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Family studies of erythrocyte complement receptor type 1 levels: reduced levels in patients with SLE are acquired, not inherited

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SUMMARY

It has been claimed that patients with systemic lupus erythematosus (SLE) have an inherited deficiency of erythrocyte complement receptor type 1 (CR_1, with ligand binding specificity for C3b, iC3b and C4b). CR_1 functions as the only cofactor for factor I-mediated cleavage of iC3b to C3c and C3dg. The activity of this receptor on red cells may be an important mechanism for handling immune complexes which have bound C3b or iC3b. Radioligand binding studies were performed using a monoclonal antibody to CR_1, E11, to enumerate these receptors accurately. The results confirmed that patients with SLE have a reduced number of CR_1 molecules per red cell, but showed no reduction in CR_1 levels amongst their consanguineous relatives. Study of 13 normal families suggested the presence of heritable factors controlling the numbers of erythrocyte CR_1 molecules; in particular there was a correlation between mean parental CR_1 numbers and CR_1 numbers in their children. However, amongst 17 families of 19 patients with SLE, four families were identified in which genotypically 'high CR_1' SLE patients had persistently low phenotypes. This is not compatible with the hypothesis that the reduction in erythrocyte CR_1 numbers in these patients is inherited.

Keywords complement receptor type 1 systemic lupus erythematosus

INTRODUCTION

Defective immune adherence by erythrocytes from patients with systemic lupus erythematosus (SLE) was first discovered by Miyakawa *et al.* (1981). This abnormality was confirmed and further characterized by Iida, Mornaghi & Nussenzweig (1982) who prepared monoclonal antibodies (MoAb) to complement receptor (CR) type 1 (ligands: C3b, iC3b and C4b) and found that CR_1 numbers were reduced on the erythrocytes of patients with SLE.

The origin of this reduction in CR_1 numbers is the subject of controversy. Miyakawa *et al.* (1981) found defective immune adherence in a small number of asymptomatic consanguineous relatives of SLE patients and suggested that the abnormality might be inherited. To test this hypothesis Wilson *et al.* (1982) counted erythrocyte CR_1 using a polyclonal antibody in families of both normal subjects and of SLE patients. On the basis of a trimodal distribution of CR_1 numbers in their normal population and their family studies they suggested the existence of a genetic locus with two

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co-dominant alleles (encoding high and low expression) determining a numerical polymorphism of CR_1. From these data they concluded that patients with SLE had an increased prevalence of the allele determining low numerical expression of erythrocyte CR_1.

In contrast, in favour of an acquired reduction in CR_1, are the findings of inverse correlations of erythrocyte CR_1 levels with immune complex levels (Iida *et al.*, 1982; Inada *et al.*, 1982) and of a direct correlation between CR_1 levels and C4 haemolytic levels in patients with SLE (Iida *et al.*, 1982). Loss of CR_1 from glomerular podocytes has been described in SLE patients with diffuse proliferative nephritis but not non-proliferative nephritis (Kazatchkine *et al.*, 1982) which is again suggestive of an acquired abnormality.

We report here the results of a study of the families of both normal subjects and SLE patients which suggest that, although there are heritable factors in the level of CR_1 expression, the reduction of erythrocyte CR_1 numbers in some SLE patients is acquired rather than inherited.

MATERIALS AND METHODS

Subjects. Forty-eight patients fulfilling the 1982 revised ARA classification cirtieria for SLE (Tan *et al.*, 1982) were studied. Fifteen of these patients were bled on two or more occasions. Forty-eight first degree consanguineous relatives and five non-consanguineous spouses of 19 SLE patients from 17 families were also studied. Family members from four of these families were studied on two occasions. Eighty-eight normal subjects, including 69 members of 12 families of laboratory personnel, had blood samples taken. Sixteen normal individuals were studied on two or more occasions.

Cells. Blood was taken from patients, their relatives and normal volunteers into 10 mM EDTA. Blood cells were washed twice in CFD/1% BSA/3 mM sodium azide and buffy coat cells were discarded after each wash. Erythrocytes were counted using a Coulter counter (model S+1) and resuspended in CFD/1% BSA/3 mM sodium azide at 5×10^8/ml.

Antibodies. A murine anti-human CR_1 MoAb, E11 (IgGκ) (Hogg *et al.*, 1984), was purified from ascites by precipitation of non-IgG proteins with octanoic acid (Steinbuch & Audran, 1969). Anti-human CR_1 MoAb, 57F (Iida *et al.*, 1982), was a gift from Dr V. Nussenzweig (New York University, New York, USA). Antiserum to CR_1 was produced in rabbits immunized with purified erythrocyte CR_1 and the $F(ab')_2$ fragments of the IgG antibody were isolated as previously described (Dobson, Lambris & Ross, 1981). Fluorescein isothiocyanate conjugated $F(ab')_2$ goat anti-rabbit IgG was obtained from N.L. Cappel Labs, West Chester, Pennsylvannia, USA. Purified MoAb were labelled with ^{125}I using Iodogen (Pierce Chemical Co., Rockford, Illinois, USA) (Fraker & Speck, 1978) to a specific activity of 0·3–0·7 μCi/μg.

Enumeration of erythrocyte CR_1. The uptake of ^{125}I-anti-CR_1 on to cells was determined as previously described (Hogg *et al.*, 1984) by a modification of the method of Iida *et al.* (1982). Briefly, $2{\cdot}5 \times 10^8$ erythrocytes were incubated in 750 μl of CFD/1% BSA/3 mM azide with varying amounts of ^{125}I-E11 or 57F for 30 min at 37°C on a tube rotator with 45° axis. Triplicate 150 μl samples of the mixture were layered on to 200 μl of a mixture of 8 parts dibutyl phthalate (BDH chemicals) and 2 parts dinonyl phthalate (BDH chemicals) in 400 μl polypropylene microfuge tubes. After centrifugation of the tubes at 8,000*g* for 1 min, the tubes were frozen in a methanol/dry ice bath, and the tips of the tubes were cut off with a wire stripper. Free and cell bound radioactivity was measured in a gamma counter.

Non-specific uptake of ^{125}I-E11 and 57F was determined as previously described and specific uptake was analysed by the method of Scatchard (1949). It was found that 250 ng of E11 was sufficient to saturate > 95% of receptors on $2{\cdot}5 \times 10^8$ erythrocytes (Hogg *et al.*, 1984). This amount was used for single point binding assays. For comparison between the uptake of E11 and 57F, 250 ng of each labelled antibody was used.

Fluorescent cell labelling. Two million erythrocytes were incubated in 5 μl of polyclonal rabbit $F(ab')_2$ anti-CR_1 (8·2 mg/ml) for 20 min at room temperature. At the end of the incubation 70 μl of PBS/1% BSA/azide was added and the cell suspension was layered on 3 ml of PBS/6% BSA/azide and spun at 400*g* for 5 min. Five microlitres of fluorescein $F(ab')_2$ goat anti-rabbit IgG was added to

the cell pellet and, after a further 20 min incubation and wash through PBS/6% BSA/azide, the cells were resuspended in 1·5 ml PBS/1% BSA/azide.

Flow cytometry. The cells were analysed with the Cambridge MRC custom built flow cytometer of which a preliminary description has been published (Watson, 1980). Modifications not only to the flow chamber, but also to the optical filtration system had to be made in order to analyse fluorescence derived from a receptor density in the range of only 100–1,000 molecules per cell. Briefly, in order to be sure that the fluorescence detector was measuring fluorescence and not a change in light scattering at 90°, the latter was monitored simultaneously with the fluorescence and forward scatter signals, with the forward scatter signal being used to trigger the system. The 164-05 Spectra Physics argon laser (Mountain View, California, USA) was tuned to the 488 nm line emitting 300 mW in order to excite fluorescein fluorescence.

Statistical analysis. For comparisons between data sets a non-parametric test, the Mann–Whitney U test, was applied. For correlations between data sets, linear regression analysis was performed and the correlation coefficient calculated.

RESULTS

Distribution of CR_1 numbers

The distribution of CR_1 numbers amongst 86 normal subjects is shown in Fig. 1. The range was 145–1,214 molecules/erythrocyte. Comparison between the distribution of CR_1 in normal subjects and SLE patients and their consanguineous relative is shown in Fig. 2. There was no significant difference between CR_1 levels in normal subjects and SLE relatives ($z=0{\cdot}24$, $P>0{\cdot}05$), in contrast to the findings of Wilson *et al.* (1982). CR_1 numbers were significantly reduced in the group of SLE patients compared with both their consanguineous relatives and normal subjects ($z=6{\cdot}28$, $P<0{\cdot}0001$). A single subject with homozygous inherited C2 deficiency and SLE had 266 CR_1/erythrocyte and an unrelated subject with SLE and heterozygous C2 deficiency had 275 CR_1/erythrocyte.

Longitudinal studies

Two normal subjects were tested repeatedly over a period of 6 months: MW was measured on 22 occasions and varied between 763 and 1,021 (mean 881, 1 s.d. = 71); RW was measured on seven occasions and varied between 145 and 214 (mean 188, 1 s.d. = 24). Thirteen other normal subjects and 15 SLE patients were studied on more than one occasion. The interval between observations ranged between 1 and 16 weeks for the normal subjects (mean 7·5 weeks) and between 4 and 32 weeks for the SLE patients (mean 13·5 weeks). The correlation between the paired observations from both the SLE patients and the normal subjects is shown in Fig. 3 ($r=0{\cdot}86$, $P<0{\cdot}001$).

Family studies

CR_1 numbers were measured in 13 normal families and 17 SLE families. In Figs 4 &5, the results in

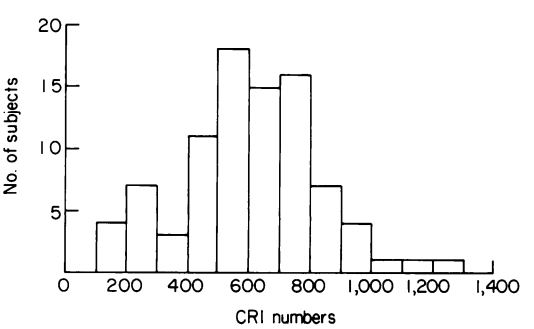

Fig. 1. Distribution of erythrocyte CR_1 numbers amongst 86 normal subjects.

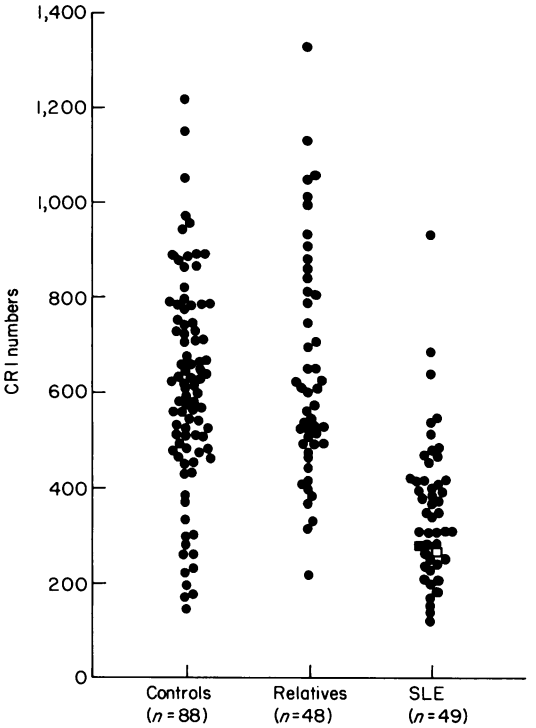

Fig. 2. Comparison between CR_1 levels in SLE patients, their consanguineous relatives and normal subjects. C2 deficient = □, C2 heterozygote = ■.

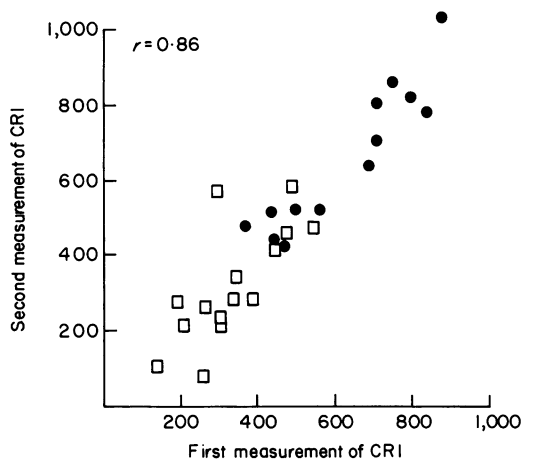

Fig. 3. Correlation between CR_1 levels measured on different occasions in normal subjects (●) and patients with SLE (□).

three normal families and in four families with SLE are shown. In the three normal families the distribution does suggest heritability of receptor numbers. However, in the four SLE families illustrated, the SLE propositus has low CR_1 numbers with: in family (a) parents and siblings who all have CR_1 numbers in the upper part of the normal range; in family (b) mother and sibling with high CR_1; in family (c) a daughter with SLE and low CR_1 and a normal daughter with high CR_1; in family (d) father with high CR_1. Families (a), (b) and (c) were studied on two separate occasions and

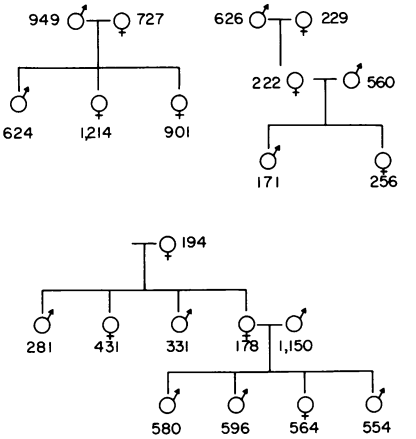

Fig. 4. CR_1 levels in three normal families.

consistent results were obtained; family (d) was assayed twice on the same samples with consistent results. Biological relationships in the families were confirmed by HLA typing.

As a test for heritability, mean parental CR_1 numbers were plotted against the CR_1 numbers of each of their children in the 10 normal families and the eight SLE families in which both parents had been studied. This is shown in Fig. 6. A strong correlation was found between mean parental CR_1 numbers and CR_1 numbers in their children in both the normal and SLE families ($r = 0{\cdot}58$, $P < 0{\cdot}001$). However the SLE patients tended to lie to the left of the distribution of the normal subjects, especially the patient from family (a), Fig. 5.

Test of Mendelian inheritance of CR_1 numbers in normal families

There was great difficulty in using the normal family data to test the model of Wilson *et al.* (1982), i.e. two co-dominant alleles at a single locus, H determining high CR_1 expression and L determining low expression. The problem lies in assigning values to what are to be considered HH and LL phenotypes. If figures are taken from the population data (Fig. 1) and the values used in the family studies they do not fit the model of inheritance. This suggests that it is necessary to allow a considerable range of values between HH and HL and between HL and LL where the genotype

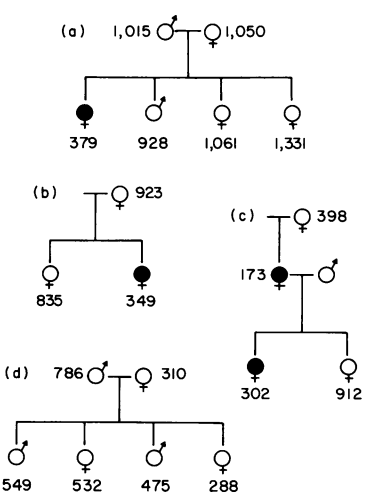

Fig. 5. CR_1 levels in four families of patients with SLE. SLE patient = ♀.

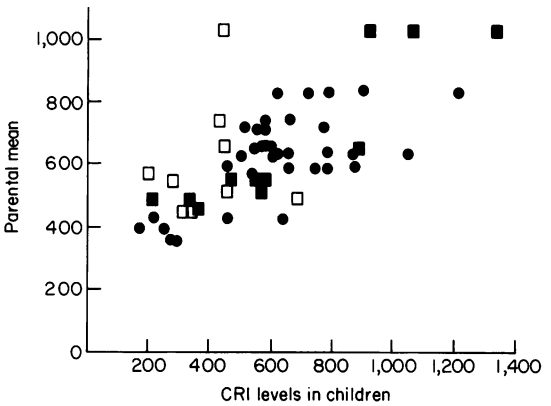

Fig. 6. Correlation between mean parental CR_1 numbers and CR_1 numbers in their children in normal and SLE families. Normal subjects = ●, SLE relatives = ■, SLE patients = □.

cannot be confidently predicted from the numbers measured. However an alternative model of inheritance that fits the data equally well is a polygenic model analogous to that involved in the control of height.

CR_1 measurement with E11 compared with 57F and polyclonal antiserum

To validate comparison of data obtained by counting CR_1 with E11 with that obtained by counting with the 57F (Iida *et al.*, 1982) and with a polyclonal antiserum (Wilson *et al.*, 1982), correlations were obtained between measurements using these various antibodies (Fig. 7). An extremely high correlation ($r = 0{\cdot}99$, $P < 0{\cdot}001$) was obtained between data obtained with E11 and 57F, and a strong correlation ($r = 0{\cdot}65$, $P < 0{\cdot}001$) between CR_1 sites measured by flow cytometry with a polyclonal antiserum and by radioligand binding assay with E11.

DISCUSSION

The data presented here suggest that amongst normal subjects there is an heritable component in the expression of erythrocyte CR_1 numbers. The strongest evidence for this is the correlation between mean parental CR_1 numbers and CR_1 numbers in their offspring. The data are insufficient to establish the precise nature of the inheritance which may be controlled by one or more genes.

The formal method to test the single gene model of Wilson *et al.* (1982) in normal subjects would

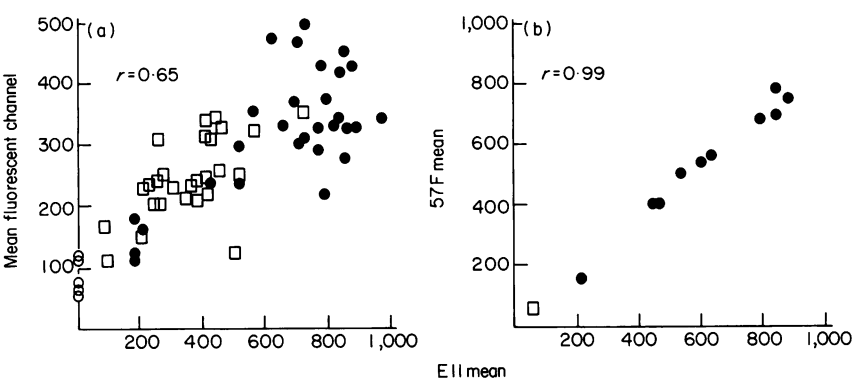

Fig. 7. Comparison between CR_1 enumerated with E11 and (a) polyclonal rabbit anti-CR_1 and (b) 57F. Fluorescent control = ○, SLE patients = □, Normal subjects = ●.

be to assign a gene frequency to the putative high or low allele based on examination of the population data. The validity of this assignment could then be tested by the examination of family data. The most important families to study would be those in which parents with opposite CR_1 extremes (i.e. obligate HH and LL subjects) had children who would then be obligate heterozygotes. An example of such a family is presented in this paper (family c, Fig. 4). Such families would provide a range of values for heterozygotes which could be compared with the values predicted by the estimated gene frequencies. However very large numbers of families might be needed to establish a satisfactory heterozygous range. It would also be expected that considerable phenotypic overlap would be found between subjects who were genotypically HH and HL and between those who were LL and HL. A further critical test of the hypothesis would be to seek discordant CR_1 numbers in healthy identical twins.

Amongst the lupus families there are two pieces of evidence that exclude genetic factors as the sole source of the reduction in erythrocyte CR_1 in SLE. Firstly, amongst consanguineous relatives there was no reduction in CR_1 levels compared with normal subjects.

This finding is at odds with the data of Wilson *et al.* (1982). It is not explained by differences in the CR_1 epitope recognized by the E11 since a close correlation was found between CR_1 levels measured with E11 and those detected both by a MoAb directed against the physiological ligand binding site (57F) (Iida *et al.*, 1982) and detected by a poyclonal antiserum (Dobson *et al.*, 1981). The second piece of evidence comes from the family studies of the SLE patients. Four families are presented in which a patient with SLE and low phenotypic expression of CR_1 has either parents (families a, b & d) or a child (family c) with high CR_1 numbers. On any model of inheritance the SLE patients in these four families are genotypically high, but phenotypically low for the numerical expression of erythrocyte CR_1.

A third piece of evidence that the reduction in CR_1 is acquired comes from the two subjects with inherited C2 deficiency. There is strong evidence that this is a sufficient risk factor for the development of SLE (reviewed in Lachmann, 1984). It would therefore not be expected that such individuals would also need to have low inherited CR_1 levels. However, both individuals studied here (one homozygous and the other heterozygous C2 deficient) had levels in the bottom 10% of the normal range.

A corollary of the suggestion that the reduction in CR_1 levels in SLE is acquired is that levels should fluctuate with disease activity. In this study no greater variation was found on repeated study of SLE patients than was found amongst normal subjects. However, erythrocytes have a long half-life and, as the mean time interval between repeat studies of SLE patients was only 13·5 weeks, it is likely that this is too short a period to demonstrate changes due to alterations in disease activity. Two previous studies have found correlations between indices of disease activity and CR_1 levels (Wilson *et al.*, 1982; Inada *et al.*, 1982). We have found a correlation between the reduction in CR_1 levels and the number of C3dg molecules present on erythrocytes, suggesting a relationship with the level of complement activation.

Genetic variation in the mol. wt of CR_1 has also recently been described (Dykman *et al.*, 1983; Dykman, Hatch & Atkinson, 1984; Wong, Wilson & Fearon, 1983) which must presumably affect the structural gene for CR_1. It is reported that this variation shows no genetic linkage to the variation in receptor number (Wong *et al.*, 1983) which in turn therefore cannot be a genetic variant in the structural gene. These observations imply the existence of at least two loci determining the expression of CR_1 on erythrocytes. The mechanism for loss of CR_1 on the erythrocytes of patients with SLE is not yet known, but a number of possibilities may be considered: (i) The presence of immune complexes may lead to the more rapid removal of red cells with larger receptor numbers, leaving those with smaller numbers circulating. (ii) CR_1 levels are greater on younger cells. If for some reason the red cell population were older than normal (perhaps due to inadequate reticuloendothelial clearance) a reduced receptor number might result. (iii) Autoantibodies might block the site of the receptors which react with C3b—and with the MoAb used to detect CR_1. It is, however, not likely that receptor occupancy by immune complexes directly affects the estimate of receptor number since the MoAb react even with occupied receptors (Hogg *et al.*, 1984). (iv) During transfer of immune complexes from erythrocytes to hepatic Kuppfer cells (Cornacoff *et al.*, 1983) there may be proteolysis of CR_1.

The relevance of this abnormality of erythrocyte CR_1 expression to the pathogenesis of SLE is not known. However, it is possible that it constitutes a significant defect in the ability of patients with SLE to handle immune complexes. In this respect it joins other abnormalities which include other complement deficiencies, both inherited and acquired (reviewed in Lachmann, 1984), and defective Fc receptor function (reviewed in Frank *et al.*, 1983). Amongst the consequences of these abnormalities are impaired immune complex solubilization (reviewed in Takahashi & Takahashi, 1981) and reduced inhibition of precipitation of immune complexes (Schifferli, Woo & Peters, 1982).

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