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Study of lymphocyte subpopulations in normal humans and patients with systemic lupus erythematosus by fractionation of peripheral blood lymphocytes on a discontinuous Ficoll gradient

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SUMMARY

Peripheral blood lymphocytes of thirty normal volunteers and fifty-two patients with systemic lupus erythematosus were fractionated using a discontinuous (5–30%) Ficoll gradient. Such fractionation permitted the isolation, identification and study of null cells, T cells and B cells. Patients with inactive SLE were found to have a cell distribution and responsiveness to PHA, Con A and Pokeweed mitogen (PWM) similar to controls. In contrast, patients with active SLE showed a significant decrease in T-cell fractions as well as a relative increase in null cells, a normal distribution of B cells, a marked reduction in responsiveness to Con A, a lesser reduction to PHA and only a minor reduction to PWM. With increasing disease activity, the number of null cells increased despite lymphopenia. Spontaneous lymphocyte transformation was observed in patients with SLE. This occurred predominantly in the fractions enriched in B cells and was observed both early (0–16 hr) and late (68–72 hr) in the lymphocyte cultures. The method of discontinuous Ficoll gradients is both versatile and reproducible with good correlations between isolated lymphoid subpopulations and disease activity.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystem disease of unknown etiology, characterized by multiple autoantibodies and immune complex deposition (Decker *et al.*, 1975). Many immunologic abnormalities have been catalogued in this disorder, and it has been proposed that a defect in lymphocyte subpopulations may be critical in disease pathogenesis. Evidence supporting such alterations in lymphocyte subpopulations is accumulating for the SLE-like disease of New Zealand mice (Garner, Gershwin & Steinberg, 1975; Stutman, 1972; Stobo, Talal & Paul, 1972).

Peripheral blood lymphocytes (PBL) from patients with SLE have been characterized with regard to distribution of surface markers (Messner, Lindstrom & Williams, 1973; Winchester *et al.*, 1974; Scheinberg & Cathcart, 1974); however, there has been no report of separation of subpopulations of these cells. Indeed, study of fractionated human PBL has been quite limited (Yu *et al.*, 1973a; Bach & Brashler, 1970; August *et al.*, 1970; Geha, Rosen & Merler, 1973). The present experiments were undertaken to separate subpopulations of PBL from normal humans, and to characterize these with regard to surface markers and responses to mitogens. With this background information, PBL from patients with SLE were similarly fractionated and studied. We herein report the successful fractionation of PBL from thirty normals and fifty-two patients with SLE.

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MATERIALS AND METHODS

Patients. Fifty-two patients with systemic lupus erythematosus (SLE) followed by the Arthritis and Rheumatism Branch, NIAMDD, NIH and thirty healthy volunteers of both sexes were studied. The healthy volunteers were age and sex matched with the SLE group such that their sex distribution, age range and mean and median age were similar. All patients met the diagnostic criteria of the American Rheumatism Association for SLE. Activity of the SLE was assessed by two unbiassed observers based upon clinical disease and graded as active, mildly active or inactive as previously described (Steinberg & Decker, 1974; Gershwin & Steinberg, 1974). No patients were graded active by one and inactive by the other observer. If a patient was graded mildly active by one observer a grade of mildly active was given. Patients receiving cytotoxic drugs (cyclophosphamide and/or azathioprine) were placed in a separate group. Those receiving high doses of steroids (> 20 mg/d prednisone) were excluded.

Separation of lymphocytes. Peripheral blood was drawn between 8.30 a.m. and 10.30 a.m. into preservative-free heparin (25 USP u/ml, Fellows Medical Manufacturing Company Incorporated, Oak Park, Michigan). The heparinized blood was diluted 1:1 with calcium- and magnesium-free isotonic Hanks's balanced salt solution (HBSS) (Grand Island Biologic Company, Grand Island, New York) and placed on a Ficoll–Hypaque gradient (Böyum, 1968). Lymphocytes, thus separated, were washed three times with HBSS, and an aliquot of these unfractionated cells was removed for use in further experiments. The remaining lymphocytes (the great majority) were resuspended in a 5% Ficoll solution in HBSS and layered onto the discontinuous Ficoll gradient.

Discontinuous Ficoll gradient. Ficoll (Sigma Lot 14C-2350), a polysucrose polymer of mol. wt 400,000, was dissolved in HBSS (Ca^{2+}- and Mg^{2+}-free) at pH 7·4, to obtain a 32% Ficoll solution. This stock solution was sterilized by passage through a Millipore filter (0·45 μ) and the following concentrations were prepared: 5, 9, 12, 15, 17, 19, 21, 23, 25 and 30%. The Ficoll concentration was controlled in each solution by measurement of refractive indices, which were linearly related to the density (Bach & Brashler, 1970; Yu *et al.* 1973a, b). Three ml of the Ficoll solutions were carefully layered in sequence, in cellulose nitrate tubes (Beckman No. 302237, 1″ × 3½″) beginning with the highest density preparation (30%) at the bottom and ending with 9% at the top. The cells, suspended in 5% Ficoll, were added as the uppermost layer. The tubes were centrifuged at 10°C for 30 min at 16,000 *g* using an SW27 swinging bucket rotor in a Beckman L2-65B ultracentrifuge. Following centrifugation the lymphocytes were distributed at the interfaces. They were removed with Pasteur pipettes and placed into separate 16 × 125 mm plastic Falcon test tubes containing 5 ml of HBSS. Each fraction of cells was named by in sequence from A to G starting with 9–12% interface and ending with the 23–30% interface. Cell viability was more than 95% in each fraction, as demonstrated by trypan blue exclusion.

Lymphocyte counts. After fractionation, the cells in each of seven fractions were washed twice in HBSS and counted. Cells were counted in a Coulter Counter Model ZBI. These counts were confirmed periodically using a haemocytometer. In addition, fractions were examined by light microscopy using a Wright–Giemsa stain.

Lymphocyte culture. The washed lymphocyte fractions were adjusted to a concentration of 2×10^6 cells/ml in RPMI-1640 medium (Grand Island Biologic Company, Grand Island, New York) supplemented with 100 u/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES buffer, 2 mM glutamine, and 2% heat-inactivated foetal calf serum absorbed with human red blood cells.

Lymphocytes (2×10^5 per well) were distributed in sterile microtitre plates in 0·2 ml of RPMI medium without mitogens or with phytohaemagglutinin, PHA-P (1 μg/ml), concanavalin A (Con A) (1 μg/ml) or Pokeweed mitogen (PWM) (1%). All cultures were performed in triplicate.

The lymphocytes were incubated in a humidified atmosphere (5% CO_2, 95% air) at 37°C for 72 hr. Four hours before harvest 0·5 μCi of tritiated thymidine, ^{3}H-TdR (New England Nuclear, Boston, Massachusetts) was added. The cultured cells were collected using an automated multiple cell harvester, washed, and the trichloroacetic acid insoluble radioactivity measured in a Nuclear Chicago liquid scintillation counter. Differences in counts per minute (ct/min) between replicate cultures, were less than 10%. Results were expressed as ct/min stimulated cultures—ct/min unstimulated cultures. The background ^{3}H-TdR incorporation in unstimulated cultures was measured between 68 and 72 hr. Additional cultures were set up simultaneously from the same pools, however, the ^{3}H-TdR was added at the onset of culture for study of early spontaneous incorporation. The cells were harvested after 16 hr of incubation and the radioactivity measured, as described above.

Identification of T cells, B cells and macrophages. The E-rosette test was used as a marker for T lymphocytes. Cells from either the Ficoll–Hypaque separation or the Ficoll gradient were placed in HBSS at a concentration of 4×10^6/ml. One hundred microlitres of this cell preparation was added to 4×10^7 SRBC (SRBC : lymphocyte ratio of 10:1) in 200 μl of 50% heat-inactivated, SRBC-absorbed foetal calf serum. The mixture was incubated at 37°C for 30 min, followed by centrifugation at 200 *g* and incubation at 4°C overnight. A rosette was defined as a lymphocyte surrounded by three or more adherent SRBC. Viability of lymphocytes was ascertained by trypan blue exclusion and was always greater than 95%. A single sheep was bled weekly for these experiments and the SRBC collected in Alsever's solution. All rosette assays were performed on SRBC less than 3 days old.

The number and percentage of lymphocytes bearing complement (C) receptors was determined by measuring the number of lymphocytes binding three or more SRBC which had been sensitized with an IgM antibody to SRBC plus C. A purified human IgM myeloma with antibody activity against SRBC was used in a subagglutinating dilution (kindly provided by Dr Richard Wistar).

An equal volume of antibody and 2% SRBC were incubated at 37°C for 45 min, washed three times in veronal-buffered saline containing calcium, magnesium and 1% bovine serum albumin (VBS) and resuspended to the original volume in VBS. Complement, 0·2 ml fresh-frozen mouse serum, was added to each 2 ml of antibody coated SRBC, incubated at 37°C for 45 min, washed three times in VBS, and diluted to a SRBC concentration of 0·5%. To 100 μl of this suspension, 4×10^5 lymphocytes in 100 μl VBS were added, incubated at 37°C for 45 min and resuspended by rotation of the test tube. The cells were drawn up in a Pasteur pipette by capillary action and enumerated.

Monocytes were identified by their ability to phagocytose 1 u latex particles (1·099±0·0059 μ diameter, Dow Chemical Company, Indianapolis, Indiana). Cell suspensions were incubated with latex particles at 37°C for 1 hr; centrifuged at 600 *g* for 10 min, 800 *g* for 10 min, and finally 1,000 *g* for 10 min in medium saving the precipitate each time. Finally, the cells were suspended in 100 μl of medium, placed in a haemocytometer and enumerated.

RESULTS

Distribution of PBL on a discontinuous Ficoll gradient

PBL, separated on Ficoll–Hypaque were further studied by determining their distribution on a discontinuous Ficoll gradient. Cells from normals were distributed primarily in fraction D (56·2%) and in fraction E (21·3%) (Table 1). The peak in fraction D was consistently observed in all normals studied. Moreover the percentage of cells in each fraction and the total recovery was very reproducible. One normal control studied five times, on different days, had values differing by less than 5%. Differences in

TABLE 1. Distribution of peripheral blood lymphocytes from patients with SLE and normal controls after fractionation on a discontinuous Ficoll gradient

		Mean percent of cells in each fraction ± s.e. mean Fraction					
Group		A	B	C	D	E	F+G
1. SLE, active*		1·9±0·22	5·6±0·70	18·5±1·87	44·4±2·28	19·3±1·72	10·3±1·01
2. SLE, inactive		1·4±0·21	2·9±0·41	10·5±1·48	58·4±2·15	19·0±1·83	7·8±1·19
3. Normal		1·7±0·22	3·2±0·37	8·0±0·83	56·2±1·65	21·3±1·30	9·8±1·33
Statistical	1 vs 2	n.s.	$P<0·01$	$P<0·005$	$P<0·001$	n.s.	n.s.
comparison	1 vs 3	n.s.	$P<0·02$	$P<0·001$	$P<0·001$	n.s.	n.s.
	2 vs 3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

* Active or mildly active.

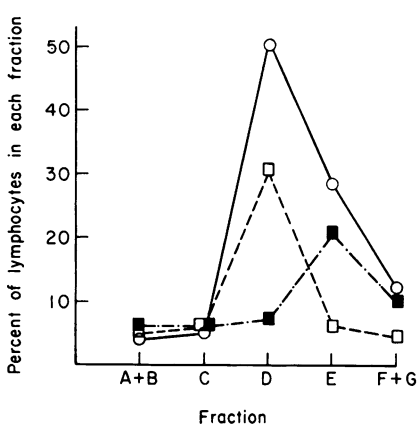

FIG. 1. Ficoll–Hypaque separated lymphocytes were fractionated on the discontinuous Ficoll gradient (○——○). Cells from fraction D were re-run on the gradient (□ – – – □) as were all of the other cells (■ – · – ■). Cells from fraction D that were re-run reappeared in fraction D, whereas non-D cells were preferentially lost from fraction D on re-fractionation.

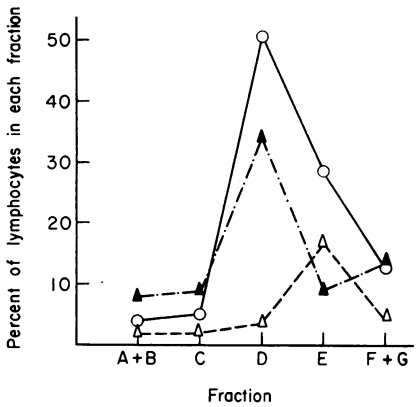

FIG. 2. Ficoll–Hypaque separated lymphocytes were fractionated on the discontinuous Ficoll gradient (○——○). Cells from fraction E were re-run on the gradient (△ - - - △) as were all of the other cells (▲ - · - ▲). Cells from fraction E were again found in fraction E following re-fractionation; non-E cells were preferentially lost from fraction E.

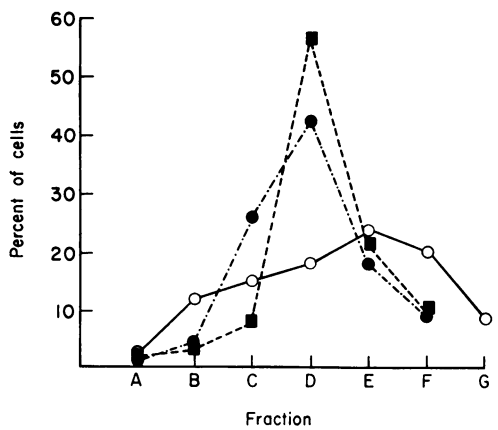

FIG. 3. Fractionation patterns from two patients with SLE and normals following discontinuous Ficoll separation of peripheral blood lymphocytes. The 'normal' pattern represents the average of twenty healthy controls (■ - - - ■). Patient BC (● - · -●) had mildly active SLE; patient DS (○——○) had very active systemic disease.

cell distribution were not observed between males and females. When fraction D cells were re-run on the discontinuous Ficoll gradient, they were again found in fraction D (Fig. 1), whereas non-fraction D cells were not found in fraction D (Fig. 1). Similarly, fraction E cells returned to fraction E, and non-E cells did not return to fraction E (Fig. 2). Patients with active SLE had an increase in percentage of cells in fractions A+B and C, and a decrease in fraction D (Table 1). The more active a patient's disease, the greater the reduction in fraction D (the T-cell peak see below). For example, patient BC with mild activity had a decrease in fraction D and an increase in fraction C (Fig. 3). An example of extreme abnormality of cell distribution is patient DS who had active SLE and a flat cell-distribution curve (Fig. 3). When her disease was less active her cell distribution returned toward normal.

Distribution of E and EAC rosette-forming cells

The cell type in each of the fractions was identified by determining the percentage of cells forming E or EAC rosettes as well as the percentage of macrophages. The majority of cells from normals in fractions

D and E formed E rosettes (Table 2). The relative percentage of cells forming EAC rosettes rose as the density of Ficoll increased. In contrast, fractions A+B and C were largely (>78%) composed of cells which did not form E or EAC rosettes (Table 2).

There were less than 5% macrophages (determined by latex-particle phagocytosis) in any of the fractions, although almost 15% of the initial isolated Ficoll–Hypaque cells were macrophages. This was attributed to the extensive number of washings in plastic tubes and the use of cellulose nitrate ultracentrifuge tubes; these cells appeared to stick to the latter. The loss of macrophages is reflected in the increased percentage of E-rosette forming cells obtained after fractionation (74·8±1·9%) compared to the percentage of E rosettes forming cells in the Ficoll–Hypaque separated PBL (66·2±1·3%) (Table 2).

TABLE 2. Distribution of E-rosette forming cells, EAC-rosette forming cells and cells forming neither rosette (termed null cells) following discontinuous Ficoll density gradient separation of PBL from patients with SLE and normal controls

			Mean percent of cells of each type in each fraction ± s.e. mean Fraction					
Group	Cell type	Unfractionated cells	A+B	C	D	E	F+G	Total
SLE, active	E	46·1±5·22*	0·4±0·19	3·1±1·07	25·9±4·27§	15·2±3·50	3·7±0·79	48·3±4·11‡
	EAC	24·9±2·35	0·1±0·03	0·6±0·21	6·6±0·90	5·6±0·89	3·6±0·32	16·4±1·91
	Null	29·1±5·46†	4·7±0·64†	13·6±2·28§	10·1±2·89‡	4·6±1·79	2·3±0·54	35·3±3·78§
SLE, inactive	E	65·8±2·73	0·4±0·29	1·7±0·84	44·6±3·92	19·5±2·54	4·6±0·49	70·8±4·25
	EAC	20·5±1·68	0·1±0·04	0·1±0·08	7·0±0·99	5·1±0·77	4·1±0·41	16·4±1·35
	Null	13·7±2·66	2·8±0·55	7·5±2·47	2·0±1·31	0·1±0·64	0·4±0·42	12·8±2·53†
Normal	E	66·2±1·31	0·7±0·37	1·2±0·37	47·6±1·63	20·1±1·87	5·2±0·32	74·8±1·92
	EAC	19·7±1·21	0·1±0·03	0·3±0·09	6·5±1·15	6·1±0·50	4·1±0·34	17·1±0·96
	Null	15·3±1·79	2·8±0·33	3·4±0·48	0·3±1·22	0·2±0·51	1·4±0·20	8·1±0·88

* Significantly different from normal controls (*t*-test).
† $P<0·05$.
‡ $P<0·01$.
§ $P<0·001$.

For ease in reading Table 2, the numbers have been expressed as percentage of the total number of cells. Almost half (47·6% of all the cells from normals were E-rosette forming cells in fraction D. Another quarter were E-rosette forming cells in fraction E. Patients with inactive SLE resembled normal controls in the relative distribution of E and EAC rosette forming cells (Table 2). In contrast, patients with active SLE had a reduction in E-rosette forming cells in fractions D, E and F with no change in EAC rosettes (Table 2), leaving a relative increase in null cells in those fractions. Patients with very active disease tended to have the greater T reduction in E rosettes (data not shown). It is apparent that the increase in cells in fraction C from patients with active SLE from Table 1 is due to an increase in null cells (Table 2).

The information was then recalculated so that it could be expressed as cells of each type per mm^3 of blood (Table 3). The absolute number of cells expressed in this way differs from the percentage of the absolute numbers shown in Table 2 since patients with active SLE had far fewer lymphocytes than did the other groups. Those with active disease had a marked reduction in total number of T cells and B cells (Table 3). Major reduction in T cells and B cells were observed in fractions, D, E and F. Despite the decrease in total cells in patients with active SLE, they had a 50% increase in null cells (Table 3). These cells were found in increased numbers in fraction C, D and E. The modest increase in null cells combined with the marked decrease in T and B cells in active SLE leads to a marked relative increase in null cells (37% in active SLE compared to 9% in normals, similar to the findings of Table 2).

TABLE 3. Absolute lymphocyte counts (cells/mm^3 blood) in normals and patients with SLE*

Group	Cell type	Mean number of cells of each type in each fraction ± s.e. mean contained in 1 mm^3 of blood Fraction A+B	C	D	E	F+G	Total
SLE, active	E	1·9±0·9†	10·8±3·5	80·2±16·9**	58·1±15·7**	16·5±3·4**	167·5±26·1**
	EAC	0·1±0·1	1·8±1·1	16·0±2·6**	15·5±3·0**	15·3±3·2**	48·7±6·1**
	Null	14·2±2·8	65·3±14·3	29·9±8·9‡	10·3±5·4	8·2±1·9	127·9±23·7
SLE, inactive	E	2·2±1·7	9·8±3·4	309·6±36·4‡	128·0±18·7‡	24·8±4·9**	474·4±39·8¶
	EAC	0·2±0·2	0·4±0·4	56·6±12·6	30·8±4·3‡	23·8±4·1¶	111·8±7·3‡
	Null	21·6±5·2	42·8±11·5	20·6±10·2	15·0±14·2	14·7±7·2	114·7±29·6
Normal	E	6·4±3·9	12·1±3·9	425·4±26·7	215·5±20·7	51·1±5·9	710·4±42·8
	EAC	0·5±0·3	3·2±0·8	66·6±4·7	56·4±6·0	42·2±4·4	168·9±12·2
	Null	25·6±3·8	35·5±7·9	7·5±4·9	−0·1±0·2	13·4±3·2	82·0±12·5

* Calculated on the basis of cell yields and percentage of each cell type in each fraction in each individual.
† Significantly different from normal controls (*t*-test).
‡ $P<0·05$.
¶ $P<0·005$.
** $P<0·001$.

Response of fractionated cells to mitogens

The response of PBL from normals and patients with SLE was first studied using unfractionated cells. Cells from patients with inactive SLE were similar to normal PBL in their response to PHA, Con A and PWM (Table 4). In contrast, patients with active SLE had a reduced response to all mitogens although the same number of cells (2×10^5) was used in each culture. The response to Con A was most impaired, whereas that to PWM was least affected (Table 4). The response to Con A was reduced to a somewhat greater extent than that to PHA.

Individual fractions from the Ficoll gradient were similarly tested. The response of normal PBL to Con A was limited to fractions D and E (Fig. 4). Patients with inactive SLE had a slight reduction in this

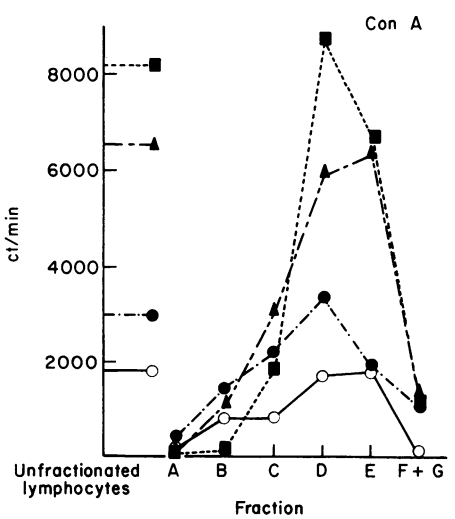

FIG. 4. Average mitogenic responses to Con A are presented for unfractionated and fractionated PBL from normals and patients with SLE with different degrees of systemic disease activity. Peak responses were observed in fractions D and E from 'normals' (■ - - - ■) and patients with inactive SLE (▲ - · - ▲). This peak was reduced in patients with mild (● - · - ●) or severe (○——○) disease activity.

TABLE 4. Lymphocyte transformation following stimulation with PHA, Con A or PWM of PBL from patients with SLE or normals

Group (no.)	Mitogen	Incorporation of ^{3}H-TdR by unfractionated lymphocytes* Mean ± s.e. mean	Range	Percent of patients with reduced response†
SLE, active	PHA	10,035 ± 2130§	3175–28,167	69
(13)	Con A	1801 ± 580**	405–7875	92
	PWM	7282 ± 880	1917–10,773	38
SLE, mild	PHA	13,092 ± 1080¶	5022–20,328	50
(18)	Con A	3221 ± 420**	973–6554	72
	PWM	6065 ± 570§	1663–8869	44
SLE, inactive	PHA	19,504 ± 2500	8290–30,128	11
(9)	Con A	6525 ± 1110	1656–13,061	11
	PWM	7576 ± 440	7226–8375	0
SLE, inactive treated with cytostatic drugs	PHA	8757 ± 1100**	3841–13,348	90
(10)	Con A	3651 ± 680**	1382–8101	50
	PWM	4303 ± 410**	2171–5741	40
Normal	PHA	19,317 ± 810	14,194–25,473	0
(20)	Con A	8164 ± 430	5831–12,213	0
	PWM	8884 ± 350	7248–12,316	0

* Significantly decreased in comparison with normal controls (*t*-test).
† More than 2 SD below the mean of normal controls.
‡ ct/min stimulated − ct/min unstimulated (background).
¶ $P < 0{\cdot}05$.
§ $P < 0{\cdot}01$.
** $P < 0{\cdot}001$.

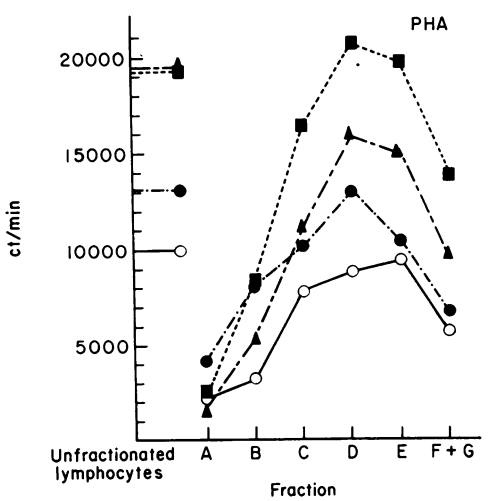

FIG. 5. Average mitogenic responses to PHA are presented for unfractionated and discontinuous Ficoll gradient fractionated PBL from normals and patients with SLE. The peak PHA response of 'normals' was broad (from fraction C to fraction F). This peak was progressively reduced in going from normal (■ – – – ■) to inactive SLE (▲ – · – ▲), to mildly active SLE (● – · – ●) to very active SLE (○——○).

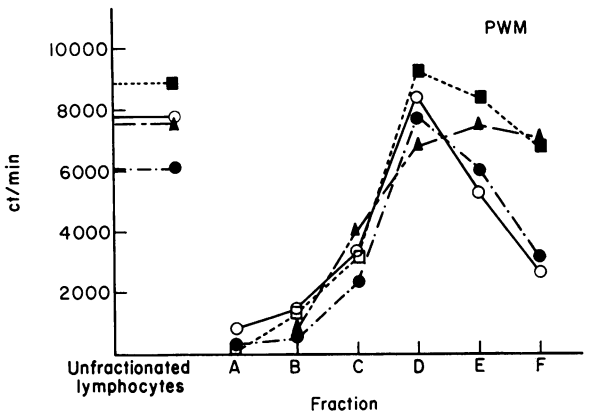

FIG. 6. Average mitogenic responses to PWM are presented for unfractionated and fractionated PBL from 'normals' and patients with SLE. The peak PWM response of normals (■ - - - ■) was found in fractions D, E and F+G. Patients with inactive SLE had a similar response (▲ - · - ▲). In contrast, patients with mildly active (● - · - ●) or very active (○——○) SLE maintained the peak in fraction D, but showed reduced responses in fractions E and F+G.

TABLE 5. Spontaneous lymphocyte transformation in the heavier fractions of PBL from patients with SLE and normals separated on a discontinuous Ficoll gradient

		Spontaneous incorporation of ^{3}H-TdR by fractionated lymphocytes			
		0–16 hr		68–72 hr	
Group (No.)	Fraction	ct/min unstimulated cultures	Percentage of patients with elevation*	Ct/min unstimulated cultures	Percentage of patients with elevation*
		Mean ± s.e. mean			
SLE, active	E	4268 ± 1130†	85	1068 ± 278‡	69
(13)	F+G	4379 ± 1026**		1396 ± 242**	
SLE, mild	E	1837 ± 353**	77	928 ± 192**	50
(18)	F+G	4638 ± 964**		1465 ± 273**	
SLE, inactive	E	2159 ± 729§	55	487 ± 179	22
(9)	F+G	3301 ± 1066**		757 ± 261	
SLE, inactive treated with	E	1694 ± 420§	50	418 ± 106	20
cytotoxic drugs	F+G	2612 ± 985¶		547 ± 197	
(10)					
Normal	E	562 ± 51	0	496 ± 61	0
(20)	F+G	410 ± 72		395 ± 55	

* More than 2 s.d. above the mean of normal controls.
† Significantly increased in comparison with normal controls (*t*-test).
‡ $P < 0{\cdot}05$.
¶ $P < 0{\cdot}01$.
§ $P < 0{\cdot}005$.
** $P < 0{\cdot}001$.

peak response. Groups of SLE patients with increasing disease activity demonstrated more marked impairment in the response of fractions D and E to Con A. PBL from patients with inactive SLE who were receiving cyclophosphamide had a reduced response to Con A in fractions D and E; whereas those patients taking azathioprine did not have such a reduction.

PHA was mitogenic for cells in fractions B, C, D, F+G (Fig. 5). Patients with active SLE had reduced responses to PHA in fractions B, C, D, E, and F+G. As was found with Con A, the more severe the disease, the greater the reduction (Fig. 5).

Responses to PWM occurred primarily in the fractions richest in EAC-rosette forming cells, the heavier fractions (Fig. 6). This response (ct/min stimulated cultures minus ct/min unstimulated cultures [background]) was reduced in fractions E and F+G patients with active SLE. However, the abnormality was not solely the result of lower ct/min in the stimulated cultures, but also of higher background ct/min in the unstimulated cultures from fractions E and F+G (right-hand half of Table 5). This represented spontaneous lymphocyte transformation by cells in fractions E and F+G from patients with active SLE. This spontaneous transformation was augmented only modestly by PWM. No increase in background incorporation at 68–72 hr was observed in fractions A, B, C or D (data not shown) suggesting that the increased transformation was a result of stimulated B cells.

The increased incorporation (background) at 68–72 hr led to analysis of the spontaneous ^{3}H-TdR incorporation early in the culture period. The same increased spontaneous incorporation observed in fractions E and F+G was repeated during this early culture period (left hand half of Table 5). In addition, patients with active SLE demonstrated a modest but significant increase in ^{3}H-TdR incorporation in unfractionated cells (2200 ct/min in active SLE; 340 ct/min in normals) and in cells from fractions A, B, C and D (1200–1900 ct/min in active SLE; 180–550 ct/min in normals) in the 0–16-hr period.

DISCUSSION

Previous workers have used Ficoll gradients to study human peripheral blood cells, thoracic duct lymphocytes, and tonsillar lymphocytes (Yu *et al.*, 1973a; Bach & Brashler, 1970; Yu *et al.*, 1973b). The present study is based upon these previous experiences with Ficoll. In addition to cell fractionation, the relative and absolute number of T, B and null cells were determined for peripheral blood from normals and patients with SLE. Patients with active SLE were round to have (a) marked decrease in absolute numbers of T cells, (b) marked decrease in absolute numbers of B cells, (c) minimal increase in absolute number of null cells, (d) decrease in percentages of T cells, (e) normal percentage of B cells, (f) increased percentage of null cells. B cells were measured by formation of EAC rosettes. This is superior to determination of surface Ig which has been found to be elevated in SLE as a result of circulating anti-lymphocyte antibodies (Winchester *et al.*, 1974; Scheinberg & Cathcart, 1974). Fortunately, such antibodies do not alter the enumeration of E rosettes (Messner, Kennedy & Jelinek, 1975), the method of determination of T cells. The percentage of E- and EAC-rosette forming cells in normals in the present study corresponds well to results of previous study (Holm *et al.*, 1975). The marked reduction in absolute number of T and B cells in active SLE agrees with recent studies (Messner, Lindstrom & Williams, 1973; Scheinberg & Cathcart, 1974). It is apparent from the present study that populations of cells in SLE might vary depending on (a) whether one is determining the percentage or absolute number of the population in question, (b) the degree of activity of the disease process.

The marked reduction of fraction D T cells in active SLE suggested that these lymphocytes may be eliminated *in vivo*. In addition, cell-membrane abnormalities might make them more sensitive to mechanical procedures leading to increased damage during separation. Control studies showed that normal lymphocytes depleted of fraction D do not reform this peak when placed a second time on the discontinuous Ficoll gradient. Furthermore, lymphocytes recovered from fraction D returned to this fraction when replaced on the gradient. Similarly fraction E, which also consists almost completely of T cells, returned to fraction E when re-run. These data suggest that the lymphocytes found in fraction D might be a distinct T-lymphocyte subpopulation, which is preferentially reduced in active SLE. This phenomenon has been further investigated (Glinski, Gershwin & Steinberg, 1976).

The rosette studies showed that 70–90% of the lymphocytes separated in fractions A+B and C are 'null' cells, both in SLE and normals. In active SLE the relative number of 'null' cells was significantly increased. Some patients with active SLE had significantly more 'null' cells than normal on an absolute basis; however, on the mean absolute 'null' cell counts were only 50% increased in active SLE. The function and cell type of null cells remain a mystery. We have operationally defined null cells in the present study as those which form neither E nor EAC rosettes. Some of these cells could be lymphocytes coated with antibody which blocked receptors necessary for rosette formation. Others may be cells without these receptors and/or activated cells. Our ability to separate cells such that a given fraction contains $>80\%$ null cells allows for investigation of the properties of these cells.

The mitogen experiments correlated with the rosette studies following Ficoll separation of normal human PBL. Con A stimulated predominantly fractions D and E, which are composed largely of T cells. The response to PHA was broader, with a peak from fractions C to F. The third mitogen, PWM, stimulated only high density lymphocyte subpopulations (fractions $\geqslant$ D) which correlated with the B-cell distribution. The lighter cells (null cells) poorly responded to all mitogens, further distinguishing this population.

Some workers have reported that PBL from patients with SLE respond normally to mitogens (Goldman *et al.*, 1972; Senyk *et al.*, 1974); others find contrary results (Scheinberg & Cathcart, 1974; Suciu-Foca *et al.*, 1974; Rosenthal & Franklin, 1975). A possible reason for this discrepancy is the variation in response with disease activity. We have found that patients with active disease have a greater impairment in responsiveness to mitogens than do patients with less active disease. Further, most previous studies have utilized the mitogen PHA. The present observations suggest that the Con A response is a more sensitive indicator of SLE activity and associated lymphoid cell disfunction. There was a strong correlation between SLE activity and impaired Con A responsiveness. A lesser association was found between SLE activity and PHA responsiveness.

The reduction in Con A-responsiveness in patients with active SLE was most prominant in fractions D and E, T-cell rich fractions which respond maximally to this mitogen in normals. Null cells were increased in fractions D and E of patients with SLE, however, the relative reduction in T cells in these fractions from patients with SLE was not sufficient to explain the marked reduction in Con A responsiveness. Anti-T cell antibodies acting *in vivo* or *in vitro* and/or shedding of antibody receptor complexes could be factors in this impaired response (Cousar & Horowitz, 1973; Winfield *et al.*, 1975). Functional defects in SLE T cells and selective loss of mitogen responsive T cells (leaving behind non-responding T cells) *in vivo* or *in vitro* are additional possibilities.

Tritiated thymidine incorporation in unstimulated cultures of PBL from patients with SLE is generally reported to be normal (Scheinberg & Cathcart, 1974); however, it has been found to be elevated by others when the ^{3}H-TdR incorporation was studied during the first hour of culture (Cooper & Firkin, 1965). This is similar to the observed blastogenic activity of PBL from patients with active SLE cultured in autologous sera (Glinski, 1974). In the present study, washed unseparated lymphoid cells from patients with active SLE did not have marked increased in ^{3}H-TdR incorporation at 72 hr. However, such increased transformation was observed in particular fractions from the Ficoll gradient in patients with active SLE. This spontaneous thymidine incorporation was present in the heavier fractions (E, F+G) at 72 hr. Spontaneous transformation was even more marked at 16 hr. Similar fractions from normals did not show such incorporation. The spontaneous transformation suggests that subpopulations of cells in patients with active SLE are stimulated excessively. In the heavier fractions this is best explained by B-cell activation. The possible role of immune complexes, autoantigens, or antilymphocyte antibodies in such activation is a question for future study.

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