A new genomic polymorphism of Rh-polypeptide genes

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To determine which Rh-polypeptide genes are related to which Rh (rhesus) antigens, genomic DNAs prepared from donors of ten kinds of different Rh phenotypes were analysed by Southern hybridization method using two probes for the 5′ moiety and 3′ moiety of an Rh-polypeptide cDNA. A polymorphism for the Rh blood-group system was observed with *Bam*HI, *Hin*dIII, *Pst*I and *Eco*RI restriction endonucleases between genomic DNAs from the RhD-positive and RhD-negative individuals as has previously been reported. Further study showed an additional genomic polymorphism. The new polymorphism was detected between the RhC/c phenotypes unrelated to the RhD/d phenotypes with *Rsa*I restriction endonuclease using the probe for the 5′ moiety of Rh polypeptide cDNA. A 1.9 kb *Rsa*I restriction fragment was missing in the genomes from individuals with the RhC phenotypes. However, this fragment was present in the genomes of Rhc phenotypes and in its half dose in the genomes of RhCc phenotypes. These results clearly demonstrate a new polymorphism between the RhC/c phenotypes regardless of the RhD-positive and RhD-negative phenotypes, and it is predicted that the Rh polypeptide genes encoding RhD, RhC/c and RhE/e antigens are different.

INTRODUCTION

The Rh (rhesus) blood-group antigens are of major clinical importance because of their involvement in haemolytic disease of the newborn [1], transfusion medicine [2] and autoimmune haemolytic anaemia [3]. The Rh antigens are very complex and include the major RhD antigen and the minor RhC/c and E/e antigens. These antigens are the components of non-glycosylated integral membrane proteins, and carried by 30–32 kDa in SDS/PAGE [4,5]. The Rh polypeptides of human erythrocytes were purified and their N-terminal sequences were determined [6–8]. Two-dimensional iodopeptide map analysis suggested that the RhD, c and E antigens were present on three distinct, but homologous, membrane proteins and all contained identical N-terminal amino acid sequences [8,9]. Moreover, a cDNA encoding one of the Rh polypeptides was isolated, and its complete primary structure was determined [10,11]. We also isolated two Rh-related cDNA clones, designated the RhPI and RhPII [12]. The RhPI cDNA showed a single nucleotide substitution, but no amino acid substitution, compared with the previously published cDNA [10,11]. The RhPII cDNA, on the other hand, was composed of the same open reading frame as the RhPI cDNA, and had 41 nucleotide substitutions resulting in 31 amino acid substitutions. It has not yet been determined which of these Rh-related cDNAs encode which Rh antigens.

A group working in Paris [13] reported that the genome of RhD-positive individuals was composed of two strong related genes, presumably RhD and RhCcEe, while one of these two genes was missing in the RhD-negative individuals as a result of the only polymorphism associated with the RhD-positive and RhD-negative phenotypes found on Southern-blot analysis. However, we found a polymorphism associated with the RhC/c phenotypes unrelated to the RhD-positive and RhD-negative phenotypes. Here we show a new polymorphism which may support the three-gene theory rather than the two-gene theory.

MATERIALS AND METHODS

DNA probes

Details of the preparation of a cDNA for Rh polypeptide (RhPI cDNA) have been previously published [12]. The Rh1(3) probe (nucleotides −30 to +674) coding the 5′ moiety of RhPI cDNA and Rh2(12) probe (nucleotides +606 to +1283) coding the 3′ moiety of RhPI cDNA were amplified by PCR as described previously [12]. These two PCR products were subcloned, and the Rh1(3) and Rh2(12) cDNAs were isolated [12]. Nucleotide +1 corresponds to the initiation methionine codon.

Genomic DNA analysis

Genomic DNA was extracted from peripheral-blood leucocytes obtained from unrelated healthy volunteers with ten phenotypes (CDe, CDEe, CcDEe, CcDe, cDE, cDe, Ccde, cde, cdEe, and cdE) in the Rh blood-group system. DNAs obtained were restricted with the endonucleases *Bam*HI, *Hin*dIII, *Pst*I, *Eco*RI (Takara Shuzo Co. Ltd., Shiga, Japan), and *Rsa*I (GIBCO BRL, Gaithersburg, MD, U.S.A.), and the restriction fragments were separated by electrophoresis in agarose gels. The fragments were transferred to nylon filters (Hybond N; Amersham International) by the method of Southern [14]. The Rh1(3) and Rh2(12) probes were each labelled using a multiprime DNA labelling kit (Amersham International). Filters were hybridized overnight at 65 °C in 5 × SSPE (1 × SSPE is 0.15 M NaCl/10 mM NaH_2PO_4/1 mM EDTA), 0.5% SDS, 5 × Denhardt's. The filters were washed in 2 × SSPE, 1 × SSPE and 0.1 × SSPE containing 0.1% SDS at room temperature in turn. Final washes were performed in 0.1 × SSPE containing 0.1% SDS at 65 °C for 30 min.

RESULTS

In order to investigate the polymorphism of human Rh locus, the genomic DNAs extracted from a RhD-positive donor (CcDEe phenotype) and a RhD-negative donor (cde phenotype) were

Abbreviation used: RFLP, restriction-fragment-length polymorphism.
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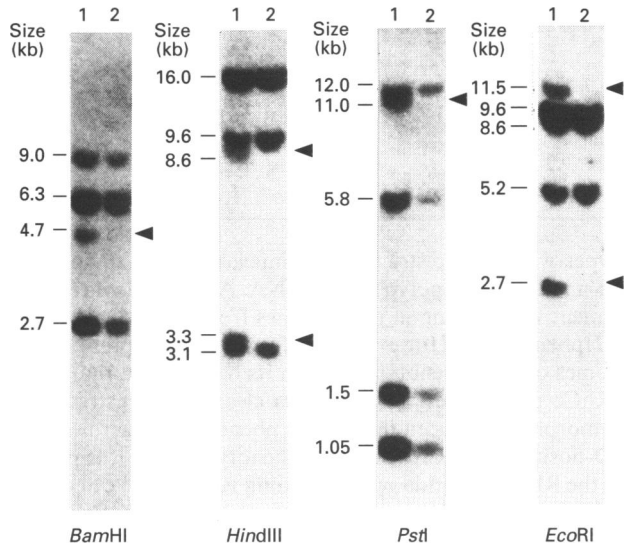

Figure 1 Southern-blot analysis of genomic DNA for an RhD-positive and an RhD-negative donor with the Rh1(3) probe

DNAs from an RhD-positive donor with the CcDEe phenotype (lane 1) and an RhD-negative donor with the cde phenotype (lane 2) were each digested with *Bam*HI, *Hin*dIII, *Pst*I and *Eco*RI restriction endonucleases and hybridized with the Rh1(3) probe. Arrowheads indicate some of the hybridization bands that are lacking in the RhD-negative DNA. The fragment size is shown in kb.

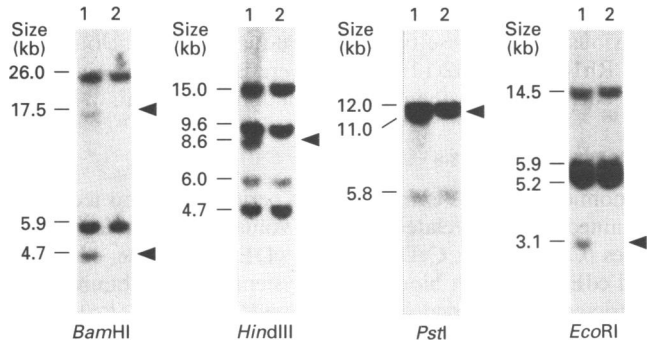

Figure 2 Southern-blot analysis of genomic DNA for an RhD-positive and an RhD-negative donor with the Rh2(12) probe

DNAs from an RhD-positive donor with the CcDEe phenotype (lane 1) and an RhD-negative donor with the cde phenotype (lane 2) were each digested as described in Figure 1 and hybridized with the Rh2(12) probe. Arrowheads indicate some of the hybridization bands that are lacking in the RhD-negative DNA.

digested with *Bam*HI, *Hin*dIII, *Pst*I and *Eco*RI and hybridized with the radiolabelled Rh1(3) probe (nucleotides −30 to +674) coding the 5′ moiety of RhPI cDNA. Figure 1 shows the restriction-fragment patterns produced by four restriction endonucleases. The lack of several fragments in a RhD-negative donor compared with a RhD-positive donor was observed using the Rh1(3) probe. The RhD-negative DNA showed the lack of a 4.7 kb fragment with *Bam*HI, 8.6 kb and 3.3 kb fragments with *Hin*dIII, a 11.0 kb fragment with *Pst*I, and 11.5 kb and 2.7 kb fragments with *Eco*RI. This result indicates that the polymorphism by Rh1(3) cDNA may be associated with the RhD-positive and RhD-negative phenotypes.

Next we attempted the detection of polymorphism using Rh2(12) probe (nucleotides +606 to +1283) coding the 3′ moiety of RhPI cDNA (Figure 2). The Rh2(12) probe, as the Rh1(3) probe, showed the lack of several fragments in a RhD-negative donor. The RhD-negative DNA showed the lack of 17.5 kb and 4.7 kb fragments with *Bam*HI, a 8.6 kb fragment with *Hin*dIII, a 11.0 kb fragment with *Pst*I, and a 3.1 kb fragments with *Eco*RI. As a result of restriction-fragment-length-polymorphism (RFLP) analyses with the Rh1(3) and Rh2(12) probes, it is presumed that the Rh polypeptide may be associated with the RhD/d antigen. Also, it would appear that the RFLP analysis by the Rh polypeptide cDNA was useful in discriminating the RhD-positive and RhD-negative phenotypes. The polymorphic pattern given by Rh polypeptide cDNA as a probe only exhibited the RhD-positive and RhD-negative phenotypes, regardless of whether they expressed the C/c and E/e antigens. Furthermore, we examined the polymorphism for the genomic DNAs of eight other kinds of donors with the phenotypes (CDe, CDEe, CcDe, cDE, cDe, Ccde, cdEe and cdE) using the above four endonucleases. The polymorphisms detected in these DNAs were all classified into the RhD-positive and RhD-negative phenotypes (results not shown). However, there was very strong possibility of the failure to detect any other polymorphism than these ones revealed with *Bam*HI, *Hin*dIII, *Pst*I and *Eco*RI.

Next we attempt to detect an RFLP related to the Rh antigens using restriction endonucleases that recognize four base-pairs. Ten kinds of DNA samples with different Rh phenotypes (CDe, CDEe, CcDEe, CcDe, cDE, cDe, Ccde, cde, cdEe and cdE) were digested with *Rsa*I and compared by Southern-blot analysis using the Rh1(3) probe coding the 5′ moiety of RhPI cDNA (Figure 3). Plural donors were examined for the Rh phenotypes of CDe, CDEe, CcDEe, CcDe, Ccde, and cde. Southern blot analysis showed the lack of a 2.35 kb fragment in the RhD-negative donors compared with the RhD-positive donors. In addition, another polymorphism in Rh polypeptide was observed by *Rsa*I. Interestingly, a 1.9 kb hybridization band was undetectable in CDe and CDEe donors and was missing in these genomes. This restriction fragment was present in double dose in the Rhc (cDE, cDe, cde, cdEe and cdE) donors, in single dose in the RhCc (CcDEe, CcDe, and Ccde) donors, and completely lacking in the RhC (CDe and CDEe) donors. Also, Southern-blot analysis using the Rh2(12) probe coding the 3′ moiety of RhPI cDNA showed the only polymorphism in the RhD-positive and RhD-negative donors (results not shown). These results clearly indicate that a new polymorphism related to the RhC/c phenotypes was observed using *Rsa*I, regardless of the RhD-positive and RhD-negative donors, and it is very useful in discriminating between the RhC/c phenotypes.

DISCUSSION

By Southern hybridization using Rh polypeptide cDNA as a probe, we have demonstrated a new genomic polymorphism for the RhC/c phenotypes in addition to the genomic polymorphism corresponding to the RhD-positive and RhD-negative phenotypes. In Southern-blot analysis using *Bam*HI, *Hin*dIII, *Pst*I and *Eco*RI, all RhD-positive genomes exhibited the same RFLP pattern regardless of whether they expressed the C or c and E or e antigens. All RhD-negative genomes, on the other hand, lacked some of the hybridization bands detected in the RhD-positive ones. This result suggests that the RhD gene differs from the genes encoding the Rh antigens of C/c and E/e as previously reported [13]. It has been debated for years how many structural

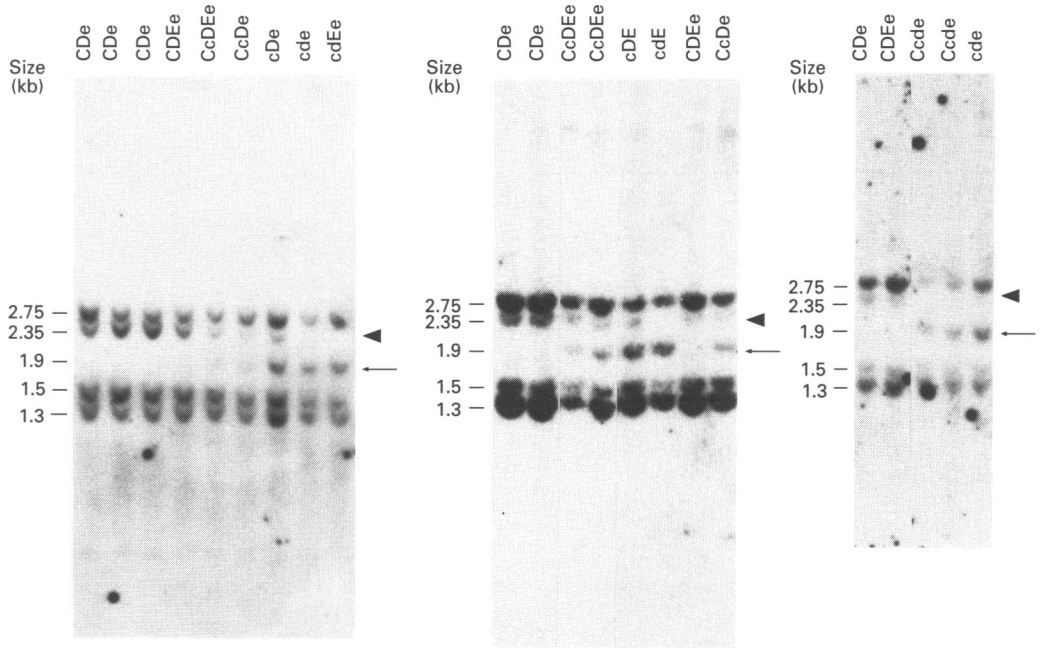

Figure 3 Southern-blot analysis of genomic DNA from ten kinds of Rh phenotypes digested by *Rsa*I

The digested DNAs by *Rsa*I were hybridized with the Rh1(3) probe. The arrowhead indicates an hybridization band that is lacking in the RhD-negative DNA. An arrow indicates an hybridization band that is lacking in DNA of the RhC phenotypes. The kinds of Rh phenotypes and number of donors examined are as follows: CDe (6), CDEe (2), CcDEe (3), CcDe (2), cDE (1), cDe (1), Ccde (2), cde (2), cdEe (1) and cdE (1).

genes are present within the Rh locus. Fisher suggested that the inheritance of Rh antigens was determined by three genes which were very closely linked. Recently, Colin et al. have argued a model of two genes at the Rh locus [13]. We previously showed that the Rh polypeptide cDNA cloned by two groups in Paris [10] and Bristol [11] was detected not only in erythroid cells with the RhD-positive phenotype, but also in ones with the RhD-negative phenotype [15]. This demonstrated that the Rh polypeptide cDNA encoded the RhC/c or E/e antigen, but not the RhD antigen. The Paris group conjectured that its Rh polypeptide cDNA encoded the E or e antigen from immunological studies performed on different Rh phenotype erythrocytes with polyclonal antibodies against synthetic peptides specific for the Rh polypeptide [16].

Recently, Le Van Kim et al. [17] reported that the primary structure of a newly found Rh polypeptide showed 36 amino acid substitutions as compared with the previously published Rh polypeptide and that the isolated cDNA clone encoded the D polypeptide [17]. It is thought that the RhPII cDNA clone isolated by us is much the same in comparison [12]. In a preliminary experiment Southern-blot analysis using the RhPII cDNA as a probe showed that its polymorphic pattern was classified into the RhD-positive and RhD-negative phenotypes in the case of the digestion with *Bam*HI, *Hin*dIII, *Pst*I, and *Eco*RI, whereas it was classified into two types of the RhC/c and RhD/d phenotypes in the case of the digestion with *Rsa*I (F. Umenishi, E. Kajii and S. Ikemoto, unpublished work). This demonstrates that the patterns of Southern hybridization has come to the same conclusion because the RhPII cDNA has a very similar character in nucleotide sequence as the RhPI cDNA. Also, we reported that the RhPII gene could be amplified from the RhD-negative phenotypes by the PCR method (the following paper [18]). This data strongly suggests that the RhPII gene encodes not the RhD antigen, but the RhC/c or RhE/e antigen. Southern hybridization patterns by the RhPI and RhPII cDNA were the same because the coding sequence of both the cDNAs exhibited 3.3% divergence. Although both the cDNAs did not encode the RhD antigen, Southern-blot analyses showed two kinds of polymorphisms associated with the RhC/c and RhD/d phenotypes. This experimental result is attributable to the considerable nucleotide sequence similarity of Rh genes which encode the closely related RhD, C/c and E/e antigens.

All these results clearly demonstrate that the Rh genes encoding the RhD, RhC/c and RhE/e antigens are different, and the genetic and biochemical data support the three-gene theory.

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