Rh_{mod} phenotype: a parentage problem solved by denaturing gradient gel electrophoresis of genomic DNA

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Initial Rh phenotyping of a man with hemolytic anemia, his wife, and son appeared to exclude paternity. No exclusion was found in other blood groups or in the human leukocyte antigen (HLA) system; excluding Rh, the paternity index was 98.58 percent. Samples from these three family members, and two other family members, were tested with additional Rh antisera. The results indicated that the propositus has an Rh_{mod} phenotype with expression of c, weak e, and very weak D, E, and G antigens. To support this hypothesis, DNA analysis of the *RHD* and *RHCE* genes was performed on the five family members. Polymerase chain reaction (PCR) products from exons 2 and 5 were analyzed by denaturing gradient gel electrophoresis (DGGE). The DNA results corroborated the serologic findings and refuted the exclusion of paternity. ***Immunohematology*** 1996;12:154–158.

The Rh antigens are encoded by at least two genes: *RHD* produces the D antigens and *RHCE* produces the C, c, E, and e antigens. *RHD* and *RHCE* display a high degree of homology, each containing ten coding exons. The D-negative phenotype generally results from homozygosity for a complete deletion of *RHD*.[1] No d antigen exists but d remains useful as a symbol representing the absence of D. Although several nucleotide substitutions within exons 1 and 2 are associated with the C/c polymorphism, the substitution encoding a Ser103Pro amino acid change in a predicted extracellular domain of the RhCcEe protein appears to be the most important change in determining C or c expression.[2] The sequence of exon 2 of the *C* allele of *RHCE* is identical to that of an *RHD* exon 2. The E/e polymorphism is associated with a single nucleotide substitution in exon 5 of *RHCE* and gives rise to a Pro226Ala amino acid change.[2]

Denaturing gradient gel electrophoresis (DGGE) is a molecular technique that can resolve fragments of DNA that differ by as little as a single base substitution. The method is based on the different partial denaturing behavior of the DNA molecules, which is sequence dependent. Alleles are resolved on a polyacrylamide gel in a gradient of chemical (urea and formamide) denaturant.[3] We have used this technique, following polymerase chain reaction (PCR) amplification of exons 2 or 5 of both *RHD* and *RHCE*, to predict D phenotype, c phenotype, and *E/e* genotype. Hence, DGGE enables us to predict the presence or absence of an *RH* gene. It can also reveal abnormal sequences within the exons being studied, signaling the presence of a possible Rh variant.[4]

Red blood cells (RBCs) lacking any serologically detectable Rh antigens were first recognized in the early 1960s. Patients with this phenotype, called Rh_{null}, are very rare and less than 50 examples have been described.[5] The parents of Rh_{null} propositi are often consanguineous,[6] and from family studies it appears that there are at least two different mechanisms that give rise to an Rh_{null} phenotype. The more common mechanism is the regulator type and is probably caused by mutations or transcriptionally silent alleles at the locus encoding Rh-associated glycoprotein.[7] Parents and children of the regulator type of Rh_{null} have normal Rh phenotypes and may be homozygous or heterozygous for *RH*. Regulator Rh_{null} genotypes can be *DD*, *Dd*, or *dd*.[8] In the other type of Rh_{null}, the amorph type, parents and children of Rh_{null} propositi appear, from serologic tests, to be homozygous for antigens at the *RH* loci, but in fact have one Rh_{null} haplotype.[9] The presence of an amorph gene is demonstrated by apparent exclusion of parentage in siblings. Recent work on the molecular aspects of the *RH* loci indicates that amorph Rh_{null} individuals have apparently normal and intact *RHCE*, which may have the C or c sequence.[10]

Chown et al.[11] first used the term Rh_{mod} to describe an RBC phenotype in which expression of all Rh anti-

gens except G was depressed due to an unlinked genetic modifier. The RBCs of several other family members showed weakening of all Rh antigens produced by both haplotypes. Other Rh_{mod} individuals have different degrees of suppression of various Rh antigens; some examples show only slightly weakened Rh antigens,[12] while Rh antigens on other examples are only detectable by adsorption and elution tests.[13] The depression of Rh antigens on RBCs of family members with a single dose of the regulator Rh_{null} or Rh_{mod} gene is variable and may depend on the Rh phenotype.

When informative families are available, Rh_{mod} appears to be due to the effect of a regulator gene unlinked to the *RH* loci. Nash and Shojania[14] have suggested that the same regulator gene, with differing penetrance, is involved in determining the phenotype in both the regulator type of Rh_{null} and in Rh_{mod}. Cherif-Zahar et al.[7] found a missense mutation in the gene encoding the Rh-associated glycoprotein (Rh50) in an Rh_{mod} individual.

Both Rh_{null} and Rh_{mod} are now referred to as Rh deficiency syndrome. The chronic hemolytic anemia and range of symptoms associated with Rh deficiency syndrome are well documented.[14] RBCs from Rh_{mod} donors display the same abnormal morphology (stomatocytic/spherocytic RBCs) seen in RBCs from Rh_{null} patients. Rh deficiency syndrome seems to indicate that the integrity of the RBC is compromised by both reduction or absence of Rh antigens.

A male patient with hemolytic anemia was found to have an unusual Rh phenotype. This led to a family study to determine his Rh phenotype. During this investigation, the presence of the Rh antigen E in his son, which was not expressed by either parent, raised questions of paternity. Further serologic investigation suggested the patient had an Rh_{mod} phenotype. DNA analysis confirmed the presence of an E producing gene in the father and hence eliminated the doubts about paternity.

Materials and Methods

Serology

RBCs of the five family members (the propositus, I-1; his wife, I-2; two children, II-1 and II-2; and his wife's sister, I-3) were tested with a wide range of antisera by standard manual serologic techniques. Control cells were obtained from Oxford Blood Transfusion Centre and from North London Blood Transfusion Centre. The polyclonal antisera used have accumulated from problems investigated by the Medical Research Council (MRC) Blood Group Unit over many years, and monoclonal antibodies used were gifts from colleagues.

Titration results were scored as follows: 4+ = 12, 3+ = 10, 2+ = 8, 1+ = 5, (+) = 3, w = 2, neg = 0. Eluates were prepared using a commercial acid-elution kit (Elukit II, Gamma Biologicals, Houston, TX).

DNA analysis

Genomic DNA was isolated from whole blood using the method of John et al.[15] PCR was performed on genomic DNA under the following conditions: 35 cycles at 94˚C for 0.7 minutes, 56˚C (exon 2) or 53˚C (exon 5) for 0.9 minutes, and 72˚C for 0.7 minutes. Primer sequences are shown in Table 1. GC-clamped PCR products were analyzed by DGGE based on the protocol of Myers et al.,[16] with modifications to allow visualization of the DNA by standard silver staining techniques.[17] The computer programs MELT87 and SQHTX were used to predict optimal conditions for amplification and DGGE focusing of exons 2 and 5 of *RHD* and *RHCE*.[18] A 10% acrylamide gel was used, with a 55 % to 70 % denaturing gradient for exon 2 and a 38 % to 68 % denaturing gradient for exon 5 (where 100% denaturant is 7 M urea and 40% formamide). The DGGE was run for 24 hours at 38V (limiting) at a constant temperature of 61˚C.

Table 1. Primers for polymerase chain reaction (PCR)

	Exon 2 primers	Exon 5 primers
5'	CAT CTC CCC ACC GAG C	TCT TCT TGT GGA TGT TC
3'	*CC AGC CAC CAT CCC AAT ACC	*A/TT GCT CAT CTT CCC/T TTG
Annealing temperature	56˚C	53˚C

*GC-clamp: GCC CCG CCC GCG CCC GCG GCC CGT CCC GCC GCC CCC G

Results

Serology

The results obtained for all family members by direct testing with Rh antisera are summarized in Table 2. These results show the initial Rh phenotypes of I-2 (wife) and II-1 (son) as D+C+E–c–e+ and D+C+E+c+e+, respectively. No C, D, or E antigen was detected on the RBCs of I-1 (Table 3), hence II-1 has an E antigen that was apparently not present in either parent, and so the question of paternity arose. Although present, the c antigen of I-1 was weaker than that of control C+c+ RBCs. Titration of his RBCs with anti-c confirmed this; RBCs of

I-1 gave a titer of 8 and a score of 33 in comparison to a titer of 64 and score of 67 for C+c+ control cells. A very weak e antigen was also detected (Table 3).

Table 2. Results of hemagglutination tests with routine Rh antisera

Red blood cells	anti-						Deduced
	D	C	E	c	e	G	phenotype
I-1 (propositus)	–	–	–	$+^{w}$	w	–	?
I-2 (wife)	+	+	–	–	+	+	DCe/DCe
I-3 (sister of I-2)	+	+	–	–	+	+	DCe/DCe
II-1 (son)	+	+	+	+	+	+	DCe/DcE
II-2 (daughter)	+	+	–	+	+	+	DCe/dce

Table 3. Rh phenotyping of the red blood cells (RBCs) of I-1 (propositus): agglutination tests with untreated- (UT) and papain-treated (Pap) RBCs.

	Anti-D		Anti-C		Anti-c		Anti-E		Anti-e		Anti-G	
	UT	Pap	UT	Pap	UT	Pap	UT	Pap	UT	Pap	UT	Pap
No. of sera	13	22	8	8	1	14	10	23	7	18	0	3
Positive	0	0	0	0	1	10	0	0	1	6	0	0
Weakened	0	0	0	0	0	4	0	0	2	1	0	0
Negative	13	12	8	8	0	0	10	23	4	11	0	3

Table 4. Comparison of reactions of red blood cells (RBCs) of the propositus (I-1) with other Rh_{mod} and Rh_{null} RBCs against immune sera from people with D-- and related phenotypes

Sera		Cells					
Name	Phenotype	I-1	Yam. Rh_{mod}	Hel. Rh_{mod}	Viv. Rh_{mod}	Tanb. Rh_{mod}	Rh_{null}
M.I.	D--	+	nt*	+	nt	nt	–
Bux	D--	–	–	nt	nt	+	–
Mrs.H.	Dc–	+	+	+	+	+	–
Joh.	Dc–	+	nt	+	nt	nt	–
Hall	DC^{w}–	+	+	+	+	+	–
H.D.	D· ·	+	–	+	+	+	–
Nuo.	D^{IVa}(C)–	+	+	+	+	+	–
Sec.	$R^{=N}$	–	–	–	–	–	–

*Not tested

The presence of E antigen on RBCs of II-1 was confirmed by adsorption and elution tests; anti-E was eluted from II-1's cells coated with anti-E. Slight depression of the C and D antigens on cells of II-2 also was noted. No weakness of II-2's c or e antigen was observed.

The cells of I-1 were coated with anti-D, anti-E, anti-G, and anti-e; weak active eluates of all these specificities were obtained. The cells of I-1 did not have the following low-incidence Rh antigens: C^{w}, C^{x}, E^{w}, VS, D^{w}, Go^{a}, Rh32, Rh33, Be^{a}, Evans, Tar, JAL, STEM, FPTT, Ol^{a}, or HOFM. The cells were LW(ab+) and S+ and s+, but had a weak atypical U antigen, since only two of six anti-U reagents gave positive reactions.

The results of testing the RBCs of I-1 with selected immune sera from D-- and related phenotypes is compared to the reactions recorded for an Rh_{null} and some Rh_{mod} samples in Table 4. His RBCs reacted with six of seven of the immune sera that had given positive reactions with other unrelated Rh_{mod} samples.[19]

DNA analysis

DGGE analysis of exon 2 is shown in Figure 1. In exon 2 the sequence of D is identical to that of C, hence cannot be separated; a band at this position is noted as CD. Exon 2 of c melts at a slightly higher denaturant concentration and is seen as a band lower on the gel. The unlabeled bands are heteroduplexes formed during PCR of heterozygotes. The double band heteroduplex in Figure 1 is seen when the CD band and the c band are present. No heteroduplexes are seen when only a CD or a c band is present. I-1, II-1, and II-2 had bands corresponding to CD and c. I-2 had a CD band only (Fig. 1).

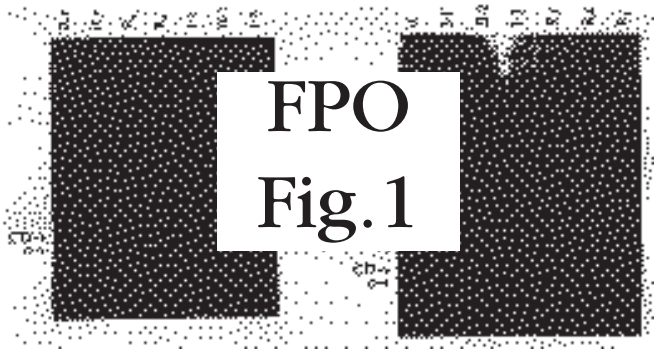

Fig. 1. Denaturing gradient gel electrophoresis (DGGE) analysis of exon 2. In exon 2 the sequence of D is identical to that of C, hence cannot be separated by DGGE; a band at this position is noted as CD. Exon 2 of c melts at a higher denaturant concentration and is seen as a band lower on the gel.

Analysis of exon 5 is shown in Figure 2. Exon 5 of *RHD* and *E* and *e* alleles of *RHCE* can all be separated by DGGE, as their sequences are not identical. E denatures first, followed by e, and then D melting at the highest denaturing concentration. I-1 was shown to have a band corresponding to E, even though the E antigen was not detected by hemagglutination methods. I-1 also had bands indicating e and D. As expected, II-1 has E, e, and D bands. In I-2 and her sister, I-3, a band melting at a higher denaturant concentration than D (and hence focusing lower on the gel) was seen. This is likely to be indicative of a D variant (Fig. 2).

The DGGE results are summarized in Table 5. They show that I-1 has genes capable of encoding D, c, E, and e antigens; no information for presence or absence of C could be deduced.

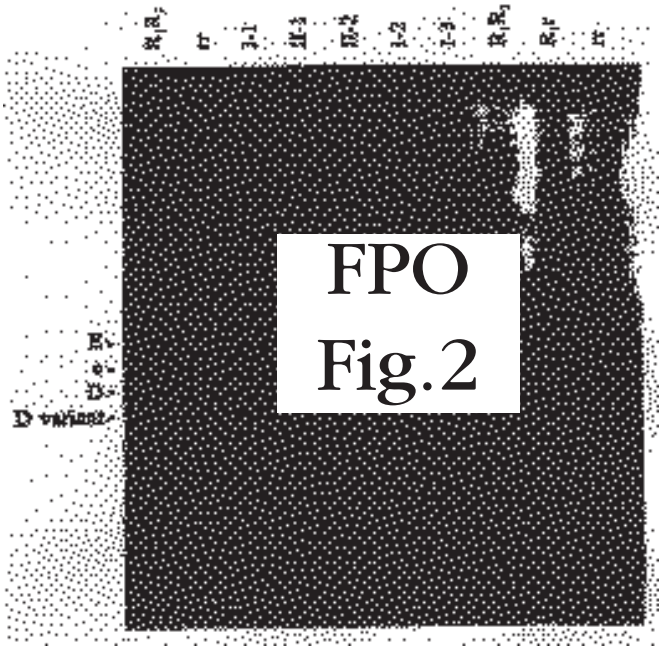

Fig. 2. Denaturing gradient gel electrophoresis (DGGE) analysis of exon 5. Exon 5 of E, e, and D can all be separated because their sequences are not identical. E denatures first, followed by e, and then D melts at the highest denaturant concentration.

Table 5. Denaturing gradient gel electrophoresis results

Samples from	Exon 2	Exon 5
I-1	CD c	E e D
I-2	CD	e D D*
I-3	CD	e D D*
II-1	CD c	E e D
II-2	CD c	e D

*Variant D band

Discussion

Since cloning of the Rh genes and identification of the molecular genetic backgrounds to the Rh polymorphisms have been described, many techniques have been devised for predicting Rh phenotype from genomic DNA.[20–28] One technique involves electrophoresis of double-stranded PCR amplification products through a gradient of chemical denaturant (DGGE).[4] Sequence differences within the amplified DNA, resulting from polymorphism or, in the case of Rh, from coamplification from two homologous genes, result in the DNA focusing at different positions within the gradient of denaturant.

The high level of homology between *RHD* and *RHCE* makes it possible to amplify equivalent segments from both genes in a single reaction. DGGE of exon 5 amplification products separates DNA representing *RHD*, the *E* allele of *RHCE*, and the *e* allele of *RHCE*. Therefore, D phenotype and *E/e* genotype can be determined. DGGE of exon 2 amplification products will not separate DNA representing *RHD* from that representing the *C* allele of *RHCE*, as they have an identical sequence, but it will distinguish DNA representing the *c* allele of *RHCE*. Therefore, by this method, C/c genotype can only be determined on D-negative samples.

DGGE is a somewhat specialized technique and not suitable for routine determination of Rh phenotype from fetal DNA. However, it is useful as a research tool because sequence changes, which may be responsible for variant antigen expression, will generally be identified by changes in the expected position of bands and by the presence of heteroduplex bands. Recognition of such changes led to the identification of the single base change responsible for the e^S variant of e and expression of VS antigen.[4] We have now used Rh-DGGE to investigate a family with unusual Rh groups and a purported exclusion of paternity.

In the initial serologic investigation, the propositus (I-1) had c and e antigens, an apparent dce/dce phenotype; his wife (I-2) was DCe/DCe and his son (II-1) was DCe/DcE. (These phenotypes are based on most probable genotypes deduced from haplotype frequencies.) Consequently, II-1 has an E antigen despite both parents being E-negative. In fact, paternity was not seriously disputed as I-1 had a weak e antigen and hemolytic anemia, suggesting that an Rh_{mod} phenotype might provide the explanation. Exon 2 DGGE of DNA from I-1 demonstrated the expected c band, but also demonstrated a CD band. Exon 5 DGGE demonstrated bands for D, E, and e. As determined from using DGGE, I-1 has the probable genotype *DCe/DcE* if C-positive or *DcE/dce* if C-negative. However, I-1 must have passed c together with e to his *DCe/dce* daughter and c together with E to his son, as their mother is *DCe/DCe*. So I-1 cannot be *DCe/DcE* and must have the probable genotype *DcE/dce*. Both I-1 and II-1 gave the same pattern of bands in DGGE of exons 2 and 5. Very weak D, E, and G antigens on the RBCs of the propositus were detected by adsorption and elution tests. In all other members of the family, DGGE results concurred with the serologic data, except for a shift of the CD band in the wife and her sister. This could represent a variant D antigen, but any serologic effect would be masked by the presence of a normal D antigen.

The term Rh_{mod} represents a heterogeneous collec-

tion of Rh phenotypes in which most or all Rh antigens, produced by both haplotypes, are expressed weakly.[11] One Rh_{mod} individual has been found to have a missense mutation within the gene encoding the Rh-associated glycoprotein.[7] It is likely, therefore, that different missense mutations in this gene, which may cause different degrees of disruption of the membrane complex involving Rh polypeptides and Rh-associated glycoproteins, account for the varying levels of Rh antigen depression in Rh_{mod}. If so, comparison of the mutations involved with the different degrees of antigen depression in a number of Rh_{mod} individuals might provide valuable information on the nature of the interaction between the Rh polypeptides and the Rh-associated glycoproteins.

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