RBCs were comparable to those reported previously.[4,11,23]

Different weak D types including weak D types 31 and 32 express phenotypes without detectable D epitope loss

Motivated by earlier reports on distinct phenotypes being associated with different weak D types,[11] the integrity of the D antigens expressed by weak D types 26, 31, and 32 (*CcDdee*) and the most frequent weak D types was investigated. For this purpose, a panel of anti-D MoAbs with known D epitope specificity[19] was employed in gel matrix technique. As shown in Table 2, all applied IgG-class anti-

TABLE 1. D antigen densities of weak D type 31, weak D type 32, and different other variant D RBCs

Rh phenotype	Molecular background	Number of D sites per cell	
		Median	Range
Weak D type 31 (*CcDdee*)	*RHD*(P6L)		
Proposita		131	
Daughter		136	
Weak D type 32	*RHD*(I374N)		
Propositus (*CcDdee*)		49	
Unrelated case, DEL (*CCDdee*)		ND*	
Weak D type 26 (*CcDdee*)	*RHD*(V9D)	70 (n = 1)†	
Weak D type 1 (*CcDdee*)	*RHD*(V270G)	978 (n = 3)	889-1197
Weak D type 2 (*ccDdEe*)	*RHD*(G385A)	557 (n = 2)	488-626
Weak D type 3 (*CcDdee*)	*RHD*(S3C)	1712 (n = 2)	1503-1920
Weak D type 4.0 (*ccDdee*)	*RHD*(T201R,‡ F223V‡)	1617 (n = 1)	
Weak D type 4.1 (*ccDdee*)	*RHD*(W16C,‡ T201R,‡ F223V‡)	2948 (n = 2)	2424-3472
Weak D type 15 (*ccDdEe*)	*RHD*(G282D)	163 (n = 2)	133-193
DIV type IV (*CcDdee*)	*RHD*(D350H,‡ G353W,‡ A354N‡)	4259 (n = 1)	
DVI type I (*ccDdEe*)	*RHD-CE(4-5)-D*	318 (n = 3)	276-378

* ND = not determined.
† Antigen density reported previously[13] and included here only for comparison.
‡ Encoded by *RHCE* sequence.

TABLE 2. Reactivity of different weak and partial D RBCs with selected anti-D MoAbs*

Anti-D MoAb	D epitope†	MoAb class	Weak D type 1	2	3	4.0	4.1	15	26	31	32‡	DIV type IV	DVI type I
LHM70/45	1.2	IgG	+	+	+	+	+	–§	+	+	+	–	–§
BS227	2	IgG	+	+	+	+	+	+	+	+	+	–	–§
P3×249	2.1	IgG	+	+	+	+	+	+	+	+	+	–	–§
H4111B7	3.1	IgG	+	+	+	+	+	+	+	+	+	–	+
P3×290	3.1	IgG	+	+	+	+	+	+	+	+	+	w+	+
LHM76/55	3.1	IgG	+	+	+	+	+	+	+	+	+	–	+
ESD1	4.1	IgG	+	+	+	+	+	+	+	+	+	–	+
BS229	5.4	IgG	+	+	+	+	+	+	+	+	+	+	–§
BS231	5.4	IgG	+	+	+	+	+	+	+	+	+	+	–§
P3×35	5.4	IgG	+	+	+	+	+	+	+	+	+	+	–§
P3×241	5.4	IgG	+	+	+	+	+	+	+	+	+	+	–§
BS221	6/7	IgG	+	+	+	+	+	+	+	+	+	+	–§
BS228	6/7	IgG	+	+	+	+	+	+	+	+	+	+	–§
BRAD3	6.2	IgG	+	+	+	+	+	+	+	+	+	+	–§
LHM169/80	6.3	IgG	+	+	+	+	+	+	+	+	+	+	–§
HM16	6.4	IgG	+	+	+	+	+	+a	+	+	+	+	–§
P3×61	6.4	IgM	+	+	+	+	+	+a	+a	+a	+a	+	–§
HM10	6.6	IgM	+/+a‖	+a	+	+	+	–§	+a	+a	+a	+	–§
BRAD5	6.8	IgG	+	+	+	+	+	+	+	+	+	+	–§
LHM59/19	8.1	IgG	+	+	+	+	+	–§	+	+	+	+	–§
P3×21211F1	8.2	IgM	+a	+a	+	+	+	+a	+a	+a	+a	+	–§
Brad2	9.1	IgG	+	+	+	+	+	+	+	+	+	–	+
MS26	9.1	IgG	+	+	+	+	+	+	+	+	+	–	+
P3 × 21223B10	9.1	IgM	+a	+a	+	+	+	+a	+a	+a	+a	–	+
LHM77/64	9.1	IgG	+	+	+	+	+	+	+	+	+	–	+
Birma D6	9.1	IgG	+	+	+	+	+	+	+	+	+	–	+

* + = positive reaction in gel matrix; – = negative reaction in gel matrix; +a = positive only by adsorption-elution; w+ = weak positive.
† D epitope nomenclature according to M. Scott.[19]
‡ Only RBCs of the propositus (*CcDdee*) were analyzed.
§ Negative also by adsorption-elution.
‖ The weak D type 1 sample with the lowest D antigen density was positive only by adsorption-elution, whereas the other two were reactive in gel matrix.

D MoAbs were reactive in gel matrix IAT with all different weak D type RBCs except for weak D type 15 samples. In contrast, a major proportion of IgM-class anti-D MoAbs failed to react with RBC samples of weak D type 1, 2, 15, 26, 31, and 32 phenotypes in gel matrix, whereas weak D type 3 and 4 RBCs featuring comparatively high D antigen densities were agglutinated by all IgM anti-D MoAbs. Nevertheless, adsorption-elution studies with these IgM MoAbs demonstrated the presence of the recognized D epitopes in all but one investigated weak D types. Only weak D type 15 RBCs were found to be negative with two IgG and one IgM anti-D MoAbs, even when using adsorption-elution techniques. No adsorption-elution procedure was performed for DIV type IV RBCs expressing more than 4000 D sites per cell; here, nonreactivity of individual anti-D MoAbs was attributed to loss of specificity owing to antigen alteration and not lack of sensitivity of gel matrix testing due to basal D antigen density.[24] Epitope mapping of partial D RBC samples yielded known reaction patterns.[19] Consequently, individual MoAb preparations or applied techniques may limit the detection of D epitope expression in weak D phenotypes. These results suggested that all novel and the most common weak D types in Central Europe expressed quantitatively altered D antigens without apparent D epitope loss.

DISCUSSION

All known weak D alleles exhibit missense mutations in *RHD* exon sequences coding for amino acid substitutions in transmembraneous or cytosolic parts of the RhD polypeptide,[10] determining phenotypes with quantitatively reduced D antigen expression. Because most D epitopes appear to be highly conformation-dependent,[25] intracellular RhD primary structure alterations may theoretically impact also on the expression of a complete set of D epitopes. This would have major implications for the anti-D alloimmunization risks of weak D individuals associated with pregnancy and transfusion. In the vast majority of weak D types, however, no alloanti-D formation was observed,[4,26] arguing against significant D epitope loss. In fact, in contrast to partial D variants, weak D types seem not to exhibit characteristic D epitope profiles, which would allow for serologic discrimination of different weak D types.

In this study, the reported apparent heterogeneity regarding the reactivity of different weak D type RBCs with anti-D MoAbs[11] was reevaluated. Epitope mapping is essential for immunohematologic characterization of novel RhD variants, although it can never completely exclude the possibility of qualitative D antigen alteration.

This is best exemplified by partial DIII RBCs reacting with all known anti-D MoAbs,[19] despite the risk of alloanti-D immunization in such individuals. Therefore, no D+ transfusion strategy can be advocated for recipients with RhD variants solely based on generally positive epitope mapping results, with or without adsorption-elution. In contrast, only documented cases of anti-D alloimmunization in variant D individuals or distinctly negative D epitope mapping results are indicative of D antigen alteration. Conventional serology may be limited, however, by MoAb quality, applied technique, or extremely low D site numbers, resulting in falsely negative reactions despite the presence of individual D epitopes. IgM-class anti-D MoAbs appear to generally perform poorly in detecting weak D by direct agglutination even in gel matrix, compared to IgG MoAbs applied in the by far more sensitive IAT.[27] Indeed, in extremely weak D type 12 and 17 RBCs with similar D antigen densities compared to weak D types 26, 31, and 32, not even one of 20 IgM anti-D MoAbs had been reactive in gel matrix.[11] This is in full accord with our findings: comparatively strongly expressed weak D types 3, 4.0, and 4.1 were readily agglutinated by all IgM anti-D MoAbs, whereas weak D types 1 and 2 with lower D antigen levels were reactive only with a fraction of the used IgM clones. None of the IgM MoAbs, however, was capable of directly agglutinating any of the novel D phenotypes characterized by extremely basal D antigen expression. In contrast, all IgG-class anti-D MoAbs employed in IAT were reactive even with the weakest D types except for weak D type 15 control samples with postulated D antigen alteration. The obvious inverse relationship of IgM MoAb reactivity and D site number suggested that the observed reactivity patterns may simply reflect the limited ability of IgM anti-D MoAbs to detect extremely weak D types rather than qualitative alteration of the respective D epitopes.[24] Moreover, it appears highly improbable that exclusively such D epitopes are altered in extremely weak D phenotypes with different molecular background which were recognized by IgM MoAbs. Adsorption-elution–based D epitope mapping was originally devised to analyze the weakest D+ phenotype termed DEL,[20,28] by definition negative with IgG anti-D in IAT. By use of this technique, DEL RBCs from individuals with the *RHD*(IVS5-38del4) allele were shown to express minute amounts of grossly intact D antigens that had induced anti-D in a D– transfusion recipient. The presence of many or all D epitopes may render very weak D variants more immunogenic, compared to D variants with similar D site numbers and major D epitope loss. The results of adsorption-elution–supported D epitope mapping could possibly aid in judging the risk of anti-D immunization associated with erroneous transfusion of extremely weak D RBCs to a D– recipient. In line with these considerations, we recently reported a case of anti-D induction in a D– patient by transfusion of extremely weak D type 26 RBCs now shown to express D antigens without apparent D epitope loss.[13]

The importance of extensive D antigen analysis of novel D variants is emphasized by the absence of a well-defined border between weak D and partial D. Weak D type 4 was originally thought to derive from one allele only.[10] Later studies, however, revealed several "suballelic" variants, with weak D type 4.2 individuals turning out to be prone to anti-D alloimmunization indicative of not only quantitative but also qualitative D antigen alteration of this D variant.[11] In addition, also weak D type 15 exhibits partial D characteristics based on cases of alloanti-D formation in such individuals.[11] With our adsorption-elution approach, we demonstrated the principal expression of all tested D epitopes by weak D type 1, 2, 3, 4.0, 4.1, 26, 31, and 32 RBCs. In contrast, negative adsorption-elution results confirmed the postulated qualitative D antigen alteration associated with weak D type 15.

To date, approximately 40 different weak D alleles are known, mostly characterized with respect to the circumstances of their first observation, their molecular background, *RHCE* haplotype association, and phenotypic and immunogenetic attributes.[26] Some weak D types appear "ethnically unspecific" in distribution such as weak D type 15 present in at least Caucasian and Asian ethnicities.[11,29] Somehow, in contrast, the common weak D types 1, 2, and 3 display significantly different allele frequencies even in geographically adjacent regions.[12] Future studies will have to define the distribution patterns of novel weak D alleles including weak D type 31 and 32. In light of the continuing development and the growing routine use of molecular *RH* typing, not only molecular but also detailed immunohematologic properties of novel D variants need to be documented. In this study, the results of a thorough D antigen analysis of the very weak D types 26, 31, and 32 are provided. We recommend the use of adsorption-elution techniques apparently having the potential to increase the reliability of conventional D epitope mapping approaches whenever qualitative D antigen alteration is suspected.

Recent evidence indicated that extremely weak RhD variants including weak D type 26 and even DEL RBCs were capable of immunizing D– transfusion recipients.[13,20] The vast majority of such RhD variants including the novel weak D types described in this study are associated with C or E.[4,13,16] Therefore, reliable *RHD* screening procedures for serologically D– but either C+ or E+ individuals were implemented in some Central European blood donation centers to minimize the risk of anti-D alloimmunization.[13]

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