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Complement receptor expression and activation of the complement cascade on B lymphocytes from patients with systemic lupus erythematosus (SLE)

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SUMMARY

It has previously been reported that the expression of the complement receptors, CR1 on erythrocytes and blood leucocytes and CR2 on B cells, is reduced in patients with SLE, and that the reduced expression of CR1 on erythrocytes is related to disease activity. We have earlier demonstrated that normal B cells are capable of activating the alternative pathway (AP) of complement in a CR2-dependent fashion. In this study we have investigated whether disturbances in this activity may be related to the altered phenotype of SLE B cells. Flow cytometry was used to measure expression of complement receptors and regulatory proteins on B cells from SLE patients, as well as the deposition of C3 fragments occurring *in vivo* or after *in vitro* AP activation. We have confirmed, for a proportion of the patients studied, reduced expression of CR1 and CR2 on B cells, and shown a consistency between low CR2 expression and reduced *in vitro* AP activation in the presence of homologous, normal serum. In addition, the B cells, like erythrocytes, bear raised levels of *in vivo*-deposited C3dg, but not C3b fragments, compared with normal B cells. The erythrocytes from SLE patients were unable to inhibit *in vitro* AP activation by B cells in homologous serum. Finally, we demonstrated an inverse relationship between SLE disease activity index (SLEDAI) and the expression of complement receptor 2 (CR2) on SLE B cells. Thus, determination of CR2 on B cells may emerge as an additional laboratory tool in the assessment of SLE activity.

Keywords systemic lupus erythematosus B cells complement

INTRODUCTION

The erythrocytes (E) of many patients with SLE display a relative deficiency of complement receptor (CR1) [1–8] as well as increased deposition of the C3-split product, C3d,g, on the E-membrane [9–12]. Both inherited [3,7] and acquired [1,4,6] factors have been reported to contribute to the deficiency of E-CR1 in SLE patients. This deficiency is related to disease activity [4,11] and one of its functional consequences may be impaired clearance of immune complexes (IC) from the circulation [12–15]. It has also been reported that expression of CR1 on blood leucocytes and of CR2 on B cells are reduced in patients with SLE [16].

We have previously shown that CR2 on normal human B cells functions as the primary acceptor site for C3b fragments in the alternative pathway (AP) activation of complement [17]. Freshly isolated normal human B cells also bear low but significant amounts of C3d,g fragments on their membranes, indicating that this AP activation probably occurs *in vivo*. Furthermore we have shown [17] that autologous E, presumably via their CR1, may be involved in down-regulating this activation *in vivo*.

The present study is a transversal study involving 17 SLE patients. We have investigated B cells from SLE patients with respect to expression of complement receptors (CR1 and CR2), *in vivo* deposition of C3 fragments and cellular uptake of C3 fragments *in vitro*, as a consequence of AP activation, in order to establish whether reduced CR2 expression could be related to reduced ability of the B cells to activate the AP. We also investigated the regulation of B cell-mediated AP activation by the patients' own E. Finally, the possible correlation between complement parameters and disease activity was studied using the recently developed SLE disease activity index (SLEDAI) [19].

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PATIENTS AND METHODS

Patients and controls

Seventeen caucasian patients (three men and 14 women, mean age 38 years, range 20–51 years) fulfilling the 1982 American

Rheumatism Association revised criteria for SLE [18], were studied. Disease duration after diagnosis ranged from 4 days to 21 years. Four of the patients did not receive any treatment at the time of blood sampling. Thirteen patients received prednisolone, in seven cases combined with azathioprine or cyclophosphamide. Disease activity at the time of blood sampling was assessed using the SLEDAI [19]. One of the SLE patients was found to have a classical complement pathway deficiency and was excluded from the statistical calculations, although the relevant data have, where pertinent, been distinctly indicated in the figures.

Plasma-C3d was measured, using rocket immunoelectrophoresis with intermediate gel as described [20]. Classical and alternative pathway haemolytic complement activity in serum (CH_{50}) was measured as described [21].

The control group consisted of 21 apparently healthy laboratory staff members (five men and 16 women) of comparable age.

Cell preparations

Leucocytes from patients and controls were prepared either as unfractionated blood leucocytes (PBL) by erythrolysis of citrate-anti-coagulated blood with isotonic NH_4Cl, or as whole blood cells (WBC) by removal of plasma and washing the cells three times in PBS at room temperature.

Human serum

Homologous normal human serum. Blood samples from three healthy AB- and Rh.-positive individuals were obtained, and the sera were separated, pooled and stored at −80°C until analysed.

Autologous human serum. A serum sample was obtained from each SLE patient and from each control donor and kept on ice until use.

Buffers and solutions

Veronal buffer (VB) pH 7·4, and VB^{2+}, VB-buffer supplemented with 0·8 mM $MgCl_2$ and 0·15 mM $CaCl_2$. PBS/bovine serum albumin (BSA) buffer: PBS pH 7·4, containing 10 mM EDTA, 0·05% globulin-free BSA (Sigma Chemical Co., St Louis, MO) and 0·05% NaN_3.

Antibodies

F(ab) fragments of FITC-conjugated rabbit IgG anti-human C3c and anti-human C3d (Dako Diagnostics, Copenhagen, Denmark) were prepared as previously described [17]. The anti-human C3d antibody preparation is known to react with epitopes expressed on native C3, C3b, iC3b and C3d,g, while the anti-human C3c antibodies react with epitopes expressed on C3, C3b, iC3b and C3c [22].

Murine MoAbs to CR1 (IgG1, HB 8592) and to CR2 (IgG2a, HB 135) were purified from supernatants of hybridoma cells (American Tissue Culture Collection, Rockville, MD) by affinity chromatography on protein G- and protein A-Sepharose, respectively. OKB-7, a monoclonal IgG2a antibody known to block the receptor site for C3d,g on CR2 [23], was a generous gift from Dr A. Fucello (R. W. Johnston Pharmaceutical Research Institute, Raritan, NJ). The IgG1 subclass MoAbs to decay accelerating factor (DAF) (BRIC-110 and BRIC-216; BPL Bio-products, Elstree, UK) and to membrane cofactor protein (MCP) TRA 2–10, a generous gift from Peter W. Andrews (The Wistar Institute, Philadelphia, PA) and E 4.3, kindly donated by Dr Bruce Loveland (University of Melbourne, Australia) were purified by affinity chromatography on protein G-Sepharose. Human IgG was obtained from Kabi (Stockholm, Sweden). Anti-human CD19 PE (Becton Dickinson, Mountain View, CA) was used as B cell marker.

Conjugation of monoclonal antibodies

The MoAbs to CR1, CR2, DAF and MCP were reacted with FITC (Sigma) as described [17], in order to achieve a conjugation level between 2 and 3 mol FITC/mol IgG.

Complement activation at the leucocyte surfaces

In vitro complement activation was carried out by incubating the cell preparation at 37°C with 25% normal human serum (NHS) for 0·5 h in VB^{2+} buffer. In order to block the classical pathway, Mg^{2+} and EGTA at final concentrations of 4·4 mM and 20 mM, respectively, were included in the relevant samples. The reaction was stopped by transfer of the samples to an ice bath and addition of EDTA at a final concentration of 20 mM. *In vivo* bound C3 fragments were measured on cells kept at 4°C after isolation. To detect cell-bound C3 fragments, either F(ab) fragments of FITC-conjugated rabbit anti-human C3c or anti-human C3d antibodies were used, and PE-conjugated anti-CD19 was employed to identify the B cells. Cells were washed, resuspended in PBS/BSA buffer at 5×10^6 cells/ml and incubated for 2 h at 4°C in saturating FITC–antibody concentrations in presence of 5 mg/ml human IgG (Kabi). After incubation, the cells were washed at 4°C with PBS/BSA buffer before they were analysed by flow cytometry.

Flow cytometry

Analyses were performed using a FACScan (Becton Dickinson) flow cytometer. For identification of the leucocyte subpopulations, cells were gated on the forward scatter and side scatter dot plot in regions containing lymphocytes, monocytes and polymorphonuclear leucocytes (PMN). A fluorescence histogram of the lymphocyte population was used for setting regions for B cells using the B cell marker, anti-CD19-PE. The $CD19^-$ lymphocyte population is referred to as 'non-B cells'.

Receptor quantification and measurement of C3 fragment deposition

FITC-conjugated MoAbs were used for quantification of complement receptors and complement regulatory proteins. Cells were washed, resuspended in PBS/BSA buffer at 5×10^6 cells/ml and incubated for 2 h at room temperature with saturating FITC–antibody concentrations containing 5 mg/ml human IgG to eliminate non-specific and Fc receptor-mediated interactions of the MoAbs with leucocytes. The cells were washed at 4°C with PBS/BSA buffer before they were analysed in the flow cytometer.

The amounts of FITC–MoAb bound to CR1, CR2, DAF and MCP on the different subpopulations of blood cells were determined from the arithmetic means of the fluorescence intensity upon calibration of the flow cytometer with 'low level quantitative fluorescein standard kit' (Becton Dickinson), using the Lysis II software [17]. The number of MoAbs bound per cell was then calculated, using values derived for the specific fluorescence of MoAb conjugates, as described [24]. Assignment of the number of receptors per cell is based on the

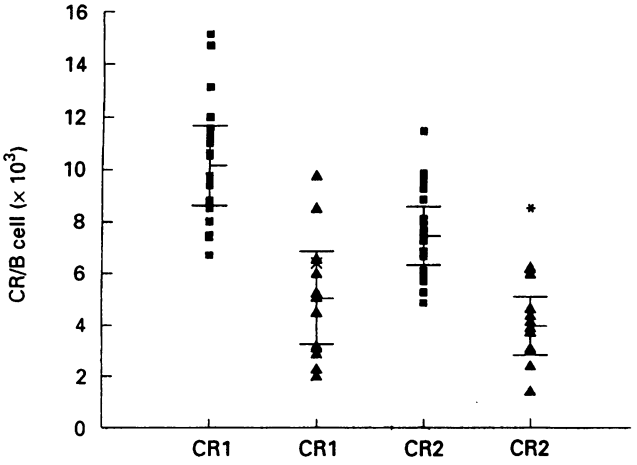

Fig. 1. CR1 and CR2 receptor expression on B cells from 19 healthy individuals (■) and in 15 SLE patients (▲), using HB135 as the probing antibody (two of the 17 patients had too few B cells to permit receptor calculation). One patient (*) had a classical complement pathway deficiency and was not included in the calculations. (Horizontal bars indicate 99% confidence limits for the population mean in all figures.)

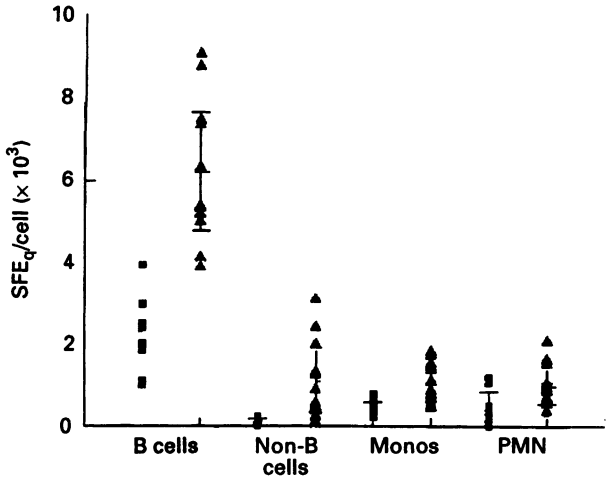

Fig. 2. Cell surface deposition of C3 fragments *in vivo* on leucocyte subpopulations from SLE patients (▲) compared with normal controls (■). The leucocytes were kept at 4°C after erythrolysis and during incubation with polyclonal anti-C3d antibodies. One patient (*) had a classical complement pathway deficiency. SFEq, Soluble fluorescein equivalents.

assumption that the MoAbs have reacted monovalently with their antigens.

The binding of FITC-conjugated anti-C3c and anti-C3d polyclonal antibodies was determined as the net geometric means of the fluorescence signal for each cell population, and re-expressed as soluble fluorescein equivalents (SFEq) using the above-mentioned calibration.

Statistical analysis

Comparison between groups was performed by calculating the 99% confidence intervals (CI) for the means, and the horizontal bars in all relevant figures indicate 95% confidence limits for the population mean. The evaluation of correlations between biological parameters (CR1 and CR2 expression, C3 fragment deposition), where a linear association might be expected, was performed by linear regression analysis, using Student's *t*-test to determine whether the correlation was significant. In the comparison of complement parameters with disease activity (SLEDAI score), non-parametric statistics (Spearman rank correlation) were employed.

RESULTS

Quantification of complement receptors on B cells from SLE patients

Expression of CR1 and CR2 was quantified on normal human B cells and B cells from SLE patients. In the SLE patients, the expression of CR1 and CR2 on B cells was significantly reduced compared with expression on B cells from healthy individuals (Fig. 1). The mean number of CR2 on SLE B cells was 60% of CR2 expression on B cells from the control group, using OKB-7, and 53% using HB135 as the probing antibody. The mean number of CR1 on SLE B cells was 50% of the control group. All except two of the patients in the SLE group displayed a number of CR1/B cells significantly lower than the controls.

The number of CR1 on monocytes from SLE patients was within the normal range (93% of the mean for the normal group), while CR1 expression on PMN was slightly elevated (132% of the mean for the normal group) (data not shown). Mean CR1 expression on SLE E was 151/E, which is 49% of the mean for the control group. The complement regulatory proteins DAF and MCP did not display significant differences in expression between SLE patients and controls on any cell type (data not shown).

Whereas decreased expression of E CR1 correlated significantly with B cell CR1 ($r = 0{\cdot}52$, $P < 0{\cdot}05$, $n = 15$) in the SLE patients, the correlation between decreased CR2 and CR1 expression on SLE B cells ($r = 0{\cdot}46$, $P < 0{\cdot}1$, $n = 15$) was not significant.

In vivo *deposition of C3 fragments on leucocyte subpopulations*

Using FITC-conjugated polyclonal anti-C3d as the probing antibody, elevated C3 fragment deposition was observed on all four SLE leucocyte subpopulations investigated (Fig. 2), as well as on the E (511 ± 699 SFEq for SLE E, 211 ± 33 SFEq for normal E). The increases were significant for B cells, non-B cells, monocytes and for E, but not for the PMN populations. Probing with anti-C3c failed to reveal significant differences between the two groups (data not shown), indicating that the C3 fragments deposited on SLE leucocytes were C3d,g.

When the SLE leucocytes were incubated for 30 min in serum-free buffer at 37°C, a significant fall ($25 \pm 11\%$) in the amount of detectable C3d,g fragments on B cells was observed, indicating that part of the *in vivo* bound material was readily dissociable from the B cell surface. For the other subpopulations (E, non-B cells, monocytes and PMN) which bore low levels of C3 fragments *in vivo* compared with B cells, no significant fall in the amount of surface-bound C3d,g fragments was observed after the same incubation.

Measurement of in vitro *complement-activating ability by B cells*

Since investigations on Epstein–Barr virus (EBV)-infected B cell lines have shown that there is a correlation between CR2 expression and degree of AP activation, we decided to measure

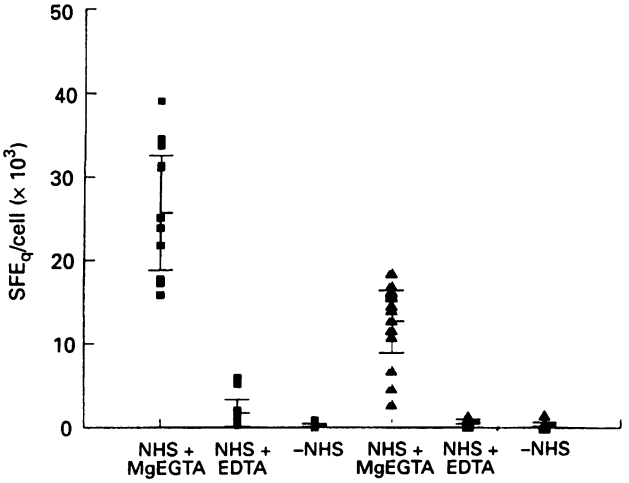

Fig. 3. C3 fragment deposition on SLE B cells (▲) and normal B cells (■) after *in vitro* alternative pathway (AP) activation in homologous normal human serum (NHS), using anti-C3c as probing antibodies. The activation was carried out in 25% NHS + MgEGTA at 37°C for 0·5 h. B cells incubated in NHS + EDTA and B cells incubated in buffer alone are shown as negative controls. Number of patients investigated = 13. SFEq, Soluble fluorescein equivalents.

in vitro complement activation in the presence of B cells from SLE patients. Using anti-C3c as the probing antibody, the degree of C3 deposition on SLE B cells incubated in NHS + MgEGTA was significantly lower than the deposition seen on normal B cells (49% of the mean of the normal group) (Fig. 3). Similar results were obtained using anti-C3d as the probing antibody (data not shown). Cells incubated in NHS containing EDTA displayed levels of fluorescence indistinguishable from those of cells incubated in the absence of serum.

A positive correlation (r = 0·72, $P < 0·01$) was observed between B cell CR2 expression and degree of C3 fragment deposition associated with *in vitro* AP activation at the B cell surface for the SLE patient group (Fig. 4). No correlation was observed between CR1, DAF or MCP expression on SLE B cells and their AP-activating capacity *in vitro* (data not shown).

Lack of regulation of B cell AP activation by SLE erythrocytes

In our previous investigations of AP activation on normal human B cells, we found that the complement-activating ability in the presence of autologous E was partially reduced, indicating that E are able to function as an extrinsic regulator of AP activation on leucocyte surfaces. To investigate whether SLE E are able to function as extrinsic regulators of AP

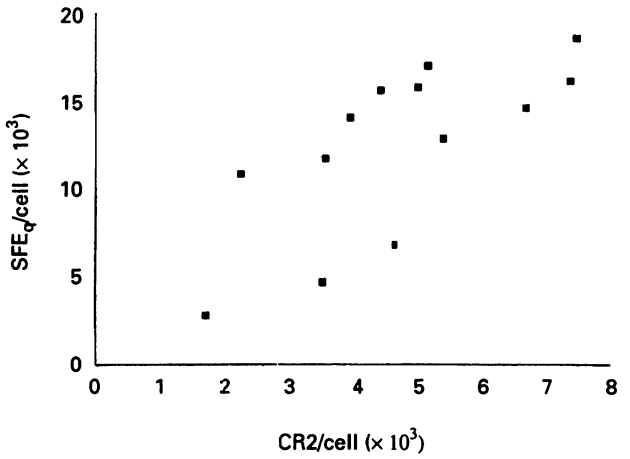

Fig. 4. The correlation between C3 fragment deposition after *in vitro* incubation in normal human serum (NHS) + MgEGTA, and CR2 expression (measured using OKB-7) on B cells from SLE patients. SFEq, Soluble fluorescein equivalents.

activation, PBL from SLE patients or from six normal control persons were incubated with homologous serum in presence and absence of autologous E. AP-mediated C3 deposition on normal B cells was decreased by 40–60% in the presence of autologous E, whereas no such effect was observed with the E from SLE patients.

Disease activity and relationship with complement receptor expression or in vivo *C3d deposition*

The expression of complement receptors and complement regulatory proteins on SLE B cells and E was investigated in relation to disease activity using the score SLE patients raised in the SLEDAI index. There appeared to be a tendency to an inverse relationship between E CR1 expression and disease activity score (Fig. 5a), which, however, was not significant ($P < 0·2$, Spearman rank correlation). No relationship between CR1 expression on B cells and the score was found ($P \geq 0·2$) (Fig. 5b). On the other hand, a non-linear, inverse relationship was observed between disease activity and the number of CR2 on B cells ($P < 0·05$) (Fig. 5c). The *in vivo* deposition of C3d,g on the patients' B cells or E showed no correlation to either disease activity or plasma C3d levels (data not shown).

DISCUSSION

Our previous investigations, using two-colour simultaneous flow cytometry of PBL for identification of B cell and C3

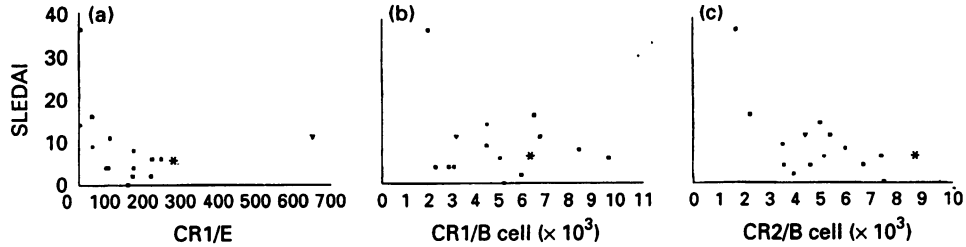

Fig. 5. Scatter diagrams. Relation between expression of E-CR1 (a), B cell-CR1 (b) and B cell-CR2 (c) and disease activity using the score SLE patients raised in the SLE disease activity score index (SLEDAI). Two of the 17 patients had too few B cells for receptor calculation. (▼) Patient investigated before receiving any medication. One patient (*) had a classical complement pathway deficiency and was excluded from the statistical calculations.

fragment deposition, demonstrated that on normal B cells, CR2 is the primary acceptor site for C3b during spontaneous *in vitro* AP activation [17]. On this basis we have investigated possible disturbances in B cell reactivity with the complement system that may be related to the altered phenotype of SLE B cells.

We found that CR1 and CR2 receptor expression on B cells from SLE patients were significantly lower than on those from normal individuals, in confirmation of a previous report [16]. In contrast to the findings of Wilson *et al.* [16], we did not find any reduction of CR1 expression on monocytes and PMN from SLE patients. However, the earlier study was performed on detergent lysates of purified cells, whereas we measured the amount of CR1 expressed on the surface of unstimulated cells. Since most CR1 of unstimulated PMN is known to reside in intracellular compartments [25], it is possible that the total amount of CR1 associated with stimulated monocytes and PMN from SLE patients could be decreased in comparison with normal cells without affecting membrane expression of the receptor.

Our results indicate a correlation between the decreased expression of E CR1 and B cell CR1. This is in contrast to the results of Wilson *et al.* [16], who found no correlation between the number of E CR1 and B cell CR1 in SLE patients, but a correlation between total CR1 of neutrophils and E CR1. On the other hand, the correlation between decreased B cell CR1 and CR2 in the SLE patients, although apparent, was not highly significant ($P < 0{\cdot}1$).

The B cells from SLE patients displayed reduced *in vitro* AP activation, in the presence of homologous, normal serum, compared with *in vitro* AP-activating capacity of normal B cells. Furthermore, significant correlation was observed within the patient group between CR2 expression on B cells and *in vitro* AP activation (Fig. 4). These findings further support our previous demonstration of an association between CR2 expression on normal B cells and AP activation of complement *in vitro* [17].

In contrast to our previous observation that E from normal individuals may be able to function as a regulator of complement activation on leucocyte surfaces [17], the E from the SLE patients were unable to inhibit *in vitro* AP activation by B cells in homologous serum, presumably as a result of their reduced level of CR1. The CR1 in SLE patients may also be functionally defective, as indicated in studies of PMN CR1 [26] and E CR1 [27].

Small amounts of C3 fragments have previously been detected on E from healthy individuals [4,9–11], while increased amounts of C3d,g have been demonstrated on E from patients with inflammatory diseases, such as SLE or rheumatoid arthritis (RA) [4,9–11]. We observed that all subpopulations of SLE blood leucocytes, as well as E, carry raised levels of *in vivo*-deposited C3d,g, but not C3b or iC3b fragments, compared with normal individuals.

A proportion of the C3d,g fragments deposited *in vivo* on the patients' B cells, but not on other blood cells, was readily dissociable from the surface during incubation with buffer. This is in contrast to our earlier observation with normal B cells, where no significant fall in the amount of detectable C3d,g fragments was observed after 30 min incubation in serum-free buffer at 37°C [17]. It would thus appear that some of the *in vivo* bound C3d,g is attached to the SLE patients' B cells as ligand for CR2, whereas C3d,g deposited on their E, PMN and non-B cells is primarily covalently attached to the cell surface. A likely explanation is that the dissociable C3d,g is bound by the B cells in the form of C3 fragment-opsonized IC, following classical pathway (CP) complement activation by the latter, whereas non-dissociable C3d,g fragments are presumably deposited as a consequence of CR2-dependent AP activation on the B cell surface. The AP activation may be enhanced in SLE patients because of the failure of their E to act as extrinsic down-regulators of this process [17].

A non-linear relationship ($P < 0{\cdot}05$) between disease activity score (SLEDAI) and decreased B cell CR2 expression was observed. Although the number of patients having high disease activity was low, both patients with high scores had very low CR2 numbers on their B cells. By employing a rather coarse statistical evaluation (non-parametric), the likelihood that the relatively low sample number might give rise to a type I error is markedly reduced. CR1 expression on the patients' E seemed to be inversely related to disease activity, in accordance with earlier investigations [4,27], but this was not significant for our patient group ($P < 0{\cdot}2$). We found no relationship between B cell CR1 expression and disease activity. These findings indicate that the reduced expression of CRs is associated with down-regulating mechanisms specifically related to the disease process [16] rather than to primary defects predisposing the individual to the disease.

Most SLEDAI descriptors are clinical, reflecting present or past involvement of tissues and organs. A few laboratory parameters are included, namely two immunologic (low complement and increased DNA binding) and two haematologic (thrombocytopenia and leukopenia), but these are all weighted low. Accordingly, only weak correlations would be anticipated between other immunologic parameters and SLEDAI scoring. Nonetheless, we did find a non-linear association between low CR2 expression on SLE-B cells and disease activity. Thus, B cell CR2 levels may emerge as an additional laboratory tool in the assessment of lupus activity.

The occurrence of polyclonal B cell activation in SLE [28–30] and the *in vitro* demonstrations that signalling via CR2 and CR1 is involved in the promotion of B cell growth [31–34] and differentiation [35,36], respectively, raise the interesting possibility that changes in CR1 and CR2 expression, and deposition of C3 fragments, on B cells from SLE patients may lead to disturbances in the complement-mediated modulation of B cell activity, thereby contributing to the pathogenesis of the disease. This question is currently under investigation.

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