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Lymphocyte reactivity to mitogens in subjects with systemic lupus erythematosus, rheumatoid arthritis and scleroderma

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SUMMARY

The mitogenic reactivity of lymphocytes from subjects with systemic lupus erythematosus, rheumatoid arthritis and scleroderma was studied. Cultures containing either unseparated or separated lymphocytes were stimulated with phytohaemagglutinin, Con A and pokeweed mitogen after inhibitory serum factors were eluted from the cell surface. Incorporation of [^{3}H]thymidine in patient cultures was compared to that of normal controls. Greatly decreased reactivity was found in SLE to all three mitogens. Significantly decreased values to some mitogens was also observed in rheumatoid arthritis and scleroderma, but the defect was less severe. Cultures of study subjects contained significantly fewer small lymphocytes than normal controls and this finding explained at least in part the decreased mitogenic reactivity.

INTRODUCTION

Certain subjects with systemic lupus erythematosus (SLE) and others with rheumatoid arthritis (RA) have impaired delayed hypersensitivity. In SLE depressed delayed hypersensitivity has been correlated with active disease (Horwitz & Cousar, 1975) and in RA with long-standing involvement (Waxman *et al.*, 1973). The factors producing this immunologic defect, however, are poorly understood.

Since lymphocytes are the specific effector cells of delayed hypersensitivity, the functional status of these cells has been the subject of numerous studies. One method employed to examine lymphocyte function has been to stimulate them with plant lectins such as phytohaemagglutinin (PHA) and measure incorporation of a radiolabelled DNA precursor. Although this has been an area of intensive investigation, results have been contradictory in both SLE and RA. Both normal (Mellbye *et al.*, 1972; Senyk *et al.*, 1974; Kacaki, Bullock & Vaughan, 1969; Reynolds & Abdou, 1973) and depressed (Horwitz & Cousar, 1975; Malavé, Layrisse & Layrisse, 1975; Suciu-Foca *et al.*, 1974; Dormont *et al.*, 1972; Lockshin *et al.*, 1975) responses have been reported.

These discrepant reports may be explained by the heterogeneity of patients studied and differences in the methodological approaches of various investigators. In the investigation of delayed hypersensitivity in SLE, it has become apparent that contradictory results could be attributed to the subjects selected for study. Investigators who have skin tested untreated subjects with very active disease have consistently reported impaired delayed hypersensitivity (Horwitz & Cousar, 1975, Paty *et al.*, 1974; Rosenthal & Franklin, 1975), whereas others who reported normal cutaneous reactivity had examined their subjects after treatment had begun (Block *et al.*, 1968; Goldman *et al.*, 1972). Similarly, investigators who have specifically studied lymphocyte mitogenic reactivity in active SLE have found decreased reactivity (Horwitz & Cousar, 1975; Lockshin *et al.*, 1975; Paty *et al.*, 1974; Rosenthal & Franklin, 1975).

It has become apparent that both cellular and humoral factors influence mitogenic reactivity. A number of reports have appeared which emphasize the effect of such variables as cell numbers and mitogen doses on the lymphocyte-proliferative response (Moorhead, Connolly & McFarland, 1967; Hersh, Harris & Rogers, 1970; Yamamura, 1973). Cellular factors include the numbers of various cell populations added to the cultures (Janossy & Greaves, 1971), the metabolic activity of the responding

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cells (Tavadia *et al.*, 1975) and the functional status of accessory cells such as macrophages (Kirchner *et al.*, 1974). The serum of subjects with both inflammatory and neoplastic diseases contain inhibitors of mitogenic reactivity (Brooks *et al.*, 1972; Horwitz & Cousar, 1975). In subjects with impaired delayed hypersensitivity two factors, immunoregulatory alphaglobulin (IRA) and lymphocyte regulatory gammaglobulin (LRG) have been characterized (Cooperband *et al.*, 1969; Horwitz & Cousar, 1975). These factors must be removed in order to assess cellular activity accurately. We have been especially concerned with the problem of distinguishing abnormalities primarily caused by cellular factors from those caused by serum factors. In many studies on mitogenic reactivity in the connective tissue diseases lymphocytes were cultured with autologous serum so that it was not possible to draw definite conclusions.

Depressed mitogenic reactivity then may be caused by: (1) reduced numbers of responding lymphocytes; (2) an intrinsic defect of the responding lymphocyte; (3) increased numbers or activity of other cells that suppress the reactivity of responders and finally (4) increased concentrations of humoral inhibitory factors. In this report we will consider certain factors which affect the assessment of cellular blastogenesis in subjects with three connective tissue diseases: SLE, RA, and sclerodoma.

Lymphocytes, isolated by two separate methods, were pretreated at 37°C to remove adsorbed serum proteins and stimulated with PHA, Con A or pokeweed mitogen in medium containing a standard lot of normal human serum. The cellular reactivity to mitogens was reduced in patients with all three connective tissue diseases. When compared to controls, these subjects had fewer small lymphocytes in the cultures, but this finding could not completely explain the observed impairment of mitogenic reactivity.

MATERIALS AND METHODS

Subjects. All subjects with SLE, RA and PSS were hospitalized for problems related to their primary disease at the time they were studied. The age, sex, race, WBC, lymphocyte counts and drug therapy of each subject is indicated in Table 1. All subjects with SLE had severely active disease as defined previously (Horwitz & Cousar, 1975) and five of eight had nephritis. Subjects receiving more than 10 mg of prednisone were not studied. Ten of eleven subjects with RA were being treated with ASA (Table 1). Two of eight subjects with scleroderma had rapidly progressive disease and died within 6 months of study. Twenty-eight healthy subjects employed in the Medical Center served as normal controls.

Lymphocyte preparations and cell counts. Cell suspensions used in these studies consisted of either unseparated or separated lymphocytes. Unseparated lymphocytes were prepared by allowing heparinized blood to stand for ½ hr and harvesting the leucocytes after the erythrocytes had settled. Dextran was added to blood from normals to accelerate RBC sedimentation (Horwitz, 1972a). To obtain the lymphocyte counts, the WBC was multiplied by the percent lymphocytes determined from a 200-cell differential count.

Separated lymphocytes were prepared by Ficoll–Hypaque density centrifugation of whole blood (Horwitz & Lobo, 1975) and the percent of small lymphocytes was determined by either a cell smear or from a wet cover-slip preparation. The wet mounts were stained with euchrysine 3RX (Chroma–Gesell Schaft Schmid & Company, Stuttgart—Untertürkheim, Distributor: Roboz Surgical Instruments, Washington, D.C.) and these slides were examined under u.v. light. This acridine fluorochrome stains nuclei green and lysosomal enzymes red-orange. Monocytes could be distinguished from lymphocytes by the presence of many brightly fluorescent lysosomal enzymes (Allison & Young, 1964). Monocytes were also identified by latex phagocytosis. Other large non-phagocytic cells with many red-orange lysosomal enzymes were observed which were probably monocyte precursors and these cells were excluded from lymphocyte counts. Small lymphocytes were defined as non-phagocytic mononuclear cells less than two-thirds the size of a typical phagocytic monocyte.

T lymphocytes were quantitated by the E-rosette method. Lymphocytes were mixed with sheep red blood cells (ratio 50:1) in media containing 5% foetal calf serum that had been absorbed with sheep red blood cells and the suspensions were incubated overnight (Horwitz & Lobo, 1975). Lymphocytes with three or more adherent RBC's were considered positive.

Culture method. Both separated and unseparated cell suspensions were incubated at 37°C for 1 hr to elute inhibitory serum factors (Horwitz, Garrett & Craig, 1977). 4×10^5 buffy coat cells or 2×10^5 mononuclear cells were added to the wells of a Falcon microtest II plate (Benton Dickenson Company, Cockeysville, Maryland) in 0·2 ml of Eagle's minimal essential media supplemented with glutamine, penicillin and streptomycin (Horwitz, 1972a), and 15% normal human serum from a pool of healthy AB^+ male donors. Various dilutions of mitogens, phytohaemagglutinin M (Difco Laboratories, Detroit, Michigan), Con A (ICN Nutritional Biochemical Corporation, Cleveland, Ohio) and Pokeweed mitogen (Grand Island Biological Company, Grand Island, New York) were added to triplicate cultures. The plates were incubated at 37°C in humidified air containing 5% CO_2 for 72 hr. Four and a half hours before termination of the cultures, one microcurie of [^{3}H]thymidine, specific activity 1·3 Ci/mM, in 0·02 ml was added to each well. After the labelling procedure, the plates were chilled at 4°C for 30 min and the supernatants carefully aspirated from each culture. 0·02 M cold thymidine was added to each well and the cultures harvested with a cell harvester (Thurman *et al.*, 1973). Incorporation of [^{3}H]thymidine was measured on a Beckman LS 250 scintillation counter.

TABLE 1. Clinical data

Patient	Sex	Age	Race	WBC	Lymphocytes	Percent T Lymphocytes	Treatment
Systemic lupus erythematosus							
Bla.	F	34	W	4000	880	65	Prednisone 10 mg
San.	F	59	W	4000	240	82	None
Zol.	M	65	W	6200	930	65	None
Cat.	F	33	W	9900	n.d.	n.d.	Prednisone 5 mg
Tho.	F	22	B	8200	1457	74	None
Den.	F	30	W	4700	987	63	None
Man.	M	58	W	5380	954	64	None
Ayl.	F	22	W	3500	770	75	Prednisone 10 mg
Mean		40		5725	888	70	
Rheumatoid arthritis							
Hei.	F	38	W	9000	2430	85	ASA 5·8 gm
Bow.	F	47	W	7200	864	55	ASA 4·2 gm
Dal.	M	54	W	11,300	2486	71	ASA 2·6 gm
Rak.	M	46	W	8400	1008	n.d.	ASA 5·5 gm
Cof.	F	45	W	9000	1080	71	Prednisone 7·5 mg
Car.	F	33	W	3800	912	85	ASA 3·9 gm
Rea.	F	41	W	8000	880	84	ASA 3·1 gm
Has.	F	47	W	8800	1144	63	ASA 4·9 gm
Meg.	F	60	B	3800	722	81	ASA 6·5 gm
Hel.	F	57	W	8200	1804	75	ASA 3·1 gm
Sta.	F	27	W	6300	1575	60	ASA 5·2 gm
Mean		45		7620	1355	74	
Scleroderma							
Cun.	F	48	W	5300	2491	73	None
Hyl.	M	57	W	6400	1536	66	None
Pay.	F	55	W	11,600	2088	68	None
Sen.	M	35	W	7400	1228	51	ASA 2·6 gm
Tho.	F	48	B	4300	1849	n.d.	None
Cre.	M	36	W	16,600	498	88	None
Lil.	M	67	W	6800	816	88	None
Hud.	F	51	W	7400	1628	60	Iboprufen 1200 mg
Mean		50		8225	1511	71	
Normals							
$N = 28$ Mean (± 1 s.d.)				6290 ± 1257	2030 ± 543	$71 \pm 9{\cdot}4$	

RESULTS

Cellular reactivity in test subjects and controls

Fig. 1 indicates that mean values for blastogenesis induced by all three mitogens was 25–50% lower in subjects with SLE and RA than in normal subjects. In those with scleroderma reactivity was reduced to a lesser extent. Cultures containing either unfractionated leucocytes or mononuclear cells responded similarly to mitogens in each group of subjects tested. Intragroup variability, however, was greater with Ficoll–Hypaque mononuclear cells (Fig. 1).

When values for both separated and unseparated leucocytes were combined and analysed with Student's *t*-test, significantly decreased blastogenesis to PHA, Con A and pokeweed mitogen was found in SLE and RA, and depressed blastogenesis to PHA was found in scleroderma (Table 2).

In approximately one-half of the patients, [^{3}H]thymidine incorporation increased to normal levels

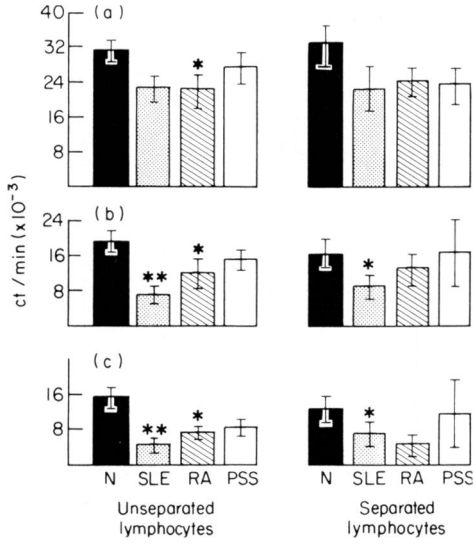

FIG. 1. The reactivity of unseparated and separated lymphocytes to phytohaemagglutinin (1:10), Con A (5 μg) and pokeweed mitogen (2·5 μl) in systemic lupus erythematosus, rheumatoid arthritis and scleroderma (PSS). The mean value of each group was compared with that of normal controls with Student's *t*-test. * Indicate $P \leqslant 0{\cdot}05$, ** $P \leqslant 0{\cdot}01$. Error bars indicate $\pm$ 1 s.e.m. (a) PHA; (b) Con A; (c) PWM.

by increasing the concentration of mitogen. At lower concentrations, differences between test subjects and normals were often greater than shown in Fig. 1.

Using another method to analyse the data, [^{3}H]thymidine incorporation by cells from each test patient was compared to that of cells from a normal control cultured on the same microplate. Fig. 2 reveals greatly depressed blastogenesis of SLE cells. Cellular reactivity to all three mitogens was less than 60% of paired controls. Reactivity of cells from those with scleroderma was 65–95% that of paired controls. In RA a decrease of 25% was found only in response to Con A. This is in contrast to Fig. 1 which revealed that the response to all three mitogens was depressed by at least 25% in RA. This discrepancy is probably explained by the small sample size.

The number of lymphocytes in unseparated and separated cell preparations

Subjects with all three connective tissue diseases had 25–50% fewer circulating lymphocytes than normals (Table 1). Quantitation of small lymphocytes in both unseparated and separated preparations revealed a significant decrease in each disease (Table 3). In the mononuclear preparations, the decrease

TABLE 2. Statistical analysis of mitogenic reactivity

	ct/min							
	PHA		Con A		PWM		Bkg.	
	Mean s.e.m.	*P* value	Mean s.e.m.	*P* value	Mean s.e.m.	*P* value	Mean s.e.m.	*P* value
Normal (74)	31,872±1504		19,480±1492		13,588±1044		405±49	
SLE (16)	24,032±2828	0·03	9232±1472	0·003	5200±980	0·001	479±79	n.s.
RA (22)	24,420±2884	0·01	14,052±1820	0·04	12,184±1080	0·004	422±37	n.s.
Scleroderma (16)	24,332±2200	0·03	14,596±2080	n.s.	10,928±2084	n.s.	356±52	n.s.

Values for both unseparated and separated lymphocytes have been pooled and analysed with Student's *t*-test.
n.s. = Not significant. Numbers in parentheses indicate the number of observations.

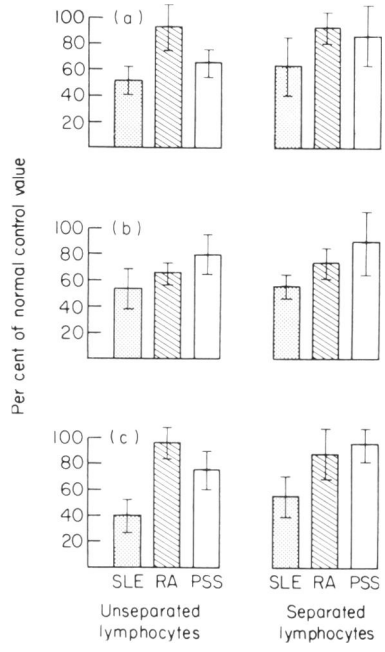

FIG. 2. The reactivity of unseparated and separated lymphocytes to phytohaemagglutinin (1:10), Con A (5 μg) and pokeweed mitogen (2·5 μl) in three connective tissue diseases. Lymphocytes from each test subject were cultured in the same microplate with a normal control and the columns indicate the mean percent of the normal control value. (a) PHA; (b) Con A; (c) PWM.

was explained by an increased percentage of phagocytic monocytes and large non-phagocytic cells with many lysosomal enzymes that were stained by euchrysine. Cultures of study subjects, then, contained fewer small lymphocytes than normal controls. The percentage of T lymphocytes quantitated by a sensitive E-rosette method was similar, however, in both patients and controls (Table 1).

The relationship between lymphocyte numbers and mitogenic reactivity

Studies were conducted to learn whether the decreased numbers of small lymphocytes found in cell suspensions of subjects with connective tissue diseases would explain the decreased mitogenic reactivity. The relationship between cell numbers and mitogenic reactivity is indicated in Fig. 3. A linear relationship was observed in Con A and PWM stimulated cultures with up to $1{\cdot}5 \times 10^5$ lymphocytes per culture, and up to $0{\cdot}8 \times 10^5$ cells with PHA. These results contrast with a previous study where cells were cultured in a 3-ml vol. With this macromethod the response to PHA was linear up to 8×10^5 lymphocytes (Horwitz & Cousar, 1975).

TABLE 3. Percentage of lymphocytes in unseparated and separated cell preparations

	Unseparated		Separated	
	Percentage	*P* value*	Percentage	*P* value
Normals	31±4		86±2	
Systemic lupus erythematosus	16±3	0·001	66±3	0·005
Rheumatoid arthritis	18±2	0·0006	72±4	0·01
Scleroderma	23±5	n.s.†	67±8	0·004

* Student's *t*-test.
† n.s. = $P > 0{\cdot}05$.

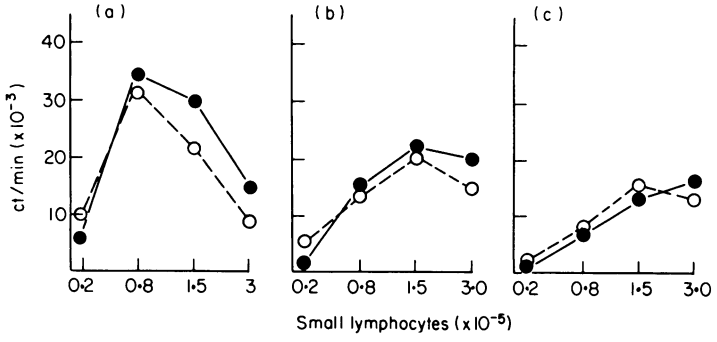

FIG. 3. The effect of the number of lymphocytes cultured on [^{3}H]thymidine incorporation induced by PHA, Con A and PWM. Values for both unseparated and separated lymphocytes are given. (●——●) Separated lymphocytes; (○- - -○) unseparated lymphocytes. (a) PHA 1:10; (b) Con A 5 μg; (c) PWM 2·5 μl.

Calculations of the actual numbers of small lymphocytes added to the microplates revealed that control cultures had a mean of $1{\cdot}3 \times 10^5$ lymphocytes in unseparated preparations. The significantly decreased numbers of lymphocytes in the cultures of patients with connective tissue diseases might contribute to decreased reactivity to Con A and PWM, but would not explain decreased reactivity to PHA.

DISCUSSION

A comparison of mitogenic reactivity in three connective tissue diseases studied with a micromethod reveals a severe impairment in SLE and a mild to moderate defect in rheumatoid arthritis and scleroderma. Reactivity in SLE was greatly decreased to PHA, Con A and PWM whereas in other diseases consistently depressed responses to all three mitogens were not found. This conclusion is based on studies of both unseparated and separated lymphocytes and was reached by two different methods of data evaluation: (1) a statistical analysis of each group compared to a normal group and (2) a comparison between the mitogenic reactivity of cells from a given patient with that of cells from a paired control cultured under identical conditions.

Subjects with all three diseases had fewer numbers of circulating small lymphocytes than normals and this fact explained, at least in part, the decreased blastogenesis observed. In view of our finding of fewer lymphocytes in cultures of unseparated preparations, it was not surprising to find decreased blastogenesis. Depressed PHA responsiveness in SLE was previously explained on this basis (Horwitz & Cousar, 1975). Depressed [^{3}H]thymidine incorporation, however, was noted using cell preparations in which the granulocytes had been removed by density centrifugation. A careful examination of these separated preparations revealed a relative increased in monocytes with a corresponding decrease in small lymphocytes. This observation was consistent with a earlier report from this laboratory on 'atypical' lymphocytes in subjects with SLE or RA. This study revealed that elevated numbers of large spontaneously proliferating mononuclears in these subjects were, in fact, monocyte precursors. Moreover, small lymphocytes in these subjects were depressed (Horwitz 1972b).

In the present experiments, studies on the effect of lymphocyte numbers on mitogenic reactivity were conducted to further explore this problem. A number-response curve was developed for each mitogen. These studies revealed that decreased numbers of small lymphocytes might contribute to the decreased Con A and PWM response, but would not explain the impaired response to PHA.

A review of the studies on mitogenic reactivity in RA revealed seven reports of normal reactivity (Kacaki, Bullock & Vaughan, 1969; Rawson & Huang, 1974; Astorga & Williams, 1969; Nelson *et al.*, 1969; Reynolds & Abdou, 1973; Lycette & Pearmain, 1965; Stratton & Peter, 1972) and two reports of decreased reactivity (Lockshin *et al.*, 1975; Lance & Knight, 1974). Groups reporting normal reactivity employed a 'macromethod' in contrast to the groups reporting decreased reactivity who have used a

'micromethod'. It is possible, then, that lymphocyte reactivity is normal in RA and that depressed responses reported may be explained by technical factors such as decreased number of cells in the cultures.

Subjects with RA in the present experiments were receiving up to 6 g of ASA. It has been reported that ASA added to lymphocytes impairs reactivity (Pachman, Esterly & Peterson, 1971; Opelz, Terasaki & Hirata, 1973), and that lymphocyte reactivity is depressed after ASA is given to subjects for 4 days (Croft, Hepburn & Ritts, 1975). Smith, Hoth & Davis (1975), however, reported that ASA given to subjects for a longer period did not significantly affect lymphocyte blastogenesis. Our studies are in agreement with this observation.

As stated previously, although both normal (Mellbye *et al.*, 1972; Senyk *et al.*, 1974; Goldman *et al.*, 1972) and decreased mitogenic reactivity have been reported in SLE (Horwitz & Cousar, 1975; Suciu-Foca *et al.*, 1974; Malave *et al.*, 1974; Lockshin *et al.*, 1975; Delbarre *et al.*, 1972; Toh *et al.*, 1973; Dormont *et al.*, 1972; Paty *et al.*, 1974; Utsinger, 1975; Rosenthal & Franklin, 1975), all groups who have examined subjects with untreated, severely active disease have found impaired reactivity.

The problem in SLE is complex because increased levels of humoral inhibitors, especially an IgG factor named lymphocyte regulatory gammaglobulin (LRG) (Horwitz & Cousar, 1975), are found in SLE serum. Although several groups have postulated a cellular defect in SLE, in many of these studies the possibility of humoral inhibitory factors was not excluded. The cultures either contained a full concentration of autologous serum, or, procedures to remove serum inhibitors from the cell surface may have been inadequate.

In the present study where appropriate precautions were taken to remove humoral factors, depressed cellular reactivity was also observed. This result could not be explained by a selective decrease in T-lymphocytes, since the percentage of T-cells in subjects was similar to that of normal controls. In an intriguing preliminary report, Utsinger (1975) found that T-cells isolated from subjects with SLE responded poorly to mitogens and that this defect could not be corrected by an overnight incubation at 37°C. Further studies on purified lymphocyte populations are in order to clarify this issue.

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