C4 null phenotypes among lupus erythematosus patients are predominantly the result of deletions covering C4 and closely linked 21-hydroxylase A genes

JUKKA PARTANEN*, SAIJA KOSKIMIES*, AND EIJA JOHANSSON†

From *the Finnish Red Cross Blood Transfusion Service, Tissue Typing Laboratory, Helsinki; and †the Department of Dermatology, University Central Hospital, Helsinki, Finland.

SUMMARY Two genes, C4A and C4B, encoding the fourth component of the complement system are linked to the HLA complex. C4 defects or C4 'null' genes can predispose to an autoimmune disease, lupus erythematosus (LE). We have used Southern blotting techniques to analyse genomic DNA from 23 patients with LE and from healthy controls, to evaluate the molecular basis of the C4 null phenotypes. In addition to the high frequencies of C4 null phenotypes and HLA-B8, DR3 antigens, confirming earlier results, we observed that among the patients both the C4A and C4B null phenotypes mostly resulted from gene deletions. Among the controls only the C4A null phenotypes were predominantly the result of gene deletions. In all cases these C4 gene deletions also extended to a closely linked pseudogene, 21-hydroxylase A (21-OHA). Altogether, 52% of the patients and 26% of the controls carried a C4/21-OHA deletion.

Genetic studies^{1,2} of patients with lupus erythematosus (LE), an autoimmune disease, have shown that certain histocompatibility complex (MHC) markers predispose to LE, especially HLA-DR3 and a haplotype HLA-B8, DR3, as well as defects in the early components of the classical complement pathway, C1, C2, and C4.³

We have approached the role of the C4 defects, or C4 'null' alleles, in LE by determining HLA and C4 allotypes and the molecular basis of C4 null phenotypes in two subgroups of LE, namely in subacute cutaneous LE (SCLE) and in systemic LE (SLE). The study groups represent the two extremes of the clinical spectrum of LE. The characteristics of SCLE² are skin manifestations, photosensitivity, and a good prognosis, whereas patients with SLE^{1,2} have more severe renal or central nervous system involvement or both with a poor prognosis. After cloning the C4 genes^{4,5} a more detailed analysis of the C4 genes and defects was possible. The C4 gene region is located in the MHC between the HLA-B and HLA class II genes. It includes the duplicated C4 genes (C4A and C4B) and steroid 21-hydroxylase

(21-OHA and 21-OHB) genes.⁵ There is evidence⁵⁻⁸ that the number of C4 genes per chromosome may vary; certain C4 null phenotypes result from a gene deletion, whereas some chromosomes express three C4 products. Particularly, the C4A null phenotype in HLA-B8, DR3 haplotype is the result of a large deletion covering the C4A and 21-OHA genes. The presence of this deletion in the haplotype associated with LE and many other autoimmune diseases prompted us to study the C4 gene region in LE in more detail using the Southern blotting technique.

Subjects and methods

PATIENTS

The first group included 11 patients (10 women, one man) with subacute cutaneous LE.⁹ Duration of the disease varied from three to 37 years (mean 14.9 years). All the patients had pronounced photosensitivity and skin changes as the main manifestation. The clinical picture corresponded to that described before⁹ and the patients fulfilled no more than four of the ARA criteria for SLE. The second group included 12 patients (11 women, one man) with severe systemic LE. Duration of the disease varied from two to 21 years (mean 9.7 years). All the

patients fulfilled more than five of the ARA criteria for SLE.

CONTROLS

DNA was prepared from 23 healthy blood donors from the Finnish Red Cross Blood Transfusion Service, Helsinki, Finland. The HLA and complement Bf and C4 frequencies did not differ from a reference Finnish population.¹⁰⁻¹¹ The reference HLA-DR frequencies were based on typing of 322 blood donors.

HLA, BF AND C4 TYPINGS

HLA,¹⁰ Bf,¹² and C4¹³ typing was performed using standard methods. The C4 null alleles were assigned according to the relative staining intensities of the C4A and C4B protein bands measured by a scanning apparatus (Helena Laboratories Ltd) as described previously.¹⁴ This method could not identify the presence of C4 null heterozygosity in both C4 loci, that is, C4A3,0 B1,0 could not be distinguished from C4A3, 3 B1,1, thus a C4 null phenotype was assigned only when the intensity of the C4A (or C4B) band was less than half of C4B (or C4A). A C4 null was also assigned if a deletion of a C4 gene could be observed with Southern blotting. The nomenclature for the C4 alleles was according to Mauff *et al.*,¹⁵ and 'complotypes' were abbreviated, for example, S31 means Bf*S, C4A*3, C4B*1.

SOUTHERN BLOTTING

Genomic DNA was digested with restriction enzymes *HindIII*, *KpnI*, *TaqI*, and *XbaI* (Promega, Madison, WI). The digested DNA samples were size separated by 0.8% agarose gel electrophoresis, denatured, and blotted on Hybond-N nylon filters.⁸ Prehybridisation and hybridisation (48 hours at 42°C) were in 50% formamide, 6×SSC, 5×Denhardt's, 0.1% SDS, 50 mmol/l phosphate buffer, and 0.1 mg/ml denatured herring DNA. Filters were washed first in 2×SSC at room temperature and then twice in 0.3×SSC at 42°C. Restriction enzyme fragments were visualised by autoradiography. A full length C4 specific cDNA probe pAT-A,⁴ and a 21-OH specific probe p21-K4,¹⁶ were used. The pAT-A probe was labelled by nick translation (Amersham) and a 0.9 kb *BglI* fragment of p21-K4 was labelled by multiprime DNA labelling (Amersham). The probes were kindly provided by Dr Michael C Carroll, Harvard University, Boston.

INTERPRETATION OF SOUTHERN BLOTS

Enzymes *TaqI* and *KpnI* produce restriction enzyme fragments which have been shown^{5, 6, 16, 17} to be, with certain exceptions,^{6, 18} diagnostic for C4A (7.0

kb *TaqI*, 12.0 kb *KpnI*), C4B (6.4, 6.0, 5.4 kb *TaqI*, 3.5 kb *KpnI*), 21-OHA (3.2 kb *TaqI*, 4.0 kb *KpnI*) and 21-OHB (3.7 kb *TaqI*, 3.0 kb *KpnI*). A deletion covering the C4A and 21-OHA genes⁵⁻⁸ generates specific fragments, for example, in *TaqI* (6.4 kb) and *HindIII* (8.0 kb) digests, and the deletion was assigned if these fragments were observed with the C4 probe. For the other C4^{5, 6} and 21-OH deletions, there are no known specific markers, so they were determined by comparing the intensities of the gene specific bands in *TaqI* and *KpnI* analysis. The *TaqI* and *KpnI* fragments represent the 5' ends of the C4 genes. The intensities were measured by an automatic scanner (Scanner Oy Ltd, Helsinki). Only the bands in a single lane, and in a single hybridisation, were compared. The results were expressed as a numerical ratio of the A specific band intensities to the B intensities. It should be noted that the term 'deletion' here indicates only the apparent lack of these fragments.

Results

HLA, BF, AND C4 PHENOTYPES

Table 1 shows the frequencies of HLA-B8, DR2 and C4 null alleles in the two patient groups (SLE and SLE), in the controls, and in the Finnish reference population. The comparison showed that in both patient groups the frequencies of HLA-B8 and DR3 were about twice those in the controls and the reference population. Furthermore, the frequency of C4AO (60% in SLE and 54% in SLE group), but not C4BO, was clearly higher among the patients than among the controls (22%, $p<0.05$). The increased frequency of HLA-DR2 (64% v 33% in SLE, NS, and 25% in the controls, $p<0.05$).

TABLE 1 Frequencies of the patients with the HLA markers and C4/21-OHA gene deletions (C4 del) associated with LE in three study groups and in the reference population.

	SLE	SLE	Control	Reference
HLA-B8	36% (4/11)	50% (6/12)	18% (4/23)	19%*
HLA-DR2	64% (7/11)	33% (3/9)	25% (5/20)	33%†
HLA-DR3	45% (5/11)	44% (4/9)	20% (4/20)	32%‡
C4AO	60% (6/10)	54% (6/11)	22% (5/23)	18%*
C4A del	36% (4/11)	50% (6/12)	22% (5/23)	—§
C4BO	40% (4/10)	45% (5/11)	43% (10/23)	29%§
C4B del	27% (3/11)	17% (2/12)	9% (2/23)	—§
C4AO or C4BO	90% (9/10)	82% (9/11)	61% (14/23)	47%‡
C4A del or C4B del	54% (6/11)	50% (6/12)	26% (6/23)	—‡

* $p<0.05$, SLE+SLE v controls.

† $p<0.05$, SLE v controls, $p=0.18$ SLE v SLE.

‡ $p<0.10$, SLE+SLE v controls.

§ $p>0.10$, NS.

seemed to be an additional marker and the only specific marker for the SLE group.

C4 AND 21-HYDROXYLASE (21-OH) GENES

Based on *TaqI*, *KpnI*, and *HindIII* analysis (see Subjects and methods), 10 of the 23 patients (cases 1 to 4 with SCLE and 12 to 17 with SLE) had the normal restriction fragment patterns suggesting an absence of deletions. An example of the normal *TaqI* and *KpnI* patterns obtained with the C4 and 21-OH probes is shown in the figure, sample C2. Five of these 10 patients were phenotyped as having a heterozygous C4 null allele.

Twelve of the 23 patients (6 to 11 and 18 to 23 in table 2) had at least one deletion in the gene region. Seven patients (6, 7, 8, and 18 to 21) showed the 6.4 kb *TaqI* (two examples are shown in the figure) and the 8.0 kb *HindIII* bands which have been reported to be specific markers for a deletion of the C4A and 21-OHA genes and to be found in HLA-B8, BfS, C4AO, B1, DR3 haplotype. The deletion was further supported by the reduced intensities of the 21-OHA gene specific bands in *TaqI* and *HindIII* patterns. An example of *TaqI* and *KpnI* band patterns of a homozygote⁸ for haplotype B is shown in the figure, sample C1.

Three patients (11, 22, 23) had both the C4A/21-OHA deletion described above and a combined

deletion of the C4B and 21-OHA genes in the other chromosome. In the *TaqI* analysis with the C4 probe, these patients had, firstly, the 6.4 kb band showing that in this chromosome they had only

TABLE 2 HLA-B, DR, Bf, and C4 phenotypes, relative intensities of the 21-OHA v 21-OHB and C4A v C4B specific bands, and suggested gene deletions in patients with SCLE (1-11) and SLE (12-23).

Phenotypes					Band intensities		Deletion
HLA-B	DR	Bf	C4A	C4B	21-OHA:A/B	C4:A/B	21-OH C4
SCLE							
1-4	Various				1.0-1.2	0.7-1.0	None
5	35.47	/1.2/FF/3.3	1.0		1.6	0.5	?
6	8.18	/2.3/SS/0 ⁺	+		0.5	0.3	A A
7	8.5	/1.3/FS/3.0	1.1		0.5	0.6	A A
8	8.51	/3.7/SS/3.0	1.0		0.6	0.3	A A
9	18.35	/1.2/FS/3.2	1.0		0.7	2.2	A B
10	35	/1/FF/3.2	0.0		0.6	3.5	A B
11	8.7	/3/SS/3.0	1.0 ⁺		0	1.0	A.A A.B
SLE							
12-17	Various				1.0-1.3	0.8-1.1	None
18	8.18	/3/SS/3.0	1.1		0.5	0.2	A A
19	8.w62/-	/SS/0 ⁺	+		0.5	0.5	A A
20	8.7	/3/SS/3.0	1.1		0.6	0.4	A A
21	8.18	/-/SS/4.0	1.2		0.6	0.4	A A
22	8.35	/1.3/SS/0 ⁺	0 ⁺		0	0.7	A.A A.B
23	8.51	/3.4/SS/3.0	1.0 ⁺		0	0.6	A.A A.B

⁺A C4 null allele determined only on the basis of DNA analysis.

⁺C4 phenotype could not be determined from the serum.

FIGURE Genomic DNA of patients 6, 7, and 11 and of two controls digested with *TaqI* (left) and *KpnI* (right) and hybridised with the C4 and 21-OH probes. The sizes of the bands are shown in kb. Samples: C1=a control homozygous for HLA-B8, DR3, BfS, C4AO (deletion), B1; C2=a control without a deletion in the gene region.

one C4B gene and no C4A gene, and, secondly, the 7.0 kb band showing that in the other chromosome they had a C4A gene without a C4B gene, because no 6.0 kb nor 5.4 kb was observed (figure). Lack of a 3.5 kb *KpnI* band (figure) confirmed this.¹⁹ Moreover, the 21-OH probe did not detect the 21-OHA bands (figure) suggesting a homozygous deletion of the gene.

Patients 9 and 10 may have a heterozygous C4B/21-OHA deletion. Their C4B specific *TaqI* band (6.0 kb in both cases) had an intensity of about half of the C4A specific 7.0 kb *TaqI* band, and, furthermore, the 21-OHA bands had a lower intensity than the 21-OHB bands (table 2). Their C4 phenotypes supported the result as both of them had C4BO. An accurate determination of the deletion would, however, require at least a family study, which was not possible.

The organisation of the C4 gene region in patient 5 could not be accurately determined. The intensity of the 21-OHA band was 1.6 times that of the 21-OHB band (table 2) suggesting that there may be three 21-OHA and two 21-OHB genes. Using the C4 probe with *TaqI*, *KpnI*, and *HindIII*, the result did not differ from the normal population. However, enzyme *XbaI* gave a band pattern suggesting a C4B gene duplication (data not shown) that was not observed in the other patients.

The frequencies of the patients and controls with the gene deletions are summarised in table 1. The two patient groups, SCLE and SLE, did not differ from each other and were combined for statistical purposes. As expected from the C4 phenotypes, the frequency of the C4A/21-OHA deletion (10/23, 43%) in the patients was higher than in the controls (22%, NS). Interestingly, although the frequency of the phenotypic C4BO between the patients and the controls did not deviate (43% v 43%), the frequency of the C4B/21-OHA deletions among the patients (22%) was more than twice that of the controls (9%). The difference was, however, not statistically significant. Of the phenotypic C4A nulls, 83% of the patients and 100% of the controls involved deletions, and the proportions for the C4B nulls were 55% and 20%, respectively.

Discussion

It is established that defects in the complement C4 genes can predispose to LE,^{1,3} and our present study in Finnish patients confirmed this: 90% of the patients carried at least one C4 null. Furthermore, we confirmed the high frequency of HLA-B8 and DR3 in both subgroups of LE. The comparison between the two groups, SCLE and SLE, showed that they had similar MHC patterns except that

HLA-DR2 might be a specific MHC marker for SCLE.

HLA-B8, SO1, DR3 haplotype has been shown^{6-8,19} to carry a large deletion of the C4A and 21-OHA genes. Therefore, we studied the nature of C4 null alleles in patients with LE in more detail using Southern blotting. With regard to C4A, the increase in the frequency of the C4AO allele could be explained by the increased frequency of the gene deletions: the proportion of C4A deletions per C4A nulls did not differ between the patients and controls. The proportion was high, about 80% of the C4A null phenotypes resulted from a deletion, and in all cases the deletion seemed to be of one simple type, that is, the same as described originally by Carroll *et al*¹⁹ in BfS, C4AO, B1 complotype. The molecular basis of the C4B null phenotypes appeared to be more heterogeneous. Firstly, in contrast to the C4A/21-OHA deletion strongly associated with HLA-B8, DR3, the C4B/21-OHA deletions were observed with various HLA-B and DR antigens which may indicate independent origin of these deletions. Secondly, the deletions explained only half of the C4B null phenotypes. We could not perform more detailed mapping of the C4B deletions, because none of the patients or the controls was homozygous. The interpretation of the C4 gene organisation on the basis of Southern blotting clearly poses some problems, because it is not easy to distinguish between a gene deletion and a duplication. This approach, when combined with phenotypic and family data, should, however, give a better picture of the role of C4 defects in diseases than C4 typing alone because, for example, variation in C4 levels does not affect the result.

With regard to the equal frequency of the C4B null phenotype in the study groups, the increased frequency of the C4B/21-OHA deletions among the patients (22% v 9%) was surprising. Undetected C4B null alleles among the patients could explain this, but the fact that the study groups were typed using the same criteria does not support this, although determination of the C4 null alleles is certainly a problem. Therefore, it is possible that the gene deletions themselves play a role in susceptibility to LE, together with other high risk factors like MHC class II genes. An interesting hypothesis is that the 21-OHA gene, which seems always to be deleted with the C4 genes, could have some unknown regulatory function. The 21-OHA is a pseudogene¹⁷ but its functional counterpart, 21-OHB, encodes an enzyme of steroid biosynthesis.^{5,17} This would provide a link between HLA and the higher risk for autoimmunity among females. Moreover, a report⁷ has shown that the 21-OHA gene, together with a C4 gene, was deleted in

two haplotypes associated with another autoimmune disease, type I diabetes. Thus, those C4 null alleles which result from a C4 and 21-OHA deletion would cause a higher risk of autoimmunity than those without a deletion, because the C4 defect impairs the clearance of immune complexes,²⁰ and the 21-OHA deletion changes the steroid balance. A possible effect of a 21-OHA deletion on the concentrations of corticosteroids and sex hormones would be worth testing. Another possibility is that only the C4 deletions would be the 'real' C4 null genes, that is, they are not expressed. The other C4 null phenotypes might result from, for instance, gene duplications actually expressing two identical C4 molecules as recently shown.¹⁸

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Correspondence and requests for reprints to Dr Jukka Partanen, FRC Blood Transfusion Service, Kivihaantie 7, SF-00310 Helsinki, Finland.