C4 concentrations and C4 deficiency alleles in systemic lupus erythematosus

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SUMMARY In a study of 66 patients with systemic lupus erythematosus (SLE) and 80 controls it was found that the presence of two deficiency (null) alleles of C4 had a significant effect on mean C4 concentrations in serum. In six controls who each had two C4 null alleles the mean C4 concentration in serum was 56% lower than in 43 controls without C4 null alleles; the nadir of the C4 concentration in four patients with SLE with two null alleles was also lower by a mean of 55% than in 32 patients who did not have null alleles. Reduced production of C4 allotypes in subjects with two null alleles may be an important determinant of total C4 concentration in patients with SLE. For optimal interpretation of C4 concentrations in SLE, C4 allotyping appears to be indicated, particularly to identify patients who have two null alleles of C4.

Systemic lupus erythematosus (SLE) is a multisystem disease that is characterised by continuing immune complex formation and depletion of complement proteins in serum. C4 concentrations in serum are widely used to monitor the clinical course of the disease. The relation between disease activity and C4 concentrations, though clinically useful, is often imprecise, possibly because multiple factors, including synthesis as well as activation, determine the concentration of C4 in serum. Studies have shown that C4 is composed of two isotypes, C4A and C4B, that both participate in the classic pathway of complement activation and are both subject to genetically controlled polymorphism.¹ C4A is co-dominantly encoded on the sixth human chromosome, and C4B is similarly encoded at a closely linked, but separate, locus.² Deficiency (null) alleles fail to code for their respective proteins in serum and occur in 60–80% of patients with SLE, as well as in 35–40% of the general population.^{3–6} We and others have shown that C4A null alleles, in particular, are associated with increased risk of SLE.^{3–6}

We report the present study because C4 concentration in serum appears to be influenced by the number of deficiency alleles present^{7,8}; this may be clinically important in SLE as the monitoring of SLE

by serum C4 concentrations would require the identification of null alleles in all patients. C4 concentrations have been studied in relation to C4 null alleles in four patients with SLE,⁸ but there have been no other similar reported studies. We therefore studied patients with SLE and controls to determine whether the presence of null alleles was a significant determinant of C4 concentration in SLE.

Patients and methods

Sixty six patients with SLE (63 female, three male, age 19 to 81 years, mean 41, 59 blacks, seven Caucasians) who had been followed up in our clinic for a minimum of two years (range 2–15) and 80 healthy controls were studied. The features of the 59 black patients have been previously described.⁶ SLE was defined by the presence of four or more of the revised American Rheumatism Association criteria. Patients had been followed up using established clinical protocols, in which clinical and laboratory data were entered into flow charts at each visit to the clinic. Records of admission to hospital were also reviewed. C4 concentration was measured when clinically indicated during follow up of the patients; in patients with SLE C4 concentrations were measured an average of 33 times (range 4 to 110) in each patient. We analysed the relation between C4 null alleles and the highest and lowest C4 concentration recorded during clinical follow up of each

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patient, as well as the C4 concentration at a point when SLE was clinically active, and again when SLE was less active. Clinically active disease was defined as a worsening of the patients' clinical status that necessitated increased levels of treatment for SLE (all patients), or admission to hospital in 52% of patients with active SLE. Patients with active SLE had two or more criteria for disease activity as defined by Urowitz *et al.*¹⁰

Eighty controls (12 male, 68 female, age 18 to 78 years, mean 36) were healthy employees and students at the medical centre. The study was approved by the institutional review board.

C3 AND C4 CONCENTRATIONS IN SERUM

Serum for the determination of C3 and C4 concentrations was obtained during clinical follow up and assayed within two weeks of venepuncture by single radial immunodiffusion in agar (Mely). Normal ranges suggested by the manufacturer for C4 are 140–680 mg/l and for C3 930–2620 mg/l.

C4 ALLOTypING

Edetic acid-plasma for C4 allotyping was stored at -70°C for a maximum of four weeks before assay and was analysed as described previously.^{1,6} Plasma was treated with neuraminidase, dialysed, and then electrophoresed in duplicate through 0.5% agarose in trometamol (TRIS)-glycine-barbital-edetic acid buffer pH 8.8, at 20 V/cm for six hours on a cooled plate.¹ This was followed by immunofixation of one sample with anti-C4 antibody (Atlantic Antibodies) and washing and staining of the gel with Coomassie blue. C4A3 B1 standards were included in each plate and a C4A6 standard was included where necessary. C4 phenotypes were assigned using published criteria.¹¹ The C4A:C4B ratio was measured by crossed immunoelectrophoresis of the excised duplicate sample from the first electrophoresis; this sample was incorporated into a new agarose gel containing 0.8% monospecific anti-C4 antibody and electrophoresed. After staining the gel the areas under the C4A and C4B precipitin peaks were measured and the C4A:C4B ratio was computed. 95% Confidence limits (0.60 to 1.40) for this ratio when four alleles were present were established by studies in 25 subjects from three families, in whom HLA-A, B, and C as well as C4 allotyping were done to establish the haplotypes that contained C4 null alleles in these families; other subjects who each had four C4 phenotypes were also included in computing the confidence limits. When indicated, some samples were also analysed by overlaying a C4 dependent haemolytic gel on the allotypes after the first electrophoresis. This haemolytic gel consisted of antibody sensitised sheep

erythrocytes (0.9%) in buffered agarose incorporating C4 deficient guinea pig serum. If the C4 concentration in plasma was too low for technically satisfactory allotyping the allotyping was repeated when the patients' C4 concentrations had returned to adequate levels. C4 null alleles were assigned as follows: Group 1—Zero null alleles present: C4A:C4B ratio was within the 95% confidence limits for four functioning alleles. Because of the possibility¹ that this group might have included subjects who were each heterozygous for coexistent C4A and C4B null alleles (balanced hemizygous nulls) we examined the C4 concentrations in a subset of group 1 subjects whom we considered were most unlikely to have balanced hemizygous nulls as they each had three or four C4 phenotypes as well as a C4A:C4B ratio within the 95% confidence limits for four functioning alleles. Although the mean C4 concentrations in this subgroup were not significantly different from those of the group 1 subjects with two allotypes, subjects with balanced hemizygous null alleles could not be completely excluded by this method. Group 2—One null allele present: C4A and C4B were both present and their ratio was outside the 95% confidence limits for four alleles. Each subject in this group was assigned one C4A or C4B null allele. Group 3—Two null alleles present: Either C4A or C4B was completely absent from serum.

Statistical methods

The significance of the differences between mean values for the various groups of subjects was examined by Student's two tailed *t* test and by Wilcoxon's rank test for non-parametric data.

Results

A wide range of C4 values was seen in both patients and controls. Patients with SLE with active disease had lower mean C3 and C4 concentrations than patients with less active disease (Table 1). The numbers of patients with SLE with zero, one, and two null alleles were 32, 30, and 4 respectively; for controls, the corresponding numbers were 43, 31, and 6 (Fig. 1, Table 1). When controls who had zero null alleles were compared with controls with one null allele there were slight and non-significant differences in mean C4 and C3 concentrations (Fig. 1, Table 1). The mean C4 concentration in 43 controls who had zero null alleles was 379 (SD 134) mg/l compared with 341 (128) mg/l in 31 controls who had one null allele of C4 (Fig. 1), $p>0.05$. During clinical follow up of the patients with SLE the mean of the highest C4 concentration was the

Table 1 Complement concentrations in serum of 66 patients with systemic lupus erythematosus (SLE) in relation to the number of null alleles of C4. Each patient had C3 and C4 concentrations determined at four points during clinical follow up. The mean of the complement concentrations (SD) that were highest and lowest as well as those during active and less active (inactive) SLE are shown

	C4 (mg/l)			C3 (mg/l)		
	Zero null alleles (n=32)	1 Null allele (n=30)	2 Null alleles (n=4)	Zero C4 null alleles (n=32)	1 Null allele of C4 (n=30)	2 Null alleles of C4 (n=4)
Highest	470 (160)	470 (140)	390 (240)	2020 (450)	2290 (500)	2530 (700)
Lowest	150 (110)	130 (80)	70 (20)**	830 (530)	880 (500)	630 (340)
Active	150 (100)	130 (90)	110 (40)	780 (380)	840 (460)	900 (550)
Inactive	340 (210)	300 (120)	290 (70)	1530 (330)	1680 (420)	1780 (280)

**p<0.001 (Student's *t* test) compared with the mean of the lowest C4 concentration in patients with zero null alleles. Differences among groups were also analysed by Wilcoxon's non-parametric rank test (see text).

Fig. 1 Serum C4 concentration in 80 controls, and the lowest serum C4 concentration during follow up of 66 patients with systemic lupus erythematosus (SLE), in relation to the number of null alleles of C4. The mean and SD for each group are shown. *p<0.002 compared with controls with zero null alleles; **p<0.001 compared with patients with SLE with zero null alleles.

same in 30 patients with one null allele as in 32 patients with zero null alleles; at the three other points during their clinical follow up non-significant decreases of less than 15% in mean C4 concentration were present in patients with one null allele compared with patients with zero null alleles (Table 1). There were also no significant differences in C4 concentrations which could be attributed to single C4A or single C4B null alleles.

Five controls each had two C4B null alleles and one control had two C4A null alleles (Fig. 1). C4 concentrations in these six controls were 167 (72) mg/l and were 56% lower than the C4 concen-

trations in 43 controls who had zero null alleles—30 (134) mg/l, p<0.002. Three patients with SLE each had two C4B null alleles and one patient with SLE had two C4A null alleles. In these four patients with SLE the mean of the lowest C4 concentration was 55% lower than in the 43 patients who did not have a null allele (p<0.001); however, the presence of two null alleles was not significantly related to mean C4 concentration at other points during the clinical follow up (Table 1). In patients with SLE the presence of two null alleles of C4 was also not significantly related to mean C3 concentrations (Table 1).

Discussion

This study is of interest because of the possibility that null alleles of C4 may be important in assessing the clinical significance of low C4 concentrations in SLE.^{7,8} We and others have previously reported that C4A null alleles are associated with risk of developing SLE,³⁻⁶ and these studies have shown that 55–75% of patients with SLE have one C4A or C4B null allele.^{3,5,6} The present study was done because of repeated and potentially important suggestions that the monitoring of SLE by C4 concentrations in serum may require C4 allotyping in all patients.^{4,7,8}

In the six control subjects who each had two null alleles the mean C4 concentration was reduced by 56% compared with controls who had zero null alleles. These observations are in agreement with those reported by Awdeh *et al.*¹ Similarly, the nadir of the C4 concentration observed during clinical follow up was 55% lower in patients with two null alleles than in patients with zero null alleles. The presence of two null alleles of C4 did not have a significant effect on C4 concentrations obtained at other points during the clinical follow up of these

patients. Patients with two null alleles comprise 7–14% of most SLE populations,^{3 5 6} but there appears to be no single clinical feature that distinguishes them from other patients with SLE. We have previously reported, however, that these patients all exhibited antibody to the soluble nucleoprotein antigen SSA (Ro), as well as cutaneous features^{6 12}; in these respects they resemble patients with total C4 deficiency, in whom C4 is genetically absent from serum.¹³

The immunochemical concentration of C4 in serum has been shown to be roughly related to the number of null alleles of C4 in individuals from several kindreds^{1 14} and in population studies of non-SLE subjects.^{1 7} In these studies, however, including the present one, there was considerable variation in the C4 concentration in subjects who had the same number of C4 null alleles,^{1 7} and the present study did not demonstrate a statistically significant association between single null alleles and the C4 concentration in serum. During clinical follow up of our patients with SLE the mean of the highest C4 concentration was the same in patients with one null allele as in patients with zero null alleles; at the three other points during their clinical follow up non-significant decreases of less than 15% in the mean C4 concentration were present in patients with one null allele, compared with patients with zero null alleles.

In the present study a number of factors may have obscured the effect of C4A and C4B null alleles on the C4 concentration in serum. Such factors include acute phase protein responses in some subjects¹⁵ and variable degrees of C4 activation and consumption in patients with SLE. Such activation may also reduce the precision with which native C4 concentration is immunochemically measured in serum, as the C4 antiserum used in immunochemical assays may also precipitate with activation (split) products of C4. It should also be noted that without family studies the assignment of null alleles of C4 by the methods used in this study is subject to some uncertainty, mainly owing to the possible presence of some subjects who had balanced hemizygous null alleles that could not be detected by the methods used in this study. The possible admixture within the groups that were assigned zero null alleles of a few subjects who had balanced hemizygous C4A and C4B null alleles would tend to obscure any apparent effects of null alleles on the C4 concentration in serum. Moreover, duplication of the C4A and C4B genetic loci has been demonstrated in occasional kindreds,^{16 17} and if present, this may also obscure the differences among the various groups of subjects. In some subjects there may also be genetic control of C4 synthesis that is independent of known C4

structural genes; in one kindred there appeared to be a genetic basis of C4 deficiency that did not segregate with HLA haplotypes.¹⁸ Our results in the control population in the present study are, however, similar to those of Awdeh *et al* who studied kindreds.¹ Using somewhat different techniques, Uko *et al*, however, found a significant reduction of approximately 35% in the mean C4 concentration, owing to the presence of single null alleles, in a mixed group of healthy subjects and patients with diabetes mellitus.⁷ Differences in techniques used to identify null alleles and in the selection of subjects, in addition to the factors discussed above, may account for these somewhat variable findings. In all the above studies, however, including the present one, the mean serum C4 concentration in the group with one null allele was in fact intermediate between the C4 concentration in the group with zero and the group with two null alleles. The use of specific assays for native C4 in plasma, as well as family studies and nucleic acid hybridisation techniques to identify balanced hemizygous null alleles and C4 gene duplications, would help to define clearly the contribution of a single C4 null allele to lowered C4 concentrations in these subjects.

In conclusion, the presence of two null alleles of C4 caused reduced mean C4 concentrations in serum in controls as well as in patients with SLE. As the presence of two null alleles of C4 appears to be associated with lower C4 concentrations in patients with SLE there may be some tendency to overtreat these patients if the C4 concentration in serum is used to monitor SLE disease activity. For optimal interpretation of C4 concentration during the clinical follow up of SLE, C4 allotyping therefore appears to be indicated, particularly to identify patients who have two null alleles.

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