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Analysis of complement C4 loci in Caucasoids and Japanese with idiopathic membranous nephropathy

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Analysis of complement C4 loci in Caucasoids and Japanese with idiopathic membranous nephropathy. Deletion of the HLA class III complement gene, C4A, has been linked with susceptibility to a number of autoimmune diseases. In this study, we show a strong positive association between C4A gene deletion and development of idiopathic membranous nephropathy (IMN) in European Caucasoids [patients, 17/27 (63%); healthy controls, 13/65 (20%); RR 6.8; $P = 0.003$]. To clarify whether C4A deletion is an independent risk factor for IMN or is increased secondarily to the Caucasoid HLA A1, B8, DR3 extended haplotype, we examined the frequency of C4A deletion in Japanese patients, in whom the disease is associated with another HLA haplotype (DR2-DQw1). Analysis of 31 Japanese patients and 46 healthy controls showed that C4A deletion was present in only one patient (3%) and one control (2%). In addition, examination of the C4B locus in Japanese patients showed that there was no significant increase in the estimated frequency of C4B deletion in patients against controls (31 vs. 27%) and no difference in the frequency of the C4B long gene (73 vs. 87%) or C4B short gene (77 vs. 78%). We conclude that although C4A deletion confers significant risk of IMN in Caucasoids, there is no significant association between C4 polymorphism, as detected here, and risk of IMN in Japanese. This suggests that either C4A deletion is irrelevant to the pathogenesis of IMN or that more than one genetic mechanism is involved.

Idiopathic membranous nephropathy (IMN) is the most common cause of primary nephrotic syndrome in adults. It leads to end-stage renal failure in about half of those affected in Europe and North America [1]. In Japan, the frequency of end stage renal disease due to IMN is reported to be much less [1]. Genetic susceptibility to IMN is associated, in northern Europeans, with the extended HLA haplotype, HLA-A1, B8, DR3 [2–8], while in Japan there is a strong association with the HLA-DR2-DQw1 haplotype [9–12]. In North America, no consistent association with the HLA class II region has been found [1, 3].

The extended caucasoid A1, B8, DR3 haplotype includes a set of alleles encoded by the class I, class II and class III regions of the HLA complex [13–17]. These include the HLA class III complement alleles, Bf*S, C2*C, C4B*1, and the non-expressed (or null) C4A allele, C4A*Q0. It is unclear, due to strong linkage disequilibrium between the allelic polymorphisms the A1, B8, DR3 haplotype represents, whether the association with IMN derives from the HLA class II region, or from immune dysfunction related to HLA class III. In systemic lupus erythematosus, for example, C4A null seems to have a primary influence on the development of SLE, and in Caucasoids is linked to the extended A1, B8, DR3 haplotype [18, 19]. SLE is also a notable cause of secondary membranous nephropathy [1].

Analysis of the C4 loci by restriction fragment length polymorphism has revealed that about 50% of all C4A null alleles in Caucasoids are due to deletion of the C4A locus [20–22]. This deletion is about 30 kilobases long and extends to the adjacent 21 hydroxylase gene [20–22]. C4A deletion in Caucasoids almost invariably occurs with the A1, B8, DR3 haplotype [20], and C4A is therefore one of a number of genes which could play a role in the genesis of IMN. In addition, it has recently been disclosed, by analysis of C4 genotypes in Black Americans, that C4A deletion may also occur with DR2, conferring increased risk of SLE [18]. We argued that since DR2 and DR3 are the main IMN disease-susceptibility alleles in Japanese and Caucasoids, a primary association with C4A deletion might explain this link.

In the present study, we carried out molecular analysis of the C4A locus in Caucasoid and Japanese patients, and healthy controls. Our first aim was to examine C4A deletion as a risk marker for IMN in Caucasoids. Secondly, we set out to examine if there was an independent association between C4A deletion and IMN in a population for whom IMN is associated with a non-DR3 haplotype.

Furthermore, analysis of the C4B locus—which is closely homologous with the C4A locus [23]—has shown that the C4B gene can be present in a long (22 kb) or short form (16 kb) [20, 21]. An increased frequency of C4B short has been found in patients with certain autoimmune disease [19]. We therefore also investigated polymorphism at the C4B locus to see if we could identify other risk markers associated with IMN in Japanese.

Methods

Patients

We studied 25 Caucasoid patients referred to Guy's Hospital Renal Unit and 65 healthy volunteers drawn from the same

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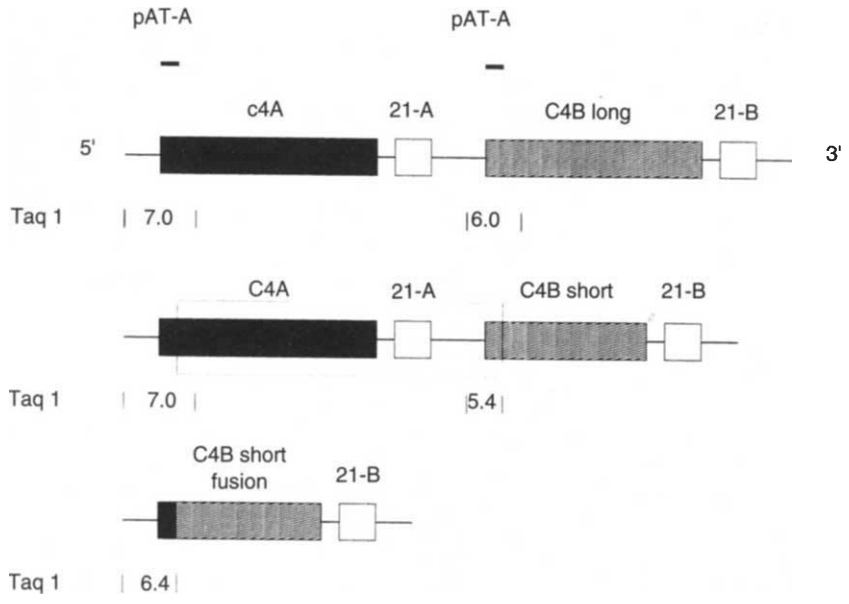

Fig. 1. *Schematic representation of the human C4 and 21-hydroxylase loci on the short arm of chromosome 6, showing Taq I restriction fragments hybridizing with the C4 probe, pAT-A.* pAT-A reveals a 7.0 kb fragment with an intact C4A gene, a 6.0 kb fragment with a "long" C4B gene and a 5.4 kb fragment with a "short" C4B gene. In the presence of a large (30 kb) C4A/210HA deletion a novel 6.4 kb fragment is revealed.

geographical area. Thirty-one Japanese patients attending Fukuoka University Hospital and 46 unrelated healthy Japanese controls from the same area were also studied. All patients had biopsy proven IMN and had no evidence of systemic disease or exposure to nephrotoxic drugs. In particular, serological investigations for systemic lupus erythematosus and chronic infection with hepatitis B were negative.

Cell lines

EBV B-lymphoblastoid cell lines were prepared from peripheral blood cells by established methods. B-cell lines from consanguineously HLA homozygous donors were used to provide information on C4 Taq 1 RFLP patterns. The HLA haplotypes of these donors were: WT49: HLA-B17, DR3; AVL: HLA-A1, B8, DR3, DQw2, Bf*S, C2*C, C4A*Q0, C4B*1; QBL: HLA-B18, DR3, DQw2, Bf*F1, C2*C, C4A*3, C4B*Q0.

HLA typing

Serological typing of HLA-A, -B and -C antigens was carried out using a standard microcytotoxicity method. HLA-DR specificities were determined by a standard cytotoxicity test on enriched B cells, and using Southern analysis of genomic DNA digested with Taq I restriction enzymes and probed with a DRB cDNA [16, 24]. HLA class II associations in the Japanese study group have been reported [11]. In brief, in the Japanese study population there were significant increases in HLA-DR2: (25/31 patients vs. 21/50 controls; $P = 0.006$; $Pc = 0.06$; RR = 5.8); −DQw1 (27/31 vs. 30/50; $P = 0.007$; $Pc = 0.03$; RR = 10.8); and −Dw2,DR2 (21/22 vs. 6/21; $P = 0.000029$; RR = 52.5).

DNA analysis

Genomic DNA was extracted from leukocytes as previously described [25] and stored at −20°C until use. DNA was digested with restriction enzymes according to previous methods [16], and then separated on 0.7% agarose gels, transferred onto nylon filters (Genescreen plus, DuPont Co., Stevenage, UK) and hybridized with ^{32}P-labeled cDNA probe as previously described [26]. Filters were washed to a high stringency (0.2 × SSC) and exposed to Kodak X-ray film at −70°C against an image intensifying screen.

DNA probes

The C4 probe was the 476 bp Bam HI/KpnI fragment of the full length probe pAT-A (provided by Dr. R.D. Campbell) [27]. This hybridizes with the 5′ ends of both C4A and C4B genes. It was used to provide information about the presence and size of C4A and C4B genes. Probes were labelled with alpha-[^{32}p] dCTP (Amersham International) by random hexanucleotide priming [28].

Interpretation of Southern blots

RFLP patterns were used to define the C4A and C4B loci as follows (Figs. 1 and 2): after Taq I digestion, the C4 probe hybridizes to a 7.0 kb fragment representing the 5′ end of the normal C4A gene. Absence of this fragment and presence of a unique 6.4 kb fragment has been shown to represent deletion of C4A [20]. The 5′ end of the C4B locus produces a 6.0 ("long" C4B) or 5.4 ("short" C4B) fragment. Absence of both these fragments corresponds to homozygous C4B deletion [20].

Scanning densitometry was used to compare the intensities of C4B and C4A bands in certain cases. The area under the curve was computed for each fragment. The C4B:C4A ratio was calculated as the sum of densitometric readings for 6.0 ± 5.4 kb bands divided by the sum for 7.0 ± 6.4 bands in the same track.

Statistical analysis

All comparisons were analyzed for statistical significance by the Chi-square method or Fisher's exact test. Where indicated, P values were multiplied by the total number of antigen comparisons made at the same HLA locus to give a corrected P (Pc), according to the method of Svejgaard et al [29].

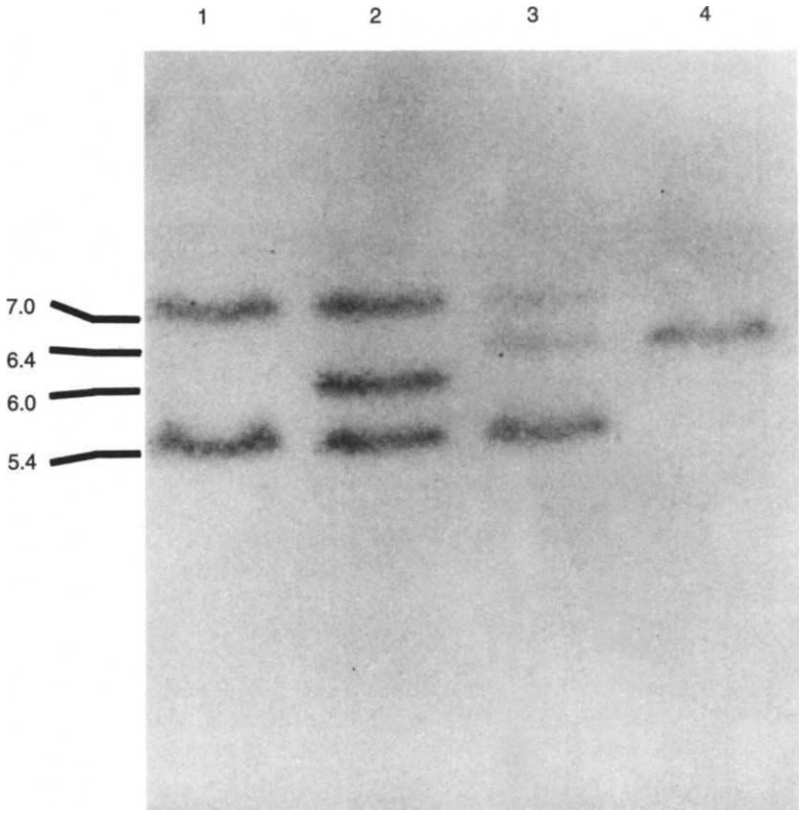

Fig. 2. *Taq I digests of genomic DNA hybridized with the C4 probe, pAT-A.* 7.0 and 6.4 kb fragments represent intact and deleted C4A respectively; 6.0 and 5.4 kb fragments represent the "long" and "short" forms of C4B respectively. Tracks 1 & 4, from HLA homozygous individuals WT49 and AVL, respectively; tracks 2 & 3, from healthy Japanese controls.

Results

Analysis of C4A and C4B genes in healthy controls

Representative Southern blots illustrating the four Taq I RFLP fragments used to describe the C4A and C4B genes are shown in Figure 2. The HLA homozygous typing cell (WT49) in track 1 exhibits the normal C4A and the short form of C4B, represented by 7.0 and 5.4 kb fragments, respectively. The individual represented in track 4 (AVL) is homozygous for C4A deletion, shown by a novel 6.4 kb fragment. Track 2 illustrates a Japanese control who has a normal C4A genotype, and both the long and short forms of C4B represented by 6.0 and 5.4 kb fragments, respectively. The healthy Japanese control in track 3 is heterozygous for the normal and deleted C4A genes, and has the C4B short gene.

IMN in European Caucasoids is associated with C4A gene deletion

C4A deletion, denoted by the presence of a novel 6.4 Taq I fragment, was present in 17/27 caucasoid patients (63%) and 13/65 (20%) healthy controls (RR 6.8; $P = 0.003$; Table 1). Five patients were homozygous for the 6.4 Taq I fragment, giving a genotypic frequency (for C4A deletion) of 22/54 (41%) in Caucasoid IMN patients. Seventeen Caucasoid patients exhibited the B8, DR3 haplotype, of whom 16 displayed the C4A deletion genotype. All 10 haplotypes of patients homozygous for A1, B8, DR3 were also homozygous for the C4A deletion genotype (Fig. 3). Conversely, only one of 17 C4A deletion genotypes found in Caucasoid patients occurred in a non-B8, DR3 subject. These data are consistent with strong positive linkage disequilibrium between C4A deletion and the extended A1, B8, DR3 haplotype. Definitive proof of linkage awaits family studies. C4B short is usually on the same haplotype as a deleted C4A gene [19, 21]. This is the probable explanation for the observed reduction in the frequency of C4B long in the patient group compared with controls.

Table 1. Frequency distribution of C4A and C4B genotypes among Caucasoid patients and controls revealed by pAT-A probe on Taq I digests of genomic DNA

	Patients $N = 27$		Controls $N = 65$			
Kilobases	N	%	N	%	P	RR
7.0 (C4A normal)	22	81	64	99		
6.4 (C4A deleted)	17	63	13	20	0.003	6.8
6.0 (C4B long)	12	44	49	75		
5.4 (C4B short)	12	44	39	60		

Analysis of C4A deletion in Japanese IMN patients and healthy controls

The frequencies of C4A gene phenotypes in 30 patients and 46 Japanese controls are shown in Table 2. There was no

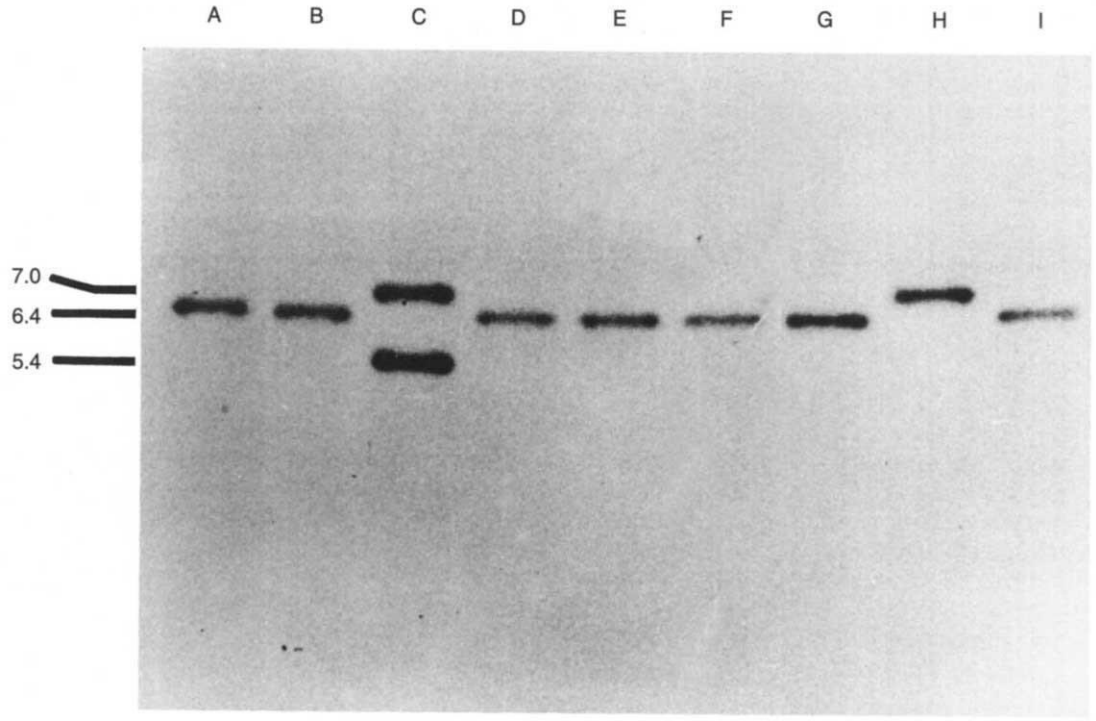

Fig. 3. *Taq I digests of genomic DNA from six caucasoid A1 B8 DR3 homozygous IMN patients (A, B, D-G) and HLA homozygous controls (H = QBL, I = AVL, C = West Indian B8 DR3), probed with pAT-A.* C4A deletion in A, B, D-G & I is revealed by absence of 7.0 fragment and presence of a novel 6.4 fragment. C4B deletion in H is shown by absence of 6.0 and 5.4 fragments.

Table 2. Frequency distribution of C4A and C4B genotypes among Japanese patients and controls revealed by pAT-A probe on Taq I digests of genomic DNA

	Patients $N = 30$		Controls $N = 46$		
Kilobases	*N*	%	*N*	%	
7.0	30	100	46	100	
6.4	1	3	1	2	
6.0	22	73	39[a]	87	NS
5.4	23	77	35[a]	78	

[a] Data on 45 patients available

significant difference in the frequency of C4A deletion between patients and controls. The overall frequency of the C4A deletion genotype in patients and controls was 2/76 (2.6%). No case of homozygous C4A deletion was identified, giving an overall genotypic frequency (for C4A deletion) of 2/152 (1.3%).

Analysis of C4B genes in Japanese patients and controls

The distribution of 6.0 and 5.4 Taq I fragments in 75 subjects analysed is shown in Table 2. This reveals no significant disease associations with the C4 RFLPs representing the long and short forms of C4B. Further, deletion of the C4B gene can be excluded in 44 subjects with both the long and short genotypes of C4B, if it is assumed that the long and short genes they represent are on different chromosomes. The remaining 31 subjects had either a 6.0 fragment *or* a 5.4 fragment, and therefore must have at least one intact C4B gene. To assess the possibility of heterozygous C4B deletion in these subjects, we examined the intensity of the C4B-associated fragments relative to the intensity of the C4A-associated fragments. In 20/28 (71%) subjects assessed, the densitometric ratio suggested there was an equivalent or slightly greater amount of DNA in C4B- compared to C4A-fragments, consistent with homozygosity at the C4B locus. The C4B:C4A ratio was inverted in 8/28 (29%) subjects examined (4/13 patients and 4/15 controls), suggesting that these individuals had only a single copy of C4B. Since no one with homozygous C4B deletion was identified, the overall genotypic frequency of C4B deletion was estimated at 8/56 haplotypes (14%).

Discussion

The results of this present study show a positive correlation between C4A gene deletion and susceptibility to idiopathic membranous nephropathy in British Caucasoids. The presence of C4A deletion increased the relative risk of IMN by about sevenfold. This compares to an increased relative risk of four- to eight-fold conferred by the HLA class II antigen, HLA-DR3, in Europeans [2–8].

One interpretation of this result is that immune dysfunction related to heterozygous or homozygous deficiency of C4A contributes to the pathogenesis of IMN. Congenital deficiency particularly of the early components of complement predisposes to a number of different types of immune complex glomerulonephritis [30, 31]. The functions of complement include enhanced clearance of immune complexes by opsonization and solubilization [30]. A plausible explanation for these current observations, therefore, is that defective clearance of immune complexes, perhaps associated with an antigen response mediated by HLA class II, leads to the evolution of immune complex glomerulonephritis.

Alternatively, C4A deletion could have no direct role in the pathogenesis of IMN, but is increased due to linkage disequilibrium with another disease susceptibility locus or loci in the HLA complex. Segregation studies in British Caucasoids have indicated that almost all of the increase in DR3 associated with IMN is due to the A1, B8, DR3 haplotype [7, 8, 16]. Our data are fully consistent with linkage of C4A deletion and A1, B8, DR3 on the same haplotype, in that there is almost complete overlap between the possession of the 6.4 Taq I fragment and the Caucasoid A1, B8, DR3 phenotype. Other candidate disease-susceptibility genes in the A1, B8, DR3 haplotype, include other HLA class III loci concerned with immunoregulatory function, such as tumor necrosis factor or heat shock protein [32, 33], the HLA class II genes [36], and non-HLA genes in the class II region involved in processing and presentation of antigen to regulatory T cells [34, 35].

To distinguish between the possibilities of a primary or secondary association of C4A deletion with IMN, we analysed the frequency of C4A deletion in another racial group in which there is known to be a strong association with HLA markers, but on a non-DR3 haplotype [11]. A similar trans-racial strategy has been useful in assessing the contribution of C4A deletion to the risk of SLE [18, 19]. Our results show that there was no independent association of C4A deletion with IMN in Japanese. Either this must mean that C4A deletion is irrelevant to the evolution of IMN, or that Japanese and Caucasoid IMN have separate genetic mechanisms.

A number of different exogenous factors, such as chronic infections, drugs and toxins, have been named as causes of secondary IMN. More than one pathogenetic mechanism may therefore exist. Moreover, there is marked geographical variation in the natural history of the disease, in that IMN rarely leads to end-stage renal failure in Japan, compared to western countries in which progressive renal disease is reported in one-half to one-third of patients with IMN. It is also relevant that no HLA-disease association in Americans has been clearly established [1, 3]. A different set of genetic and environmental factors may therefore combine to produce a similar disease in different parts of the world.

It should be realised that our results do not exclude the possibility of an association with those C4 null phenotypes which are due to non-expression rather than deletion of a C4A gene. Only about 50% of null alleles have been estimated to arise from actual deletion of the C4A gene [20]. Our examination will only identify those due to C4A deletion. Studies of complement protein allotype are clearly needed to address this issue in Japanese and other study groups.

The results of the current analysis also indicate that C4B deletion is unlikely to provide an alternative disease association in Japanese. C4B and C4A have some functional similarities [23], and C4B null alleles occur in about 7% of the Japanese population, compared with only 2% for C4A null [37]. However, we found no increase in the frequency of C4B deletion in our study population. It can be difficult to assess heterozygous C4B deletion by measurement of the intensities of bands revealed by autoradiography. However, we found no difference between patients and controls in these measurements. Moreover, the frequency of C4B deletion estimated in the present study was remarkably close to the reported prevalence of C4B null in the general Japanese population [37].

The C4B short gene has also been found with increased frequency in patients with SLE from more than one racial group [19]. This is probably because C4B short is usually present on the same haplotype as deleted C4A gene [20, 22]. Our data do not support an independent association of C4B length polymorphism with DR2-associated autoimmune disease.

In conclusion, we have shown a significant association between C4A deletion and risk of IMN in Caucasoids, of similar proportions to the risk associated with class II markers on the same haplotype [2–6]. However, we found no significant association between C4 polymorphism, as detected by RFLP, and risk of IMN in Japanese. This could favor a primary influence of HLA class II genes on disease susceptibility, and imply that the association with HLA class III in Caucasoids might be due to linkage disequilibrium with HLA class II. An alternative explanation which seems also tenable at this point is that more than one genetic pathway could be involved, which in Caucasoids might be dependent on C4A and HLA class II genes.

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