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DIII type 7 is likely the original serologically-defined DIIIb

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Abstract

Background—Due to their homology, close proximity and opposite orientation, *RHD* and *RHCE* can exchange nucleotides giving rise to variant alleles. Some of these variants encode the so-called partial phenotypes. The DIII partial D category has been subdivided into DIIIa, DIIIb, DIIIc, DIII type 4, DIII type 6, and DIII type 7. During DNA-based screening tests, we identified a second example of DIII type 7 in a Dce donor from South Africa. Our study describes hemagglutination tests on this sample and raises a question regarding the molecular basis of the originally defined DIIIb category.

Materials and methods—Hemagglutination and DNA testing were performed by standard techniques.

Results—RBCs from this DIII type 7 donor typed D+C–E–c+e+G–, DAK+ and did not react with anti-D made by people with the DIII phenotype. The allele is *RHD***DIII* 150C, 178C, 201A, 203A, 307C, 410T, 455C, 602G, 667G).

Conclusions—Based on the serotype and ethnicity (black African), it is likely that DIII type 7 is the originally defined DIIIb category.

Keywords

DIII phenotype; RHD allele; Rh blood group system; RHD variant; partial D phenotype

Introduction

The Rh blood group system is diverse in terms of both antigens and alleles.¹ The genes, *RHD* and *RHCE*, encoding the Rh antigens are homologous and reside on chromosome 1 (1p36.11). Due to their homology as well as their close proximity and opposite orientation, these two genes sometimes exchange nucleotides thereby giving rise to variant alleles. The variant alleles encode variant proteins, some of which are the so-called partial D phenotypes. Numerous partial D phenotypes, which are usually identified when a D-positive person makes an alloanti-D, have been described.^{1,2}

Based on serological tests and ethnicity, Tippett and Sanger subdivided the partial DIII phenotype into three categories. DIIIa and DIIIb phenotypes are found in people of African heritage in a Dce complex; DIIIa red blood cells (RBCs) are G+ whereas DIIIb RBCs are G–. The DIIIc phenotype is found in Caucasians in a DCE complex.³ When RH alleles were sequenced, further subdivisions of DIII occurred.^{1,2} *RHD***DIII* type 4 (ISBT provisional

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allele name *RHD*03.04* shared with *RHD*DIIIa* (*RHD*03.01*) three nucleotide (nt) changes (186G>T, 410C>T, 455A>C) but lacked two other changes: 602C>G and 667T>G.^{2,4} *RHD*DIII type 5* was made obsolete after it was shown to be the *RHD*DIIIa* allele.⁵ Grootkerk-Tax *et al.* found that *RHD*DIII type 6* (*RHD*03.06*) differs from *RHD*DIIIa* in that it lacks the nucleotide change of 186G>T and that *RHD*DIII type 7* is identical to the *RHD*DIII type 6* for the nucleotide changes except it has exon 2 from *RHCE*c* in place of *RHD*.⁶ They found that RBCs from a donor with the *DIII type 6* phenotype were agglutinated by 59 monoclonal (mab) anti-D, a minority giving weaker reactions than an R_{1r} control RBC sample. They were not agglutinated by one mab anti-D, which weakly agglutinated (1+) the control RBCs.⁶ Grootkerk-Tax *et al.*, found *RHD*DIII type 7* in one donor from South Africa. RBCs from this donor were agglutinated to a strength of 2+ with mab anti-D and 1+ with polyclonal anti-D. Insufficient RBCs were available for extended hemagglutination testing and the G type was not noted.⁶

In 1995, two samples with a replacement of *RHD* exon 2 by *RHCE*c* exon 2 were reported, and were presumed to be *DIIIb*.⁸ However, as both samples were from Caucasians, the RBCs not only had a very weak expression of D (detectable only by the indirect antiglobulin test) but were not agglutinated by all monoclonal anti-D⁹, and the variant D antigen traveled with cE, the samples did not meet the characteristics defined by Tippett and Sanger for a category *DIIIb*.³ In retrospect, it is likely that these RBCs were weak D, G- and not *DIIIb*.

During DNA-based screening tests on people of African ancestry, we identified a second example of *DIII type 7*, also in a South African donor. This finding prompted this study, the purpose of which was to define the serologic characteristics of RBCs with the *DIII type 7* phenotype. Our findings lead us to propose that the *DIII type 7* phenotype is more likely the *DIIIb* phenotype of Tippett and Sanger than the *DIIIb* reported by Rouillac and coworkers.⁸

Materials and Methods

Sample and hemagglutination

The sample described herein (MA113-09) was from a South African blood donor (code name Marcus). The various RBC samples and reagents used in the hemagglutination testing were from our collection. Hemagglutination was performed by standard methods.^{11,12}

RNA extraction, cDNA synthesis, amplification, and sequencing

A cryopreserved blood sample from MA113-09 was thawed and RNA isolated from the reticulocytes by standard methods using TriZol and PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA). Reverse transcription was carried out with gene-specific *RHD* and *RHCE* primers and Superscript III according to manufacturer's instructions (Superscript III first-strand synthesis SuperMix, Invitrogen), and polymerase chain reaction (PCR) amplification was carried out with primers to amplify exons 1 to 4 and exons 5 to 10 in *RHD* and *RHCE* as described previously.¹³ RT-PCR products were directly sequenced by GENEWIZ, Inc. (South Plainfield, NJ). Sequences were aligned, and protein sequence comparisons were performed with Sequencher v4.9 (GeneCodes, Ann Arbor, MI).

Genomic DNA extraction, amplification, and sequencing

Standard methods were used to extract genomic DNA from 200µl of the thawed blood sample (MA113-09) with a commercial kit (QIAamp, Qiagen, Inc., Valencia, CA). To overcome messy sequences, PCR amplification of *RHD* exons 7 and 8 was conducted separately on genomic DNA. PCR was carried out using HotStarTaq Master Mix (Qiagen, Inc., Valencia, CA). The following primers were used: for exon 7: RhD-I6F (5'-cttcattcaacaaactccccga-3') and DNB-R (5'-gtgataaatccatccaaggtaggggccggccagaat-3'); and

for exon 8: RhD-I7F (5'-ctggaggctctgagaggttgag-3') Rh-I8-R (5'-catagacatccagccacaggca-3').

Results

DNA Testing

Molecular analyses of *RHD* showed the proband had *RHD*DIII type 7* (either homozygous or hemizygous) with the following nucleotide changes: 150T>C, 178A>C, 201G>A, 203G>A, 307T>C in exon 2 (*RHCE*c*), and 410C>T in exon 3; 455A>C in exon 3; 602C>G in exon 4; 667T>G in exon 5. The GenBank accession number for this allele (ISBT provisional name *RHD*03.02*) is JF436967.

Molecular analyses of *RHCE* showed the proband was homozygous for *RHCE*ceS* (*RHCE*ceVS.04*; *RHCE*ce48G>C*; 733C>G; 1006G>T).

Hemagglutination

When tested with 24 monoclonal anti-D, the proband's RBCs were agglutinated (2+ to 4+) by those that reacted with a strength of 2+ or greater with control RBCs but were not agglutinated by those giving a 1+ reaction with the control RBCs. This is consistent with the results reported by Grootkerk-Tax, et al.⁶ RBCs from MA113-09 were also tested with a collection of alloanti-D made by D+ people. Five anti-D made by DIIIa people were non-reactive. Three anti-D made by DIVa people, one anti-D made by a DVa person, and six of seven anti-D made by DVI people were reactive. These results indicate that the DIII type 7 RBCs lack the same RhD epitopes as RBCs with the DIIIa phenotype.

The proband's RBCs were tested with anti-G: two polyclonal anti-G (EBS, GH) and one monoclonal anti-G (MS1) were non-reactive. Anti-DAK (AK, Riz) agglutinated the proband's RBCs. Taken together the donor's RBCs typed D+C-E-c+e+G-DAK+.

Conclusions

We identified a second example of *RHD*DIII type 7* in a donor of African heritage. This allele carries missense nucleotide changes identical to *RHD*DIII type 6* in exon 3 (410C>T, 455A>C), exon 4 (602C>G), exon 5 (667T>G), but differs in that exon 2 derives from *RHCE*c* in place of *RHD* (150T>C, 178A>C, 201G>A, 203G>A, 307T>C). Fortuitously, as the allele was homozygous (or hemizygous) we were able to perform extensive serological testing on the RBCs.

The reactivity of the proband's RBCs with reagent anti-D and with anti-D made by people with DIVa, DVa, and DVI partial D antigens, and the absence of reactivity with anti-D made by DIIIa people is strong evidence that DIII type 7 belongs in the original classification of DIII. Based on the serological similarity and nucleotide sequence, we conclude that DIII type 7 is more likely to be DIIIb as defined by Tippett and Sanger in 1977,³ than the samples analyzed by Rouillac, et al. in 1995.⁸ (see table 1) Evidence for this conclusion is as follows: (i) both probands are African, whereas the two 'DIIIb' samples of Rouillac and colleagues were from Caucasians, (ii) in both probands, DIII type 7 travels with ce (a characteristic of the original DIIIb classification), whereas in the probands reported as DIIIb by Rouillac et al, the altered D traveled with cE (R₂r^{cc} and R₂r); (iii) the reactivity of the original DIIIa, DIIIb and DIIIc RBCs with polyclonal and monoclonal anti-D was similar in direct testing, while the samples reported by Rouillac et al., were only agglutinated in the indirect antiglobulin test.;

In conclusion, four DIII phenotypes are considered to belong in the DIVa cluster^{6,7}, namely, DIIIa (encoded by *RHD*03.01*), DIII type 4 (encoded by *RHD*03.04*), DIII type 6 (encoded by *RHD*03.06*), and DIII type 7 (encoded by *RHD*03.02* and so named by the ISBT working party because it is likely the original DIIIb category³). (Figure 1) These alleles have been so far only found in people of African Ancestry. The *RHD*D-c(exon 2)-D* hybrid, said to encode DIIIb⁸, is actually a weak D phenotype found in Caucasians. The DIIIc phenotype (encoded by *RHD*03.04*) also has been found only in Caucasians and does not belong in the DIVa cluster. The ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology has given provisional numerical names to these allele.

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Figure 1.
Family of DIII alleles: Comparison of *RHD* exons; relevant nucleotides changes, and amino acids changes and selected antigen typing for RhD.
RHD exons are represented by black boxes; *RHCE* exons are represented by white boxes. Silent nucleotides are not given. Antigen typings are given where the information was recorded.

Table 1

Comparison of DIIIb of Tippett and Sanger, 'DIIIb' of Rouillac, et al, and DIII type 7

Characteristic	DIIIb of Tippett and Sanger ³	DIIIb of Rouillac, et al ⁸	DIII type 7 ⁶ (and this paper)
Ethnicity	African	Caucasian	African
D antigen travels with	ce	cE	ce
D type	D+	D ⁺ ^W	D+
Reagent anti-D	Agglutinated in direct tests	Not agglutinated in direct tests	Agglutinated in direct tests
Anti-D from DIIIa people	Not agglutinated	Not reported	Not agglutinated
Anti-D from people with DIVa, DVa, or DVI partial D phenotypes	Agglutinated	Not reported	Agglutinated