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Total C4B Deficiency Due to Gene Deletion and Gene Conversion in a Patient with Severe Infections

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Deficiencies of the early components of the classical complement pathway impair the actions of innate and humoral immunity and may lead to increased susceptibility to infections. We have studied the genetic basis of total C4B deficiency in a Finnish patient with recurrent meningitis, chronic fistulas and abscesses. The maternal chromosome carried a four-gene deletion including the C4B gene, and a conversion from C4B to C4A gene was found on the paternal chromosome resulting in complete deficiency of C4B. In the converted C4A gene, mutation screening did not reveal any amino acid changes or prominent mutations, yet a large number of nucleotide variations were found. Further, the patient was heterozygous for structural deficiency of mannan binding lectin (MBL) associating with medium levels of serum MBL. Our data provides new information on the genetic instability of the C4 gene region, and on the association of homozygous C4B deficiency and variant MBL genotype with increased susceptibility to recurrent and chronic infections. Importantly, plasma therapy induced a prompt clinical cure with long-term effects.

The four consecutive genes RP, C4, CYP21, and TNX form two modules in 69% of human major histocompatibility complex (MHC) haplotypes (8). Only the C4 gene is functional in both modules. RP, CYP21, and TNX genes can be either functional or nonfunctional. The size of the four-gene segment is either 32.7 or 26.4 kb, depending on the length polymorphism of the C4 gene caused by an endogenous retrovirus HERV-K(C4) in intron 9 (12, 40, 42, 43). The genes and their pseudogene counterparts in the C4 region share a great homology and they are susceptible to deletions and conversions through nonhomologous recombination, thus adding to the diversity.

The two isotypic forms of C4, C4A and C4B, have over 99% gene homology. The isotype specificity is determined by four amino acids; C4A carries $P^{1100}C^{1105}L^{1105}D^{1106}$, whereas the sequence $L^{1105}S^{1102}T^{1105}H^{1106}$ is specific for C4B (5, 23). The two isotypes also differ in their covalent binding activities. The binding reaction of C4B is very fast, with a half-life less than 1 s, compared to the 10 s for C4A (14). C4B has higher hemolytic activity than C4A, and it binds preferentially to free hydroxyl groups on bacterial surface polysaccharides. In contrast to C4A, C4B is found in all species of mammals studied and can be regarded as more important in defending against pathogens (13, 32). C4B deficiency, manifested as low serum concentration or total absence of the protein, may result from gene deletion or nonfunctionality of the gene due to conversion or mutation. In the North American Caucasian population, partial C4B deficiency is quite common, with a gene frequency of 18.3%, whereas total C4B deficiency was found only in two individuals among the 150 subjects studied (8). In Finland, the estimated frequency for total C4B deficiency is 8% (M.-L. Lokki, unpublished data).

Complete C4B deficiency predisposes for bacterial meningitis and impairs the host response for enveloped viruses (7, 33). Finnish patients with C4B deficiency manifest urticaria eruptions and vasculitis-related joint symptoms, indicating an inadequacy in immune complex clearance (S. Meri, personal communication). In viral infection, the complement activation and resulting complement cleavage products enhance the neutralizing function of immunoglobulin M (IgM) and IgG antibodies (30). Thus, complement provides an essential link between innate and adaptive immunity. Complement binding increases immunogenicity of an antigen and B cells become stimulated through complement receptors without T-cell-dependent antibody responses. Studies of C4-deficient mice show impaired B-cell priming, reduced number of germinal centers, and features of immune complex disease (E. Paul, O. O. Pozdnyakova, and M. C. Carroll, Abstr. 8th Eur. Meet. Complement Hum. Dis., Strasbourg, France, 2001) (16). Human mannan binding lectin (MBL) is involved in defense against bacterial, fungal and viral pathogens activating complement through the classical pathway. Low levels of MBL in serum due to variant alleles is associated with susceptibility to acute respiratory tract infections during childhood (22).

In this study, we report homozygous C4B deficiency and variant MBL genotype in a patient with increased susceptibility to severe infections. The patient suffered from recurrent meningitis during infancy and chronic draining fistulas and perirectal abscesses later on. Analysis of the C4 gene region revealed a deletion of CYP21A-TNXA-RP2-C4B genes on the maternal chromosome. On the paternal chromosome, cloning and sequencing showed the presence of C4A genes only due to the conversion of C4B to C4A gene resulting in total C4B
deficiency. The patient also had a structurally heterozygous MBL genotype, which is known to have a diminishing effect on the activation of classical complement pathway through MBL binding. The data concur with our hypothesis of genetic instability as the reason for total C4B deficiency in this patient, and an increased susceptibility to infections due to defects of C4B and MBL.

**CASE REPORT**

A female baby was born in 1977 by normal vaginal delivery after a full-term and uncomplicated pregnancy. The infant’s birth weight was 2,960 g. The histories of family members included viral type meningitis in the father and the mother’s sister. The infant was well until 3 months of age, when she was admitted to hospital due to a fever. A bacterial type culture negative meningitis (7,000 leukocytes/liter with 85% granulocytes in the spinal fluid) was diagnosed, and at the age of 6 months a third purulent bacterial, culture-negative meningitis (7,000 × 10⁶ leukocytes with 89% granulocytes in the spinal fluid) was diagnosed. The clinical response to antibiotic treatments was delayed. A small sacral fistula was found and operated. At the age of 11 months chronic sacral fistulas were developed. The child suffered from persistent fever and had the erythrocyte sedimentation rates constantly between 60 to 100 mm/h. Pararectal abscesses with chronic draining were developed and she had poor weight gain. During the next 3 years the child was constantly ill, she was treated with many different antibiotics, and several operations to correct the draining fistulas were carried out. At the age 46 months, an intravenous fresh frozen plasma treatment (30 ml/kg) every 4 weeks was initiated with prompt clinical response. To correct the draining fistulas were carried out. At the age of 11 months chronic sacral fistulas were developed. The child did well. Therefore, the treatment was stopped. Except for occasional urinary tract infections, no other infections were recorded thereafter. At the age of 17 years she developed a sacral abscess which was operated and successfully treated with antibiotics and fresh frozen plasma (26 ml/kg) infusions.

Immunologic evaluations were carried out numerous times. Normal numbers of peripheral blood leukocytes as well as T lymphocytes (CD3⁺), B lymphocytes (CD19⁺) and NK cells (CD16/56⁺) were detected. Serum immunoglobulin levels, IgG subclasses, serum pneumococcal IgG antibodies, and antibody responses to tetanus toxoid were normal, showing intact B-cell function. T cells responded normally to phytohemagglutinin, concanavalin A, and pokeweed mitogen. The lymph node biopsy showed normal histology. Phagocytosis, killing test, and nitroblue tetrazolium test measuring granulocyte function were within normal limits when determined using routine clinical laboratory methods.

At the age of 17 years in 1994, total hemolytic complement of the patient’s serum was undetectable three times. The dose of 500 ml of fresh frozen plasma made the total hemolytic complement detectable, 18 U/ml, while after the infusion of 1,000 ml (22 ml/kg) it was 28 U/ml (reference interval 50 to 150 U/ml). The hemolytic complement values of the mother, father, brother, and sister were 30, 72, 29, and 53 U/ml, respectively. The patient’s C4 concentrations were persistently low, 0.11 to 0.16 g/liter, being approximately 60% of normal values. C4 concentrations for the mother, father, brother and sister were 0.18, 0.22, 0.16, and 0.29 g/liter, respectively. C4 allotyping showed total C4B deficiency in the patient, as well as in the mother and brother (Fig. 1). All other complement components (C1q, C1r, C1s, C2, C3, C5, C6, C7, C8, and C9) were normal when measured by nephelometry and electroimmunoassay at three different times (February 1980, May 1997, and December 1997).

**MATERIALS AND METHODS**

**Study subjects.** A patient having suffered from recurrent and severe infections and her parents and two siblings were studied. Informed consent was obtained from the patient and her family members by the attending physician at Turku University Central Hospital, Turku, Finland.

**HLA and complement typing.** The HLA-A, -B, and -C typing was performed using the standard microlymphocytotoxicity test (1). The HLA-B locus was sequenced to determine the subtype of B35 alleles as previously described (6). The HLA-DRB1 alleles were genotyped using a LIPA HLA-DRB1 kit (Immgenetics, Zwijndrecht, Belgium). Complement factor B and C4 allotypes were determined by immunofixation electrophoresis as previously described (27, 34). Factor B alleles F and S, as well as the subtypes FA and FB, were identified using a PCR-based method utilizing restriction polymorphism in the codon seven of exon two (21).

**DNA preparation.** Genomic DNA was extracted from acid-citrate dextrose anticoagulated peripheral blood samples by standard salting out method (28).

**Analysis of MBL gene polymorphism.** Variants of MBL due to mutations at codon 52, 54, and 57 in exon 1 of the MBL gene was determined by PCR amplification and restriction enzyme digestion of amplified DNA according to the method of Madsen et al. (25). Promoter variants at position –550 (H/L) and –221 (I/K) were determined by allele specific PCR amplification (26).

**RFLP analysis.** Restriction fragment length polymorphism (RFLP) analysis was performed using probes for RP, C4, CYP21, and TNX genes and the Southern hybridization protocol described previously (19).

**Isotype specific PCR.** C4A/C4B specific PCR was performed according to the method of Barba et al. using primers L3, A-UP, and B-UP (4). PCR products were analyzed on 2% agarose gel and visualized by ethidium bromide staining.

**PCR amplification of the C4d-region.** A 2.9-kb fragment including the C4d-region was amplified with C4-specific primers C4E 22.5 and C4E 31.3 (24) using the Expand High-Fidelity PCR system (Boehringer Mannheim, Indianapolis, Ind.). The PCR conditions in a final volume of 100 µl were 0.2 mM concentrations of deoxynucleoside triphosphates (dNTPs), a 0.3 µM concentration of each primer, 500 ng of genomic DNA, and 2.6 U of High-Fidelity enzyme mix. The following PCR program was used: 1 cycle at 94°C for 2 min; 10 cycles at 94°C for 30 s, 66°C for 1 min, 72°C for 2 min; 20 cycles at 94°C for 30 s, 57°C for 1 min, 72°C for 3 min; final extension at 72°C for 7 min, followed by a 4°C dwell. An
aliquot of each PCR product was analyzed on agarose gel using ethidium bromide staining.

**Cloning and sequencing the C4d region.** The amplification products were purified with QiAquick spin columns (Qiagen, Chatsworth, Calif.) and cloned into TA-cloning vector using the Original TA Cloning Kit (Invitrogen, Carlsbad, Calif.). The plasmid DNA was extracted from 5 ml overnight cultures using QiAprep Spin Plasmid kit (Qiagen). The C4d insert was amplified by PCR and sequenced using the BigDye Terminator cycle sequencing ready reaction kit. Electrophoresis was performed with ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.). The primers used in sequencing were C4E 25.3, C4E 26.5, C4E 28.5, and C4E 29.3 (24).

**Restriction enzyme digestions with NlaIV and Eco0109I.** The NlaIV restriction enzyme digestion was performed to further determine C4 isotypes. PCR products encompassing the C4d region were purified with ethanol precipitation and restriction digested with 4 U of NlaIV. The restriction fragments were analyzed on 2% agarose gel and detected with ethidium bromide. Specific samples were sequenced for unique isotype differences.

**Analysis of C2 gene.** Screening for the four known mutations of C2 gene in exons 2, 5, 6, and 11 was performed as described earlier (19).

**Screening for known C4 mutations.** The recently found site of a novel cystein deletion at codon 522 in exon 13 was studied using a mutation specific primer C4del13 (5’ CATCACCTGGCACTCCTTCTTA 3’) and reverse primer C4E14.3R (5’ CTTGCCATGTGGAGGGICT G3’) (K. L. Rupert, J. M. Moulds, Y. Yang, R. Warren, J. Reveille, F. C. Arnett, and C. Y. Yu, abstract from the XVIIIth Int. Complement Workshop, Immunopharmacology, 49:29, 2000). The PCR conditions were 100 ng of genomic DNA, 0.2 μM (each) primer, 100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl2, 0.1% gelatin, 0.1 mM dNTPs, and 2.5 U of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, N.J.). The following PCR program was used: 1 cycle at 94° C for 10 min; 35 cycles at 94° C for 30 s, 64° C for 45 s, 72° C for 1 min; final extension at 72° C for 10 min, followed by a 4° C dwell. An aliquot of the PCR product was analyzed using 1% agarose gel and ethidium bromide staining.

The amplification of exon 20 was performed using primers Xc-9 and C420up described by Nordin Fredrikson et al. to detect a 1-bp deletion which abolishes an AciI restriction site (29). The amplification product was purified with ethanol precipitation, digested with AciI and separated on 3% MetaPhor agarose (FMC BioProducts, Rockland, Maine) to reveal the presence or absence of a cleavage site in the fragment.

To detect a 2-bp insertion in exon 29 originally described by Barba et al., a mutation specific primer C4ins29 (5’ GCTGTGAAACGCTGAGGAA 3’) was designed to be used with isotype specific primers A-down and B-down (3, 4). PCR was performed using primers to detect an AciI restriction site (29). The amplification product was purified and sequenced for unique isotype differences.

**Screening for new C4 mutations.** PCR amplification of all 41 exons of C4 genes was performed using a 0.2 μM concentration of each primer, 100 ng of genomic DNA, 0.1 mM dNTPs and AmpliTaq, AmpliTaq Gold, or DyNAzyme EXT DNA polymerase using the following program: 10 min at 94° C, 45 s at 64° C for 38 cycles; ten minute hold at 72° C. Screening for the mutation in exon 29 was also confirmed by the PCR method described by Sullivan et al. (36).

**Southern blot analysis.** To determine the genetic basis of the total C4B deficiency, the organization of MHC class III genes RP1 and RP2, C4A and C4B, CYP21A and CYP21B, as well as TNXA and TNXB, was studied. DNA samples of all family members were subjected to restriction enzyme mapping (Fig. 2). The 3.7- and 3.2-kb TaqI fragments for CYP21B and CYP21A genes, respectively, could be seen in all family members. In the patient and the mother, the 3.7-kb fragment was twice as intense as the 3.2-kb fragment. Thus, they carried a heterozygous deletion of CYP21A gene. The intensities of the 6.5- and 5.0-kb BamHI fragments representing TNXB and TNXA genes, respectively, showed a deletion on TNXA gene in the patient and the mother. The intensity of the 5.0-kb BamHI fragment identifying RP2 gene was decreased in the patient and the mother, indicating a deletion of RP2 gene on the maternal chromosome inherited by the patient. The 7.0-kb TaqI fragment specifying RP1-C4 long gene locus was present in all family members, as well as the fragment of 6.0 kb corresponding to RP2-C4 long locus. The 5.4-kb fragment for RP2-C4 short locus was seen in father and sister only. According to the intensities of the TaqI fragments, the patient had a missing long C4B gene inherited from the mother. In conclusion, the restriction data indicate that the patient has a deletion of four genes, CYP21A, TNXA, RP2, and C4B, inherited on the maternal chromosome, and two long C4 genes on the paternal chromosome (Fig. 3).

**Isotype-specific PCR and restriction analysis.** All family members had the C4A type verified by PCR and by NlaIV restriction. The C4B isotype was absent in the patient, and detected in father and sister only. The patient’s C4d clones carried the Rg1 determinant identified by Eco0109I digestion.
Hence, only C4 genes with C4A isotype were present, corresponding to the total C4B deficiency on protein level.

**Sequence analysis of C4d region.** The known C4 allotype specific determinants are carried in exons 25, 28, and 29. In order to define the allotypes of two C4A genes on the chromosome inherited from the father, the C4d region of the patient and the father was cloned and sequenced. As a result, all the clones studied were of the same allotype C4A3a. Nucleotide alterations in intron 28 and exon 29 of these C4A3a clones specified one C4A3a gene and one long C4B gene converted to C4A3a-like.

**Screening for mutations.** The converted C4B gene of the patient was screened for new mutations in the coding region by SSCP and sequencing. In all, 10 alterations were observed in the patient compared to the published C4A sequences M59816 and M59815 (42). However, none of these caused amino acid changes or prominent mutations (Table 1). Nucleotide 5494 in exon 28 was found to be either a T or a C, and a substitution, 5911G→A, in exon 29 was revealed. All the other alterations found were of intronic origin. The segments without variation in SSCP were subjected to further screening with DHPLC, TMHA, and sequencing, which revealed the presence of four additional alterations. One single nucleotide polymorphism (SNP) was found in exon 12, all other point mutations were in intronic regions (Table 1). The sequence variation detected in the patient has been recorded in Human Genome Variation Database HGVbase (9) (http://hgvbase.cgb.ki.se/) under identification numbers IND/SNP001026494-IND/SNP001026518. Previously described C2 and C4 mutations were not detected in this family.

**DISCUSSION**

The patient with severe infections had total deficiency of C4B. The MHC region on the maternal chromosome carried a common deletion with C4B gene and on the paternal chromosome the C4B gene was converted to C4A. The presence of two C4A genes on a chromosome, instead of one C4A and one C4B, has been observed in 13.3% of the Caucasian population (8). The most prevalent homoexpressed C4 is C4A3,3, and the resulting partial deficiency of C4B is among the most common variations in human genome. When the amount of C4A proteins in allotyping has been correlated to the number of C4A genes in RFLP analyses, the results indicate that the converted genes are indeed functional. In our study, the patient showed one C4A3 gene and one C4B gene converted to the C4A3-like gene on the paternal chromosome with HLA-B51. The maternal haplotype with CYP21A-TNXA-RP2-C4B deletion carried
HLA-B*35011, as did the other maternal haplotype. HLA-B51 and HLA-B35 form a serologically cross-reacting antigen group, and three subtypes from this group have recently shown to predispose to rapid progression of AIDS. Subtypes HLA-B*3502 and HLA-B*3503 have the accelerating influence in whites, and subtype HLA-B*5301 has the accelerating influence in African Americans (17). HLA-B*3501 had no influence on AIDS progression, while susceptibility to other pathogenic microbes is not yet known.

In the studied family, the expressed C4A allotypes do not support strong protein expression of the converted C4B gene. Mutation screening revealed no mutations in the converted gene that would conclusively explain its nonfunctionality. However, several alterations in intronic regions were found. Insertion 1314-1315insC in intron 4 resides in a very close proximity to the branch point upstream of the 3' splicing site. The replacement 626C→T in intron 11 is located right after the 5' consensus sequence involved in the splicing reaction, and the 5840T/C heterozygosity in intron 28 is seen near the 3' branch point. It is not fully known how sequence variation affects the splicing of introns. A retroposon-like insertion in intron 13 generating abnormal mRNA splicing has been shown to lower C4 production in mice (44). In neurofibromatosis type 1, an intronic Alu sequence insertion causes defective gene expression due to interference of branch point recognition (41). In a study describing nonexpressed C4A and C4B genes due to a frameshift mutation in exon 29, the size of mRNA was normal yet the amount was reduced (24). Even though greater flexibility in the consensus sequences have been observed in higher eukaryotes, intronic point mutations may impede splicing or lead to the generation of cryptic splicing signals and exceptional splicing of precursor RNA. In this patient, the alterations found in the intronic regions may reduce if not prevent the expression of the converted C4A gene.

Congenital absence of C4 is most often associated with systemic lupus erythematosus-like syndrome (37). Also, an increased risk for bacterial infections has been reported. Of 46 children with bacterial meningitis, 11% were homozygous deficient for C4B versus 3% in 223 controls (33). In 29 white patients with bacteremic Streptococcus pneumoniae, Haemophilus influenzae, or Neisseria meningitidis infections, 14% had homozygous C4B deficiency compared to 2% in race-matched controls (7). However, there are studies that do not indicate C4B deficiency as a significant predisposing factor in bacterial meningitis (11, 15). Our patient had suffered from recurrent meningitis and chronic draining sacral fistulas. Intensive antibiotic and surgical treatments were ineffective. After empirical treatment with fresh frozen plasma, correcting the complement function temporarily, a prompt clinical recovery was noted. Interestingly, the plasma dose of 20 to 30 ml/kg of body weight, also used in replacement therapy of primary hypogammaglobulinemia (10), made the total hemolytic complement just detectable. In a patient with C2 deficiency and systemic lupus erythematosus, plasma therapy has been shown to induce a full clinical remission for 6 to 8 weeks and a brief restoration of hemolytic complement activity (35). In our pa-
tient, plasma therapy was given for 12 months and the patient has stayed free of severe infections thereafter. The treatment may have induced substitutive mechanisms to accommodate immune responses as it has had long-term effects without a continuous supply of C4B or MBL. A similar observation has been made in patients with C4B deficiency: a recent study shows that a small proportion of bone marrow cells is sufficient to restore normal C1q levels in serum (31).

In northern Europe, N. meningitidis is the major cause of meningitis (20). In the present study, the patient had a structurally heterozygous MBL genotype in addition to the homozygous C4B deficiency. Besides the normal MBL allele, the patient had a variant allele carrying the codon 54 substitution. In vivo studies, a recombinant protein of the variant allele has been proved unstable (39). Moreover, from the heterozygous MBL genotype in addition to the homozygous C4B deficiency, a recent study shows that a small proportion of bone marrow cells is sufficient to restore normal C1q levels in serum (31).

### Table 1. Novel nucleotide alterations found in patient’s C4 sequence

<table>
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<th>Exon or intron</th>
<th>Nucleotide(s)</th>
<th>Amino acid</th>
<th>Codon sequence</th>
<th>Variation</th>
<th>Origin</th>
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a Human C4A3 sequences M59816 (exons 1 to 9) and M59815 (exons 10 to 41) were used as reference sequences.
b Variations are found in HGVbase under identification numbers IND/SNP001026494–IND/SNP001026518.

### ACKNOWLEDGMENTS

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