Methods for Obtaining Purified Lymphocytes, Glass-adherent Mononuclear Cells, and a Population Containing Both Cell Types From Human Peripheral Blood

By William R. Levis and Jay H. Robbins

Methods are presented for obtaining simultaneously or separately two populations of cells from human peripheral blood, lymphocytes and monocytes, both of which are required to obtain blastogenesis and DNA synthesis in human leukocyte cultures. A simple 5-min centrifugation of heparinized blood yields a mononuclear leukocyte culture fluid containing 70–90% lymphocytes with few granulocytes but with sufficient numbers of monocytes so that the latter cell is not a limiting factor in the blastogenic reaction. A method is also presented for removing both granulocytes and monocytes from lymphocyte populations in a manner that permits monitoring and choice of the degree of lymphocyte purification. A method is also presented for obtaining glass-adherent mononuclear cells that do not undergo blastogenesis but will enable suitably stimulated "purified" lymphocytes to undergo blastogenesis. Studies of the function and morphology of these different cell populations are presented.

THE HUMAN SMALL LYMPHOCYTE is the cell that undergoes blastogenesis and DNA synthesis in peripheral blood leukocyte cultures in response to various blastogenic factors.1–3 It has, however, recently been shown that a glass-adherent (g-a) population of mononuclear cells, derived primarily if not entirely from the blood monocyte, is necessary for the human peripheral blood lymphocyte to undergo blastogenesis in response to specific antigens4–7 and to allogeneic cells.4–5–8–12 G-a cells are also required to obtain an early maximum blastogenic response to pokeweed mitogen13 and to all concentrations of phytohemagglutinin (PHA).14 To study the function of the g-a cell and lymphocyte populations in the response of human peripheral blood lymphocytes to blastogenic factors, it is useful to have techniques that will adequately separate these cell types and make them available in separate populations. We have utilized a method in which lymphocytes are separated from other leukocytes in a manner that permits constant monitoring of the degree of purification being attained, thereby providing a means for determining when the desired purity has been achieved.

The realization that g-a cells are required for the lymphocytes’ response to blastogenic factors in leukocyte cultures and that granulocytes, the most abundant leukocyte in human peripheral blood, perform no known useful function and may even be injurious in such cultures15 prompted us to employ
a simple and rapid method for obtaining plasma with sufficient quantities of both lymphocytes and monocytes and as few granulocytes as possible for use in routine, commonly employed leukocyte cultures. This method, which is a simplified adaptation of that of Jago, requires only a 5-min centrifugation of heparinized whole blood and yields a mononuclear leukocyte population in which the ratio of monocytes to lymphocytes is sufficiently above that currently known to be a limiting factor in the lymphocytes' response to blastogenic factors.

In this paper, we present in detail our methods for obtaining "purified" lymphocytes, g-a cells, and a "mononuclear leukocyte culture fluid" containing adequate numbers of both cell types.

**MATERIALS AND METHODS**

**Obtaining Mononuclear Leukocytes for Culture and as a Source of G-A Cells**

Peripheral venous blood was drawn from normal donors whose last meal was between 2 and 4 hr prior to venipuncture. Ten-milliliter quantities of this blood were placed in 10-ml screw-capped conical centrifuge tubes (Cat. No. 2993-H25, A. H. Thomas) each of which contained 100 U of preservative-free heparin (10 μl of Panheparin, Abbott Lab.). The tubes were then gently inverted to mix the contents and were placed, within 10 min, in Type 3 centrifuge carriages (International Equipment Co.) held on the No. 269 rotor of the Model PRB centrifuge (International Equipment Co.). Centrifugation at room temperature was begun by switching the speed control rapidly up nine notches from its "off" position. As soon as the rotor attained a speed of 1500 rpm, the speed control was reduced four notches to provide a setting at which the speed of rotation remained at 1500 rpm. Under these conditions, a constant centrifugal force of approximately 300 g existed at the 7.5 ml level of each tube, which is the usual level of the resulting plasma-erythrocyte interface. After 5 min, the speed control was returned to its off position, and the rotor permitted to slow down and stop of its own accord. After 1–2 min, the tip of a Pasteur pipette was inserted into the resulting supernatant plasma to a level approximately 1.0–1.5 mm above the buffy coat which is situated upon the packed erythrocytes. By sucking up the top layer of the buffy coat into the pipette at this level with approximately 1.5–2 ml of the plasma, a population of leukocytes was obtained that usually contained 2–10 × 10^6 cells/ml and was comprised of at least 90% mononuclear leukocytes. A "mononuclear leukocyte culture fluid" at a final concentration of 0.4–2 × 10^6 cells/ml was obtained by diluting this mononuclear-rich plasma with 4 volumes of tissue culture medium 199 (Code 5477; Difco Laboratories) that contains 87.5 U/ml of penicillin and 87.5 μg/ml of streptomycin (Code 51082; BBL, Division of BioQuest). A mononuclear leukocyte culture fluid with a higher density of cells can be obtained by recentrifuging the mononuclear-rich plasma for 6 min at 900 rpm (150 g) and then dispersing the resultant cell pellet in an appropriate volume of a mixture comprised of one part of cell-free plasma and four parts of the medium 199.

The mononuclear leukocyte culture fluid was cultured at 38°C with room air as the gas phase in 0.5 ml aliquots in 12 × 35 mm screw-capped vials (No. 9802-G, A. H. Thomas Co.) or in 0.4–2.0-ml aliquots in stoppered Leighton culture tubes (Cat. No. 1928, Belco Glass, Inc.) containing 9 × 9 mm glass cover slips. Stimulants used were phytohemagglutinin (PHA, 0.5–2.5 μg/ml of culture fluid; batches E118 and E316A, Burroughs Welcome and Co.) and tuberculin-purified protein derivative (PPD, lyophilized, without preservative, 1.25 μg/ml of culture fluid, Parke-Davis). Mixed leukocyte cultures were obtained by mixing equal parts of mononuclear leukocyte culture fluid from each of two cell donors.

G-a cells were obtained attached to the glass cover slips after incubating the latter in Leighton tubes with unstimulated mononuclear leukocyte culture fluid obtained directly
from the 5-min centrifugation or after concentration of the cells by recentrifugation.

The cover slips were removed from the Leighton tubes after incubation periods of 2 hr-6 days. Nonadherent cells were removed from the g-a cells by dipping the cover slips into three batches of 38°C medium 199 and then subjecting each of the two surfaces of the cover slips to a forceful flow of 2 ml of 38°C medium 199 dispensed from a Pasteur pipette. For morphologic studies of the g-a cells, the cover slips were air dried, and their g-a cells stained with Wright's stain. For studies on the function of the g-a cells, the cover slips were recultured in cell-free culture fluid or added to Leighton tubes containing purified lymphocytes obtained as described below. Phagocytic activity was evaluated by incubating the g-a cells with polystyrene latex particles (1.3 μm in diameter; Dow Chemical). Twenty-five microliters of a 1:400 dilution of the original stock solution of these particles was placed in each milliliter of culture fluid.

Obtaining Purified Lymphocytes

In the procedure for obtaining purified lymphocytes, seven 15-ml aliquots of peripheral venous blood were placed in round-bottomed, 16 × 125 mm screw-capped glass tubes (Cat. No. 9210-E44, A. H. Thomas Co.), each of which contained 150 U of the Panheprin. The purified lymphocytes were subsequently obtained from the leukocyte-rich plasma resulting after 1-1 1/2 hr of gravity sedimentation of this heparinized blood at 38°C. This leukocyte-rich plasma was diluted with an equal volume of medium 199 and placed in a separatory funnel held above a glass column 1 inch in diameter containing a tapered opening at the lower end. The glass column was tightly packed to a height of 14 cm with 12 gm of dry, sterile, nylon fiber17 (Leukopak, Fenwal Laboratories) that had previously been washed in several batches of boiling, distilled water to remove soluble contaminants. Up to 250 ml of the diluted leukocyte-rich plasma was then slowly dripped from the separatory funnel into the nylon fiber column followed by an equal volume of medium 199. The flow rate from the separatory funnel was adjusted so that all the filtering was completed within 1 1/2-2 hr. The column effluent, which contained small lymphocytes, erythrocytes, and platelets, was collected in an upright, 16 oz, glass prescription bottle. The bottle was then tightly capped and laid horizontally at 38°C for 2 hr on one of its two flat sides to permit any contaminating g-a cells to adhere to the bottle while the nonadherent lymphocytes remained in the supernate. Wright-stained smears of the pellets from spun aliquots of the lymphocyte-rich supernate served to monitor the degree of remaining contamination of the lymphocyte population with monocytes and/or granulocytes. If any leukocytes other than lymphocytes were observed on these smears, the still contaminated lymphocyte-rich supernate was gently decanted into clean, 16-oz bottles, and the incubation process was repeated until the supernate was free of morphologically indentifiable nonlymphoid leukocytes. Only occasionally was it necessary to utilize more than three such bottles.

When more than 99% of the resulting supernate's leukocyte population had the morphology of lymphocytes, the supernate was centrifuged at 150 × g for 6 min. The cell pellets were dispersed to a final concentration of 0.5-3 × 10^9 lymphocytes/ml of a fluid comprised of one part autologous cell-free plasma and four parts medium 199 containing the antibiotics. Four-tenths milliliter aliquots of these lymphocyte suspensions, cultured in stoppered Leighton tubes with air as the gas phase, are hereafter referred to as "purified" lymphocyte cultures. When glass cover slips containing g-a cells are placed in the Leighton tubes with these purified lymphocytes, the cultures are referred to as "reconstituted" cultures.

Evaluation of Radioactive Uridine and Thymidine Incorporation

DNA synthesis was measured by scintillation spectroscopy utilizing the Millipore filter assay method18 after the cells were incubated for 3 hr at 38°C with thymidine-methyl-3H (HTdR, specific activity 17-22 Ci/mM, Amersham-Searle) at a concentration of 10 μCi/ml of culture fluid.

Radioautographic studies of 3H-uridine (uridine-5-T; specific activity; 16.3 Ci/mM;
1.25 μCi/ml of culture fluid, Nuclear Chicago Corp.) and 3HTdR incorporation into g-a cells and into nonadherent leukocytes were performed after 2-24 hr of incubation with the radioactive compounds. The incubated cells were then washed in three batches of medium 199 after which the g-a cells, still on their cover slips, and the nonadherent leukocytes, pelletized and then smeared on cover slips, were air dried and then fixed in methanol for 10 min. After a rinse in distilled water, the cover slips were air dried, mounted cell-side up on regular 1 X 4 inch glass slides and coated with a thin layer of 43°C, 0.5% gelatin solution to promote adherence of the NTB-3 radioautographic emulsion (Eastman Kodak) that was then applied. After exposure at 4°C for periods up to 3 wk, the radioautographs were developed and placed in running tap water for 30 min, rinsed in a 1:1 methanol-Giordano phosphate buffer solution (pH 6.4; Fisher Scientific Co.), then in the Giordano buffer alone, and then in absolute methyl alcohol. The slides were then stained with Wright's stain.

RESULTS

Mononuclear Leukocyte Culture Fluid

Morphology: Figure 1 shows a representative field containing the mononuclear leukocytes obtained by the 5-min centrifugation technique. Differential analyses of leukocytes obtained in 20 consecutively performed experiments

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>% Lymphocytes</th>
<th>% Monocytes</th>
<th>% Granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>91.5 (±1.5)</td>
<td>8.0 (±1.0)</td>
<td>0.5 (±0.5)</td>
</tr>
<tr>
<td>2</td>
<td>91.9 (±1.3)</td>
<td>4.9 (±0.4)</td>
<td>3.1 (±0.9)</td>
</tr>
<tr>
<td>3</td>
<td>79.9 (±0.9)</td>
<td>14.0 (±0.2)</td>
<td>6.2 (±1.0)</td>
</tr>
<tr>
<td>4</td>
<td>86.3 (±6.0)</td>
<td>12.7 (±5.3)</td>
<td>1.2 (±0.6)</td>
</tr>
<tr>
<td>5</td>
<td>71.5 (±4.3)</td>
<td>23.2 (±2.2)</td>
<td>9.2 (±0.1)</td>
</tr>
<tr>
<td>6</td>
<td>80.7 (±2.6)</td>
<td>16.4 (±3.3)</td>
<td>2.9 (±0.7)</td>
</tr>
<tr>
<td>7</td>
<td>77.7 (±0.7)</td>
<td>12.4 (±0.2)</td>
<td>10.0 (±0.5)</td>
</tr>
<tr>
<td>8</td>
<td>83.9 (±0.3)</td>
<td>13.0 (±0.0)</td>
<td>3.1 (±0.3)</td>
</tr>
<tr>
<td>9</td>
<td>82.5 (±2.4)</td>
<td>12.2 (±1.8)</td>
<td>5.4 (±0.5)</td>
</tr>
<tr>
<td>10</td>
<td>85.0 (±1.3)</td>
<td>7.6 (±1.8)</td>
<td>7.6 (±0.2)</td>
</tr>
<tr>
<td>11</td>
<td>84.8 (±1.2)</td>
<td>10.3 (±1.1)</td>
<td>5.0 (±0.2)</td>
</tr>
<tr>
<td>12</td>
<td>81.1 (±1.9)</td>
<td>12.3 (±1.9)</td>
<td>6.7 (±0.1)</td>
</tr>
<tr>
<td>13</td>
<td>88.8 (±0.7)</td>
<td>9.6 (±0.0)</td>
<td>1.7 (±0.7)</td>
</tr>
<tr>
<td>14</td>
<td>82.9 (±2.7)</td>
<td>12.1 (±2.5)</td>
<td>4.9 (±0.3)</td>
</tr>
<tr>
<td>15</td>
<td>90.1 (±0.2)</td>
<td>7.4 (±0.1)</td>
<td>2.5 (±0.1)</td>
</tr>
<tr>
<td>16</td>
<td>90.4 (±1.0)</td>
<td>7.7 (±0.9)</td>
<td>2.0 (±0.1)</td>
</tr>
<tr>
<td>17</td>
<td>81.6 (±2.4)</td>
<td>14.2 (±2.6)</td>
<td>4.2 (±0.2)</td>
</tr>
<tr>
<td>18</td>
<td>87.1 (±2.9)</td>
<td>15.2 (±2.2)</td>
<td>2.6 (±0.0)</td>
</tr>
<tr>
<td>19</td>
<td>78.2 (±0.1)</td>
<td>17.7 (±0.8)</td>
<td>4.1 (±0.9)</td>
</tr>
<tr>
<td>20</td>
<td>85.0 (±1.2)</td>
<td>12.6 (±0.6)</td>
<td>2.5 (±0.5)</td>
</tr>
</tbody>
</table>

Mean ± SD of differential cell count† 84.0±5.4 12.2±8.5 3.9±4.9

* Four cover-slip smears were prepared from each donor's mononuclear leukocyte-rich plasma obtained by the 5-min centrifugation method. Total of 500 leukocytes were analyzed (125 from each of four cover slips), and percentages of these cells that were lymphocytes, monocytes, and granulocytes were calculated. At a later time, differential cell counts were repeated on another 500 cells from same four cover slips without the analyzer's having knowledge of previous results. Results for each of 20 experiments are expressed as mean percentage (±SE) of results of two 500-cell analyses.

† Average of mean percentages of differential cell counts of 20 experiments; this SD represents distribution of the 20 means about their average.
Fig. 1. Mononuclear leukocytes obtained by 5-min centrifugation method. Of 97 leukocytes in field, three are polymorphonuclear leukocytes (P), 19 are monocytes (M), and 75 are lymphocytes (L). Platelets (Plt) and erythrocytes (E) are also present. × 160.

are presented in Table 1. Lymphocytes comprised, on the average, 84% (range 71.5–91.9%), monocytes 12.2% (range 4.9–23.2%), and granulocytes 3.9% (range 0.5–10.1%).

Table 2. DNA Synthesis in PHA-containing Mononuclear Leukocyte Culture Fluid

<table>
<thead>
<tr>
<th>Donor No.</th>
<th>3HThdR Incorporation in Cultures* (cpm)</th>
<th>No Blastogenic Factor (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHA</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>87.901 ± 9,469</td>
<td>1057 ± 178</td>
</tr>
<tr>
<td>2</td>
<td>208.487 ± 29,843</td>
<td>547 ± 264</td>
</tr>
<tr>
<td>3</td>
<td>173.003 ± 613</td>
<td>808 ± 98</td>
</tr>
<tr>
<td>4</td>
<td>99.901 ± 731</td>
<td>399 ± 45</td>
</tr>
<tr>
<td>5</td>
<td>65.989 ± 4,863</td>
<td>2447 ± 170</td>
</tr>
<tr>
<td>6</td>
<td>204.939 ± 22,687</td>
<td>206 ± 54</td>
</tr>
<tr>
<td>7</td>
<td>51.765 ± 3,849</td>
<td>508 ± 98</td>
</tr>
<tr>
<td>8</td>
<td>314.791 ± 48,538</td>
<td>178 ± 38</td>
</tr>
<tr>
<td>9</td>
<td>66.543 ± 3,629</td>
<td>304</td>
</tr>
<tr>
<td>10</td>
<td>143.544 ± 1,250</td>
<td>702</td>
</tr>
</tbody>
</table>

* Each culture contained 3–6 × 10⁵ leukocytes and was assayed with 3-hr 3HThdR pulse on third or fourth day of culture. Values are expressed as mean (±SE) of cpm obtained from duplicate or triplicate cultures. In each of experiments 9 and 10, only one unstimulated control culture was assayed.
Blastogenesis: When appropriately stimulated, lymphocytes in the mononuclear leukocyte culture fluid developed into blastoid cells capable of DNA synthesis and mitotic division. Table 2 shows the incorporation of $^3$HTdR into DNA in PHA-containing mononuclear leukocyte culture fluids obtained from ten consecutive donors. These mononuclear leukocyte culture fluids responded well also to blastogenic factors other than PHA. For example, when a 0.5 ml. mixture of culture fluids from donors 9 and 10 was incubated in the absence of PHA, a mixed leukocyte reaction occurred that gave over 4000 cpm on the third day; in 0.5 ml aliquots of the culture fluid from donor 8 cultured with tetanus toxoid, 4300 cpm were obtained at the 96th hr of culture.

G-A Cells

Morphology: By the 36th hr of culture (Fig. 2A), all the g-a cells derived from the centrifugation technique were mononuclear, and no intact polymorphonuclear leukocytes were seen. Some of the g-a cells already had the peripheral, wedge-shaped nucleus and foamy cytoplasm characteristic of macrophages. By the sixth day of culture (Fig. 2B) most of the g-a cells had more abundant foamy cytoplasm.

RNA Synthesis: When $^3$H-uridine was added to the mononuclear leukocyte culture fluid for the first 2 hr of culture, radioautograms revealed labeling
Fig. 3. Radioautograms of incorporation of $^3$H-uridine into g-a cells. (A). Predominantly nuclear labeling found in g-a cells fixed after 2-hr exposure to $^3$H-uridine at start of culture; (B). Nuclear and cytoplasmic labeling in 5-day-old g-a cells fixed at end of 17-hr exposure to $^3$H-uridine. In latter experiment, polystyrene latex particles, appearing as tiny clear spheres in one of the cells, were added simultaneously with $^3$H-uridine. As shown by the other cell, even those g-a cells that did not phagocytose the polystyrene latex particles incorporated $^3$H-uridine. × 1000.

primarily over the nucleus in most of the cells (Fig. 3A), as has been reported to occur in human macrophages, obtained by a different technique, when fixed immediately after such a short pulse. When $^3$H-uridine was added for the last 17 hr of a 120-hr culture period, grains were found over both the nucleus and cytoplasm in phagocytizing and nonphagocytizing g-a cells (Fig. 3B).

DNA Synthesis: Cover slips containing g-a cells from 2- to 6-day-old cultures of mononuclear leukocyte culture fluid were dipped and then sprayed

Table 3. Ability of G-A Cells to Stimulate Allogeneic Purified Lymphocytes

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>1</td>
<td>207 (± 9)</td>
<td>118 (± 39)</td>
<td>2,820 (± 367)</td>
</tr>
<tr>
<td>2</td>
<td>123 (±13)</td>
<td>248 (±152)</td>
<td>18,828 (±1,062)</td>
</tr>
<tr>
<td>3</td>
<td>202</td>
<td>140</td>
<td>1,044</td>
</tr>
<tr>
<td>4</td>
<td>194</td>
<td>148</td>
<td>1,684</td>
</tr>
<tr>
<td>5</td>
<td>235 (±33)</td>
<td>119 (± 1)</td>
<td>2,849 (±1,339)</td>
</tr>
<tr>
<td>6</td>
<td>145 (±26)</td>
<td>217 (± 69)</td>
<td>1,785 (± 585)</td>
</tr>
<tr>
<td>7</td>
<td>198 (± 4)</td>
<td>184</td>
<td>1,706 (± 146)</td>
</tr>
</tbody>
</table>

* Values for $^3$HTdR incorporation are expressed as mean (±SE) of cpm obtained from duplicate, 0.4 ml cultures, except where only one culture was assayed. Thymidine incorporation was determined after incubating culture fluid with 4 μCi of $^3$HTdR for 3-hr period sometime between 84th and 132nd hr of culture.

† Each culture containing g-a cells alone consisted of 0.4 ml of cell-free culture fluid overlying a glass cover slip containing the g-a cells that had been washed by dipping and spraying procedure. Cultures of purified lymphocytes alone and plus g-a cells contained 0.4 ml of culture fluid containing 0.4-1.0 × $10^6$ purified lymphocytes.
Table 4. Ability of Autologous G-A Cells to Restore DNA Synthesis in Purified Lymphocyte Cultures in Response to PPD

<table>
<thead>
<tr>
<th>Contents of Cultures*</th>
<th>3HTdR Incorporation (cpm)+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes alone</td>
<td>114 (± 18)</td>
</tr>
<tr>
<td>Lymphocytes + PPD†</td>
<td>110 (± 14)</td>
</tr>
<tr>
<td>Lymphocytes + g-a cells</td>
<td>254</td>
</tr>
<tr>
<td>G-a cells alone</td>
<td>286</td>
</tr>
<tr>
<td>G-a cells + PPD</td>
<td>417 (± 159)</td>
</tr>
<tr>
<td>Lymphocytes + g-a cells + PPD</td>
<td>42,915 (±5227)</td>
</tr>
</tbody>
</table>

* Each culture with lymphocytes contained 10^6 purified lymphocytes.
† Values for 3HTdR incorporation are expressed as mean (±SE) of cpm obtained from duplicate, 0.4 ml cultures, except where only one culture was assayed. Thymidine Incorporation was determined after incubating culture fluid with 4 μCi of 3HTdR for 3-hr period beginning at 84th hr of culture.
‡ PPD, 1.25 μg of tuberculin-purified protein derivative/ml of culture fluid.

with medium 199 and reincubated in cell-free culture fluid containing PHA or antigens. A 3-hr incubation with 3HTdR on the second, third, or fourth day thereafter did not result in radioautographically detectable labeled g-a cells. To determine whether any DNA-synthesizing g-a cells could become detached from the cover slips and thereby escape radioautographic detection, the radioactive culture fluid was passed through a Millipore filter to trap any such detached cells. A small amount of thymidine incorporation (about 1100 cpm) was thus found in response to PHA in only one of ten experiments in which the cover slips had been dipped and sprayed. The necessity for spraying was shown by the finding that considerable DNA synthesis was often detected when the cover slips were dipped but not sprayed.

Stimulation of Allogeneic Lymphocytes: Table 3 shows that the purified lymphocytes underwent DNA synthesis when cultured with g-a cells from allogeneic donors. In other experiments with g-a cell and purified lymphocyte mixtures in which the donors were of opposite sex, sex chromosome analysis indicated that all the dividing cells were derived from the lymphocyte population.11

Table 5. Ability of G-A Cells to Increase DNA Synthesis in Purified Lymphocyte Cultures in Response to PHA

<table>
<thead>
<tr>
<th>Contents of Cultures*</th>
<th>3HTdR Incorporation (cpm)+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes + PHA†</td>
<td>5,166 (± 152)</td>
</tr>
<tr>
<td>Lymphocytes + PHA‡</td>
<td>106,602 (±818)</td>
</tr>
<tr>
<td>G-a cells + PHA</td>
<td>52 (± 6)</td>
</tr>
</tbody>
</table>

* Each culture with lymphocytes contained 1.1 x 10^6 lymphocytes. Glass cover slips with g-a cells, removed after 36-hr incubation in mononuclear leukocyte culture fluid, were dipped and sprayed; they were then added to purified lymphocytes or to cell-free culture fluid containing PHA.
† Values for 3HTdR incorporation are expressed as mean (±SE) of cpm obtained from duplicate 0.4 ml cultures. 3HTdR incorporation was determined by incubating culture fluid with 4 μCi of 3HTdR for 3-hr period beginning at 59th hr of culture.
‡ PHA, 2.5 μg/ml of culture fluid.
Obtaining Lymphocytes and Mononuclear Cells

Purified Lymphocytes

The leukocytes obtained from the nylon fiber columns were comprised almost exclusively of small lymphocytes (Fig. 4A). Occasionally a contaminating polymorphonuclear leukocyte or monocyte was observed, but most were removed by incubating the lymphocyte preparations in the glass bottles (Fig. 4B).

Two additional techniques were employed that provided more sensitive indications of minor degrees of contamination than the use of cell smears. The first consisted simply of incubating $4 \times 10^5$ or more purified lymphocytes for up to several days at $38^\circ C$ in Leighton culture tubes containing clean cover slips, which serve as “concentrating” attachment sites on which contaminating monocytes eventually settle. At the end of the incubation period the cover slips were removed, air dried, stained, and examined for the presence of g-a cells. One or more macrophages could thereby be detected among $4 \times 10^5$ lymphocytes, despite the fact that in smears of the original lymphocyte population no monocytes had been observed.

The most sensitive index of “purity” was a functional one and consisted of determining the degree of impairment of the blastogenic responses to specific antigens and PHA. Thus, lymphocytes of sufficient purity failed to give any
detectable blastogenic response to antigens but did respond when cultured in the presence of a glass cover slip containing autologous g-a cells (Table 4). Purified lymphocytes still gave some response to highly blastogenic concentrations of PHA (Table 5, line 1). However, addition of both PHA and g-a cells to these lymphocytes resulted in a much greater response (Table 5, line 2).

DISCUSSION

One of the first steps frequently required in obtaining leukocytes for in vitro studies is to effect a substantial separation of the leukocytes from erythrocytes. It was for this purpose that phytohemagglutinin was used, prior to the discovery that PHA preparations also contained a blastogenic factor(s). Numerous other substances have been utilized to separate erythrocytes from leukocytes, e.g., fibrinogen, dextran, polyvinylpyrrolidone, gelatin, and gum acacia. The methods we use involve either a simple centrifugation or a gravity sedimentation of the erythrocytes, thereby obviating the necessity of exposing the leukocytes to any of these substances.

Investigators have utilized several methods that separate lymphocytes from phagocytic cells (monocytes and polymorphonuclear leukocytes). In some of these methods the phagocytic cells were removed after they had ingested iron particles. In most other methods, however, the separation is based on the tendency of the phagocytic cells, but not the lymphocytes, to be retained by certain surfaces and matrices, e.g., siliconized glass beads, glass wool, nylon fiber, and cotton wool. None of these methods is well suited to providing cells for the study of blastogenesis, since all of them may remove most of the monocyte-containing fraction (which contains the g-a cells essential for the blastogenic response) together with the polymorphonuclear leukocytes that perform no known useful function in, and may be detrimental to, blastogenic responses in vitro. Another disadvantage of many of these methods, when utilized for obtaining purified lymphocytes, is that the methods may not reliably produce the required high degree of separation and generally do not provide a method for monitoring and thus determining when the desired purity has been attained. Each of these disadvantages can be easily circumvented by the cell preparation methods we have described herein and discuss below.

Our 5-min centrifugation of freshly drawn heparinized peripheral blood and collection of the leukocytes in the upper portion of the resulting buffy coat are conducted almost exactly as described by Jago. However, her subsequent 30-min, 37°C sedimentation of the leukocytes is entirely omitted from our procedure, which yields a leukocyte population (Table 1) containing approximately 70–90% lymphocytes, 5–20% monocytes, and less than 10% polymorphonuclear leukocytes, thus essentially eliminating the unnecessary and possibly deleterious latter cell type. Because of the high percentage of mononuclear leukocytes in this leukocyte preparation, we refer to the culture fluid resulting from its addition to medium 199 as the "mononuclear leukocyte culture fluid." In view of its very adequate responses to PHA (Table 2) and other blastogenic factors, it is apparent that this mononuclear leukocyte culture fluid contains sufficient numbers of viable lymphocytes, as well as suffi-
OBTAINING LYMPHOCYTES AND MONONUCLEAR CELLS

sufficient numbers of the required g-a cells, the latter presumably being derived from the monocytes.

Jago reported obtaining in 16 of 23 experiments leukocyte populations comprised 100% of lymphocytes. Mangi and Mardiney, using an adaptation of Jago's technique, stated that they almost invariably obtained lymphocyte purity greater than 96% and at times attained a purity of 99%. It is known that when populations of leukocytes are comprised of approximately 98–100% lymphocytes, the blastogenic response to antigens, allogeneic cells, and PHA will be limited by the extremely low numbers of g-a cells. The technique, as employed precisely as described by Jago and by Mangi and Mardiney, will, in view of their reported results, in many instances provide insufficient numbers of g-a cells to permit the leukocyte populations to be used as measures of the lymphocytes’ ability to respond to blastogenic factors. In our adaptation of Jago's technique, we have never obtained a leukocyte population in which the lymphocytes comprised more than 92% of the cells (Table 1), a degree of purity that is below that at which the percent of g-a cells is known to become a limiting factor.

Our method for obtaining purified lymphocytes from human peripheral blood differs from most other methods in the following respect: we utilize two procedures in the purification process, the latter of which permits monitoring and choice of the purity attained. Thus, the partially purified column effluent is incubated on the inner surfaces of glass bottles to permit the remaining g-a cells to adhere to the glass. By removing periodically tiny aliquots of the nonadherent supernatant lymphocyte population, concentrating them via centrifugation, and making smears of the resulting pellets for microscopic examination, it is possible to monitor the degree of purification being achieved and to continue the purification process until the desired purity has been attained.

Most, if not all, of the cells in the g-a mononuclear leukocyte population that restores detectable blastogenic responses in cultures of purified lymphocytes are derived from the monocytes. However, it is not known whether it is these monocyte-derived cells that restore the response or whether the g-a cell restoring the response is derived from a lymphoid cell that had, or developed, glass-adherent characteristics. In either event, removal of virtually all the monocytes, i.e., achieving a leukocyte population composed more than 99% of lymphocytes, results in the depletion of the required g-a cell, indicating that even if the required cell is not derived from the monocyte it is removed when the monocytes are removed.

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REFERENCES

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Methods for Obtaining Purified Lymphocytes, Glass-adherent Mononuclear Cells, and a Population Containing Both Cell Types From Human Peripheral Blood

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