Purification and Characterization of Granulocytic Progenitor Cells (CFU-C) From Human Peripheral Blood Using Immunologic Surface Markers

By Carol M. Richman, Leonard Chess, and Ronald A. Yankee

The concentration of committed granulocytic progenitor cells (CFU-C) in functionally unique subpopulations of human peripheral blood mononuclear cells has been determined by the in vitro methylcellulose assay. Using immunoabsorbent column chromatography and rosette-depletion techniques, we have demonstrated that CFU-C, although not present in either purified T or B lymphocyte populations, are highly concentrated in the "null" cell population, which lacks sheep erythrocyte receptors and surface immunoglobulin. Further fractionation of this null subset has demonstrated that CFU-C do not bear complement receptors, but require the presence of peripheral blood mononuclear cell feeder layers for maximum proliferation.

The regulation of granulopoiesis in vivo is a complex process which, at present, is not well understood. Development of a clonal assay for quantitation of granulocytic progenitor cells (CFU-C) has, however, provided a number of important observations concerning regulation of granulocyte production in vitro. Current evidence suggests, for example, that both monocytes and activated T lymphocytes can produce substances that may stimulate proliferation and differentiation of granulocytic progenitors in vitro. Mature polymorphonuclear leukocytes, on the other hand, appear to produce factors that may inhibit colony growth in vitro.

Since the proportion of CFU-C is small relative to the other cells in bone marrow and peripheral blood, purification of hematopoietic progenitor cells is an important step in studying the interaction between these progenitor cells and other cell populations. Previous attempts to isolate CFU-C have employed techniques for separating cell populations on the basis of cell size or density. Using albumin density gradients, Dicke et al. and Moore et al. have achieved up to 100-fold purification of bone marrow granulocytic progenitors in primates. Messner et al. have fractionated human marrow by velocity sedimentation at unit gravity and have studied cellular interactions in the resulting purified populations. Although the cells obtained by these techniques are homogeneous in cell size and/or density, they are heterogeneous in function with CFU-C comprising only a small percent of the total cells.

The use of surface membrane properties to obtain functionally unique subpopulations of mononuclear cells offers an alternative approach to purifying CFU-C and studying cell-to-cell interactions. Recent studies have identified a var...
the precursor cells for both mature B and T lymphocytes in a population of mononuclear cells that lack intrinsic surface immunoglobulin (SIg) and sheep erythrocyte (E) receptors (null cells). One might predict that the progenitor cells for the granulocytic series are also present in the null cell population. Barr et al. have provided preliminary evidence to support this prediction by demonstrating good diffusion chamber growth in vivo with human peripheral blood cells lacking surface receptors for sheep erythrocytes and complement.

In the present study, specific functional subpopulations of peripheral blood mononuclear cells have been evaluated for the presence of CFU-C using the in vitro methylcellulose technique. We have demonstrated that CFU-C, although not present in either purified T or B lymphocyte populations, are highly concentrated in the SIg-negative, E-negative, null subset. Moreover, further fractionation of this null subset has demonstrated that the CFU-C do not bear EAC receptors, but require the presence of peripheral blood mononuclear cells (probably EAC-positive cells) for maximum proliferation.

**MATERIALS AND METHODS**

**Cell Preparation and Fractionation**

Human peripheral blood mononuclear cells were obtained by Ficoll-Hypaque density gradient separation of heparinized whole blood from normal volunteers (Table 1). A semicontinuous-flow cell separator (Haemonetics, Natick, Mass.) was used in some experiments (Tables 2, 3) to obtain larger numbers of cells. Using the latter technique, a buffy-coat fraction was obtained that consisted of 64% of the mononuclear cells present in each 600 ml of whole blood processed. The buffy-coat concentrates were diluted with medium and separated on Ficoll-Hypaque. The immunologic properties and CFU-C concentrations of buffy coat-cells were essentially identical to those of whole blood mononuclear cells.

Imunoabsorbent column chromatography was used to separate the cells into surface im-

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>CFU-C/2 × 10^5 Cells*</th>
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</thead>
<tbody>
<tr>
<td>1. Ficoll–Hypaque separated mononuclear cells</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>2. Anti-FAB column adherent (B) cells</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>3. Anti-FAB column nonadherent (T + null) cells</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>4. Cells from No. 3 following E-rosette depletion (null cells)</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>5. Cells from No. 4 following EAC-rosette depletion (EAC-negative null cells)</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

*Mean ± SEM using leukocyte conditioned medium.

**Table 2. Effect of Leukocyte Conditioned Medium (LCM) and Autologous Feeder Layers on CFU-C Proliferation (CFU-C/2 × 10^5 Cells) in EAC-negative Null and EAC-positive Subpopulations**

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>LCM</th>
<th>Feeder Layer</th>
<th>LCM</th>
<th>Feeder Layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 ± 0</td>
<td>83 ± 7</td>
<td>—</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>1 ± 0</td>
<td>29 ± 2</td>
<td>1 ± 0</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>3</td>
<td>0 ± 0</td>
<td>178 ± 32</td>
<td>0 ± 0</td>
<td>17 ± 10</td>
</tr>
<tr>
<td>4</td>
<td>0 ± 0</td>
<td>48 ± 7</td>
<td>0 ± 0</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Mean</td>
<td>0 ± 0</td>
<td>84 ± 33</td>
<td>0 ± 0</td>
<td>6 ± 4</td>
</tr>
</tbody>
</table>

*Mean ± SEM.
CHARACTERIZATION OF HUMAN CFU-C

Table 3. Effect of Conditioned Media and Autologous Feeder Layers on CFU-C Proliferation

<table>
<thead>
<tr>
<th>Subpopulations</th>
<th>CFU-C/2 × 10⁶ Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ficoll-Hypaque separated mononuclear cells</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>2. Cells from No. 1 following E- and EAC-rosette depletion (E-/EAC-)</td>
<td>2 ± 1 5 ± 2 67 ± 14</td>
</tr>
</tbody>
</table>

*Mean ± SEM of 11 experiments.
†HEKS was tested in dilutions up to 1:32 and the highest value obtained was used to calculate the mean.

mucoglobulin positive (Ig+) and immunoglobulin negative (Ig-) subpopulations, as previously described. In brief, mononuclear cells were applied to a column containing Sephadex G-200 to which purified rabbit antibody to the FAB (fragment, antibody binding) component of human Ig had been linked covalently. The cells passing through the column routinely contained less than 2% Ig+ cells, as measured by immunofluorescent staining with polyclonal fluorescein-labeled anti-FAB reagent. Cells with Ig on their surface, which adhered to the column, were then eluted by competitive inhibition with 1% human gamma globulin. Both Ig+ and Ig- populations contained 2%–5% phagocytic cells as judged by latex ingestion.

The Ig- cells were then depleted of sheep erythrocyte rosetting (E+) T lymphocytes by a technique previously described. After Ficoll-Hypaque separation, the resulting Ig-, E-negative population, termed “null” cells, contained less than 2% Ig+ cells and less than 10%, E-rosette positive cells.

Null cells were further fractionated by depletion of EAC rosetting cells on Ficoll-Hypaque gradients, thus leaving a residual population of EAC-negative null cells at the interface and EAC-positive cells in the red cell button. The EAC-negative null cell population contained less than 5%, EAC-rosette-positive cells. Osmotic lysis of erythrocytes was used to obtain the EAC-positive cells. The immunologic functions of these null cell subpopulations have been analyzed in detail in previous reports.

In the experiments reported in Tables 2 and 3, EAC-negative null cells were obtained by E- and EAC-rosette depletion, without the use of column chromatography. The sum of E- and EAC-rosetting cells remaining in the EAC-negative null subset was an average of 10^-3.

**CFU-C Assay In Vitro**

The number of granulocytic colony-forming cells in each mononuclear cell subset was determined by the CFU-C assay. The cells were plated in a methylcellulose culture system in vitro at a concentration of 2 × 10⁵ mononuclear cells/ml. Each plate contained 40%, methylcellulose (Dow, Midland, Mich.), 20%, fetal calf serum (Flow Laboratories, Rockville, Md.), 20%, cells in alpha medium (Flow), and 20%, colony-stimulating factor (CSF) provided by a supernatant of a 7-day culture of human peripheral blood leukocytes. Control plates without CSF were also included. In several experiments, a partially purified substance derived from human embryonic kidney cell conditioned medium (HEKS) was employed as a source of CSF. This standard material (kindly provided by Abbott Laboratories) has been shown by many investigators to be active in stimulating colony formation for both murine and human bone marrow. In addition, the subpopulations were plated on feeder layers of autologous peripheral blood cells prepared by Ficoll-Hypaque separation and were plated in 0.5% agar (Difco Bactoagar) at a concentration of 1 × 10⁶ cells/ml.

Triplicate plates were incubated at 37°C in a moist 10% CO₂ atmosphere and counted at 14 days using an inverted microscope at 50×. Aggregates greater than 20 cells were designated as colonies. Results were expressed as mean CFU-C/2 × 10⁵ mononuclear cells plated ± SEM (Tables 1-3).

**RESULTS**

In seven normal donors, the mean CFU-C concentration per 2 × 10⁵ Ficoll-Hypaque separated mononuclear cells was 4 ± 1 (Table 1). Cells that adhered...
to the anti-FAB column (primarily B cells) had a CFU-C concentration of 1 ± 1. The nonadherent cells passing through the anti-FAB column (T plus null cells) contained 6 ± 2 colonies. After E-rosette depletion of this population, the resulting null cells had a CFU-C concentration of 26 ± 3 (a sevenfold increase over the initial mononuclear cell fraction). Since the null cell population is known to be heterogeneous with respect to EAC receptors, the null fraction was further depleted of EAC-rosetting cells in three experiments (Table 1, subpopulation 5). This subpopulation of EAC-negative null cells yielded no colonies, using leukocyte conditioned medium as the source of CSF.

Since no colony growth was observed with EAC-negative null cells, we evaluated the colony-forming capacity of EAC-positive cells obtained by osmotic lysis of rosetted erythrocytes in the red cell button following EAC rosetting and separation on Ficoll-Hypaque. In the presence of conditioned medium, no CFU-C were obtained from this EAC-positive cell population (Table 2, column 3). Because of this failure to detect CFU-C activity in either the EAC-negative null or EAC-positive subpopulations when there was clearly CFU-C growth prior to EAC rosetting, we altered our culture conditions by using feeder layers of peripheral blood leukocytes as the source of CSF. In a pilot experiment, a marked increase in the number of colonies was observed when EAC-negative null cells were plated on feeder layers, as compared to the results with either leukocyte conditioned medium (LCM), or a partially purified CSF derived from HEKS. A parallel experiment was performed in which the EAC negative null cells were prepared by E- followed by EAC-rosette depletion without using the anti-FAB column. These results were similar to the results with cells separated by column chromatography followed by E and EAC rosetting. Feeder layers markedly increased the number of colonies observed compared to the results using LCM or HEKS. Since the two techniques yielded similar information, additional experiments were performed using cells obtained by E- followed by EAC-rosette depletion.

In these studies the source of CSF was either LCM, HEKS, or autologous feeder layers (Table 3). The addition of LCM to Ficoll-Hypaque separated mononuclear cells resulted in a CFU-C concentration of 2 ± 1; when autologous feeder layers were used, the concentration was 7 ± 3, a difference that was not statistically significant (p > 0.05 by Student's t test). The cell population that underwent both E- and EAC-rosette depletion (Table 3, subpopulation 2) demonstrated 2 ± 1 colonies with LCM and 5 ± 2 with HEKS. There was no significant difference between these two values (p > 0.05). However, using feeder layers, the concentration of CFU-C in the EAC-negative null cells was 67 ± 14 [significantly higher (p < 0.001) than that observed with either LCM or HEKS].

To establish further that the CFU-C activity was contained in the EAC-negative null population and not in the EAC-positive fraction, EAC-positive cells were plated with either LCM or autologous feeder layers. In four experiments (Table 2) no colonies were observed in either the EAC-negative null or the EAC-positive cell populations with LCM. However, with feeder layers, 84 ± 33 colonies were observed with EAC-negative null cells and only 6 ± 4 with EAC-positive cells.
DISCUSSION

Previous studies have demonstrated that precursor cells for both B and T lymphocytes are contained in a subpopulation of peripheral blood mononuclear cells lacking intrinsic surface immunoglobulin and sheep erythrocyte receptors. Both B- and T-cell precursors appear to bear complement receptors. In the present study, we have shown that granulocytic progenitor cells (CFU-C) from human peripheral blood reside in a subpopulation of mononuclear cells that lack surface receptors for immunoglobulin, sheep erythrocytes, and complement.

Our results confirm the studies of Barr et al., who demonstrated that peripheral blood cells lacking E and complement receptors proliferate well in a diffusion chamber assay in vivo. The in vitro assay system employed in the present study offers several advantages over the in vivo system used by Barr et al. First, there is general agreement that the cell proliferating in the in vitro system is a committed granulocyte progenitor. However, with the diffusion chamber assay, there is controversy over the identity of the proliferating cells (i.e., pluripotent and/or committed progenitor cells). In addition, the clonal in vitro system allows precise quantitation of the concentrating capacity of various fractionation techniques and permits analysis of cell-to-cell interactions not possible in an in vivo assay. Our rosette-depletion method concentrated CFU-C from approximately 1/30,000 cells in the unseparated mononuclear cell population to 1/3,000 cells in the EAC-negative null population. It is apparent that the latter population is heterogeneous and may contain other precursor cells whose nature and function remain to be discovered.

The failure of the EAC-negative null cells to proliferate in the presence of conditioned media was an unexpected finding. Considerable spontaneous colony growth was observed in both the unseparated mononuclear and the null (Ig-negative, E-negative) populations. This result, described previously, was probably due to the large number of monocytes or other cell types in the plating mixture serving as an endogenous source of CSF. However, following EAC-rosette depletion, optimum CFU-C growth was dependent on feeder layers of peripheral blood leukocytes. We initially felt that perhaps our conditioned medium was inadequate. However, the EAC-negative null cells also failed to grow in the presence of a standard partially purified HEKS preparation shown by several investigators to be active in stimulating human bone marrow CFU-C.

An inherent difference between the type of CFU-C occurring in normal peripheral blood and bone marrow may partially explain the failure of the EAC-negative null cells to grow optimally with conditioned medium. Granulocytic progenitor cells in the marrow are known to vary in their sensitivity to CSF, whereas peripheral blood may consist predominantly of a subpopulation of CFU-C requiring very high or continuous levels of CSF to initiate division. In the erythroid system there appear to be several types of committed progenitors. The erythrocytic CFU (CFU-E) is capable of a limited number of self-replications and of responding to low doses of erythropoietin. The erythropoietic burst-forming unit (BFU-E), on the other hand, is presumed to be a more primitive cell requiring higher levels of erythropoietin to
induce proliferation. Recent information suggests that while human bone marrow contains both CFU-E and BFU-E, the erythroid colonies present in Ficoll-Hypaque separated peripheral blood are primarily of the BFU-E type. If a parallel situation is true for the granulocytic series, one might predict that stimulating substances, though adequate for bone marrow, might not effect equal stimulation of peripheral blood CFU-C. The latter cells may require either a higher dose of CSF or a continuous source of stimulating factor in order to initiate division. Monocytes (and perhaps other presumably EAC-positive cell types) present in feeder layers and in unseparated mononuclear preparations may provide this continuous source of stimulating factor.

The failure of EAC-negative null cells to form colonies with conditioned medium may have several additional explanations. First, the separation procedure itself may have altered the cells in some way, impairing the ability of CFU-C to respond normally to conditioned medium. A second, more attractive, hypothesis is that the separation procedure depletes a critical regulator cell population that may ordinarily facilitate the action of conditioned medium in inducing CFU-C proliferation. In the absence of such regulator cells, conditioned medium alone might not provide an adequate stimulus for colony formation, although the medium might be effective when tested with unfractonated cells. Further studies will be required to investigate these possibilities. In the present report, the method of purifying specific functional subpopulations of mononuclear cells provides a means of determining the interrelationships of cells in the presence or absence of added stimulator substances.

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