Cytofluorometric Analysis of the Kinetics of Lymphocyte Transformation After Phytohemagglutinin Stimulation: Comparison With the Kinetics of Thymidine Incorporation

By Peter D. Utsinger, William J. Yount, Janet G. Fallon, Marshall J. Logue, C. Randall Fuller, and Delores Elliott

The technique of flow cytofluorometry has been employed to assess the response of unfractionated and highly purified human lymphocyte subpopulations to phytohemagglutinin (PHA) and pokeweed mitogen. Normal values for cytofluorometric responses were established and compared to the uptake of tritiated thymidine in simultaneous experiments. Cytofluorometric analysis offered the advantages of increased sensitivity and direct measurement of DNA content per cell, and provided percentages and absolute numbers of responding cells. B-cell responses to pokeweed mitogen were absent, but brisk T-cell responses were noted. Between 4% and 8% of highly purified human B cells were found to respond to PHA by increasing their DNA content; modest but significant uptake of tritiated thymidine by B cells following PHA stimulation was also observed.

The transformation of lymphocytes that results from lymphocyte stimulation with phytohemagglutinin (PHA) was described by Nowell in 1960. The cellular interactions which develop after stimulation in vitro with mitogens is currently under intensive investigation. It is not clear what the relative contribution of lymphocyte subpopulations is to the transformation response, and contradictory results about the response of human T- and B-lymphocyte populations exist. In the great majority of investigations, the marker system for detecting transformation has been the increased uptake of tritiated thymidine (\(^{3}\)H-TdR) by the stimulated lymphocyte. There are, however, several criticisms of this technology. First, lymphocytes themselves are not auxotrophic for thymidine. An 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.

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Second, addition of a radiolabeled precursor and scintillation counting is an indirect or "blind" technique and speaks only in terms of net synthesis of protein, DNA, or RNA in the whole cell population. Information about individual cells or subpopulations of cells is lacking. Some of this information is obtainable from autoradiography, but the number of cells observed is small and the method is extremely time consuming and relatively insensitive.

Some of these problems can be bypassed by using flow cytofluorometry. Biophysical analysis of flow cytofluorometry has been extensively described. Briefly, cells to be studied are stained in suspension with the desired fluorochrome. Stained cells are allowed to flow through a small-diameter laser light beam. Each cell generates a pulse of scattered light and a fluorescent pulse, dependent on DNA or protein content. The resultant light scatter/absorption is measured and electronically developed as a dot pattern distribution on an oscilloscope. If the optical properties of the cells are similar, the cells will cluster. Approximately 10,000 cells can be counted in 15 sec. The main advantages of the instrument include direct staining of intracellular constituents with data about relative DNA or protein content per cell, rapid counting of large numbers of cells, and the assessment of percentages and absolute numbers of responding cells.

In the present report, we have examined the responsiveness of highly purified populations of human B and T lymphocytes to PHA. This transformation response was measured simultaneously by the uptake of $^3$H-TdR and by flow cytofluorometry of DNA-stained lymphocytes. Flow cytofluorometry appeared to be somewhat more sensitive than uptake of $^3$H-TdR in the assessment of blastogenic responses to lectins. In highly purified B-cell populations a small percentage of B cells was found to respond to PHA, and minimal or no response to pokeweed mitogen (PWM) was observed.

MATERIALS AND METHODS

Mononuclear cell isolation. Peripheral blood from healthy human donors was drawn into a syringe with preservative-free heparin and sedimented for 90 min at room temperature. The leukocyte-rich supernatant was centrifuged and the cells were washed with 10% fetal calf serum (FCS)-RPMI 1640 (Grand Island Biological, Grand Island, N.Y.). Washed cells were recentrifuged and suspended in 5 ml of cold 10% FCS-RPMI 1640, and layered over 3 ml of Ficoll-Hypaque (F-H, Bionetics Laboratory Products, Kensington, Md.) solution (12 parts of 14% Ficoll mixture with 5 parts of 32.8% Hypaque) and centrifuged at 300 g at room temperature for 25 min. Theuffy coat interface was then washed three times in cold 10% FCS-RPMI 1640 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 macroglobulinemia. Monoclonal proteins were isolated by preparative zone electrophoresis, using polystyrene co-polymer as supporting medium in barbital buffer, pH 8.6, t 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. A polyvalent antiserum specific for $\gamma$, $\mu$, $\lambda$, and $\kappa$ chains was prepared in a goat immunized with a mixture of purified IgM $\lambda$ and IgG $\kappa$ proteins. The antiserum obtained showed reactions of identity for a panel of purified IgG proteins, IgM proteins, and $\kappa$ or $\lambda$ Bence Jones proteins. Monospecific antisera to IgG were prepared in New Zealand albino rabbits by immunization with purified IgG, $\lambda$ myeloma proteins and absorption with purified $\lambda$ 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 antiserum by DEAE-
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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 (w/w) of up to 3% for 72 hr to obtain virtually complete digestion of IgG. Digests were 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 (PBS), pH 7.2, for separation of undigested IgG, F(ab')2, Fab', Fc', and peptides. The purified F(ab')2 was harvested from fractions of approximately 100,000 molecular weight with exclusion on 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 μg/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 G25 Sephadex. The F/P ratios for antisera were between 2 and 3. Specificity was reassessed and 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.15

Enumeration

Aggregate (Fc) receptor-bearing cell enumeration. The Fc receptor was identified as described by Dickler,16 employing the following modifications (H. B. Dickler, personal communication). Cohn fraction II (Pentex, Miles Laboratories, Kankakee, Ill.) was conjugated in 50-ml lots at 10 mg/ml using sufficient fluorescein isothiocyanate (FITC) to achieve an O.D. ratio 495/280 nm of at least 0.5. Then 5- or 10-ml aliquots were heat aggregated at 63°C for 20 min in a 10-ml polycarbonate tube and then pelleted at 145,000 g for 1 hr at 4°C. The supernatant was discarded, and the pellet homogenized in a 7-ml Dounce homogenizer. The pH was corrected to 8.3 rather than 8.1 with 0.1 N NaOH for maximum solubility of aggregated IgG. Just prior to use, aggregated material was centrifuged at 600 g at room temperature, not 4°C as originally described. The aggregated material was titered on normal lymphocytes, and could often be used initially at 1:64, and at progressively lesser titers for 1-3 wk. Staining was carried out for 1 hr 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 described17 using the purified F(ab')2 antisera as described by Winchester et al.18 Lymphocytes in a concentration of 1-2 x 10^6 in 0.05 cc 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 x 75-mm glass tubes for 1 hr at 4°C, washed three times with a total of 10 ml of cold FCS-RPMI, and mounted on glass slides. The cells were overlayed with a coverslip, sealed, and 1000 cells were counted with a Zeitz ultraviolet microscope equipped with a mercury arc HBO-200 lamp, using BG 38 and 490 nm (FITC) exciter filters and a K 510-nm barrier filter, by epillumination and simultaneous phase contrast microscopy to identify all nonstaining cells.

T-cell enumeration. Spontaneous cold sheep erythrocyte (E) rosettes were assayed as previously described17,19 by Ouchterlony analysis and confirmed by specific blocking of direct and indirect immunofluorescence only with purified proteins of the designated specificity as previously described.15

Complement receptor-bearing lymphocyte enumeration. Complement receptor-bearing (EAC-bearing) lymphocytes were determined by a modification of the method of Ross.19,20 To 2 ml of a 5%, SRBC (E) suspension, 2 ml of rabbit IgM anti-SRBC (A) (Cordis, Miami, Fla.) diluted 1:1000 in PBS was added, incubated for 15 min, at 37°C, centrifuged at 300 g at 4°C in PBS, and resuspended to 1% (in 10 ml of RPMI). Then 1 ml of fresh human AB serum (complement (C) source), taken from the same donor throughout the study, was absorbed with 0.5 ml of SRBC for 30 min at room temperature, diluted 1:10 in minimum essential medium (MEM)-10% decomple-
mented FCS, added to the EA suspension and incubated at 37°C for 30 min, centrifuged twice at 200 g at 37°C in 10% FCS-MEM, and adjusted to 0.5-1.0 × 10^8 cells/ml. Equal volumes of lymphocytes at 0.5 × 10^6-1 × 10^6 cells/ml and EAC were incubated for 15 min at 37°C in 10 × 75-mm round-bottom glass tubes, and centrifuged at 37°C for 15 min at 400 g. The pellet was resuspended in the remaining saline (about 0.05 ml), a drop aspirated in a Pasteur pipette, and 1000 cells counted. Lymphocytes with 3 or more RBC attached were considered to be positive. E and EA controls were carried out with each experiment. E controls were always less than 2%, and EA controls less than 5%.

Monocyte enumeration. Monocytes were identified by size (>10μ), alpha-naphthyl acetate staining, and latex phagocytosis. For the latex phagocytosis enumeration a modification of the method of Zucker-Franklin was employed.2 One drop of a suspension of 0.81-μ latex beads (Dow Chemical Co., Midland, Mich.) was added to 1 × 10^6 F-H-separated mononuclear cells in 1 ml RPMI-50% FCS, incubated 45 min at 37°C on a shaking table, and washed twice at 400 g at room temperature in 50% FCS-RPMI. The alpha-naphthyl acetate staining was done according to the method of Yam et al.22 Cell smears fixed in buffered formalin were air dried and incubated in a medium of phosphate buffer, hexazotized pararosanilin, and alpha-naphthyl acetate in ethylene glycol monomethyl ether, washed in distilled water, counterstained with 1% methyl green, washed with distilled water, dried, and mounted for examination under light microscopy.

Purification

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.23 In this procedure 300 mg nylon wool (Fenwal-Leukopak, Travenol Laboratories, Thetford, England) was soaked for 4 days at 37°C in normal saline and loosely packed into a 3-ml syringe and washed with 20 ml RPMI and 10 ml RPMI-10% FCS. The outflow was sealed, 10 ml RPMI-10% FCS added, and the syringe placed upright in an incubator at 37°C for 30 min. The fluid was then allowed to drain, 5 × 10^7 lymphocytes in 1 ml RPMI were added dropwise, and the fluid was in-
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Cubated for 30 min at 37°C. The cells were then eluted with 10 ml of 37°C RPMI, each drop of eluate added as each drop of lymphocyte solution was eluted. The eluted cells were then resuspended and again layered over the column, exactly as described above. The final elution of cells was resuspended to 1 x 10^6/ml RPMI. These cells were designated E-RFC (a).

The second purification was a modification of the method of Parish and Hayward.24 Aliquots of 5 x 10^6 E-rosetted lymphocytes/ml RPMI were layered over 3 ml of F-H (density 1.09 g/ml) at room temperature and centrifuged at 1200 g for 30 min. The pelleted cells were collected, the red cells lysed by addition of distilled water followed rapidly by flooding with RPMI, and the mononuclear cells resuspended over F-H. The interface cells were washed twice in RPMI 1640 and resuspended to 1 x 10^6/cc. These cells were designated E-RFC (a).

The third purification was also a modification of the method of Parish.24 Aliquots of 5 x 10^6 EAC-rosetted lymphocytes/ml RPMI were layered over 3 ml of F-H (density 1.09 g/ml) at room temperature and centrifuged at 1200 g for 30 min. The interface cells were washed once at room temperature in RPMI 1640 at 300 g, rosetted with SRBC exactly as described above, and recentrifuged over F-H. The interface cells were collected, layered over 3 ml of room-temperature F-H, and centrifuged at 1200 g for 30 min. The interface cells were then washed twice in RPMI 1640 and resuspended to 1 x 10^6/ml. These cells were designated E-RFC (b).

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.25 Aliquots of 5 x 10^6 E-rosetted lymphocytes/ml RPMI-10% FCS at room temperature were layered over 3 ml of F-H (density 1.09 g/ml) at room temperature and centrifuged at 1200 g for 30 min. The interface cells were washed once at room temperature in RPMI 1640 at 300 g, rosetted with SRBC exactly as described above, and recentrifuged over F-H. The interface cells were collected, layered over 3 ml of room-temperature F-H, and centrifuged at 1200 g for 30 min. The interface cells were then washed twice in RPMI 1640 and resuspended to 1 x 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.24 Aliquots of 5 x 10^6 EAC-rosetted lymphocytes/ml RPMI-10% FCS at room temperature were layered over 3 ml F-H (density 1.09 g/ml) at room temperature and centrifuged at 1200 g for 30 min. The pelleted cells were resuspended, and the red cells were lysed by addition of distilled water, followed rapidly by flooding with RPMI, and recentrifuged over F-H. The interface cells were washed twice in RPMI 1640 and resuspended to 1 x 10^6/ml with RPMI. These cells were designated B enriched (e).

PHA and PWM Dose Response

The optimal dose of PHA and PWM and the optimal number of lymphocytes had been previously determined by culturing for 2, 3, and 4 days with PHA concentrations of 0.05, 0.5, 1, 2, 5, and 50 μg and with PWM concentrations of 1, 2, 5, and 10 μg, lymphocytes with a final cell concentration of 1 x 10^5, 3 x 10^5, 5 x 10^5, 1 x 10^6, and 2 x 10^6 cells/ml. All cultures were done in triplicate and the optimal dose of PHA and PWM and the optimal concentration of lymphocytes were selected by determining the cell number and lectin concentration that resulted in the highest uptake of 3H-TdR on either day 2, 3, or 4. The highest uptake of 3H-TdR was obtained using 5 x 10^5 cells/ml and a concentration of 0.5 μg of PHA and 2 μg of PWM. In most situations, the maximal uptake of 3H-TdR occurred on day 3, though on occasion it occurred on day 4.

After determining the optimal dose of lectin and cell number, all cell cultures were done as described. RPMI 1640 with 10% heat-inactivated fresh-frozen pooled human AB positive serum and 50 μg/ml of gentamycin (Microbiological Associates, Bethesda, Md.) was the medium used in all cell cultures. Quadruplicate cultures of 0.5 x 10^6 lymphocytes or lymphocyte subpopulations in 1 ml medium were made in 10 x 75-mm sterile capped tissue culture tubes (Falcon Plastics, Oxnard, Calif.). PHA (Burroughs Wellcome, Research Triangle Park, N.C.) at 0.5 μg/culture was added in a volume of 0.5 ml medium. Culture were terminated on days 0 (after 2 hr), 1, 2, 3, 4, and 5 after a 5-hr pulse with 1 μCi 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.

Cell Staining and Cytofluorograph Instrumentation

Mononuclear cells were stained with ethidium bromide, a general nucleic acid stain, by the method of Göhde and Dittrich.26 For analysis of cellular DNA content, 1 x 10^5 cells were
treated with 10 ml ribonuclease (Worthington Biochemical Corp., Freehold, N.J.) for 30 min at 37°C to hydrolyze RNA prior to ethidium bromide staining.

To 1 x 10^6 lymphocytes/ml, 10 ml of a solution of 5 mg ethidium bromide (Calbiochem, San Diego, Calif.) in 100 ml 1.12% aqueous sodium citrate were added, incubated for 30 min at room temperature, and centrifuged at 400 g for 5 min. The supernatant was discarded and the cells were resuspended in RPMI and recentrifuged at 400 g for five min, and the supernatant was discarded. The cells were resuspended in distilled water for counting; 10,000 cells were counted.

All counting was done with a Model 4802A cytofluorograf (Bio-Physics Systems, Mahopac, N. Y.). This cytofluorograf has been described in detail elsewhere. Each cell in the cell suspension, as it passes through a 488-nm argon-ion laser beam, generates fluorescence and light scatter signals which are converted to an electronic pulse by a multiplier phototube. The signals are then amplified and analyzed for intensity. The intensity distribution is stored in a multichannel pulse-height analyzer. Thus, the analyzer memory accumulates a record of the DNA content/cell of 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 within 1.1%. Duplicate cultures had an error of less than 3.2% from the mean. Fluorescent microscopic 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.

RESULTS

Separation of Lymphocyte Subpopulations

Lymphocyte preparations obtained twice from peripheral blood of nine subjects were separated into populations enriched for T or B lymphocytes by F–H density sedimentation of SRBC-rosetted lymphocytes and by Fenwal-Leukopak filtration of F–H-separated peripheral blood lymphocytes, as shown in Fig. 1. A variety of separation techniques was used to help eliminate the possibility that a cell population purified by one technique differed from a cell population purified by another. The separation methods used yielded highly enriched subpopulations of lymphocytes, as determined by surface receptors, esterase activity, and latex phagocytosis. The recovery (total number of cells recovered after each separation procedure divided by the total number of cells added) was greater than 65.8% in the B-purified fractions and greater than 55.4% in the E-purified fractions. Viability was greater than 90% in all reported experiments; in several experiments viability after cell separation was less than 90% and the experiments were not continued.

The unseparated populations and separation of peripheral blood lymphocytes had a distribution of lymphocytes and monocytes as outlined in Table 1.

The B-enriched (d) and (e) populations had a significant increase in B lymphocytes as determined by a FITC-conjugated F(ab')2 anti–human antisera, and EAC-rosetting. The percentage would have been higher, had monocytes not been present. There was a marked depletion of E-rosetting cells. In contrast, the E-RFC-enriched fractions (a,b,c) had low percentages of Ig-bearing and EAC-rosetting lymphocytes and an increase in E-RFC. Aggregate or Fc receptor lymphocytes were determined in four subjects; an increased percentage in the B-enriched fractions was found.

The B-enriched (f) population after three E-rosetting steps had less than
Table 1. Surface Markers of Separated Mononuclear Cell Populations

<table>
<thead>
<tr>
<th>Lymphocyte Population*</th>
<th>F(ab')2 Anti-lg</th>
<th>EAC</th>
<th>Fct†</th>
<th>E</th>
<th>Latex Phagocytosis</th>
<th>Esterase Activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unseparated</td>
<td>10.8 ± 0.6</td>
<td>10.0 ± 0.8</td>
<td>12.0 ± 1.0</td>
<td>71.6 ± 1.5</td>
<td>21.7 ± 2.8</td>
<td>24.1 ± 1.6</td>
<td>86.7 ± 1.9</td>
</tr>
<tr>
<td>E-RFC (a)</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>5.3 ± 0.6</td>
<td>97.5 ± 0.5</td>
<td>2.0 ± 1.0</td>
<td>2.9 ± 0.4</td>
<td>55.4 ± 3.2</td>
</tr>
<tr>
<td>E-RFC (b)</td>
<td>5.1 ± 0.4</td>
<td>4.2 ± 0.3</td>
<td>6.5 ± 1.7</td>
<td>88.8 ± 0.7</td>
<td>6.5 ± 0.8</td>
<td>8.0 ± 0.8</td>
<td>58.1 ± 2.6</td>
</tr>
<tr>
<td>E-RFC (c)</td>
<td>3.8 ± 0.6</td>
<td>1.9 ± 0.4</td>
<td>4.5 ± 0.3</td>
<td>94.6 ± 0.7</td>
<td>5.7 ± 0.6</td>
<td>5.1 ± 0.6</td>
<td>84.8 ± 0.9</td>
</tr>
<tr>
<td>B-enriched (d)</td>
<td>83.7 ± 1.0</td>
<td>81.3 ± 1.7</td>
<td>82.3 ± 1.5</td>
<td>1.1 ± 0.2</td>
<td>17.4 ± 0.6</td>
<td>15.9 ± 1.4</td>
<td>82.4 ± 1.7</td>
</tr>
<tr>
<td>B-enriched (e)</td>
<td>90.6 ± 0.2</td>
<td>90.5 ± 1.6</td>
<td>84.3 ± 1.3</td>
<td>1.3 ± 0.2</td>
<td>8.4 ± 1.5</td>
<td>7.7 ± 0.9</td>
<td>66.8 ± 3.9</td>
</tr>
<tr>
<td>B-enriched (f)</td>
<td>91.3 ± 0.4</td>
<td>90.8 ± 0.5</td>
<td>NDI &lt;0.5</td>
<td>8.3 ± 0.5</td>
<td>7.1 ± 0.2</td>
<td>65.1 ± 2.6</td>
<td></td>
</tr>
</tbody>
</table>

Values are percentages ± SD.

* (a) Nylon passage (twice); (b) E-rosettes pelleted by density sedimentation, red cells lysed, lymphocytes resup 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 rero setted and pelleted by density sedimentation, and interface cells washed; (e) EAC pelleted by density sedimentation, red cells lysed, lymphocytes resup on F-H, and interface cells washed and resuspended; (f) same as (d) but with a third E-RFC rosetting.
† Done on four subjects only.
‡ Not done.
<table>
<thead>
<tr>
<th>Lymphocyte Population*</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unseparated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>89 ± 21</td>
<td>914 ± 469</td>
<td>24,941 ± 5,821</td>
<td>37,877 ± 6,506</td>
<td>36,421 ± 6,201</td>
<td>33,824 ± 6,100</td>
</tr>
<tr>
<td>Bckg</td>
<td>93 ± 20</td>
<td>141 ± 29</td>
<td>641 ± 98</td>
<td>691 ± 101</td>
<td>685 ± 121</td>
<td>695 ± 142</td>
</tr>
<tr>
<td>E-RFC (a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>86 ± 19</td>
<td>843 ± 358</td>
<td>22,212 ± 5,324</td>
<td>29,435 ± 5,829</td>
<td>32,521 ± 5,924</td>
<td>31,422 ± 5,621</td>
</tr>
<tr>
<td>Bckg</td>
<td>89 ± 23</td>
<td>158 ± 32</td>
<td>652 ± 96</td>
<td>681 ± 99</td>
<td>741 ± 88</td>
<td>781 ± 108</td>
</tr>
<tr>
<td>E-RFC (b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>91 ± 21</td>
<td>894 ± 365</td>
<td>28,419 ± 6,214</td>
<td>32,541 ± 6,491</td>
<td>30,621 ± 6,350</td>
<td>25,241 ± 6,205</td>
</tr>
<tr>
<td>Bckg</td>
<td>104 ± 35</td>
<td>124 ± 39</td>
<td>624 ± 86</td>
<td>653 ± 91</td>
<td>621 ± 92</td>
<td>621 ± 98</td>
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<tr>
<td>E-RFC (c)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Stim</td>
<td>98 ± 29</td>
<td>902 ± 321</td>
<td>26,248 ± 7,413</td>
<td>31,452 ± 6,205</td>
<td>30,851 ± 6,090</td>
<td>24,391 ± 5,921</td>
</tr>
<tr>
<td>Bckg</td>
<td>91 ± 32</td>
<td>151 ± 33</td>
<td>532 ± 109</td>
<td>542 ± 112</td>
<td>551 ± 108</td>
<td>681 ± 95</td>
</tr>
<tr>
<td>B-enriched (d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>124 ± 35</td>
<td>175 ± 53</td>
<td>2,431 ± 295</td>
<td>3,185 ± 384</td>
<td>4,092 ± 328</td>
<td>2,519 ± 230</td>
</tr>
<tr>
<td>Bckg</td>
<td>116 ± 29</td>
<td>168 ± 42</td>
<td>701 ± 43</td>
<td>741 ± 132</td>
<td>724 ± 116</td>
<td>751 ± 124</td>
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<tr>
<td>B-enriched (e)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>126 ± 41</td>
<td>189 ± 106</td>
<td>2,149 ± 325</td>
<td>3,839 ± 341</td>
<td>4,924 ± 321</td>
<td>2,259 ± 316</td>
</tr>
<tr>
<td>Bckg</td>
<td>121 ± 39</td>
<td>173 ± 39</td>
<td>641 ± 151</td>
<td>674 ± 129</td>
<td>594 ± 142</td>
<td>741 ± 143</td>
</tr>
</tbody>
</table>

Values are cpm ± SE.
*See footnote to Table 1. Stim, stimulated; Bckg, background.
LYMPHOCYTE TRANSFORMATION AND PHA

Table 3. PHA and PWM Response of Lymphocyte Population B-enriched (f) and E-RFC (c)

<table>
<thead>
<tr>
<th>Lymphocyte Population</th>
<th>PHA 3H-TdR (cpm) t</th>
<th>3H-TdR (cpm) t</th>
<th>S-G2-M (%)t</th>
<th>PWM S-G2-M (%)t</th>
<th>E-RFC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-enriched (f)</td>
<td>2,845</td>
<td>1,060</td>
<td>6.3</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>E-RFC (c)</td>
<td>22,584–39,421</td>
<td>11,525–27,241</td>
<td>20–27</td>
<td>95–99</td>
<td></td>
</tr>
</tbody>
</table>

*(f) E pelleted by density sedimentation, interface cells resuspended and pelleted by density sedimentation, and interface cells washed and collected (cultured for 3, 4, and 5 days); (c) E pelleted by density sedimentation, interface resuspended and pelleted by density sedimentation, and interface cells washed (cultured for 0, 3, 4, and 5 days).

†Stimulated sample-background sample.

0.5% E-RFC and greater than 90% SIg-bearing cells and EAC-RFC. At least 7% of the cells were monocytes.

Mitogen Response, ³H-TdR

The PHA response as measured by ³H-TdR incorporation of the unseparated peripheral blood lymphocytes (PBL), the three E-RFC enriched fractions, and the three B-enriched fractions is outlined in Table 2. The unfractionated PBL and the three E-RFC-enriched fractions had minimal incorporation of ³H-TdR on day 1, but underwent high levels of ³H-TdR incorporation on day 2, maximizing on days 3 and 4, and decreasing on day 5 after PHA stimulation. The incorporation in the E-RFC fractions was less than in the unfractionated PBL fraction on all days of the culture. In contrast, the three B-enriched fractions had much less incorporation of ³H-TdR on days 2–5. The very highly purified B-enriched fraction with less than 0.5% E-RFC still had a slight but definite incorporation of ³H-TdR as outlined in Table 3. After PWM stimulation, there was almost no incorporation of ³H-TdR in the B-enriched (f) population, but brisk incorporation in the E-RFC (c) population.

There was close similarity between each of the E-RFC-enriched populations and each of the B-enriched populations, despite the variety of methods used for cell purification, making a loss or enhancement of a particular subpopulation of lymphocytes less likely.

PHA Response. Cytofluorography

The PHA response as measured by the percentage of interploid and tetraploid DNA cells is outlined in Table 4. In general, the DNA synthesis response, as measured cytofluorographically, paralleled the incorporation of ³H-TdR. In the unseparated PBL and the three E-RFC-enriched populations, there was a marked increase in S–G2 and M cells on day 2, peaking on days 3 and 4, and decreasing on day 5; though small, there was a definite increase in cells in S–G2 and M as early as day 1. The percentage of cells in S–G2 and M was less in the E-RFC-enriched population as compared to the unseparated PBL population, again paralleling ³H-TdR incorporation. E-RFC (c) had a mean of 24% of cells in S–G2–M after PWM stimulation.

The B-enriched populations had a much smaller percentage of cells in S–G2 and M on days 2, 3, 4, and 5, and no detectable increase on day 1. However, even B-population (f), with less than 0.5% E-RFC at the start of culture, had
<table>
<thead>
<tr>
<th>Lymphocyte Population*</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
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<tbody>
<tr>
<td>Unseparated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>92.0 ± 8.3</td>
<td>8.0</td>
<td>91.5 ± 8.5</td>
<td>8.5</td>
<td>71.5 ± 7.9</td>
<td>28.5</td>
</tr>
<tr>
<td>Bkg</td>
<td>92.1 ± 8.2</td>
<td>7.9</td>
<td>93.4 ± 8.4</td>
<td>6.6</td>
<td>92.6 ± 8.3</td>
<td>7.4</td>
</tr>
<tr>
<td>E-RFC (a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>93.8 ± 9.1</td>
<td>6.2</td>
<td>90.7 ± 8.3</td>
<td>9.3</td>
<td>74.4 ± 8.3</td>
<td>25.6</td>
</tr>
<tr>
<td>Bkg</td>
<td>93.3 ± 8.7</td>
<td>6.7</td>
<td>93.6 ± 7.9</td>
<td>6.9</td>
<td>92.2 ± 8.8</td>
<td>7.8</td>
</tr>
<tr>
<td>E-RFC (b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>94.1 ± 7.8</td>
<td>5.9</td>
<td>91.2 ± 8.3</td>
<td>8.8</td>
<td>79.3 ± 8.1</td>
<td>20.7</td>
</tr>
<tr>
<td>Bkg</td>
<td>94.2 ± 8.6</td>
<td>5.8</td>
<td>94.1 ± 8.2</td>
<td>5.9</td>
<td>92.2 ± 8.8</td>
<td>7.4</td>
</tr>
<tr>
<td>E-RFC (c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>93.3 ± 9.1</td>
<td>6.7</td>
<td>92.1 ± 9.3</td>
<td>7.9</td>
<td>76.6 ± 7.9</td>
<td>23.4</td>
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<tr>
<td>Bkg</td>
<td>93.2 ± 8.6</td>
<td>6.6</td>
<td>93.6 ± 8.7</td>
<td>6.4</td>
<td>91.5 ± 8.1</td>
<td>8.5</td>
</tr>
<tr>
<td>Slg (d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>92.2 ± 9.5</td>
<td>7.8</td>
<td>92.6 ± 8.1</td>
<td>7.4</td>
<td>84.3 ± 7.5</td>
<td>15.7</td>
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<tr>
<td>Bkg</td>
<td>92.1 ± 7.3</td>
<td>7.9</td>
<td>92.9 ± 7.9</td>
<td>7.1</td>
<td>92.6 ± 8.1</td>
<td>7.9</td>
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<tr>
<td>EAC (e)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stim</td>
<td>91.9 ± 9.4</td>
<td>8.1</td>
<td>92.1 ± 8.0</td>
<td>7.9</td>
<td>95.1 ± 6.8</td>
<td>14.9</td>
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<tr>
<td>Bkg</td>
<td>92.0 ± 9.1</td>
<td>8.0</td>
<td>92.2 ± 8.1</td>
<td>6.8</td>
<td>92.1 ± 7.3</td>
<td>7.9</td>
</tr>
</tbody>
</table>

*See footnote to Table 1. Stim, stimulated; Bkg, background.
LYMPHOCYTE TRANSFORMATION AND PHA

at least 4% of its cells in interploid-tetraploid on day 5, as outlined in Table 3. Of interest was the maximal uptake of \(^{3}\)H-TdR on day 5, as compared to days 3 and 4 in the less highly purified B-cell preparations. To eliminate the possibility that any contaminating E-RFC present at the commencement of culture were accounting for the increased DNA content, E-RFC were searched for in the stimulated cell populations. No E-RFC were found. In contrast, greater than 95% of the cells in an E-RFC-purified sample were able to be rerosetted after PHA stimulation. Consequently, the ability to form E-RFC seemed to be maintained by a PHA-stimulated lymphocyte. This finding suggested that a small population of B-cells was capable of being stimulated by PHA. As was the case with the uptake of \(^{3}\)H-TdR, there was a close similarity between the E-RFC-enriched and B-enriched populations, again making loss or enrichment of a particular subpopulation of lymphocytes unlikely. The minimal increase in S-G2 and M cells in the PWM-stimulated B-enriched (f) populations paralleled the low incorporation of \(^{3}\)H-TdR.

DISCUSSION

The methodology needed to separate human T- and B-lymphocyte populations has been an area of increasing interest over the past several years, and a variety of techniques are now available. These include rosette sedimentation, immunoabsorbent columns, nylon wool columns, and a fluorescence-activated cell sorter. We have utilized a combination of rosette sedimentation and nylon wool columns to enrich cell populations in an attempt to avoid artifactually enriching or depleting a B- or T-lymphocyte subpopulation. Our criteria for calling cells “T lymphocytes” are the presence of E-receptors, for “B lymphocytes” the presence of EAC receptors and SIg, and for “monocytes” the presence of esterase activity and the ability to phagocytose latex particles. Aggregate or Fc receptors may be found on both B and T lymphocytes and other mononuclear cells, primarily monocytes, and EAC receptors may be found on monocytes, B lymphocytes, and K cells. Separation methods utilizing rosette formation are simple to accomplish, require readily available equipment, and provide high yields of cells with enrichment of the desired populations. The nylon wool column separation technique also has the virtue of simplicity, but the cell yield is somewhat less. Because of cell loss and the multiple steps utilized to achieve an enriched cell population, the relationship of one surface marker to another in our experiments cannot be easily compared. However, in B-enriched population (f), formed by sedimenting (presumably leaving the non-E-RFC relatively unchanged), there are minimal numbers of T-cells and similar percentages of SIg and EAC cells. Since only 8% of the mononuclear cells present are monocytes (as determined by latex phagocytosis and esterase activity), this purified population indicates that the majority of SIg-bearing cells are also EAC-RFC, and vice versa, as recently proposed by Ehlenberger et al. The use of F(ab')\(_2\) fragment antisera eliminates the problem of passive adsorption of Ig via the Fc receptor. This problem may explain prior reports on the lower frequency of EAC-RFC than SIg-bearing cells in B-cell populations.

Most studies on the nonhuman lymphocyte response to PHA suggest that
only T cells respond to PHA by increasing DNA synthesis. A number of possibilities can explain the discrepant results. Different separation procedures could result in different subpopulations of enriched T or B cells. This postulate seems an unlikely explanation, since different groups using identical separation techniques have had discrepant results. Purification of the responding cell population could affect results. A small percentage of T cells can result in significant incorporation of 3H-TdR. This contamination has been thought to be the explanation for increased uptake of 3H-TdR by B-enriched populations in a number of studies. In highly purified B-enriched (f), small but significant incorporation of 3H-TdR is noted in the presence of less than 0.5% T cells. It seems unlikely that it can be ascribed to T-cell stimulation since there are no E-RFC detectable at the onset and after 5 days of culture. Loss of ability to rosette during the course of cell culture also seems unlikely, since in three experiments in the E-RFC (c) population, the percentage of E-RFC ranged from 95% to 99% after 5 days. Cytofluorographic analysis has indicated that 4%-8% of the cells are in the inter- or tetraploid stage of the cell cycle, strongly suggesting that a small percentage of B lymphocytes synthesized DNA in the presence of PHA, supporting data previously obtained by other groups. Whether or not very small percentages of residual T cells are necessary for this response is impossible to say. Recruitment of B lymphocytes by T lymphocytes has, however, been invoked as an explanation for the finding, observed in this and other studies, of a decreased incorporation of 3H-TdR in an enriched T-cell population in comparison to an unseparated PBL population.

It is also unclear what cell type responds to PWM. Animal studies emphasize the thymus dependency of the response. In some human immunodeficiency diseases where circulating B lymphocytes are depleted, such as sex-linked agammaglobulinemia, normal PWM responses are found. However, the maximal response of T cells to PWM may require the presence of non-T cells. Our study strongly suggests that T-cell-depleted B lymphocytes are unable to initiate DNA synthesis or incorporate 3H-TdR after PWM stimulation. The presence and percentage of monocytes may also be important for maximal response to mitogen, although it is unclear if an absolute requirement for monocytes exists. A mean of at least 2.0% monocytes is present in all of our E-RFC-enriched populations, and a mean of at least 8% monocytes is present in all of our B-enriched populations. A close similarity in the incorporation of 3H-TdR and percentage of S-G2-M cells is found in the different cell populations, regardless of the minor differences in the percentage of monocytes present.

Although discrepancies between incorporation of labeled thymidine into DNA and the rate of DNA synthesis have been observed in several cell culture systems, we found a notable parallel between the percentage of cells in S-G2 and M and the uptake of 3H-TdR in normal donors. The measurement of uptake of 3H-TdR in PHA-stimulated cultures has been recommended as a test of cellular immunity in a variety of human diseases. It appears that flow cytometry may also be useful. Advantages of the latter technique include the ability to count large numbers of cells rapidly, with information about the
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relative DNA content per cell. The added information of percentages and absolute numbers of responding cells is useful. Experience must be acquired in clinical diseases before the applicability of cytofluorometric analysis of DNA content as a gauge of cellular immunity in man can be fully ascertained.

ACKNOWLEDGMENT

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Cytofluorometric analysis of the kinetics of lymphocyte transformation after phytohemagglutinin stimulation: comparison with the kinetics of thymidine incorporation

PD Utsinger, WJ Yount, JG Fallon, MJ Logue, CR Fuller and D Elliott