Peripheral blood lymphocyte response to phytomitogens in systemic lupus erythematosus

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Foad, B., Adams, L. E., Litwin, A., and Hess, E. V. (1976). *Annals of the Rheumatic Diseases*, 35, 407-414. Peripheral blood lymphocyte response to phytomitogens in systemic lupus erythematosus. The response of peripheral blood lymphocytes to the phytomitogens, PHA, Con A, and PWM, was evaluated in 30 SLE patients and in 30 age, sex, and race-matched controls using dose and time responses. The proliferative response to the three phytomitogens was not depressed in this group of subacute and chronic SLE patients. Active lupus nephritis and a slow acetylator phenotype were associated with a decreased lymphocyte response. The incidence of a slow acetylator phenotype in spontaneous SLE was 68%. In interpreting the lymphocyte response to phytomitogens, the importance of a clear definition of the SLE group under study, the activity of the disease, and treatment status are emphasized.

Various parameters of cell-mediated immunity in systemic lupus erythematosus (SLE) have been reported by several workers with conflicting results. These have included the study of delayed hypersensitivity responses to various antigens (Friedman and others, 1960; Ores and Lange, 1964; Fardal and Winkelman, 1965; Chieragato and Faldarini, 1964; Azoury and others, 1966; Block and others, 1968; Abe and Homma, 1971; Goldman and others, 1972; Bitter and others, 1971; Horwitz, 1972; Hahn, Bagby, and Osterland, 1973; Rosenthal and Franklin, 1975), the *in vitro* response of peripheral blood lymphocytes to phytomitogens and antigens (Parruccio, Rothfield, and Hirschhorn, 1967; Bitter and others, 1971; Horwitz, 1972; Goldman and others, 1972; Senyk and others, 1974; Suciu-Foca and others, 1974; Rosenthal and Franklin, 1975; Lockshin and others, 1975; Malavé, Layrisse, and Layrisse, 1975), the elaboration of macrophage inhibitory factor (Rocklin, Lewis, and David, 1970; Galanaud and others, 1971; Moulias and others, 1972; Abe and others, 1973; Rosenthal and Franklin, 1975), blastic transformation of lymphocytes in mixed leucocyte culture (Hedberg and others, 1971; Wernet and others, 1973; Suciu-Foca and others, 1974), and recently the absolute number and per-

centage of circulating T and B lymphocytes (Messner, Lindstrom, and Williams, 1973; Scheinberg and Cathcart, 1974; Lockshin and others, 1975).

The reasons for the conflicting data are not clear though there are a number of possible explanations. The SLE syndrome covers a wide spectrum of disease subsets varying in mode of presentation and severity of disease activity. In many of the above studies a careful definition of the SLE population was not given. The influence of treatment with corticosteroids and/or immunosuppressive agents on the immune response of SLE patients is difficult to distinguish from aberrations of the immune response caused by the disease itself. Also, these drugs may obscure disease activity. Another major difficulty in comparing data from various laboratories is the lack of standardization of the *in vitro* techniques utilized for measuring cell-mediated immunity. Several factors influence the proliferative response of peripheral blood lymphocytes to phytomitogens. Foad and others (1974) stressed the need for using age-matched controls in comparing populations and for performing dose and time response curves. Most other investigators have used undiluted phytohaemagglutinin and a culture duration of 3 days.

Accepted for publication March 27, 1976.

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The present study of a group of SLE patients took many of these variables into account. The clinical status of the patient population was well defined. Dose and time response curves of peripheral blood lymphocytes to the three phyto mitogens, phytohaemagglutinin-M (PHA-M), concanavalin-A (Con-A), and pokeweed mitogen (PWM), were performed and these data were correlated with disease activity, treatment status, kidney disease, and hepatic acetylation rate. The *in vitro* lymphocyte response data expressed both as counts per minute (cpm) and as ratios of mitogen stimulated cultures divided by control cultures.

Materials and methods

MITOGENS

The concentration of mitogens was similar to that used previously (Foad and others, 1974). PHA-M was used at concentrations of 50 µg, 200 µg, and 1000 µg. Con A was used at concentrations of 50 µg and 200 µg. 1000 µg Con A proved to be toxic. PWM was used undiluted, in 1:5 and 1:20 dilutions.

CELL PREPARATION, CULTURE MEDIUM, AND ASSAY SYSTEM

The method used was that described previously (Foad and others, 1974). Briefly, heparinized venous blood was sedimented with 5% dextran. Leucocytes from the plasma were washed three times with minimum essential medium (MEM) and the cells diluted with the appropriate amount of MEM with gentamicin which contained 20% fetal calf serum and L-glutamine to yield a final concentration of 500 000 mononuclear cells/ml. 4 ml aliquots of the cell suspension were placed into sterile disposable glass culture tubes containing the proper concentration of the three mitogens. The cultures were incubated at 37°C in a moist atmosphere of 95% air and 5% CO₂. After the proper incubation period (3 and 5 days for mitogens and controls), the cells were pulsed for 2 hours with 4 µCi ³H-thymidine (6.0 Ci/mmol per h). After the 2-hour pulse, the cultures were chilled, precipitated with 5% trichloroacetic acid, washed, and solubilized with hydroxide of Hyamine (10×). The vials were counted in a liquid scintillation counter.

PATIENTS AND CONTROLS

Thirty SLE patients and 30 controls matched for age, sex, and race were studied. The age range in the SLE group was 17–73 years with a mean of 36.7 years, and in the control group 17–66 years with a mean of 36.7. There were 24 females and 6 males in the SLE group, and 23 females and 7 males in the control group. 16 were white and 14 black in both the SLE and the control groups.

The clinical features of the SLE group are shown in Table I. The SLE patients were considered to have active disease if two or more of the following were present. (1) Persistent arthritis and/or arthralgias. (2) Persistent skin rash or alopecia and/or mucous membrane lesions. (3) Significant proteinuria, haematuria, or diffuse proliferative glomerulonephritis on

Table I Clinical features of the 30 SLE patients at time of study

	No. of patients
<i>Clinical manifestations</i>	
Skin and mucous membrane lesions	17
Arthritis or arthralgias	16
Serositis	2
Haemolytic anaemia and/or thrombocytopenia	3
Active kidney disease	8
<i>Laboratory data</i>	
Positive antinuclear factor*	30
Antibodies to native DNA†	8
Low C3 complement level	7
<i>Treatment</i>	
Untreated	10
Corticosteroids	20
Prednisone 15 mg or more	5
Prednisone 10 mg or less	15
Immunosuppressive agent‡ and corticosteroids	4
<i>Activity</i>	
Clinically active	16
<i>Duration of disease</i>	
4 months–20 years (mean 4.2 years)	
< 1 year	2
1–5 years	15
5–10 years	8
> 10 years	5
<i>Acetylation rate (21 tested)</i>	
Slow	14
Rapid	7

* ANF by indirect immunofluorescence.

† Determined by a modified Farr technique.

‡ Azathioprine (3), cyclophosphamide (1).

kidney biopsy. (4) Pericarditis or pleural effusion. (5) Convulsions and/or psychosis. (6) Haemolytic anaemia and/or thrombocytopenia.

Sixteen patients were considered to have active SLE and 14 patients to have inactive 'controlled' disease. 20 patients were on corticosteroids and 4 of these were on either azathioprine or cyclophosphamide in addition to corticosteroids. 10 patients were receiving no medications at the time of study. Aspirin was discontinued 48–72 hours and prednisone 24–48 hours before performing the lymphocyte cultures. Most patients had had the disease for more than one year; only 2 patients were studied 4 and 6 months after the onset of symptoms. Thus this series consisted mainly of SLE patients with subacute and chronic disease. 8 patients were considered to have active kidney disease at the time of study either because they had a significant proteinuria and/or haematuria on examination of the urinary sediment or because diffuse proliferative glomerulonephritis was present histologically. Kidney biopsy was performed in 21 of 30 patients at some time during the course of the disease, usually at the time SLE was diagnosed. Focal glomerulonephritis was present in 9 patients, diffuse proliferative glomerulonephritis in 8, membranous nephropathy in 2, and glomerulosclerosis in 2.

The acetylation rate was determined in 21 patients using a modification of the test described by Price Evans and others (1972). 14 of the 21 patients were slow hepatic acetylators of sulphamethazine and 7 of 21 were rapid acetylators.

ANALYSIS OF RESULTS

The response values for the incorporation of ^3H -thymidine into DNA was expressed both as the ratio of

cpm mitogen cultures — background

cpm control cultures — background

and as the difference in counts per minute of mitogen stimulated cultures—cpm in control cultures (Δcpm). Mitogen and control cultures were done in triplicate in most cases, but occasionally in duplicate because of a low cell count. For each concentration of mitogen, the data obtained at 3 and 5 days were expressed as a mean or median value. Student's 't' test was performed on sample means of the ratios in controls and SLE patients and the values were expressed as the mean $\pm$ SD; a P value of 0.05 or less was considered to be significant. Differences within the SLE group relating to status of treatment, disease activity, presence or absence of active kidney disease, and acetylation rate were analysed using Student's 't' test. Because the assumptions underlying the use of this test may not be met by the data, the distribution of the Δcpm and ratios in the SLE and control groups were also analysed by the Mann-Whitney test. In the data analysed by the Mann-Whitney test, the

values for both groups were expressed as median ratios and Δcpm .

Results

CONTROL CULTURES

Due to the wide variations in spontaneous transformation found in control cultures in both SLE and control groups, statistical analysis of the results was performed using the Mann-Whitney test. In 3-day cultures the median cpm for the control group was 503 and for the SLE group 379. This difference was not significant. In 5-day cultures the median cpm was 1437 for controls and 666 for SLE patients. This was significant ($P < 0.05$). No significant differences were noted between treated and untreated SLE patients in 3- and 5-day cultures.

MITOGEN STIMULATED CULTURES

PHA-M

Table II gives the results of lymphocyte transformation to PHA-M as median ratios and median Δcpm . Using the Mann-Whitney test, no statistical differences were noted between the SLE and control groups except to PHA-M at a concentration of 1000 μg and 5 days of culture. However, if the Δcpm is compared, even this difference is not seen. Analysis of data by Student's 't' test gave the same findings.

Table II Lymphocyte response to PHA-M in controls and SLE patients (median ratio and Δcpm)

Culture duration (d)	Group	PHA-M					
		50 μg		200 μg		1000 μg	
		Ratio	Δcpm	Ratio	Δcpm	Ratio	Δcpm
3	Control	22.7	11 500	55.0	31 000	43.2	24 900
	SLE	40.0	12 800	81.5	26 200	54.4	19 200
5	Control	6.9	7 400	14.5	22 200	10.9	15 500
	SLE	7.7	8 800	22.5	18 200	28.0	20 200

* $P < 0.005$.

Table III Lymphocyte response to Con A in controls and SLE patients (median ratio and Δcpm)

Culture duration (d)	Group	Con A			
		50 μg		200 μg	
		Ratio	Δcpm	Ratio	Δcpm
3	Control	39.1	10 100	68.0	40 700
	SLE	68.2	23 800	85.5	32 300
5	Control	9.5	14 500	24.7	37 700
	SLE	17.2	7 100	27.5	35 100

* $P < 0.05$.

Table IV *Lymphocyte response to PWM in controls and SLE patients (median ratio and Δ cpm)*

Culture duration (d)	Group	PWM					
		50 μ g		200 μ g		1000 μ g	
		Ratio	> cpm	Ratio	> cpm	Ratio	> cpm
3	Control	18.2	9 600	36.9	15 200	32.8	15 900
	SLE	22.5	8 500	35.5	12 100	29.7	9 600*
5	Control	17.7	28 800	20.0	29 600	8.5	11 300
	SLE	16.0	14 700	33.5	21 100	21.4	16 300

* $P < 0.05$.

Con A

Table III gives the results of lymphocyte formation to Con A in SLE and control groups as median ratios and Δ cpm. Using the Mann-Whitney test, the SLE group showed a significantly higher response compared to the control group at a Con A concentration of 50 μ g in 3-day cultures ($P < 0.05$). However, no differences between the two groups were observed when the results were expressed as Δ cpm. Analysis of data by Student's 't' test again showed the same findings.

PWM

Table IV gives the results of lymphocyte transformation to PWM. Using the Mann-Whitney test, the SLE group showed no significant differences compared to the control group using median ratios. However, using the Δ cpm, the SLE group showed a significantly higher response compared to the control group at the PWM concentration of 1000 μ g

FIG. 1 *Mean lymphocyte response to PHA-M in SLE patients with active lupus nephritis (●) and inactive lupus nephritis (○). A Culture duration 3 days; B culture duration 5 days*

FIG. 2 *Mean lymphocyte response to Con A in SLE patients with active lupus nephritis (●) and inactive lupus nephritis (○). A Culture duration 3 days; B culture duration 5 days*

and 5-day cultures ($P < 0.05$). Also, no differences were noted using Student's 't' test.

COMPARISON WITHIN THE SLE GROUPS

No significant differences were noted between treated and untreated SLE patients and between those with active versus inactive disease. However, when kidney disease alone is used to divide SLE patients into two groups (active and inactive lupus nephritis), significant differences were noted. SLE patients with active kidney disease showed a decreased lymphocyte response to PHA-M at a concentration of 200 μ g in 5-day cultures ($P < 0.05$), to Con A at a concentration of 200 μ g in 3-day cultures ($P < 0.05$) and 5-day cultures ($P < 0.05$), and to PWM at 200 μ g in 5-day cultures ($P < 0.05$). These results are shown in Figs. 1–3.

FIG. 3 Mean lymphocyte response to PWM in SLE patients with active lupus nephritis (●) and inactive lupus nephritis (○). A Culture duration 3 days; B culture duration 5 days

Slow acetylators of sulphamethazine had a lower lymphocyte response to PHA-M, Con A, and PWM, both at 3- and 5-day cultures compared to rapid acetylators (Fig. 4). This difference was seen with all concentrations of phytomitogens; $P = 0.06$ and 0.08 for PHA-M at 1000 and 200 µg and 3-day cultures; $P = 0.08$ for Con A at 50 µg and 3-day cultures; and $P = 0.06$ for PWM at 200 µg and 3-day cultures.

FIG. 4 Mean lymphocyte response in 3-day cultures to PHA-M, Con A, and PWM in SLE patients. Rapid acetylators (○), slow acetylators (●)

Discussion

In this group of SLE patients (mainly subacute and chronic cases with onset of disease several years before study) the lymphocyte responses to PHA-M, Con A, and PWM were not depressed as compared to controls. Activity in the SLE patients as defined previously did not alter lymphocyte responsiveness

to phytomitogens except for those patients with active lupus nephritis or a slow acetylation rate of sulphamethazine where depressed responses were found.

The sulphamethazine test is a reflection of the activity of the enzyme acetyl transferase which is a genetically determined trait. Hydralazine-induced SLE is seen almost entirely in those individuals who are 'slow acetylators'. Thus, activity of this enzyme was sought in our SLE patients as a potential genetic marker for SLE. A high incidence of 'slow acetylators' was present in our SLE group; 68% compared to the reported incidence of 50% in the general population (Price Evans and White, 1964). Reidenberg and Martin (1974) have also determined the acetylator phenotype by measuring the ratio of monoacetyl dapsone to dapsone in plasma of SLE patients 3 hours after they received a single oral dose of 100 mg dapsone. 10 of their 14 SLE patients were slow acetylators. The slow acetylator phenotype in our study was associated with a depressed lymphocyte response to phytomitogens. The reason for this association is not clear. The difference in lymphocyte culture responses within the SLE group were statistically significant only at some concentrations of phytomitogens in 3- or 5-day cultures. Without performing dose and time response curves, some of the significant differences would have been missed.

Table V summarizes the lymphocyte responses to PHA in SLE patients reported by various workers, including this study. When interpreting these data, many variables should be considered. The SLE population under study must be clearly defined especially as to whether the patients have chronic or acute disease, whether the disease is active, and their treatment status. In the studies reported in the literature on the lymphocyte responses to PHA the data were usually expressed either as a ratio or total cpm.

In the present study the ratios were analysed by two statistical tests, Student's 't' test and the Mann-Whitney test. For PHA-M, the only significant difference between SLE and controls using both statistical tests was observed with a PHA concentration of 1000 µg and 5-day cultures. The lymphocyte response to Con A was significantly different according to both tests at concentrations of 1000 and 50 µg. The two tests gave the same results with the PWM concentration of 1000 µg at 5 days of culture. Therefore, these two tests gave identical results when the ratios were used. However, when the ratios and Δ cpm were compared using the one test (the Mann-Whitney), different results were obtained. The ratios gave significant differences on 3 occasions while the Δ cpm did not show a significant difference. On one occasion, the Δ cpm showed a significant difference between the SLE and

Table V *Lymphocyte response to PHA-M (published studies)*

Author	SLE group (n)	PHA-M concentration (μg)	Culture duration (d)	Ratios or cpm	Response
Patrucco and others (1967)	Not defined (14)	1000	3	Morphology	Normal
Goldman and others (1972)	Chronic, treated (24)	1000	3	Ratio	Normal
Bitter and others (1972)	Acute, untreated (10)	1000	3	Ratio	Depressed
Horwitz (1972)	Acute SLE (14)	1000	3 & 4	cpm	Normal
Senyk and others (1974)	50% clinically active (16)	10-500	5	cpm	Normal
Suciu-Foca and others (1974)	Not defined (40)	5	5	cpm	Depressed
Malavé and others (1975)	Active, untreated (8)	5-20	3	cpm	Depressed
Rosenthal and Franklin (1975)	Active and inactive (12)	150	3	Ratio	Depressed in 2/6 active SLE, normal in inactive SLE
Lockshin and others (1975)	Not defined (21)	0.5-300	3	cpm	Depressed
Present study	Chronic (30)	50-1000	3	Ratio and cpm	Normal
Paty and others (1975)	Untreated (23)	1000	3	cpm	Depressed

control groups which was not confirmed by using the ratios. These different results are due to the decreased spontaneous transformation observed in 5-day cultures in SLE patients compared with controls. This decreased spontaneous transformation tends to increase the value for the ratios in the SLE group compared to the control group. We therefore feel that a more accurate way of expressing the results would be Δ cpm.

In the present study lymphocyte responsiveness to phyto mitogens was unimpaired. This finding agrees with the previous report from this laboratory (Goldman and others, 1972) and the finding of

Senyk and others (1974), *i.e.* that patients with 'controlled' SLE or inactive SLE do not have depressed lymphocyte responses to phyto mitogens. Studies of patients with acute untreated SLE (Rosenthal and Franklin, 1975) do show impairment. This discrepancy may be partially explained by the study of Grossman and others (1975) which showed that acutely ill patients with a variety of diseases have transient depression of cell-mediated immunity.

This work was supported by NIAMDD Training Grant AM 05509 (B.F.), a GCRC Grant RR 00068, and an Arthritis Foundation Clinical Research Center Grant.

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