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Spontaneous plaque forming cells in the peripheral blood of patients with systemic lupus erythematosus

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SUMMARY

A reverse haemolytic plaque assay using staphylococcal protein A coupled to sheep red blood cells was set up in Cunningham chambers. Using this method, the numbers of Ficoll-Hypaque isolated peripheral blood lymphocytes (PBL) secreting IgG, IgA or IgM without preceding culture or mitogen stimulation were estimated in patients with systemic lupus erythematosus (SLE) and control subjects. Seven patients with clinically inactive SLE at the time of the study had values similar to those of the control subjects. In contrast, eight patients who had clinically active SLE had markedly increased numbers of PBL secreting IgG, IgA and IgM. Control experiments confirmed that the plaques were due to Ig secretion by lymphoid cells rather than to immune complexes adsorbed onto Fc receptor bearing cells or to passively adsorbed Ig. The results confirm the expected polyclonal B cell activation in patients with SLE and serial measurements showed that clinical relapses occurred only when the numbers of immunoglobulin secreting cells were high. Experiments in three patients with active SLE using native DNA prepared from T_2 bacteriophage as the 'developing antigen' suggest that PBL secreting nDNA antibody can also be demonstrated by this method.

INTRODUCTION

Polyclonal B cell activation evidenced by diffuse hyperglobulinaemia, presence of autoantibodies of multiple specificities and increased levels of anti-viral antibodies is a well recognised feature of SLE. However, although there is an overall correlation between disease activity on the one hand and the extent of derangement of certain laboratory measurements on the other—particularly levels of nDNA binding, immune complexes and serum complement components—changes in these measurements may fail to parallel activity or predict relapse in the individual patient (Grennan *et al.*, 1977; Davis, Cumming & Verrier-Jones, 1977; Appel *et al.*, 1978; Chubick *et al.*, 1978; Webb, 1978). Thus Liebling, Chia & Barnett (1978) found that none of these parameters alone or in combination gave less than a 50% false positive rate in predicting significant deterioration of lupus nephritis although all were capable of predicting stability with reasonable accuracy.

The recent introduction of the staphylococcal protein A-coated sheep red blood cell (SPA-SRBC) plaque forming cell (PFC) assay in agar gel (Gronowicz, Coutinho & Melchers, 1976) provides a simple method for demonstrating Ig production by single cells thus affording another way of assessing the degree of B cell activation. We have adapted the SPA-SRBC plaque assay for use in Cunningham chambers and have used it for two purposes: first, to assess B cell activation and

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its fluctuations in patients with SLE and second to attempt to demonstrate cells secreting antibody to nDNA. The studies we report here have been done using unstimulated peripheral blood lymphocytes and the plaque forming cells are therefore referred to as spontaneous plaque forming cells (SPFC).

MATERIALS AND METHODS

Patients studied. There were 15 patients who met at least four of the preliminary criteria for SLE (Cohen *et al.*, 1971). Eight were considered to have active disease at the time of the initial study based on the presence of one or more of the following: arthritis (five patients), pleurisy or pericarditis (two patients), renal involvement (four patients, biopsy proven with continuing active urinary sediment), CNS involvement (one patient). Three of the eight patients were studied before steroid therapy was begun, in the remainder the dose ranged from 10 mg to 40 mg prednisolone a day. The seven patients who were regarded as having inactive disease, had none of the manifestations listed above although mild arthralgia without arthritis and a raised ESR were present in some. Four of the seven were receiving prednisolone in doses ranging from 2 mg to 10 mg. CNS involvement had occurred more frequently in the patients with inactive SLE (three patients) but renal disease had not been present. No patient in this group had a relapse during the course of the study. The control subjects were healthy blood donors and hospital staff.

Methods. SPFC were determined using sheep red blood cells coated with staphylococcal protein A (SPA-SRBC) according to the method of Gronowicz *et al.* (1976). Peripheral blood mononuclear cells (MNC) were separated by Ficoll-Hypaque density gradient centrifugation and incubated for 60 min at 37°C in RPMI 1640 with 10% fetal calf serum (FCS) to allow shedding of passively adsorbed Ig. In some experiments latex particles of 0·81 μm diameter were added for the period of incubation to allow later recognition of phagocytic cells. No attempt was made to remove adherent or phagocytic cells.

SPA-SRBC were prepared by coupling three times washed SRBC with SPA as described by Gronowicz *et al.* (1976) with minor modifications. SPA and the washed SRBC were allowed to interact for 10 min at room temperature before the $CrCl_3$ was added in small aliquots with constant mixing (Goding, 1976). The mixture was incubated for 10 min at 37°C and for a further 20 min at room temperature after which the SPA-SRBC were washed three times in Hank's balanced salt solution (HBSS) and used at a concentration of 20–25%.

To 25 μl of the SPA-SRBC suspension were added 25 μl of antiserum at a previously determined optimum dilution (rabbit anti human IgG, IgA, IgM and IgGAM, Dako, Copenhagen), 100 μl of the mononuclear cell suspension adjusted to contain 2×10^5 cells with the morphological appearance of lymphocytes and 50 μl of fresh guinea-pig serum diluted 1:7 which had been absorbed three times with washed SRBC. The monospecificity of the antisera to IgG and IgA was verified by direct immunofluorescence of bone marrow smears from patients with IgG or IgA myeloma. The reagents were mixed in microtitre wells and the mixture was transferred to Cunningham chambers (Cunningham & Szenberg, 1968) which were sealed and incubated for 30–60 min at 37°C with a further 30–60 min at room temperature. Plaques were counted at $\times 20$ magnification. Controls omitting the mononuclear cells, antiserum or complement were included in each assay. In some experiments the incubation step following density gradient separation was omitted for an aliquot of the mononuclear cells so that cells which had or had not been incubated and washed could be assayed in parallel. Anti-nDNA secreting cells were sought in five patients using T_2 bacteriophage nDNA in the assay *in lieu* of anti-Ig serum. In these experiments nDNA was used in concentrations increasing from 0·2 ng/ml to 6 ng/ml in a semi-logarithmic manner.

Immunoglobulin in the cytoplasm of or adsorbed to mononuclear cells was demonstrated by direct immunofluorescence using cytosmears of MNC fixed for 10 min in 3% paraformaldehyde and for a further 5 min in acetone at −20°C with washes in PBS after both fixations. FITC-conjugated antisera to human Ig, IgG and IgM and C3 (Wellcome) and to human IgA (Orion Diagnostics, Helsinki) were used for staining at 1 in 20 dilution and the preparations were viewed under incident u.v. light.

Serum nDNA binding was measured using ^{125}I-labelled nDNA (Amersham).

RESULTS

Plaques appeared as circular areas of lysis with a central lymphoid cell (Fig. 1). Patients who had clinically active SLE when first studied had markedly increased numbers of SPFC compared with normal subjects and patients with clinically inactive disease. The increase was evident in plaque forming cells of all three major Ig subclasses (Table 1) and the values of the patients with active disease did not overlap those of the other two groups. IgA SPFC were the most numerous in all groups studied and also provided the best discrimination (Student's *t* values comparing active SLE

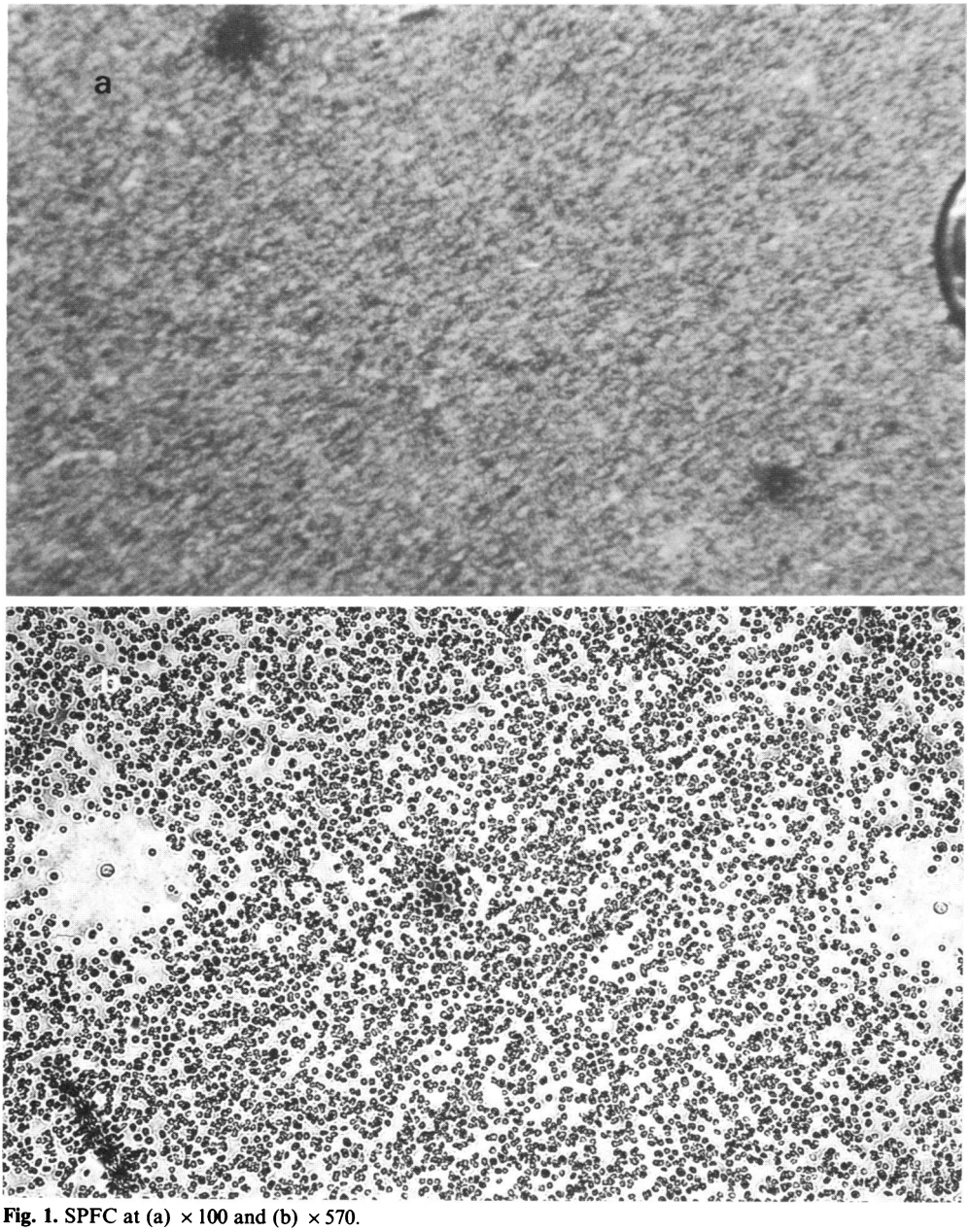

Fig. 1. SPFC at (a) × 100 and (b) × 570.

Table 1. SPFC (per 10^6 lymphocytes) in control subjects and in patients with clinically active or inactive SLE (mean ± s.d.)

	n	IgG	IgA	IgM	Σ IgG,A,M
Active SLE	8	926 ± 1335*	1450 ± 719*	516 ± 539*	2893 ± 1967*
Inactive SLE	7	34 ± 41	150 ± 99	17 ± 25	201 ± 144
Control group	29	23 ± 20	124 ± 97	41 ± 36	185 ± 116

* $P < 0{\cdot}001$ compared to control group.

to the control group: 5·93 for SPFC detected with polyvalent anti-GAM, 3·89 for anti-IgG, 9·98 for anti-IgA, 4·87 for anti-IgM and 7·67 for the sum of SPFC detected with the three monovalent antisera).

Three patients, initially classified as having clinically active disease were studied serially for 6–11 months with a total of 35 tests. Nine relapses occurred during this time, in each case whilst SPFC numbers were high. However, SPFC were as numerous during relapses of joint symptoms as during potentially more serious complications such as serositis or nervous system involvement (Fig. 2). An increase in steroid dose sufficient to control arthritis or serositis reduced SPFC numbers to normal and this sometimes occurred within 24 hr. On the other hand, even moderately high doses of prednisolone failed to bring SPFC values to normal if the disease remained clinically active. nDNA binding and serum levels of C3 and C4 did not correlate with clinical activity in these three patients.

Four patients initially classified as having clinically inactive disease were also studied serially for 6–8 months. None had a clinical relapse and SPFC numbers remained normal in all as did nDNA binding and serum C3 and C4.

The proportion of B lymphocytes engaged in Ig secretion was estimated in one patient with active SLE. She had 7% B cells (estimated by FITC conjugated $F(ab)_2$ antiserum to human IgG, IgA and IgM) and 6000 SPFC/10^6 lymphocytes so that approximately 8% of the B cells were activated and secreting Ig.

The predominance of IgA secreting cells was confirmed in six patients with active SLE by staining fixed cytosmears with monovalent FITC conjugated antisera to IgG, IgA and IgM (data

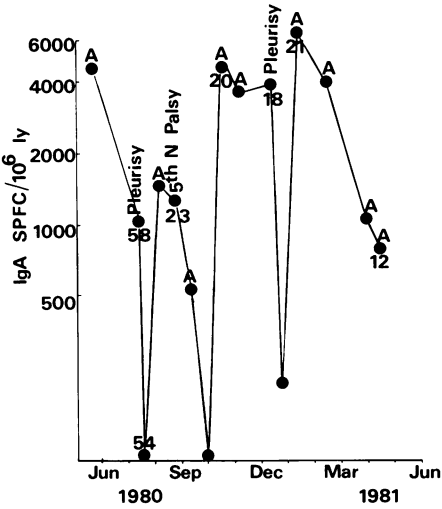

Fig. 2. SPFC during clinical relapses and remissions of SLE in one patient. Numbers in graph denote ESR (mm/hr). (A = arthritis; 5th N palsy = bilateral trigeminal palsy).

Table 2. Effect of omission of antiserum, mononuclear cells or complement and of incubating (60 min at 37°C) and washing mononuclear cells before the assay

SpA-SRBC	Anti-IgA	Mononuclear cells	Complement	SPFC/10^6 lymphocytes
+	+	+	+	1090
+	−	+	+	0
+	+	−	+	0
+	+	+	−	0
+	+	incubated and washed	+	960

not shown). Ig synthesis, as judged by the presence of cytoplasmic Ig was also far more common than Ig secretion as measured by the plaque assay.

Control studies

Controls were included in each assay to check that plaques were due to secretion of Ig by the central lymphoid cell and not to release of immune complexes or Ig passively adsorbed onto cells (Table 2). Except in one instance, plaques could not be demonstrated if anti-Ig was omitted from the reaction mixture. The exception was an assay done in a patient with untreated active lupus nephritis. Incubation of this patient's mononuclear cells with latex particles before the plaque assay showed that many of the PFC were phagocytic. When mononuclear cells from this patient were stained with FITC-conjugated antiserum to human Ig, many showed homogeneous cytoplasmic fluorescence but others showed granular surface fluorescence both with FITC anti-Ig and FITC anti-C3. The granular fluorescence was not seen in similar preparations made in other patients with high SPFC numbers although cytoplasmic Ig was often noted and slides stained with May-Grunwald-Giemsa showed numerous plasmacytoid cells.

Incubation of the mononuclear cells in RPMI 1640–10% FCS for 1 hr at 37°C followed by washing to allow passively adsorbed serum Ig to be shed before the plaque assay did not usually cause reduction in SPFC. No effect on SPFC numbers was seen in ten control subjects tested in this manner whilst the greatest change in any of the patients with active SLE was a reduction of 12% (Table 2).

Table 3. Effect of metabolic inhibitors on plaque formation. Cells from a patient with active SLE or EB virus transformed Ig secreting lymphoid cells from a normal donor were incubated with different inhibitors for 1 hr at 37°C. The PFC assay was then performed immediately or after a 2 hr recovery period

Incubation with	Recovery	SLE cells IgA SPFC/10^6ly	EBV cells IgM PFC/10^6ly
RPMI-FCS		850	30,000
Cycloheximide 100 μg/ml	nil	15	2,100
	2 hr		0
Mitomycin C 25 μg/ml	nil	70	8,000
	2 hr		6,400
NaN_3 1 mg/ml	nil	15	30,800
	2 hr		29,600
NaN_3 2 mg/ml	nil		11,000
	2 hr		17,000
NaN_3 4 mg/ml	nil		800
	2 hr		6,400

Table 4. Demonstration of anti-nDNA SPFC. Native DNA was used in final concentrations increasing in half log steps from 0·2 ng/ml to 6 ng/ml. The mononuclear cells were incubated at 37°C for 60 min with 0·81 μm latex particles before the assay

Patient	Diagnosis	Serum nDNA binding (units/ml)	SPFC (ΣGAM) per 10^6 lymphocytes	Anti-nDNA SPFC per 10^6 lymphocytes
1	Active SLE	>114	5,540	3
2	Active SLE	>114	1,510	11
3	Active SLE	85	720	3
4	Inactive SLE	15	415	0
5	Bacteroides empyaema	12	3,400	0

Plaque formation by SPFC could be almost completely inhibited by incubating the mononuclear cells with cycloheximide, mitomycin C or sodium azide before the assay (Table 3). Plaque formation by *Epstein Barr* virus transformed Ig secreting B lymphocytes from a normal donor could also be inhibited by cycloheximide; the effect of azide was reversible.

Demonstration of cells secreting anti-nDNA antibody

Three patients with active SLE, high nDNA binding and SPFC numbers, one patient with inactive SLE and normal SPFC and one patient who had high SPFC associated with a Bacteroides empyaema but who did not have SLE were tested for anti-nDNA secreting cells. Using the plaque assay as described but with anti-Ig replaced by nDNA, plaque forming cells were demonstrated only in patients with active SLE (Table 4). The anti-nDNA SPFC were few in number and there was no obvious correlation with the total number of SPFC. None of the plaque forming cells contained latex beads and no plaques were present in control preparations from which mononuclear cells, nDNA or complement had been omitted.

DISCUSSION

Some technical aspects of the assay need brief consideration. The plaques were not pseudoplaques (Muchmore *et al.*, 1976) but had clearly visible central MNC. Aggregates which formed in the anti-Ig sera after prolonged storage were however, capable of producing pseudoplaques unless removed by Millipore filtration. Even the presence of a central MNC in a plaque is not conclusive proof that the central cell is secreting Ig. Plaques could also be due to immune complexes released from Fc receptors on lymphocytes or macrophages, to Ig passively adsorbed onto L cells (Horwitz & Lobo, 1975) or to anti-lymphocyte antibodies released from lymphocytes during the assay (Winchester *et al.*, 1974). However, these three explanations are unlikely. First, except in one instance, no plaques formed in preparations from which anti-Ig had been omitted; in the one exception the identity of some of the PFC as monocytes was confirmed by their phagocytic property and surface bound complement fixing Ig was demonstrated by immunofluorescence. Second, incubation of MNC before the assay did not cause a consistent reduction in the number of SPFC as might have been expected had the shedding of Ig from L cells or of anti-lymphocyte antibodies been the cause of the plaques. Third, metabolic inhibitors caused a reduction or abolition of plaque formation both by cells from patients with SLE and by EBV transformed cells suggesting that the plaques were indeed due to active secretion of Ig by B lymphocytes.

The predominance of IgA-SPFC was unexpected and although noted in normal subjects in one previous report (Pryjma *et al.*, 1980) and confirmed in this study by direct immunofluorescence staining in six patients with SLE, other possibilities need consideration. Technical factors may

contribute in that IgG plaque preparations underwent generalized non-specific lysis more rapidly than IgA and IgM preparations and IgG plaques therefore had less time to develop. It is also possible, though conjectural that IgA is secreted at a faster rate than IgG by lymphoid cells activated *in vivo*.

The demonstration of increased numbers of SPFC in SLE confirms, by an independent method, the B cell activation demonstrated by other workers using measurements of Ig secreted into culture medium (Jasin & Ziff, 1975; Nies & Louie, 1978; Okudaira *et al.*, 1980) or of cells secreting IgM class antibody to haptenated or native SRBC (Budman *et al.*, 1977). It also confirms the results obtained with a solid phase SPA plaque assay by Ginsburg, Finkelman & Lipsky (1979) and Blaese, Grayson & Steinberg (1980). In the present study, SPFC were increased only during clinically active disease which is in agreement with previous reports (Jasin & Ziff, 1975; Budman *et al.*, 1977; Ginsburg *et al.*, 1979; Blaese *et al.*, 1980).

The correlation of high SPFC with clinical activity and the occurrence of clinical relapses—albeit of widely varying severity—only in patients with high SPFC raises the possibility that monitoring of SPFC may help in predicting relapses. This might be of clinical use since serial measurement of nDNA binding, ssDNA binding, complement component levels and immune complex levels are of limited value in this regard (Liebling *et al.*, 1978). The ESR, nDNA binding and serum levels of C3 and C4 were not helpful in predicting relapses in those patients whom we followed serially. The disadvantage of the SPFC assay as used in this study is its relative complexity and the variability inherent in different batches of complement and SRBC and minor variations in technique when SPA is coupled to SRBC. Nevertheless, the reasonably close scatter of values in the control group, collected over a period of 15 months, suggests that with due care the method is capable of producing consistent results. It is possible that a plaque system using solid phase agar (Gronowicz *et al.*, 1976) or poly L-lysine plates (Kennedy & Axelrad, 1971) may be more stable than the Cunningham fluid phase assay which however, is quicker to perform. It should also be pointed out that B cell activation is not exclusive to SLE. It has been reported in patients with juvenile rheumatoid arthritis (Strelkauskas *et al.*, 1978), infectious mononucleosis (Bird & Britton, 1979), and we have noted it in sarcoidosis, Wegener's granulomatosis, Henoch–Schonlein purpura and in viral and Gram negative infections (unpublished observations). The latter two conditions need to be excluded before a rise in SPFC numbers is interpreted as heralding a relapse of SLE and estimation of serum C reactive protein may serve this purpose (Honig, Gorevic & Weissmann, 1977).

Anti-nDNA SPFC were unexpectedly few, maximally 0·7% of the SPFC. It is possible that the assay is not particularly efficient if one of the components of the immune complex causing lysis of the indicator cells is of a non-Ig nature although Primi, Lewis & Goodman (1979) were able to show secretion of T cell products by an agar gel reverse haemolytic plaque assay. We are not aware of previous enumerations of anti-nDNA secreting cells although anti-DNA has been detected in the supernatant of pokeweed mitogen-stimulated MNC from patients with SLE (Nies *et al.*, 1978). It should also be possible to quantitate cells secreting other autoantibodies such as anti-ssDNA and anti-Ro (SS-A) which are thought to be capable of forming pathogenic immune complexes and which may be found in patients who have SLE but who are negative for antinuclear antibodies and anti-nDNA by conventional tests (Andres *et al.*, 1970; Koffler *et al.*, 1971; Maddison & Reichlin, 1979; Reichlin, Maddison & Provost, 1980). Although quantitation of cells secreting specific pathogenic antibodies should be a more appropriate method for monitoring disease activity and predicting relapse, such an approach is technically more difficult to apply than the simpler measurement of total Ig secreting cells. Moreover, the number of antibody producing cells in the peripheral blood may not reflect the extent and rate of antibody synthesis in lymph nodes and spleen.

This study has not provided an indication of whether the B cell activation in SLE is a primary event or secondary to an imbalance of regulatory T cells. It is well recognized that suppressor cell numbers and function are reduced in active SLE (Bresnihan & Jasin, 1977; Horowitz *et al.*, 1977; Sakawa & Abdou, 1978; Sakane, Steinberg & Green, 1980; Krakauer *et al.*, 1980). However, there is also evidence that lupus prone NZB mice have a subpopulation of hyperactive B cells whose number and Ig production are genetically determined (Manny, Datta & Schwartz, 1979) and that they have no splenic suppressor cell defect at a time when autoimmune responses are already present

(Lebman & Calkins, 1980). In man, decreased suppressor function has been described in relatives of patients with SLE (Miller & Schwartz, 1979) but B cell activation was not studied. It would be of interest to ascertain whether such activation is present and whether it pre-dates alterations in the numbers or function of immunoregulatory cells.

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