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Reference ranges for serum C4 concentrations in subjects with and without C4 null alleles

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SUMMARY Serum C4 concentrations were measured in 102 healthy subjects and 90 subjects with type I diabetes mellitus. A wide range was observed in the group as a whole (0·08–0·67 g/l; mean = 0·26 g/l; SEM = 0·01 g/l). After C4 allotyping it was possible to subgroup 134 of these subjects according to the number of C4 null alleles present. C4 concentrations in the group with two null alleles were lower than in the group without null alleles (mean 0·2 *v* 0·37 g/l; $p < 0·001$). C4 concentrations in the group with one C4 null allele were intermediate and significantly different from those of the group without null alleles (mean 0·24 *v* 0·37 g/l; $p < 0·001$). Appropriate analysis has defined reference ranges for serum C4 concentrations in subjects with two, one, or zero C4 null alleles. Interpretation of low serum C4 concentrations should take account of the number of C4 null alleles present.

It is often assumed that low serum concentrations of the fourth component of complement (C4) are due to consumption and therefore reflect activity of diseases such as systemic lupus erythematosus. It is now clear, however, that genetic factors influence serum C4 concentrations and contribute to the wide scatter seen in health and disease.

In 1978 we reported that the presence of HLA B8 DR3 was associated with lower serum C4 concentrations in patients with systemic lupus erythematosus and myasthenia gravis and suggested that genetic factors may be important in determining C4 concentrations in various autoimmune diseases.[1 2] At the same time O'Neill *et al*[3] showed that C4 is controlled by two HLA linked genes, and it soon became clear that these genes encode multiple allelic forms of C4A and C4B.[4] Null alleles are common at both loci, and in an extensive family study Alper *et al* clearly showed that serum C4 concentrations varied inversely with the number of null alleles at C4A and C4B.[5] Any interpretation of serum C4 concentrations must take account of the C4 genotype,[6 7] and this is true in diseases such as systemic lupus erythematosus[8] and insulin dependent diabetes mellitus.[9]

A subject's C4 genotype can be determined by C4 allotyping of the family,[5] but family studies are often not possible and generally not practical. We have shown elsewhere that densitometric comparison of the C4A and C4B bands can sometimes obviate the need for a family study.[8] Furthermore, major histocompatibility complex supratypes can be used as markers for many C4 null alleles.[10] For example, A1 B8 DR3 contains the C4A null allele and B18 BfF1 DR3 contains the C4B null allele. Indeed, in at least some autoimmune diseases the reported lowering of serum C4 concentrations can be largely or wholly accounted for by the increased incidence of these supratypes.

If serum C4 concentrations are to help in the assessment of classical pathway activation of complement and indicate severity of disease or the effectiveness of treatment it will be necessary to define reference ranges that take account of the number of C4 null alleles. As a minimum the importance of "low" serum C4 concentrations may only be clear if the effect of C4 null alleles is considered.

Material and methods

The study included 192 subjects of caucasoid origin drawn from two groups. The first group of 102 was drawn from 200 healthy subjects who comprised a representative sample of an adult population, which has been used in numerous health and immunogenetic surveys.[11]

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The second group comprised 54 men and 36 women with type I (insulin dependent) diabetes, as defined by non-obesity, a tendency to ketosis, and absolute insulin requirements within two years of diagnosis. Age at onset of disease ranged from 5 to 68 years. Most (80 patients) had an age of onset of less than 40 years.

C4 CONCENTRATIONS

Sera were tested immediately or stored at −20°C. Serum C4 concentration was measured by rate nephelometry using the Beckman ICS Autoanalyser II and Beckman anti-C4 antiserum. All results were read from a standard curve and accepted only if the appropriate quality control conditions were met. The use of the standard curve minimised the variations seen with reagent changes.

C4 ALLOTYPING AND ASSIGNMENT OF NULL ALLELES

C4 allotypes were determined by immunofixation following electrophoresis of edetic acid plasma treated with neuraminidase.[4] In a previous study we showed that in most cases the presence of null alleles could be predicted by comparing the relative densities of the C4A and C4B bands.[8] The number of null alleles was determined from the C4 phenotype as follows. Two: homozygous deficiency at one locus and one or two separate alleles present at the other. Where only one allele was identified the possibility of a third null allele could not be excluded in this group. One: three separate alleles with C4A to C4B densitometric ratios of either about 2 or 0·5. Zero: four separate alleles, or three separate alleles with a C4A to C4B densitometric ratio of about 1. Indeterminate: only one allele present at each locus and a densitometric ratio of 1. Not possible: densitometric ratio impossible due to overlapping C4A and C4B loci bands.

The number of null alleles was assigned in 74 of the 102 healthy subjects, but was indeterminate in 26, and not possible in the remaining two subjects. Similarly, the number of null alleles was assigned in 60 of the 90 patients, but was indeterminate in 18 subjects, and not possible in the remaining 12, mainly due to the presence of the C4B2·9 allele, which is known to be associated with insulin dependent diabetes mellitus.[12] Exclusion of these 28 healthy subjects and 30 patients did not affect the distribution or mean concentration of C4 in either group. Duplicated C4 genes were unlikely to be important in more than a few cases since associated supratypes[13 14] had been found in only four of the study subjects.

C4 concentrations were compared using Student's two tailed t test. A cumulative frequency plot was used to derive the 5th and 95th percentiles for C4 concentrations.

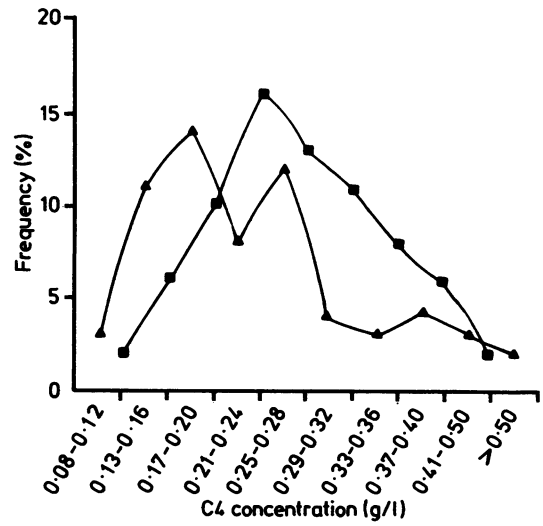

Fig. 1 *Frequency curves (%) of serum C4 concentration in study subjects in whom C4 null alleles could be assigned or excluded. Group 1 subjects (closed squares) showed an apparently symmetrical distribution of C4 concentration, while bimodal distribution was observed in group 2 subjects (closed triangles). Bimodality may reflect an increase in subjects with two null alleles in patient group.*

Results

Fig. 1 shows the serum C4 concentrations in the two groups. Results for all subjects are given without regard to the number of null alleles. There was a wide range of concentrations in both groups, but the means were similar. There seemed to be a bimodal distribution in the patient group but a relatively symmetrical distribution in the controls.

Fig. 2 shows the distribution of C4 concentrations in patients with two, one, or zero null alleles. Those with two or one null alleles had lower concentrations with relatively symmetrical distributions which could have accounted for the bimodality seen in the total patient group. Those with zero null alleles were shifted to the right. In the healthy subjects rather similar distributions were seen (data not shown), although the proportion with two null alleles was reduced as expected from the more symmetrical distribution of C4 concentrations (Fig. 1).

As the serum C4 concentrations of subjects with the same number of C4 null alleles were similar, irrespective of health or disease, the data could be pooled

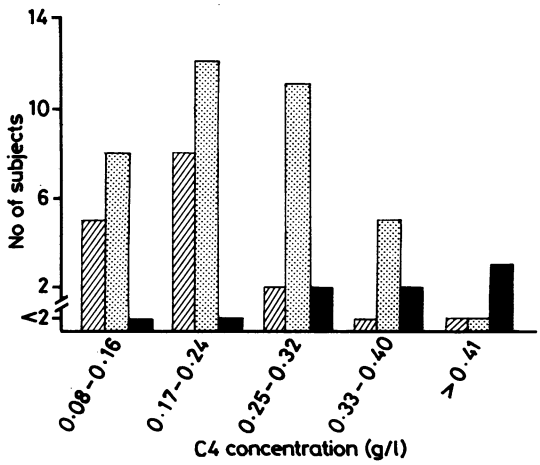

Fig. 2 *Frequency histogram of serum C4 concentration within group 2 subjects (patients with insulin dependent diabetes mellitus) with two, one, or zero null alleles. Subjects with two (striped columns) or one null alleles (dotted columns) have a relatively symmetrical distribution of C4 concentration with some skewing. A shift to the right was observed in subjects with zero (closed columns) null allele.*

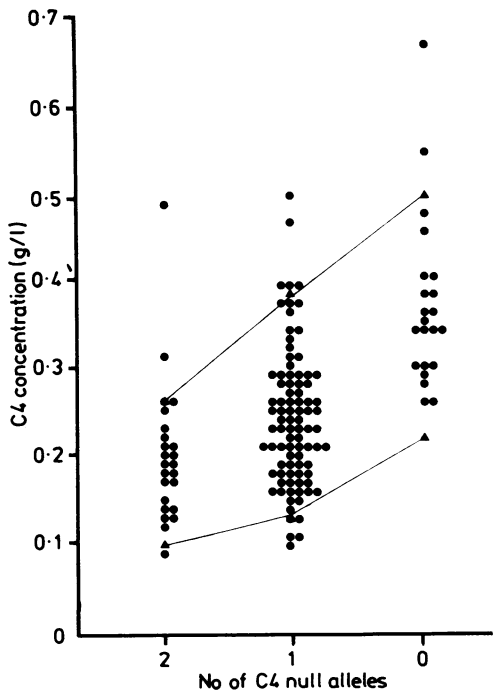

Fig. 3 *Serum C4 concentration in all study subjects with two, one, and zero null alleles. C4 concentrations in subjects with two or zero C4 null alleles are different, with little overlap and intermediate in those with one C4 null allele. Lines joined by closed triangles represent 5th and 95th percentiles for each subgroup.*

(Fig. 3). After excluding two points greater than three standard deviations from the mean C4 concentrations were between 0·09 and 0·31 g/l; 0·10 and 0·50 g/l; 0·26 and 0·55 g/l in subjects with two, one, and zero null alleles, respectively. Cumulative frequency curves were drawn and the 5th and 95th percentiles determined. For those with no null alleles the lowest cumulative frequency point was 9%, and the 5th percentile was estimated by extrapolation of the curve. Interim reference ranges suggested by this approach are as follows: two null alleles 0·10 to 0·26 g/l; one null allele 0·13 to 0·38 g/l; and zero null alleles 0·22 to 0·50 g/l.

Discussion

This study shows that serum C4 concentrations in both patients with insulin dependent diabetes mellitus and healthy subjects are influenced by the number of C4 null alleles present. These observations are important for several reasons. Firstly, wide reference ranges used by diagnostic laboratories should be qualified. Reference ranges should take account of the number of null alleles present. In patients with low serum C4 concentrations the possibility of C4 null alleles should be considered as an alternative to consumption. HLA typing may be helpful in suggesting the presence of supratypes which include null alleles.[10] In some cases C4 allotyping will be required. Family studies may sometimes be necessary, especially if C4 concentrations are very low.

Secondly, this study highlights possible errors in studies in which C4 concentrations in disease are compared with those in reference populations. Such studies must take account of the presence of C4 null alleles. Lower serum C4 concentrations reported in diseases such as Graves' disease,[15] insulin dependent diabetes mellitus,[16] active chronic hepatitis,[17] or associated with disease complications[18] may simply reflect an increased incidence of C4 null alleles in these diseases. Supratypes containing C4 alleles have previously been reported in type I diabetes,[19-21] chronic hepatitis,[17] and in some patients with low C4 concentrations in Graves' disease.[15]

Thirdly, these results should predict HLA associations with C4 concentrations. The lower serum C4 concentrations in B8 DR3 positive patients with myasthenia gravis[1] can be explained by the presence of the supratype HLA B8 C4AQ0 B1 DR3 which bears a C4 null allele. Any disease or subgroup of disease associated with this supratype or other C4

null bearing supratypes is likely to have reduced serum C4 concentration.

Fourthly, the presence of C4 null alleles may be important in predisposing to several autoimmune diseases. Early complement components do have a role in immune complex clearance[22] and viral neutralisation,[23] and lower C4 concentration might impair these functions. Alternative explanations, however, should be considered. C4 null alleles may merely be markers present on several supratypes associated with disease. The important genetic factor predisposing to disease may not be C4 null genes and could be anywhere along the segment of the chromosome defined by the supratype.[7 8 10]

Finally, these data provide the basis for a practical approach to assessing a C4 concentration. Regardless of the number of null alleles present, C4 concentrations below the reference range for two null alleles are clearly abnormal, while those within the reference range for zero null alleles exclude excess consumption. Interpretation of intermediate results will be influenced by the number of null alleles present. In most cases this can be determined by C4 allotyping alone, but HLA typing and sometimes family studies may be required. Once the number of null alleles has been determined, C4 concentrations can be assessed in relation to the appropriate reference range. Such an approach is likely to be of value in the assessment of the severity or effectiveness of treatment of several diseases such as systemic lupus erythematosus.

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