Molecular characterisation of C4 null alleles found in Felty's syndrome

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Abstract

A higher prevalence of C4B null alleles is found in Felty's syndrome. The molecular basis of C4 null alleles was investigated by studying restriction fragment length polymorphisms (RFLPs) obtained with C4 and 21-hydroxylase (21-OH) DNA probes and by pulsed field gel electrophoresis in 30 subjects with Felty's syndrome. C4A null alleles were found in 10 subjects, and in five of these were associated with a deletion that included C4A and adjacent 21-OHA gene sequences. A 6.4 kilobase C4B-5'-specific *Taq I* fragment usually provided a reliable guide to the presence of a C4A deletion but unusually in one instance this fragment was found to be a marker of a functioning C4A gene. A C4B null allele was found in 17 subjects and was associated with a deletion involving C4B and 21-OHA gene sequences on only two occasions. There were no instances in which deletion of the 21-OHB gene occurred.

The class III major histocompatibility complex (MHC) genes, complement C2, Bf, C4A, and C4B, map between HLA-B and HLA-DR on the short arm of chromosome 6. Both C4 genes are associated with a steroid 21-hydroxylase gene (21-OH or CYP21), the chromosomal order being C2, Bf, C4A, 21-OHA(CYP21A), C4B, 21-OHB (CYP21B). The 21-OHB gene is normally functional in steroid 21-hydroxylation, but the closely homologous 21-OHA gene is a pseudogene that is defective in expression.¹ Null alleles associated with absence of the circulating protein occur at both C4 loci. In previous studies we found no association between any C4A or C4B variant and susceptibility to rheumatoid arthritis itself,² but we found an association between the C4B null allele (C4B Q0) and the extra-articular disease feature of Felty's syndrome.³ The clinical importance of this association is unknown but might represent a direct effect of the partial deficiency of C4 itself or the effect of a linked gene, including the 21-OHB gene. At a molecular level the C4B null allele may result from either a deletion of C4B gene sequence plus adjacent 21-OHA or 21-OHB gene sequence, non-expression of a non-deleted C4B gene, or expression of the 'C4B' protein as a product electrophoretically indistinguishable from a C4A protein.⁴

In this study we investigated the molecular basis of the C4 null alleles found in patients with Felty's syndrome firstly by examining C4 and 21-OH restriction patterns after hybridisation of C4 specific and 21-OH specific DNA probes

to *Taq I* DNA digests.⁵ Apparent deletions and duplications inferred from *Taq I* mapping were confirmed by studying variations in the large DNA fragment sizes which are generated by digesting genomic DNA samples with an infrequently cutting restriction enzyme and then by separating the fragments obtained by pulsed field gel electrophoresis (PFGE).

Patients and methods

Thirty subjects with Felty's syndrome were studied. Felty's syndrome was defined as classical or definite rheumatoid arthritis plus current or previously documented total white cell counts of $3.5 \times 10^9/l$ or less and granulocyte counts under $2 \times 10^9/l$ over periods of at least six months which were not attributable to drug toxicity, plus an enlarged spleen reported by a clinician or an ultrasound scan. Family material was available for 21 subjects. The results of C4 allotyping in 20 subjects have been reported previously.³

Techniques for HLA typing and C4 allotyping were as defined previously.³

Genomic DNA extracted from circulating white cells by a standard method⁶ was used for studying C4 and 21-OH specific restriction fragment length polymorphisms (RFLPs). High molecular weight DNA for PFGE was prepared in agarose blocks by standard methods.⁷

PULSED FIELD GEL ELECTROPHORESIS

High molecular weight genomic DNA was digested with *Bss HII* at 50°C overnight before size fractionation by field inversion gel electrophoresis. Gels were run for 16 hours in four cycles at 20°C at 7 V/cm. The forward switch time for each cycle was set for one to 15 seconds with an exponential ramping to reach 50% of the required switch time in 40% of the total run. Backward switch time was set to 33% of forward time with a 2% pause. Separated DNA fragments were transferred to Hybond N filters as below. The C4B probe hybridised to a fragment of about 110 kb when both genes were present, and to fragments of 80 and 140 kb in the case of deletion or duplications, respectively.^{7,8}

DNA HYBRIDISATION ANALYSIS

DNA samples were digested by the restriction enzyme *Taq I* or *Bss HII* before size fractionation by conventional agarose gel electrophoresis or by field inversion gel electrophoresis respectively, and then transferred to Hybond N filters by vacuum blotting. The immobilised DNA

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Figure 1 Map of the human C4 and 21-hydroxylase genes showing the Taq I restriction fragment pattern. (A) 7.0 kb associated C4A gene with 6.0 kb associated C4B gene and both 21-OH genes. (B) 7.0 kb associated C4A gene with 5.4 kb associated (short) C4B gene. (C) 6.4 kb associated C4B gene where deletion of C4A and 21-OHA genes has occurred. (D) 7.0 kb associated C4A gene and 3.7 kb associated gene where deletion of C4B and 21-OHA genes has occurred. (E) 6.4 kb associated C4A gene with a 6.0 kb associated C4B gene and both 21-OH genes.

was hybridised with a C4B-5' DNA probe, C4B550, or a 21-OH specific DNA probe, 21A-1.8, as described previously.⁷ Hybridising restriction fragments were subsequently visualised by autoradiography. As noted previously,⁵ a 7.0 kb Taq I fragment was seen, which is associated with the C4A gene, and 6.0 and 5.4 kb fragments were seen, which are markers of the long and short C4B genes respectively (fig 1). In the presence of deletions which eliminate the C4A gene sequence a 6.4 kb C4B fragment was seen (fig 2). The 21-OH probe hybridised to 3.2 and 3.7 kb Taq I fragments which are associated with the 21-OHA and 21-OHB genes respectively. Where a C4B deletion was suspected, band densities were determined by microdensitometry with a Beckman Du8 gel scanner at a wavelength of 500 nm.

Results

Protein electrophoresis showed that 10 of 30 (33%) subjects with Felty's syndrome typed for a C4A null allele (all were heterozygous) and 17 (57%) for a C4B null allele (two were homozygotes). Control frequencies have been previously reported as 40% for the C4A null and 25% for the C4B null alleles.³ Eight of the subjects with Felty's syndrome typed for both a C4A and C4B

null allele, with the two null alleles appearing on opposite haplotypes in all instances. Five of the C4A null alleles were associated with deletion of the C4A gene sequence, shown by the finding of a 6.4 kb band after hybridisation of the C4-5' DNA probe to Taq I DNA digests (figs 1 and 2). These were also associated with deletion of the 21-OHA gene sequence, shown by a decreased intensity of the 3.2 kb 21-OHA associated band (fig 1). Fresh DNA samples were available from four of the five subjects and field inversion gel electrophoresis showed the approximate 80 kb hybridisation band expected in the case of a deletion of a 30 kb fragment in all four subjects. Haplotypes were assigned in six of the 10 subjects with electrophoretic C4A null alleles (table). The most common haplotype associated with a C4A null allele is the haplotype B8-Bf*S-C4A*Q0-C4B*1-DR3, which is usually, but not always, associated with a deletion. Other haplotypes which carried the C4A null allele did not show deletions.

Only two of the 17 C4B null alleles were associated with deletion of the C4B gene, as shown by a decreased intensity of the 6 kb C4B associated Taq I hybridisation band. Both were associated with deletions of the 21-OHA and not the 21-OHB gene. Fresh lymphocytes were available from one of the two subjects with a

Figure 2 Autoradiographs of *Taq I* restriction fragment length polymorphisms using C4 and 21-OH specific probes (fig 1). (A) Lanes 1 and 2 show a heterozygous deletion of C4A and 21-OHA gene sequences. Lane 3 shows a subject with a long (6 kb) and a short (5.4 kb) C4B gene and two normal C4A (7 kb) gene sequences. (B) Lane 1 shows a subject (fig 4, I-3) heterozygous for a 6.4 kb band associated with an active C4A gene. Lane 2 shows a subject who has a heterozygous deletion of the C4B gene sequence. Lane 3 shows a heterozygous duplication of a long C4B gene sequence. (C) Lane 1 shows a heterozygous deletion of the 21-OHA gene sequence (associated with the C4B deletion shown in lane 2, fig 2B). Lane 2 shows a heterozygous duplication of the 21-OHA gene sequence (associated with the C4B duplication shown in lane 3, fig 2B).

Haplotypes carrying C4 null alleles in subjects with Felty's syndrome with and without deletions

Number	HLA-B	Bf	C4A	C4B	DR
<i>C4A/21-OHA deletion</i>					
3	8	S	0	1	3
<i>C4B/21-OHA deletion</i>					
1	40	S	3	0	4
<i>No deletion</i>					
1	8	S	0	1	3
1	40	S	0	1	5
1	44	S	0	1	6
5	44	S	3	0	4
2	8	S	3	0	4
1	8	S	3,2	0	1
1	7	S	3	0	4*
1	17	S	3	0	6
1	45	S	3	0	4
1	39	F	3	0	1
1	62	S	3	0	4

*This haplotype has a 6.4 kb C4A fragment.

deletion, and PFGE showed bands of about 80 and 110 kb, confirming that a deletion of about 30 kb was present on one haplotype (fig 3). The haplotype bearing the deletion was assigned in one of the two subjects with a C4B deletion: B40-Bf*S-C4A*3-C4B*Q0-DR4. One other subject with Felty's syndrome, who carried a non-deleted C4B null allele on one haplotype, carried a duplication of the C4B and 21-OHA genes on the opposite haplotype, as shown by increased intensities of the 6 kb C4B and 3.2 kb 21-OH bands, as well as by a band of about 140 kb, in addition to the usual band of about 110 kb on reverse field electrophoresis. The haplo-

type carrying the duplication typed as follows: HLA-A1-B15-Bf*F-C4A*3-C4B*1-DR1. In another subject with Felty's syndrome, who typed for a C4B null allele by protein electrophoresis, DNA hybridisation studies showed 7, 6.4, and 6 kb bands (fig 2b, lane 1). Microdensitometry showed that the 6 kb hybridisation band was about twice as intense as that of the 7 or 6.4 kb bands, while the 3.7 and 3.2 kb 21-OH specific bands showed equal intensities. Field inversion gel electrophoresis showed a single *Bss HII* hybridisation band of about 110 kb, as would be expected of a haplotype in which there was no evidence of gene deletion. Family studies (fig 4) showed that the 6 and 6.4 kb C4 bands were inherited on the haplotype HLA-A24-B7-Bf*S-C4A*3-DR4. In this patient there was probably an unusual C4A associated 6.4 kb *Taq I* fragment in addition to a C4B associated 6.0 kb *Taq I* band. In one other patient typing as a C4A3,2 duplication, together with a C4B null allele on one haplotype, C4 and 21-OH hybridisation studies showed no evidence for gene deletion or duplications at either C4 or 21-OH loci, and field inversion gel electrophoresis showed a single band of about 110 kb, excluding the presence of a deletion. Equal density 6.0 and 5.4 kb *Taq I* C4B hybridisation bands were present. The 'C4B locus' was probably synthesising a protein which has the electrophoretic properties of C4A, and such observations are consistent with the occurrence of a C4A/C4B fusion gene.^{4 9}

Figure 3 Autoradiography of Bss HII restriction fragment length polymorphisms using a C4 specific probe. Lane 1 shows a normal homozygous 110 kb band, which is found when none of the C4 or 21-OH genes are deleted. Lane 2 shows a subject heterozygous for a 30 kb deletion associated with a deletion of the 21-OHA, together with the C4B gene sequences (lane 2 of fig 2B).

Figure 4 Family tree of the subject (I-3) who has a 6.4 kb Taq I fragment associated with a functional C4A gene. Family studies showed this 6.4 kb fragment to be on haplotype d, which also contains a non-deleted C4B null gene. Subject I-1 has a recombinant haplotype. HLA-A24-B7-Bf*S-C4A*3 (7 kb)-C4B*Q0 (6 kb)-DR4.

Discussion

The molecular basis for the C4 null alleles determined by protein electrophoresis in subjects with Felty's syndrome has been defined both by hybridisation of C4 and 21-OH probes to Taq I DNA digests and by PFGE. As has been shown previously,^{5 10} deletions of the

C4A and 21-OHA genes are seen mainly (in this study exclusively) with the haplotype B8-Bf*S-C4A*Q0-C4B*1-DR3, although in the present study we have also found one instance in which this haplotype did not seem to be associated with this deletion. Most of the C4B null alleles were not associated with deletions and are thus presumably the result of transcription or translation defects. In the two instances where deletions occurred this included the 21-OHA and not the 21-OHB gene. One of the apparent C4B null alleles found on protein electrophoresis may be accounted for by an active gene which encodes a product electrophoretically identified as a C4A protein. This mechanism has been described previously, also on a haplotype typing for C4A3,2-C4BQ0, and such fusion genes may not be uncommon.^{4 9}

The 6.4 kb Taq I C4B fragment is usually a reliable guide to the presence of a C4A deletion and is produced by deletion of two Taq I restriction sites, one in the interval between the 21-OHA and C4B genes and one in the C4A gene. An unexpected finding in our study was the probable association of a 6.4 kb Taq I restriction fragment with a functioning C4A gene and a present but non-functional C4B gene. PFGE excluded a deletion in this haplotype. This might have arisen by previous multiple recombination events exchanging a portion of the 5' region of a short C4B gene for the corresponding region of the C4A gene containing the Taq I site (fig 1).

Deletion of the 21-OHB gene was not found in this group of patients, thus excluding the possibility that the association of the C4B null allele with Felty's syndrome may be secondary to a deletion of the adjacent active 21-OHB gene, though we cannot discount the possibility that the C4B null allele may be acting as a marker for a more subtle effect at the 21-OHB locus. A further possibility is that the C4B null allele may be acting as a marker for alleles at other class III or class II loci. A possible candidate in the class II subregion is the DQB variant DQw7, which was also found in association with DR4 positive Felty's syndrome.¹¹ In the class III subregion several new non-complement, but potentially immunologically important, genes have recently been identified¹² and would be worth investigating in Felty's syndrome. The last possibility for the association of Felty's syndrome with the C4B null allele is that the partial deficiency of the C4 protein itself has a direct effect on increasing the risk of this complication of extra-articular disease. This is in keeping with the variety of molecular mechanisms by which C4B null alleles have been shown to result. Early components of the classical pathway of complement activation, including C4, have a crucial role in preventing the precipitation of circulating immune complexes,¹³ and circulating immune complexes are thought to contribute to the pathogenesis of Felty's syndrome.¹⁴ Studies of C4 null alleles in other extra-articular complications of rheumatoid disease would be of interest.

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