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CR1 deficiency in SLE: acquired or genetic?

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SUMMARY

Erythrocytes from 30 patients suffering from systemic lupus erythematosus (SLE) were tested for CR1 activity by an immune adherence haemagglutination technique. Defective CR1 activity (CR1D) was found in 11 (37%) of the patients on initial testing. On repeat testings, however, CR1 activity often varied from time to time and was shown to be inversely related to serum anti-DNA binding and apparent complement activation *in vivo*. Two of the 19 patients with normal CR1 activity acquired CR1D during the study. One patient with previously defective CR1 attained normal activity in the course of the study. The increased occurrence of CR1D in patients with SLE is largely or wholly acquired rather than genetically determined.

Keywords systemic lupus erythematosus complement receptor deficiency anti-DNA antibodies complement activation

INTRODUCTION

The complement receptor for C3b and C4b (CR1) is thought to be important in the binding of immune complexes to erythrocytes (Nishioka & Linscoff, 1963; Cooper *et al.*, 1969) and may be critical in the clearing of circulating complexes (Siegel, Liu & Gleicher, 1981; Cornacoff *et al.*, 1983). CR1 has also been shown to be important in regulating degradation of at least C3 and C3b (Fearon, 1979) and thus influence generation of ligands for complement receptors CR2 and CR3.

Previous authors (Miyakawa *et al.*, 1981; Wilson *et al.*, 1982) have shown that CR1 deficiency (CR1D) is relatively common in patients with systemic lupus erythematosus (SLE) and have suggested that the defect is genetically determined, and possibly a factor predisposing to disease susceptibility. By contrast, it has been suggested that increased CR1D in SLE patients may be a consequence of the disease process (Iida, Mornaghi & Nussenzweig, 1982; Ross *et al.*, 1984).

SLE is known to be associated with multiple autoantibodies, hypocomplementaemia and circulating immune complexes. It is clear that complement is consumed, and possible that CR1 is largely occupied by C3b or C4b fragments (or split products) or by immune complexes bearing these ligands. Receptor sites may not be available to react with the indicator immune complexes used in the immune adherence assay without necessarily implying blocking of these receptors.

Using a monoclonal antibody to CR1, Iida *et al.* (1982), observed an increase in CR1 levels during remission in patients with SLE. Using competitive inhibition of immune adherence haemagglutination, Inada *et al.* (1982), observed that erythrocytes from such patients showed defective CR1 activity when immune complexes were found in serum. These observations appear to suggest an acquired rather than a genetic explanation for the observed increase in CR1D in SLE.

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To explore this phenomenon further, we examined immune adherence function in erythrocytes from the patients in a long-term study, and related our findings to sequential serum anti-DNA titres and apparent degree of *in vivo* activation of C3.

MATERIALS AND METHODS

Patients. Thirty patients comprising 27 females and three males were included in the study, which lasted over a period of 9 months. Their ages ranged from 17 to 65 years. All were of Caucasoid origin and fulfilled at least four of the American Rheumatism Association criteria for SLE (Cohen *et al.*, 1971). All had positive antinuclear antibodies and a majority had elevated anti-DNA antibody on several occasions. The clinical details of the majority have been reported previously (Rigby *et al.*, 1978).

Reference group. The reference group comprised 72 'healthy' individuals. 54 of these were drawn from a group of 200 which has been used in numerous health and immunogenetic surveys (Hawkins, Houliston & Dawkins, 1979). The remaining 18 were staff volunteers. A subgroup of 20 age and sex-matched reference subjects were selected for further comparative studies.

Immune adherence haemagglutination (IAHA). Venous blood was collected from participants and transferred immediately to a tube containing two volumes of sterile Alsever's solution and stored at 4°C until required for testing.

IAHA was performed using the method of Miyakawa *et al.* (1981), except that local human IgG was purified by chromatography on DEAE-Sephadex A50 and dithiothreitol was omitted since non-specific reactions were observed with some RBCs from reference and patient subjects who were IAHA positive. Briefly, guinea pig serum was diluted with gelatin veronal buffer containing $1{\cdot}5 \times 10^{-4}$ M Ca^{2+} and 5×10^{-4} M Mg^{2+} (GVB^{2}) to give 3CH50 units. 25 μl was added to an equal volume of doubling dilutions of heat aggregated human IgG(AHG) in GVB^{2+} in a U-bottom polystyrene microtitre plate. Initial concentration of the AHG was 100 μg/ml. After incubation at 37°C for 45 min, 5×10^{6} red cells contained in 25 μl gelatin veronal buffer (containing 40 mM EDTA) were added, mixed and incubated for 1 h at room temperature. Red cells from the same reference subject with an intermediate IAHA titre were used as a positive control and included in every test run. Agglutination patterns were read by two independent observers. IAHA titre was expressed as the reciprocal to the base 2 (2^{n}) of the highest AHG dilution that induced haemagglutination.

Detection of C3 activation products ($C3_{act}$): Activation of C3 can be measured by an immunofixation electrophoresis technique. Details of the method will be reported elsewhere (Kay *et al.*, in preparation) and are similar to those reported by Whicher *et al.* (1980).

EDTA plasma was frozen to −70°C within 2 h of collection. 3 μl plasma was subjected to high voltage electrophoresis at a constant current, 80 mA/gel, in 1% ICN agarose using the buffer system described by O'Neill *et al.* (1978). The electrophoresis was terminated when a haemoglobin A marker had migrated 4·5 cm. The gel was overlaid with anti-human C3 (Atlantic/Scarborough, Maine, USA) resulting in precipitation of both apparently native C3 and C3 degradation fragments. After 15 min the gel was pressed then washed overnight in 0·15 M sodium chloride at 4°C and stained with Coomassie brilliant blue. Two sets of bands were obtained. Since $C3_{act}$ are anodal to native C3, an estimate of the degree of C3 activation was obtained by comparing the densitometric ratio of cathodal and anodal bands. By this technique serum incubated with 5% zymosan at 37°C for 30 min prior to electrophoresis showed approximately 75% $C3_{act}$. This method has been developed primarily to study *in vitro* activation of C3. However, by subjecting samples from patients with SLE or reference subjects to the above procedures an estimate of the '*in vivo* C3 activation' was obtained.

Anti-DNA. Antibodies to double-stranded DNA were measured by a modification of the method of Ginsberg & Keiser (1973), using ^{14}C-DNA of *Escherichia coli* origin (Amersham). Briefly 10 μl of heat-inactivated serum was incubated with 100 μl borate buffer and 50 μl ^{14}C-DNA for 1 h at 37°C. After incubation at 4°C overnight, the reactants were filtered through a 0·45 millipore membrane. The amount of ^{14}C-DNA retained on the millipore filter was determined by scintillography. The percent DNA binding was calculated by reference to a standard curve. The normal reference range is 0–15% binding.

HLA typing. HLA typing was performed by microlymphocytotoxicity using sera characterized against cells in previous international workshops. DR typing was performed on B lymphocytes separated on nylon wool columns (Danilovs, Ayoub & Terasaki, 1980).

Bf and C4 allotyping. Bf alleles were determined by immunofixation electrophoresis using a standard method (Alper, Boenisch & Wilson, 1972). C4 allotypes were determined using immunofixation following electrophoresis of neuraminidase-treated EDTA plasma (Awdeh & Alper, 1980). The method for the detection of null alleles at the C4 loci has been described previously (Christiansen *et al.*, 1983).

Statistical methods. The significance of difference in distribution of CR1D in the reference and patient groups was examined by χ^2 analysis.

RESULTS

IAHA in patients and reference subjects

Erythrocytes which did not show agglutination in any of the dilutions of AHG (IAHA negative) were termed CR1 deficient (CR1D). Of 30 patients with SLE, erythrocytes from 11 (37%) showed CR1D, whereas this was observed in only nine (13%) of the 72 reference subjects ($P = 0{\cdot}006$). More importantly, IAHA titres were decreased in the patient group as a whole (geometric mean 5 versus 8). This shift to the left resulted in a reduction of the extreme bimodality seen in health (Fig. 1).

In an attempt to explain the bimodality in IAHA titres especially in reference subjects, in separate experiments, erythrocytes negative in the IAHA assay were mixed in variable proportions with IAHA positive cells from three different individuals and titres determined (Fig. 2). At low dilutions of positive IAHA erythrocytes, an intermediate titre was observed which approximately related directly to the proportion of positive IAHA cells. However, this relationship did not hold at higher dilutions of positive IAHA cells. IAHA titres between 0 and 5 could not be demonstrated. These data suggest a critical number of CR1 sites are required for haemagglutination as suggested by Minota *et al*, 1984 and may explain the marked biodality of IAHA titres seen in both reference and patient groups.

Variability of IAHA findings

Erythrocytes used as a positive control repeatedly gave a titre of 7 or 8 with a coefficient of variation less than 7%. Sequential testing performed on the erythrocytes from seven of the reference subjects

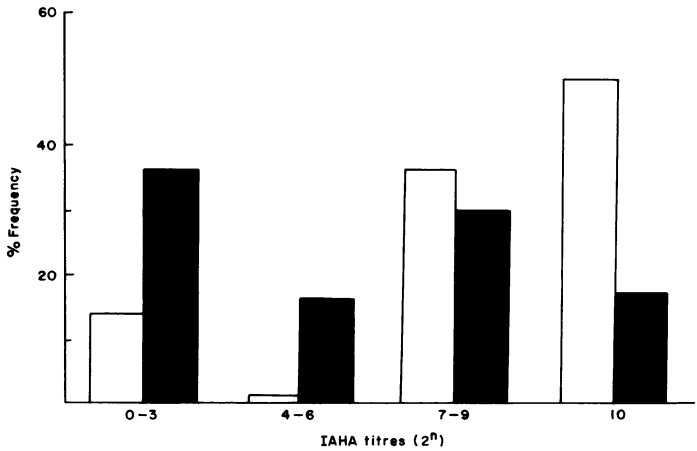

Fig. 1. Frequency histogram of immune adherence haemagglutination titres in 30 patients with SLE (■) and 72 reference subjects (□). It can be seen that patients are often deficient and that the patient group as a whole is shifted to the left.

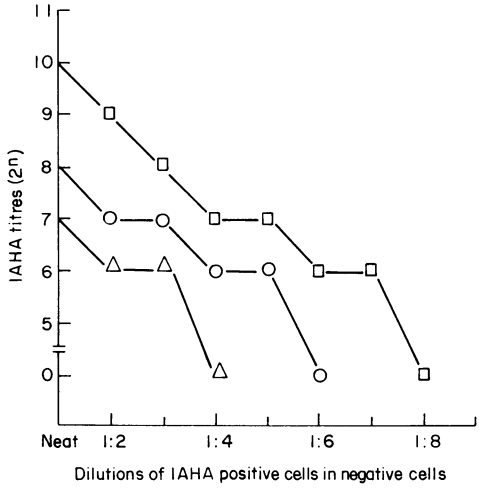

Fig. 2. Three samples of IAHA positive cells were diluted serially with IAHA negative cells and titres were then determined. Titres were directly related to the proportions of positive and negative cells at low dilutions of IAHA positive cells. Note the apparent threshold of numbers of CR1 sites required for positive haemagglutination.

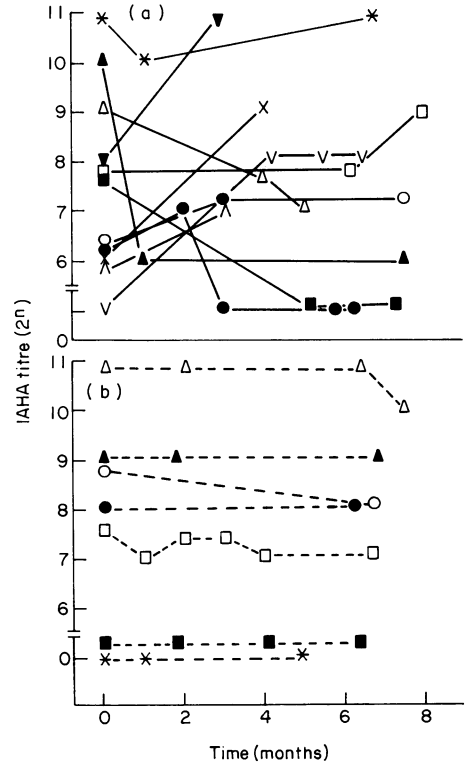

Fig. 3. Sequential IAHA titres in 11 patients with SLE (top) and in seven reference subjects (bottom). IAHA titres fluctuated in the patient group but were stable in the reference group.

showed similar minimal variation in IAHA titres (Fig. 3b). In contrast, the IAHa titres of many of the patients varied considerably with time. In three extreme instances there was a complete change in status from negative (CR1D) to positive and vice versa (Fig. 3a). However six patients were persistently negative by IAHA on sequential testing (Fig. 4a).

$C3_{act}$

The relationship of IAHA titres to an estimate of the degree of 'C3 activation *in vivo*' was examined in those SLE patients (Fig. 4a) and reference subjects (Fig. 4b) in whom repeated simultaneous estimations were available. As shown in Fig. 4, most patients can be subgrouped according to the relationship between IAHA and $C3_{act}$. In one subgroup IAHA appears to be independent of $C3_{act}$. In the second subgroup IAHA decreases as $C3_{act}$ increases, suggesting that low IAHA titres may sometimes be due to complement activation.

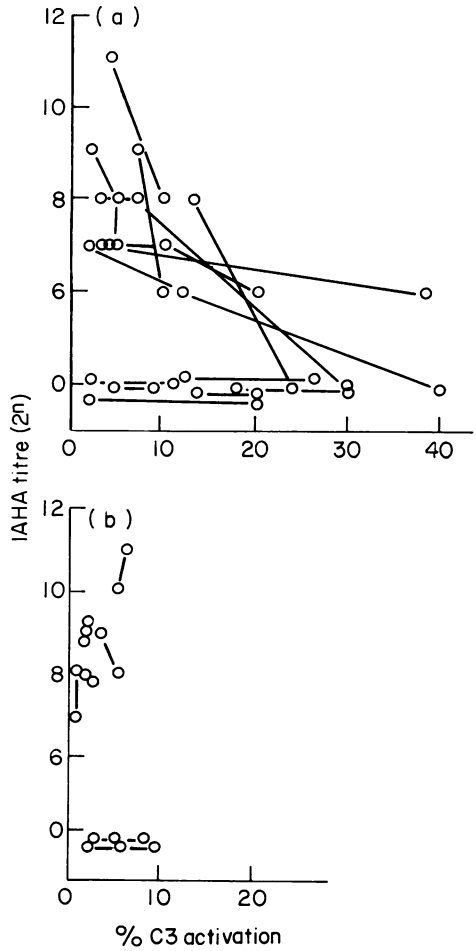

Fig. 4. The relationship between IAHA titres and 'C3 activation *in vivo*' in 14 patients with SLE (a, top) and in seven reference subjects (b, bottom) in whom repeated simultaneous IAHA titres and percentage C3 activation were available. Points joined together represent repeated studies on the same subject but without regard to temporal sequence. In some subjects with SLE the IAHA titre varies inversely with C3 activation. In others the titre remains low irrespective of the degree of apparent complement activation. In the reference subjects there is less complement activation and IAHA titres show minimal variation.

DNA binding and IAHA

Sequential studies revealed that serum anti-DNA binding and the degree of C3 activation generally moved in parallel and appeared to correlate with changes in clinical activity (Fig. 5). In general IAHA titres were negatively correlated such that increases in anti-DNA antibody and C3 activation were associated with decreases in IAHA titres. In one patient (Patient N) C3 activation and IAHA titre showed the expected inverse relationship, but anti-DNA remained relatively constant suggesting the possibility of complement activation due to other autoantibodies.

CR1D and HLA

As reported in detail elsewhere, there is an association between SLE and A1,B8,C4AQ0,C4B1,DR3 (Christiansen *et al.*, 1983). It was therefore of interest to determine whether HLA B8 and CR1D were associated. Interestingly, CR1D was not found in any patient (or reference subject) with HLA B8 regardless of the degree of apparent C3 activation (data not shown). This observation requires further investigation.

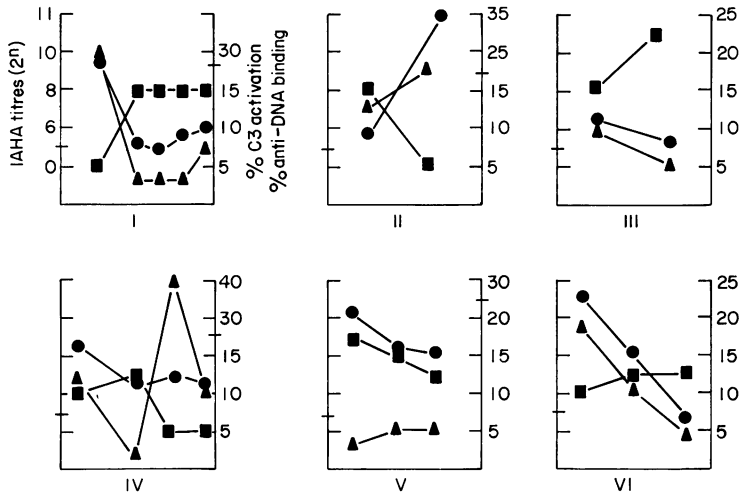

Fig. 5. An inverse relationship between percent activated C3 (▲) or percent DNA binding (●) and IAHA titres (■) in six patients serially studied over same period of time as in Fig. 2. All axes are the same as in Patient 1, unless otherwise indicated.

DISCUSSION

The immune adherence of erythrocytes has been used previously to assess CR1 activity in patients with SLE (Miyakawa *et al.*, 1981; Inada *et al.*, 1982; Minota *et al.* 1984). Results can be affected by many factors including, for example, anti-erythrocyte antibodies in guinea pig serum (Mayumi, Okochi & Nishioka, 1971) or human gammaglobulin. Since there are numerous variables, we were concerned to determine reproducibility of the assay. As indicated, the coefficient of variation was less than 7% in healthy subjects. Accordingly, the sequential changes in the patients can be attributed to factors such as the degree of complement activation rather than to inadequacy of the assay.

The present data reveal that CR1 is deficient in approximately 13% of healthy subjects, and reduced or deficient in a larger proportion of patients with SLE. In these respects we have essentially confirmed the findings of other workers (Miyakawa *et al.*, 1981; Wilson *et al.*, 1982; Iida *et al.*, 1982; Inada *et al*, 1982; Minota *et al.*, 1984). Given the extreme bimodality and the stability of IAHA titres in health, it is likely that genetically determined CR1D is relatively common in the population as a whole. In several families we have seen the apparent inheritance of CR1D but no evidence of linkage to HLA (data not shown).

Currently it is unclear whether the increased CR1D in SLE is acquired or genetically determined. The following results in patients appear to clarify this controversy:

(1) CR1 activity is reduced in the patient group as a whole.
(2) When compared to the healthy reference group, patients with SLE show little bimodality of CR1 activity.
(3) There were substantial changes in CR1 activity from time to time, with reductions generally being associated with rises in serum anti-DNA binding and increased '*in vivo* complement activation'.

Taken together, these results suggest that the excess of deficient or defective CR1 in SLE is at least largely acquired, possibly as a consequence of *in vivo* C3 activation. Other workers, using different assays, have reported evidence of increased C3 activation in SLE (Perrin, Lambert & Miescher, 1975; Ross *et al.*, 1984). Elsewhere (Kay *et al.*, in preparation), we describe in more detail the assay used to estimate the degree of C3 activation. However, it appears that several factors can affect results of this assay when used to measure apparent '*in vivo* C3 activation'. These factors include collection, storage and pH of samples (Ingham, Gowland & Taylor, 1985) and the allotype of C3. It is not yet clear to what extent these various factors may be operative in SLE.

A further finding of some interest arose from the examination of the relationship between HLA phenotype and presence or absence of CR1D. HLA B8 apparently provides some protection from the development of CR1D. Since HLA B8 will generally reflect the presence of the A1, B8, C4AQ0,C4B1,DR3 supratype (Christiansen *et al.*, 1983), and since this supratype is often regarded as a marker of impaired suppression (Zilko, Dawkins & Carrano, 1980) and high titres of some autoantibodies (Garlepp, Kay & Dawkins, 1984), we assumed that we would find an association with increased 'C3 activation *in vivo*' and reduced CR1 activity. Paradoxically, although C3 activation was relatively high in those patients with B8, CR1D did not occur in this subgroup. Several possible explanations for this unexpected finding deserve consideration. Since B8 is associated with a null gene at the C4A locus (Christiansen *et al.*, 1983) and since the products of the two C4 loci may have different functional characteristics (Isenman & Young, 1984), it is possible that complete or even partial deficiency of the C4A locus product may result in increased complement activation but reduced occupation of CR1 receptors. Further studies are required to examine this and related possibilities.

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