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Unique Phenotypes of C1s Deficiency and Abnormality Caused by Two Compound Heterozygosities in a Japanese Family

Katsuaki Abe,* Yuichi Endo,2† Naomi Nakazawa,† Kazuko Kanno,† Mitsuo Okubo,‡ Tadashi Hoshino,* and Teizo Fujita†

A deficiency in the early components of complement is associated with an increased susceptibility to pyrogenic infections and multiple autoimmune diseases. We previously reported a Japanese case of selective C1s deficiency resulting from a compound heterozygosity for a 4-bp deletion in exon X and a nonsense mutation Glu597X in exon XII of the C1s gene. In this previous case, the patient suffered from unique symptoms including virus-associated hemophagocytic syndrome and died after a long period of loss of consciousness. In the present study, we report another patient from the same family, with C1s abnormality caused by a distinct compound-heterozygous genotype and who had a novel missense mutation Gly630Glu transmitted from the mother’s side and a previously identified nonsense mutation Glu597X from the father’s side. Thus three distinct mutations of the C1s gene were clustered and resulted in two distinct genotypes for C1s deficiency and C1s abnormality within this one family. The present patient showed symptoms that were similar in part to our previous patient, which were different from those of the cases reported in other families. The biochemical properties of C1s in the patient’s serum and the recombinant form were closely related to the undetectable or very low activity of complement activation. These results suggested that the uniqueness and severity of the symptoms observed here in the two patients might be under the control of a common C1s allele and distinct counterparts, respectively. The Journal of Immunology, 2009, 182: 1681–1688.

The serum serine protease C1s is a subcomponent of the complement C1 complex, which combines with two other subcomponents C1q and C1r (1, 2). Upon binding immune complexes by C1q, the proenzyme form of C1s is converted by C1r to the active form by limited proteolysis into the H chain and L chain. In turn, the active C1s triggers subsequent steps of the classic pathway of complement activation by formation of C3 convertase, C4b2a.

Several cases of human complement C1 deficiency have been reported (3–16), in which the deficiency was caused by the absence of the subcomponents C1q (6, 9–13), C1r (3, 4, 8), C1s (7, 14–17), or both C1r and C1s (5). The molecular basis for hereditary C1q deficiency is well defined, where homozygosity for nonsense mutations in the C1q A-chain and B-chain genes (6, 10, 12, 13) and deletion, nonsense, and missense mutations in the C1q C-chain gene (10, 11) have been demonstrated. There have been a few reports on the molecular basis for C1r or C1s deficiency (14–17), possibly because of their low incidence (18, 19) or less severe symptoms. In general, patients with a deficiency in complement C1 have immune complex-related diseases, autoimmune disorders and recurrent bacterial infections (18, 19).

Previously, we reported a Japanese case of selective C1s deficiency resulting from a compound heterozygosity of the C1s gene (14). One of the two mutations, a 4-bp deletion in exon X, was also identified in another Japanese family as a homozygote, and the patient had symptoms like systemic lupus erythematosus (SLE) and chronic glomerulonephritis (15). In a Caucasian patient with selective C1s deficiency, a third mutation was identified as homozygosity and was a nonsense mutation, Arg534X, in exon XII (16). This case had multiple autoimmune diseases such as SLE-like symptoms, Hashimoto’s thyroiditis, and autoimmune hepatitis. Recently, it was reported that two of four C1s-deficient siblings in a Brazilian family, who were all homozygous for a new nonsense mutation Tyr204X in exon VI, developed SLE-like symptoms (17).

In the present study, we report another compound-heterozygous patient in the subfamily of our previously reported family. This patient had symptoms that were similar in part to those observed in our first patient, but had no clear autoimmune disorder. Using the genetic analysis of the C1s gene and biochemical analyses of C1s in patient’s serum and the recombinant form as a basis, we discuss the etiology of C1s deficiency and C1s abnormality in this family.

Materials and Methods

Patients

The pedigree of a Japanese family, including two patients with C1s deficiency or C1s abnormality, is shown (see Fig. 1A). We previously reported a subfamily of this family, including a patient (III-5 in Fig. 1A) who died after 6 mo of loss of consciousness at age 7 in 1997 (14). His medical history included virus-associated hemophagocytic syndrome (VAHS), fever of unknown origin and convulsive fits as well as loss of consciousness. He was deficient in C1s resulting from a compound heterozygote with a 4-bp deletion in the paternal allele and a nonsense mutation in the maternal allele.

‡Transfusion Medicine and Cell Therapy, Saitama Medical School, Saitama, Japan

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2 Address correspondence and reprint requests to Dr. Yuichi Endo, Department of Immunology, Fukushima Medical University School of Medicine, 1-Hikarigaoka, Fukushima 960-1295, Japan. E-mail address: yendo@fmu.ac.jp

1 Abbreviations used in this paper: SLE, systemic lupus erythematosus; SSCP, single-strand conformation polymorphism; VAHS, virus-associated hemophagocytic syndrome.

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The Journal of Immunology
Table I. Level of C1q, C3, C4, CH50, and autoantibodies in sera from members of the subfamily including patient III-1

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Patient Gender</th>
<th>C1q</th>
<th>C3</th>
<th>C4</th>
<th>CH50</th>
<th>Autoantibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-1</td>
<td>M</td>
<td>37</td>
<td>94.5</td>
<td>21.3</td>
<td>39.7</td>
<td>ND</td>
</tr>
<tr>
<td>II-2</td>
<td>M</td>
<td>39</td>
<td>101.1</td>
<td>16.1</td>
<td>36.6</td>
<td>ND</td>
</tr>
<tr>
<td>III-1</td>
<td>F</td>
<td>13</td>
<td>6.5</td>
<td>101.4</td>
<td>19.2</td>
<td>&lt;10</td>
</tr>
<tr>
<td>III-2</td>
<td>M</td>
<td>11</td>
<td>111.2</td>
<td>21.6</td>
<td>&lt;10</td>
<td>ANA 40</td>
</tr>
<tr>
<td>III-3</td>
<td>M</td>
<td>6</td>
<td>122.2</td>
<td>30.8</td>
<td>45.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Normal level of C1q, C3, C4, and CH50 ranges from 8.8 to 15.3 mg/dl, 85 to 135 mg/dl, 13 to 35 mg/dl, and 30 to 45 U/ml, respectively. ANA, antinuclear autoantibody; ND, not determined.

The patient in the present study (III-1 in Fig. 1A), a 13-year-old girl, was one of the previous patient’s cousins on his mother’s side. She had a high fever with acute gluteal pain in July 2006. She was hospitalized in Chiba Children’s Hospital (Chiba City, Japan) because she had a convulsive fit followed by a short-term disturbance of consciousness. Magnetic resonance imaging revealed sacroiliac arthritis on her right side. After i.v. administration of antibiotics, she was transiently free from fever and pain. Since this episode, however, she has frequently suffered from a fever of unknown origin and recurrent gluteal pain. In addition, she showed hyper-sensitivity for multiple antibiotics. This patient had undetectable or sometimes very low levels of CH50 in her serum, which ranged from less than 10 to 13.2 IU/ml (Table I). She showed normal levels of C2 to C9 and C1 inhibitor in her serum, whereas her C1q was slightly lower than normal. An antinuclear autoantibody of titer 640 was detected in her serum, whereas she was negative for anti-DNA autoantibodies.

A signed consent form for this study was obtained from the patient’s parents. The DNA recombination studies were reported and approved by the committee of Fukushima Medical University (Fukushima, Japan).

**PCR single-strand conformation polymorphism (SSCP) analysis and nucleotide sequencing**

PCR was conducted to amplify each exon of the human C1s gene (exons I to XII) using genomic DNA as a template and primers designed from flanking intron sequences (14). The SSCP analysis and nucleotide sequencing of the PCR products were performed as previously described (14). The frequency of the three mutated alleles of the C1s gene, which have been identified in this family, was estimated by a similar PCR-SSCP analysis in a cohort study with genomic DNA prepared from 100 healthy Japanese volunteers. Signed consent forms were obtained from all individuals.

The abnormality of the C1r gene was screened in patient III-1 by both PCR-SSCP and direct sequencing of the PCR products, where PCR was performed to amplify each exon (exons I to XII) of this gene using the genomic DNA as a template and exon-specific primers (see Table III). The SSCP profiles of patient III-1 were compared with those of the healthy subject, and the nucleotide sequences were referred to the sequence of C1r cDNA deposited in GenBank (accession no. NM 001733.4).

**Rocket immunoelectrophoresis, SDS-PAGE, and Western blotting**

Serum C1s and C1r levels were estimated by rocket immunoelectrophoresis using goat anti-C1s (Affinity Biologicals) and sheep anti-C1r (AbD Serotec) Abs, respectively, as previously described (14). SDS-PAGE was performed using serum samples of 0.2 μl or purified C1 complex, prepared as below, on a 10–12% SDS-polyacrylamide gel under reducing conditions. After electrophoresis, the gel was transferred to a polyvinylidene difluoride membrane filter (Millipore) and the filters were treated with Blocking One (Nakarai Tesque) and probed with rabbit anti-C1s (Behringwerke), goat anti-C1s, sheep anti-C1r, and mouse anti-His tag (Qiagen) Abs in PBS containing 0.1% Tween 20. After washing, the filters were further incubated either with HRP-conjugated second Abs or with biotinylated second Abs and avidin-biotinylated HRP complex (Vector Laboratories), and developed using a chemiluminescent substrate (ECL; Amer sham Biosciences). The chemiluminescence image was analyzed using a LAS-3000 (Fuji film).

**Figure 1. Levels of C1s and C1r in serum.** A, Pedigree of a family with C1s deficiency and C1s abnormality. Patients in the present study (arrow) and the previous study (arrowhead) (14) are indicated. Subjects who have died are indicated (+). Objects in black Symbols indicate subjects with a nonsense mutation in exon XII (black filled), a novel missense mutation in exon XII (hatched), and a 4-bp deletion in exon X (checkered). B, Serum C1s (left) and C1r (right) levels in the subfamily including patient III-1. The level was determined by rocket immunoelectrophoresis, in which a serial dilution of the healthy subject’s (H) serum was used as a standard, and the levels in the family member are expressed as a percentage ratio to that in the healthy subject.

**Purification of C1 complex**

The C1 complex was purified from sera of patient III-1 and an unrelated healthy subject by affinity chromatography with an anti-C1s IgG-coupled Sepharose column. The column was then washed extensively with TBS containing 2 mM CaCl₂ and 0.05% Tween 20 (TBS-Ca/T), and the bound fraction was eluted with 0.1 M glycine-HCl (pH 2.5), immediately neutralized with one-tenth volume of 1 M Tris-HCl (pH 8.0), and analyzed by Western blotting for C1q, C1r, and C1s as described. The protein concentration of the C1 complex was determined using a BCA protein assay kit (Pierce).

**Preparation of recombinant C1s (rC1s) proteins**

cDNA encoding the mature proteins of C1s, which covered between 203–2266 (Accession no. J04080) was obtained by RT-PCR-based cloning. The mutant C1s cDNA harboring Gly630Glu was prepared by site-directed mutagenesis. These cDNAs were subcloned into pMT/Bip/V5-His A expression vector (Invitrogen) at the BgIII and AgeI sites. The His-tagged rC1s proteins, designated rC1sWT and rC1sG630E for the wild-type and the mutant forms, respectively, were expressed in *Drosophila* S2 cells according to the manufacturer’s instruction (Invitrogen). The rC1s secreted into the culture medium was purified by affinity chromatography with a column of Ni-NTA agarose (Qiagen). The eluate from the column was dialyzed against TBS and concentrated in a centrifugal filter (Amicon Ultra-4; Millipore). The purity of rC1s was estimated by SDS-PAGE followed by staining with Coomassie brilliant blue. The protein concentration of rC1s was determined using a BCA protein assay kit (Pierce). The antigenicity of rC1sG630E was compared with C1sWT by Western blotting with goat anti-C1s Ab, where the amount of rC1s used was adjusted based on both the protein concentration and Western blotting with anti-His tag Ab.

**Activation of rC1s by patient serum and recombinant C1r (rC1r)**

The 70 ng of rC1sWT and rC1sG630E were incubated with 1 μl of serum from patient III-1 in 10 μl of TBS containing 2 mM CaCl₂ (TBS-Ca) on ice overnight and then at 37°C for 1 h. The mixture was then subjected to Western blotting with anti-C1s and anti-C1r Abs. In another experiment, 70 ng of rC1s was incubated with 150 ng of the active form of rC1r (R&D Systems) in 10 μl of TBS-Ca containing 500 μg/ml BSA at 4°C overnight and then at 37°C for 1 h. The mixture was then subjected to Western blotting with anti-C1s and anti-C1r Abs. In both experiments, the activation of rC1s was monitored by appearance of the H chain and L chain converted from the proenzymes.
Complex formation of rC1s with rC1r

The 70 ng of rC1sWT and rC1sG630E were incubated with 100 ng of rC1r in 20 µl of TBS containing 3 mg/ml BSA on ice overnight. The generated immune complex was pulled down by adding 30 µg of anti-C1s IgG at 4°C for 1 h. The generated immune complex was eluted by adding 0.1 M glycine-HCl (pH 2.5) followed by immediate neutralization with a tenth volume of 1 M Tris-HCl (pH 8.0). The eluate was further incubated by adding 0.1 M glycine-HCl (pH 2.5) followed by immediate neutralization with a tenth volume of 1 M Tris-HCl (pH 8.0). The eluate was subjected to Western blotting with anti-His tag Ab to simultaneously detect the trypsin-activated rC1s-WT and rC1sG630E. The serum samples were also subjected to immunoblotting with anti-C1s Ab. In another experiment the immune complex was further treated with endoglycosidase F (EMD Biosciences) as described (20), and then subjected to Western blotting for C1s.

ELISA for C4a generated from C4 by activated rC1s

The 70 ng of rC1sWT and rC1sG630E were incubated with 140 ng of rC1r in TBS-Ca containing 500 ng/ml BSA on ice overnight. The mixture was further incubated by adding 70 ng of purified human C4 at 37°C for 1 h. After the incubation, the generated C4a, which was split from C4 by proteolysis by activated rC1s, was determined by using an ELISA specific for C4a and C4a des Arg (BD Biosciences).

Treatment of rC1s with trypsin and its characterization

To further evaluate the biochemical properties of rC1sWT and rC1sG630E, the susceptibility of rC1s to proteolytic degradation and the intrinsic serine protease activity of rC1s, rC1sG630E and rC1sWT were treated with 10 U/ml trypsin (Type I; Sigma-Aldrich) at 37°C for 0–30 min. The mixture was subjected to Western blotting with anti-C1s Ab. In another experiment to assess the molecular size of peptide chain without the carbohydrate moiety, the trypsin-activated rC1s-WT were further treated with endoglycosidase F (EMD Biosciences) as described (20), and then subjected to Western blotting for C1s.

To determine the N-terminal amino acid sequences of the active forms of rC1s, the trypsin-treated rC1s were separated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane filter (Millipore). The membrane filter containing the H chain and L chain were cut and subjected to amino acid sequencing in a Procise cLC Protein Sequencing System (Applied Biosystems).

To determine the serine protease activity of activated rC1s, the mixture of rC1s and trypsin was subjected to gel chromatography with a Superose 6 column connected to AKTA purifier system (Amersham Biosciences). The trypsin-free and activated rC1s was recovered from the column and pooled. The serine protease activity of activated rC1s was determined by using a synthetic substrate (Boc-Leu-Ser-Thr-Arg-methylcoumarylamide; Peptide Institute) as previously described (21). The activity was expressed as fluorescence intensity determined at 380 nm for excitation and 460 nm for emission.

Results

Levels of C1s and C1r in serum

An estimation of C1s by rocket immunoelectrophoresis showed a low amount of C1s in serum from patient III-1 (Fig. 1B). The level among the serum specimens varied slightly in a range from 5 to 15% of that in the unrelated healthy subject. A similar low level of C1s was also observed in one of the two brothers (Fig. 1B, patient III-2), who had an undetectable level of CH50. The levels of C1s in the parents (patients II-1 and II-2) and another brother (patient III-3) were approximately half that of the healthy subject. In addition, patient III-1 and her brother patient III-2 showed low levels of C1r in their sera, being 15 and 25% of the healthy subject, respectively, whereas the C1r level of her parents and brother patient III-3 was more than 60% of the healthy subject (Fig. 1B).

Mutations of the C1s gene

The PCR products obtained from patient III-1, her parents, and two brothers were subjected to PCR-SSCP analysis to screen for abnormalities in the C1s gene. When using the PCR products corresponding to exon XII, an abnormal pattern was observed in the patient, her father, and two brothers (Fig. 2A). By sequencing the PCR products, we identified a nonsense mutation at codon 597 (corresponding to codon 608 of the C1s variant sequence in Ref. 14), which was the same mutation as identified previously in the subfamily that included patient III-5 (Fig. 1A). This mutation was caused by a nucleotide substitution from G (GAA for Glu) to T (TAA). The abnormal gene encodes a truncated form of C1s, which lacks the C-terminal 80 aa. In this study, four individuals were all heterozygous for this mutation as shown by SSCP patterns and sequencing of the PCR product clones (Fig. 2C). This result also indicates that the mutation was transmitted from the father’s side.

Table II. Frequency of the mutated alleles of the C1s gene in the Japanese population

<table>
<thead>
<tr>
<th>Allele</th>
<th>Exon</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size (bp)</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>delTTTG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>X</td>
<td>5′-CTCCCTCTTTTCTGCTTGTTG-3′</td>
<td>5′-CTGCAAGTAGAAGAACCT-3′</td>
<td>129</td>
<td>0/200</td>
</tr>
<tr>
<td>GAA→TAA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>XII</td>
<td>5′-CTGCTGTTGCTCCCTTCAA-3′</td>
<td>5′-ACAGCTATCCATGGCCCTT-3′</td>
<td>151</td>
<td>0/200</td>
</tr>
<tr>
<td>GGG→GAG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>XII</td>
<td>5′-GCTATTGTTTCTTCACCTT-3′</td>
<td>5′-ACACAGGACCGACCTGGT-3′</td>
<td>136</td>
<td>0/200</td>
</tr>
</tbody>
</table>

<sup>a</sup> Allele frequency was determined by PCR-SSCP with genomic DNA from 100 healthy volunteers. DNA specimens from family members of patient II-4 harboring delTTTG, patients II-2 and III-1 harboring GAA→TAA, and patients II-1 and III-1 harboring GGG→GAG were used as positive controls.

<sup>b</sup> The 4-bp deletion detected in the subfamily, including patient III-5 in the previous study (Fig. 1A).

<sup>c</sup> Nonsense mutation Glu597X.

<sup>d</sup> Missense mutation Gly630Glu.
The SSCP analysis showed another abnormality in exon XII in the patient, her mother, and brother III-2 (Fig. 2B). We identified a novel missense mutation at codon 630, which was caused by a nucleotide substitution from G (GGG for Gly630 to A (GAG for Glu)). Interestingly, it is known that Gly630 is positioned at 2 aa upstream of the active center serine and completely conserved among the serine protease superfamily, including digestive serine proteases, complement serine proteases including C1s and C1r, and coagulation serine proteases (22, 23). The SSCP patterns and nucleotide sequencing of the PCR product clones showed that these three individuals were all heterozygous for this mutation (Fig. 2B). Thus the patient and her brother III-2 were compound heterozygous for the paternal nonsense mutation Glu597X and maternal missense mutation Gly630Glu. The subfamily, including patient III-1, had no other mutations (i.e., they did not have the 4-bp deletion in exon X, was identified in 200 loci of 100 healthy individuals in the Japanese population, genomic DNA from healthy volunteers was screened by PCR-SSCP and direct sequencing of the PCR products of patient III-1.

To estimate the frequency of the mutated alleles of the C1s gene in the Japanese population, genomic DNA from healthy volunteers was subjected to PCR-SSCP. As shown in Table II, none of the three alleles in question, Glu597X and G630E in exon XII or the 4-bp deletion in exon X, was identified in 200 loci of 100 healthy volunteers. This result indicated that these alleles are very rare, with the frequency of less than 0.5% in Japanese population.

Patient III-1, and her brother III-2, also showed a detectable but low level of serum C1r as described. To screen for abnormalities in the C1r gene in patient III-1, we performed PCR-SSCP and direct sequencing of the PCR product derived from each exon of this gene. No abnormality was found in the nucleotide sequences of any of the eleven exons, indicating that at least the coding sequence of the C1r gene is normal in patient III-1 (Table III).

**Biochemical property of C1s protein in patient’s serum**

Western blotting was used to visualize C1s in the serum from patient III-1 and in the affinity-purified C1 complex. Several bands of C1s were demonstrated in the C1 complex, which were different in molecular weight as compared with C1s in the healthy subject (Fig. 3A). Using the primary structure of C1s protein deduced from the gene sequence as a basis, a faint band of 81 kDa could be the proenzyme form of C1s generated by the paternal abnormal allele. It is also possible that several low molecular mass bands of less than 16 kDa were the truncated L chain of paternal mutated C1s and its degraded products. Interestingly, the patient had a 30-kDa band that was slightly larger than the corresponding one in the healthy subject. Similarly, the larger band was observed in the patient’s mother and one of her brothers (III-2) (Fig. 3B), suggesting that this band was the L chain of C1s derived from the maternal abnormal allele. The results also indicated that the 90-kDa band should be the proenzyme form of maternal mutated C1s because it should be similar in the size to that in the healthy subject.

Western blotting of the affinity-purified C1s from patient III-1 showed that this preparation contained C1q and C1r (Fig. 3C), indicating that the patient’s C1s still had the ability to form the C1 complex. It has been suggested that the N-terminal region consisting of a CUB domain and an EGF-like domain are essential for homodimerization of C1r and C1s (24). It is possible that two mutated C1s associated to form a C1s2 dimer in the patient because

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**Table III. Screening for abnormalities in the C1r gene in patient III-1 by PCR-SSCP and direct sequencing**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Coding Domain</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size (bp)</th>
<th>SSCP</th>
<th>Sequencing</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>5’ UTR/SIG</td>
<td>5’-TTCCCTCGGGAATGGTTCC-3’</td>
<td>5’-TTCCCTCGGGAATGGTTCC-3’</td>
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<td>no</td>
<td>no</td>
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<tr>
<td>II</td>
<td>SIG/CUB</td>
<td>5’-CCCTAGCTGAGATACCT-3’</td>
<td>5’-CCCTAGCTGAGATACCT-3’</td>
<td>299</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>III</td>
<td>CUB</td>
<td>5’-ATGATCTTCTTCACCTCC-3’</td>
<td>5’-ATGATCTTCTTCACCTCC-3’</td>
<td>276</td>
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<td>no</td>
</tr>
<tr>
<td>IV</td>
<td>EGF</td>
<td>5’-AGTGAATCGCTGCTGGTT-3’</td>
<td>5’-AGTGAATCGCTGCTGGTT-3’</td>
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<td>V</td>
<td>CUB</td>
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<td>VII</td>
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<td>VIII</td>
<td>CUB</td>
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<td>X</td>
<td>CUB</td>
<td>5’-AGTGAATCGCTGCTGGTT-3’</td>
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<td>XIa</td>
<td>PROT/3’ UTR</td>
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<td>XIc</td>
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</table>

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**FIGURE 3.** Western blotting of C1s in the C1 complex purified from sera of patient III-1 and a healthy subject (H). A, The 6 μg of protein of the complex was subjected to SDS-PAGE on 10% gel (top panel) or 12% gel (bottom panel). The H chain of Ig (IgH) was nonspecifically detected due to a cross-reaction of the anti-C1s Ab used. B, Western blot of the C1s L chain in sera from the subfamily including patient III-1. C, Western blotting of C1q and C1r in the C1 complexes purified from sera of patient III-1 and a healthy subject (H). The 6 μg of the C1 complex was subjected to SDS-PAGE on a 10% gel. In the blotting of C1q (left), a 30-kDa band represents the doublet consisting of A- and B-chains of C1q, and a 25-kDa band represents the C-chain. In the blotting of C1r (right), 55- and 27-kDa bands represent the H chain and L chain, respectively, of C1r. A 70-kDa band could correspond to the proenzyme form of C1r.
the N-terminal regions of these molecules should be intact. The homodimers of C1r and C1s interact to form a C1r2C1s2 tetramer, which can bind to the collagen-like domain of polymeric C1q to form the C1 complex (25). The possibility of C1 complex formation in the patient’s serum is also supported by the presence of the L chains derived from two abnormal C1s, which were probably cleaved by the complexed C1r.

Biochemical property of rC1s harboring Gly630Glu

To elucidate the effect of Gly630Glu on the structure and function of C1s, we prepared rC1s protein harboring this mutation and compared its biochemical properties with the wild-type protein rC1s. As shown in Fig. 4A, the purity of purified rC1s was high (upper blot). The 2 μg of purified rC1sWT and rC1sG630E protein were subjected to SDS-PAGE on 10% polyacrylamide gel under the reducing conditions, followed by staining with Coomassie brilliant blue (CBB). Antigenicity of rC1sG630E against anti-C1s Ab in comparison with those of rC1sWT (middle blot). The amount of rC1s used was adjusted based on the protein concentration and the intensity by Western blots with anti-His tag Ab (lower blot). B, Activation of rC1s in the presence of serum from patient III-1. Mutant form rC1sG630E or rC1sWT was incubated in the presence (+) or absence (−) of serum on ice overnight. Western blotting was performed using anti-His tag Ab to distinguish rC1s from the native C1s in the mixture, where the proenzyme form and the L chain of rC1s, but not the H chain, were detected.

FIGURE 4. The purity, antigenicity, complex formation and complement activation activity of purified rC1s harboring mutation Gly630Glu (rC1sG630E). A, Purity of the purified rC1s (upper blot). The 2 μg of purified rC1sWT and rC1sG630E protein were subjected to SDS-PAGE on 10% polyacrylamide gel under the reducing conditions, followed by staining with Coomassie brilliant blue (CBB). Antigenicity of rC1sG630E against anti-C1s Ab in comparison with those of rC1sWT (middle blot). The amount of rC1s used was adjusted based on the protein concentration and the intensity by Western blots with anti-His tag Ab (lower blot). B, Activation of rC1s in the presence of serum from patient III-1. Mutant form rC1sG630E or rC1sWT was incubated in the presence (+) or absence (−) of the serum on ice overnight. Western blotting was performed using anti-His tag Ab to distinguish rC1s from the native C1s in the mixture, where the proenzyme form and the L chain of rC1s, but not the H chain, were detected. C, Activation of rC1s by rC1r. Protein rC1sWT or rC1sG630E was incubated in the presence (+) or absence (−) of rC1r on ice overnight and further incubated at 37°C for 1 h. The mixture was then subjected to Western blot with anti-C1s Ab (top blot) and with anti-C1r Ab (bottom blot). D, Complex formation of rC1s with rC1r. Protein rC1WT or rC1sG630E was incubated with (+) or without (−) rC1r. The formed rC1s-rC1r complex was pulled down by adding anti-C1s Ab and protein A-Sepharose and was subjected to Western blotting with anti-His tag Ab. E, C4a released from C1s by activated rC1s. Protein rC1sG630E or rC1sWT was incubated in the presence (+) or absence (−) of rC1s on ice overnight and at 37°C for 1 h with C4. The mixture was subjected to ELISA for C4a in quadruplicate determination.
To further clarify the biochemical properties of rC1sG630E, we incubated it with an unrelated and not complexed serine protease, trypsin, instead of rC1r. As shown in Fig. 5A, the respective Western blotting profiles of rC1sG630E and rC1sWT treated by trypsin were similar to that activated by patient’s serum and rC1r. Again, the difference in molecular mass of the L chain was observed between rC1sWT and rC1sG630E. To elucidate the reason for the enlargement of L chain of rC1sG630E, the contribution of carbohydrate moiety was first investigated by the treatment with endoglycosidase F. As shown in Fig. 5A, the removal of N-linked carbohydrates reduced the molecular sizes of proenzyme and the H chain with little or no protease activity. The wild type, and that prolonged proteolysis generated a truncated L chain with little or no protease activity.

In a Superose 6 gel chromatography of the mixture of trypsin and either rC1sG630E or rC1sWT, both rC1s were eluted into the fraction with the molecular mass of ~200 kDa, indicating that, like serum C1s, each rC1s forms a homodimer (data not shown). The eluate containing the trypsin-free active form of rC1s was then subjected to an assay to determine its serine protease activity. As shown in Fig. 5D, the activity of rC1sG630E was significantly lower than that of rC1sWT, being ~60% of the wild-type rC1sWT.

Discussion

A detectable, but significantly reduced level of C1s resulted from a compound heterozygous genotype of the C1s gene in patient III-1 and her brother patient III-2. The mutated C1s gene, transmitted from the paternal side, encodes a functionally inactive protein, which has the mutation Glu597X and therefore lacks the C-terminal 80 aa including the active center Ser632. Another mutated C1s gene, transmitted from the maternal side, encodes an abnormal C1s protein bearing the missense mutation Gly630Glu, which is located in a highly conserved sequence CXG630DSG that includes the active center Ser632. In the analysis using rC1s, we found that rC1sG630E had a similar antigenicity against anti-C1s Ab as compared with the wild-type rC1sWT. This result suggests that patient III-1 and her brother patient III-2 have substantially lower levels of C1s in their sera (~50%). The level of serum C1s appears to be dependent on the gene dosage of the mutated allele Gly630Glu, although we have no data about any homozygote for this mutation. There are two possibilities to explain a reduced amount of serum C1s in the heterozygotes. One possibility is that the mutated gene is expressed at a lower level at the primary expression site or is poorly secreted into the circulation. The main expression site of C1s is known to be liver hepatocytes. No study was performed on the expression of C1s in the patient, because the tissue specimen of patient III-1 was not available in the present study. However, in our study on rC1s, rC1sG630E appeared to be produced in Dro sophila S2 cells without any bias, because the amount of rC1sG630E secreted into the culture medium was similar to that of rC1sWT. Another possibility is that the secreted, abnormal C1s disappears rapidly due its susceptibility to proteolysis. The rC1sG630E form was demonstrated to be more susceptible to proteolysis by trypsin than rC1sWT (Fig. 5C), supporting the latter possibility. Although we have no information on the susceptibility of C1r-C1s complex to proteolysis, it is likely that the rapid degradation of C1s harboring Gly630Glu is caused before its association with C1r. We also found that rC1sG630E showed a reduced C4 activation activity. In addition, rC1sG630E formed the complex with rC1r, although its quantitative data was not available in the present study. The rC1sG630E form was activated by limited proteolysis at the same site as rC1sWT, as indicated by using trypsin-treated rC1s. These results might suggest that the mutation Gly630Glu directly affects the active center of C1s and reduces the C4 activation activity of this protein. This suggestion is supported by the reduced serine protease activity of noncomplexed, trypsin-activated rC1sG630E determined using a synthetic substrate. This functional abnormality of rC1sG630E would be due to the structural alteration of the L chain, the catalytic subunit of C1s, which was expected from its slightly slower mobility on SDS-PAGE.
However, further studies are needed to provide a clear explanation because no difference was observed in the processing with carbohydrates between rC1sG630E and rC1sWT. Thus the allele Gly630Glut encodes a structurally and functionally abnormal C1s, which is responsible for a reduced amount of serum C1s in the heterozygous individuals. Taken together with the deduced property of C1s coded by the allele harboring Glu597X, the present study demonstrated that the compound heterozygosity for Glu597X and Gly630Glut were associated with the low concentrations of serum C1s and the undetectable or threshold levels of CH50 activity in patient III-1 and her brother patient III-2, whereas a 50% reduction in the amount of normal C1s appeared to be sufficient for full CH50 activity (Table I).

In addition to a low level of serum C1s, patient III-1 and her brother patient III-2 showed significantly lower levels of serum C1r. Several previous reports showed that C1s deficiency was sometimes accompanied with C1r deficiency or abnormally low C1r (5, 7). In the present study, we screened for abnormalities in the C1r gene, and demonstrated that patient III-1 has no abnormalities, at least in the coding sequence of this gene. In addition, it is unlikely that the mutations in the C1s gene affect the expression of C1r at transcription or translation levels. One possibility is that the structurally abnormal C1s encoded by both alleles harboring Gly630Glut and Glu597X, are rapidly degraded by facilitated proteolysis and result in a decreased amount of the C1 complex and an increased amount of the free form of C1r in the circulation. The unbound C1r might have a shortened half-life due to an increased susceptibility against proteolysis, thus resulting in its low level in the sera of patient III-1 and her brother patient III-2. Similarly, the low levels of C1s and C1r might be responsible for the reduced level of C1q in the serum of patient III-1.

Several symptoms observed in patient III-1, such as fever with an unknown cause, convulsive fits followed by disturbances of consciousness, were similar to those observed in patient III-5. These conditions of the patients in this family appear to be different from those of previously reported cases (15–18). It has been reported that a patient with a homozygous 4-bp deletion in exon X had SLE-like symptoms and chronic glomerulonephritis (15). A patient with a homozygous Arg534X in exon XII had SLE-like symptoms, Hashimoto’s thyroiditis, and autoimmune hepatitis (16). Thus the previous cases had in common immune complex-related diseases or autoimmune diseases, especially SLE-like symptoms. The mechanism underlying the association of SLE-like symptoms with complement deficiency has not yet been established. An important role of complement appears to be removal of immune complexes from the circulation and tissues. Therefore, the increased susceptibility of the previous cases to immune complex-related diseases and autoimmune disorders can be explained by the presence of increased levels of immune complexes resulting from the reduced ability of the complement system to remove the immune complexes. Immune complexes deposited in tissues induce inflammation that allows the release of autoantigens into the circulation as well as inflammatory cytokines, and in turn the production of autoantibodies is stimulated. Thus increased autoantibodies, immune complexes, and cytokines will develop immune complex-related diseases and autoimmune disorders in patients. However, this observation seems unable to explain the symptoms of the patients in our study.

Patient III-1 showed no clear signs of autoimmune disorders including SLE-like symptoms. She appears to have a pathological background of developing autoimmune disorders because she showed a certain level of antinuclear Ab. It is possible that sacroiliac arthritis, convulsions, and disturbances of consciousness, with the latter two possibly having been caused by an impaired CNS, were the visible signs of autoimmune disorders in this patient. Patient III-5 also had no clear autoimmune symptoms, except for VAHS. It is possible that VAHS was a phenotype of autoimmune disorder in this patient because hemophagocytosis is observed as a complication of severe autoimmune disorders, for example, in macrophage activation syndrome. In this study, the brother patient III-2 had no clear symptoms, except for recurrent infectious diseases, despite the fact that he shared the same genotype as patient III-1. It is possible that all compound heterozygous individuals were too young to develop obvious autoimmune symptoms or that typical autoimmune disorders are of a late-onset type in the present family. With regard to genotype-phenotype correlation in our patients, one possibility is that a particular molecule derived from mutated C1s genes, especially from the mutant allele encoding Glu597X, is responsible for the unique symptoms because this allele was common to the two patients III-1 and III-5. The difference in the severity of symptoms between the patients might be interpreted as the result of distinct counterpart alleles existing. The detectable, threshold level of CH50 that was occasionally observed in patient III-1 could have been contributed to by a counterpart allele encoding Gly630Glut, resulting in the relatively mild conditions of this patient. This is also supported by the result that rC1sG630E exhibited low, but detectable C4 activation.

The present study showed that this family is a very rare case, as highlighted by the accumulation of three distinct mutated alleles of the C1s gene and two different compound heterozygous genotypes causing C1s deficiency or C1s abnormality in three generations. The present study also showed that the frequency of each mutated allele is less than 0.5% in the Japanese population. As shown in Fig. 1A, the origin of the mutation Glu597X could be traced back to at least one of the grandfathers, patient I-1, although his genotype was not experimentally confirmed. The origin of the 4-bp deletion in exon X was traced back to at least one of the grandmothers, patient I-4. An early origin of a 4-bp deletion was also supported by identification of this mutation in an unrelated patient in Japan (15). The mutation Gly630Glut was traced back to at least patient’s mother II-1. Because the genotypes of grandparents at the mother’s side were not determined, the possibility remains that this mutation had an origin at patient’s mother, being of very recent origin. If the mutated alleles have been distributed widely in the Japanese population and the equilibrium of the genotype was established, all three alleles in question should be classified as pathogenic mutations, rather than gene polymorphisms. In general, the latter category is defined as a nucleotide variation with a frequency of more than 1%. The very low frequency of the allele Gly630Glut is supported by the abnormal biochemical properties of rC1sG630E.

In conclusion, two patients with C1s deficiency or abnormality were found in a Japanese family that had distinct compound heterozygous genotypes derived from three distinct C1s gene mutations including a novel missense mutation in exon XII. The two patients shared some similar symptoms, which were different from those of previously reported cases. The biochemical properties of mutated C1s were closely related to undetectable or very low activity of complement activation via the classical pathway in these patients. The uniqueness of the symptoms and the differences in severity in this family might have been associated with the mutated alleles of the C1s gene, in a special relationship with the common allele and distinct counterpart alleles, respectively.

Disclosures
The authors have no financial conflict of interest.
References


