

Brief report

DNB: a partial D with anti-D frequent in Central Europe

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To improve routine D typing and define transfusion strategy, it is important to establish the frequency of partial D alleles and their susceptibility to anti-D alloimmunization due to transfusion or pregnancy. We identified the partial D DNB that was caused by an *RHD*(G355S) allele associated with a *CDe* haplotype and whose phenotype presented a nor-

mal D in routine typing. The antigen density was about 6000 D antigens per red blood cell, and the Rhesus index was 0.02. Five anti-D immunization events with allo-anti-D titers up to 128 were observed. Twelve carriers of DNB were whites of Central Europe; the only Danish proband had Austrian ancestry. DNB was the most frequent partial D recognized so

far in whites, occurring with frequencies of up to 1:292 in Switzerland. DNB was the underlying partial D phenotype in a relevant fraction of anti-D immunizations occurring in whites. (Blood. 2002;100:2253-2256)

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Introduction

Anti-D immunizations may occur in “partial D” carriers. This designation relates to more than 30 alleles differing in molecular structure, phenotype, population frequency, and lenience to anti-D production.¹ The molecular bases were gene conversions,² single missense mutations in the exofacial protein segments,³ and multiple missense mutations dispersed throughout the RhD protein.⁴ A partial D needs to be considered for transfusion strategies if its carriers are frequent, easily anti-D immunized, and likely to receive D-positive units in the case of transfusion. D category VI⁵ is the classical example. In several countries, typing methods⁶ were introduced that assured D-negative transfusion and anti-D prophylaxis for DVI mothers to prevent anti-D immunizations. Despite the large number of known partial D alleles, a relevant portion of anti-D immunizations is still occurring in individuals who did not carry any known partial D.⁷⁻⁹ Thus, the characterization of the unknown alleles underlying such case reports is remaining important.

Study design

Blood samples with anti-D

Two D-positive samples (RIR-2 from Bern, Switzerland; RIR-3 from Bavaria, Germany) with anti-D were referred to our laboratory to elucidate the cause of anti-D immunization. Later, 1 additional blood sample (RIR-41 from Denmark) and 2 DNA samples (RIR-7, RIR-8 from Austria) were referred to the Rhesus Immunization Registry (RIR) because of anti-D in these D-positive probands. The Danish proband RIR-41 had an Austrian grandfather.

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Further information for this *RHD* allele and the Rhesus Immunization Registry [RIR] is available online at <http://www.uni-ulm.de/~wfliegel/RH/> and with Internet links

RHD nucleotide sequencing

DNA was handled as described previously.¹⁰ The 10 *RHD* exons were sequenced by an *RHD* allele-specific method¹¹ in the 2 index samples (RIR-2, RIR-3). In the remaining samples, exon 7 was sequenced only.

PCR-SSP

A polymerase chain reaction with sequence-specific priming (PCR-SSP) to confirm or detect the 1063G>A substitution in DNB was devised as modular extension of a PCR-SSP system previously developed for *RHD* typing.^{10,12} Specific primers were re77 (TCTCCACAGCTCCATCATGGG) and DNBb (CAGTGACCCACATGCCATTACT) at a concentration of 3.5 μmol; *HGH* control primers were used at 0.75 μmol.

Immunohematology

Routine D typing was done in tube tests using commercial monoclonal anti-D (Seraclone anti-D 226, clone BS226; and Seraclone anti-D [232], clone BS232; Biotest, Dreieich, Germany; and ImmuClone anti-Dfast, clone D1-4E11; and ImmuClone anti-Drapid, clone RUM-1; Immucor, Norcross, GA). A commercial panel of monoclonal anti-D (D-Screen; Diagast, Loos, France) was tested in gel matrix technique (LISS-Coombs 37°C, DiaMed-ID Micro Typing System; DiaMed, Cressier sur Morat, Switzerland). D epitopes were determined by agglutination, and antigen density and Rhesus index were determined by flow cytometry as previously described.¹³ DIV type III¹³ and DNU¹⁴ controls derived from donors in Baden-Württemberg, Germany.

Population screens for DNB

Ethylenediaminetetraacetic acid (EDTA)- or citrate-anticoagulated blood samples were collected from blood donors in Baden-Württemberg (Germany, 1118 donors of CcDee phenotype, 1010 donors of ccDEe phenotype), Ticino (Italian-speaking Switzerland, 500 D-positive donors), Bern region

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(German-speaking Switzerland, 693 donors of CcDee phenotype), and Denmark (768 donors of CcDee phenotype). The donors were tested with the immunoglobulin G (IgG) monoclonal anti-D LOR17-6C7 (Germany and Denmark) or HIRO-7 (Switzerland).¹⁵ Suspected DNB samples were confirmed by sequencing of exon 7 or by PCR-SSP. Haplotype frequencies used for calculation of DNB allele frequencies were CDe 0.431, cde 0.394, cDe 0.021, and Cde 0.011 in Germans¹⁶; 0.4236, 0.3792, 0.0145, and 0.0059 in Danes¹⁷; and 0.4442, 0.3617, 0.0232, 0.0113, and cDe 0.049 in Swiss.¹⁷ Phenotype frequencies for CcDee were compared using the Fisher exact test for a 4 × 2 contingency table; for this calculation the 500 D-positive samples in Ticino were inferred to comprise 187 CcDee samples.

Nomenclature

The name DNB derived from DNU-like and Bayern (Bavaria, Germany) or Bern, because DNB was similar to DNU,¹⁴ whose point mutations were closely adjacent.

Results and discussion

Molecular structure

The molecular structure of 2 D-positive samples with anti-D (RIR-2, RIR-3) was determined by *RHD*-specific sequencing from genomic DNA. In *RHD* exon 7, a single G to A exchange at position 1063 was detected that resulted in a Gly to Ser substitution at codon 355. The affected amino acid was located in the exofacial loop 6, adjacent to the mutations observed in DII (Ala to Asp at position 354)¹⁴ and DNU (Gly to Arg at position 353).¹⁴ The nucleic acid and amino acid sequence data were deposited in EMBL/GenBank/DBJ under accession number AJ417868.

Immunohematology

In routine D typing, both DNB samples typed as CcDee without noticeable weakening of the antigen D. All 9 monoclonal anti-Ds of a commercial partial D classification kit (D-Screen) were reactive. An antigen density of 5908 antigens per cell and a Rhesus index^{13,18} (RI) of 0.02 were determined in RIR-2. These results were comparable to those obtained with a DNU sample (8073 antigens per cell, RI 0.06) and a CcDee DIV type 3 sample (4544 antigens per cell, RI 0). The D epitope pattern was determined for both index samples and was unique (Table 1): 6 of 83 anti-Ds tested did not agglutinate DNB, indicating a loss of epitopes epD6 and epD31 as well as part of epD18 and epD23. The crossmatch with DNU red cells was faintly positive; there are no DII red cells available anymore.

Population frequencies

We screened random samples of CcDee blood donors in 4 European populations (Table 2). With the exception of Danes, DNB was found frequently. The highest frequency was observed in the Ticino population. DNB was the most frequent partial D in whites described so far; its frequency even exceeded that of DVII.¹⁹ Further testing of other populations may be of potential benefit.

Including the probands with anti-D, we presented 12 unrelated carriers of DNB who were whites of Central Europe; the only Danish proband had an Austrian grandfather, from whom he may have inherited the *DNB* allele. All DNB probands were of CcDee phenotype. The *CDe* haplotype association was further corroborated by testing the parents of the Danish proband RIR-41, who inherited his DNB allele from his CcDee father.

Table 1. Reactivity patterns of monoclonal anti-D

Pattern*		Anti-D tested, n†	Partial D			
1-9	1-37		DNB‡	DII§	DNU‡	DIV type 3‡
1	1	1	+	+	+	-
1	2	2	+	+	+	-
2	3	2	+	+	+	-
2	4	1	+	+	+	-
3	5	3	+	+	+	-
4	6	1	-	-	+	-
5	7	2	+	+	+	+
5	10	4	+	+	+	+
5	11	1	+	+	+	+
6/7	12	4	+	+	+	+
6/7	13	6	+	+	+	+
6/7	15/16	8	+	+	+	+
		4	+	+	+	-
6/7	17	8	+	+	+	+
6/7	18	6	+	+	+	+
		1	-	+	+	+
6/7	20/21	3	+	+	+	+
		1	+	+	+	-
8	22	2	+	+	+	+
9	23	3	+	-	+	-
		2	-	-	+	-
		2	+	-	-	-
N/A	31	2	-	+	+	-
N/A	32	1	+	+	+	+
N/A	33	1	+	+	+	+
N/A	34	1	+	+	-	-
N/A	35	1	+	+	+	-
N/A	36	3	+	+	+	-
		1	+	+	-	-
N/A	37	5	+	+	+	+

N/A indicates not applicable; +, a normal or weak positive result; and -, a negative result.

*Pattern is as described previously by Lomas et al²⁴ (1-9) and Scott²¹ (1-37).

†A complete listing of the antibodies was published previously¹³ (Table 2). The following antibodies failed to agglutinate DNB: LOR17-6C7 (epD6), P3F20 (epD18), HIRO-7, HIRO-8 (epD23), NAU6-1G6, and NOU (epD31). In addition, the reactivity of the following anti-Ds differed from that of the majority assigned to the respective epitope: D-90/7, SAL20-12D5, LHM50/2B, BIRMA-DG3 (epD15/16), as well as VOL-3F6 (epD20/21) did not agglutinate DIV type 3; LHM77/64, BIRMA-D6 (epD23), and NAU6-4D5 (epD36) did not agglutinate DNU.

‡The DNB pattern was established with samples RIR-2 and RIR-3 and the DNU pattern with red cells of the original DNU donor.¹⁴ The DIV type 3 data are a reevaluation of the original pattern using a CcDee DIV type 3 donor from our local population.

§The results depicted for D category II were determined by Scott²¹ and are shown for comparison.

Anti-D immunization

Including the 2 index probands, 5 cases of allo-anti-D immunization submitted to the Rhesus Immunization Registry occurred in DNB probands (RIR-2, RIR-3, RIR-7, RIR-8, and RIR-41).²⁰ These cases represented a sizable fraction of the 32 confirmed allo-anti-D immunizations reported to this registry until February 2002. Although any Rhesus-positive blood sample with anti-D— with or without known Rhesus variant—may be sent to the registry for analysis, no other *RHD* allele was involved in a similar number of cases. Two additional carriers of DNB with anti-D were from Slovakia (Dr Martin Pisacka, personal communication, 1999) and from Germany (submitted as RIR-49 after we had finished the data analysis).

Anti-D titers ranged from 4 to 128, indicating that DNB carriers were generally able to produce strong anti-D. However, no clinical data like signs of transfusion reactions or hemolytic disease of the newborn were reported. In concordance with the assumption that

Table 2. Population frequencies of DNB

Population	Phenotype checked*	Samples tested	DNB detected, n	Phenotype frequency among CcDee†	Phenotype frequency among all phenotypes	Haplotype frequency
Switzerland (Lugano)	All D-positive	500	2	N/A‡	1:292	1:220
Switzerland (Bern)	CcDee	693	4	1:173	1:538	1:389
Germany (Ulm)	CcDee	1118	2	1:559	1:1644	1:1295
	ccDEe (control)	1010	0	N/A	N/A	N/A
Denmark (Aarhus)	CcDee	768	0	< 1:256§	< 1:798§	< 1:605§

*Samples were checked for DNB using monoclonal anti-D LOR17-6C7 (Germany and Denmark) and HIRO-7 (Switzerland).

†The phenotype frequencies among CcDee were statistically significantly different, assuming that the 500 D-positive samples tested in Ticino corresponded to 187 CcDee samples ($P < .023$, Fisher exact test; 4×2 contingency table).

‡Not applicable. The frequency among D-positive was 1:250; both carriers were of CcDee phenotype.

§Upper limit of the 95% confidence interval according to the Poisson distribution.

these anti-Ds were triggered by transfusion or pregnancies, 4 of the probands were female; the male and at least 2 of the female probands were transfused prior to the detection of the anti-D. The anti-D was still detectable up to 8 years following the latest possible anti-D immunization event.

Impact on typing strategies

The repeated observations of anti-D immunizations probably reflected the combination of a high population frequency with a phenotype that triggered D-positive transfusions and with a moderate anti-D immunization potential. Hence, we recommended D-negative transfusions if a recipient is known to carry a DNB phenotype.

A serologic recognition of DNB would be demanding because it has a normal antigen density and is agglutinated by most anti-Ds, including almost all commercial anti-D typing reagents. All tested high-affinity monoclonal IgM anti-Ds that did not bind DVI but that agglutinated most weak D phenotypes^{13,21} agglutinated DNB also (Table 1). Therefore, a serologic strategy for detecting DNB would have to rely on a separate anti-D especially introduced to discriminate DNB from normal D. By careful selection of this third antibody (eg, HIRO-7 or HIRO-8), the strategy could be tuned to discriminate also DII, DIV, and R₀^{Har} from normal D.²¹ Such a major change in D typing would be costly and might induce uncertainties that outweigh the benefit by far. The anti-D immunization risk in DNB carriers may safely be estimated to be lower than 1% per D-positive transfusion. This immunization index was less than known for anti-K and anti-c²² but may be comparable to that of anti-Fy(a) and anti-Jk(a).

Genotyping strategies are increasingly utilized for blood grouping²³ and may be devised to meet predefined specificity criteria. For

example, *RHD* PCR in whites may be tuned to a specificity of greater 0.9999 and allows the identification of D-positive units missed by routine serology.¹⁰ Likewise, *DNB* or *DIIIa* alleles may be detected specifically without any limitations imposed by the lack of suitable monoclonal anti-D. Because the frequency of partial D with relevant anti-D immunization risk is low in whites, their D-negative transfusion would not compromise the D-negative blood supply. Hence, a specific recognition and D-negative transfusion strategy for DNB may be perceived advantageous and become feasible with genotyping strategies in the future.

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