Fractionation of Cells on a Discontinuous Ficoll Gradient

STUDY OF SUBPOPULATIONS OF HUMAN T CELLS USING ANTI-T-CELL ANTIBODIES FROM PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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ABSTRACT Patients with active systemic lupus erythematosus (SLE) had a decrease in a subpopulation of cells (fraction D) when peripheral blood lymphocytes were separated on a discontinuous Ficoll gradient. Preincubation of SLE cells at 37°C for 30 min led to a marked decrease in this fraction, composed primarily of thymus-derived (T) cells. Supernates of such preincubations were found to cause a reduction in fraction D cells from normal humans. The active factor in the supernate was found to be an IgG antibody. Similarly, serum from patients with active SLE produced a reduction in fraction D cells from normal donors. This activity was also found in the IgG fraction, and could be absorbed with a pure T-cell population.

Depletion of macrophages and complement did not reduce the SLE anti-T-cell antibody-mediated loss of cells from fraction D; however, heat-aggregated human gamma globulin led to impairment of the reaction. These findings suggest that antibody-dependent direct lymphocyte-mediated cytotoxicity may play a role in T-cell lymphopenia of SLE.

It was further noted that the SLE anti-T-cell antibodies, in contrast to rabbit antihuman thymocyte serum, recognized fraction D cells but not fraction E cells from normals. Since both fractions are largely T cells, it appeared that the SLE serum was directed against cell-membrane antigenic determinants present on fraction D T cells, which were absent or reduced in quantity on fraction E T cells. Thus, evidence was presented indicating the presence of at least two subpopulations of T cells in man. This was supported by differential absorption of the anti-T-cell sera with fractions D and E.

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Received for publication 30 July 1975 and in revised form 23 October 1975.

INTRODUCTION

In an earlier study, peripheral blood cells from normal humans and patients with systemic lupus were fractionated on discontinuous Ficoll gradients.[1] Two fractions contained the great majority of thymus-derived (T) cells: fractions D and E. Patients with active systemic lupus erythematosus (SLE)[2] had a selective reduction in cells from fraction D. Previous observations of antilymphocyte antibodies in patients with active SLE (1-5) suggested to us that cells from fraction D might be reduced by such antibodies. This was a particularly appealing hypothesis in view of the T-cell specificity of some of these antibodies (6, 7) as well as their ability to alter T-cell functions (8, 9).

In addition, the relatively greater loss of T cells from fraction D as compared to T cells from fraction E suggested that subpopulations of human T cells might be recognized by SLE anti-T-cell antibodies. The present study was, therefore, undertaken to further investigate these possibilities. The relative loss of cells from fraction

[1] Glinski, W., M. E. Gershwin, and A. D. Steinberg. 1975. Fractionation of cells on a discontinuous Ficoll gradient. Study of peripheral blood lymphocyte subpopulations in normal humans and patients with systemic lupus erythematosus. Submitted for publication.

[2] *Abbreviations used in this paper:* ADDLC, antibody-dependent direct lymphocyte-mediated complement-independent cytotoxicity; ATS, antihuman thymocyte serum; HBSS, Hank's balanced salt solution; PBL, peripheral blood lymphocytes; SLE, systemic lupus erythematosus; SRBC, sheep red blood cells; VBS, Veronal-buffered saline.

D was found to be produced by an IgG antibody found in SLE sera. This antibody activity could be completely absorbed by T cells. In addition, it recognized antigenic determinants present on normal T cells in fraction D but not in fraction E, indicating that at least two T-cell subpopulations exist in humans.

METHODS

Patients. 56 patients with SLE followed by the Arthritis and Rheumatism Branch, National Institute of Arthritis and Metabolic Diseases, and 20 healthy volunteers of both sexes were studied. All patients met the diagnostic criteria of American Rheumatism Association for SLE. Activity of the SLE was assessed by two observers based upon clinical disease and graded as active or inactive. Only patients with clearly active or clearly inactive disease were studied for this report. Patients receiving cytotoxic drugs or high doses of corticosteroids (> 15 mg/day prednisone) were excluded.

Separation of lymphocytes. Peripheral blood was drawn between 8:30 and 10:30 a.m. into preservative-free heparin (25 USP U/ml, Fellows Medical Mfg. Co., Oak Park, Mich.). The heparinized blood was diluted 1:1 with calcium- and magnesium-free isotonic Hank's balanced salt solution (HBSS) (Grand Island Biological Co., Grand Island, N. Y.) and placed on a Ficoll-Hypaque gradient as previously described (10). 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 generally 30×10^6 ($25–40 \times 10^6$) cells per tube.

Discontinuous Ficoll gradient. Ficoll, (Sigma Chemical Co., St. Louis, Mo., lot 14C-2350), a polysucrose polymer of mol wt 400,000, was dissolved in HBSS (Ca^{++}- and Mg^{++}-free) at pH 7.4, to obtain a 32% Ficoll solution. This stock solution was sterilized by passage through a 0.45-μm Millipore filter (Millipore Corp., Bedford, Mass.) and the following concentrations (wt/vol) 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 (11, 12). 3 ml of the Ficoll solutions was carefully layered in sequence, in cellulose nitrate tubes (model 302237, $1 \times 3\frac{1}{2}$ inches, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) 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* (force at bottom of tube) using an SW27 swinging-bucket rotor in a model L2-65B ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). After centrifugation the lymphocytes were distributed at the interfaces. They were removed with Pasteur pipettes and placed into separate 16×125-mm plastic test tubes (Falcon Plastics, Oxnard, Calif.) containing 5 ml of HBSS. The fractions were named sequentially from A to G for each interface starting at 9–12% Ficoll and ending at 23–30% Ficoll. Cell viability was more than 95% in each fraction, as demonstrated by trypan blue exclusion. The cells in fractions C, D, and E looked relatively homogeneous under the microscope.

Lymphocyte counts. After fractionation, the cells in each of eight fractions were washed twice in HBSS and counted. Cells were counted in a Coulter Counter (model ZBI, Coulter Electronics, Inc., Hialeah, Fla.). These counts were confirmed periodically using a hemocytometer.

Identification of T cells, bone marrow-derived (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 in HBSS. 100 μl of this cell preparation was added to 4×10^7 sheep red blood cells (SRBC), (SRBC: lymphocyte ratio of 10:1) in 2,200 μl of 50% heat-inactivated, SRBC-absorbed fetal 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 receptors were determined by measuring the number of lymphocytes binding three or more SRBC which had been sensitized with a IgM antibody to SRBC plus complement. 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 (VBS) containing calcium, magnesium, and 1% bovine serum albumin, 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-μm latex particles (1.099±0.0059 μm diameter, Dow Chemical Co., Indianapolis, Ind.). Cell suspensions were incubated with latex particles at 37°C for 1 h; 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 hemocytometer, and enumerated.

Preincubation of peripheral blood lymphocytes (PBL) of SLE patients. 60–100-million lymphocytes from each of 10 patients with SLE were placed in two to four separate tubes in the same absolute number. The cells were incubated at a concentration of 2×10^7/ml at either 37°C or at room temperature (22°C) for 30 min, and then separated on the discontinuous Ficoll gradient.

The supernates from the 22° and 37°C preincubation experiments were saved for further investigations and are called 22° and 37°C supernates. Normal lymphocytes preincubated under the same conditions were similarly studied.

Preincubation of normal lymphocytes with SLE supernates, SLE sera, or antihuman thymocyte serum (ATS). The effect of SLE supernates (22° and 37°C), SLE sera, and ATS on the distribution of normal lymphocytes on the discontinuous Ficoll gradient was next studied. The 22° and 37°C supernates (each 2.0–2.5 ml) were used undiluted. SLE sera were inactivated at 56°C for 30 min and used in various dilutions. ATS, prepared by weekly injections of human thymocytes (obtained during cardiac surgery), was the gift of Dr. A. Ahmed. The ATS had

been depleted of complement activity by heat inactivation and exhaustively absorbed with human tissues including erythrocytes and B cells from a patient with chronic lymphocytic leukemia. It was cytotoxic only for human T cells. It was used in dilutions of 1:50–1:600. Typically, 4×10^7 normal PBL (from the Ficoll-Hypaque separation) were suspended in 1 ml of HBSS. The test serum was added and the volume brought to 2 ml with additional HBSS. Thus, addition of 0.25 ml SLE serum would represent a dilution of 1:8 (0.25 ml serum in a total volume of 2 ml). Controls consisted of (*a*) medium only and (*b*) normal human serum, type AB, which was previously heat inactivated.

The normal lymphocytes at a concentration of 2×10^7/ml were incubated at 37°C for 1 h with one of these additional substances and then separated on the discontinuous Ficoll gradient. The total number of cells recovered as well as the number of cells in each fraction was determined for each treatment.

Depletion of macrophages. Macrophages were removed by iron particle phagocytosis. Suspensions of lymphocytes, isolated initially with Ficoll-Hypaque, were mixed 2:1 (vol:vol) with lymphocyte-separator reagent (Technicon Instruments Corp., Tarrytown, N. Y.) which contains iron particles. After 30 min at 37°C, the cells were suspended and the tube placed over a magnet. Iron particles and particle-containing cells were pulled to the bottom of the tube. The remainder of the cells were aspirated and placed on a Ficoll-Hypaque gradient for reisolation of the lymphocytes, free of remaining iron particles. As a control for this experiment, an aliquot of cells was carried through the same procedures, but without the addition of the lymphocyte-separator reagent.

Complement. To restore complement activity to the medium containing SLE serum, 5% fresh human AB serum was added.

Blocking of cytotoxicity with aggregated human gamma globulin. Human gamma globulin (human Cohn fraction II, E. R. Squibb & Sons, Princeton, N. J.) was dissolved in phosphate-buffered saline (pH 7.2) at a concentration of 10 mg/ml and heated to 63°C for 20 min. The resultant aggregated gamma globulin was diluted to 1 mg/ml, and 0.25 ml was added to 2.0 ml of a suspension of normal lymphocytes (2×10^7/ml). The mixture was incubated at 37°C for 15 min after which 0.25 ml of SLE serum was added. The cells were incubated at 37°C for an additional hour and then separated on the discontinuous Ficoll gradient. Control lymphocytes were incubated in the medium (without aggregated gamma globulin) after which 0.25 ml of SLE serum was added and the same procedures carried out. The number of lymphocytes in each fraction and the total number of lymphocytes was determined for both control and aggregated gamma globulin preincubated cells. These were compared to a third aliquot of cells treated with medium only instead of the SLE serum.

Study of IgG and IgM fractions of SLE sera. Serum proteins were precipitated with 50% saturated ammonium sulfate. The precipitate was centrifuged at 1,000 *g* for 30 min and dissolved in distilled water. The solution was dialyzed against Tris-buffered saline (pH 7.4) for 48 h and concentrated to 0.5 ml with a collodion bag apparatus (Schleicher and Schuell, Inc., Keene, N. H.—bag 100, lot 79432). The sample was subjected to gel filtration (Bio-Gel A-5m, operating range 10,000–5,000,000 mol wt, Bio-Rad Laboratories, Richmond, Calif.). The serum protein concentration in each tube was determined in a spectrophotometer. The purity of the IgG and IgM fractions was determined by gel diffusion with goat antihuman IgG and goat antihuman IgM antisera (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.). The uncontaminated individual fractions were concentrated to approximately the original concentration in serum: IgG, 10 mg/ml, and IgM, 1 mg/ml. In addition, individual SLE sera (one part) were mixed with four parts of rabbit antiserum to human IgG (purchased from Microbiological Associates, Bethesda, Md., and found to precipitate only the IgG fraction of human serum) and incubated at 4°C for 48 h. The precipitate was removed by centrifugation at 2,000 *g* for 1 h and the supernate ultracentrifuged at 105,000 *g* for 30 min; then the supernate of this procedure was studied. Similarly, the supernate of 37°C incubation of SLE cells was mixed with rabbit antihuman IgG serum (one part antiserum to five parts supernate), incubated at 4°C for 48 h, centrifuged at 2,000 *g* for 1 h, ultracentrifuged as above, and the supernate used. The removal of IgG was confirmed by radial diffusion.

Absorption of SLE and ATS sera. Sera from three SLE patients were diluted 1:5 and absorbed with 10^9 lymphocytes/ml. The lymphocytes were derived from a patient with Sézary syndrome, kindly provided by Dr. R. Edelson. The leukocyte count of this patient was 48,000/mm^3 and 99% of these cells were lymphocytes with T-cell markers. The cells were incubated for 1 h at room temperature and for 24 h at 4°C.

For study of the specificity of anti-T-cell antibodies in SLE sera and in ATS, a 1:5 dilution of SLE serum and a 1:50 dilution of ATS were absorbed at 22°C for 1 h and at 4°C for 24 h with cells of fraction D or fraction E (5×10^8/ml) from a normal individual.

RESULTS

Distribution of E and EAC rosette-forming cells in normals. The cell type in each of these 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 in fractions D and E formed E rosettes (Fig. 1). The relative percentage of cells forming EAC rosettes rose as the density of Ficoll increased (Fig. 1). In contrast, fractions B and C were largely (> 80%) composed of cells which did not form E or EAC rosettes (herein called null cells).

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.

Distribution of E and EAC rosette-forming cells in SLE. Patients with inactive SLE resembled normal controls in the relative distribution of E and EAC rosette-forming cells (Fig. 1). In contrast, patients with active SLE had a reduction in E rosette-forming cells in fractions D, E, and F + G with no change in EAC rosettes (Fig. 1), leaving a relative increase in null cells in those fractions.

Preincubation of PBL of SLE patients. Aliquots of PBL from 10 patients with SLE were incubated at either 22° or 37°C for 30 min and then separated on a dis-

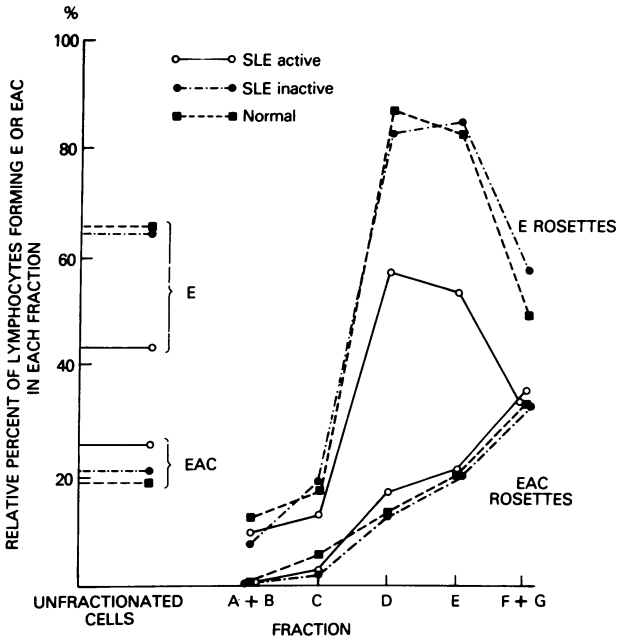

FIGURE 1 Average values for three groups of patients are presented. The percentage of lymphocytes in a given fraction forming E or EAC rosettes is shown in the main figure, while the unfractionated results are presented to the left. The majority of cells in fraction A+B and C formed neither E nor EAC rosettes. Most cells in fractions D and E formed E rosettes. Normals (20 patients) and patients with inactive SLE (12 patients) had similar values. In contrast those with active SLE (16 patients) had reduced E rosettes in the heavier fractions as well as in unfractionated cells.

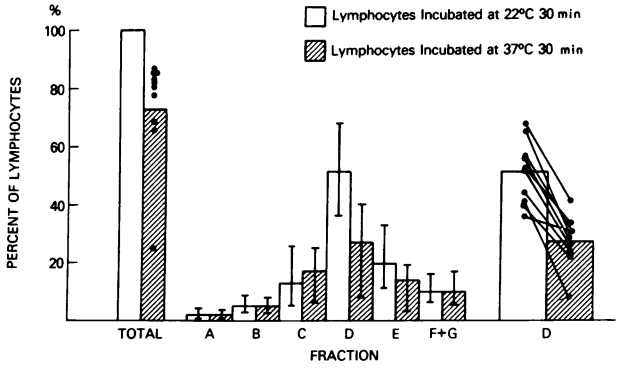

FIGURE 2 Washed lymphocytes from 10 patients with active SLE obtained by Ficoll-Hypaque separation of peripheral blood were incubated at either 22° or 37°C for 30 min and then fractionated on a discontinuous Ficoll gradient. The total number of cells and the distribution of cells was not altered by the 22°C incubation. In contrast, incubation at 37°C led to a reduction in total number of cells (left-hand side of figure) and an alteration in the distribution (center of figure). The pronounced loss of cells from fraction D seen in the center of the figure is magnified on the right-hand side where individual values for the 10 patients are presented.

continuous Ficoll gradient. Cell counts were performed before and after. No significant changes were observed after incubation at 22°C; however, a reduction in total lymphocyte count was observed after the 37°C incubation (left-hand part of Fig. 2). It was found that the cells were being lost almost entirely from fraction D (center of Fig. 2). In 9 of the 10 patients, the reduction in PBL from fraction D was significant and is illustrated in the right-hand part of Fig. 2.

Preincubation of normal PBL. Normal lymphocytes incubated at 22° or 37°C did not change in cell number or distribution on the discontinuous Ficoll gradient in 12 separate experiments. However, incubation of normal lymphocytes with serum from a patient with active SLE led to a marked reduction in the number of cells in fraction D (illustrated in Fig. 3) without a concomitant increase in other fractions. This observation suggested that the SLE serum was eliminating cells from fraction D in the same manner as cells were eliminated from fraction D of SLE patients by 37°C incubation. Furthermore, the supernate of the 37°C incubation of PBL from patient B. A. also reduced the number of cells in fraction D (Fig.

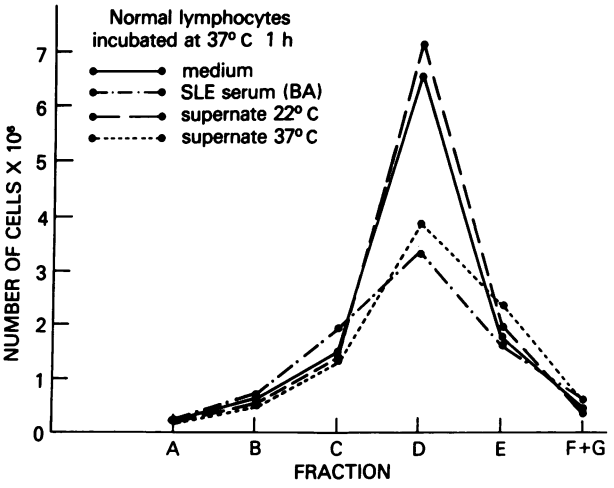

FIGURE 3 Washed lymphocytes from a patient (B. A.) with active SLE obtained by Ficoll-Hypaque separation of peripheral blood were incubated at either 22° or 37°C. Each supernate was subsequently incubated for 1 h at 37°C with washed lymphocytes from a normal volunteer (obtained by Ficoll-Hypaque separation of peripheral blood). For comparison, serum from patient B. A. was also incubated with an aliquot of the normal washed cells. Each aliquot of treated cells (as well as one incubated with medium only) was then fractionated on a discontinuous Ficoll gradient. The number of cells in each fraction is presented in the figure. The 22°C supernate had no effect on the cell distribution (compared to the medium control). In contrast, both the 37°C supernate and the serum markedly reduced the number of cells in fraction D.

TABLE 1

Effect of SLE Serum, 37°C Supernate and IgG-depleted 37°C Supernate on the Distribution of Normal PBL from a Single Donor

Normal lymphocytes incubated at 37°C for 1 h with	Cell distribution on discontinuous Ficoll gradient* Fractions A + B + C	D	E	F + G	Total	Reduction in cells in fraction D
						%
Medium	495,000 (3.1%)‡	8,250,000 (50.8%)	5,360,000 (33.0%)	2,120,000 (13.1%)	16,225,000 (100.0%)	—
Donor D. S. SLE serum	624,000 (3.9%)‡	3,875,000 (23.9%)	5,808,000 (35.8%)	2,512,000 (16.2%)	12,819,000 (79.8%)‡	52.9
37°C supernate	660,000 (4.1%)	4,988,000 (30.7%)	5,538,000 (34.1%)	2,791,000 (17.2%)	13,977,000 (86.1%)	39.6
37°C supernate precipitated with rabbit antihuman IgG	827,000 (5.1%)	7,880,000 (48.6%)	6,180,000 (38.0%)	1,670,000 (10.3%)	16,557,000 (102.0%)	4.3

* Absolute number of cells in each fraction.

‡ Percentage of cells given in parentheses; the total number of cells in medium control taken as 100%.

3). It was noted that the supernate of the 22°C incubation of B. A. cells did not alter the normal cell distribution (Fig. 3). The same results were obtained in 40 experiments using 16 different sera from patients with

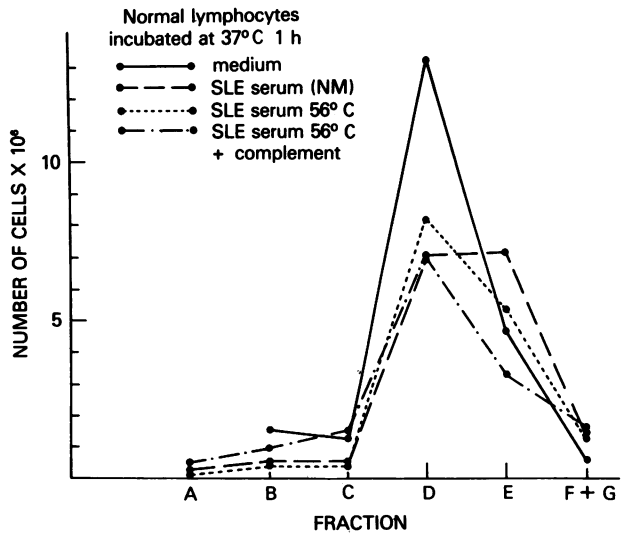

FIGURE 4 Washed PBL from a normal volunteer were incubated at 37°C for 1 h with (*a*) serum from a patient N. M. with active SLE, (*b*) serum N. M. previously heated to 56°C for 1 h to remove complement activity, (*c*) serum N. M. previously heated to 56°C plus a fresh source of complement, or (*d*) medium only, and the cells fractionated on a discontinuous Ficoll gradient. All treatment groups containing serum N. M. had a reduction in cells in fraction D in comparison with the medium only treatment. Normal serum and serum from a patient with inactive SLE led to a distribution very similar to that of medium only (data not shown).

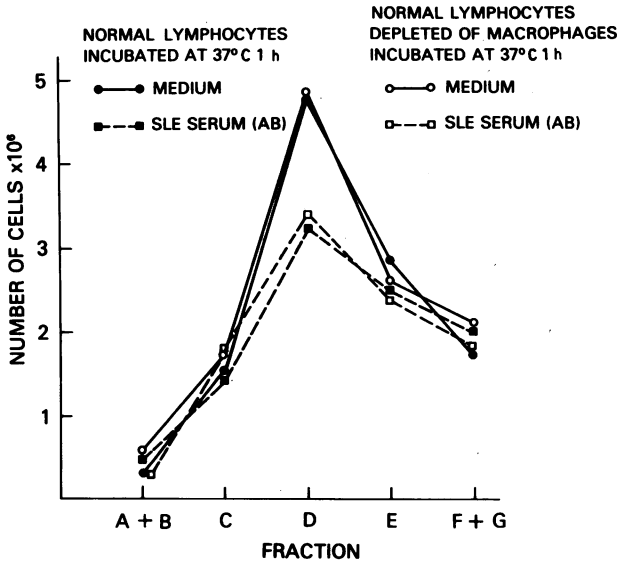

FIGURE 5 Washed cells from a normal volunteer obtained by Ficoll-Hypaque separation of peripheral blood were divided into two aliquots. The first was depleted of macrophages by iron-particle phagocytosis and reseparated free of iron particles by a second Ficoll-Hypaque treatment. The second aliquot was carried through the same procedures without the addition of iron particles. Each aliquot was then divided in half. One-half was incubated with serum from patient A. B., with active SLE, at 37°C for 1 h. The other half was incubated with medium only. All four aliquots were then fractionated on the discontinuous Ficoll gradient. The number of cells in each fraction following each treatment is presented in the figure. The aliquot depleted of macrophages showed a reduction in fraction D following treatment with serum A. B. in comparison with medium only. This reduction was almost identical to that observed in the aliquot not depleted of macrophages.

TABLE II

Effect of Serum from Patients with Active SLE on the Distribution of Normal PBL

Normal lymphocytes incubated at 37°C for 1 h with	Cell distribution on discontinuous Ficoll gradient*				
	Fractions				
	A + B + C	D	E	F + G	Total
Medium	7.4	60.6	21.2	10.7	100.0
Serum A. B. (1:10)	7.7	31.1	25.2	12.5	76.5
Reduction‡, %	−0.3	29.5	−4.0	−1.8	23.5
Medium	11.1	53.1	20.2	15.6	100.0
Serum M. N. (1:10)	12.8	14.7	15.9	13.4	56.8
Reduction, %	−1.7	38.4	4.3	2.2	43.2
Medium	17.0	47.9	26.2	8.8	100.0
Serum D. S. (1:10)	14.4	22.2	31.7	12.1	80.4
Reduction, %	2.6	25.7	−5.5	−3.3	19.6
Medium	4.2	61.1	20.2	14.5	100.0
Serum A. S. (1:10)	3.4	47.0	19.4	10.9	80.7
Reduction, %	0.8	14.1	0.8	3.6	19.3
Medium	6.1	52.7	27.9	13.3	100.0
Serum J. U. (1:5)	5.5	15.9	28.6	13.7	63.7
Reduction, %	0.6	36.8	−0.7	−0.4	36.3
Medium	7.4	58.0	22.8	11.8	100.0
Serum B. K. (1:10)	6.8	30.4	21.6	10.6	69.4
Reduction, %	0.6	27.6	1.2	1.2	30.6
Medium	6.8	58.5	22.4	12.3	100.0
Serum B. C. (1:10)	5.8	41.2	23.0	12.7	82.7
Reduction, %	1.0	17.3	−0.6	−0.4	17.3
Medium	14.3	44.8	20.2	20.7	100.0
Serum B. H. (1:10)	12.5	21.1	23.6	18.8	76.0
Reduction, %	1.8	23.7	−3.4	1.9	24.0

* Percentage of total cells of medium control in each fraction.

‡ Reduction = Percentage of all the medium-treated cells (total) that are reduced in a given fraction (a minus sign indicates an increase).

active SLE. An individual serum gave reproducible results when run on separate occasions.

When the 37°C supernate was depleted of IgG (by precipitation with a rabbit antibody to human IgG), it no longer produced a reduction in cells in fraction D (Table I). This suggested that the antibody shed from the surface of T cells, rather than a nonspecific toxic substance in the supernate, was responsible for the reduction in fraction D cells. The IgG class of the antibody correlates with the serum anti-T-cell antibody as discussed below.

In several experiments, it was noted that serum from patients with active SLE reduced fraction D, whereas serum from patients with inactive SLE did not. It was repeatedly observed that although both fractions D and E are enriched in T cells, the SLE sera and supernates markedly reduced the cells in fraction D, but had little effect on cells from fraction E.

The distribution of normal PBL on the discontinuous Ficoll gradient was altered by SLE serum so as to resemble the distribution of PBL from the serum donor. However, this was almost completely accounted for by the changes in fraction D, with rather little change in the other fractions (Table II).

Studies of the mechanism of reduction in lymphocytes in fraction D complement. In the first study, the requirement for the complement was investigated. SLE serum N. M. reduced the number of cells in fraction D of normal PBL. Heating the serum to 56°C to inactivate the complement, or adding the complement, did not alter the result (Fig. 4). These observations, which were repeated three times, suggested that the mechanism of loss of cells from fraction D was not complement mediated.

Macrophages. Normal peripheral blood was depleted of macrophages by the technique of iron-particle phagocytosis and sedimentation in a magnetic field. Depletion

TABLE III

Distribution of Normal PBL after Incubation with SLE Serum Plus Heat-Aggregated Human Gamma Globulin (HGG) or SLE Serum Absorbed with T Cells from a Patient with Sézary Syndrome. Three Different SLE Sera Varying in Ability to Reduce Cells in Fraction D Were Studied

Normal lymphocytes incubated at 37°C for 1 h with	Cell distribution on discontinuous Ficoll gradient*					Reduction in cells in fraction D
	A + B + C	D	Fractions E	F + G	Total	
						%
Donor A. S.						
Medium	4.2	61.1	20.2	14.5	100.0	—
SLE serum	3.4	47.0	19.4	10.9	80.7	23.0
SLE serum + heat-aggregated HGG	2.6	56.4	27.9	12.8	99.7	7.7
SLE serum absorbed with Sézary (T) cells	3.5	57.2	22.2	12.5	95.2	6.7
Donor B. K.						
Medium	7.4	58.0	22.8	11.8	100.0	—
SLE serum	6.8	30.4	21.6	10.6	69.4	47.6
SLE serum + heat-aggregated HGG	6.2	45.7	24.0	10.2	86.1	20.8
SLE serum absorbed with Sézary (T) cells	4.0	57.1	22.4	12.3	95.8	1.6
Donor B. C.						
Medium	6.8	58.5	22.4	12.3	100.0	—
SLE serum	5.8	41.2	23.0	12.7	82.7	33.0
SLE serum + heat-aggregated HGG	4.1	53.1	24.1	10.8	92.1	9.2
SLE serum absorbed with Sézary (T) cells	6.5	60.2	21.0	13.6	101.3	2.9

* Percentage of total cells of medium control in each fraction.

of macrophages had no effect upon the reduction of cells in fraction D by SLE serum in several experiments as illustrated in Fig. 5.

Antibody-dependent direct lymphocyte-mediated cytotoxicity (ADDLC). Numerous experiments indicate that nonsensitized lymphocytes are able to induce cytotoxicity of cells coated with specific antibody (13-18). This reaction is inhibited by aggregated gamma globulin (18, 19). We, therefore, pretreated the normal PBL with aggregated gamma globulin before addition of the SLE serum in an attempt to inhibit ADDLC. Such treatment markedly reduced the loss of cells from fraction D in three separate experiments (Table III). The serum that most effectively reduced fraction D (B. K.) was still partially active despite the aggregated gamma globulin.

Specificity and antibody nature of the factor in the SLE serum leading to reduction in fraction D. Since fraction D contained primarily T cells, it seemed most likely that the SLE-serum factor responsible for loss of T cells from fraction D was an antibody to T cells. Accordingly, the SLE serum was absorbed with T cells from a patient with the Sézary syndrome, a T-cell malignancy. This absorption eliminated the active factor from three different SLE sera (Table III), but did not alter normal sera.

The antibody nature of the factor was investigated next. The IgG and IgM fractions of three patients with active SLE and three normals were obtained by gel filtration. The IgM fraction had no effect on the distribution of normal PBL. In contrast, the IgG fraction of each SLE serum was found to be active (Table IV); the IgG fractions were as active as the whole sera from the same patients (Table IV).

Throughout these studies, we were impressed that fraction D, but not fraction E, was recognized by the anti-T-cell antibody in SLE sera. Two possible explanations immediately presented themselves: (*a*) fraction E cells for some reason were not susceptible to killing or (*b*) fraction E cells had a reduction in the membrane antigen(s) being recognized by the SLE anti-T-cell antibody. To investigate this further, normal PBL were treated with either an SLE serum or a rabbit antiserum specific for human T cells (ATS). Both sera were first heated to 56°C for 1 h to inactivate complement. They

TABLE IV

Effect of the IgG Fraction of SLE Serum on the Distribution of Normal PBL

Normal lymphocytes incubated at 37°C for 1 h with	Cell distribution on discontinuous Ficoll gradient*					Reduction in cells in fraction D
	Fractions					
	A + B + C	D	E	F + G	Total	
						%
Donor B. C.						
Medium	6.9	57.5	21.5	14.1	100.0	—
SLE serum	8.8	35.0	23.0	13.4	80.2	39.1
SLE serum IgG	9.9	21.2	26.7	15.0	72.8	59.7
SLE serum IgM	10.6	53.8	24.1	12.5	101.0	6.4
Normal serum IgG	14.6	51.2	23.5	13.2	102.5	11.0
Donor A. B.						
Medium	11.6	52.6	20.0	15.8	100.0	—
SLE serum	8.7	30.9	21.2	12.5	73.3	41.3
SLE serum IgG	10.8	31.9	22.1	17.9	82.7	39.4
SLE serum IgM	9.4	48.1	20.5	16.2	95.2	8.5
Normal serum IgG	7.7	50.2	20.9	17.4	96.1	4.8
Donor B. H.						
Medium	14.3	44.8	20.2	20.7	100.0	—
SLE serum	12.5	21.1	23.6	18.8	76.0	52.9
SLE serum IgG	11.3	15.0	26.1	19.6	72.0	66.5
Normal serum IgG	11.1	46.7	21.3	21.8	100.9	−4.2

* Percentage of total cells of medium control in each fraction.

were then incubated with normal lymphocytes at 37°C for 1 h and the cells were fractionated on the discontinuous Ficoll gradient. The ATS led to a significant reduction in cells in both fractions D and E. This was repeatedly true in a number of experiments and over a wide range of dilutions of this antiserum (1 : 50-1 : 600). In contrast, the SLE serum significantly reduced the cell number only in fraction D, again consistently and over a wide range of serum dilutions (1 : 4-1 : 128).

To further study differences between fractions D and E, the ATS and SLE sera were first absorbed with fraction D or fraction E from a normal donor. These absorbed sera were then incubated with normal PBL at 37°C and the cells fractionated on the discontinuous Ficoll gradient (Table V). Control unabsorbed SLE serum reduced the cell number in fraction D, but not in fraction E. ATS reduced the number of cells in fraction D and E by approximately the same amount. Absorption of the ATS with either fractions D or E markedly reduced its activity (Table V). This suggested that this antiserum was able to recognize cell surface-membrane determinants common to both fractions. In contrast, absorption of the SLE serum with the different fractions gave different results. Absorption of the SLE serum with fraction D reduced its activity towards fraction D, whereas absorption with fraction E had no significant effect upon its activity toward fraction D (Table V). This suggested that the SLE serum recognized surface-membrane antigeneic determinants of cells in fraction D that were diminished on or absent from fraction E cells.

DISCUSSION

It has been known that patients with active SLE have reduced numbers of total leukocytes (20-22) and lymphocytes (22-24). Furthermore, they have been found to have a reduction in T cells (24, 25) and T-cell functions (26-30). Finally, anti-lymphocyte and anti-T cell-antibodies have been found in patients with SLE (1-9). The present study links these various observations.

We have observed that patients with active SLE have a reduction in the percentage of cells in fraction D, a fraction obtained by separating PBL on a discontinuous Ficoll gradient. Furthermore, this fraction is composed primarily of T cells. In the present study, the mechanism of this reduction was investigated.

Preincubation at 37°C of PBL from patients with active SLE resulted in a loss of cells from fraction D. Supernates from this incubation were found to reduce the number of cells in fraction D of PBL from normal donors. Similarly, SLE serum reduced the number of cells in fraction D from normals. An IgG antibody with anti-T-cell specificity present in the SLE serum was required for this effect. The reaction was independent of both complement and macrophage. Heat aggregated gamma globulin markedly inhibited the anti-T-cell cyto-

TABLE V

Differential Absorption of SLE Serum and Rabbit ATS with Two T-Cell Subpopulations (Fractions D and E) of Normal PBL. The Effect of These Absorbed Sera on the Distributions of PBL from a Single Normal Donor Was Studied

Normal lymphocytes incubated at 37°C for 1 h with	Cell distribution on discontinuous Ficoll gradient*					Reduction in cells in	
	Fractions						
	A + B + C	D	E	F + G	Total	Fraction D	Fraction E
						%	
Medium	6.1	52.7	27.9	13.3	100.0	—	—
SLE serum J. U. (1:5)							
Unabsorbed	5.5	15.9	28.6	13.7	63.7	69.8	−2.5
Absorbed with fraction D	6.1	42.1	28.7	15.5	92.4	20.1	−2.8
Absorbed with fraction E	5.0	19.2	27.0	15.9	67.1	63.6	3.2
ATS (1:50)							
Unabsorbed‡	5.7	22.6	14.8	12.1	55.2	57.2	47.0
Absorbed with fraction D	6.7	51.3	30.6	14.9	103.5	2.7	−9.7
Absorbed with fraction E	7.9	43.6	24.4	13.9	89.8	17.3	12.6

* Percentage of totol cells of medium control in each fraction.

‡ Unabsorbed with fraction D or E. This serum had previously been absorbed as described in the methods.

toxicity, implicating Fc receptor-bearing cells. These observations suggested that ADDLC (10-16) was an important mechanism for T-cell loss in SLE patients, particularly since the reactions were carried out at body temperature, 37°C. The possibility of additional mechanisms for T-cell loss was suggested by only partial reduction in activity of an active serum (B. K.) with the aggregated gamma globulin.

It was further observed that the SLE anti-T-cell antibodies recognized antigeneic determinants on cells of fraction D that were absent from or much reduced in density on cells of fraction E. Since both fractions contained primarily T cells, it appeared that the SLE antibodies could distinguish among cells in the two fractions leading to loss of cells only from fraction D. This is evidence for two subpopulations of T cells in normal human peripheral blood. The antihuman T-cell antibodies in SLE sera were distinguishable from rabbit antihuman T-cell antibodies. The activity of the former was absorbed to a much greater extent with fraction D than fraction E, providing further evidence for membrane differences between cells in fractions D and E. In contrast, the rabbit antibody activity was equally absorbed with fractions D and E. Thus the rabbit anti-T-cell antibodies recognized all human T cells, whereas the SLE anti-T-cell antibodies recognized preferentially a subpopulation of human T cells.

Since only some of the T cells in fraction D were eliminated by the SLE serum, fraction D itself may contain more than one subpopulation of T cells. Alternatively, soluble immune complexes in SLE sera might have inhibited the ADDLC reaction against antibody-coated fraction D T cells, especially since immune complexes have been shown to inhibit ADDLC (31) and ADDLC is reduced in SLE (32).

The IgG anti-T-cell antibodies active at 37°C which were studied herein appear to be different from the IgM anti-T-cell antibodies which are often more active at low temperatures (2, 4). The anti-T-cell specificity is analogous to that previously described (6, 7) and may be related to the antibodies which inhibit the mixed-lymphocyte culture (8, 9). The present studies are also in agreement with a previous observation, suggesting that IgG anti-T-cell antibodies in SLE are only minimally cytotoxic (4). Previous reports of complement-dependent IgG antilymphocyte antibodies in SLE (3, 5) may involve sera with specificities different from those studied in the present report. In fact, the existence of antilymphocyte antibodies with different specificities in SLE sera has recently been emphasized (4).

The effect of supernates from the present study may be related to the "shedding" phenomenon previously described (33-35). The IgG nature of the supernatant antibody is in agreement with the elution of IgG antibody from SLE lymphocytes (36).

Multiple antibodies may be responsible for lymphocyte depletion in SLE. Different mechanisms may be responsible for lymphocyte depletion in SLE. Different mechanisms may be responsible for the loss of different types of cells. Patients with SLE may lose cells from fraction E by a mechanism different from that responsible for the loss of a subpopulation of cells from fraction D. Nevertheless, the observations reported herein suggest that ADDLC is a likely mechanism for loss of at least one

T-cell subpopulation in SLE. The abnormalities in SLE-PBL distribution observed in a previous study using this technique[1] appear to be caused at least partially by effects of antilymphocyte antibodies. The relatively greater reduction in cells of fraction D is explainable on the basis of specificity of SLE anti-T-cell antibodies for cells in fraction D.

New Zealand mice represent animal models of human SLE (37). These mice spontaneously produce antibodies to T cells (38), as well as antierythrocyte and antinuclear antibodies. Before the appearance of overt autoimmune disease manifestations, New Zealand mice appear to lose a subpopulation of T cells, which serves a regulatory or suppressor function (39-43). It is possible that a similar loss of regulatory T cells occurs in patients with SLE. Investigations of this question are currently in progress.

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