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Reactivity patterns of anti-phospholipid antibodies in systemic lupus erythematosus sera in relation to erythrocyte binding and complement activation

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SUMMARY

We studied 51 sera from patients with systemic lupus erythematosus to determine the relationship of their anti-phospholipid activity to their anti-erythrocyte and complement activation properties. Forty-nine per cent of the sera had anti-phospholipid activity as demonstrated by ELISA using a panel of anionic phospholipids, and most of these also bound to neutral phospholipids, albeit to a lesser extent. A cellular radioimmunoassay was used for the detection of immunoglobulin and C3 binding to normal erythrocytes (intact, enzyme-treated or glutaraldehyde-fixed) following incubation with patient sera. The levels of IgG anti-phospholipid correlated with hypocomplementaemia and with immunoglobulin binding (paralleled by a deposition of C3 fragments) to the three types of erythrocytes, although most strongly to fixed cells. Immunoglobulin binding to intact erythrocytes correlated primarily with reactivity against neutral phospholipids. The specificity for phospholipid epitopes of immunoglobulin adsorbed onto erythrocytes was confirmed by acid elution followed by testing in ELISA. These data suggest that some anti-phospholipid antibody subsets may bind to erythrocytes *in vivo*, thus accounting for the observed association of these antibodies with positive direct antiglobulin tests.

Keywords anti-phospholipid antibodies systemic lupus erythematosus erythrocytes complement

INTRODUCTION

Lupus anti-coagulants, antibodies to cardiolipin and to mitochondrial type 5 antigen belong to the heterogenous group of autoantibodies against anionic phospholipids (Mackworth-Young, 1990). Putative interactions of anti-phospholipid antibodies (APLA) with platelet (Rauch, Meng & Tannenbaum, 1987) and/or endothelial cell (Vismara *et al.*, 1988) membrane lipids might explain the occurrence of thrombosis, fetal loss and thrombocytopenia which have been associated with circulating levels of these antibodies in patients with systemic lupus erythematosus (SLE) or related autoimmune disorders (Harris *et al.*, 1986). In SLE patients, APLA have also been associated with a positive direct Coombs' test (Harris *et al.*, 1986; Hazeltine *et al.*, 1988) and with reduced levels of erythrocyte complement receptor type 1 (CR1) (Hammond *et al.*, 1989). It has been suggested that APLA may bind directly to erythrocytes and fix complement to their membranes but no formal evidence for this exists.

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In order to evaluate the validity of the latter hypothesis, we examined the relationships between the characteristics of the APLA response, erythrocyte binding *in vitro* and complement activation in 51 SLE sera. Our results indicate that at least some subpopulations of APLA, showing a preferential reactivity with neutral phospholipids, have the potential to bind to the surface of intact human erythrocytes.

MATERIALS AND METHODS

Patient and control sera

The sample included 51 sera from consecutive patients with SLE, classified by the revised 1982 ARA criteria (Tan *et al.*, 1982). Aliquots of plasma and serum, the latter obtained after spontaneous coagulation, were rapidly frozen at −70° C. Only those sera that had not been thawed before were used for complement determinations. Sera from 20 healthy blood donors were used as normal controls.

Antibodies against mitochondrial type 5 antigen and dsDNA

These were identified by indirect immunofluorescence using cryostat sections of rat kidney, liver, mouse stomach (anti-M5;

Norberg *et al.*, 1984) and smears of *Crithidia luciliae* (anti-dsDNA; Aarden, de Groot & Feltkamp, 1976).

ELISA for anti-phospholipid antibodies

The following phospholipids were used (all from Sigma): cardiolipin (CL) in methanol; phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in chloroform; phosphatidylglycerol (PG) and phosphatidylserine (PS) in 95:5 chloroform/methanol; and phosphatidic acid (PA) as solid.

The ELISA for APLA was carried out basically according to Harris *et al.* (1987). Polystyrene microtitre plates (Nunc) were coated overnight at 4°C with 50 μl/well of either a 50 μg/ml solution of the individual phospholipids in 1:4 chloroform/methanol or a mixture of CL, PA and PS each at a concentration of 50 μg/ml in methanol. The plates were blocked, without prior washing, by incubation with phosphate-buffered saline (PBS)/10% fetal calf serum (FCS) for 1 h at 20°C. Then, 100 μl of test sample were applied to replicate antigen-coated and control wells for 2 h at 20°C. Patient and control sera were assayed at 1/50 in PBS containing 10% FCS and acid eluates from erythrocytes (previously incubated in patient or control sera) were tested undiluted. After washing with PBS, peroxidase-conjugated rabbit anti-human IgG, IgM and IgA antibodies (Dako) or $F(ab')_2$ fragments of goat anti-human immunoglobulin antibodies (Cappel) were added at a 1/2000 dilution in PBS/10% FCS. After washing, peroxidase activity was measured using H_2O_2 and OPD and the optical density was read at 492 nm using a microplate reader (Titertek, Flow). Samples were assayed for antibodies to the different phospholipids simultaneously using the same plate and the absorbance of control wells was substracted from the absorbance in the antigen-coated wells to account for non-specific binding. In each assay, the same positive control was included at various dilutions in addition to the control and patient sera or eluates. Anti-phospholipid levels were expressed as s.d. above the mean of normal controls; levels >3 s.d. above this mean were considered positive.

Erythrocyte isolation and treatment

Erythrocytes from heparinized type O blood samples were washed five times with PBS within 24 h after being drawn from healthy volunteers and the buffy coat was removed along with the upper layer of red cells by aspiration. Erythrocytes were resuspended at 2×10^8/ml in PBS and fixed with 0·12% glutaraldehyde for 5 min at 20°C. The reaction was stopped with the addition of 1/10 volume of 10% bovine serum albumin (BSA) and the cells were washed three times. For treatment of erythrocytes with enzymes, packed cells were pipetted into twice their volume of solutions of bromelain (2·5 mg/ml), papain (10 mg/ml) or trypsin (10 mg/ml). After 15 min at 20°C, the cells were washed and used immediately.

Cell surface RIA

An indirect binding assay was used to quantify immunoglobulin and C3 antigenic sites on $0{\cdot}5 \times 10^8$ erythrocytes (intact, fixed or enzyme-treated) following a 1-h incubation at 37°C with 50 μl/well of patient sera, serially diluted in PBS/0·5% BSA (for immunoglobulin determinations) or in a fresh serum pool from 15 healthy subjects (for C3 determinations).

Sensitized erythrocytes were washed, incubated with saturating levels of specific second antibody (rabbit anti-human immunoglobulin or clone 3, a rat monoclonal antibody to C3d, donated by P. Lachmann, MRC Centre, Cambridge, UK) and then with ^{125}I-labelled goat $F(ab')_2$ anti-rabbit or anti-rat immunoglobulin (2×10^5 ct/min per well; specific activity 4×10^7 ct/min per μg) as appropriate.

Background values for non-specific binding were determined by substitution of the pool of control sera for patient sera and were substracted.

Elution of antibodies from sensitized erythrocytes

Sensitized erythrocytes (8×10^8) were washed five times with PBS and incubated with 1 ml of elution buffer (0·1 M glycine, 0·1 M NaCl, pH 3) at 20°C for 1 min while vortexing. The eluates recovered after centrifugation were adjusted to 0·5% BSA and to pH 7·2 with Tris, and kept frozen.

Complement determinations

Serum levels of complement components C3 and C4 were measured by nephelometry using commercially available monospecific antisera. Haemolytic titres of total comlement (CH_{50}) were assessed with a modification of the method described by Mayer (1972). A timed assay using optimally sensitized sheep erythrocytes in the presence of Mg^{2+} and Ca^{2+} was performed. The functional activity of the alternative pathway of complement (APH_{50}) was measured at physiological ionic strength in the presence of Mg^{2+} and EGTA, using a timed assay and rabbit erythrocytes as indicator cells (Mayer, 1972). The limits of normal are as follows: CH_{50} and APH_{50}, 82–102% and 84–150% of the value with a pool of 30 normal sera, respectively; C3, 550–760 mg/*l* C4, 175–285 mg/*l*. Anti-complementary activity was determined by inhibition, by the patient sera, of the CH_{50} of a guinea pig serum pool (Johnson, Mowbray & Porter, 1975); it was considered present if $>28\%$.

Statistical analysis

Non-parametric comparisons were performed throughout. Correlations were evaluated using Spearman's rank correlation test, based on the numerical data and not on the assignment of positive or negative assay results. Probability values $<0{\cdot}05$ were considered to indicate statistical significance.

RESULTS

Isotype distribution and specificity of anti-phospholipid antibodies from SLE patients

Twenty-five (49%) of the 51 patients had APLA of one or more isotype. The binding of IgG, IgM and IgA from the APLA-positive sera in the standard ELISA (mixture of the three anionic phospholipids, CL, PA and PS) is shown in Fig. 1a. Three and four patients had only IgG or IgM, respectively; IgG and IgM co-existed in eight patients, IgG and IgA in three; and seven patients possessed the three isotypes.

We studied the specificity of APLA by measuring their individual reactions with a variety of anionic and neutral phospholipids (Fig. 1b). The highest levels of APLA binding were to anionic phospholipids with a similar prevalence to the standard ELISA. Reactivity against the neutral phospholipids, PC and PE, was found, respectively, in 84% and 72% of the APLA-positive sera, but in none of the negative sera in the standard ELISA ($P<0{\cdot}001$).

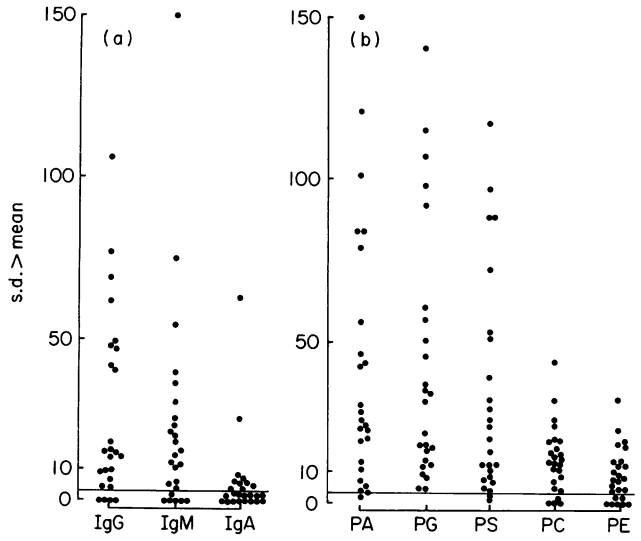

Fig. 1. (a) IgM, IgG and IgA binding to a mixture of cardiolipin (CL), phosphatidic acid (PA) and phosphatidylserine (PS); (b) anti-phospholipid antibody (APLA, any isotype) binding to individual phospholipids. Results concern only APLA-positive sera and are expressed in s.d. above a normal mean derived from 20 healthy controls.

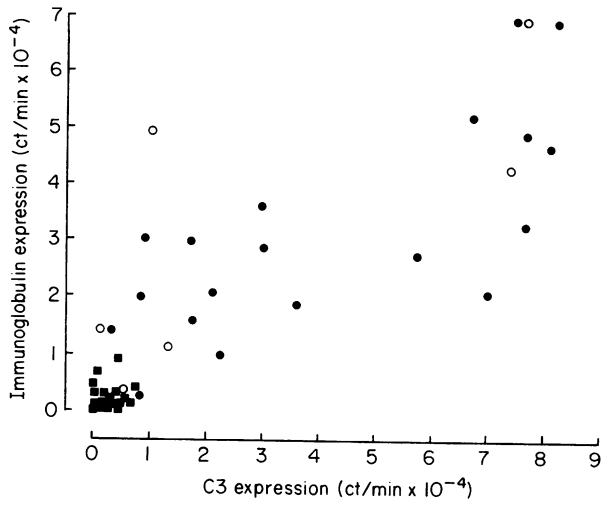

Fig. 2. Correlation between immunoglobulin and C3 expression on glutaraldehyde-fixed erythrocytes following incubation with 49 SLE sera ■, APLA-negative sera; ○, APLA-positive sera containing IgG ± IgA, or ●, IgM ± IgG ± IgA. Each point represents the mean of duplicate samples. Spearman's correlation coefficient (r_s) was 0·72 ($P < 0·001$).

Table 1. Correlations between IgG, IgM and IgA anti-phospholipid antibody (APLA) levels in the standard ELISA and erythrocyte (E) immunoglobulin and C3 expression following incubation with SLE sera

Correlation (n)	r_s	P
IgG APLA and		
Intact E immunoglobulin (51)	0·49	< 0·001
Bromelain-treated E immunoglobulin (48)	0·47	0·001
Glutaraldehyde-fixed E immunoglobulin (51)	0·87	< 0·001
Glutaraldehyde-fixed E C3 (50)	0·73	< 0·001
IgM APLA and		
Intact E immunoglobulin (51)	0·25	0·087
Bromelain-treated E immunoglobulin (48)	0·40	0·005
Glutaraldehyde-fixed E immunoglobulin (51)	0·68	< 0·001
Glutaraldehyde-fixed E C3 (50)	0·69	< 0·001
IgA APLA and		
Intact E immunoglobulin (51)	0·20	0·157
Bromelain-treated E immunoglobulin (48)	0·31	0·029
Glutaraldehyde-fixed E immunoglobulin (51)	0·56	< 0·001
Glutaraldehyde-fixed E C3 (50)	0·54	< 0·001

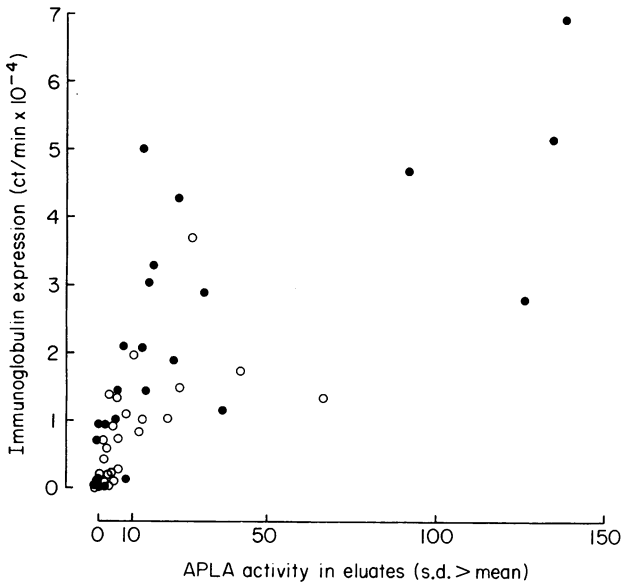

Fig. 3. Correlations between immunoglobulin binding to sensitized erythrocytes (○, intact; ●, glutaraldehyde-fixed) and anti-phospholipid antibody (APLA) reactivity of the corresponding acid eluates in the standard ELISA. Each point represents the mean of duplicate samples. The Spearman's correlation coefficients were 0·74 (○) and 0·83 (●) ($P < 0·001$ in both cases).

Immunoglobulin and C3 binding to erythrocytes sensitized with SLE sera

An indirect radioactive binding assay was used to quantify immunoglobulin and C3 antigenic sites on normal erythrocytes following incubation with patient and control sera. A 1/2 dilution of sera, that provided the highest binding activities to erythrocytes, was selected. Of note was the presence of unexpected alloantibodies to erythrocytes in five sera; these were reassayed, for immunoglobulin (and C3) binding, on erythrocytes of the panel devoid of the corresponding alloantigen(s) to exclude any possible interference. Owing to the fact that about one out of three of APLA-positive sera gave negligible immunoglobulin binding to intact erythrocytes, experiments were also carried out with cells either treated with enzymes or fixed with glutaraldehyde.

Correlations (and their significance values) between APLA levels of each isotype and expression of erythrocyte immuno-

Table 2. Correlations between immunoglobulin binding to sensitized erythrocytes (E) and serum levels of antibodies to different phospholipids

Correlation (*n*)	r_s	*P*
Intact E immunoglobulin and antibodies to		
Phosphatidylserine (46)	0·34	0·020
Phosphatidylcholine (46)	0·46	0·001
Phosphatidylethanolamine (46)	0·51	<0·001
Bromelain-treated E immunoglobulin and antibodies to		
Phosphatidylserine (44)	0·36	0·015
Phosphatidylcholine (44)	0·47	0·001
Phosphatidylethanolamine (44)	0·51	<0·001
Glutaraldehyde-fixed E immunoglobulin and antibodies to		
Phosphatidylserine (46)	0·86	<0·001
Phosphatidylcholine (46)	0·82	<0·001
Phosphatidylethanolamine (46)	0·73	<0·001

globulin and C3 are shown in Table 1. For intact erythrocytes, there was a correlation (highly significant although relatively weak) between immunoglobulin expression and the titres of APLA of the IgG but not of the IgM or IgA isotype. Fixed erythrocytes expressed significantly higher immunoglobulin (and C3) levels when APLA were present in the serum and the strongest correlations were again with IgG APLA. Somewhat weaker correlations existed between immunoglobulin binding to enzyme-treated (e.g. bromelain) erythrocytes and APLA levels of each isotype. Incubating patient sera and erythrocytes at 37°C induced deposition of C3 fragments that roughly paralleled immunoglobulin binding and occurred even though IgG and IgA were the unique APLA isotypes, as shown in Fig. 2 for glutaraldehyde-fixed cells. Cleaved C3 could be demonstrated by crossed-immunoelectrophoresis in several selected sera following incubation at 37°C with intact erythrocytes (not shown).

Specificity of immunoglobulin binding to erythrocytes

The strong correlations between APLA levels and immunoglobulin and C3 deposition on sensitized erythrocytes led us to attempt a direct demonstration that bound immunoglobulin may be reactive with phospholipids by performing elution experiments. It can be seen from Fig. 3 that significant positive correlations existed between erythrocyte immunoglobulin expression and APLA activity in the acid eluates from both intact and glutaraldehyde-fixed erythrocytes. Acid elution from enzyme-treated erythrocytes has not been performed.

We then investigated whether the effects of the binding specificities of serum antibodies to the panel of phospholipids could be distinguished with respect to immunoglobulin expression on each type of erythrocytes used. The results show (Table 2) that, for intact and bromelain-treated erythrocytes, immunoglobulin expression correlated most strongly with the level of binding to the neutral phospholipids, i.e. PC and PE. In contrast, immunoglobulin expression on glutaraldehyde-fixed erythrocytes was similarly correlated with reactivity to all of the anionic (e.g. PS) and neutral phospholipids tested. Furthermore, the acid eluates from intact erythrocytes exhibited higher anti-PC/anti-PS binding ratios than the ones from fixed erythrocytes (not shown).

Table 3. Correlations between IgG, IgM and IgA anti-phospholipid antibody (APLA) levels, anti-dsDNA antibodies and complement determinations in SLE sera

Correlation (*n*)	r_s	*P*
IgG APLA and		
C3 (48)	−0·48	0·001
C4 (48)	−0·34	0·021
CH_{50} timed assay (46)	−0·44	0·003
Alternative pathway assay (37)	−0·31	0·067
Anti-complementary activity (44)	0·52	<0·001
IgM APLA and		
C3 (48)	−0·30	0·044
C4 (48)	−0·23	0·128
CH_{50} timed assay (46)	−0·42	0·005
Alternative pathway assay (37)	−0·06	0·731
Anti-complementary activity (44)	0·26	0·101
IgA APLA and		
C3 (48)	−0·41	0·004
C4 (48)	−0·18	0·217
CH_{50} timed assay (46)	−0·23	0·131
Alternative pathway assay (37)	−0·38	0·020
Anti-complementary activity (44)	0·33	0·032
Anti-dsDNA antibodies and		
C3 (48)	−0·35	0·014
C4 (48)	−0·28	0·055
CH_{50} timed assay (46)	−0·39	0·007
Alternative pathway assay (37)	−0·38	0·020
Anti-complementary activity (44)	0·38	0·010
APLA (any isotype) (51)	0·13	0·360

Only four sera, belonging to the APLA-positive group, were positive for anti-mitochondrial type 5 antibodies and all of them mediated immunoglobulin binding to the three types of erythrocytes. However, these numbers were too small to permit statistical analysis.

Complement determinations in relation to phospholipid and dsDNA binding activities

In view of the possibility for APLA of acting as anti-erythrocyte antibodies *in vivo* and despite the diversity of autoantibodies that fix complement in patients with SLE, it was important to examine the relationships between signs of complement activation, APLA levels and also antibody titres to dsDNA, possibly reflecting disease activity (Davis, Cumming & Verrier-Jones, 1987).

C3 and C4 levels tended to parallel each other and were depressed, respectively, in 37% and 48% of the sera evaluated. Anti-complementary activity (present in 32% of the sera) was best correlated with activation of the classical pathway (evident in 39% of the sera), whereas activation through the alternative pathway was found in 38% of the sera. As shown in Table 3, various degrees of negative correlations were found between APLA reactivity and the levels of C4 (for IgG APLA only) and C3 (for the three APLA isotypes but primarily IgG). Interestingly, a negative correlation was observed between IgA APLA levels and the activity of the alternative pathway, but not the classical pathway, and conversely for IgG and IgM APLA levels. Anti-complementary activity was positively correlated

with IgG APLA levels and to a lower degree with IgA APLA levels. The signs of complement activation were largely independent of the binding patterns of serum antibodies to individual phospholipids (not shown).

Anti-dsDNA activity was present in 31 out of 51 (61%) sera studied. There were weak correlations between anti-dsDNA antibody titres and C3 levels, as well as classical pathway, alternative pathway and anti-complementary activities, but none between levels of anti-dsDNA antibodies and APLA.

DISCUSSION

The associations of APLA in SLE with positive Coombs' and reduced numbers of erythrocyte CR1 were the rationale for investigating the *in vitro* reactivity of these antibodies with erythrocytes. In this study, APLA levels in SLE sera were correlated with anti-erythrocyte reactivity and antibodies eluted from sensitized erythrocytes reacted with solid-phase phospholipids in ELISA. Although we did not address the molecular nature of the erythrocyte membrane epitopes recognized by APLA, the specificity of APLA was studied by measuring their reactions with a variety of anionic and neutral phospholipids. A different behaviour was found between APLA recognizing glutaraldehyde-fixed erythrocytes on the one hand and intact or enzyme-treated erythrocytes on the other hand, this latter group showing preferential binding to neutral phospholipids, i.e. PC and PE.

These findings can be related to the phospholipid composition of the outer leaflet of the erythrocyte membrane. In normal human erythrocytes, the choline-containing phospholipids, i.e. PC and sphingomyelin (35% and 20% of the total phospholipids, respectively), are mainly located in the outer half of the lipid bilayer, whereas the two remaining major phospholipid components, PE (25%) and PS (11%) are essentially in the inner half of the bilayer (van Deenen & de Gier, 1964; Marchesi & Furthmayr, 1976).

There have been numerous and partly conflicting reports on the pattern of reactivity to various phospholipids of sera from patients with chronic infections and autoimmune disorders. Our study differs from previous reports (Koike *et al.*, 1984; Harris *et al.*, 1988), but confirms others (Weidmann *et al.*, 1988; Loizou *et al.*, 1990), finding significant binding of SLE sera to neutral phospholipids. The reason for these differences is unclear, but the observation by Harris *et al.* (1988) of high background binding of some sera to wells coated with organic solvent alone may provide an explanation, because slight binding of these sera to neutral phospholipids would be difficult to determine. The lack of correlation in SLE sera between APLA and anti-dsDNA activities is in agreement with most studies and contrasts with the reported cross-reactivity of IgM APLA and anti-DNA monoclonal antibodies, the most likely explanation for these differences being the low affinity of the monoclonal reagents that favoured cross-reactivity (Mackworth-Young, 1990).

Although correlations were demonstrated between APLA levels and immunoglobulin binding to any type of erythrocytes, these were very strong only in the case of fixed erythrocytes. While the widespread procedure of aldehyde fixation of cells is often assumed to maintain cells in their original condition, fixation with even small concentrations of glutaraldehyde (e.g. 0·1% w/v) changes the charge-associated surface properties of erythrocytes as detected by cell partitioning (Walter & Krob, 1989). Conceivably, it is these differences between intact and fixed erythrocytes that APLA are sensitive to. The relatively weak correlations of immunoglobulin expression on intact erythrocytes with APLA reactivity, even to neutral phospholipids, make it possible that additional serum factors are operative in the binding of these antibodies to the surface of intact erythrocytes. In line with this hypothesis, Galli *et al.* (1990) have purified from plasma and characterized a protein cofactor which was required for APLA to bind to liposomes containing anionic phospholipids.

The structural configuration of the phospholipid antigen may be as important as its charge and primary structure. Indeed, Rauch *et al.* (1986) showed that monoclonal lupus anticoagulants could bind to the hexagonal but not to the lamellar form of PE, this latter state being that present in solid-phase assays and in most cell membranes. In another study, however, Rauch *et al.* (1987) found that the anti-platelet activity of these lupus anti-coagulants was most strongly correlated with anti-PE reactivity, reminiscent of our results using intact erythrocytes and SLE sera.

Norberg *et al.* (1984) noted in 11 out of 15 sera positive for anti-mitochondrial antibodies type M5 the presence of cold agglutinins that may be relevant to our findings, although the APLA specificity of these anti-erythrocyte antibodies was not established. In addition, the same authors found that low C4 levels were common in patients with anti-mitochondrial antibodies type M5. Several, but not all (Hammond *et al.*, 1989) previous studies have noted an association between APLA and reduced levels of serum C4 or C3 (Norberg *et al.*, 1987; Hazeltine *et al.*, 1988). Despite the fact that reduced C4 is considered to be a more sensitive indicator of minor episodes of *in vivo* complement activation than is C3 and that C4 was more frequently depressed than C3 in the present study, the correlations of APLA with C3 levels were higher than with C4 levels. However, persistently reduced C4 might indicate a heterozygous C4 deficiency, a condition which was not assessed in our patients. Whether APLA contributed to the hypocomplementaemia, observed in about 40% of our patients, cannot be decided from this study. However, it must be pointed out that IgA was the sole APLA isotype the levels of which were negatively correlated with the activity of the alternative pathway, but not the classical pathway, whereas the reverse was true for IgG and IgM APLA. Alternatively, the association of APLA with hypocomplementaemia may simply reflect the underlying disease activity, although the value of APLA as an indicator of active disease is disputed (Norberg *et al.*, 1987; Weidmann *et al.*, 1988; Hazeltine *et al.*, 1988).

The finding that both APLA-positive SLE patients (Hammond *et al.*, 1989) and patients with autoimmune haemolytic anemia (Ross *et al.*, 1985) have an acquired reduction of erythrocyte CR1 levels that parallel disease activity, suggests that antibody binding to erythrocytes may play a part in this pathogenic process. To our knowledge, no study has addressed the question of the potential for APLA to shorten erythrocyte survival. Some evidence of such a mechanism is provided by the observation that the anti-erythrocyte activity in the eluate from erythrocytes of a patient with IgG warm-type autoimmune haemolytic anemia was totally inhibited by adsorption with phospholipids (manuscript in preparation).

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