Influence of C4 null alleles on C4 activation in systemic lupus erythematosus

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Abstract

Deficiencies of early components of the classical complement pathway are known to be associated with systemic lupus erythematosus (SLE). C4 null alleles, C4A Q0 and C4B Q0, are prime candidates for the major histocompatibility complex associated factor which determines susceptibility to SLE. There is poor correlation, however, between the presence of low concentrations of C4 and possession of C4 null alleles, and thus the basis of the association between C4A Q0, C4B Q0 and SLE remains obscure. The possibility that activation of C4 may be related to the possession of C4 null alleles was examined. C4 phenotypes were investigated, and C4 concentration and activation were estimated in patients with SLE. C4 activation was determined by measuring the concentration of C4d—a split product of C4. Twenty five of 35 patients had C4 phenotypes which include null alleles. No association between low C4 concentrations and C4 null alleles was found, but a significant association between low C4d concentrations and C4 phenotypes including null alleles, particularly those with C4A Q0, was noted. No correlation between concentrations of C4 and C4d was found. These results show an influence of C4 null alleles on the activation of the C4 molecule, which is independent of the concentration of C4. The possession of silent genes coding for C4 null alleles might predispose to SLE by conditioning poor C4 activation, a critical event for the clearance of immune complexes mediated by the classical complement pathway.

Expression of C4, the pivotal component of the classical pathway of the complement system,¹ is controlled by duplicated genes located within the major histocompatibility complex (MHC). Two isotypes, C4A and C4B, exist,² which can be differentiated by their electrophoretic mobility,³ antigenicity,⁴ and function.^{3 5–7} C4A is more efficient than C4B in prevention of the formation of insoluble immune complexes, an important physiological function of the classical complement pathway.^{7–9} Both C4 genes show a high degree of structural polymorphism, and a relatively high frequency of null, or non-expressed, alleles (C4A Q0 and C4B Q0).¹⁰

In systemic lupus erythematosus (SLE)—the prototype immune complex disorder¹¹—continuing classical pathway activation, which handles and disposes of pathological loads of immune complexes, can be shown during active as well as inactive phases of the disease.¹² Acti-

vation of the classical pathway can be assessed by measurement of the concentration of C4d, a conversion fragment of C4.¹³

There is a strong association between SLE and genes in the MHC, especially DR3 and C4A Q0,^{14–18} but strong linkage disequilibrium makes it difficult to determine which, if either, is the primary disease marker. Although an association between MHC class II type and autoantibody status has been established,¹⁹ recent data implicate C4A Q0 as the primary MHC susceptibility marker for SLE.²⁰ This is consistent with the high prevalence of patients with lupus who have complete deficiencies of early components of the classical pathway.²¹ As low concentrations of C4 are not a necessary consequence of C4 null alleles,²² however, the basis for the association between C4 null alleles and SLE remains obscure. It has been suggested that the functional difference between C4A and C4B in their ability to prevent immune complex precipitation⁷ explains the high prevalence of C4A Q0 in this disease.^{16–18} The purpose of this study was to examine a further hypothesis—namely, that the possession of C4 null alleles might predispose to SLE by affecting activation of C4.

Subjects and methods

SUBJECTS

Thirty five patients with SLE were studied, chosen at random from those attending the lupus clinic held weekly in the Bloomsbury Rheumatology Unit. Each patient met four or more of the revised criteria of the American Rheumatism Association for the classification of the disease.²³

BLOOD COLLECTION

Blood (5 ml) collected in a final concentration of EDTA of 10 mmol/l was immediately separated by centrifugation at 1000 g for 15 minutes at 4°C and the plasma stored at –70°C.

C4 PHENOTYPING

Analysis of C4 polymorphism was performed by immunofixation of electrophoresed desialated plasma according to Awdeh and Alper.³ C4 isotypes were assigned by analysing their haemolytic function.³ Null alleles were defined on the basis of the C4A/C4B ratio, as assessed by densitometric scanning of the C4 gels; this allows identification of single null alleles and double null alleles if both are at the same C4 locus, not if they are one at each C4 locus. Thus

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Table 1 C4 phenotypes* in the patients with systemic lupus erythematosus studied

Phenotype group‡	Phenotype§		Band intensity	
	C4A	C4B	% C4A	% C4B
1	3 3	1 1	46	54
1	3 3	2 1	54	46
1	3 2	1 1	52	48
1	3 3	2 2	60	40
1	3 3	1 1	55	45
1	3 3	1 1	46	54
1	3 3	1 1	51	49
1	3 3	1 1	49	51
1	3 3	2 1	51	49
1	4 2	2 1	50	50
2	0 3	1 1	38	62
2	0 3	1 1	27	73
2	0 3	1 1	39	61
2	0 3	1 1	27	73
2	0 3	1 1	19	81
2	0 2	1 1	34	66
2	0 3	1 1	33	67
2	0 3	1 2	24	76
2	0 6	3 1	27	73
2	0 3	1 1	29	71
2	0 3	1 1	25	75
2	0 3	1 1	32	68
2	0 3	1 1	38	62
2	0 3	1 1	†	†
2	0 3	1 1	38	62
2	0 3	1 1	25	75
2	0 3	1 1	34	66
2	0 3	1 1	32	68
3	3 3	0 1	64	36
3	3 2	0 1	†	†
3	3 3	0 1	†	†
3	3 3	0 1	61	39
4	0 0	1 1	0	100
4	0 0	1 1	0	100
4	0 0	1 1	0	100

*C4 phenotypes were assigned from the combination of structural polymorphism (electrophoretic mobility) data and ratio of C4A to C4B isotypes.
†C4A/C4B ratio not determined.
‡The phenotype groups 1-4 were as follows 1=A B; 2=A0 BB; 3=AA B0; 4=00 BB.
§0 refers to a null allele, derived from the proportion of the two isotypes.

Table 2 Median and range values of C4 and C4d in the phenotype groups of patients

Phenotype group*	Median (range)	
	C4	C4d
1	0.31 (0.10-0.43)	94 (58-157)
2	0.23 (0.12-0.47)	54 (24-135)
3	0.25 (0.21-0.51)	44 (30- 98)
4	0.19 (0.13-0.24)	43 (22-43)

*The phenotype groups 1-4 were as follows: 1=A B; 2=A0 BB; 3=AA B0; 4=00 BB.

Figure 1 Concentrations of C4 (left, ●) and of C4d (right, ○) in patients with systemic lupus erythematosus having C4 non-null phenotypes (A B), and C4 null phenotypes containing heterozygous C4A Q0 (A0 BB), heterozygous C4B Q0 (AA B0), and homozygous C4A Q0 (00 BB). C4 concentrations are in g/l. C4d values are given as percentages of a 100% C4d standard. Closed and open arrows point to the median values of C4 and C4d respectively.

C4 phenotypes including one C4A Q0 and one C4B Q0 are indistinguishable from phenotypes which do not contain any null alleles. Five C4 phenotype groups are therefore derivable: (a) A B=no detectable null alleles; (b) A0 BB=a single null allele at the C4A locus; (c) AA B0=a single null allele at the C4B locus; (d) 00 BB=homozygous C4A null allele; (e) AA 00=homozygous C4B null allele, where A and B refer to any expressed allele of the respective isotype.

C4 MEASUREMENT

C4 concentrations were determined in plasma by laser nephelometry, using specific antiserum (Behring Diagnostics, Hounslow, Middlesex) in a Behring laser nephelometer, according to the manufacturer's instructions. Results were expressed in grams per litre.

C4d MEASUREMENT

C4d concentrations were determined by laser nephelometry.¹³ Briefly, plasma was brought to 12% final concentration of polyethylene glycol 6000 and centrifuged at 1500 g for 30 minutes at 4°C. This manoeuvre precipitates C4 and its larger fragments, leaving free C4d in the supernatant. The concentration of C4d was then measured by laser nephelometry, using anti-C4 intact antiserum (Behring Diagnostics). C4d results were expressed as percentages of a 100% C4d standard, obtained by exhaustive activation of complement through the classical pathway.

STATISTICAL ANALYSIS

Statistical evaluation of the results was carried out with Wilcoxon's rank sum test and Spearman's rank correlation analysis.

Results

C4 PHENOTYPES

Table 1 shows the C4 phenotypes of the patients with SLE. Twenty five of 35 patients had C4 null alleles. Distribution of the null alleles among the C4 phenotype groups of the patients was as follows: 10 patients had non-null phenotypes (A B), 18 heterozygous C4A Q0 (A0 BB), four heterozygous C4B Q0 (AA B0), and three homozygous C4A Q0 (00 BB).

C4 AND C4d CONCENTRATIONS

Figure 1 shows the C4 and C4d concentrations in the patient groups as defined by the distribution of the C4 phenotypes A B, A0 BB, AA B0, 00 BB. Table 2 gives the median and range values.

Concentrations of C4 possessed by patients in the C4 null phenotype groups were all within or above the range of C4 concentrations in patients in the non-null phenotype group. In contrast, the concentrations of C4d were lower in patients with C4 null phenotypes than in the patients with non-null phenotypes (p<0.02). In particular, C4d concentrations were significantly lower in patients with either single C4A Q0 or

Figure 2 Lack of correlation between concentrations of C4 and C4d. C4 concentrations are in g/l. C4d values are given as percentages of a 100% C4d standard.

double C4A Q0 phenotypes ($p < 0.03$, $p < 0.01$, respectively) than in those with non-null phenotypes. Ten of 18 patients with the single C4A Q0, 3/4 with the single C4B Q0, and 3/3 with the double C4A Q0 had C4d values below the range of C4d in patients with non-null phenotypes.

No significant correlation was found between concentrations of C4 and C4d either in the patients as a whole or in the patients divided into the C4 phenotype groups (fig 2), as assessed by Spearman's rank correlation analysis.

Discussion

This study shows that C4 null alleles, prime candidates for the MHC associated factor which confers susceptibility to SLE,²⁰ are associated with impaired activation of the C4 molecule.

The prevalence of C4 null alleles in patients with SLE has been shown to be high,^{16-18 24} and our observations are consistent with this. We suggested that a defect in C4 activation might be of pathogenetic relevance in SLE and explored this in relation to C4 null alleles. In this paper we present data showing that in SLE the degree of C4 activation, as indicated by the concentration of C4d, is indeed dependent on C4 null alleles, being reduced in patients with null phenotypes. This implies an influence on C4 activation by C4 genes, which cannot be explained by an association of C4 null alleles with low C4 concentrations because, firstly, low C4 concentrations were not associated with C4 null phenotypes, and, secondly, C4d concentrations were unrelated to the concentrations of C4. We think it unlikely that the association between low C4 activation and C4 null alleles is due to linkage disequilibrium between C4 null genes and a locus encoding factors regulating C4 activation because C1 proteins, which directly activate C4,¹ are determined by genes on chromosomes 1 and 12,^{25 26} and no other relevant genes are found in the MHC.

A current theory to explain the susceptibility to SLE conferred by the inheritance of null

genes at the C4A locus is based on the observation that C4A and C4B gene products display differential abilities to inhibit immune complex precipitation.⁷ In this respect C4A is more efficient than C4B, hence the assumption that by influencing the concentration of the C4A isotype the C4A null gene determines a partial defect in this process. Our results suggest an additional mechanism by which the C4 null genes might predispose to SLE—that is, the impairment of C4 activation. Impairment of inhibition of immune complex precipitation by the C4A null genes may therefore take place in two stages, with additive effect. Although impaired C4 activation seems to be associated with both C4A Q0 and C4B Q0, it may be that subjects with C4B null alleles can compensate for this defect at a later stage in the classical pathway by exploiting the ability of C4A to prevent the formation of insoluble immune complexes. This consideration would help to explain why in SLE the prevalence of C4A Q0 is higher than that of C4B Q0.¹⁶⁻¹⁸

In conclusion, this study shows a relation between C4 activation and possession of C4 null alleles in patients with SLE. Further investigation is required to understand whether this is specific to this disease or applies equally to disorders such as systemic sclerosis,^{27 28} which are also characterised by accelerated C4 turnover and increased prevalence of C4 null alleles.

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- Cooper N R. The complement system. In: Stites D P, Stobo J D, Wells V J, eds. *Basic and clinical immunology*. 6th ed. East Norwalk: Appleton and Lange, 1987: 114-27.
- Robinson M A, Carroll M C, Johnson A H, Hartzman R J, Belt K T, Kindt T J. Localization of the C4 genes within the HLA complex by molecular genotyping. *Immunogenetics* 1985; 21: 143-52.
- Awdeh Z L, Alper C A. Inherited structural polymorphism of the fourth component of the human complement system (C4). *Proc Natl Acad Sci USA* 1980; 77: 3576-80.
- O'Neill G J, Dupont B. Serum C4 levels, Chido, Rodgers, and allotypes of C4 component of complement. *Transplant Proc* 1979; 9: 1102-6.
- Law S K A, Dodds A W, Porter R R. A comparison of the properties of two classes, C4A and C4B, of the human complement component C4. *EMBO J* 1984; 3: 1819-23.
- Isenman D E, Young J R. The molecular basis for the difference in immune hemolysis activity of the Chido and Rodgers isotypes of human complement component C4. *J Immunol* 1984; 132: 3019-27.
- Schifferli J A, Steiger G, Paccaud J P, Sjöholm A G, Hauptman G. Difference in the biological properties of the two forms of the fourth component of human C4. *Clin Exp Immunol* 1986; 63: 473-7.
- Takahashi M, Takahashi S, Brade V, Nussenzweig V. Requirements for the solubilisation of immune aggregates by complement. The role of the classical pathway. *J Clin Invest* 1978; 62: 349-58.
- Schifferli J A, Woo P, Peters D K. Complement inhibition of immune precipitation. I. Role of the classical and alternative pathways. *Clin Exp Immunol* 1982; 47: 555-62.
- Mauff G, Alper C A, Awdeh Z, et al. Statement on the nomenclature of human C4 allotypes. *Immunobiology* 1983; 164: 184-91.
- Koffler D, Agnello V, Thoburn R, Kunkel H G. Systemic lupus erythematosus: prototype of immune complex nephritis in man. *J Exp Med* 1971; 134 (suppl): 169-79.
- Senaldi G, Makinde V A, Vergani D, Isenberg D A. Correlation of the activation of the fourth component of complement (C4) with disease activity in systemic lupus erythematosus. *Ann Rheum Dis* 1988; 47: 913-7.
- Davies E T, Nasaruddin B A, Alhaq A, Senaldi G, Vergani D. Clinical application of a new technique that measures C4d for assessment of activation of the classical complement pathway. *J Clin Pathol* 1987; 41: 143-7.
- Black C M, Welsh K I, Fielder A H L, et al. HLA antigens and Bf allotypes in systemic lupus erythematosus: evidence for the association being with specific haplotypes. *Clin Exp Immunol* 1982; 19: 115-20.
- Shur P H, Meyer I, Garavoy M, Carpenter C B. Associations between systemic lupus erythematosus and the major

- histocompatibility complex: clinical and immunological considerations. *Clin Immunol Immunopathol* 1982; 25: 263-75.
- 16 Fielder A H L, Walport M J, Batchelor J R, *et al*. A family study of the major histocompatibility complex of patients with SLE: null alleles of C4A and C4B may determine disease susceptibility. *BMJ* 1983; 286: 425-8.
 - 17 Howard P F, Hochberg M C, Bias W B, Arnett F C, McLean R H. Relationship between C4 null genes, HLA-D region antigens, and genetic susceptibility to systemic lupus erythematosus in caucasian and black Americans. *Am J Med* 1986; 81: 187-93.
 - 18 Kemp M E, Atkinson J P, Skanes V M, Levine R P, Chaplin D D. Deletion of C4A genes in patients with systemic lupus erythematosus. *Arthritis Rheum* 1987; 30: 1015-22.
 - 19 Ahearn J M, Provost T T, Dorsch C A, *et al*. Interrelationship of HLA-DR, MB, and MT phenotypes, autoantibody expression and clinical features in systemic lupus erythematosus. *Arthritis Rheum* 1982; 25: 1031-40.
 - 20 Batchelor J R, Fielder A H L, Walport M, *et al*. Family study of the major histocompatibility complex in HLA DR3 negative patients with systemic lupus erythematosus. *Clin Exp Immunol* 1988; 70: 364-71.
 - 21 Rynes R I. Inherited complement deficiency states and SLE. In: Hughes G R V, ed. *Clinics in rheumatic diseases*. Vol 8. Philadelphia: Saunders, 1982.
 - 22 Mauff G, Bender K, Giles C M, Goldman S, Opferkuch K, Wachauf B. Human C4 polymorphism: pedigree analysis and qualitative, quantitative, and functional parameters as a basis for phenotype interpretations. *Hum Genet* 1984; 65: 362-72.
 - 23 Tan E M, Cohen A S, Fries J F, *et al*. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; 25: 1271-7.
 - 24 Wilson W A, Armatis P E, Perez M C. C4 concentrations and C4 deficiency alleles in systemic lupus erythematosus. *Ann Rheum Dis* 1989; 48: 600-4.
 - 25 Morton N E, Bruns G A. Report of the committee on the genetic constitution of chromosomes 1 and 2. Ninth International Workshop on Human Gene Mapping. *Cytogenet Cell Genet* 1987; 46: 102-30.
 - 26 Kazazian H H Jr, Junien C. Report of the committee on the genetic constitution of chromosomes 10, 11 and 12. Ninth International Workshop on Human Gene Mapping. *Cytogenet Cell Genet* 1987; 46: 188-212.
 - 27 Senaldi G, Lupoli S, Vergani D, Black C M. Activation of the complement system in systemic sclerosis: relationship with clinical severity. *Arthritis Rheum* 1989; 32: 1262-7.
 - 28 Briggs D C, Pereira R S, Black C M, Welsh K I. A strong association between null alleles at the C4A locus in the major histocompatibility complex and systemic sclerosis. *Arthritis Rheum* 1986; 29: 1274-7.