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*RHCE*ceTI* encodes partial c and partial e and is often *in cis* to *RHD*DIVa*

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Abstract

Background—In the Rh blood group system, variant RhD and RhCE express several partial antigens. We investigated *RH* in samples with partial DIVa that demonstrated weak and variable reactivity with anti-C.

Material and methods—Standard hemagglutination techniques, PCR-based assays, and *RH* sequencing were used.

Results—DNA analysis showed that six RBC samples with weak and inconsistent reactivity with anti-C lacked *RHCE*C*, but all had *RHD*DIVa*, which encodes partial D and Go^a. We then tested RBCs from 19 Go(a+) cryopreserved samples (confirmed to have *RHD*DIVa*) with four anti-C and observed weak variable reactions. *RHCE* genotyping found all but one of the samples with *RHD*DIVa* also had *RHCE* nt 48G>C and 1025C>T; named *RHCE*ceTI*. Look-back of samples referred for workup and found to have either allele revealed 47/55 had both *RHD*DIVa* and *RHCE*ceTI*; four had *RHD*DIVa* without *RHCE*ceTI*; four had *RHCE*ceTI* without *RHD*DIVa*. Alloanti-c was found in a patient with c+ RBCs and *RHCE*ceTI in trans* to *RHCE*Ce*, and alloanti-e was found in a patient with e+ RBC and *RHCE*ceTI in trans* to *RHCE*cE. RHD*DIVa in trans* to *RHD* erroneously tested as *RHD* hemizygous.

Conclusions—*RHD*DIVa* and *RHCE*ceTI* almost always, but not invariably, travel together. This haplotype is found in people of African ancestry and the RBCs can demonstrate aberrant reactivity with anti-C. *RHCE*ceTI* encodes partial c and e antigens. We confirm that *RHD* zygosity assays are unreliable in samples with *RHD*DIVa*.

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Web Resources

http://www.patientsville.com/medical-device/blood-grouping-reagent-immucor-quality.htm Rhesus Base: http://www.uni-ulm.de/~fwagner/RH/RB/mutind.htm. Accessed 5/01/2012.

ISBT Blood Group Terminology: http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/blood-group-terminology/blood-group-allele-terminology/. Accessed 5/01/2012

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Keywords

Blood groups; RH alleles; Rh blood group system; blood transfusion; partial antigen

Introduction

The Rh blood group system is complex[1] due not only to the more than 50 discrete antigens[2] but also to the fact that the common antigens, D, C, c, E and e, are carried on proteins that may have amino acid changes resulting in qualitatively altered forms of the antigens; the so-called partial antigens.

The D antigen encompasses the entire RhD protein and is known to have numerous epitopes. These epitopes were first recognized by cross-testing the alloanti-D made by people with D+ RBCs against the RBCs from each other,[3] and later by testing these RBCs with various monoclonal anti-D.[4,5] Pertinent to this report is the partial D antigen originally named category DIV. This was later divided into DIVa and DIVb: DIVa, but not DIVb, RBCs express the low prevalence Rh antigen, Go^a (RH30), and this phenotype is found in black people of African descent. In sequencing a sample from one Go(a+) donor (Bel.),[6] the DIVa allele was reported in 1995 to have three nucleotide changes (186G>T, 455A>C, 1048G>C) encoding the amino acid changes (Leu62Phe, Asn152Thr, Asp350His). An allele with one additional change to those above (nt 410C>T; Ala137Val), and also encoding a partial DIVa and expression of Go^a, was subsequently reported in 2009 and named DIVa-2.[7]

Altered forms of RhCE proteins also express partial antigens which are revealed when an antigen-positive patient makes either alloanti-C, -c, -E, -e, or less often, anti-ce (f), -Ce (rh_i), or -cE (Rh27). The antibodies can appear to be, for example, anti-Rh17, $-hr^S$ (Rh19), or $-hr^B$ (Rh31), and testing of the serum against RBCs from people whose RHCE alleles have been defined by DNA testing has enabled further refinement of the specificity of some of these antibodies, including anti-CEST, -CELO, -CEAG.[8–11]

We report here an investigation of samples with *RHD*DIVa* and *RHCE*ceTI* that were initially studied because of aberrant reactivity with some anti-C reagents.[12] RH genotyping of a large cohort of samples referred over the years from African Americans revealed that *RHD*DIVa* is often found with *RHCE*ceTI*. That *RHCE*ceTI* encodes both a partial c and partial e was demonstrated by the production of alloanti-c in a patient with *RHCE*ceTI in trans* to *RHCE*Ce*, and alloanti-e in another with *RHCE*ceTI in trans* to *RHCE*cE.*[13]

Material and Methods

Blood Samples

Blood samples were from patients and donors referred to our laboratories in three main categories: samples referred because of typing discrepancies, historically Go(a+) samples, and samples typing D+ with anti-D in the serum or plasma.

Serologic Testing

Reagents for serologic testing were commercial reagents and from our library of antibodies collected over many years and included the use of an outdated polyclonal anti-C no longer available commercially. Hemagglutination tests were performed by standard tube methods that were appropriate for the reagent used. Commercial anti-C reagents were used according to the manufacturers' instructions and included Immucor Gamma-clone and Immucor Series anti-C (Immucor, Inc. Norcross, GA), Ortho BioClone (Ortho-Clinical Diagnostics, Inc.

Raritan, NJ), Bio-Rad Seraclone (Biotest Medical Diagnostics, GmbH, Dreich, Germany), and Immucor polyclonal.

Genomic DNA extraction, amplification, RFLP, and sequencing

Genomic DNA was extracted by standard methods from white blood cells from the buffy coat layer of peripheral blood using a commercial kit (QIAamp, Qiagen, Inc., Valencia, CA). DNA was subjected to polymerase chain reaction (PCR) amplification using *RHD*-specific and *RHCE*-specific primers designed to target the flanking intronic regions and the products were analyzed by direct sequencing of specific exons and/or a combination of multiple PCR-restriction fragment length polymorphism (RFLP) assays, as described in detail previously.[14] The presence of the 1025 C>T change in *RHCE*ce* was confirmed by PCR-RFLP with *Hph*I or by sequencing. Known Go(a+) samples were analyzed by sequencing exons 3 and 7 of *RHD* to confirm the presence of *RHD*DIVa*, and exon 7 of *RHCE* to detect *RHCE*ceTI. RHD* zygosity assays were performed using an allele specific assay for the hybrid *Rhesus box*[15] and an RFLP assay with *Pst*I.[16]

RNA extraction and Rh-cDNA cloning and sequencing

RNA was isolated from the reticulocytes by standard methods using TriZol or 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). Alternatively, reverse transcription was carried out with Superscript II and random hexamers and oligo(dT) primer, according to the manufacturer's instructions (Superscript First Strand Synthesis System, Invitrogen, Carlsbad, CA). PCR amplification was carried out for 35 cycles with primers to amplify exons 1 to 4 and exons 5 to 10 in *RHD* and *RHCE,* or as full-length transcripts, followed by cloning and sequencing as described previously.[14] PCR products were checked for purity on agarose gels, purified using PCR product clean-up kits according to manufacturers' instructions (ExoSAP-IT, USB Corporation, Cleveland, OH or Quiagen PCR purification Kit) and directly sequenced by GENEWIZ, Inc. (South Plainfield, NJ) or by the University of Pennsylvania (Philadelphia, PA). Sequences were aligned, and protein sequence comparisons were performed with Sequencher v4.9 (GeneCodes, Ann Arbor, MI) or CLUSTALX.

Results

Discrepant C typing

RBCs from three antibody identification panel donors and three blood donors who were thought to be D+C−E−c+e+ were referred for RH genotyping because of weak and variable reactivity with some anti-C. Fresh RBCs were not agglutinated by the anti-C reagent from Ortho, reacted weakly (1+ or 2+) or not agglutinated by anti-C from Immucor, and reacted variably from $+^{w}$ to as much as 2+ by Gamma-clone anti-C (Table 1). The commercial monoclonal anti-C contain the same source clone MS24, but the formulations vary in the potentiator(s) present and or concentration of the antibody (personal communication). DNA testing by the multiplex assay[17] which detects the intron 2 polymorphism characteristic of the RHCE*C allele, showed all were *RHCE*c/c,* and that they lacked *RHCE*C*. All were negative for alleles encoding partial C associated with the expression of C^{W}, C^{X}, or with the r'^{S} haplotype or R^{N}. RH genotyping found these six samples all had *RHD*DIVa*, known to encode Go^{a}, and an altered RHCE*ce allele, named *RHCE*ceTI* (nt 48G>C encoding 16Cys and 1025C>T encoding 342Ile).[12]

Testing of Go(a+) samples

This finding prompted us to investigate other Go(a+) samples. We isolated DNA from 19 cryopreserved Go(a+) samples, and RH genotyping confirmed all 19 had *RHD*DIVa* and 18 also had *RHCE*ceTI*. (Table 2) RBCs were recovered from storage in liquid nitrogen (from a few months to four decades) and tested on the day of recovery with four commercial anti-C reagents. Twelve samples were very weakly (microscopic to 1+) agglutinated by Gamma-clone anti-C (C+c+ control reacted 4+), six were agglutinated very weakly (microscopic to 1+) by the Ortho BioClone reagent, and four were very weakly (microscopic to 2+) agglutinated by Immucor polyclonal reagent. None were agglutinated by the Bio-Rad Seraclone reagent. These Go(a+) samples were historically C-negative from testing performed by multiple technologists in multiple institutions using various reagents.

Which allele encodes the aberrant reactivity with anti-C?

We reviewed our referred cases with either *RHD*DIVa* or *RHCE*ceTI* for C typing reactions. RBCs from one sample with *RHD*DIVa* without *RHCE*ceTI* gave a 2+ reaction with Gamma-clone anti-C when tested by the referring laboratory on day one, but they were non-reactive with Gamma-clone anti-C when shipped and subsequently tested by the reference laboratory. RBCs from two freshly collected samples with *RHCE*ceTI* without *RHD*DIVa* were not agglutinated by seven monoclonal and single source anti-C (data not shown). These findings suggest that the aberrant C reactivity is associated with *RHD*DIVa*.

Linkage of RHD*DIVa and RHCE*ceTI

The RH genotypes and referral reasons of samples with *RHD*DIVa* or *RHCE*ceTI* are summarized in Table 3. *RHD*DIVa* was found with *RHCE*ceTI* in 47/55 samples and two of these were homozygous for both alleles, strongly suggesting linkage. Only four had *RHD*DIVa* without *RHCE*ceTI*, and four had *RHCE*ceTI* without *RHD*DIVa*. Taken together, among 80 samples (Tables 1, 2, and 3), 71 had both *RHD*DIVa* and *RHCE*ceTI* which is strong evidence for linkage.

RHD zygosity testing by PCR-RFLP or hybrid Rhesus box assay in samples with RHD*DIVa is unreliable

Of 60 referred samples that were tested for *RHD* zygosity, 25 were shown by exon specific DNA sequence analyses to be homozygous for *RHD* (or variant or hybrid alleles), however, the hybrid *Rhesus box* assay[15] and/or the RFLP assay[16] indicated, falsely, a deletion of *RHD*. Zygosity testing in three samples with *RHCE*ceTI* without *RHD*DIVa* was not discordant between sequencing and zygosity assays, confirming the discordant results are not travelling with *RHCE*ceTI*.

RHCE*ceTI encodes a partial c and a partial e antigen

A 26 year-old African American female with sickle cell disease who had previously been multiply transfused presented with alloanti-c-like in her plasma. Her RBCs typed D+C+E−c+e+, and were strongly agglutinated by seven anti-c; one of which gave a slightly weaker reaction than a single dose c positive control. Analyses of both genomic DNA and cDNA revealed that the patient was *RHD*DIVa/*D* and *RHCE*ceTI/RHCE*Ce.*

A 54 year-old multiply transfused African American female was admitted to hospital with chest pain and pneumonia. Her plasma contained alloanti-e-like antibody that was non-reactive with R_2R_2 or hr^B– RBCs. She had been recently transfused and no pre-transfusion extended RBC phenotyping was done. RH genotyping of her sample predicted a C−E+c+e+ phenotype. Analyses of genomic DNA and cDNA revealed that this patient was *RHD*DIVa/*D* and *RHCE*ceTI/RHCE*cE.*

Discussion

We report that *RHD*DIVa* and *RHCE*ceTI* very often, but not invariably, travel together. Among eighty samples with either allele, seventy-one (89%) had both *RHD*DVIa* and *RHCE*ceTI* (two of which were homozygous), five (7%) had *RHD*DIVa* without *RHCE*ceTI* and four (5%) had *RHCE*ceTI* without *RHD*DIVa. RHD*DIVa* and *RHCE*ceTI* are not uncommon in African Americans referred for antibody investigations. Indeed, with regards to *RHD*DIVa*, in our experience, 20% (33/162) of the samples referred because the patient's RBCs typed D+ with anti-D identified in the serum had *RHD*DIVa* (personal observations). Although prevalent in problem samples, testing of random African Americans (donors who self-declared and patients with sickle cell disease) revealed *RHD*DIVa* in 9/488 samples. All nine with *RHD*DIVa* also had *RHCE*ceTI*, however, 18/488 had *RHCE*ceTI* without *RHD*DIVa. RHCE*ceTI* encodes a partial c and a partial e, which are only apparent if the *in trans* allele encodes, respectively, C or E. Weak, variable, and unstable positive reactions of RBCs with some anti-C appears to be associated with the protein encoded by *RHD*DIVa*. Gamma-clone anti-C gave the strongest reactions and variable reactivity of panel donor RBC samples with this reagent reported by customers to the manufacturer (web resources) was the impetus for the initial investigation. This aberrant C typing was not stable: reactive monoclonal anti-C (containing clone MS24) tended to agglutinate only RBCs that were freshly collected. However, upon storage, the reactivity with the anti-C quickly faded. The aberrant reactivity appears to be related to not only the condition of the RBCs but also to the reagent, including the length of time the vial has been opened (personal communication). The relevant factors are not fully understood. However, if examined closely, a large proportion of samples with both *RHD*DIVa* and *RHCE*ceTI* gave weak, variable, and unstable C typing. Of samples freshly collected and tested with anti-C from a freshly opened vial, one sample with *RHD*DIVa* without *RHCE*ceTI* gave a 1+ reaction, and three samples with *RHCE*ceTI* without *RHD*DIVa* were non-reactive. Furthermore, three Riv+ samples that have the rare complex, DIVa(C)-, composed of *RHD*DIVa* and a hybrid *RHCE* allele with exons 2 and 3 from *RHD*DIVa* and exon 5 from *RHD* and, thus without *RHCE*ceTI*, have been shown to have robust expression of the C antigen.[18] Taking together, the data support that the RHD*DIVa allele is associated with the weak, variable, and unstable C expression rather than the RHCE*ceTI allele. The observation of loss of reactivity on storage together with the variable reaction from nonreactive to 2+ with many DIVa/ceTI samples, and results obtained using reagents with the same clone, collectively suggest that C expression is unstable. The practical impact includes weak 'false' positive C typing and potential discrepancy between phenotype and DNA testing.

It has previously been reported that *RHD* zygosity testing in samples from black Africans can be unreliable.[19–21] Specifically, we confirm that *RHD*DIVa in trans* to *RHD* often erroneously tests as *RHD* hemizygous by PCR[15] and/or PCR-RFLP.[16] *RHD* zygosity determination in African American samples should include assays for common partial D for improved accuracy. Lastly, we found no examples of the original *RHD*DIVa*, which is identical to the alleles reported here with the exception that it lacks 410C>T.[6] This raises the question as to whether the sample reported by Rouillac et al[6] was a very rare example of the *RHD*IVa* family of alleles, or whether the 410C>T change was inadvertently overlooked and if *RHD*DIVa.1* (original *RHD*DIVa*) exists. In 80 samples with *RHD** 186T, 455C, 1048C (respectively, 62Phe, 152Thr, 350His) investigated, all also had a 410C>T change in *RHD*. Attempts to obtain a sample from the original proband, Bel,[6] were unsuccessful, and discussions with colleagues on both sides of the Atlantic have not revealed any examples of *RHD* with 186T, 455C, 1048C changes, but without 410T. Furthermore, the curators of the Rhesus Base database have not observed examples of the *RHD*DIVa* without 410C>T

(Web Resources). We suggest that the *RHD** 186T, 455C, 1048C, 410T allele be called *RHD*DIVa* and have used the name *RHD*DIVa* throughout this manuscript.

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Table 1

RH alleles and reactivity of the RBCs with anti-C reagents in samples referred for C typing discrepancies. For clarity *RHD** and *RHCE** have been excluded from the allele name.

Sample	Alleles		Anti-C reagent		
	RHD	***RHCE***	**Ortho Bioclone**	**Immucor Series**	**Immucor Gamma-clone**
Panel cell 1	*DIVa* No D	*ceTI ce48C*	Neg	2+	2+
Panel cell 2	*DIVa DAU0*	*ceTI ce48C*	Neg	Neg	$+^{W}$
Panel cell 3	*DIVa D*	*ceTI ce*	NT	1+	NT
Donor 1	*DIVa* No D	*ceTI ce*	Neg	NT	$+^{W}$
Donor 2	DIVa *No D*	*ceTI ce48C*	Neg	2+	NT
Donor 3^	*DIVa D*	*ceTI ce48C*	Neg	Neg	NT

^ = a single source polyclonal anti-C reacted 2+

Table 2

Go(a+) samples. RH haplotypes and reactivity of RBCs with anti-C reagents. For clarity *RHD** and *RHCE** have been excluded from the allele name.

n	Presumed *RH* haplotypes		Historic typing	Ortho Bioclone	Immucor polyclonal	Immucor Gamma-clone	Bio-Rad Seraclone
8	*DIVa-ceTI*	*D-ce*	0	0 (n=5) wk (n=3)	0 (n=7) wk (n=1)	0 (n=2) wk (n=4) $1+^{W}$ (n=2)	0
6	*DIVa-ceTI*	*ce*	0	0 (n=3) wk (n=2) 1+ (n=1)	0 (n=4) wk (n=1) $2+^{W}$ (n=1)	0 (n=3) wk (n=2) 1+ (n=1)	0
2	*DIVa-ceTI*	*DIIIa-ceS*	0	0	0	0 (n=1) ± (n=1)	0
2	*DIVa-ceTI*	*ceS*	0	0	0 (n=1) ± (n=1)	± (n=1) $1+^{W}$ (n=1)	0
1	*DIVa-ce*	*ceS*	0	Trace	0	0	0

ceS = ce 48C, 733G, 1006T

Table 3

Summary of RH genotyping results by referral reason. For clarity *RHD** and *RHCE** have been excluded from the allele name.

I. *RHD*DIVa* with *RHCE*ceTI* (n=47)					
D+ with anti-D (n=32)			**Weak & variable e typing (n=3)**		
***RH* haplotypes**		**n**	***RH* haplotypes**		**n**
DIVa-ceTI	*ce*	14	*DIVa-ceTI*	*DcE*	2
DIVa-ceTI	*Pseudo RHD-ce48C*	6	*DIVa-ceTI*	*DAU0-ceMO*	1
DIVa-ceTI	*DIVa-ceTI*	2	**Multiple antibodies (n=1)**		
DIVa-ceTI	*ce48C*	2	*DIVa-ceTI*	*D-ce 733G*	1
DIVa-ceTI	*DIVa-ce*	1	**Donor D typing discrepancy (n=1)**		
DIVa-ceTI	*Ce*	1	*DIVa-ceTI*	Under investigation	1
DIVa-ceTI	*ce254G*	1	**Screening Random Blacks (n=7)**		
DIVa-ceTI	*ce733G*	1	*DIVa-ceTI*	*ce733G*	1
DIVa-ceTI	*(C)ceS (r'S type 1)*	1	*DIVa-ceTI*	*D-ce48C*	1
DIVa-ceTI	*ceS*	1	*DIVa-ceTI*	*D-Ce(RN)*	1
DIVa-ceTI	*ceEK*	1	*DIVa-ceTI*	*ce*	1
DIVa-ceTI	Under investigation	1	*DIVa-ceTI*	*DAU0-ce 48C*	1
Weak C and/or weak e (3)			*DIVa-ceTI*	*D-ceTI*	1
DIVa-ceTI	*(C)ceS (r'S type 1)*	2	*DIVa-ceTI*	*(C)ceS (r'S type 1)*	1
DIVa-ceTI	*(C)ceS (r'S type 2)*	1			
II. *RHD** *DIVa* without *RHCE*ceTI* (n=4)					
DIVa-ce	*Pseudo RHD-ce*	1			
DIVa-ce 733G	*ce 48C, 733G*	1			
DIVa-ce-48C	*ce*	1			
DIVa-CE-D(5–7)-CE	*(C)ceS (r'S type 1)*	1			
III. *RHCE*ceTI* without *RHD*DIVa* (n=4)					
D+ with anti-D		n	**Random Black screening**		n
D-ceTI D-Ce		1	*DIIIa-ceTI*	*(C)ceS (r'S type 1)*	1
D-ceTI or *ceTI D-Ce*		1	*DIIIa-ceTI*	*ce 48C*	1

ceS = ce 48C, 733G, 1006T