Molecular basis of hereditary C1q deficiency associated with SLE and IgA nephropathy in a Turkish family

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Molecular basis of hereditary C1q deficiency associated with SLE and IgA nephropathy in a Turkish family. Two siblings (case 1 and case 2) with homozygous C1q deficiency are described. Both presented with a photosensitive rash, and during follow-up case 1 developed SLE with nephrotic proteinuria. Case 2 had microscopic hematuria with a past history of macroscopic hematuria. Renal biopsies revealed mesangio-proliferative glomerulonephritis in case 1 and IgA nephropathy in case 2, a new finding in association with C1q deficiency. Since the classical pathway of complement plays a role in the development of antibody responses, the family was also evaluated for the immune response to hepatitis B vaccine. Antibody response to hepatitis B vaccine was normal in both affected members and the rest of the family. The A-, B- and C-chain genes of C1q were amplified by PCR and directly sequenced. A homozygous C to T point mutation was identified in genomic DNA isolated from the patients at codon 186 in the A chain that resulted in a premature stop codon. This mutation was present in both parents and both unaffected sibs in the heterozygous state. This mutation was identical to that previously described in a Slovakian family with C1q deficiency. Because of this finding, a series of 92 genomic DNA samples was screened from ethnically distinct patient groups with SLE to test the hypothesis that this mutation of C1q may be a widespread disease susceptibility gene. No further examples of this mutation were found.

Hereditary homozygous C1q deficiency is a rare disease that was first described in a ten-year-old Turkish boy by Berkel and colleagues in 1977 [1]. Since then 32 cases of C1q deficiency associated with recurrent skin lesions, chronic infections, systemic lupus erythematosus (SLE) or SLE-like diseases, and mesangial proliferative glomerulonephritis have been reported [reviewed in 2–4].

C1q is composed of 18 polypeptide chains of three different chain types: A, B and C. The A- B- and C-chain genes are tandemly arranged 5' to 3' in the order of A, C, B at chromosome lp34.1 to lp36.3 [5–7]. The molecular basis of C1q deficiency has been described in six cases [4, 8–10]. All the mutations have been point changes. In three cases there were premature stop codons [4, 8, 9], in one case a single base deletion [4], and in two cases a glycine to arginine amino acid substitution at codon 6 of the C-chain, causing expression of a dysfunctional protein [4, 10].

Complement plays a role in the generation of antibody responses and immunological memory [11, 12]. There is evidence from studies in mice depleted of complement using cobra venom factor [13] and in C4, C2 and C3 deficient guinea pigs [12] that C3 activation via the classical pathway is an important step in generating a normal humoral immune response, particularly to low doses of T cell-dependent antigens. Early components of the classical pathway of complement play a role in the processing and removal of immune complexes from the circulation and tissues. In humans with homozygous deficiency of one of the early components of classical complement pathway, the excess formation and defective clearance of immune complexes may predispose to the development of glomerulonephritis and autoimmune disease including especially SLE [14, 15]. IgA nephropathy has been described in patients and families with partial deficiency of regulatory factors properdin (P) or H and in a patient with homozygous deficiency of C3 [16–18].

In this report we describe a Turkish family in which two siblings had homozygous C1q deficiency associated with SLE and IgA nephropathy. We examined the response of the patients and other family members to immunization with hepatitis B vaccine. We identified the molecular defect causing the C1q deficiency in this family and screened 92 genomic DNA samples from patients with SLE for this particular mutation, using the polymerase chain reaction (PCR) and restriction enzyme digestion.

Methods

Complement assays

CH50 and AP50. Classical pathway hemolytic complement activity was measured by plate assay, using antibody-sensitized sheep erythrocytes as previously described [19]. The alternative pathway hemolytic complement activity was measured by plate assay, using guinea pig erythrocytes in Mg-EGTA buffer as previously described [19].

C1q. Plasma samples were initially screened by double immunodiffusion assay using sheep polyclonal anti-C1q (The Binding Site, Birmingham, UK). Antigenic C1q levels were measured by ELISA as previously described [20].
Products were used as template DNA for sequence analysis using the Clean-Up System (Promega, Madison, WI, USA). The purified PCR products were purified using the Wizard DNA Clean-Up System (Promega, Madison, WI, USA). The purified products were used as template DNA for sequence analysis using the fmol DNA sequencing system according to manufacturer’s instructions (Promega).

Restriction enzyme analysis of exon 2 of A-chain
We amplified a 260 bp fragment from exon 2 of the A-chain from genomic DNA samples of all six family members and a panel of 102 SLE patients by PCR (using the primers C1QA-REV' and C1QA23'; Table 1). PCR reactions (20 µl final volume) were performed with 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 100 µM of each dNTP, 150 ng of each primer, 50 ng genomic DNA and 1 unit of Taq DNA polymerase (Gibco BRL). The reaction conditions were 30 amplification cycles of 94°C for 30 seconds, 58°C for one minute and 72°C for 45 seconds. The PCR products were digested at 37°C for two hours in a 20 µl volume containing 1×Rae buffer 6 buffer (Gibco BRL), 5 µl of PCR product, and 10 units PvuII (New England Biolabs, Beverly, MA, USA). The fragments were separated on a 3% metaphor agarose gel (Flowgen, Sittingbourne, Kent, UK) in 1× TBE buffer by electrophoresis at 50 V for one hour. The mutation observed in this family causes the loss of a PvuII site such that the PCR product remains at 260 bp. Normal samples are digested, producing 160 bp and 100 bp fragments.

Case reports
Case 1. A girl aged 3.5 years presented with a two year history of photosensitive skin and scalp eruptions, associated with fatigue and arthralgia. Except for an older sister (Case 2) who had similar skin lesions, the rest of the family members were healthy. The parents were not consanguinely related.

Physical examination revealed erythematous desquamatative and hyperkeratotic skin and scalp lesions. There were monilial and aphthous lesions of her mouth and palate, and finger and toenail deformity secondary to moniliasis. The rest of the physical examination was normal. Urinalysis was normal.

Blood tests showed hemoglobin 9 g/dl with an MCV of 71.7 fl (normal 80 to 100 fl) and serum ferritin level of 84.8 ng/ml (normal > 100 ng/ml). Immunoglobulins, direct Coombs' test, anti-nuclear antibodies (ANA), anti-dsDNA, and anti-cardiolipin antibodies measured by ELISA and ANA were normal or negative.

Complement assays showed absent CH50 and no C1q was detectable by single radial immunodiffusion (Table 2). Following
mixing her serum with a known C1q deficient serum (from another patient) the CH50 remained at 0%. However, mixing her serum with 10% of a known C2 deficient serum, or normal serum (NHS) restored the CH50 to the normal range. These functional measurements correlated with the antigenic absence of C1q. Assay results of other complement proteins in the patient and her relatives are presented in Table 2.

Her rash persisted and at the age of 5.5 she developed peripheral edema, +4 proteinuria on stick testing and reduced serum albumin concentration of 25 g/liter during a febrile episode associated with a culture-negative bronchopneumonia. Her ANA and anti-dsDNA levels had risen to 335 arbitrary ELISA units respectively. Her ANA (1101, 452, 362, and 335 AEU) and anti-dsDNA levels (385, 342, 126, and 100 AEU) remained high on four subsequent occasions during a four month period. She was treated with fresh frozen plasma, IVIG, and antibiotics; however, on four subsequent occasions during a four month period. She was aged 17 an autoimmune serological analysis was repeated at Hammersmith Hospital. ANA, anti-dsDNA, anti-ENA and anti-cardiolipin antibodies were all within normal limits. When she was aged 17 an autoimmune serological analysis was repeated at Hammersmith Hospital. ANA, anti-dsDNA, anti-ENA and rheumatoid factor were all negative. IgG anticardiolipin antibodies were weakly positive (IgG ACA = 18.8 AEU, normal < 9.0 AEU); IgM ACA were negative. Complement findings were identical to those of her sister (Table 2).

During follow-up to the age of 17 she did not have any recurrence of macroscopic hematuria, however, on five occasions microscopic hematuria was detected and a renal biopsy was performed. Light microscopy showed similar findings to those seen in case 1 (Fig. 2A). Immunohistology showed mesangial deposits of IgA (Fig. 2B), IgM, C3 and C5b-9. By electron microscopy only very small osmiophilic deposits along the mesangial basement membrane were found (Fig. 3). The final diagnosis was of IgA nephropathy in a case of mesangioproliferative glomerulonephritis by light microscopy.

### Results of sequence analysis of A-B-C chain genes

The complete DNA sequence of A-, B- and C-chain genes of C1q, including the splice donor and splice acceptor sites was determined in genomic DNA derived from Case 1. A homozygous C to T transition at codon 186 in the A chain was identified. The same mutation was present in homozygous form in the other patient (case 2) and both of the parents and the two unaffected sibling were found to be heterozygous for this mutation by sequence analysis. Figure 4 shows the mutation in homozygous form in one of the affected siblings and in a heterozygous form in the mother in comparison with the sequence from a normal individual. This mutation causes a change from CAG to TAG at codon 186 that results in a premature stop codon, and also causes a loss of a recognition site for the restriction enzyme PvuII. No other coding abnormalities were found in any remaining C1q A-gene or the B- or C-chain genes.
Fig. 1. A and B. Peripheral glomerular loop with adjacent mesangium. There are numerous osmiophilic deposits on the outside of the basement membrane which are partly surrounded by newly formed basement material.
Fig. 2. A. *Mesangioproliferative glomerulonephritis*. There is mesangial enlargement with mesangial hypercellularity. B. Extensive IgA deposits in the mesangium. The deposits for IgM, C3 and C5b-9 showed a similar pattern of distribution. Immunohistochemistry was performed on paraffin-embedded tissue according to standard methods after pronase pre-treatment.

**Results of restriction enzyme digestion analysis of the A chain PCR product**

Digestion with PvuII of the PCR product from exon 2 of A chain confirmed that both patients are homozygous for the mutation. The 260 bp PCR products were resistant to digestion with PvuII, consistent with the loss of the PvuII site, due to the C to T transition at codon 186 (Fig. 5). Both parents and both unaffected sibs were confirmed as heterozygous for this mutation as there were three bands observed of 260 bp, 160 bp and 100 bp.

This corresponds to the presence of one wild type and one mutant allele (Fig. 5).

This mutation is the same as that previously reported in a Slovakian patient with Clq deficiency [9, 22]. The finding of the same mutation responsible for cases of homozygous Clq deficiency in geographically-disperse patients suggests that the mutation might be of some antiquity. This prompted us to ask whether the same mutation might be found in other patients with SLE and conceivably could cause increased disease susceptibility when expressed in heterozygous form. We screened genomic DNA samples from 92 patients with SLE by PvuII digestion of the A-chain exon 2 PCR. The samples from the homozygous patient and heterozygous parents were used as positive controls. All of the 92 samples produced the 100 and 60 bp products, which indicates the presence of the recognition site for PvuII. It is therefore unlikely that the mutation identified in this family is a significant disease susceptibility gene in the population of lupus patients as a whole.

**Immunization of the patients with hepatitis B vaccine**

The two patients with Clq deficiency were vaccinated against hepatitis B with recombinant hepatitis B vaccine (Gen Hevac B, Pasteur Merieux, France). Both had no specific anti-HBS antibody before vaccination, and both had a normal IgG antibody
Fig. 4. DNA sequence of a normal individual, mother of case 1, and case 1 using sequencing oligonucleotide C1QA23'. The PCR product of the complete A-chain gene was used as template DNA. The gel was electrophoresed for 2.5 hours at 1.1 kV. The autoradiogram was exposed for 24 hours. Case 1 shows a C to T nucleotide change marked by an asterisk, resulting in a premature stop codon, TAG. The mother is a C/T heterozygote at this position.

Fig. 5. Digestion of PCR product from exon 2 of the A-chain with restriction enzyme PvuII. The family tree is shown at the top, with the affected status displayed. The PCR fragments were separated on a 3% Metaphor agarose gel in 1 x TBE, electrophoresed at 40 V for 90 minutes and stained with ethidium bromide. DNA size standards are 250 ng PhiX 174 DNA digested with HaeIII. The lanes contain digested PCR product from a normal individual, father, case 2, two unaffected siblings, case 1, mother and a negative control for the PCR reaction. The uncut product is 260 bp and the two fragments produced by digestion are 160 and 100 bp. The parents and unaffected sibs are confirmed as heterozygotes for the loss of the PvuII site and the two patients are homozygous for the loss of the PvuII site.

response with levels of 128300 and 131690 IU/liter (normal > 1250 IU/liter) four weeks after the last of the three injections.

Discussion
There are three points for discussion in relation to this new family with homozygous C1q deficiency: (i) the finding of a mutation identical to that previously reported in a Slovakian family; (ii) the presence of IgA nephropathy in case 2; and (iii) the normal response to hepatitis B vaccination.

C1q deficiency presents in two different forms, absent C1q protein or presence of a dysfunctional molecule. The molecular basis of both forms of deficiency has been characterized in six cases [4, 8–10]. The first molecular lesion, reported by McAdam, Goundis and Reid [8], was a homozygous G to A mutation at residue 150 in the B-chain resulting in a premature stop codon. The second report was of a point mutation at codon Gln186 (CAG to TAG) in the globular region of the A chain, which also caused a premature stop codon [9, 22]. The other four mutations were found in C chain. One patient had a homozygous deletion of a C nucleotide at position 43 of the C chain, resulting in a frame shift with premature stop codon in frame at position 108 [4], the other had a homozygous C to T mutation at position 41 of the C-chain resulting in a premature stop codon [4]. Two further cases presented with dysfunctional C1q deficiency from two racially distinct groups but showed the same homozygous point mutation (G to A) at position 6 of the C chain [4, 10]. Thus mutations have been found in all three chains of C1q and all were either single base pair deletions or single base pair mutations. No example has been identified of dominant C1q deficiency caused by expression of a poisonous polypeptide chain, as has been described for
The patients presented here have the same mutation to that previously reported in a Slovakian family [9, 22]. The molecular basis of Clq deficiency has now been determined in patients from seven families. It is remarkable that among this small number of families, two mutations have each been found in two families. The two families sharing each mutation were widely separated, in one case geographically (Central Turkey and Slovakia), and in the other geographically and ethnically (Punjabi from India and Sudeten-German). There are two explanations for these findings: (i) that the mutations are ancient and therefore widely distributed; or (ii) that the same mutations have arisen spontaneously on two occasions. We performed a limited test of the first hypothesis by screening a population of predominantly Western European Caucasoid patients with SLE to see if further examples of either of these mutations could be identified, and to test the hypothesis that heterozygous deficiency of Clq might increase disease susceptibility to the development of SLE. We did not find further examples of either mutation [4, and the present report]. We are currently collecting samples from other Turkish patients with SLE to see how widespread the mutation is in the local population. In this context it is interesting to note that the parents of the two patients described in this report were not known to be consanguineously related, and that other Turkish patients with the Clq deficiency have been described [1, reviewed in 3].

There have been a number of reports of associations of complement deficiency with the development of IgA nephropathy. A 23-year-old Japanese male with a homozygous C3 deficiency presented with a three-year history of proteinuria and microhematuria, and a renal biopsy showing marked deposition of IgA in glomerular mesangium [18]. IgA nephropathy has also been described in patients with less well characterized, familial abnormalities in expression of complement Factor H and properdin [16, 17]. Three patients have been described with hereditary angioedema from a single family who all developed IgA nephropathy [24]. Hereditary angioedema is associated with severe and persistent hypocomplementemia of C4 and C2 secondary to the failure of regulation of activated C1s in the presence of heterozygous C1 inhibitor deficiency. Hereditary angioedema is also associated with the development of SLE [25]. The etiology of IgA nephropathy is unknown, however, these rare associations of IgA nephropathy with hereditary and acquired complement deficiencies implies that a normally functioning complement system may provide some protection against the development of this disease.

There is a substantial body of evidence that complement plays an important role in the antibody response [reviewed in 11, 12, 26–28]. In animal models it was found that there was a defect in antibody responses that was a function of the immunizing dose of antigen. By using high doses of bacteriophage ΦX174 as an immunogen, the titers of the primary and secondary antibody responses in complement-deficient guinea pigs approached normal, but the normal switch from IgM to IgG did not occur as part of the secondary response and the IgG anti-ΦX174 antibody response was markedly impaired [27, 28]. Experimental studies of immunization of complement deficient patients are few and far between. The South African patient with C3 deficiency studied by Alper and colleagues [29] showed a normal anamnestic response to diphtheria toxoid and a normal primary antibody response to the polynucleotide phosphate antigen of H. influenzae in terms of quantitative antibody response. Two C3 deficient subjects were immunized with ΦX174 and these patients demonstrated normal titers of antibody following both primary and secondary immunizations, but a failure to switch the antibody response from IgM to IgG [30]. A further patient with C3 deficiency was given a primary immunization with hemocyanin and was reported to show a reduced response [31]. This same subject showed a fourfold increase in titer in response to secondary immunization with diphtheria, tetanus, and pertussis vaccine, which was considered normal [31]. A further patient showed a normal anamnestic response to typhoid immunization [32].

Our two patients with Clq deficiency were vaccinated against hepatitis B with recombinant hepatitis B vaccine. Both had no specific anti-HBS antibody before vaccination, and both had a normal IgG antibody response four weeks after the last of the three injections. Thus, in these two Clq deficient patients, the total lack of classical pathway function did not impair a normal immune response towards HBSAg vaccine.

This family takes the total number of patients with homozygous Clq deficiency reported to 34. All but one of the patients had SLE or an SLE-like illness. Clq deficiency, although very rare, is a powerful disease susceptibility gene for the development of SLE.

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