Complete Complement Components C4A and C4B Deficiencies in Human Kidney Diseases and Systemic Lupus Erythematosus

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Complete Complement Components C4A and C4B Deficiencies
in Human Kidney Diseases and Systemic Lupus Erythematous

Yan Yang,*† Karl Lhotta,§ Erwin K. Chung,** Paula Eder,¶ Friedrich Neumair,‖ and C. Yung Yu*†**‡

Although a heterozygous deficiency of either complement component C4A or C4B is common, and each has a frequency of ~20% in a Caucasian population, complete deficiencies of both C4A and C4B proteins are extremely rare. In this paper the clinical courses for seven complete C4 deficiency patients are described in detail, and the molecular defects for complete C4 deficiencies are elucidated. Three patients with homozygous HLA A24 Cw7 B38 DR13 had systemic lupus erythematosus, mesangial glomerulonephritis, and severe skin lesions or membranoproliferative nephropathy. Immunofixation, genomic restriction fragment length polymorphisms, and pulsed field gel electrophoresis experiments revealed the presence of monomodular RP-C4-CYP21-TNX (RCCX) modules, each containing a solitary, long C4A mutant gene. Sequencing of the mutant C4A genes revealed a 2-bp, GT deletion in exon 13 that leads to protein truncation. The other four patients with homozygous HLA A30 B18 DR7 had SLE, severe kidney disorders including mesangial or membranoproliferative glomerulonephritis, and/or Henoch Schoenlein purpura. Molecular genetic analyses revealed an unusual RCCX structure with two short C4B mutant genes, each followed by an intact gene for steroid 21-hydroxylase. Nine identical, intronic mutations were found in each mutant C4B. In particular, the 8127 g→a mutation present at the donor site of intron 28 may cause an RNA splice defect. Analyses of 12 complete C4 deficiency patients revealed two hot spots of deleterious mutations: one is located at exon 13, the others within a 2.6-kb genomic region spanning exons 20–29. Screening of these mutations may facilitate epidemiologic studies of C4 in infectious, autoimmune, and kidney diseases. The Journal of Immunology, 2004, 173: 2803–2814.

In the past 30 years, complete complement components C4A and C4B deficiencies have been identified and studied clinically in 13 males and 13 females from 18 families of different racial backgrounds (1, 2). Although 15 HLA haplotypes were present in these patients, nearly three-quarters of them were homozygous in HLA alleles. All but one of the complete C4-deficient subjects experienced symptoms related to immune complex clearance disorders such as systemic lupus erythematosus (SLE),

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Abbreviations used in this paper: SLE, systemic lupus erythematosus; Herv-K(C4), endogenous retrovirus located in intron 9 of a long C4 gene; PFGE, pulsed field gel electrophoresis; RCCX, RP-C4-CYP21-TNX, a four-gene duplication module consisting of genes for serine/threonine nuclear protein kinase RP, complement component C4, steroid 21-hydroxylase CYP21, and extracellular matrix protein tenasin; RFLP, restriction fragment length polymorphism; SSF-PCR, sequence-specific primer PCR. Gene symbols are italicized throughout the text. For DNA sequences, exon sequences are in uppercase, and intron sequences are in lowercase. For RCCX modular variants: mono-L, monomodular RCCX with one long C4 gene; LL, bimodular RCCX with two long C4 genes; LS, bimodular RCCX structure with one long C4 gene and one short C4 gene; SS, bimodular RCCX structure with two short C4 genes. RP2 and TNXA are linked, partially duplicated gene segments present in all bimodular, trimodular, or quadrimodular RCCX structures. CYP21A is a nonfunctional mutant gene.

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(7–10). Considering the relevant roles of C4A and C4B in immunonclearance, memory, and effector functions of the humoral immune response, it is not unexpected that a deficiency of C4A or C4B is frequently associated with infectious and/or autoimmune diseases.

An elucidation of the molecular basis of complete C4A and C4B deficiencies may help in designing a comprehensive screening strategy to determine the prevalence of C4A and C4B mutations in autoimmune, infectious, and kidney diseases. To date, the molecular defects of complete C4A and C4B deficiencies have been elucidated in only five subjects from three families residing in Scandinavia and the U.S. (11–13). These patients had single C4A mutant genes in monomodular long (mono-L) RCCX haplotypes or one mutant C4A and one mutant C4B genes from bimodular long-short (LS) haplotypes. Among the molecular defects found, a 2-bp TC insertion into codon 1213 has been detected in C4A mutants from several families as well as a C4B mutant from HLA A2 Cw7 B39 DR15 (12). In addition, a C nucleotide deletion at codon 811 was discovered in a long C4A mutant gene of HLA A30 B18 DR3 (11), and another C nucleotide deletion at codon 522 was detected in a short C4B mutant gene from HLA A2 B12 DR6 (13). In this study the clinical histories of seven patients with complete C4A and C4B deficiencies from four European families are described together with detailed molecular genetic analyses to determine the RCCX modular variation and defects in the C4A and C4B mutant genes.

Materials and Methods

Human subjects and peripheral blood samples

Seven complete C4-deficient patients from four unrelated families residing in the alpine region close to the border of Austria and northern Italy were recruited for this study after giving their informed consent. The parents from patient families 1 and 3 were also included. Peripheral blood samples were used to isolate genomic DNA and EDTA plasma following established procedures (14). The protocol of this study was approved by the institutional review boards of Columbus Children’s Hospital in the U.S. and Innsbruck Medical University in Austria.

Allotyping of human complement components C4A and C4B proteins

EDTA plasma samples were digested with neuraminidase and carbonyl-peptidase B, resolved by high voltage agarose gel electrophoresis, and fixed by goat antiserum against human C4 (DiaSorin, Stillwater, MN) (15). C4A and C4B electrophoretic variants were stained by Simply Blue (Invitrogen Life Technologies, Carlsbad, CA). Complement C3 protein polymorphisms in EDTA plasma were determined by the immunofixation technique similar to that used for C4, with goat antiserum against human C3 (DiaSorin).

Genomic restriction fragment length polymorphisms (RFLPs)

Three RFLP strategies were used to elucidate the number and size of C4A and C4B genes in each human subject. TaqI genomic Southern blot analysis was applied to elucidate the RP-C4-CYP21-TNX modular length variants, especially the presence of long and short C4 genes linked to RP1 or RP2. The relative quantities of CYP21B and of TNX1 and TNXB, PsaAI and PvuII genomic RFLP analysis was applied to determine the relative dosages of C4A and C4B genes, using a C4d probe spanning exons 22–25 for hybridization. Finally, a long-range mapping technique was applied to give independent and confirmative information of the RCCX modules. Pmel-digested genomic DNA trapped in agarose plaus was resolved by pulsed field gel electrophoresis on a CHEF Mapper (Bio-Rad, Hercules, CA) and was subjected to Southern blot analysis using a C4d-specific probe (16).

Specific oligonucleotide primers used in this study

For long-range PCR of DNA fragments, the following primers were used: C4E1.5, 5'-TCC AAG AGA GGT TAG ATC CG-3'; C4E9.3, 5'-CTG GAG ACT AAT GAT GGC T-3'; YVE10, 5'-GGAG GCC AGA GCT CAC ATC CGT GTA-3'; Y303N, 5'-CAG GAA GGA GTG CTG CGG GGA-3'; C4E29.52, 5'-GCT TCT CTC CCT GCC TTC CT-3'; C4E41.3, 5'-TGG TCC CAG GCT GTG TTC AT-3'; C4E25.3, 5'-CAG GTG CTG TCC GTG GA-3'; and Y231N, 5'-CTC TGA CAC AGA GTC ATG AGA CC-3'.

For mutation detection using sequence-specific PCR, the following primers were used: C4E13D5, 5'-ATC CGG AGG GCA GAT GTG CT-3'; C4E14G, CT-3'; C4E12F, 5'-CTG CAC ATC TTA CTA CTA CAC TAT TCG CAC CT-3'; C4E13D3, 5'-GGG CTC TCG ATT CAT GAA CGA-3'; I27F, 5'-CAG GAC CCT CCT CCC GTT TCC-3'; and MBO-28R, 5'-GGC AGA GCC CCT CAC CCC TGA-3'.

For genetic polymorphisms in the two short C4B mutant genes, the following primers were used: E95, 5'-CTG GAG ACG ATC GTA TGG-3'; and C4I113, 5'-CCA TGG ATC CTT GGC ACC CCA-3'.

PCR amplification, cloning, and sequencing of the long C4A mutant gene from HLA A24 B38 DR13

The long C4A mutant gene was amplified in three fragments using PCR. The first fragment was 2.3 kb in size and spanned exons 1–9. It was generated using primer sets C4E1.5 and C4E9.3. The PCR conditions were one cycle at 94°C for 2 min; 33 cycles at 94°C for 45 s, 58°C for 45 s, and 72°C for 3 min; and one cycle at 72°C for 10 min. The second fragment was 6.6 kb in size and covered exons 10–30. It was amplified by primer sets Y2310 and Y303N. The PCR conditions were one cycle at 98°C for 2 min; 40 cycles at 95°C for 45 s, 66°C for 40 s, and 72°C for 4 min; one cycle at 94°C for 45 s, 59°C for 60 s, and 72°C for 9 min; and one cycle at 72°C for 15 min. All PCR were performed using the Falsafe PCR amplification kit (Epitect Technologies, Madison, WI). The 2.3-kb exon 1–9 DNA fragment was purified using a PCR purification kit (Qiagen, Valencia, CA) and was directly sequenced. The 6.6-kb exons 10–30 fragment and the 5.7-kb exons 29–41 fragment were purified by gel filtration, cloned into the pCR4-TOPO vector (Invitrogen Life Technologies), and then sequenced. Sequencing reactions were performed using the version 3 BigDye kit (Applied Biosystems, Foster City, CA). The sequencing products were purified by a spinning device (EDGE Biosystems, Gaithersburg, MD), vacuum-dried and processed by a 16-channel capillary sequencing machine (Applied Biosystems) operated by the Sequencing Core Facility of the Columbus Children’s Research Institute. DNA sequences were assembled and analyzed using GCG software (Genetics Computer Group, Madison, WI). The polymorphisms and mutations discovered in plasmid clones were further confirmed by direct sequencing of the original genomic PCR products.

Sequence-specific primer PCR (SSP-PCR) for the 2-bp deletion at exon 13

The presence of the 2-bp deletion at exon 13 of the C4 gene was detected in SSP-PCR using primers C4E13D5 and C4E14G or using primers C4E12F and C4E13D3. PCRs were performed using the Falsafe PCR amplification kit (Epitect Technologies). The PCR conditions were one cycle at 94°C for 3 min; 30 cycles at 94°C for 30 s, 60°C for 45 s, and 72°C for 1 min; and one cycle at 72°C for 10 min.

PCR amplification, cloning, and sequencing of two short C4B mutant genes from HLA A30 B18 DR7

The short C4B mutant genes were amplified using two PCR and cloned into the pCR4-TOPO vector. The first fragment of 7.3 kb spanning exons 1–25 was amplified with primers C4E13S and C4E13S, and the second fragment of 7.4 kb spanning exons 23–41 was amplified with primers Y323N and C4E14I. Both PCRs were performed using the Falsafe enzyme. The PCR conditions were one cycle at 98°C for 2 min; eight cycles at 94°C for 45 s, 64–60°C (–0.5°C/cycle) for 60 s, and 72°C for 9 min; 30 cycles at 94°C for 45 s, 59°C for 60 s, and 72°C for 9 min with a increase of 10 s/cycle; and one cycle at 72°C for 15 min. The isolated plasmids from both PCR products were purified and sequenced to completion. To separate these two mutant C4B genes, multiple clones from each group were sequenced. The polymorphisms and mutations were further confirmed by direct sequencing of PCR products.

SSP-PCR-MboI RFLP to detect mutation at the 5° splice site of intron 28

SSP-PCR was used to determine the g→a mutation in intron 28 of the C4B gene using primers I27F and MBO-28R. The PCR was performed using the Falsafe PCR amplification kit. The PCR conditions were one cycle at 96°C
for 3 min; 33 cycles at 96°C for 45 s, 62°C for 45 s, and 72°C for 2 min; and one cycle at 72°C for 5 min. The PCR products were digested overnight with restriction enzyme *MboI* at 37°C and resolved by electrophoresis with a 1.5–2% agarose gel.

**PCR for exons 9–11 to segregate two mutant C4B genes**

To detect the t/c polymorphism in intron 9, DNA fragments spanning exons 9–11 from patients with two mutant C4B genes were amplified using primers E95 and 2C4I113. The PCR conditions were one cycle at 94°C for 2 min; 35 cycles at 94°C for 45 s, 58°C for 60 s, and 72°C for 2 min; and one cycle at 72°C for 7 min. The PCR products were digested with *Hinfl* at 37°C and resolved by agarose gel electrophoresis.

**Results**

**Clinical histories of the patients**

Seven complete C4 deficiency individuals from four independent families residing in Austria or Sudtirol, an alpine area in northern Italy close to Austria, were recruited for the current study. The clinical histories of these patients are listed in Table I and described as follows.

**Family 1: 1P.** The 42-year-old male patient suffered from severe Henoch Schoenlein purpura at the age of 17 years, with involvement of the skin, intestines, and kidneys (17–19). Six years later he developed macrohematuria and nephrotic syndrome. A renal biopsy showed mesangial glomerulonephritis with fibrous crescents and tubular atrophy. At the age of 23 years, hemodialysis had to be started. One year later he received a renal allograft. After 2 years, hematuria and proteinuria were noted, and a biopsy of the transplanted kidney showed recurrence of mild mesangial glomerulonephritis and chronic allograft nephropathy. Five years after transplantation dialysis had to be resumed. After 8 years of hemodialysis the patient received a second renal allograft. Six years later his serum creatinine is 130 μmol/l, and urinalysis is normal without signs of recurrent disease. Recent treatment regimen includes tacrolimus, azathioprine, and prednisolone.

The 37-year-old brother of the patient also has complete C4 deficiency. He was unavailable for clinical investigation, but was reported to be healthy.

**Family 2: 2P.** The now 20-year-old male presented at the age of 10 years with recurrent attacks of fever, vomiting, and macrohematuria. A renal biopsy showed mild mesangioproliferative glomerulonephritis with immune deposits in the mesangium. He was treated with low dose steroids and amoxicillin (20, 21). At the age of 15 years, after a wound infection, he developed a nephrotic syndrome with proteinuria of 10 g/day. Renal histology revealed a membranous-type glomerulonephritis with large epimembranous immune deposits. He responded well to treatment with i.v. Ig (1 g/kg body weight monthly for 10 mo) with reduction of protein excretion to <1 g. However, after that treatment was stopped, proteinuria recurred and the patient remained unresponsive to Ig infusion. Treatment with mycophenolate mofetil was initiated, and a partial response with reduction of proteinuria to 2.5 g/day was achieved. Renal function remains normal.

**Family 3.** The three children in family 3 suffered primarily from proliferative glomerulonephritis. Two of them developed end-stage renal failure. The third sibling had a life-threatening cerebral involvement.

**3P1:** This 33-year-old female patient developed a lupus-like disease at age 6 years. She had hypertension and erythema of the face, hands, and arms. Urinalysis showed microhematuria and proteinuria of 3 g/day. A renal biopsy showed a membranoproliferative-like glomerulonephritis with immune complex deposition predominantly in the mesangium. She received azathioprine and steroids. Despite the treatment, she developed slowly progressive, chronic renal failure. Hemodialysis had to be started when she was 26 years of age. After 5 years of hemodialysis she received a renal allograft. Two years later she has normal transplant function and no signs of recurrence of glomerulonephritis in the allograft. Her current immunosuppressive regimen consists of cyclosporine A, mycophenolate mofetil, and prednisolone (22).

**3P2:** This male patient is currently 26 years old. At the age of 5 years he developed lupus-like skin lesions, microhematuria, and proteinuria of 2 g/day. When he was 9 years old, a renal biopsy was performed, which showed severe membranoproliferative-like glomerulonephritis with mesangial proliferation. All glomerular capillaries showed proliferative changes of variable severity. Despite immunosuppressive treatment with azathioprine and

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex and Age (years; disease onset/current)</th>
<th>Disease and Organ Involvement</th>
<th>Medical Procedures</th>
<th>Recent Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA A24 Cw7 B38 DR13</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2P M; 10/20</td>
<td>Kidney (mesangial GN; membranous GN)</td>
<td>Mycophenolate mofetil (1.5 g), prednisolone (5 mg)</td>
<td>Hydroxychloroquine (200 mg), prednisolone</td>
<td></td>
</tr>
<tr>
<td>4P1 M; 5/29</td>
<td>SLE, skin, kidney (mesangial GN)</td>
<td>Skin autotransplantation (chin)</td>
<td>Hydroxychloroquine (200 mg), azathioprine (100 mg), prednisolone (8 mg)</td>
<td></td>
</tr>
<tr>
<td>4P2 F; 2/40</td>
<td>SLE, skin, kidney (mesangial GN)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA A30 B18 DR7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1P M; 17/42</td>
<td>Schoenlein Henoch purpura, kidney (mesangial GN), skin, gut</td>
<td>Two kidney transplantations</td>
<td>Tacrolimus (6 mg), azathioprine (50 mg), prednisolone (5 mg)</td>
<td></td>
</tr>
<tr>
<td>3P1 F; 6/33</td>
<td>SLE, kidney (MPGN)</td>
<td>Kidney transplantation</td>
<td>Cyclosporin A (150 mg), mycophenolate mofetil (2 g)</td>
<td></td>
</tr>
<tr>
<td>3P2 M; 5/26</td>
<td>SLE, kidney (MPGN), skin</td>
<td>Kidney transplantation</td>
<td>Hemodialysis, sevelamer (4.8 g), furosemide (500 mg), mycophenolate mofetil (1 g), prednisolone (4 mg)</td>
<td></td>
</tr>
<tr>
<td>3P3 F; 5/23</td>
<td>SLE, kidney (MPGN), skin, cerebral vasculitis</td>
<td></td>
<td></td>
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</tr>
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</table>

*GN, glomerulonephritis; MPGN, membranoproliferative GN.*
prednisolone, his renal functions deteriorated. Cyclophosphamide bolus therapy stabilized renal disease for some time, but at the age of 16 years the patient was started on hemodialysis. After 2 years a cadaveric renal transplantation was performed. The patient was treated with tacrolimus, azathioprine, and steroids. Because of proteinuria and increasing serum creatinine levels 5 years after transplantation, a graft biopsy was performed. The biopsy showed no signs of recurrence of lupus nephritis, but indicated chronic allograft nephropathy. Six years after transplantation hemodialysis again became necessary. After 3 mo of hemodialysis the patient suffered from *Aspergillus fumigates* meningitis. He was treated with liposomal amphotericin B and made a full recovery. Currently, patient 3P2 is receiving hemodialysis and is on the waiting list for a second renal transplantation (22–24).

3P3: This female patient is 23 years of age. At 5 years of age she was noted to have hematuria and proteinuria of 4 g/day. A renal biopsy showed mesangial and focal endocapillary proliferative glomerulonephritis, and the patient was diagnosed with SLE. Treatment with azathioprine and prednisolone was started and resulted in marked improvement of proteinuria. The patient was well until the age of 22 years, when she developed a febrile illness with a facial maculopapular rash. A skin biopsy revealed vasculitis with immune complex deposits. The patient’s mental status deteriorated rapidly, and magnetic resonance imaging showed severe cerebral vasculitis. The patient was unresponsive to high dose steroids, plasma infusion and exchange, and i.v. Igs. However, she responded to immunoadsorption treatment and mycophenolate mofetil. An almost complete recovery of cerebral function was achieved. At present she is maintained on mycophenolate mofetil and low dose steroids without signs of glomerulonephritis or vasculitis (25).

**Family 4.** The prominent clinical presentation in the patients of family 4 is lupus-like skin disease. The two patients also suffered from mild glomerular disease.

4P1: This male patient is now 29 years old. At the age of 5 years he suffered from acute oliguric renal failure. A renal biopsy revealed mild mesangial glomerulonephritis. Steroid treatments led to a complete resolution and normalization of renal functions. Serum creatinine and urine analyses were normal at the time of this report. The patient developed skin manifestations of SLE primarily on the face. For that condition he was treated with hydroxychloroquine (200 mg daily) and low dose prednisolone (22, 23, 26).

4P2: The female patient is 40 years of age. When she was 2 years old she developed recurrent attacks of fever; rashes on the face, trunk, and extremities; and oral ulcers. She was treated with systemic and later topical steroids. Skin lesions became atrophic and scarring. At the age of 24 years a renal biopsy was obtained because of microscopic hematuria. Histologic studies showed mesangial glomerulonephritis with an increase in mesangial matrix and extensive mesangial immune complex depositions. At present renal function is normal, and there is no hematuria or proteinuria. However, the patient continues to suffer from skin involvement. She required plastic surgery with autologous skin transplantation to her chin at age 29 years. Her current medications are azathioprine (100 mg), hydroxychloroquine (200 mg), and low dose steroids to control skin disease (22, 23).

A brother of patients 4P1 and 4P2 died at the age of 3 years from cerebral vasculitis and sepsis caused by *staphylococci* and *streptococci* infections (23).

**Additional immunologic and hematologic observations of the complete C4 deficiency patients**

The seven patients tested positive for antinuclear Abs, but subtypes, including anti-dsDNA, were negative. Patients 3P3 and 4P1 had leucocytopenia and thrombocytopenia, but these were not constant features. Interestingly, IgG and IgA deficiencies were observed in patients 2P and 3P3, which probably reflected a defect in Ig class switching.

The patients were vaccinated against various pathogens without complication. They also appeared to have normal responses to tetanus toxin vaccination. However, repeated vaccinations of patient 1P against hepatitis B surface Ag were not successful, because the patient did not develop a specific Ab response. Other patients were successfully vaccinated against hepatitis B surface Ag.

**RCCX modules and C4A and C4B mutant genes in the seven complete C4 deficiency patients**

HLA typing revealed that two common haplotypes were present among the seven patients described above. Patients 2P, 4P1, and 4P2 were homozygous with HLA A30 B18 DR7; patients 1P, 3P1, 3P2, and 3P3 were homozygous with HLA A24 B38 DR13 (18, 22, 27). Immunofixation experiments of EDTA plasma confirmed the complete absence of complement C4A and C4B proteins in these seven patients (Fig. 1A, upper panel). The results also showed that the mother of 1P expressed C4A3 and C4B5, and both parents of patients 3P1, 3P2, and 3P3 expressed C4A3 and C4B1. Complement C3 proteins were detectable in all patient samples and their relatives using the same EDTA plasma samples and immunofixation technique (Fig. 1A, lower panel), suggesting that the absence of C4 proteins was not likely to have been caused by protein degradation.

The organization of the MHC complement gene cluster and the characteristic RFLP patterns of the RCCX modular variants are depicted in Fig. 1B (5, 16). The RCCX structures of the patients and relatives were determined by TaqI RFLP (Fig. 1C) and further confirmed by PmeI-PFGE (Fig. 1D) of genomic DNA samples. The dosages of C4A and C4B genes were determined by PshAI-PvuII RFLP (Fig. 1E).

The three patients with homozygous HLA A24 B38 DR13 (2P, 4P1, and 4P2) had the identical TaqI restriction patterns that are characteristic of the mono-L RCCX structures. As shown in Fig. 1C, left panel, each patient had RP1 linked to a long C4 gene (7.0 kb), followed by a CYP21B gene (3.7 kb) and a TNXB gene (2.5 kb) in the RCCX modules. PmeI PFGE revealed the presence of a 113-kb fragment, confirming the presence of homozygous (L/L) RCCX structures (Fig. 1D, left panel). PshAI-PvuII RFLP using a C4d probe further revealed that the mutant C4 gene present in 2P, 4P1, and 4P2 is C4A, because only the 1.7-kb C4A-specific restriction fragments were detectable (Fig. 1E, left panel). In essence, there is a solitary, long C4A mutant gene (C4AQ0) present in the monomodular RCCX structure with RP1-C4AQ0 (L)-CYP21B-TNXB between HLA A24-B38 and HLA DR13.

For the four patients with homozygous HLA A30 B18 DR7 haplotypes from families 1 and 3, TaqI RFLP showed the presence of bimodular RCCX structures. These structures were characterized by RP1 linked to a short C4 gene (6.4 kb), followed by CYP21B (3.7 kb) and TNXA (2.4 kb) in the first module, and then RP2 linked to another short C4 gene (5.4 kb), followed by CYP21B (3.7 kb) and TNXB (2.5 kb) in the second module. The presence of such a bimodular RCCX structure with two short C4 genes was further confirmed by the 139-kb PmeI fragment in the PFGE (Fig. 1D, right panel).
A Immunofixation of EDTA-plasma proteins

![Immunofixation of EDTA-plasma proteins](image1)

B A map of MHC complement gene cluster with selected RCCX modules

![A map of MHC complement gene cluster with selected RCCX modules](image2)

C TaqI RFLP - RCCX length variants

![TaqI RFLP - RCCX length variants](image3)

D PmeI PFGE - haplotypes of RCCX modules

![PmeI PFGE - haplotypes of RCCX modules](image4)

E PshAI-PvuII RFLP - ratio of C4A and C4B genes

![PshAI-PvuII RFLP - ratio of C4A and C4B genes](image5)

**FIGURE 1.** Phenotypic and genotypic analyses of the complete complement C4 deficiency patients. Patients 2P, 4P1, and 4P2 are homozygous with HLA A24 Cw7 B38 DR13. Patients 1P, 3P1, 3P2, and 3P3 are homozygous with HLA A30 B18 DR7. 1M is the mother of patient 1P; 3M and 3F are parents of patients 3P1, 3P2, and 3P3. A, Immunofixation of EDTA plasma complement proteins. EDTA plasma samples treated with neuraminidase and carboxypeptidase B were resolved by high voltage agarose gel electrophoresis, fixed with goat serum with Abs against human C4 (upper panel) or Abs against human C3 (lower panel), and stained. B, A map of the MHC complement gene cluster with selected RCCX modular variants and their defining restriction enzyme fragment sizes. Horizontal arrows represent gene transcriptional orientations; inverted arrows represent locations of DNA probes hybridized in a Southern blot analysis. The white box in the shaded C4 genes represents the endogenous retrovirus HERV-K(C4). 21A, CYP21A; 21B, steroid 21-hydroxylase CYP21B. An asterisk on bimodular SS highlights the presence of two CYP21B genes. C, TaqI RFLP elucidates the number and length variants of RCCX modules. Genomic DNA samples digested with TaqI restriction enzyme were hybridized to three probes in a Southern blot analysis that distinguished RPI linked to a long or a short C4 gene, RP2 linked to a long or a short C4 gene, CYP21A or CYP21B, and TNXA and TNXB. D, PmeI pulsed field gel electrophoresis (PFGE) to determine the RCCX haplotypes. Intact genomic DNA from peripheral blood lymphocytes in agarose plugs was digested with PmeI and resolved by PFGE, processed by Southern blotting, and hybridized to C4d (exons 28–31) genomic DNA probe. RCCX haplotypes deduced from the autoradiographs were labeled on top of the lanes. E, PshAI-PvuII genomic RFLP to determine the relative gene dosages of C4A and C4B. Genomic DNAs digested with PshAI and PvuII were hybridized to a C4d genomic probe (exons 22–25). The DNA sequence for the C4A isotypic residues contains a PshAI restriction site and is therefore used to define the presence of C4A and C4B genes in the RFLP.
CYP21A-speciﬁc patients. Based on these genotypic and phenotypic fi-
cseven complete C4 deﬁciency patients. This is unusual because a haplotypes contained two short B38 DR13 rm the absence of CYP21A ﬁlotype. To further con-
intensities of cient patients engaged in this study. The relative band C4B-de-
C4B-fragments were detectable in any of the complement C4A- and C
Taq CYP21A together with

![Image](http://www.jimmunol.org/)

FIGURE 2. Bsal RFLP to determine the presence of CYP21A and CYP21B. An 8-bp deletion in exon 3 of the CYP21A pseudogene generates a novel restriction site for Bsal. Genomic Southern blot analysis of Bsal-digested DNAs was hybridized to a CYP21 genomic DNA probe. The 0.37-kb fragment is common to both CYP21A and CYP21B.

PshAI-PvuII RFLP of the C4d region revealed that the C4 genes in 1P, 3P1, 3P2, and 3P3 were all C4B, because only the 2.2-kb C4B-speciﬁc restriction fragments were detectable (Fig. 1E, right panel). Therefore, the four patients with homozygous HLA A24 B38 DR13 haplotypes contained two short C4B mutant genes (C4BQ0) and no C4A genes.

TaqI RFLPs showed that CYP21A was present in none of the seven complete C4 deﬁciency patients. This is unusual because a bimodular RCCX structure often contains a CYP21A pseudogene together with TNXA-RP2 gene fragments between the two C4 genes. As shown in Fig. 1C, no CYP21A-speciﬁc TaqI restriction fragments were detectable in any of the complement C4A- and C4B-deﬁcient patients engaged in this study. The relative band intensities of CYP21B:CYP21A in the mother of family 1 and the parents of family 3 both showed a ratio of 3:1, which suggested a bimodular RCCX structure often contains a C4A pseudogene generates a novel Bsal restriction site (28). As shown in Fig. 2, no CYP21A-speciﬁc Bsal fragments were detectable in the complete C4 deﬁciency patients. Based on these genotypic and phenotypic analyses, the RCCX modules in HLA A30 B18 DR7 haplotype. To further conﬁrm the absence of CYP21A, Bsal RFLP was performed. The 8-bp deletion in exon 3 of the CYP21A pseudogene creates a novel Bsal restriction site (28). As shown in Fig. 2, no CYP21A-speciﬁc Bsal fragments were detectable in the complete C4 deﬁciency patients. Based on these genotypic and phenotypic analyses, the RCCX modules in HLA A30 B18 DR7 are interpreted as bimodular short-short (SS) with the following conﬁguration: RP1-C4BQ0 (S)-CYP21B:TNXA-RP2-C4BQ0 (S)-CYP21B:TNXB.

Genotype analysis of the mother from patient 1P revealed LS/SS heterozygous RCCX structures (1M; Fig. 1, C and D). In addition, the SS structure with two mutant C4B genes, her other two C4 genes from the SS structure (Fig. 1, C and E) coded for C4A3 and C4B5 proteins (Fig. 1A). The parents of patients 3P1, 3P2, and 3P3 were also heterozygous in RCCX structures. The mother of family 3 (3M) had long-long (LL)/SS haplotypes, and the father (3F) had LS/SS haplotypes (Fig. 1, C and D). Both of the LL haplotype from 3M and the LS haplotype from 3F expressed C4A3 and C4B1 (Fig. 1A).

A 2-bp deletion in exon 13 of the long C4A mutant gene from HLA A24 Cw7 B38 DR13

Sequencing determination and analyses were ﬁrst initiated on pa-
patient 2P at the polymorphic C4d region that spanned 2.3 kb. It was found that the mutant gene contained sequences characteristic of C4A, which included the sequences coding for D1054, PCPVLD 1101–1106, and VDIL 1188–1191. In addition, the mutant gene had the typical indels in introns 28 and 29 that are present in long C4A genes. However, no deleterious mutations were detected in the C4d genomic region of the mutant gene.

To identify the nucleotide mutations contributing to the nonex-
pression of complement protein, the long C4A mutant gene from patient 2P was ampliﬁed by PCR in three fragments. These frag-
ments corresponded to exons 1–9 (2.3 kb), exons 10–31 (6.6 kb), and exons 29–41 (5.7 kb). The 6.4-kb endogenous retrovirus HERV-K(C4) located in intron 9 was not included. The three ampliﬁed fragments were cloned and sequenced using 60 primers that allowed sequence determination of all 41 exons and their intervening introns in both orientations (Fig. 3A) (3, 29, 30).

Compared with the annotated sequence for C4A and C4B (3, 30), 10 nucleotide substitutions or indels present in the C4A mutant gene that deserve special attention are listed in Table II. The most conspicuous change is a deleterious 2-bp deletion at the sequence for codon 497 from exon 13 (Fig. 3B). At nt 9968–9969, the sequence from patient 2P was missing the GT dinucleotide. Such a deletion would change the protein reading frame and generate terminations at codons 607 and 613 from exon 15 (Fig. 3C). Two other novel nucleotide changes were C977ST (from exon 12), which was a synonymous mutation at the sequence for codon 476, and the c→a substitution at nucleotide 14,701 (from intron 28).

Subsequently, genomic fragments at exon 13 from patients 2P, 4P1, and 4P2 were independently ampliﬁed by PCR and subjected to sequencing. The identical mutation was found in genomic DNA samples from all three patients with HLA A24 Cw7 B38 DR13.

To facilitate screening of the 2-bp deletion at exon 13 from genomic DNA samples, speciﬁc forward (E13D5) and reverse (E13D3) PCR primers were designed for two independent experiments. The application of E13D5 and E14.3 yielded a 492-bp fragment from patients 2P, 4P1, and 4P2 (Fig. 5A). The application of 12f and E13D3 yielded 391-bp fragments from the same subjects. In each set of experiments, the positive control was a 757-bp fragment ampliﬁed by 21A5 and 21A3.

Identical mutations in the short C4B mutant genes from HLA A30 B18 DR7

Genomic DNA fragments corresponding to the two short C4B genes from patient 1P were ampliﬁed together by PCR in two independent experiments as shown in Fig. 4B. Each of these DNA fragments was sequenced to completion. Variant sequences were identiﬁed by comparison with C4B sequences in public databases and are listed in Table III.

Nine novel nucleotide changes were detected in the mutant C4B genes. However, none of these changes was located in the coding sequences (Table III). Peculiarly, ﬁve novel single-nucleotide mutations were clustered in intron 19. The remaining four mutations were present in introns 20, 28, 30, and 31, respectively. Remarkably, the g→a substitution at intron 28 was present at the intron donor site (position 8127; Fig. 4D). Such a substitution (i.e., g→a) would abrogate the correct splicing of C4 RNA transcripts. A new potential splice junction is present in seven nucleotides downstream of the original donor site. If the C4 heteronuclear RNA were spliced according to this cryptic donor site, a new termination codon, TAA, would be generated nine nucleotides downstream of Gly1206.

The homogeneity of the genomic DNA sequences at the 3’ region of exon 28 from patient 1P (Fig. 4C, right panel) suggested that both short C4B genes in the PCR product had the same mutation. In contrast, direct sequencing of the corresponding genomic region ampliﬁed for subject 1M (the mother of patient 1P) yielded both g and a sequences. The latter was expected because subject 1M had two functional C4 genes coding for C4A3 and C4B5 in addition to the two mutant C4B genes (Fig. 1A).

Sequencing of the mutant C4B genes from patients 3P1, 3P2, and 3P3 also revealed homogeneous and identical sequences with
Screening of mutation g8127a

To facilitate screenings of genomic DNA samples for the g→a mutation at the donor site of intron 28 (nt 8127), a new PCR strategy was created that used a reverse primer (primer MBO-28R) with one mutagenized nucleotide to create a novel MboI restriction site after amplification across the intron 28 donor site (Fig. 4D). As shown in Fig. 5B, the DNA fragments corresponding to intron 27-intron 28 from 1P, 3P1, 3P2, and 3P3 were homogeneous with the presence of the g8127a mutation. As expected, samples from subjects 1M and 3F were heterozygous, and the normal control gave rise to the uncleaved fragment only.
Two different short C4B genes in HLA A30 B18 DR7

Sequence determination of the amplified DNA fragments showed that both short C4B genes had virtually identical sequences, except for a single nucleotide at intron 9. At position 2601, both t and c nucleotides were detectable, suggesting a possible diversion between the two mutant C4B genes. The C4BQ0 gene with 2601c is

Table II. The sequence changes in the mutant long C4A gene in families 2 and 4

<table>
<thead>
<tr>
<th>Exon/Intron</th>
<th>Position&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change (if any)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 9</td>
<td>2296</td>
<td>C→A (TCT to TAT)</td>
<td>S 328 Y</td>
<td>nc</td>
</tr>
<tr>
<td>Exon 12</td>
<td>9775 (3401)</td>
<td>C→T (GCC to GCT)</td>
<td>476 A</td>
<td>nc; frameshift and premature stop codon</td>
</tr>
<tr>
<td>Exon 20</td>
<td>12169 (5796)</td>
<td>T→C (GT to GTC)</td>
<td>806 V</td>
<td></td>
</tr>
<tr>
<td>Intron 20</td>
<td>12209 (5923)</td>
<td>g→a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 21</td>
<td>12526 (6150)</td>
<td>A→G (ACC to GCC)</td>
<td>T 888 A</td>
<td></td>
</tr>
<tr>
<td>Intron 28</td>
<td>14510 (8140)</td>
<td>g→c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 28</td>
<td>14514 (8144)</td>
<td>+ c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 28</td>
<td>14701 (8332)</td>
<td>c→t</td>
<td></td>
<td>nc</td>
</tr>
<tr>
<td>Intron 29</td>
<td>14980 (8611)</td>
<td>c→g</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Nucleotide numbering is based on the long C4A gene; the nucleotide numbering in a short C4 gene is in parentheses. nc, novel change in sequence. An update and annotated sequence for a short C4 gene can be found in Ref. 2.

FIGURE 4. RCCX structure, PCR amplification, cloning, and sequence determination of the bimodular structure of patient 2P with HLA A30 B18 DR7. A. Gene organization in the bimodular SS structure. 21B, CYP21B.

B. PCR strategy to amplify the mutant C4B genes. C, Mutation at the donor splice site of intron 28. Left panel, Heterozygous sequences with both G and A nucleotides (marked by a red arrow) from the patient’s mother, 1M; right panel, sequence of the patient. D, The probable splice defect in intron 28 leads to a stop codon (asterisk) in exon 29. The g→a mutation in 1P is marked by a red arrow. A cryptic splice signal is present seven nucleotides downstream of the original donor sequence. A specific reverse primer (MBO-28R) designed to detect the g→a mutation at the splice junction is shown in purple (see Fig. 5B).
detectable by restriction enzyme HinPI, which recognizes the DNA sequence gcgc. To confirm the presence of two different C4BQ0 genes in the HLA A30 B18 DR7 haplotype, a 931-bp genomic DNA fragment spanning exons 9–11 was amplified by PCR and digested by HinPI. If a HinPI site is present, the restriction enzyme-digested products would be 585 and 346 bp in size. Fig. 5C showed the result of such an experiment. With respect to the presence of 2601c, the control sample (lane C) was homogeneously positive. In contrast, patient members of families 1 and 3 all yielded heterogeneous results, because both 2601c and 2601t alleles were present.

By long-range PCR, a genomic DNA fragment spanning between Rp1 and C4B exon 11 was amplified. The mutant C4B gene from the first RCCX module was found by HinPI RFLP and Southern blot analysis to contain the 2601t allele (data not shown).

**Discussion**

In this study we described the clinical histories and the molecular bases of seven patients with complete complement C4 deficiencies. These patients are from four independent families, and they are homozygous in two different HLA haplotypes. The first group with HLA A24 B38 DR13 contains the monomodular RCCX structures, each with a single long C4A mutant gene. No C4B gene is present. The molecular defect leading to the absence of C4A protein production is a 2-bp deletion at exon 13. The three patients with this molecular defect have either SLE with severe skin lesions and frequent infections, or membranous nephropathy.

The second group with HLA A30 B18 DR7 contains the bi-modular RCCX configurations with two short mutant C4B genes. No C4A gene is present in this haplotype. In each of the two C4B mutant genes, the molecular defect is probably a point mutation at the donor site of the intron 28 splice junction. The four patients with this defect have SLE or Henoch Schoenlein purpura with severe glomerulonephritis. Three of these patients had end-stage kidney failure and required hemodialysis and kidney transplantation. Two of these grafts were eventually lost due to allograft nephropathy. A successful second renal transplantation was performed in one of the patients (1P). The SLE disease in another patient (3P3) progressed to a life-threatening cerebral vasculitis, an illness that claimed the life of another complete C4 deficiency patient who we had studied previously (13). A complex treatment regimen, which included the application of mycophenolate mofetil to suppress B cell proliferation and a potential alloimmune response and immunoadsorption using a column coated with polyclonal sheep Abs against human IgG, appeared to have reversed the disease course, and the patient regained much of her CNS function (21).

For therapies of complete C4 deficiency patients with severe organ involvements, such as glomerulonephritis or cerebral vasculitis, mycophenolate mofetil could be used as a basic treatment. In addition, either i.v. Ig or immunoadsorption procedures could be applied. Low dose steroids appeared effective toward lesser problems, such as skin involvement. Vigorous treatments of all infections in the C4-deficient patients are essential. As severe glomerulonephritis does not recur in a renal allograft, C4 deficiency is not a contraindication for kidney transplantation.

The roles of complement C4A and C4B in immunity, autoimmunity, and kidney physiology remain perplexing. Current thoughts are that the complete absence of C4 proteins probably impaired the clearance of immune complexes and apoptotic materials, which contributes to inflammatory and vasculitic lesions in various organs, including skin and kidneys. It is postulated that the presence of complement C4-decorated self-Ags would facilitate the deletion of autoreactive B cells in the bone marrow in the process of central tolerance. It is also suggested that the deposition of activated C4 on foreign Ags facilitates the activation of Ag-specific B cells and enhances the class switching of Igs in the peripheral lymphoid system (31, 32). Thus, it is plausible that there is a strong association between complete C4 deficiency with systemic autoimmune diseases and kidney disorders.

Multiple investigators observed very low levels of complement C4 in patients with lupus nephritis (33–36). Such a phenomenon could be explained by high consumption rates caused by pathogenic immune complexes, inherited deficiencies (or low gene dosages) of C4A or C4B, and possibly lower C4 biosynthesis rates. The surface deposition of C4d, which is a split product of inactivated C4 containing the thioester residues, has been found recently to be one of the most consistent markers for acute and chronic renal allograft rejections caused by the humoral immune response (2, 37). These phenomena reflect the complement-mediated tissue injuries caused by effector functions of C4 in the complement activation pathways.
To date, six deleterious mutations in the \(C4A\) or \(C4B\) gene have been detected in 12 human subjects with complete \(C4A\) and \(C4B\) deficiencies. All except one of these mutations are 1- or 2-bp insertions or deletions (indels) in coding sequences that lead to frameshift and nonsense mutations (Fig. 6). SSP-PCR and SSP-PCR plus RFLP techniques to screen mutations of \(C4\) genes have now been created, and these would help clarify the roles of \(C4A\) and/or \(C4B\) deficiencies in infectious and autoimmune disease patients. The molecular basis of \(C4\) mutations in 10 other HLA haplotypes are yet to be elucidated (Table IV). It is worthwhile to point out that deleterious nonsense mutations tend to be race- or ethnic group-specific (38). For example, the presence of the 2-bp insertion in exon 29 of the \(C4A\) genes have been detected in healthy Caucasians (39) and in Caucasian and black SLE patients (40). Such a mutation has not been detectable in Asians (41). Although considerable progress has been made in understanding the molecular basis of \(C4A\) and/or \(C4B\) deficiencies in European and Northern American Caucasians, very little or no knowledge is available on the basis of \(C4A\) or \(C4B\) deficiencies in any other ethnic group (in the U.S.).

As established previously, a bimodular RCCX is regularly characterized by the presence of an \(RP1\) gene, followed by \(C4A\), the

<table>
<thead>
<tr>
<th>HLA RCCX (if known)</th>
<th>Ref. No.</th>
</tr>
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<tbody>
<tr>
<td>A11 Cw5 B18 DR2</td>
<td>n.d. b</td>
</tr>
<tr>
<td>A1 Cw7 B7 DR2</td>
<td>43</td>
</tr>
<tr>
<td>A2 Bw15 Dw8</td>
<td>n.d.</td>
</tr>
<tr>
<td>(A2 B12 Dw2) c</td>
<td>44–47</td>
</tr>
<tr>
<td>A26 B49 DR2</td>
<td>n.d.</td>
</tr>
<tr>
<td>A11 Cw4 B35 DR1</td>
<td>49, 50</td>
</tr>
<tr>
<td>A1 B17</td>
<td>L</td>
</tr>
<tr>
<td>A2 B15 DR2</td>
<td>50, 51</td>
</tr>
<tr>
<td>A2 B15 DR3</td>
<td>52</td>
</tr>
<tr>
<td>A31 B51 DRw9</td>
<td>n.d.</td>
</tr>
<tr>
<td>A24 B52 DR2</td>
<td>53</td>
</tr>
</tbody>
</table>

* Modified from Ref. 2.

b n.d., not determined.

c Mutations in \(C4AQ0\) and \(C4BQ0\) genes of HLA A2 B12 DR6 were determined (see Fig. 6).

To date, six deleterious mutations in the \(C4A\) or \(C4B\) gene have been detected in 12 human subjects with complete \(C4A\) and \(C4B\) deficiencies. All except one of those mutations are 1- or 2-bp insertions or deletions (indels) in coding sequences that lead to frameshift and nonsense mutations (Fig. 6). SSP-PCR and SSP-PCR plus RFLP techniques to screen mutations of \(C4\) genes have now been created, and these would help clarify the roles of \(C4A\) and/or \(C4B\) deficiencies in infectious and autoimmune disease patients. The molecular basis of \(C4\) mutations in 10 other HLA haplotypes are yet to be elucidated (Table IV). It is worthwhile to point out that deleterious nonsense mutations tend to be race- or ethnic group-specific (38). For example, the presence of the 2-bp insertion in exon 29 of the \(C4A\) genes have been detected in healthy Caucasians (39) and in Caucasian and black SLE patients (40). Such a mutation has not been detectable in Asians (41). Although considerable progress has been made in understanding the molecular basis of \(C4A\) and/or \(C4B\) deficiencies in European and Northern American Caucasians, very little or no knowledge is available on the basis of \(C4A\) or \(C4B\) deficiencies in any other ethnic group (in the U.S.).

As established previously, a bimodular RCCX is regularly characterized by the presence of an \(RP1\) gene, followed by \(C4A\), the

![FIGURE 6](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)
pseudogene CYP21A1, gene fragments TXNA and RP2, C4B, the steroid 21-hydroxylase CYP21B1, and then the extracellular matrix protein TNXB. Two major bimodular RCCX haplotypes are present in Caucasians, LL and LS. With some exceptions, the first long gene usually codes for C4A. The second gene may be long or short; it generally codes for a C4B protein, but sometimes for a C4A protein. The C4 genes and RCCX constituents in the HLA A30 B18 DR7 haplotype contain some distinct features. The first is the presence of two short C4B genes in a row (i.e., bimodular SS). These two C4B genes share the identical mutations, and only one nucleotide change is detectable between the two genes, of which each spans 14.2 kb. The second is the presence of CYP21B-CYP21B instead of CYP21A-CYP21B in the bimodular RCCX structure. The presence of this unusual configuration could be explained by a recent gene duplication event or by a genetic process, such as a long-range gene conversion that homogenized both genes. The presence of this unusual configuration could be explained by a recent gene duplication event or by a genetic process, such as a long-range gene conversion that homogenized both genes.

The four complete C4 deficiency patients with HLA A30 B18 DR7 each have four intact and probably functional CYP21B genes in a genome. Our ongoing epidemiologic study of the RCCX module variations in autoimmune diseases reveals that a considerable percentage of healthy subjects and patients have more than two functional CYP21B genes in a diploid genome, particularly subjects with trimodular RCCX structures (4, 39). Although the homozygous deficiency of CYP21B2 leads to congenital adrenal hyperplasia (42), the possible impact of the presence of high gene dosage of functional CYP21 on steroid biosynthesis, including cortisol, mineralocorticoids, and sex hormones, deserves further study.

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References


