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RhCE protein variants in Southwestern Germany detected by serologic routine testing

Peter Bugert, **Erwin A. Scharberg**, **Christof Geisen**, **Inge von Zabern**, and **Willy A. Flegel**

German Red Cross Blood Donor Service, Baden-Württemberg–Hessen; the Institute of Transfusion Medicine and Immunology, Medical Faculty of Mannheim, University of Heidelberg, Heidelberg; the Institute for Transfusion Medicine and Immunohematology, Baden-Baden; the Institute of Transfusion Medicine and Immunohematology, Frankfurt/Main; the Institute for Transfusion Medicine, University Hospital Ulm, Ulm; and the Institute for Clinical Transfusion Medicine and Immunogenetics, Ulm, Germany

Abstract

BACKGROUND—Variant *RHCE* alleles with diminished expression of C, c, E, and e antigens have been described and indicate the genetic diversity of this gene locus in several populations. In this study the molecular background of variant RhCE antigens identified by standard serologic routine testing in German blood donors and patients was determined.

STUDY DESIGN AND METHODS—Samples from blood donors and patients were routinely analyzed for RhCE phenotype using the PK7200 analyzer with two sets of monoclonal anti-C, -c, -E, and -e reagents. Samples with confirmed variant RhCE antigens were analyzed by nucleotide sequencing of the 10 *RHCE* exons. A multiplex polymerase chain reaction with sequence-specific priming (PCR-SSP) method was established for rapid typing of the rare *RHCE* alleles.

RESULTS—We identified 43 samples with serologic RhCE variants. Molecular analysis revealed variant *RHCE* alleles in 34 samples. Altogether 22 *RHCE* alleles were detected; 10 have not been published before. Twenty alleles harbored distinct single-nucleotide substitutions, 18 of which encoded amino acid changes and 2 of which occurred in noncoding regions. Two samples represented *RHCE-D-CE* hybrid alleles involving different segments of the *RHCE* Exon 5. A multiplex PCR-SSP screening for 17 *RHCE* alleles was negative in 1344 samples of the DNA bank GerBS. The cumulative phenotype frequency was estimated between 1 in 488 (0.20%) and 1 in 8449 (0.012%).

CONCLUSION—Single-amino-acid substitutions were the molecular basis for variant RhCE antigen expression in most samples. Nucleotide substitutions in *RHCE* exons were excluded as

Address reprint requests to: Willy A. Flegel, MD, Department of Transfusion Medicine, Building 10, Room 1C711, NIH, Bethesda, MD 20892; flegelwa@cc.nih.gov.

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CONFLICT OF INTEREST

The authors declare no competing interests relevant to this article.

possible mechanism of diminished RhCE antigen expression in one-fifth of the serologically identified samples.

Rh is the most polymorphic of all 30 blood group systems.[1] The Rh blood group system is encoded by the two highly homologous genes, *RHD* and *RHCE*. The two polymorphic genes encode the five major antigens: *RHD* encodes the D antigen and *RHCE* the C, E, c, and e antigens. Forty-five additional low- and high-prevalence Rh antigens are defined to date. Many *RHCE* alleles were identified by molecular analyses in different populations.[2,3] Variant RhCE phenotypes may be caused by single- or multiple-nucleotide substitutions in the *RHCE* gene or by *RHCE-D-CE* hybrid alleles at the *RHCE* gene locus. Rare RhCE phenotypes were found either by antibodies against low-prevalence[4-7] and high-prevalence[8,9] RhCE antigens or by weak and partial expression of the major RhCE antigens.[5,10-12]

In early studies the serologic patterns established by monoclonal anti-E allowed categorization of four variants of the E antigen (cat EI to EIV).[13] The molecular basis in *RHCE* alleles are the single-amino-acid substitution M167K (500T>A) for cat EI,[13] later recognized to express the low-prevalence antigen E^w;[14] the *RHcE-D(2-3)-cE* hybrid allele for cat EII;[13] the two D-specific amino acid substitutions Q233E and M238V (697C>G, 712A>G) for cat EIII;[13] and the single D-specific amino acid substitution R201T (602G>C) for cat EIV,[15] which is occurring in a *cE* haplotype and located suspiciously close to the amino acid 223 encoding the E/e antigens.

Another way to discover RhCE variants is the expression of low-prevalence antigens, some of which occur in RhCE proteins whose major antigens are altered. For example, the *Rhce-D(5)-ce* hybrid allele encodes an RhCE protein expressing the low-prevalence antigens RH33 (Har) and RH50 (FPTT) and a weakened e antigen.[5] An *RHCe-D(4)-Ce* hybrid allele encodes an RhCE protein expressing the low-prevalence antigens RH32 (R^N) and RH54 (DAK) and weakened C and e antigens, but lacking the high-prevalence antigen RH46 (Sec).[10] The ceCF variant expresses the low-prevalence RH43 (Crawford) antigen that reacted with a polyclonal antibody inadvertently occurring in former commercial polyclonal anti-D reagents and is cross-reactive with a currently available monoclonal anti-D.[16] Similarly, the RhCE variants ceRT and ceSL[17,18] associated with the *ce* haplotype were found by the unexpected expression of D epitopes. If the same ceSL amino acid substitution occurs in the *Ce* haplotype, it induces the expression of the low-prevalence antigen RH53 (JAHK) and is associated with significantly weakened C and e antigens.[19]

Hence, minor modifications, like single-amino-acid substitutions typically occurring in the extracellular part of the RhCE protein but also in transmembraneous or intracellular segments, may induce the expression of neoantigens, recognized as low-prevalence antigens, or weakened epitope and antigen expression, which may involve conformational changes of the RhCE protein, reduce membrane integration, or hamper the interaction with other proteins of the Rh complex in the red blood cell (RBC) membrane.

In this study we investigated the RhCE variants encountered in the routine serologic workup of blood donors and patients in four reference laboratories of a large blood donor service in

Southwestern Germany. Our screening procedure allowed detecting the weakened expression of the major RhCE antigens. We found 22 *RHCE* alleles among which 10 have not been published before.

MATERIALS AND METHODS

Blood samples and immunohematology

Blood samples were collected from blood donors in Southwestern Germany, which comprises a population of 16.5 million people. The first- and second-time donors were typed for ABO, D, CcEe, and K with an automated analyzer (PK7200; Olympus, Hamburg, Germany) as a routine serologic method. Two sets of monoclonal antibodies (MoAbs) diluted in 0.9% NaCl with 0.1% bromelin were used: anti-C (MS24 at a final dilution of 1 in 300, Ortho, Neckargmünd, Germany; and 392/P3x25513G8 at 1 in 175, Biotest, Dreieich, Germany), anti-E (MS258/906 and MS260/MS12, both at 1 in 150, Biotest), anti-c (MS33 at 1 in 100, Immucor, Rödermark, Germany; and MS42 at 1 in 100, Ortho), and anti-e (MS63/MS16/MS21 at 1 in 30, Ortho; and BS260/267 at 1 in 8, Biotest). The incubation time was 60 minutes at room temperature.

We examined samples in tube or column agglutination techniques that showed discrepant, weak, or unclear results for any of the antigens CcEe with the PK7200 analyzer and blood samples from patients with aberrant CcEe phenotypes that had been sent to our reference laboratories. Tube tests were performed for 15 minutes at room temperature with the same monoclonal reagents used for the PK7200 analyzer (MS24, 392/P3x25513G8; MS258/906, MS260/MS12; MS33, MS42; MS63/MS16/MS21, BS260/267). CcEe typing with column agglutination techniques was performed using the same MoAbs at room temperature (ID-Card, DiaMed, Ottobrun, Germany; and ScanGel, BioRad, München, Germany) or polyclonal (ID-Card Rh-subgroups plus K; DiaMed) RHCE typing cards. For the polyclonal typing gel card (DiaMed) the RBCs were suspended in Diluent 1 (bromelin solution, DiaMed) and in Diluent 2 for monoclonal typing cards. If a serologically variant RhCE phenotype was confirmed, the sample was subjected to molecular investigation of the *RHCE* gene.

RHCE nucleotide sequencing

For specific polymerase chain reaction (PCR) amplification and nucleotide sequencing of the 10 *RHCE* exons we used primers and PCR conditions as previously described[20] except for the *RHCE*-specific reverse primer in Exon 5. This primer was replaced by the *RHD/RHCE*-specific primer rb15.[21]

RHD zygosity

The presence of the hybrid *Rhesus box* was determined by PCR (Ready Gene D neg, inno-Train, Kronberg, Germany).

Multiplex PCR for rare *RHCE* alleles

A PCR with sequence-specific priming (PCR-SSP) with five multiplex reactions was established for the rapid detection of the observed rare *RHCE* alleles. All primers were used

at a final concentration of 1 mmol/L, except the control primers HBB-F and HBB-R, which were added to each multiplex PCR mix at 0.4 mmol/L (Table 1). The total volume of each PCR-SSP was 10 mL containing 10 ng of sample DNA. The cycling conditions were 2 minutes at 95°C, followed by 10 cycles with 15 seconds at 95°C and 1 minute at 65°C, followed by 20 cycles with 15 seconds at 95°C, 1 minute at 61°C, and 30 seconds at 72°C. The total thermocycling time was 1.5 hours. Amplification products were examined in 2% agarose gels with 0.5 ng/mL ethidium bromide (ultraviolet documentation device with charged coupled device camera, UVP, Upland, CA).

Using the multiplex PCR-SSP method, the DNA bank GerBS (German Blood Service) control series[22] was screened. GerBS consists of 1344 DNA samples of healthy, unrelated blood donors from the southwestern area of Germany, which corresponds to the geographical origin of the blood donors and patients of the current study. Equal numbers of female and male subjects were investigated with a median age of 50 years (range, 18-68 years); their ABO, Rh, and Kell blood group phenotypes were random (Table 2).[23]

Nomenclature

Novel *RHCE* alleles were named after the involved amino acid substitution, such as *RHcE*(R10W). In case of variations in noncoding regions, the allele was named after the involved nucleotide change. Hence, *Rhce*(-10C>T) represents a single-nucleotide substitution in the untranslated region (UTR) located 10 nucleotides upstream of (5′ to) the start codon, and *RHCe*(IVS3-5G) represents a substitution in Intron 3 located five nucleotides 5′ to the first nucleotide of Exon 4.

RESULTS

We identified 43 samples with weak expression of one or two major RhCE antigens (Table 3) by testing blood donors and patients with routine serologic methods. For automated blood donor testing, diluted monoclonal antisera were used. In approximately four of five samples, nucleotide sequencing revealed a variant *RHCE* allele, while nine samples showed no deviation from one of the regular *RHCE* exon nucleotide sequences. Between June 2005 and October 2007, a total of 1,208,162 whole-blood donations were collected in Baden-Württemberg (Southwestern Germany) at our DRK Blood Donor Service, of which 143 donations were from the 34 blood donors (Table 3).

RHCE alleles

Among 34 samples (Table 3), we encountered 22 different *RHCE* alleles, 10 of which have previously not been published (Table 4). In a parallel study by Doescher and colleagues[24] describing RhCE variants in Northern Germany, only 10 of these 22 *RHCE* alleles were observed, although the variety of alleles was similar. However, none of our donors and patients overlapped with those found in the Northern German study, published in this issue of **TRANSFUSION.**[24]

Immunohematology

The pattern of serologic reactivity with the major RhCE antigens confirmed the haplotype association for known *RHCE* alleles and allowed to identify the probable haplotypes for the 10 new *RHCE* alleles (Table 5). The *RHCe*(S122P) allele in the *Ce* haplotype had diminished C and e antigens but was not found to encode RH53 (JAHK), although RH53 is known to be encoded by the *RHCe*(S122L) allele in the *Ce* haplotype with significantly diminished antigens C and e.[19]

Population frequencies

We devised a multiplex PCR-SSP method for detection of 17 of the 20 rare *RHCE* alleles with single-nucleotide polymorphisms (Table 6). The variant alleles ce(C48), C^X, and C^W were not included. This multiplex PCR facilitated the rapid screening of DNA samples (Fig. 1). We validated our multiplex PCR-SSP method by testing of the applicable DNA samples (Table 5), which corroborated their nucleotide sequencing data (data not shown). All 1344 blood donor samples of the GerBSDNA bank,[22] which represented regular RhCE phenotypes (Table 2), were negative for any of the tested *RHCE* alleles.

The frequency estimate for each rare *RHCE* allele is less than 1 in 488 donors (0.2%; upper limit of 95% confidence interval, one-sided Poisson distribution). Because none of the 17 rare *RHCE* alleles in our assay (Table 6) were found in the 1344 samples, even the cumulative population frequency estimate for these rare *RHCE* alleles may be less than 1 in 488. The documented cumulative phenotype frequency was approximately 1 in 8449 (0.012%) for the RhCE variants detected in our serologic routine testing.

Samples carrying the regular *RHCE* allele

The DCcEe and DccEE phenotypes may be encoded by *DCE/dce* and *DCE/cDE* genotypes, respectively. The *DCE* haplotype is known to be associated with a diminished C antigen.[25-27] Four samples with regular *RHCE* alleles had diminished C but, if any, a normal e antigen; all four samples could be explained by a *DCE* haplotype, as shown by *RHD* zygosity analysis (Table 5). With this observation we confirmed that this long recognized *DCE* haplotype is composed of a regular *RHD* and a regular *RHCE* allele with normal exon and splice site nucleotide sequences. Five samples also harbored regular *RHCE* alleles but carried diminished C antigen or e antigen or both, which cannot be explained by the mechanism of this *DCE* haplotype. Our study allowed identifying these unusual samples for further investigation toward their underlying molecular cause(s) that did not fit any known mechanism.

DISCUSSION

We identified 43 samples with variant RhCE phenotypes in a serologic routine setting. Among these, 34 carried variant *RHCE* alleles detectable by nucleotide sequencing of the 10 *RHCE* exons. Ten of the encountered alleles have not been published previously. Nine samples showed regular *RHCE* nucleotide sequences within the 10 exons and their adjacent intron regions. The occurrence of the *DCE* haplotype underlay the diminished C antigen in four samples with regular *RHCE* alleles, which left five samples in our study carrying a

regular *RHCE* allele whose molecular basis for variant RhCE expression remained unexplained. In contrast to *RHCE*, a diminished D antigen expression is rarely associated with an apparently regular *RHD* allele.[21,28]

The proportion of novel *RHCE* alleles was unexpected. A large number of alleles have been discovered in previous population screens. Furthermore, a parallel study in Northern Germany[24] revealed a similar proportion of novel alleles, but barely half of them were congruent with the alleles found in this study in Southwestern Germany. Our results otherwise correlated well with the observations in Northern Germany (Table 4). The differing E^w phenotype frequencies may be explained by the use of distinct serologic routine methods or by actual frequency variations.

Because most samples were from Caucasian donors and patients, two approaches allowed us to gauge the prevalence of variant *RHCE* alleles in the Southwestern German population. The calculated cumulative frequency of approximately 1 in 8500 (0.012%) may well represent a lower limit for variant RhCE phenotypes in the population. The cumulative frequency of 1 in 488 (0.20%) was the estimated upper limit for the distinct *RHCE* alleles as determined by screening 1344 samples of a DNA bank. The molecular screening that we applied was more powerful than our serologic routine screen, because the rare alleles may be detected in heterozygous fashion even when the variant phenotype is masked by a regular RhCE antigen. Almost all identified *RHCE* alleles will neither be novel mutations nor limited to isolated cases or families. Hence, the presented multiplex PCR tool (Table 6 and Fig. 1) may be used for population screening.

The accumulated evidence indicates a huge variety of *RHCE* alleles in populations, much of which remains to be explored. Although data on definite frequencies for variant *RHCE* alleles are missing to date, the available estimates are surprisingly similar to population frequencies of sporadically occurring alleles in other blood group genes. For example, the Bombay allele frequency was estimated to be 1 in 347 (0.28%) by a population genetics approach.[29] For the D antigen, the cumulative phenotype frequency of weak D is approximately 1 in 227 (0.44%)[23] and the most prevalent *weak D type 1* allele frequency is approximately 1 in 277 (0.29%).[21]

It will be interesting to explore whether the variant RhCE proteins express novel low-prevalence antigens, which may cause immunizations in transfusion recipients. Examples of variant RhCE proteins are known that express low-prevalence antigens of appreciable immunogenicity. C^w,[4] C^x,[4] E^w,[14] JAHK,[19] Crawford,[16] and JAL[30-32] antigens are all caused by single-amino-acid substitutions in the RhCE protein. For example, the S122L substitution (365C>T) in the *Ce* haplotype encodes the JAHK antigen and is associated with a suppression of the C and e antigens. An RhCE protein with the S122P substitution (364T>C) in the *Ce* haplotype may lack JAHK expression and is associated with a suppression of the C and e antigens (Table 5).

The mechanisms of diminished antigen expression may include conformational changes in the RhCE protein, missplicing of the *RHCE* gene transcript, and reduced translation. Similar to RhD, single-amino-acid substitutions were the most frequent molecular bases of

diminished RhCE expression. While in RhD variants *RHD* Exons 5 to 7 are predominantly affected,[33] we found many RhCE variants with single-nucleotide substitutions in *RHCE* Exons 1, 3, and 4 (Table 4). In 12 of 18 observed alleles, the single-amino-acid substitution were located in the transmembraneous sections of the RhCE protein (Table 4 and Fig. 2). Our samples with unresolved molecular bases exemplified that additional molecular mechanisms exist and can be addressed now, for example, using the samples identified in the current study.

Three RhD and three RhCE variants harbor amino acid substitutions at positions 114 and 115, which are located in the transmembraneous section of Helix 4 (Fig. 2). The three RhD variants are weak D Type 17 (114W), weak D Type 47 (114G), and weak D Type 25 (114Q) with weakened D antigen expression. Both 114W and 114Q substitutions are known to be associated with JAL antigen expression,[30-32] which has been documented so far in RhCE only. The L115R substitution introduces a basic residue into the putatively hydrophobic transmembraneous segment and the L115P substitution introduces proline, which is known to disturb the regular helix structure. Further examples observed by us for disruption of helical structures by proline are the substitutions S122P and L297P.

At the extracellular RhD vestibule several amino acid substitutions cause antigen loss and permit anti-D immunization.[34] Similarly, the amino acid substitutions H166L, M167K, and L169Q in RhCE are predicted to lie at the extracellular RhCE protein vestibule and the corresponding RhCE variants likely represent “partial RhCE.” The partial D variant DFW (H166P) also carried an amino acid substitution at Position 166.[35] We confirmed that the M167K amino acid substitution in cat EI (E^w) is associated with a weakened expression of antigen E but with a normal antigen c,[13] which was also observed for the novel L169Q substitution.

Single-nucleotide substitutions in the promoter region or in proximity to splice sites can influence protein translation. First, *RHce*(5′-UTR-10C>T) may be explained by inhibiting ribosomal binding to the mRNA, which would decrease translation. The second mechanism may be exemplified by *RHCe*(IVS3-5G) rendering splicing less efficient, which reduces protein expression representing a quantitative effect, or causing missplicing of the transcript, which leads to an altered protein representing a qualitative effect. Although the nucleotide substitution in *RHCe*(M267K) lies at the splice site it is unlikely to affect the splicing process, because it represents a templated mutation, that is, deriving from the normal *RHD* gene in which it is not known to affect splicing.

The rare RhCE phenotypes observed in Southwestern Germany do not frequently permit a clinically relevant immunization in their carriers. While the humoral immune response in carriers of RhCE variants appears almost always to be left mute, the identification of rare RhCE variants enables to examine the possible cellular immune response in the carriers when they were transfused with blood harboring the regular RhCE protein.

Most of the RhD and RhCE protein is integrated into the RBC membrane and also most of the amino acid substitutions occur in this transmembrane part. Similar to weak D caused by substitutions in the transmembraneous section, the number of examples for such weak CE is

increasing. Substitutions in the much smaller extracellular parts of these proteins are inherently rarer but make the carrier of the variant prone to immunization. Hence, the consequences with respect to immunization are expected to be the same for D and CE: as weak D, most weak CE will have no consequence for the carrier while carriers of partial D and partial CE can get immunized against the normal protein. Although the discrimination between weak and partial will never be sharp, the predominant amount of alleles confers to this scheme which is of great help to the practically oriented physician. A considerable amount of information has been collected with regard to the immunization of individuals with partial D; comparable information is now accumulating for CE.

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ABBREVIATIONS

SSP	sequence-specific priming
UTR	untranslated region

REFERENCES

1. Daniels GL, Castilho L, Flegel WA, Fletcher A, Garratty G, Levene C, Lomas-Francis C, Moulds JJ, Moulds JM, Olsson ML, Overbeeke MA, Poole J, Reid ME, Rouger P, van der Schoot CE, Scott M, Sistonen P, Smart E, Storry JR, Tani Y, Yu L-C, Wendel S, Westhoff CM, Yahalom V, Zelinski T. International Society of Blood Transfusion Committee on terminology for red cell surface antigens: Macao report. Vox Sang. 2009; 96:153–6. [PubMed: 19152607]
2. Wagner FF, Flegel WA. Review: the molecular basis of the Rh blood group phenotypes. Immunohematol. 2004; 20:23–36.
3. Ansart-Pirenne H. Stratégie d'identification des variants du gène RHCE au Centre national de référence pour les groupes sanguins: impact sur la sécurité transfusionelle [Identification strategy of RHCE gene variants at the National Blood Group Reference Laboratory: impact on transfusion safety]. Transfus Clin Biol. 2006; 13:13–8. [PubMed: 16563835]
4. Mouro I, Colin Y, Sistonen P, Le Pennec PY, Cartron JP, Le Van Kim C. Molecular basis of the RhC^{w} (Rh8) and RhCx (Rh9) blood group specificities. Blood. 1995; 86:1196–201. [PubMed: 7620172]
5. Beckers EA, Faas BH, von dem Borne AE, Overbeeke MA, van Rhenen DJ, van der Schoot CE. The R_0^{Har} Rh:33 phenotype results from substitution of exon 5 of the *RHCE* gene by the corresponding exon of the RHD gene. Br J Haematol. 1996; 92:751–7. [PubMed: 8616049]
6. Daniels GL, Faas BH, Green CA, Smart E, Maaskant-van Wijk PA, Avent ND, Zondervan HA, von dem Borne AE, van der Schoot CE. The VS and V blood group polymorphisms in Africans: a serologic and molecular analysis. Transfusion. 1998; 38:951–8. [PubMed: 9767746]
7. Faas BH, Beckers EA, Wildoer P, Ligthart PC, Overbeeke MA, Zondervan HA, von dem Borne AE, van der Schoot CE. Molecular background of VS and weak C expression in blacks. Transfusion. 1997; 37:38–44. [PubMed: 9024488]
8. Noizat-Pirenne F, Mouro I, Le Pennec PY, Ansart-Pirenne H, Juszczak G, Patereau C, Verdier M, Babinet J, Roussel M, Rouger P, Cartron JP. Two new alleles of the RHCE gene in Black individuals: the RHce allele ceMO and the RHcE allele cEMI. Br J Haematol. 2001; 113:672–9. [PubMed: 11380457]

9. Noizat-Pirenne F, Lee K, Pennec PY, Simon P, Kazup P, Bachir D, Rouzaud AM, Roussel M, Juszczak G, Menanteau C, Rouger P, Kotb R, Cartron JP, Ansart-Pirenne H. Rare RHCE phenotypes in black individuals of Afro-Caribbean origin: identification and transfusion safety. Blood. 2002; 100:4223–31. [PubMed: 12393640]
10. Rouillac C, Gane P, Cartron JP, Le Pennec PY, Colin Y. Molecular basis of the altered antigenic expression of RhD in weak D (Du) and RhC/e in R^N phenotypes. Blood. 1996; 87:4853–61. [PubMed: 8639859]
11. Noizat-Pirenne F, Le Pennec PY, Mouro I, Rouzaud AM, Juszczak G, Roussel M, Lauroua P, Krause C, Rouger P, Cartron JP, Ansart-Pirenne H. Molecular background of D(C)(e) haplotypes within the white population. Transfusion. 2002; 42:627–33. [PubMed: 12084172]
12. Westhoff CM, Silberstein LE, Wylie DE, Skavdahl M, Reid ME. 16Cys encoded by the RHce gene is associated with altered expression of the e antigen and is frequent in the R0 haplotype. Br J Haematol. 2001; 113:666–71. [PubMed: 11380456]
13. Noizat-Pirenne F, Mouro I, Gane P, Okubo Y, Hori Y, Rouger P, Le Pennec PY, Cartron JP. Heterogeneity of blood group RhE variants revealed by serological analysis and molecular alteration of the RHCE gene and transcript. Br J Haematol. 1998; 103:429–36. [PubMed: 9827916]
14. Strobel E, Noizat-Pirenne F, Hofmann S, Cartron JP, Bauer MF. The molecular basis of the Rhesus antigen Ew. Transfusion. 2004; 44:407–9. [PubMed: 14996199]
15. Noizat-Pirenne F, Mouro I, Le Pennec PY, Roussel M, Boulard P, Rouger P, Cartron J. Molecular basis of category EIV variant phenotype [abstract]. Transfusion. 1999; 39:103S.
16. Flegel WA, Wagner FF, Chen Q, Schlanser G, Frame T, Westhoff CM, Moulds MK. The *RHCE* allele *ceCF*: the molecular basis of Crawford (RH43). Transfusion. 2006; 46:1334–42. [PubMed: 16934069]
17. Chen Q, Hustinx H, Flegel WA. The *RHCE* allele *ceSL*: the second example for D antigen expression without D-specific amino acids. Transfusion. 2006; 46:766–72. [PubMed: 16686844]
18. Vege S, Lomas-Francis C, Kosanke J, Westhoff CM. The RHCE*ceSL allele encodes a weak e antigen [abstract]. Transfusion. 2007; 47:160A.
19. Scharberg EA, Green C, Daniels G, Richter E, Klüter H, Bugert P. Molecular basis of the JAHK (RH53) antigen. Transfusion. 2005; 45:1314–8. [PubMed: 16078918]
20. Wagner FF, Ladewig B, Flegel WA. The *RHCE* allele *ceRT*: D epitope 6 expression does not require D-specific amino acids. Transfusion. 2003; 43:1248–54. [PubMed: 12919427]
21. Wagner FF, Gassner C, Müller TH, Schönitzer D, Schunter F, Flegel WA. Molecular basis of weak D phenotypes. Blood. 1999; 93:385–93. [PubMed: 9864185]
22. Erdmann J, Großhennig A, Braund PS, König IR, Hengstenberg C, Hall AS, Linsel-Nitschke P, Kathiresan S, Wright B, Tregouet D-A, Cambion F, Bruse P, Aherrahrou Z, Wagner AK, Stark K, Schwartz SM, Salomaa V, Elosua R, Melander O, Voight BF, O'Donnell CJ, Peltonen L, Siscovick DS, Altshuler D, Merlini PA, Peyvandi F, Bernardinelli L, Ardissino D, Schillert A, Blankenberg S, Zeller T, Wild P, Schwarz DF, Tiret L, Perret C, Schreiber S, El Mokhtari NE, Schäfer A, März W, Renner W, Bugert P, Klüter H, Schrezenmeir J, Rubin D, Ball SG, Balmforth AJ, Wichmann HE, Meitinger T, Fischer M, Meisinger C, Baumert J, Peters A, Ouwehand WH, Italian Atherosclerosis, Thrombosis, and Vascular Biology Working Group; Myocardial Infarction Genetics Consortium; Wellcome Trust Case Control Consortium; Cardiogenics Consortium. Deloukas P, Thompson JR, Ziegler A, Samani NJ, Schunkert H. Novel susceptibility locus for coronary artery disease on chromosome 3q22.3. Nat Genet. 2009; 41:280–2. [PubMed: 19198612]
23. Wagner FF, Kasulke D, Kerowgan M, Flegel WA. Frequencies of the blood groups ABO, Rhesus, D category VI, Kell, and of clinically relevant high-frequency antigens in South-Western Germany. Infusionsther Transfusionsmed. 1995; 22:285–90. [PubMed: 8924742]
24. Doescher A, Vogt C, Bittner R, Gerdes I, Petershofen EK, Wagner FF. *RHCE* alleles detected in automated RH blood grouping in Northern Germany. Transfusion. DOI: 10.1111/j.1537-2995.2009.02221.x.
25. Murray J, Race RR, Taylor GL. Serological reactions caused by the rare human gene Rh_z. Nature. 1945; 155:112–3.
26. Race RR, Sanger R, Lawler SD. *Rh* genes allelomorphic to *C*. Nature. 1948; 161:316.

27. Race RR, Sanger R, Lawler SD. The Rh antigen called c-v: a revocation. Vox Sang. 1960; 5:334–6. [PubMed: 13738896]

28. von Zabern I, Flegel WA. IVS5-38del4 deletion in the *RHD* gene does not cause a DEL phenotype: relevance for RHD alleles including *DFR-3*. Transfusion. 2007; 47:1552–5.

29. Wagner FF, Flegel WA. Polymorphism of the h allele and the population frequency of sporadic nonfunctional alleles. Transfusion. 1997; 37:284–90. [PubMed: 9122901]

30. Ong J, Walker PS, Schmulbach E, Storry JR, Vege S, Westhoff C, Lomas-Francis C, Reid ME. Alloanti-c in a c-positive, JAL-positive patient. Vox Sang. 2009; 96:240–3. [PubMed: 19076333]

31. Westhoff CM, Vege S, Wylie D, Nickle P, Lomas-Francis C, Hue-Roye K, Reid ME. The JAL antigen (RH48) is the result of a change in *RHCE* that encodes Arg114Trp. Transfusion. 2009; 49:725–32. [PubMed: 19170983]

32. Hustinx H, Poole J, Bugert P, Gowland P, Still F, Fontana S, Scharberg EA, Tilley L, Daniels G, Niederhauser C. Molecular basis of the Rh antigen RH48 (JAL). Vox Sang. 2009; 96:234–9. [PubMed: 19207167]

33. Wagner, FF. The RhesusBase. DRK-Blutspendedienst Baden-Württemberg—Hessen; Ulm: 1998-2009.

34. Flegel WA, von Zabern I, Doescher A, Wagner FF, Vytiskova J, Pisacka M. DCS-1, DCS-2 and DFV share amino acid substitutions at the extracellular RhD protein vestibule. Transfusion. 2008; 48:25–33. [PubMed: 17900276]

35. Flegel WA, von Zabern I, Doescher A, Wagner FF, Strathmann KP, Geisen C, Palfi M, Pisacka M, Poole J, Polin H, Gabriel C, Avent ND. D variants at the RhD vestibule in the weak D type 4 and Eurasian D clusters. Transfusion. 2009; 49 in press.

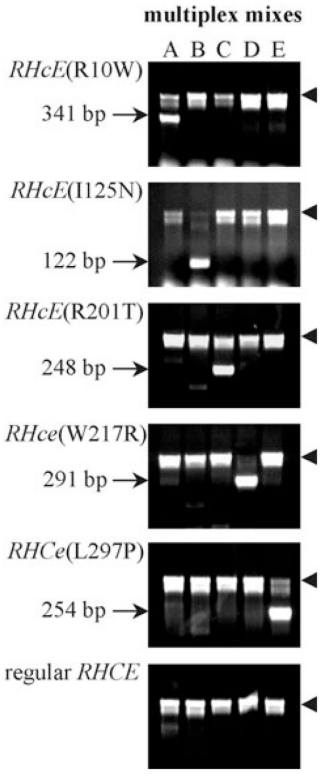

Fig. 1.

Multiplex PCR for rare *RHCE* alleles. Representative results are shown for the multiplex PCR-SSP typing of samples with five rare *RHCE* alleles and a sample with the regular *RHCE* genotype that is positive for antigens CcEe. The multiplex PCR-SSP reactions in the lanes comprised the Primer Mixes A to E (see Table 6). In all PCR-SSP procedures a 536-bp fragment of the *HBB* gene was coamplified as internal control (arrowhead).

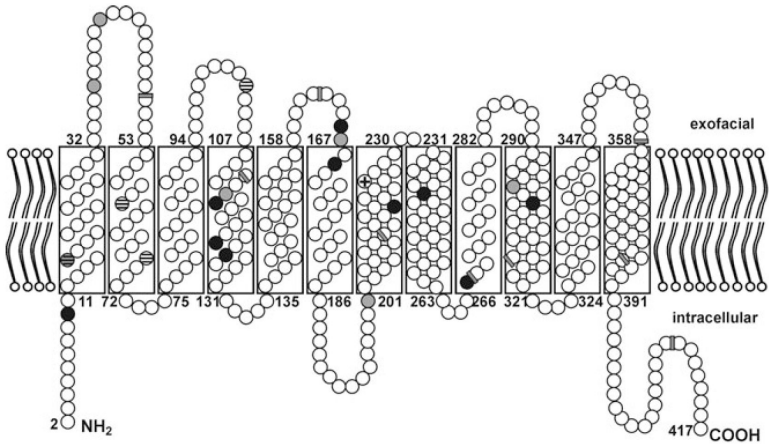

Fig. 2.

Model of the RhCE protein topology in the RBC membrane. Amino acids are depicted as circles. Four amino acid positions differ between C and c antigens (horizontally striped circles) and one amino acid differs between E and e antigens (crossed circle). Previously known amino acid substitutions (gray circles) and novel variants identified in this study (black circles) are predominantly located within the N-terminal half of the protein. The 9 exon boundaries in the *RHCE* cDNA, as reflected in the amino acid sequence, are indicated (gray bars).

TABLE 1

Primers for PCR-SSP typing of rare *RHCE* alleles

Name	Nucleotide sequence (5′-3′)	Genomic region	Direction	Specificity
F-10T	TGGAACCCCTGCACAGAGAT	5′-UTR Exon 1	Sense	*RHCE*(−10T)
F28T	AAGTACCCGCGGTCTGTCT	Exon 1	Sense	*RHCE*(28T)
RIVS1	acactgttgrctgaatttcggtgc	Intron 1	Antisense	*RHD/RHCE*
F341A	ccttctcacccccagTATTCA	Exon 3	Sense	*RHCE*(341A)
F344C	tctcacccccagTATTCGGCC	Exon 3	Sense	*RHCE*(344C)
F344G	tctcacccccagTATTCGGCG	Exon 3	Sense	*RHCE*(344G)
F364C	GGCCACCATGAGTGCTATGC	Exon 3	Sense	*RHCE*(364C)
F374A	GAGTGCTATGTCGGTGCTGAA	Exon 3	Sense	*RHCE*(374A)
R455C	TACTGATGACCATCCTCAGGG	Exon 3	Antisense	*RHCE*(455C)
FIVS3-5G	ctctactgctcttactgggttttatg	Intron 3	Sense	*RHCE*(IVS3-5G)
F497T	ggttttattgcagACAGACTACCT	Exon 4	Sense	*RHCE*(497T)
F500A	tttattgcagACAGACTACCACAA	Exon 4	Sense	*RHCE*(500A)
F506A	gACAGACTACCACATGAACCA	Exon 4	Sense	*RHCE*(506A)
F602C	GAACGGAGGATAATGATCAGAC	Exon 4	Sense	*RHCE*(602C)
RIVS4	gaggtccctaaaaggagtgc	Intron 4	Antisense	*RHCE*
F649C	ccagGCGCCCTCTTCTTGC	Exon 5	Sense	*RHCE*(649C)
F722T	GGAAGAATGCCATGTTCAACAT	Exon 5	Sense	*RHCE*(722T)
F800A	CCCCAAAGGAAGATCAGCAA	Exon 5	Sense	*RHCE*(800A)
RIVS5	agctccaccacccggcatgt	Intron 5	Antisense	*RHCE*
FIVS5	gccccaacacaggggagag	Intron 5	Sense	*RHD/RHCE*
R890G	CCCAGCCACAAGACCCG	Exon 6	Antisense	*RHCE*(890C)
R908T	GCTCCCCCGATGGAGATCT	Exon 6	Antisense	*RHCE*(908A)
HBB-F	GGTTGGCCAATCTACTCCCAGG	5′-UTR Exon 1	Sense	*HBB* *
HBB-R	GCTCACTCAGTGTGGCAAAG	Exon 2	Antisense	*HBB* *

* Internal control primers specific for conserved sequences of the β-hemoglobin gene (*HBB*).

TABLE 2

Blood group distribution among the samples of the GerBS control series

Blood group		Blood donors	
System	**Phenotype**	**Number**	**%**
ABO	O	527	39.2
	A	556	41.4
	B	177	13.2
	AB	84	6.2
Rh	DCcee	487	36.2
	DCCee	267	19.9
	DCcEe	156	11.6
	DccEe	156	11.6
	DccEE	22	1.6
	Dccee	16	1.2
	DCcEE	1	0.1
	ddccee	227	16.9
	ddCcee	7	0.5
	ddccEe	5	0.4
Kell	kk	1245	92.6
	Kk	99	7.4
	KK	0	0
Total		1344	100

TABLE 3

Blood donor and patient samples in this study

	Blood donors		Patients		
Rhesus phenotype	**Variant *RHCE* allele (n)**	**Regular *RHCE* allele (n)**	**Variant *RHCE* allele (n)**	**Regular *RHCE* allele (n)**	**Total**
DCcEe	17	6	1	1	25
DCcee	3	0	4	1	8
DccEe	7	0	2	0	9
DCcEE	0	1	0	0	1
Total		34		9	43

TABLE 4

RHCE alleles identified in samples with aberrant RhCE antigen expression

Allele	Trivial name	Nucleotide change	Genomic region	Amino acid change	Membrane localization*	Original report†	Observed by Doescher et al.[24]	Accession number
Single-nucleotide polymorphisms								
RHce(5′-UTR-10C>T)		−10C>T	5′-UTR Exon 1	none	NA	2008	Yes	FM866412
RHcE(R10W)		28C>T	Exon 1	R10W	IC	This study	No	FJ486155
RHce(W16C)	ce(C48)	48G>C	Exon 1	W16C	TM	Westhoff et al., 2001[12]	Yes	DQ266400
RHCe(A36T)	C^x	106G>A	Exon 1	A36T	EF	Mouro et al., 1995[4]	No	NA
RHCe(Q41R)	C^W	122A>G	Exon 1	Q41R	EF	Mouro et al., 1995[4]	Yes	NA
RHce(R114Q)	ce JAL+	341G>A	Exon 3	R114Q	TM	2003	No	AJ548432
RHCe(L115R)		344T>G	Exon 3	L115R	TM	2004	Yes	AJ867774
RHcE(L115P)		344T>C	Exon 3	L115P	TM	This study	Yes	FJ486156
RHCe(S122P)		364T>C	Exon 3	S122P	TM	This study	No	FJ486157
RHcE(l125N)		374T>A	Exon 3	I125N	TM	This study	No	FJ486158
RHCe(IVS3-5G)		487-5T>G	Intron 3	none	NA	2008	Yes	FM866415
RHCe(H166L)		497A>T	Exon 4	H166L	EF	This study	No	FJ486159
RHcE(M167K)	cat El (E^w)	500T>A	Exon 4	M167K	EF	Strobel et al., 2004[14]	Yes	NA
RHcE(L169Q)		506T>A	Exon 4	L169Q	TM	this study	No	FJ486160
RHcE(R201T)	cat EIV	602G>C	Exon 4	R201T§	IC	1999[15]	Yes	FJ486161
RHce(W217R)		649T>C	Exon 5	W217R	TM	This study	No	FJ486162
RHCE(T241I)		722C>T	Exon 5	T241I	TM	This study	No	FJ486163
RHCe(M267K)		800T>A	Exon 5	M267K§	TM	This study	No	FJ486164
RHCe(L297P)	CE-OL10	890T>C	Exon 6	L297P	TM	2006	Yes	AM295501
RHcE(L303Q)		908T>A	Exon 6	L303Q	TM	This study	No	FJ486165
Gene conversions								
RHCe-D(5)-Ce	RHCeVA	667G>T; 697G>C; 712A>G; 733C>G; 744T>C; 787A>G; 800T>A	Exon 5	V223F; Q233E; M238V; L245V; R263G; M267K	TM	Noizat-Pirenne et al., 2002[11]	Yes NA	

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Allele	Trivial name	Nucleotide change	Genomic region	Amino acid change	Membrane localization*	Original report†	Observed by Doescher et al.[24]	Accession number
RHCe-D(5$_{667-712}$)-Ce	RHCeVF	667G>T; 697G>C; 712A>G	Exon 5	V223F; Q233E; M238V	TM	2005	No	AJ867777

NA = not applicable.

*Amino acid changes located in the intracellular (IC), exofacial (EF), or one of the 12 transmembrane (TM) regions of the RhCE protein.

†Reference for original publication or year of GenBank nucleotide sequence database submission.

TABLE 5

Serologic characteristics of samples with *RHCE* alleles

Trivial name or allele	Rhesus antigen expression*					*RHD* zygosity	Phenotype†	Probable variant haplotype	Number of probands
	D	C	c	E	e				
RHce(5′-UTR-10C>T)	++++	−	++++	++++	++	Dd	DccEe	*dce*	1
RHcE(R10W)	++++	++++	+++/++	+++/+	++++	DD	DCcEe	*DcE*	1
ce(C48)	++++	−	++++	++++	++/+	Dd	DccEe	*dce*	1
	++++	++++	++++	++++	±	DD	DCcEe	*Dce*	1
C^X	++++	++++/+++	++++	++++	++++/+++	DD	DC^xCcEe	DC^xe	1
C^W	++++	++++/+++	++++	++++	++++	Dd	DC^wCcEe	DC^we	1
RHce(R114Q)‡	++++	−	++++	++++	+	Dd	DccEe	*dce*	1
RHCe(L115R)	++++	++/−	++++	−	++++	DD	DCcee	*DCe*	1
RHcE(L115P)	++++	++++	+++/++	+++/−	++++	DD	DCcEe	*DcE*	1
RHCe(S122P)	++++	++++/+++	++++	++++	+++/++	DD	DCcEe	*DCe*	1
	++++	++++/+++	++++	−	++++	Dd	DCcee	*DCe*	
RHcE(I125N)	++++	++++	++	+/−	++++	DD	DCcEe	*DcE*	2
RHCe(IVS3-5G)	++++	+++	++++	++++	+	DD	DCcEe	*DCe*	1
	++++	+++	++++	−	++++	Dd	DCcee	*DCe*	
RHCe(H166L)	++++	++/−	++++	−	++++	DD	DCcee	*DCe*	1
cat El [E^w]	++++	−	++++	+++/+	++++	DD	DccEe	*DcE*	
RHcE(L169Q)	++++	++++	++++	++++/++	++++	DD	DCcEe	*DcE*	1
cat EIV [*RHcE*(R201T)]	++++	−	++++	++++/++	++++	Dd	DccEe	*DcE*	4
	++++	++++	++++	++++/++	++++	DD	DCcEe	*DcE*	5
RHce(W217R)	++++	−	++++	++++	+++/++	Dd	DccEe	*dce*	
RHCE(T241I)	++++	++/+	++++	+++/−	++++	Dd	DCcEe	*DCE*	
RHCe(M267K)	++++	+++/+	++++	−	++++	Dd	DCcee	*DCe*	
RHCe(L297P)	++++	++++/++	++++	++++	++/±	DD	DCcEe	*DCe*	
RHcE(L303Q)	++++	++++	++	++	++++	DD	DCcEe	*DcE*	1
*RHCe*VA	++++	+++	++++	−	++++	Dd	DCcee	*DCe*	1
*RHCe*VF	++++	++++/+++	++++	−	++++	Dd	DCcee	*DCe*	1
Regular RHCE	++++	++++/+++	++++	++++	++++	Dd	DCcEe	*DCE*	3

Trivial name or allele	Rhesus antigen expression* D	C	c	E	e	*RHD* zygosity	Phenotype†	Probable variant haplotype	Number of probands
	++++	+++	++++	++++	−	DD	DCcEE	*DCE*	1
Regular RHCe	++++	++++/++	++++	++++	+++/±	DD	DCcEe	*DCe*	3
	++++	++++/+++	++++	++++	++++/+++	Dd	DCcEe	*DCe*	1
Regular RHce	++++	++++	++++/+++	−	++++	DD	DCcee	*Dce*	1

*Some reactions patterns, indicated by two agglutination strengths like +++/−, varied among different undiluted routine monoclonal and polyclonal antisera in gel matrix antiglobulin technique.

†Antigens that are underlined indicate weak or absent reactions with some undiluted routine monoclonal antisera.

‡*RHce*(R114Q) represents a second sample and independent observation of the same allele reported by Hustinx et al.[32] It carried W16C heterozygously and lacked L245V. Although *RHCE* Exon 3 sequences were deposited only, both samples of the initial documentation of the molecular bases of JAL in 2003 were heterozygous for C48 (GenBank Accession Number AJ548431 Ce JAL+ for *RHCe*(R114W)[31,32] and AJ548432 ce JAL+ for *RHce*(R114Q)[32]); heterozygous W16C was expected for Ce JAL+ but unusual for ce JAL+.

TABLE 6

Multiplex PCR-SSP typing for rare *RHCE* alleles

Multiplex primer mix*	Allele specificity	Primers		PCR product size (bp)
		Forward	Reverse	
A	*RHce*(−10C>T)	F-10T	RIVS1	341
	RHcE(R10W)	F28T		303
B	*RHce*(R114Q)	F341A	R455C	155
	RHcE(L115P)	F344C		152
	RHCe(L115R)	F344G		152
	RHCe(S122P)	F364C		131
	RHcE(I125N)	F374A		122
C	*RHCe*(IVS3-5G)	FIVS3-5G	RIVS4	371
	RHCe(H166L)	F497T		355
	RHcE(M167K)	F500A		352
	RHcE(L169Q)	F506A		343
	RHcE(R201T)	F602C		248
D	*RHce*(W217R)	F649C	RIVS5	291
	RHCE(T241I)	F722T		221
	RHCe(M267K)	F800A		141
E	*RHCe*(L297P)	FIVS5	R890G	254
	RHcE(L303Q)		R908T	274

*Each multiplex primer mix contained primers HBB-F and HBB-R to amplify a 536-bp fragment of the HBB gene as an internal control.