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The *in vitro* production and regulation of anti-double stranded DNA antibodies by peripheral blood mononuclear cells from normals and patients with systemic lupus erythematosus

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SUMMARY

The *in vitro* production of anti-double stranded DNA antibodies (anti-DNA) by peripheral blood mononuclear cells (PBMC) was investigated in 19 patients with systemic lupus erythematosus (SLE) and in 12 normal individuals, using a micro solid phase enzyme immunoassay. PBMC from SLE patients spontaneously produced anti-DNA with a higher frequency (16 of 19) than did PBMC of controls (three of 12). In addition SLE patients produced predominantly IgG antibodies. PWM and DNA enhanced anti-DNA synthesis in spontaneously low and non-producers, but acted as inhibitors in spontaneously high producers. The partial removal of T cells decreased or abolished anti-DNA synthesis in four of nine SLE patients. In contrast the B cell enriched fractions of five of nine SLE and five of seven normal patients produced the same or higher anti-DNA levels than did the corresponding unseparated PBMC. These results suggest evidence for autoreactive B cells in SLE as well as in normals, and therefore the combination of these autoreactive B cells with helper and/or suppressor T cell disorders could lead to the over production of anti-DNA seen in different patients with SLE.

Keywords anti-DNA antibodies systemic lupus erythematosus

INTRODUCTION

Patients with systemic lupus erythematosus (SLE) manifest a variety of immune abnormalities leading to the over production of autoantibodies including anti-nuclear antibodies (ANA). However uncertainties remain regarding the cellular immune basis for this human disease. Indeed extensive studies using non specific Ig synthesis by peripheral blood mononuclear cells (PBMC), or their responses to mitogen stimulation and to conventional antigens, have demonstrated hyper-reactivity of B cells (Jasin & Ziff, 1975; Budman *et al.*, 1977; Ginnsburg, Finkel & Lipsky, 1979), B cell defects (Pelton & Denman, 1982), diminished number and/or impaired function of suppressor T lymphocytes (Messner, Lindström & Williams, 1974; Abdou *et al.*, 1976; Bresnihan & Jasin, 1977; Fauci *et al.*, 1978; Krakauer *et al.*, 1979, 1980) and helper T cell defects with normal suppressor activity (Tan, Pang & Wilson, 1981; Delfraissy *et al.*, 1980).

In view of these conflicting results, specific cellular disorders responsible for the over production of ANA were studied. In the preceeding communication, using the *in vitro* production of ANA by

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PBMC, we suggested evidence for the existence of helper T cells promoting ANA synthesis in SLE patients. However we reported that the immunofluorescence technique (IF) used for the detection of ANA was not sensitive enough to detect low anti-DNA antibody levels released in culture supernatants. The question is whether or not cell populations involved in the production of anti-DNA are the same ones regulating the synthesis of other ANA as detected by IF. In this report, the *in vitro* production of anti-DNA antibodies was investigated using a sensitive solid phase microenzyme immunoassay.

Data reported here suggest that the combination between different but separate cellular disorders could lead to abnormal anti-DNA production seen in different SLE patients.

MATERIAL AND METHODS

Patients. Nineteen SLE patients either acute or in remission according to the criteria indices previously described (Delbarre, Mery & de Gery, 1977) were investigated. All these patients had circulating anti-nuclear antibodies (ANA) and were receiving corticosteroid therapy. The control group consisted of 12 adults with no apparent autoimmune disease and negative circulating ANA.

Methods. Peripheral blood mononuclear cells were obtained from plasma of venous heparinized blood by Ficoll-Hypaque density centrifugation and washed three times with RPMI 1640 containing 80 mg/l gentalline.

B and T cells enriched fractions were obtained by one step rosette technique (AET rosette) using sheep red blood cells pre-treated with 2-amino-ethyl-isothio-uronium (Saxon, Feldhaus & Robbins, 1976).

Culture conditions. Unseparated PBMC (2×10^6 cells) and enriched B cells ($0{\cdot}5 \times 10^6$ cells) were cultured with and without stimulation by pokeweed mitogen (PWM, 5 μg/ml, final concentration) or by DNA (Sigma, 0·1 μg/ml, final concentration) in 1 ml RPMI 1640 (Flow Laboratories), supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories), 1% glutamine, 1% non-essential amino acids, 1% sodium pyruvate and 80 mg/l gentalline, and incubated at 37°C in 5% CO_2 humidified environment.

Anti-DNA antibodies detection. Anti-DNA were measured in unconcentrated culture supernatants at days 7–10, using a modification of a solid phase microimmunoassay (Fish & Ziff, 1982). In our experiments alkaline phosphatase enzyme linked anti-human IgG and IgM (Behringwerke) antibodies were used instead of ^{125}I-radiolabelled antibodies. Briefly microtitration plates were coated with DNA (25 μg) diluted in 0·1 M Tris, HCl, $CaCl_2$ buffer (pH 8). Control wells (antigen blank) were not coated with antigen. After 1 h incubation antigen excess was removed by washing, 50 μl of 20% heat inactivated fetal calf serum in PBS buffer was added and left for 30 minutes. After washing 50 μl of DNAase treated culture supernatants were introduced into antigen coated and antigen free wells and incubated for 1 h at 37°C and overnight at 4°C. The plates were washed five times with phosphate buffer (pH 7) containing 20% Tween 20. In the final step the wells were coated with a solution of alkaline phosphatase enzyme linked anti-human IgG and IgM diluted in a buffered 2% bovine globulin. The plates were incubated 2 h at room temperature, extensively washed and dried. Anti-DNA were revealed by incubating with 50 μl of *p*-nitrophenylphosphate in 10% diethanolamine $MgCl_2$ (0·5 mmol/l) buffer (pH 9·8) for 45 min, the reaction stopped by addition 10 μl of 2N NaOH (Enzyme conjugate AP and supplementary reagents Enzygnost Behring) and the intensity of the reaction measured using a Titertek spectrophotometer at 405 nm. Titres of anti-DNA were expressed as OD × 1,000.

RESULTS

Measurement of anti-DNA antibodies

Employing a modification of the technique described by Fish & Ziff (1982) using a solid phase microradioimmunoassay for the detection of anti-DNA in supernatants of spleen cell cultures of experimental mice, it was possible to measure anti-DNA in human PBMC cultures by a solid phase

microenzyme immunoassay. We confirm the observations reported by these authors: culture supernatants exhibited high binding to plastic surface giving high background. To facilitate the measurement of antibodies, the high background could be reduced by digesting supernatants with DNAase before testing. Further reduction in background was achieved by covering the solid phase with 20% heat-inactivated fetal calf serum and by diluting the enzyme linked anti-human antibodies in a buffered 2% bovine γ-globulin.

Spontaneous production of anti-DNA antibodies

When unseparated PBMC were cultured without stimulation spontaneous production of anti-DNA was observed in 16 of 19 SLE patients and in three of 12 controls (Fig. 1). In addition SLE patients secreted predominantly IgG antibodies.

Results shown in Tables 1 & 2 indicate that, after partial removal of T cells, six of nine patients with SLE and five of seven normal B cell enriched fractions could produce anti-DNA without T cell

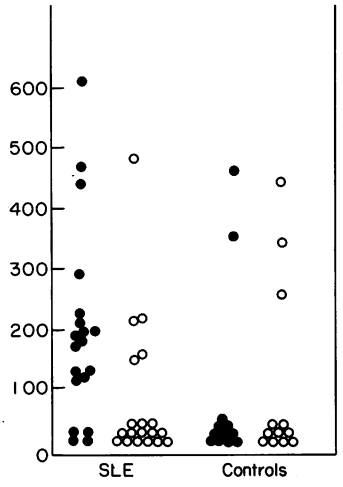

Fig. 1. PBMC from SLE (16 of 19) spontaneously produced anti-DNA antibodies with a higher frequency than did PBMC from controls (three of 12). In addition: SLE patients synthesized predominantly IgG antibodies. ● = IgG anti-DNA; ○ = IgM anti-DNA.

Table 1. Comparison between anti-DNA produced by unseparated PBMC and B enriched fractions from normal individuals.

	Unseparated PBMC supernatants		B enriched fractions	
	IgG	IgM	IgG	IgM
1	—	—	—	115
2	—	—	154	—
3	—	—	—	—
4	—	—	—	—
5	—	—	—	705
6	—	—	—	147
7	—	324	—	280

In normal individuals B cell enriched fractions produced anti-DNA in five of seven experiments, whereas anti-DNA were detected only in one unseparated normal PBMC. These results indicate that autoreactive B cells exist in normals, but are kept under control. Furthermore normal individuals synthesized predominantly IgM antibodies.

Table 2. Comparison between anti-DNA produced by unseparated PBMC and B enriched fractions from SLE patients

	Unseparated PBMC supernatants		B enriched fractions	
	IgG	IgM	IgG	IgM
1	470	230	342	160
2	441	—	—	—
3	350	—	—	—
4	218	—	—	—
5	175	—	198	116
6	195	—	151	278
7	239	487	235	430
8	119	—	445	—
9	—	—	600	230

In SLE patients anti-DNA were detected in six of nine B cell enriched fractions and in eight of nine unmanipulated PBMC. The comparisons between anti-DNA produced by B cell enriched fraction and by unseparated PBMC allows the differentiation of two groups of patients. In the first group (1–4) the partial removal of T cells decreased or abolished anti-DNA production, suggesting that helper T cells could trigger or enhance anti-DNA synthesis by B cells. In the second group (5–8), B cell enriched fractions produced anti-DNA at the same or higher levels than unseparated PBMC, suggesting a partial or total loss of suppressor activity. In one patient (9) activated B cells were kept under strict control.

help. SLE patients produced IgG and IgM antibodies whereas controls synthesized predominantly IgM anti-DNA. In four of nine SLE patients the removal of T cells decreased or abolished anti-DNA synthesis. It is unlikely that in our conditions anti-DNA detected in B cell enriched fractions were solely due to residual helper T cells since anti-DNA levels produced by these B cell fractions were the same or higher than the corresponding unseparated PBMC. Furthermore anti-DNA were demonstrated in some B cell supernatants with no detectable anti-DNA in PBMC. In contrast no detectable anti-DNA was demonstrated in other B cell enriched fractions whereas unmanipulated PBMC produced high anti-DNA levels. The comparison between anti-DNA levels of unseparated PBMC and B fractions shows that low anti-DNA levels (titre < 200) produced by unseparated PBMC were generally associated with autoreactive B cells, whereas helper T cells associated or not with activated B cells were involved in the production of high anti-DNA levels by PBMC (titre > 200).

Effects of PWM and DNA stimulations

After PWM and DNA stimulation two contrasting responses were observed. PWM and DNA stimulated anti-DNA synthesis in low and non-producers (titre < 200), whereas in high producers (titre > 200) PWM and DNA acted as inhibitors (Figs 2 & 3). Decreased anti-DNA seen in high producers after DNA stimulation was not due to *in vitro* binding of antibodies with added DNA, since all supernatants were previously treated by DNAase before testing. However it should be noted that like spontaneous anti-DNA production, PWM and DNA stimulated SLE PBMC low producers to synthesize anti-DNA with a higher frequency than normal PBMC.

The enhancing and particularly the inhibiting effect observed after PWM or DNA stimulation on the production of anti-DNA suggest that anti-DNA released in supernatants were synthesized and secreted by the cells. These results further confirm experiments in the preceding report demonstrating that autoantibodies detected under our conditions were not simply due to cytophilic antibody.

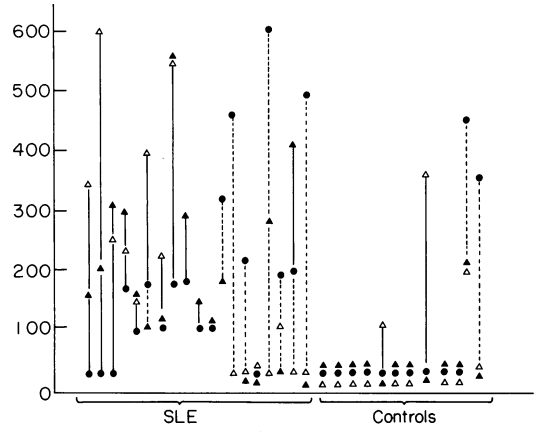

Fig. 2. PWM and DNA *in vitro* enhanced anti-DNA production in spontaneously PBMC low or non-responders (titre < 200) but inhibited anti-DNA synthesis in spontaneously PBMC high producers (titre > 200). ● = Spontaneous IgG anti-DNA; ○ = spontaneous IgM anti-DNA; △ = anti-DNA after stimulation by DNA; ▲ = anti-DNA after stimulation by PWM; — = increase anti-DNA production; - - - = decrease anti-DNA production.

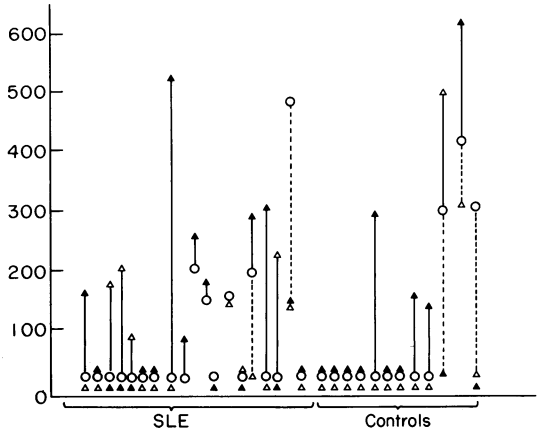

Fig. 3. Effects of PWM and DNA on the production of anti-DNA (IgM) by PBMC. See Fig. 2 for explanation of symbols.

DISCUSSION

This investigation shows that PBMC from SLE patients spontaneously produced anti-DNA with a higher frequency than did PBMC of controls. These data are consistent with studies recently reported (Cairns & Bell, 1983; Hoch, Schen & Schwaber, 1983).

Analysis of these results allows the differentiation of two groups of patients depending on the cellular basis of anti-DNA synthesis. In some SLE patients the removal of T cells decreased or abolished the spontaneous production of anti-DNA, suggesting the existence of activated helper T cells promoting anti-DNA synthesis. In the second group, activated B cells capable of producing anti-DNA without T cell help were demonstrated in SLE patients and in normal individuals. However, the comparison between anti-DNA levels released in B cell enriched fractions and in supernatants of unmanipulated PBMC suggests that in normals these activated B cells are kept under control whereas in SLE patients of this group anti-DNA spontaneously produced by PBMC was related to activated B cells associated with a partial or total loss of suppressor activity. These

findings suggesting evidence for autoreactive B cells in SLE and in normal individuals independently of suppressor cell defects in SLE are in accord with data reported by Clough, Frank & Calabrese (1980). In SLE it is notable that suppressor defects were more frequently observed for IgG than for IgM antibodies (Table 2). These observations may explain the higher frequency of IgG anti-DNA produced by PBMC from SLE patients.

The different cellular abnormalities responsible for anti-DNA production may explain the two opposite responses seen after PWM stimulation. When anti-DNA was mainly due to activated B cells, particularly in low responders (titre < 200) PWM *in vitro* could stimulate helper T cells to enhance anti-DNA synthesis. By contrast when anti-DNA was triggered by *in vivo* activated helper T cells, in high producers (titre > 200), these activated helper T cells become incapable of being further stimulated *in vitro* by PWM which in turn could activate suppressor cell subsets. Indeed in this *in vitro* model, if it is well accepted that the helper function of T cells is essential for B cell differentiation after PWM stimulation, it has been also shown that under certain conditions PWM could exert a suppressive effect via T cells bearing the FcγR (Keightley, Cooper & Lawton, 1976; Jones, 1983; Moretta *et al.*, 1977). The profound decline of anti-DNA synthesis seen after PWM stimulation indicates that in these patients, cell populations involved in the production of anti-DNA were still sensitive to suppressor signals.

If PWM is considered as a polyclonal activator capable of stimulating helper and suppressor T cells regulating anti-DNA, the effects of DNA on the production of anti-DNA shown in this report remain to be elucidated. In this *in vitro* model DNA could act as a polyclonal activator (Reeves, Taurog & Steinberg, 1981) or may have an antigen specific effect, since it was demonstrated that nucleic acids could be recognized by the immune system as antigens (Steinberg, Daley & Talal, 1970; Scher, Frantz & Steinberg, 1973).

From the results reported in this communication, it may be concluded that multiple but separate cellular abnormalities occurred in different SLE patients. In addition to the existence of autoreactive B cells in SLE and in normal individuals, the combination with helper and/or suppressor T cell disorders demonstrated in SLE patients increases the chance of abnormal anti-DNA production. These data contrast with our previous findings demonstrating helper T cell dependency for the production of other ANA as detected by IF. The separate abnormalities shown in this report, leading to the overproduction of anti-DNA, might arise from a common stem cell defect (Morton & Sergel, 1971). Other studies have suggested that different clinical syndromes may be associated with different T cell disorders in patients with SLE (Smolen *et al.*, 1982).

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