

Phytohemagglutinin Response in Systemic Lupus Erythematosus

RECONSTITUTION EXPERIMENTS USING HIGHLY PURIFIED LYMPHOCYTE SUBPOPULATIONS AND MONOCYTES

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ABSTRACT The phytohemagglutinin (PHA) response of lymphocytes from untreated patients with systemic lupus erythematosus (SLE) was studied using highly purified subpopulations of cells involved in the transformation response: T lymphocytes, B lymphocytes, and monocytes. Cell transformation was quantitated using both tritiated thymidine ($[^3\text{H}]\text{TdR}$) incorporation into DNA and cytofluorographic determination of cellular DNA content. Dose-response curves using six concentrations of PHA and five concentrations of cells over 0–5 days revealed a decrease in $[^3\text{H}]\text{TdR}$ by stimulated lymphocytes from some SLE patients. This decrease in $[^3\text{H}]\text{TdR}$ was paralleled by a decreased percentage of cells in S, G_2 , and M phases of the cell cycle. However, abnormal response occurred entirely in those SLE patients who were hypocomplementemic. The etiology of the impaired response was further examined. Lymphocyte receptors for concanavalin A were studied using cytofluorography of lymphocytes stained with fluorescein-conjugated concanavalin A. The frequency distribution of concanavalin A receptors was similar in the normocomplementemic and hypocomplementemic lupus patients and in normals. The latex phagocytic activity of lupus macrophages was similar to normals when allogeneic normal plasma was used in the culture medium. Phagocytic activity became abnormal in the presence of SLE plasma. However, there was no difference in the $[^3\text{H}]\text{TdR}$ response or the percentage of cells in S, G_2 , and M phases when T lymphocytes from the

hypocomplementemic patients were stimulated on either autologous or normal allogeneic monocyte monolayers. Likewise, normal lymphocytes incorporated similar amounts of $[^3\text{H}]\text{TdR}$ and had similar percentages of cells in S, G_2 , and M phases whether their T lymphocytes were stimulated on autologous or SLE monocyte monolayers. Highly purified subpopulations of B and T lymphocytes were obtained by density sedimentation or Fenwal Leuko-Pak passage of lymphocyte populations. The response to PHA by lymphocytes from the hypocomplementemic lupus patients could be seen to involve at least two abnormalities. One, in reference to normal lymphocytes, SLE T lymphocytes plus monocytes had an impaired response; two, SLE B lymphocytes plus SLE T lymphocytes plus SLE monocytes had an impaired response. Two patients in the hypocomplementemic group were treated with steroids. 5 days after steroid treatment was initiated, the percentage of cells in S, G_2 , and M phases and the $[^3\text{H}]\text{TdR}$ response of PHA-stimulated lymphocytes returned to normal. The normalization of the $[^3\text{H}]\text{TdR}$ response was explained both by a return of purified T cells plus monocytes, purified B cells plus monocytes, and whole lymphocyte populations to normal responsiveness. These studies suggest that a steroid-correctable defect exists in T and B lymphocytes in SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE)¹ was originally described as an inflammatory disorder of connective

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¹ *Abbreviations used in this paper:* ARA, American Rheumatism Association; E, erythrocyte; EAC, erythrocyte antibody complement; FCS, fetal calf serum; F-H, Ficoll-Hypaque; PHA, phytohemagglutinin; SLE, systemic lupus erythematosus; $[^3\text{H}]\text{TdR}$, tritiated thymidine.

tissue, blood vessels, and serosal surfaces (1). Following the insight provided by the elucidations of the immunologic mechanisms involved in the formation of the LE cell, autoantibodies with binding affinities for various nuclear, cytoplasmic, cellular, and protein antigens have been described (2), and there is strong evidence that antigen-antibody complexes play a central role in the induction of tissue injury (3).

It has been suggested that defects in cell-mediated immunity may play a role in the development of autoimmune disease. Studies of the NZB-NZW mouse indicate that autoantibody formation is preceded by T-cell dysfunction (4) suggesting a primary immunoregulatory abnormality of cellular immunity.

We have examined cellular immunity in an untreated group of SLE patients. Lymphocyte responsiveness to phytohemagglutinin (PHA) was assessed using purified populations of T lymphocytes, monocytes, and B lymphocytes in the PHA response of cells from 14 patients. Responsiveness was assessed by both tritiated thymidine ($[^3\text{H}]\text{TdR}$) incorporation and relative cellular DNA content determined by cytofluorography.

METHODS

Patients. All of the 14 patients studied had at least four of the preliminary criteria of the American Rheumatism Association (ARA) for the diagnosis of SLE (5). Their mean age was 25 yr, and 13 patients were females. At the time of study, no patient had taken any drug other than aspirin or birth control pills for the previous 3 wk. In particular, no patient was being treated with cytotoxic drugs or steroids. All patients agreed to take no medication (including aspirin and oral contraceptives) for 2 days before the study. The control group consisted of 15 normal females with a mean age of 29 yr. None had taken any drugs for 5 days before the study.

The patients were arbitrarily divided into two groups on the basis of the serum complement. As outlined in Table II, those patients with hypocomplementemia (defined as a serum C3 < 90 mg/dl) had a longer disease duration, a higher mean number of preliminary ARA diagnostic criteria, a larger number with fever, anti-DNA antibodies, higher serum creatinine, lower peripheral blood lymphocyte counts, and a larger number with lymphocytotoxic antibodies. In general, the disease seemed more active in hypocomplementemic patients.

Mononuclear cell isolation. Peripheral blood from healthy human donors and patients was drawn into a syringe with preservative-free heparin or heparinized tubes and sedimented for 90 min at room temperature. The leukocyte-rich supernate was centrifuged and the cells were washed with 10% fetal calf serum (FCS)-RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.). Washed cells were re-centrifuged and suspended in 5 ml of cold 10% FCS-RPMI 1640, layered over 3 ml of Ficoll-Hypaque (F-H) (Bionetics Laboratory Products, Kensington, Md.) solution (12 parts of 14% F-H mixture with 5 parts of 32.8% Hypaque), and then centrifuged at 300 *g* at room temperature for 25 min. The buffy interface was then washed three times in cold 10% FCS-RPMI 1640 and centrifuged at 300 *g* at 4°C for 10 min.

Antisera. Serum and urine were obtained from patients with an established diagnosis of multiple myeloma or macro-

globulinemia. Monoclonal proteins were isolated by preparative zone electrophoresis, using polyvinyl copolymer as supporting medium in barbital buffer pH 8.6, T/2 0.1. Proteins were further purified on Sephadex G-200 or Bio-Gel A 1.5 m 100–200 agarose gel (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 0.1 M Tris-0.5 M NaCl. Protein concentrations were determined by the Folin-Lowry technique (6). A polyvalent antiserum specific for γ -, μ -, κ -, and λ -chains was prepared in a goat immunized with a mixture of purified IgM, λ -, and IgG, κ -proteins. The antiserum obtained showed reactions of identity for a panel of purified IgG, IgM, and κ -, or λ -Bence-Jones proteins. Monospecific antisera to IgG were prepared in New Zealand albino rabbits by immunization with purified IgG1, λ -myeloma proteins and absorption with purified λ -Bence-Jones protein. Antisera obtained gave a reaction of identity with 16 purified IgG myeloma proteins (four from each IgG subclass), and showed heavy chain specificity for both Fc and Fab fragments. IgG was purified from monospecific antisera by DEAE-Sephadex ion-exchange chromatography using elution with 0.005 M phosphate buffer, pH 7.2. Pepsin digestion of purified rabbit or goat IgG was carried out in acetate buffer at pH 4.5 using ratios of crystalline pepsin/protein (wt/wt) of up to 3% for 72 h to obtain virtually complete digestion of IgG. Digestion was monitored by Ouchterlony analysis using antisera to rabbit and goat IgG having specificity for the Fc region. Fragments were then purified on a calibrated Sephadex G-150 column equilibrated with phosphate-buffered saline, pH 7.2, for separation of undigested IgG, F(ab')₂, Fab', PFC', and peptides. The purified F(ab')₂ was harvested from fractions of $\approx 100,000$ mol wt with exclusion of all overlap fractions containing undigested IgG. Balanced specificity for γ , μ , κ , and λ was achieved by the addition of purified rabbit F(ab')₂ with κ specificity. The polyvalent and IgG F(ab')₂ antisera were conjugated with fluorescein or rhodamine using 30 $\mu\text{g}/\text{mg}$ of protein in saline-carbonate-bicarbonate buffer 0.5 M, pH 9.0, with removal of unbound fluorochrome by exhaustive dialysis or filtration through G-25 Sephadex. The fluorescein/protein ratios for antisera were between 2 and 3. Specificity was reassessed, titered by Ouchterlony analysis, and confirmed by specific blocking of direct and indirect immunofluorescence only with purified proteins of the designated specificity as previously described (7).

Aggregate (Fc) receptor-bearing cell enumeration. The Fc receptor was identified as previously described using the modifications of Yount et al. (7) and Dickler (8). The pelleted aggregated IgG was homogenized in a 7-ml Dounce homogenizer and the pH corrected with 0.1 N NaOH to 8.3 rather than 8.1 for maximum solubility of aggregated IgG. Just before use, aggregated material was centrifuged at 600 *g* at room temperature, not 4°C as originally described. Staining was carried out for 1 h at pH 8.3 at room temperature rather than 4°C. Under these conditions, a more uniform speckled staining pattern over the entire cell surface was noted.

Surface Ig-bearing cell enumeration. Staining for surface immunoglobulins was carried out as previously described (9) using the purified F(ab')₂ antisera as described by Winchester et al. (10). Lymphocytes in a concentration of $1-2 \times 10^6$ in 0.5 cm³ of 10% FCS-RPMI 1640 were incubated with 0.05–0.10 ml of fluorochrome-conjugated F(ab')₂ polyvalent anti-human immunoglobulin in $10 \times 75\text{-mm}$ glass tubes for 1 h at 4°C, washed three times with a total of 10 ml of cold FCS-RPMI, and mounted on glass slides. The cells were overlapped with a cover slip, sealed, and 1,000 cells were counted with a Leitz ultraviolet microscope equipped with a mercury arc HBO-200 lamp (E. Leitz, Inc., Rockleigh, N. J.), using BG 38- and

490-nm (fluorescein isothiocyanate) exciter filters and a K510-nm barrier filter, by epi-illumination and simultaneous phase-contrast microscopy to identify all nonstaining cells.

T-cell enumeration. Spontaneous cold sheep erythrocyte (E) rosettes were assayed by a variation (9) of the method of Ross et al. (11).

Complement receptor-bearing lymphocyte enumeration. Complement receptor-bearing (EAC-bearing) lymphocytes were determined as previously reported by a modification of the method of Ross et al. (11) and Utsinger et al. (12). Fresh normal human serum from a single donor was used as a source of complement. The method detects receptors for both C3b and C3d. E and EA controls were carried out with each experiment. E controls were always <2% and EA controls <5%.

Monocyte enumeration. Monocytes were identified by size (>10 μ m), α -naphthylacetate staining, and latex phago-

cytosis but they were not included in counts of lymphocytes. Latex phagocytosis was measured by a modification (12) of the method of Zücker-Franklin (13). The α -naphthylacetate staining was done according to the method of Yam et al. (14).

Purification of T lymphocytes. T cells were purified by three techniques, as outlined in Fig. 1. The first purification method was a modification of the method of Julius et al. (15). 300 mg of nylon wool (Leuko-Pak, Fenwal Laboratories, Morton Grove, Ill.), soaked in normal saline for 4 days at 37°C, was loosely packed into a 3-ml syringe, and then washed with 20 ml RPMI and 10 ml RPMI-10% FCS. The outflow was sealed, 10 ml RPMI-10% FCS was added, and the syringe was placed upright in an incubator at 37°C for 30 min. After draining this medium, 5×10^7 mononuclear cells in 1 ml RPMI were added dropwise, and incubated for 30 min at 37°C. The cells were eluted

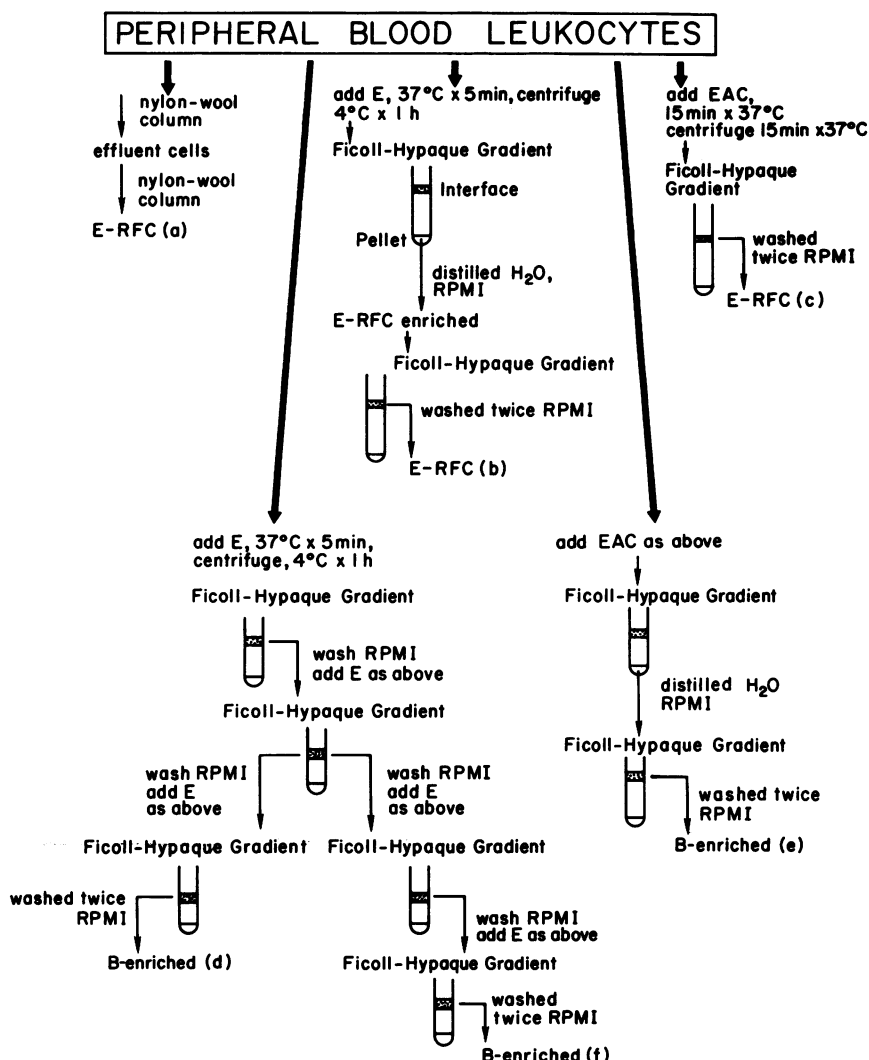


FIGURE 1 Lymphocyte separation scheme. Ficoll-Hypaque-separated peripheral blood leukocytes were divided into five aliquots. The cells of one aliquot were serially passed over nylon wool columns to effect E-RFC(a). The cells of the remaining aliquots were individually rosetted with either sheep erythrocytes (E) or (EAC) and sequentially passed over Ficoll-Hypaque to enhance or remove E- or EAC-rosetting cells, effecting E-RFC(b-f).

with 10 ml of 37°C RPMI, one drop of medium added as each drop of eluate came off. The eluted cells were resuspended, again layered over the column, and eluted exactly as described above. The final elution of cells was resuspended to 1×10^6 /ml RPMI. These cells were designated E-RFC(a).

The second purification was a modification of the method of Parish and Hayward (16). Aliquots of 5×10^6 E-rosetted lymphocytes/ml RPMI were layered over 3 ml of F-H (*d*, 1.09 g/ml) at room temperature and centrifuged at 1,200 *g* for 30 min. The pelleted cells were collected, the erythrocytes were lysed by addition of distilled water followed rapidly by flooding with RPMI, and the mononuclear cells were respun on F-H. The interface cells were washed twice in RPMI-1640 and resuspended to 1×10^6 /cm³. These cells were designated E-RFC(b).

The third method of purification was a modification of the method of Parish and Hayward (16). Aliquots of 5×10^6 EAC-rosetted lymphocytes/ml RPMI-10% FCS at room temperature were layered over 3 ml F-H (*d*, 1.09 g/ml) at room temperature and centrifuged at 1,200 *g* for 30 min. The interface cells were collected, washed twice in RPMI-1640, and resuspended to 1×10^6 /ml with RPMI. These cells were designated E-RFC(c).

Purification of B lymphocytes. B cells were purified by three techniques, as outlined in Fig. 1. The first and second purification methods were modifications of the method of Greaves and Brown (17). Aliquots of 5×10^6 E-rosetted lymphocytes/ml RPMI-10% FCS at room temperature were layered over 3 ml of F-H (*d*, 1.09 g/ml) at room temperature and centrifuged at 1,200 *g* for 30 min. The interface cells were washed once at room temperature in RPMI-1640, spun at 300 *g*, and rosetted with sheep erythrocytes exactly as described above, and recentrifuged over F-H. The interface cells were collected, layered over 3 ml of F-H at room temperature, and centrifuged at 1,200 *g* for 30 min. The interface cells were then washed twice in RPMI-1640 and resuspended to 1×10^6 /ml. These cells were designated B-enriched(d). In some cases, a third E-rosetting was performed; these cells were designated B-enriched(f).

The third purification method was a modification of the method of Parish and Hayward (16). Aliquots of 5×10^6 EAC-rosetted lymphocytes/ml RPMI-10% FCS at room temperature were layered over 3 ml F-H (*d*, 1.09 g/ml) at room temperature and centrifuged at 1,200 *g* for 30 min. The pelleted cells were resuspended, the erythrocytes lysed by addition of distilled water followed rapidly by flooding with RPMI, and then recentrifuged over F-H. The interface cells were washed twice in RPMI-1640 and resuspended to 1×10^6 /ml with RPMI. These cells were designated B-enriched(e).

Monocyte isolation and preparation. Monocytes were isolated by peripheral blood mononuclear cells by attachment to the plastic surface of tissue culture tubes. 1×10^6 peripheral blood mononuclear cells were added to each culture tube in RPMI-1640 and placed in a 95% air-5% CO₂ incubator for 2 h. The cell suspension was discarded and the tubes were washed four times with RPMI to remove nonadherent cells; recovery was $\approx 10\%$. More than 98% of the adherent cells (removable by vigorous washing) were monocytes, having used latex phagocytosis and α -naphthylacetate staining as criteria. In two experiments, 1×10^5 , 5×10^5 , 1×10^6 , and 5×10^6 peripheral blood mononuclear cells were added to different culture tubes (effecting monocyte layers of roughly 1×10^4 , 5×10^4 , 1×10^5 , and 5×10^5 cells).

In some experiments, peripheral blood mononuclear cells were irradiated with 2,500 rads before use in culture as a

monocyte source. Mitomycin C treatment was performed by adding 50 μ g/ml mitomycin C to 5×10^6 peripheral blood mononuclear cells/ml for 30 min at 37°C, and washing three times in RPMI-1640.

Mitogen culture. The optimal dose of PHA and the optimal number of lymphocytes was initially determined by culturing for 2, 3, 4, and 5 days with PHA concentrations of 0.05, 0.5, 1, 2, 5, and 50 μ g and SLE or normal mononuclear cells with a final cell concentration of 1×10^4 , 1×10^5 , 3×10^5 , 5×10^5 , and 1×10^6 cells/ml. Before culture, the mononuclear cells were washed three times at 37°C and incubated at 37°C for 12 h. All cultures were done in duplicate at 37°C in a 5% CO₂ atmosphere, and the optimal dose of PHA and the optimal concentration of lymphocytes was selected by determining the cell number and lectin concentration which resulted in the highest uptake of [³H]TdR on either day 2, 3, 4, or 5. The highest uptake of [³H]TdR was obtained using 1×10^5 cells/ml and a concentration of either 1.0 or 0.5 μ g PHA in all experiments. The [³H]TdR uptake was similar with 0.5 and 1.0 μ g of PHA. In most situations, the maximal uptake of [³H]TdR occurred on day 3, though on occasion, the maximal uptake of [³H]TdR occurred on day 4.

After determining the optimal dose of lectin and cell number, all cultures using purified mononuclear cells and lymphocyte subpopulations were performed as described. RPMI 1640 with 10% heat-inactivated, fresh frozen pooled human AB+ serum and 50 μ g/ml of gentamycin (Microbiological Associates, Bethesda, Md.) was the medium. The mononuclear cells and purified lymphocyte subpopulations were always washed three times at 37°C and incubated at 37°C for 12 h before culture. Quadruplicate cultures of 1.0×10^5 mononuclear cells or purified lymphocyte subpopulations in a 1-ml medium were made in 10×75 -mm, sterile, capped tissue culture tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in a 37°C, 5% CO₂ atmosphere. PHA (Burroughs Wellcome, Co., Research Triangle Park, N. C.) at 0.5 μ g/culture was added in a volume of 0.5 ml medium. Duplicate cultures were terminated on days 0 (after 2 h), 1, 2, 3, 4, and 5 days after a 5-h pulse with 1 μ Ci of [³H]TdR (New England Nuclear, Boston, Mass.). The trichloroacetic acid-insoluble radioactivity of cells was collected on Millipore filters (HAMK, Millipore Corp., Bedford, Mass.) and counted in a liquid scintillation counter. All data are expressed as the maximum difference between the counts per minute (cpm) in the stimulated culture and the cpm in the control culture. To determine the maximal difference in cpm, the difference in cpm for each day of culture and each concentration of mitogen was determined and the largest (maximal) differences in cpm used for comparison. Thus, the cpm in each figure represent the group mean derived from the maximal difference in cpm of each individual determination.

Cell staining and cytofluorograph instrumentation. Duplicate tubes of the PHA-stimulated mononuclear cells or lymphocytes were stained as previously described with ethidium bromide, a general nucleic acid stain, by the method of Gohde and Dittrich (18). For analysis of cellular DNA content, 1×10^5 cells were treated with 1 ml ribonuclease (Worthington Biochemical Corp., Freehold, N. J.) for 30 min at 37°C to hydrolyze RNA before ethidium bromide staining.

The cells were resuspended in distilled water and agitated in a Vortex-Genie (Scientific Industries, Inc., Bohemia, N. Y.) at speed control 5 for approximately 5 s before counting. 10,000 cells were counted. All counting was done with a BioPhysics Systems, Inc. (Mahopac, N. Y.) cytofluorograph, model 4802A. This cytofluorograph has been described in detail elsewhere (19). Each stained cell, in suspension, is

passed in single file through a 488-nm argon-ion laser beam. During transit through the beam, a stained cell generates a fluorescent light flash which is converted to an electronic pulse by a multiplier phototube. The light flashes are amplified and analyzed for intensity. The intensity distribution, which is directly proportional to the DNA content, is stored in a multichannel pulse-height analyzer which can accumulate a record of the DNA content per cell for 10,000 cells in several minutes.

The percentage of diploid (2n) G₁ cells, tetraploid (4n) G₂ + M cells, and interploid (2n > DNA > 4n) S-phase cells was calculated from the DNA histograms. G₁ was defined as the first peak of cells in the histogram of the DNA spectra of the control (unstimulated) or stimulated cells. S-G₂-M was defined as those cells outside the G₁ locus. Replicate determinations of 10,000 cells from the same sample were in agreement with 1.1%. Duplicate cultures have an error of <3.2% from the mean. Fluorescent microscope examination of cell samples after stimulation with PHA failed to reveal any pairs of agglutinated cells, supporting the concept that the DNA content measured reflected individual cell DNA content.

Other studies. C3 was performed by an automated turbidimetric (nephelometric) technique. Hemagglutinating antibodies to DNA were detected by incubating test sera with human O+ cells coated with native DNA according to the method of Koffler et al. (20). Less than 0.3% of normal individuals have antibody to native DNA by this method. The purification, and fluorescein-conjugation of concanavalin A and the specificity of the reaction was done by the method

of Kraemer et al. (21). Macrophage adherence was performed by the method of Krikorian et al. (22) with two variations; only 2×10^6 F-H-separated mononuclear cells were added to the plastic chambers (with a 12-mm internal diameter), and then were adhered to a glass slide with Vaseline. Lymphocytotoxic antibodies were detected as previously described (23) by the method of Terasaki and McClelland (24) and Terasaki et al. (25). Cytotoxicity was considered to be significant if cell death was >15%. For statistical analysis the Student's *t* test was employed.

RESULTS

Surface markers on normal and SLE lymphocytes. The absolute peripheral blood lymphocyte count was lower in the 14 SLE patients than in the 15 control subjects ($1,474 \pm 162$ vs. $2,432 \pm 175$) ($P < 0.0005$). The surface characteristics of the F-H-separated lymphocyte populations and the purified B- and T-cell populations obtained by the different separation methods are outlined in Table I. The percentage of unseparated cells bearing the four surface markers studied and the percentage of latex phagocytic cells was similar in the normal and lupus populations. The percentage of cells bearing surface immunoglobulin, and complement, Fc, and sheep erythrocyte

TABLE I
Surface Markers on Unseparated and Purified Mononuclear Cell Populations

| Lymphocyte populations | F(ab') Anti-Ig | EAC | Fc | E | LP* |
|--------------------------|-------------------|----------------|----------------|----------------|----------------|
| mean % \pm SEM | | | | | |
| Normal E-RFC enriched(a) | 1.0 \pm 0.2 | 0.8 \pm 0.1 | 5.4 \pm 0.5 | 97.4 \pm 0.3 | 2.1 \pm 1.0 |
| SLE E-RFC enriched(a) | 1.1 \pm 0.3 | 0.9 \pm 0.2 | 6.1 \pm 0.3 | 96.5 \pm 0.4 | 2.2 \pm 0.9 |
| Normal E-RFC enriched(b) | 5.2 \pm 0.3 | 4.0 \pm 0.3 | 5.1 \pm 0.3 | 91.3 \pm 0.4 | 6.6 \pm 0.7 |
| SLE E-RFC enriched(b) | 5.3 \pm 0.4 | 4.1 \pm 0.4 | 6.6 \pm 0.4 | 92.3 \pm 0.5 | 5.8 \pm 0.6 |
| Normal E-RFC enriched(c) | 3.6 \pm 0.7 | 1.8 \pm 0.3 | 4.7 \pm 0.3 | 92.3 \pm 0.6 | 5.9 \pm 0.7 |
| SLE E-RFC enriched(c) | 3.5 \pm 0.8 | 1.9 \pm 0.3 | 4.3 \pm 0.4 | 93.1 \pm 0.7 | 6.0 \pm 0.6 |
| Normal B-enriched(d) | 83.6 \pm 1.1 | 81.2 \pm 1.6 | 82.2 \pm 1.4 | 1.1 \pm 0.3 | 15.8 \pm 1.3 |
| SLE B-enriched(d) | 83.5 \pm 1.0 | 80.3 \pm 1.5 | 83.1 \pm 1.5 | 1.0 \pm 0.3 | 14.6 \pm 1.2 |
| Normal B-enriched(e) | 90.5 \pm 0.3 | 90.4 \pm 1.5 | 85.2 \pm 1.3 | 1.2 \pm 0.2 | 7.6 \pm 0.9 |
| SLE B-enriched(e) | 90.6 \pm 0.2 | 90.2 \pm 1.4 | 86.3 \pm 1.4 | 1.3 \pm 0.4 | 8.3 \pm 0.9 |
| Normal B-enriched(f) | 91.2 \pm 0.5 | 90.7 \pm 0.4 | 88.4 \pm 1.5 | <0.5 | 7.2 \pm 0.3 |
| SLE B-enriched(f) | 91.3 \pm 0.5 | 91.6 \pm 0.5 | 91.3 \pm 1.6 | <0.5 | 7.1 \pm 0.2 |
| Normal unseparated | 10.7 \pm 0.5 | 10.1 \pm 0.4 | 12.1 \pm 1.1 | 71.5 \pm 1.3 | 21.6 \pm 2.4 |
| SLE unseparated | 10.5 \pm 0.6 | 10.3 \pm 0.5 | 15.3 \pm 1.6 | 68.6 \pm 1.4 | 20.9 \pm 2.3 |

* LP, latex phagocytic cells, excluded when enumerating Ig-, EAC-, Fc-, and E-bearing cells.

(a) Nylon passage twice; (b) E rosettes pelleted by density sedimentation, erythrocytes lysed, lymphocytes respun on F-H, and interface cells washed and resuspended; (c) EAC pelleted by density sedimentation, interface cells sedimented on F-H, washed, and resuspended; (d) E pelleted by density sedimentation, interface cells re-rosetted and pelleted by density sedimentation, and interface cells washed; (e) EAC pelleted by density sedimentation, erythrocytes lysed, lymphocytes respun on F-H, and interface cells washed, and (f) Same as (d), but with a third E-RFC rosetting.

receptors was similar in each of the separated B- and T-cell preparations.

[³H]TdR incorporation by normal and SLE lymphocytes. In Fig. 2, a significantly decreased incorporation of [³H]TdR can be noted in the lupus population on culture days 2, 3, 4, and 5 ($P < 0.0025$). The thymidine doubling time was estimated to be 12 h in the normal population and 16 h in the lupus population, when calculated from several points on the curve during the 2nd day of culture.

Cytofluorography of normal and SLE lymphocytes. To eliminate the possibility that the decrease in the incorporation of [³H]TdR in the lupus population was simply due to an abnormality in the thymidine transport system, the cellular DNA content of the lupus lymphocytes was determined cytofluorometrically. In Fig. 3, a marked discrepancy is seen between the lupus population and the normal mononuclear cells. The kinetics of cell proliferation using cytofluorography closely parallel the kinetics when using thymidine incorporation, suggesting that this method may be used alternatively to thymidine incorporation as a means of assessing DNA synthesis.

Effect of disease activity on lymphocyte transformation in hypo- and normocomplementemic patients. The SLE patients were divided into two groups, based on the presence of hypocomplementemia (Table II). The hypocomplementemic group of seven patients differed in other respects from the normocomplementemic group as outlined in Table II. When [³H]TdR incorporation and DNA synthesis determined

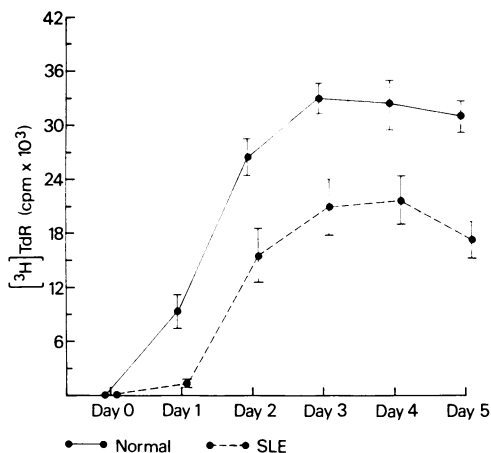


FIGURE 2 Comparison of [³H]TdR incorporation by normal (15 subjects, 15 experiments) and SLE (14 subjects, 14 experiments) lymphocytes (Ficoll-Hypaque-separated mononuclear cells) after PHA stimulation. Each datum point represents the group mean \pm SE derived from the maximum [³H]TdR incorporation-³H]TdR background in individual dose-response curves using six concentrations of PHA and five concentrations of cells. Significant impairment in PHA responsiveness in SLE patients was noted from days 1 to 5.

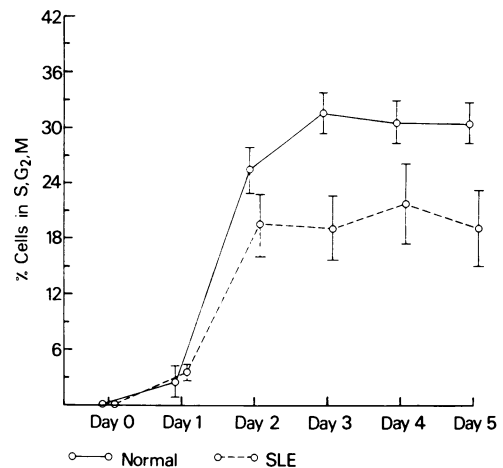


FIGURE 3 Comparison of the percentage of normal (15 subjects, 15 experiments) and SLE (14 subjects, 14 experiments) lymphocytes (Ficoll-Hypaque-separated mononuclear cells) in S, G₂, or M phases of the cells cycle after PHA stimulation. Each datum point represents the group mean \pm SE derived from the maximum number of S, G₂, and M cells in individual dose-response curves using six concentrations of PHA and five concentrations of cells. The SLE patients showed significantly fewer cells in an interloid or tetraploid state on days 2-5.

cytofluorographically were studied (Fig. 4), a striking difference between the two populations of patients was seen ($P < 0.0005$). The kinetics of thymidine incorporation and DNA production was markedly abnormal in the hypocomplementemic group of lupus patients. More striking was the fact that the normocomplementemic group of patients have a [³H]TdR incorporation and DNA proliferative response almost identical to that of normal age- and sex-matched controls.

Density distribution of lectin receptor. To eliminate the possibility that the defect in the hypocomplementemic lupus patients was due to receptor binding of the lectin, the lymphocytes of two of the hypocomplementemic patients with low [³H]TdR incorporation and two of the normocomplementemic patients with normal [³H]TdR incorporation were stimulated with pokeweed mitogen and concanavalin A. Proliferative responses were diminished in comparison to normal subjects. Fluorescein-conjugated concanavalin A, by cytofluorographic analysis, had a similar density distribution in both groups of patients (two experiments on each patient in each group).

Monocyte function. To examine the role of monocytes in the abnormal proliferative response we determined the percentage of macrophages which developed from peripheral blood mononuclear cells in these patients. When autologous SLE mononuclear cells were cultured in the presence of autologous serum, a decrease (but not statistically significant) in glass-adherent cells was found in comparison to 10

TABLE II
Analysis of Two Groups of SLE Patients

| Clinical and laboratory features | Normocomplementemic C3 > 90 mg/dl | Hypocomplementemic C3 < 90 mg/dl |
|--|--------------------------------------|-------------------------------------|
| Patient number | 7 | 7 |
| Age (mean) | 25.8 | 24.4 |
| Sex (F:M) | 6:1 | 7:0 |
| Race (B:W) | 6:1 | 6:1 |
| Prior therapy: | | |
| Aspirin | 3/7 | 5/7 |
| Oral contraceptives | 2/7 | 3/7 |
| Disease duration before study (Mean months±SE, range) | 2.6±0.5 (1-4) | 4.7±0.9 (1-8) |
| ARA diagnostic criteria for SLE | | |
| Mean criteria per patient: | 4.4 | 6.3 |
| Facial erythema | 3/7 | 6/7 |
| Discoid lupus | 1/7 | 1/7 |
| Raynaud's phenomenon | 3/7 | 4/7 |
| Alopecia | 4/7 | 3/7 |
| Photosensitivity | 3/7 | 4/7 |
| Oral or nasopharyngeal ulceration | 2/7 | 2/7 |
| Arthritis without deformity | 6/7 | 6/7 |
| LE cells | 2/5 | 3/6 |
| Chronic false-positive STS | 1/7 | 1/7 |
| Profuse proteinuria | 1/7 | 5/7 |
| Cellular casts | 2/7 | 3/7 |
| Pleurisy/pericarditis | 1/7 | 3/7 |
| Psychosis/convulsion | 1/7 | 1/7 |
| Hemolytic anemia/leukopenia/ thrombocytopenia | 1/7 | 2/7 |
| Fever | 1/7 | 4/7 |
| Mean erythrocyte Sedimentation rate±SE (range) | 37.0±2.8 (29-41) | 48.7±3.9 (31-61) |
| Mean serum C ₃ ±SE (Normal > 90 mg/dl) (range) | 98.4±2.1 (91-107) | 68.1±5.8 (45-88) |
| Mean anti-DNA antibody (I/titer) (range) | 0/7 | 5/7 (14.4) (4-32) |
| Mean serum creatinine (mg/100 dl)±SE (range) | 1.6±0.1 (0.9-2.1) | 1.8±0.1 (1.4-2.2) |
| Mean lymphocyte Count/mm ³ ±SE (range) | 1,941±185 (1,160-2,845) | 1,008±1.37 (550-1,480) |
| Lymphocytotoxic antibodies (<15% cytotoxicity) | 3/5 | 6/6 |
| Mean±SE (range) | 56.0±16.9 (25-83) | 66.8±3.3 (58-80) |

normals ($102,932 \pm 95,620$ vs. $195,523 \pm 86,521$, $P > 0.05$). In addition, these glass-adherent cells phagocytized fewer latex particles per cell in comparison to normals (mean four particles vs. nine particles in normals, $P < 0.05$). However, when SLE glass-adherent mononuclear cells were cultured in allogeneic normal serum, phagocytic capabilities were similar to normals. To completely eliminate the possibility that monocyte dysfunction was playing a role, two series of experiments were performed. In one series of experiments, monocytes (tissue culture tube-adherent cells) from seven lupus patients (four normocomplemen-

temic and three hypocomplementemic) and seven normal subjects were individually cultured with allogeneic normal T lymphocytes. In these experiments, outlined in Fig. 5, it can be seen that lupus monocytes are able to function as well as allogeneic normal monocytes in the role of helper monocytes in a PHA response. In our culture system, monocytes potentiate the lymphocyte proliferative response to PHA. In 10 experiments using five normal donors, the PHA-stimulated E-RFC(a) population of cells incorporated $24,108 \pm 2,843$ less cpm [^3H]TdR than the E-RFC(a) population reconstituted with autologous

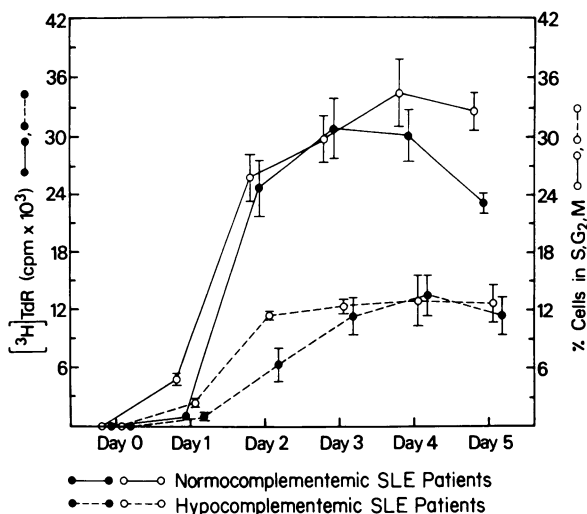


FIGURE 4 Comparison of $[^3\text{H}]\text{TdR}$ incorporation and the percentage of lymphocytes (Ficoll-Hypaque-separated mononuclear cells) in S, G_2 , M by normocomplementemic SLE patients (seven subjects, seven experiments) and hypocomplementemic SLE patients (seven subjects, seven experiments). Individual data points are maximum mean points derived from the dose-response curves. Significant impairment of PHA responsiveness was noted only in hypocomplementemic SLE patients.

monocytes. These studies have been reported in detail elsewhere.² The monocytes of one patient did not potentiate a PHA response. Normal monocytes, cultured with allogeneic lupus T lymphocytes (from the hypocomplementemic group), did not effect a change in the proliferative response of the SLE cells either in terms of DNA content of cells or thymidine incorporation (Fig. 6). The striking single exception to this general observation was found in the same SLE patient whose monocytes were unable to potentiate the proliferative response of an allogeneic normal subject. To further eliminate a contribution of monocyte dysfunction, a second series of two experiments were performed reconstituting 1×10^5 SLE T lymphocytes on different concentrations of adherent monolayers of allogeneic and autologous monocytes (1×10^4 , 5×10^4 , 1×10^5 , and 5×10^5). Differing lymphocyte to monocyte ratios had no effect on the proliferative response. Similar augmentation was obtained after culturing 1×10^5 SLE T lymphocytes with allogeneic and autologous mononuclear cells which were treated (either irradiated or incubated with mitomycin C) to render lymphocytes inactive.

B-Cell helper effect. The contribution of B lympho-

² Utsinger, P. D., and W. J. Yount. Cytofluorometric analysis of the kinetics of lymphocyte transformation after phytohemagglutinin stimulation: monocyte-macrophage potentiation of phytohemagglutinin-induced DNA synthesis. Manuscript in preparation.

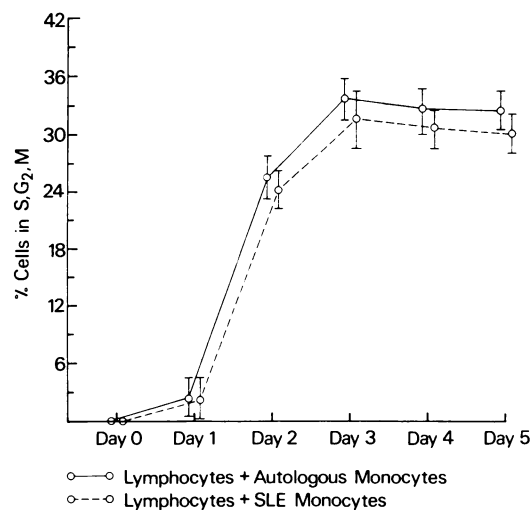


FIGURE 5 A comparison of the percentage of normal T lymphocytes in S, G_2 , and M after PHA stimulation based on the monocyte source. 1×10^5 normal T lymphocytes (E-RFC[a]) were added to either 1×10^4 SLE monocytes or 1×10^4 autologous monocytes (three experiments). A similar percentage of cells is seen in S, G_2 , and M regardless of the monocyte source.

cytes to the abnormal proliferative response was then examined. The effect of adding normal B lymphocytes to autologous T lymphocytes cultured on autologous monocyte monolayers was studied (Fig. 7). In this experiment, it can be seen that a statistically significant increase in thymidine incorporation occurs when B lymphocytes are added to the culture system ($P < 0.0005$ on day 3). This B-cell helper effect can be expressed by the increase in $[^3\text{H}]\text{TdR}$ uptake of

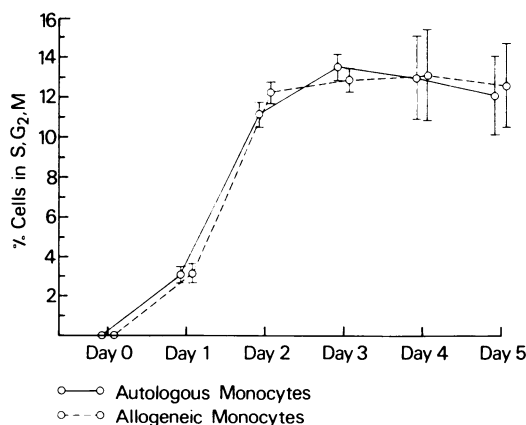


FIGURE 6 A comparison of the percentage of hypocomplementemic SLE T lymphocytes (seven subjects, seven experiments) (E-RFC[a]) in S, G_2 , and M after PHA stimulation based on the monocyte source. 1×10^5 SLE T lymphocytes were added to either 1×10^4 SLE monocytes or 1×10^4 allogeneic monocytes. A similar percentage of cells is seen in S, G_2 , and M regardless of the monocyte source.

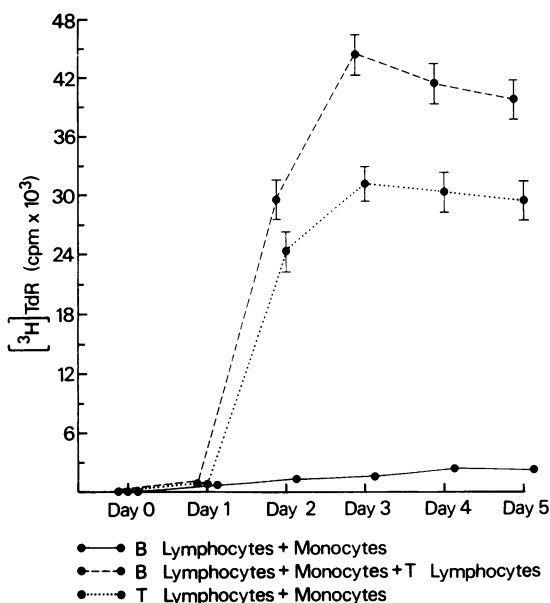


FIGURE 7 Influence of normal B lymphocytes (B-enriched[d]) on the normal T lymphocyte (E-RFC[a]) plus monocyte response to PHA (15 subjects, 15 experiments). 0.5×10^5 B lymphocytes were cultured with 0.5×10^5 T lymphocytes with 1×10^4 monocytes. In the B lymphocyte plus monocyte and T lymphocyte plus monocyte experiments, 1×10^5 lymphocytes were added to 1×10^4 monocytes. The helper effect of highly purified B lymphocytes was noted.

cultures containing B cells related to culture containing only T cells by the formula:

$$\text{Helper effect} = 100 \times \frac{\text{cpm}(T + xB) - \text{cpm}(T + xT)}{\text{cpm}(T + xT)},$$

where x B and x T correspond to an equal number of cells. This mean B-cell helper effect in normals was 44% (range 29–65%) and in the SLE group 21% (range 0–30%) ($P < 0.05$). This increase in $[^3\text{H}]\text{TdR}$ cannot be solely accounted for by proliferation of B lymphocytes because in 10 experiments mitomycin-treated B lymphocytes, which are incapable of proliferating, were also able to enhance thymidine incorporation, though not to the extent of nonmitomycin-treated B lymphocytes. (The mitomycin-treated B lymphocytes increased the incorporation of $[^3\text{H}]\text{TdR}$ by the E-RFC(a) population from $28,421 \pm 1,762$ to $41,593 \pm 1,685$). The effect of adding lupus B lymphocytes from the hypocomplementemic group to autologous T-lymphocyte monocyte monolayer cultures was also studied (Fig. 8). It can be seen that no increase in thymidine incorporation was found. Corroborative evidence for the lack of effect of lupus B lymphocytes was obtained by cytofluorography; no increase in the number of cells entering an interloid or tetraploid state was found. The effect of adding

lupus B lymphocytes from the normocomplementemic group was similar to the effect of normal B lymphocytes.

Effect of steroid treatment. Two of the original group of seven hypocomplementemic lupus patients were subsequently treated with steroids (1 mg/kg oral prednisone) because of persistent abnormalities in urine sediment and rising serum creatinine. These patients were studied serially after the institution of corticosteroid therapy. Normalization of the kinetics of thymidine incorporation and DNA synthesis, which occurred in the lymphocytes of both patients within 5 days after treatment was initiated (Fig. 9), was striking. This normalization of the kinetic response of the cells was independent of any change in clinical status since the urinalysis and serum creatinine remained abnormal for several weeks after treatment was started. In both of these patients, purified T and B lymphocytes responded normally to PHA and B-lymphocyte helper function was normal. A similar increase in lymphocyte $[^3\text{H}]\text{TdR}$ incorporation was not noted in three patients with rheumatoid vasculitis and decreased lymphocyte $[^3\text{H}]\text{TdR}$ incorporation who were also treated with 1 mg/kg prednisone.

DISCUSSION

Studies of cell-mediated immunity in SLE reveal significant discrepancies (26–34). Some of the reasons for these discrepancies have become clear over the last several years. Various drugs such as aspirin and the sera from some SLE patients are known to interfere

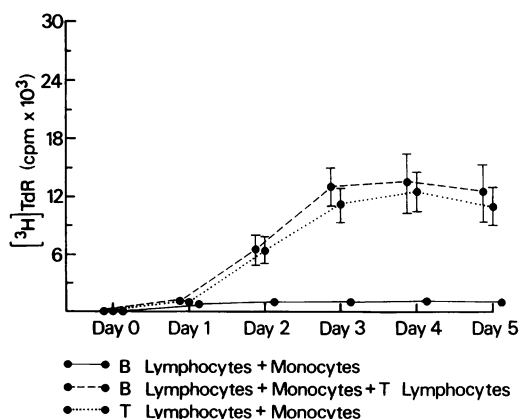


FIGURE 8 Influence of hypocomplementemic SLE B lymphocytes (B-enriched[d]) on the hypocomplementemic SLE T lymphocyte (E-RFC[a]) plus monocyte response to PHA (seven subjects, seven experiments). 0.5×10^5 B lymphocytes were cultured with 0.5×10^5 T lymphocytes with 1×10^4 monocytes. In the B lymphocytes plus monocytes and T lymphocytes plus monocytes experiment, 1×10^5 lymphocytes were added to 1×10^4 monocytes. No helper effect of highly purified SLE B lymphocytes was noted.

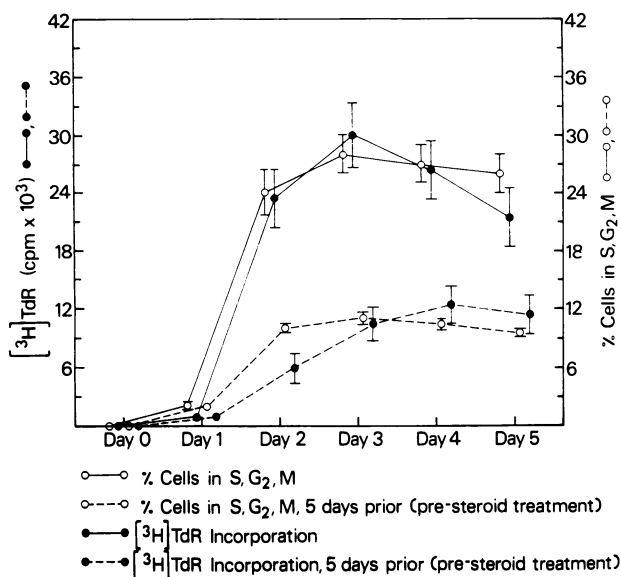


FIGURE 9 Effect of oral prednisone (1 mg/kg) on $[^3\text{H}]\text{TdR}$ incorporation and DNA synthesis by SLE lymphocytes. 1×10^6 Ficoll-Hypaque-separated mononuclear cells were cultured with $0.5 \mu\text{g}$ PHA. A significant improvement in $[^3\text{H}]\text{TdR}$ incorporation and the percentage of cells in S, G₂, and M is noted after steroid treatment (one experiment on each subject on each day).

with lymphocyte transformation (32, 35, 36). To exclude these factors lymphocytes should be cultured in normal allogeneic serum and patients should, so far as can be ascertained, be taking no drugs. We originally studied patients on their initial visit to our Rheumatic Disease Clinic who were not receiving any drugs other than aspirin or oral contraceptives. The SLE patients in this study took no drugs for 2 days before the study.

The hypocomplementemic SLE group was lymphopenic in comparison to the normocomplementemic group and to age- and sex-matched controls. This lymphopenia was accounted for by absolute decreases in SIg, EAC, Fc, and E-rosetting cells; however, the percentages of these cells were not significantly different from normals. Previously, we, as well as others, had found a significant percentage decrease in T cells in SLE (23, 37, 38). However, our previously studied SLE patients had disease of much longer duration than the patients reported here, and disease duration and activity may play a role in the T lymphopenia.

To help eliminate the possibility that potentially inhibitory serum factors were adhering to the mononuclear cells of the lupus patients, F-H-separated mononuclear cells were washed extensively and cultured at 37°C for 12 h. To further investigate T- and B-cell responsiveness, T and B lymphocytes were purified from peripheral blood. Three different separation methods for both T and B lymphocytes were

used to avoid possible selective depletion of subpopulations during purification. We found that the proliferative response to PHA was similar with different methods, and therefore have presented data only for the separation method which we termed E rosette-forming cell "a" (E-RFC[a]) and for the separation methods for B lymphocytes which we call B-enriched(d).

Both cytofluorography and thymidine incorporation were used for the following reasons. First, lymphocytes themselves are not auxotrophic for thymidine. A small intracellular pool of thymidine monophosphate as a DNA precursor exists, produced by synthetic reactions from simple precursors such as aspartic acid. Thymidine added to a culture system uses an unknown transport system to enter the cell. The relative contribution of these two metabolic pathways to the pool of thymidine monophosphate is usually not known, and discrepancies between incorporation of labeled thymidine into DNA and the actual rate of DNA synthesis have been observed in several systems (39, 40). Secondly, addition of a radio-labeled precursor and scintillation counting is an indirect or "blind" technique and speaks only in terms of net synthesis of DNA in the whole population. Information about individual cells or subpopulations of cells is lacking.

The decreased incorporation of $[^3\text{H}]\text{TdR}$ by the SLE group of patients was consonant to a decreased number of cells with an interpoloid or tetraploid DNA content making the possibility that the decreased $[^3\text{H}]\text{TdR}$ uptake was due to prolonged transit time of a uniformly responsive population of cells unlikely. Within the SLE group, the relation of the abnormal transformation response to disease activity was striking. This previously had been noted in SLE, in this and other laboratories (41–43). The striking relation of PHA responsiveness to disease activity may explain partly the previously noted discrepancies of PHA responsiveness in SLE. The mechanism for the relationship of disease activity and lymphocyte transformation appears to be extremely complex.

The purified T-lymphocyte population of the hypocomplementemic group responded poorly to PHA. It is impossible to determine whether this abnormal response is due to a mixture of nonresponding T cells and normally responding T cells. However, the ratio of $[^3\text{H}]\text{TdR}$ incorporation to interpoloid-tetraploid cells is consistent with two populations of T cells, one with normal cell transit time and one with absent or severely decreased cell transit time. Cell synchronization "thymidine suicide" studies, now in progress, should clarify the kinetic status of the cell populations. Present data appear consistent either

with functional suppressor cells, intrinsic T-cell defects, or both. The failure to find the normally affected increase in PHA stimulation after 12 h of overnight culture in the hypocomplementemic SLE group, as we previously noted (43), suggests that long-lived suppressor cells or long-lived nonresponder cells may be present. Experimental conditions, however, may also play a role since Stobo and co-workers have found a restoration of some SLE lymphocyte functions after elution of anti-lymphocyte antibodies (44).

The striking association of lymphopenia with lymphocytotoxic antibodies, and of lymphopenia with impaired mitogen responsiveness, suggests that some cells in our culture system may have been coated with lymphocyte membrane-determinant antigen antibodies (32, 44). We attempted to eliminate this possibility by 37°C washing of the cells followed by 37°C incubations. We cannot exclude the possibility that residual cell-bound antibody or antigen-antibody complexes may be present and effect anchorage modulation of the cytoskeleton (46). Our data concerning the density distribution of concanavalin A receptors strongly suggest that steric phenomena do not cause masking of the lectin receptor.

To clarify the abnormal transformation response further, we studied the other two cell types which are needed for an optimal response to PHA: monocytes and B lymphocytes. SLE monocytes in hypocomplementemic autochthonous serum demonstrated diminished phagocytosis. Although the potentiating effect of monocytes on a PHA response may involve internalization and subsequent presentation of the mitogen (47–49), this abnormality does not seem likely to be responsible for the impaired transformation response. First of all, cultures were performed in normal allogeneic serum. Secondly, these monocytes with one exception were able to reconstitute responses in normals. Thirdly, normal allogeneic monocytes did not stimulate SLE T lymphocytes to incorporate more [³H]TdR. The single patient whose monocytes were abnormal did not differ clinically from the other hypocomplementemic patients. This striking exception may represent a minor subpopulation of SLE patients with monocyte function impaired through a mechanism yet to be defined. Staining properties of the monocytes in this patient for α -naphthylacetate and latex phagocytosis were similar to other patients.

B lymphocytes have been shown to have a helper cell function in PHA responsiveness (50). This helper cell function is largely independent of B-lymphocyte proliferation since mitomycin-treated B lymphocytes (data not shown) also can function as helper cells, though not as effectively as nonmitomycin-treated B lymphocytes. Lupus B lymphocytes from the hypo-

complementemic group of patients were unable to function as helper cells. This abnormality may be related to an intrinsic B-cell defect or to an absence or dysfunction of that subpopulation of T lymphocytes which the B-cell "helps."

The response of the whole mononuclear cell population to PHA was markedly improved by steroid treatment; this improvement involved both B-lymphocyte helper function and T-lymphocyte function, occurred early in the course of treatment, and was independent of clinical improvement. It is tempting to speculate that the prompt response to the steroids was due to a depletion of a suppressor cell population of cells, a population that has both T- and B-lymphocyte suppressor activities, or to removal of cell surface-bound protein, or immune complexes, similar to what has been postulated to occur in steroid-treated idiopathic thrombocytopenic purpura (51).

The study of cellular contribution to abnormal lymphocyte proliferation in SLE may help in dissecting out the cellular contribution of abnormal responsiveness in a variety of disease states. There is strong evidence for abnormal T-cell function in SLE. However, abnormalities in B lymphocytes also appear to contribute to the decreased incorporation of [³H]TdR noted. Monocytes exhibit abnormal phagocytic capabilities in the presence of lupus serum but appear to maintain at least some functional capabilities when cultured in autologous serum and appear only rarely to contribute to the abnormal PHA responsiveness noted.

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