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A Non-Sense Mutation at Arg^{95} Is Predominant in Complement 9 Deficiency in Japanese[1]

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Deficiency of the ninth component of complement (C9D) is one of the most common genetic abnormalities in Japan, with an incidence of one homozygote in 1000. Although C9D individuals are usually healthy, it has been shown that they have an significantly increased risk of developing meningococcal meningitis. In the present study we report the molecular bases for C9D in 10 unrelated Japanese subjects. As a screening step for mutations, exons 2 to 11 of the C9 gene were analyzed using exon-specific PCR/single-strand conformation polymorphism analysis, which demonstrated aberrantly migrating DNA bands in exon 4 in all the C9D subjects. Subsequent direct sequencing of exon 4 of the C9D subjects revealed that eight of the 10 C9D subjects were homozygous for a C to T transition at nucleotide 343, the first nucleotide of the codon CGA for Arg^{95}, leading to a TGA stop codon (R95X). R95X is a novel mutation different from those recently identified in a Swiss family with C9D. Cases 6 and 7 were heterozygous for the R95X mutation. Family study in case 10 confirmed the genetic nature of the defect. In case 6, the second mutation for C9D of the C9 gene was identified to be the substitution of Cys to Tyr at amino acid residue 507 (C507Y), while the genetic defect(s) in the other allele in case 7 remains unknown. Our results indicate that a novel mutation, R95X, is present in most cases of C9D in Japan. ***The Journal of Immunology*****, 1998, 160: 1509–1513.**

The ninth component of complement (C9) is one of five constituents of the membrane attack complex (MAC)[3] that is assembled on the membrane of target cells and gradually inserted into the lipid bilayer, leading to eventual lysis of susceptible cells. The formation of MAC is the outcome of sequential addition of one molecule each of C5b, C6, C7, and C8, followed by subsequent oligomerization of six to nine C9 molecules (1, 2). C9 is a single-chain polypeptide of 538 amino acid residues and is structurally related to C6, C7, C8α, and C8β (3, 4). All these proteins have a mosaic structure consisting of protein modules apparently derived from diverse protein families. The genes for C9 as well as those of C6 and C7 are located on chromosome 5p13, while the genes for C8α and C8β are on the short arm of chromosome 1 (5–7). The gene for C9 is approximately 100 kb in length and is composed of 11 exons (4). The C9 structure, especially the exon-intron boundaries, have recently been revised (8).

Inherited C9 deficiency (C9D) is a common genetic abnormality in Japan, with an incidence of about one homozygote in 1000, while only a few C9D cases have been reported in Caucasians (9–15). Although C9D individuals were originally been reported to be healthy, it was subsequently shown that they carried a much higher risk for developing meningococcal meningitis than normal controls (16). However, deficiencies of the other components of MAC; C5, C6, C7, and C8, are much more frequently associated with neisserial infections than C9 deficiency (17, 18).

Molecular defects leading to inherited deficiencies of most of the components of the MAC such as C5, C6, C7, and C8β have been reported (19–24). Recently, the genetic defects leading to C9D in a Swiss family have also been described (8). Considering the extreme ethnic predominance of C9D in Japan, it is important to study Japanese cases to understand the molecular mechanism for C9D in detail. In the present study we investigated 10 unrelated Japanese cases of C9D for mutations of the C9 gene using exon-specific PCR/single-strand conformation polymorphism (SSCP) analysis (25, 26) as a first step of screening, followed by sequencing exons of interest. We have identified a novel non-sense mutation at Arg^{95} that is predominant (18 of 20 null alleles) in our C9D subjects. A missense mutation, Cys^{507} to Tyr^{507}, was identified that may explain another molecular defect in one of the remaining two null alleles, while the mutation(s) of the other allele was not determined. As in the case of C2 deficiency (C2D), which is common among Caucasians (27), there seems to be a founder effect for individuals with C9D among Japanese.

Materials and Methods

C9D subjects

Ten unrelated Japanese individuals from two different geographic areas (seven from Osaka and three from Fukuoka) were included in this study. The cases from Osaka were identified during a large-scale screening for inherited complement deficiencies among healthy blood donors (11). One of three subjects from Fukuoka was identified during screening for C9D reported previously (13). One of the other two was a 32-yr-old female

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[3] Abbreviations used in this paper: MAC, membrane attack complex; C9D, complement 9 deficiency; SSCP, single-strand conformation polymorphism; C2D, complement 2 deficiency SRID, single radial immunodiffusion.

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Table I. *PCR fragment lengths and restriction enzymes used for SSCP analysis*

Exons	PCR Fragments (bp)	Restriction Enzymes	Restricted Fragments (bp)
2	257	—	—
3	304	—	—
4	362	*Pst*I	198, 154
5	328	*Hin*fI	103, 225
6	431	*Bbv*I	232, 199
7	474	*Hin*dIII	221, 120, 133
8	328	*Fok*I	71, 131, 126
9	282	—	—
10	351	*Hin*fI	202, 149
11[a]	969	*Rsa*I	138, 344, 239, 216, 32

[a] Primers 11Ado and 11Bup were used for the amplification of exon 11.

presenting with arthralgia, positive anti-nuclear Ab, and Hashimoto's thyroiditis. The other case was a 25-yr-old female who visited our affiliated hospital for a common cold. None of the 10 C9D subjects had a history of meningitis. The total complement activity (CH50) level of our subjects was approximately 30% that of normal human serum. The serum concentration of C9 in all these Fukuoka subjects was determined by single radial immunodiffusion (SRID) as previously described (11) and was below the level of detection in all cases. The serum C9 concentration of the seven Osaka C9D subjects was below detectable levels using the hemolytic assay for C9 activity or the sensitive sandwich ELISA as described previously (11).

PCR/SSCP analysis

Primer sequences for exon-specific PCR for exons 2 to 11 of the C9 gene were prepared as previously described (8). These primer pairs were designed to include at least 20 nucleotides of flanking intronic sequences. Exon 1, which encodes the leader peptide and the amino-terminal five residues of the mature polypeptide, was not studied, because its flanking sequences have not been determined. Genomic DNA was prepared from peripheral blood of C9D individuals and healthy controls as previously described (28). PCR was performed using 50 ng of genomic DNA as template, 0.2 μM of each primer, 25 μM of dNTP, 2 μCi of [α-^{32}P]dCTP (Amersham International, Aylesbury, U.K.), 0.125 U of Taq polymerase, and the standard buffer provided by the supplier (Perkin-Elmer, Norwalk, CT) in a total reaction mixture of 5 μl (21, 22, 29). Reactions were conducted for 30 cycles, consisting of 1 min at 95°C and 2 min at 60°C for exons 3, 4, 6, 7, and 8 and for 30 cycles consisting of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C for exons 2, 5, 9, 10, and 11, using a thermal cycler PJ2000 (Perkin-Elmer). The PCR products were diluted 10 times with formamide dyes (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) and heat denatured at 80°C for 5 min. Electrophoresis was conducted at 13 V/cm on a 5% polyacrylamide gel containing 5% glycerol at 25°C or without glycerol at 4°C using 45 mM Tris-borate and 1 mM EDTA buffer, pH 8.3. DNA fragments were visualized by exposing gels to Kodak XAR film (Eastman Kodak, Rochester, NY). PCR products of exons 4, 5, 6, 7, 8, 10, and 11 that were longer than 300 bp were digested with appropriate restriction enzymes before SSCP analysis (Table I). Two to five DNA fragments were generated from each of those PCR products.

Nucleotide sequencing

The DNA fragments of interest were amplified directly from the genomic DNA by PCR, electrophoresed in 1.5% agarose gels, excised from the gel, and purified on SUPREC-01 columns (Takara Shuzo Co. Ltd., Otsu, Japan). They were reamplified for 20 cycles, consisting of 1 min at 95°C and 2 min at 60°C in a total reaction volume of 25 μl, purified on Microcon-100 (Amicon, Beverly, MA), and directly sequenced using the Amplicycle sequencing kit (Perkin-Elmer). The primers for sequencing were radiolabeled using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-^{32}P]ATP at 37°C for 20 min. PCR was conducted for 25 cycles, consisting of 1 min at 95°C, 1 min at 68°C, and 1 min at 72°C.

Results

Detection of C9 gene mutations by PCR/SSCP analysis

Aberrant bands were detected in exon 4 of all C9D subjects as shown in Figure 1. Eight C9D individuals (*lanes 1*, *2*, *3*, *4*, *5*, *8*, *9*, and *10*) displayed two bands migrating differently from those of C9-sufficient controls (C1 and C2). In cases 6 and 7 (*lanes 6* and *7*), four bands were identified, two corresponding to those of controls and the other two to those of the C9D subjects. These results suggested that cases 1 to 5 and 8 to 10 were homozygous for a mutation in exon 4, while cases 6 and 7 were heterozygous for the same mutation.

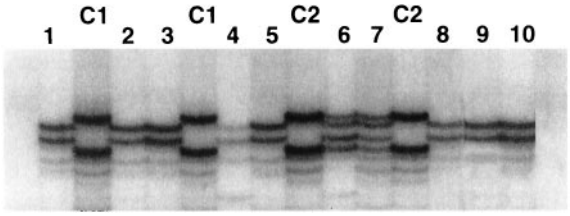

FIGURE 1. PCR/SSCP analysis of 10 unrelated C9D individuals. Exon 4-specific PCR products from genomic DNA were digested with *Pst*I and subjected to 5% polyacrylamide gel without glycerol at 4°C. DNA bands corresponding to the larger fragments (198 bp) are shown. *Lanes 1* to *10* are C9D individuals, and C1 and C2 are C9-sufficient controls.

Determination of the mutation in exon 4

The amplified PCR products of exon 4 from the 10 C9D subjects were directly sequenced in their entirety. For cases 1 through 5 and 8 through 10, the nucleotide sequence was identical with that reported previously (3, 4), except that the reported C at position 343 (Fig. 2*a*, *III*) was mutated to T (Fig. 2*a*, *I*), indicating a homozygous mutation. Cases 6 and 7 were heterozygous for the mutation,

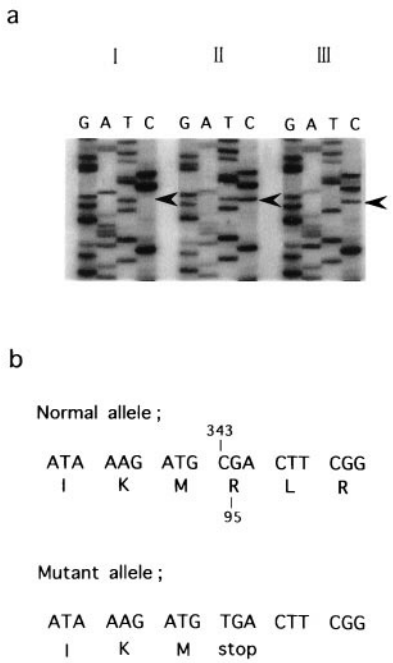

FIGURE 2. Definition of exon 4 mutation. *a*, The genomic DNA from the C9D individuals as well as C9-sufficient controls was amplified by PCR and directly sequenced in its entirety. The results for cases 1 to 5 and those for cases 8 to 10 were identical (homozygous C to T transition at nucleotide 343) as shown in *I*, and those for cases 6 and 7 were identical (heterozygous C to T transition) as shown in *II*. *III* shows the results for C9-sufficient individuals. *b*, Nucleotide sequences and deduced amino acid sequence (one-letter code) around nucleotide 343. The C to T transition at 343 generates a stop codon at Arg[95], which results in the truncation of the C9 protein.

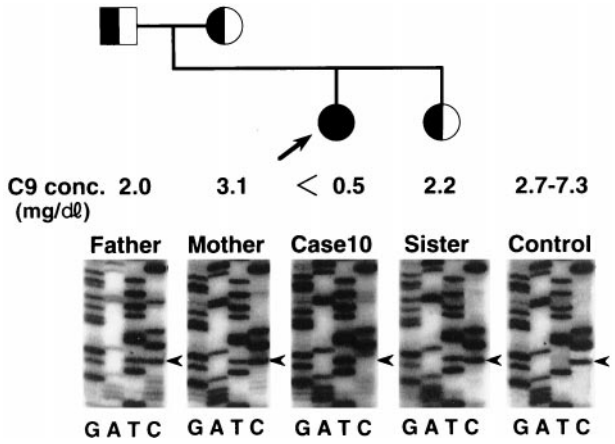

FIGURE 3. Pedigree of case 10 and demonstration of homozygosity for the R95X mutation. Squares indicate male family members, and circles indicate females. C9 concentrations estimated by SRID assay are shown below. Arrows indicate the position at nucleotide 343.

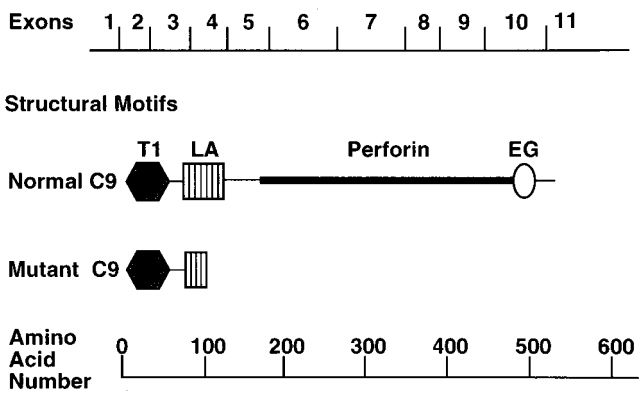

FIGURE 4. Schematic diagram of the molecular structure of normal C9 (adapted from Ref. 31) and truncated C9 in the C9D individuals. Modules are designed, according to the recommendations of a recent workshop (32), as follows: T1, thrombospondin, type 1; LA, low density lipoprotein receptor, type A; EG, epidermal growth factor-like.

as both C and T were identified at position 343 (Fig. 2*a*, *II*). Nucleotide 343 is the first nucleotide of the codon CGA for Arg^{95}. The C to T transition would cause the generation of a stop codon and truncation of the C9 polypeptide (Fig. 2*b*). This mutation at nucleotide 343 was observed in 18 of the 20 alleles of the C9D individuals. It is therefore likely that we have identified a common mutation that causes the majority of C9D in Japan.

Family study of case 10

The serum C9 concentrations of both parents and a sister of case 10 were just below the normal range, whereas no C9 was detected in the serum of the proband (Fig. 3). Sequencing of exon 4 of the C9 gene of the family members revealed that in both parents as well as in the sister, both the native nucleotide C and the mutated T were present at position 343. This result confirms that in case 10 the mutation at nucleotide 343 is homozygous and is the cause of the complete C9D.

Identification of the second mutation in case 6

Aberrant bands were also detected in exon 10 of case 6. Direct sequencing of the amplified PCR product of exon 10 of case 6 revealed that the nucleotide sequence was identical with that previously described, except the reported G and the mutated A were both identified at nucleotide 1580, suggesting that the mutation was heterozygous (data not shown). Nucleotide 1580 is the second nucleotide of the codon TGT for Cys^{507}. The G to A transition resulted in the substitution of Cys by Tyr and in the disruption of a disulfide bridge between Cys^{492} and Cys^{507} (30).

Discussion

We studied the molecular basis for C9D in 10 unrelated Japanese cases and identified a novel mutation as the most common cause. The mutation was caused by the C to T transition at nucleotide 343, the first nucleotide of codon CGA for Arg^{95}. The mutation generated a stop codon (R95X). Of 20 unrelated null alleles studied, 18 (90%) carried this mutation. The putative polypeptide chain encoded by this gene would have 94 amino acid residues, and even if translated it would be nonfunctional because it lacks the entire domain essential for the insertion of C9 in the membrane (1, 2) (Fig. 4). Non-sense mutations in human disease genes frequently cause a severe reduction in mRNA levels, and even when normally transcribed, truncated proteins are quickly degraded (33–36). As reported previously (11), C9 was not detected in sera from the individuals homozygous for the non-sense mutation, R95X, by hemolytic assay, SRID assay, or sandwich ELISA. Further study is necessary to clarify which mechanism is responsible for the absence of C9 in our C9D individuals.

C9D is a common genetic abnormality in Japan, with an incidence of one homozygote in 1000, which was estimated from large-scale screenings for C9D of healthy blood donors or hospitalized patients (11–13). No distinct difference in the incidence of C9D has been reported throughout eight areas of Japan. In our study, seven were from the Osaka area, and three were from the Fukuoka area. The two areas are separated with each other by >600 km. As 12 of 14 unrelated null alleles in the Osaka area and all six unrelated null alleles in the Fukuoka area carried the same non-sense mutation (R95X), it is obviously predominant in Japanese C9D subjects. In contrast to the common occurrence in Japan, only a few cases with C9D have been identified in Europe and the United States. Recently, the molecular bases for C9D of a Swiss family have been reported (8). The proband was shown to be a compound heterozygote for the non-sense mutation at Cys^{33} and Arg^{133}. The former mutation was caused by a C to A transversion at nucleotide 166, the third nucleotide of the codon TGC for Cys^{33}, and the latter by C to T transition at nucleotide 464, the first nucleotide of the codon CGA for Arg^{133}. Considering the extreme ethnic predominance of C9D in the Japanese and the absence of this common mutation in the Swiss family, R95X has probably been caused by a founder effect, an inheritance of an ancient mutation by successive generations. The alternative possibility, that the C at nucleotide 343 is a mutational hot spot in the C9 gene, seems unlikely. To investigate the presence of the C to T transition at nucleotide 343 in the C9 gene in ethnic groups different from the Japanese, a large-scale population study is currently underway. The extreme predominance of C9D in Japan might be caused by a selective advantage. Consistent with this speculation, the mortality of meningococcal disease in individuals with deficiencies of MAC proteins is much lower than that in the general population (37). In addition, less endotoxin release was demonstrated in C6-deficient sera compared with that in complement-sufficient sera (38). It is possible that the severity in inflammatory diseases is attenuated in the absence of MAC proteins. Another possible explanation for the selective advantage is the association of complement system and reproduction. Several regulators of complement system, such as membrane cofactor protein, decay-accelerating factor, and CD59,

are strongly expressed by trophoblast and amniotic epithelium (39). Considering that membrane cofactor protein and decay-accelerating factor inactivate C3 and C5 convertases, and CD59 prevents the addition of C9 to the C5b-8 complex, regulation of complement activation might be important for the development of normal pregnancy, as allogeneic interaction is inevitable. The potential activation of the complement system in reproduction might be reduced in the absence of C9, which would be advantageous for fertility. To address the issue, further study of the role of the complement system in reproduction is needed.

Ethnic predominance, such as that of C9D, has been reported in the other inherited complement deficiencies as well. C2D is the most common complement deficiency in Caucasians, with the incidence of one homozygote in 10,000 (27), while no C2D individuals have been reported in Japan. More than 90% of null alleles of C2D carry a common mutation, a 28-bp deletion at the exon 6-intron 5 boundary of the C2 gene, which was assumed to be a founder mutation in Caucasians (40). C8β deficiency is also primarily found in Caucasians, and the founder effect caused by a non-sense mutation at exon 9 of the C8B gene is the major cause of the defect (23, 24).

CpG dinucleotides are hot spots for mutation in vertebrate genomes. When single base pair substitutions that cause human genetic diseases were collected, 32% of point mutations are caused by CG to TG or CG to CA transitions consistent with a chemical model of mutation by methylation-mediated deamination (41, 42). A transition of C to T in the CGA codon results in a TGA stop codon (non-sense mutation). In patients with hemophilia A, which is one of the best characterized human inherited diseases for which the molecular basis is known, non-sense mutations occur in every CGA codon in the factor VIII gene (43). In the C9 gene, there are altogether five CGA codons; Arg^{65}, Arg^{95}, Arg^{133}, Arg^{173}, and Arg^{425}. The fact that only one of these Args, Arg^{95}, was affected in our cases might reflect the difference in the methylation in these CpGs, but suggests the view of a founder effect.

We also identified another putative molecular defect in the other allele of case 6 that was heterozygous for the R95X mutation. The G to A transition at nucleotide 1580 resulted in the substitution of Cys by Tyr at amino acid residue 507. All the 24 half-cystines of C9 are engaged in the intramolecular disulfide bond formation, and the bridge between Cys^{492} and Cys^{507} has been assigned (30). The possibility that the amino acid substitution from Cys to Tyr is a rare polymorphism is unlikely, because the mutated C9 of case 6 would result in the disruption of proper folding of the C-terminal epidermal growth factor-like domain and the failure of protein secretion. There are several explanations for our failure to find the second mutation in case 7. 1) The defect might reside in the exon 1 that we were not able to investigate because its flanking sequence has not been determined. 2) It is possible that a mutation was not detected by our PCR/SSCP condition. The sensitivity of the PCR/SSCP analysis in our experiment is about 90% because the DNA fragments used for the SSCP analysis were $<$300 bp long (Table I) (44). 3) A mutation in an intron away from a exon-intron boundary may result in a defect in splicing. Such a case has been reported in the neurofibromatosis type 1 gene (45). A de novo insertion of Alu sequence, 44 bp upstream of exon 6, resulted in the skipping of exon 6, a shift of the reading frame, and the truncation of neurofibromatosis type 1 protein. 4) There might be a mutation in the promoter region of the C9 gene that affects transcription of the C9 gene. Although further study is needed to evaluate in detail the extent of the genetic bases predisposing individuals to C9D, our results have presented the first evidence that most of the C9D in ethnic Japanese might be caused by a founder effect arising from the R95X mutation.

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