Studies of Human Pluripotential Hemopoietic Stem Cells (CFU-GEMM) In Vitro

By Robert C. Ash, Robert A. Detrick, and Esmail D. Zanjani

An in vitro clonal assay for a class of human hemopoietic progenitors (CFU-GEMM) with several characteristics of pluripotential stem cells has been previously described. In the presence of medium conditioned by leukocytes stimulated with phytohemagglutinin (PHA-LCM) and erythropoietin (Ep), CFU-GEMM give rise to mixed hemopoietic colonies containing granulocytic, erythroid, monocyte-macrophage, and megakaryocytic elements. In initial studies we found that CFU-GEMM were present in equal but low frequencies in blood (B) and bone marrow (M) mononuclear cell populations. However, when the culture system was modified by the substitution of Iscove's modified Dulbecco's medium for α-MEM and the addition of mercaptoethanol, a significant enhancement of mixed colony formation occurred, and an approximately 3–4-fold difference in the frequency of CFU-GEMM between B and M emerged. Repeating studies showed the formation of secondary differentiated hemopoietic colonies and at least a limited capacity for self-renewal of CFU-GEMM. The in vitro growth of normal CFU-GEMM was highly dependent on hemopoietin(s) present in PHA-LCM. In vitro detection of CFU-GEMM, however, requires only relatively low permissive concentrations of Ep, in contrast to the high Ep requirement for optimal BFU-E growth in vitro. These and other data described demonstrate CFU-GEMM to be a distinct multipotential stem cell class whose assay may prove useful in the study of human blood dyscrasias.

THE DEVELOPMENT of in vitro clonal assays for hemopoietic progenitor cells has allowed investigation of factors influencing hematopoietic stem cell differentiation and proliferation and has provided a tool for exploring mechanisms of disease involving disordered blood cell production. For human study, clonal assays for granulocyte-macrophage progenitors1,2 and erythroid precursors3–5 have been utilized for several years, but investigations have been limited by lack of a functional assay for the multipotential stem cell comparable to the spleen colony-forming unit (CFU-S) of the murine model.6 The development by Fauser and Messner7,8 of a human in vitro culture system for growth of colonies of single cell origin containing granulocytic, erythroid, monocyte-macrophage, and megakaryocytic elements offered this potential. In this study we have observed that minor modifications of this methylcellulose culture system have resulted in detection of significantly enhanced mixed colony formation as well as an increased frequency of such progenitors in bone marrow mononuclear cell populations compared to peripheral blood. This increased plating efficiency has facilitated more detailed quantitative investigation of the properties of this pluripotential progenitor cell class, which are reported here.

MATERIALS AND METHODS

Blood and Marrow Samples

Paid normal volunteers (18 healthy adult men and 2 healthy adult women with normal blood counts) served as control subjects. After informed consent was obtained, 30–60-mI blood samples and 3–5-mI bone marrow aspirates (from posterior iliac crest) were drawn into heparinized springs. Samples were diluted 2–3 times in media (RPMI 1640, α-MEM, or Iscove’s modified Dulbecco’s medium), layered over Ficoll-Hypaque gradients (density, 1.077), centrifuged at 400 g for 25–30 min, and interface mononuclear cells collected.

Interface cells were washed 3 times in media, counted, and resuspended in either α-MEM or Iscove’s medium containing 20% fetal calf serum (FCS) and held at 4°C until used for culture.

Preparation of PHA-LCM

Media conditioned by human peripheral blood leukocytes for 7 days in the presence of 1% (v/v) phytohemagglutinin (PHA-LCM) and 10% fetal calf serum was prepared as previously described.9

Culture Systems for CFU-GEMM

Two sets of culture conditions were employed. In the first, following the procedure outlined by Fauser and Messner,10–12 blood or marrow mononuclear cell preparations were suspended in media containing methylcellulose as viscous support (0.9% final concentration), 30% FCS (Reheis Biologicals), 5% PHA-LCM, and α-MEM to a final cell concentration of 2 × 10⁵ cells/ml. Quadruplicate 1-mI aliquots were cultured in 35-mm plastic dishes at 37°C in an environment of 5% CO₂ and 100% humidity. Erythropoietin (Ep, 2.5 IU/plate) was added on day 4 and colonies were scored on day 15 of culture.

In the second procedure the system was modified to reflect the following. (1) After preliminary experiments in which the effects of various concentrations of the reducing agents α-thioglycerol and 2-mercaptoethanol on CFU-GEMM growth were examined, 5 × 10⁻³ M 2-mercaptoethanol was routinely added to all cultures. (2) Iscove’s modified Dulbecco’s media was substituted for α-MEM. (3) One IU Ep/plate was used. (4) Because preliminary experiments had demonstrated that similar numbers of recognizable mixed colonies were formed when Ep was added to the plate on day 0 to day 4 of culture, Ep was routinely added at the start of culture.

From the Veterans Administration Medical Center, Departments of Medicine and Physiology, University of Minnesota School of Medicine, Minneapolis, Minn.

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Address reprint requests to Robert C. Ash, M.D., Veterans Administration Medical Center (151), 54th Street and 48th Avenue South, Minneapolis, Minn. 55417.

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The remaining conditions were similar to those outlined above. Thus, 0.9% methylcellulose, 30% FCS, and 5% PHA-LCM, were used throughout; colonies were scored on day 15. Both sheep (Connaught Step III) and human urinary Ep were used. Human Ep (15 IU/mg protein) used in these studies was prepared from urine of anemic patients, as previously described.10

**Staining Procedures and Examination of Individual Colonies**

Individual colonies were removed from the plate by micropipetting into thin-tipped Pasteur pipettes, applied to slides directly or by spinning on a cytocentrifuge, and stained. Colonies were routinely stained with Wright-Giemsa for morphological analysis. Selected colonies were also examined after chloroacetate esterase,12 "nonspecific" esterase,12 acid phosphatase,13 or hematoxylin-benzidine14 staining to confirm their cytologic composition.

**Cell Transfer Experiments**

In these studies, individual mixed colonies were removed from the plate on day 14 by micropipetting under sterile conditions, disrupted to obtain a single-cell suspension, and transferred to a second culture media in microtiter wells. The appearance of secondary hemopoietic colonies was scored after an additional 14-16 days of culture. This second growth media consisted of 0.9% methylcellulose, 30% FCS, 5 x 10^-2 M 2-mercaptoethanol, Iscove's media, 5% PHA-LCM, and 1 U/ml Ep. To some transfer cultures an additional "support cell" population (2000 cells/0.1 ml) of peripheral blood mononuclear cells (PBMC) previously irradiated with 2000 rads was added.

**Cell Irradiation**

PBMC suspended at 10^5 cells/ml in Iscove's modified Dulbecco's medium supplemented with 10% FCS were irradiated with a 4 meV linear accelerator at a dose rate of 200 rads/min. After irradiation, cells were immediately washed once in Iscove's-10% FCS and added in appropriate aliquots to microtiter wells.

**RESULTS**

When plates were examined with an inverted microscope on day 15 of culture, several types of hemopoietic colonies were recognized by their distinct color and morphology in situ. Figure 1A shows a granulo-
cyte macrophage colony (CFU-C derived) with a flat arrangement of nonhemoglobinized cells; Fig. 1B shows an erythroid burst colony (BFU-E derived) with densely packed configuration of hemoglobinized cells; and Fig. 1 C and D demonstrate representative “mixed” colonies (CFU-GEMM derived) with a compact, usually central, hemoglobinized area and a peripheral “lawn” of nonhemoglobinized small and large cells.

Figure 2 shows representative areas of mixed colonies as they appear in Wright’s stained slide preparations, demonstrating the heterogeneous cell composition of such colonies. In Fig. 2A are shown large “foamy” macrophages surrounded by erythroblasts, a densely granulated myeloid precursor, and a monocyte. In Fig. 2B is a closer view of a monocytoid cell and a hemoglobinized erythroblast. Megakaryocytes, found both individually and in small clusters within mixed colonies, were also seen (Fig. 2 C and D). The presence of each of these cell types was confirmed in selected colonies by use of selective histochemical stains, including myeloperoxidase and chloroacetate esterase for myelocytic elements, nonspecific esterase for monocyte-macrophages, benzidine for erythroblasts, and acid phosphatase for megakaryocytes.

Table 1 summarizes the morphological assessment of 231 mixed colonies scored for content of recognizable cells of the respective lineages as seen in Wright’s stained smears of individual colonies. All mixed colonies contained at least granulocytic and erythroid elements, with approximately one-quarter containing only these lineages. A majority of mixed colonies (62%) also contained monocytes or macrophages; 19% contained easily characterized megakaryocytes, and 5% contained eosinophils.

In order to improve the yield of the mixed colony assay, we examined the effects of various culture media, reducing agents, and other additives. Table 2 compares the number of “pure” erythroid and mixed colonies formed under culture conditions originally...
described by Fauser and Messner7'8 (group A: 30% FCS, α-MEM, 5% PHA-LCM, 2.5 IU Ep, and 0.9% methyl cellulose) with the number of similar colonies produced under the modified conditions (group B). The modified system substitutes Iscove’s modified Dulbecco’s media for α-MEM, adds 5 × 10^5 M 2-mercaptoethanol, and uses less Ep. In both systems, PHA-LCM and 30% FCS are required. The assay modifications (group B) resulted in a significant increase in the yield of mixed colonies for both blood (p < 0.05) and bone marrow (p < 0.025).

The results presented in Table 2 also show that, whereas the frequency of CFU-GEMM in blood and marrow was similar in group A cultures, cultures employing the modified media revealed the presence of greater numbers of CFU-GEMM in bone marrow than blood. The values shown in table 2 represent the mean of all experiments performed under each set of conditions. Although the colony growth of most donor samples was consistent and closely distributed about the mean values shown, three donors in group B deviated significantly from the norm, as reflected in the magnitude of the standard error shown in Table 2. One of the donors demonstrated poor colony growth (14 CFU-GEMM and 25 BFU-E per 10^6 BM cells), and two demonstrated high colony growth (576 and 387 CFU-GEMM and 389 and 550 BFU-E per 10^6 BM cells, respectively). The greater frequency of CFU-GEMM in bone marrow was apparent over a wide range of cell concentrations tested (Fig. 3).

Figure 3 also demonstrates that the number of mixed colonies detected by the modified assay has a linear relationship to the number of cells plated over a limited range, and that this relationship extrapolates to the origin. At low plating densities the assay efficiency is decreased (as shown by the dotted line). Also important at higher plating densities was the problem of colony crowding and the difficulty distinguishing overlapping granulocytic and erythroid colonies from true mixed colonies. For this reason, in all other studies, cultures were plated at cell concentrations at the lower end of this linear range of cell densities.

The data presented in Fig. 4 show that, in contrast to the relatively higher Ep requirement of BFU-E, CFU-GEMM required less Ep for in vitro growth. Thus, optimal numbers of mixed colonies were detected at about 0.5 IU Ep/ml, whereas maximal

### Table 1. Cytologic Analysis of Mixed Colonies From Normal Donors*

<table>
<thead>
<tr>
<th>Number of colonies containing</th>
<th>Number of Mixed Colonies Examined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
</tr>
<tr>
<td>Granulocytic and erythroid only</td>
<td>24</td>
</tr>
<tr>
<td>Macrophages</td>
<td>69</td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td>19</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>3</td>
</tr>
</tbody>
</table>

*Combined data of 12 normal subjects. Individual colonies were obtained from methylcellulose culture on day 15 and analyzed in slide preparations for content of easily characterized cells of the respective lineages, as seen with Wright-Giemsa staining.

### Table 2. Effect of Culture Media and 2-Mercaptoethanol on Plating Efficiency of CFU-GEMM and BFU-E*

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Colonies/10^6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
</tr>
<tr>
<td><strong>A (n = 8)</strong></td>
<td></td>
</tr>
<tr>
<td>Alpha medium</td>
<td>125 ± 12</td>
</tr>
<tr>
<td>30% FCS</td>
<td></td>
</tr>
<tr>
<td>5% PHA-LCM</td>
<td></td>
</tr>
<tr>
<td>2.5 U Ep/plate</td>
<td></td>
</tr>
<tr>
<td><strong>B (n = 12)</strong></td>
<td></td>
</tr>
<tr>
<td>Iscove’s modified</td>
<td>86 ± 29</td>
</tr>
<tr>
<td>Dulbecco’s medium</td>
<td></td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td></td>
</tr>
<tr>
<td>30% FCS</td>
<td></td>
</tr>
<tr>
<td>5% PHA-LCM</td>
<td></td>
</tr>
<tr>
<td>1 U Ep/plate</td>
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</table>

*Combined data of multiple experiments with normal subjects. In group A, cells were cultured at 2 × 10^5 cells/ml and numbers of “pure erythroid” (BFU-E derived) and “mixed” (CFU-GEMM derived) colonies scored in cytocentrifuge-prepared slides made of all erythroid-containing colonies individually obtained from quarter sectors of plates. In group B, numbers of BFU-E and CFU-GEMM were scored by counting entire plates based on in situ colony morphology, with cytologic confirmation obtained for selected individual colonies. In group B, cells were cultured over a varying concentration range (1-400,000/ml) and colony numbers calculated from those groups cultured at the lowest cell density showing consistent growth (i.e., in the linear range of assay)—usually 2 × 10^6 cells/ml for marrow and 3-4 × 10^6 cells/ml for blood samples.

†Significant difference (p < 0.05).
‡Significant difference (p < 0.025).
Fig. 3. CFU-GEMM plating efficiency. Cells were cultured at concentrations from \(5 \times 10^4\) to \(10^6/\text{ml}\) under "group B" conditions (0.9% methylcellulose, 30% FCS, Iscove's media \(5 \times 10^{-6}\) M mercaptoethanol, 5% v/v PHA-LCM, and 1 U Epo/ml added day 0 of culture). Results are combined data of multiple experiments with 3 normal subjects, shown as means ± SE of available data at each point.

In contrast, the requirement for a factor(s) present in PHA-LCM was significantly greater for CFU-GEMM than BFU-E. Figure 5 demonstrates that there was little or no growth of mixed colonies in the absence of PHA-LCM and that there was a dose-dependent increase in mixed colony growth up to a concentration of 5% PHA-LCM. BFU-E growth, on the other hand, occurred in the absence of PHA-LCM, but was enhanced approximately 50% when PHA-LCM was added to cultures (Fig. 5).

Table 3 shows the results of experiments in which cell suspensions prepared from individual mixed colonies were transferred to a second culture media in a microtiter well. When transfers were made into an appropriate environment, which included irradiated nonproliferating "support" cells, approximately one-quarter of such transfers gave rise to secondary hemopoietic colonies, both of differentiated types (granulocyte-macrophage and erythroid), and less frequently, to mixed colonies. In the absence of a support cell population, however, only rare granulocyte-macrophage colonies (2/74 transfers) developed. Irradiated
feeder cells alone did not give rise to colonies, and no successful transfers occurred when PHA-LCM and Ep were omitted from the secondary media.

DISCUSSION

Fauser and Messner\textsuperscript{7,8} have described an in vitro clonal assay for a class of human hemopoietic progenitor cells that exhibit at least some features similar to those that characterize the pluripotent stem cell of the mouse (CFU-S). The human CFU-GEMM are functionally identified by their ability to form mixed hemopoietic colonies in which differentiated granulocytic, erythroid, monocyte-macrophage, and megakaryocytic elements can be recognized. That such mixed colonies arise from single cells (and are not the result of overlapping erythroid and granulocytic colonies) has been demonstrated by Y chromatin analysis of individual mixed colonies formed in coculture experiments involving male and female donor cells,\textsuperscript{7,15} and has been further supported by sedimentation velocity studies showing that these "colony-forming units" have size characteristics of single cells similar to CFU-C and BFU-E.\textsuperscript{7}

The results of transfer experiments such as those described here also support the concept that CFU-GEMM have "stem cell" properties. These experiments show that the CFU-GEMM not only give rise to differentiated blood cells of multiple lineages, but can, under appropriate in vitro conditions, give rise to committed progenitor cells (as demonstrated by the formation of erythroid and granulocytic secondary colonies) as well as possessing at least a limited capacity for self-renewal (demonstrated by formation of secondary mixed colonies).

In the studies reported here, using culture procedures as described by Fauser and Messner,\textsuperscript{7,8} we confirmed the finding that CFU-GEMM were present in blood and bone marrow of normal individuals (in equal numbers but at the relatively low frequency of about 1 CFU-GEMM/10\textsuperscript{5} nucleated cells). However, the slight modifications of the culture procedure recently reported\textsuperscript{16} and described here resulted in significant enhancement of mixed colony formation. Moreover, this improved CFU-GEMM plating efficiency revealed the presence of greater numbers of CFU-GEMM in bone marrow mononuclear cell populations than in blood. If one also accounts for the much higher concentration of mononuclear cells in marrow...
aspirate samples compared to blood (in our studies, approximately 20–50 × 10^6 mononuclear cells/cc for normal marrow aspirates and 1–1.5 × 10^6 mononuclear cells/cc blood), this higher number of identifiable CFU-GEMM in marrow is in general accord with the expectation of a higher concentration of stem cells in hematopoietic organs, compared to circulating blood, as demonstrated for the murine CFU-S.17

This improved assay efficiency has facilitated quantitative study of biologic regulators of CFU-GEMM growth and differentiation in this system. The primary requirement for CFU-GEMM growth in vitro, in addition to routine culture components, appears to be a factor(s) present in PHA-LCM.7 PHA-LCM also contains factors that promote the formation of CFU-C-derived colonies (colony-stimulating factor, CSF)9 and, in the presence of Ep, BFU-E-derived colonies (burst-promoting activity, BPA).18,19 The presence of megakaryocytic elements in mixed colonies suggests that the conditioned medium may also contain agents capable of supporting the growth of megakaryocyte progenitors. Whether the dependence of CFU-GEMM on the presence of PHA-LCM is indicative of the presence of a distinct hemopoietic factor in PHA-LCM whose activity is permissive for the growth of CFU-GEMM, or can be attributed to the effects of these previously recognized factors (CSF, BPA, etc.), cannot conclusively be stated. In the experiments described here, however, the very different effects of PHA-LCM on CFU-GEMM growth compared to BFU-E (i.e., no observed CFU-GEMM-derived colony formation without PHA-LCM and significant direct increase with PHA-LCM addition, compared to only modest enhancement of BFU-E-derived colony formation with PHA-LCM addition) suggests that a factor(s) other than BPA may have a role in this promotion of CFU-GEMM growth in vitro. Only by biochemical isolation and characterization of the various hemopoietins in PHA-LCM will it be possible to provide a clearer answer to this question. The studies described here do clearly show that the effect of PHA-LCM upon CFU-GEMM growth is not attributable to a direct influence of the mitogen PHA on the stem cell itself, as PHA alone was unable to support mixed colony formation. Rather, the role of PHA is probably in the stimulation of a population of cells among peripheral blood leukocytes which, during preincubation, are capable of producing these various stimulatory factors.

The high Ep requirement for optimal in vitro BFU-E growth demonstrated in these studies is consistent with observations previously reported3–5,20 and is in contrast to the relatively low Ep requirement of CFU-GEMM. This low Ep threshold for in vitro CFU-GEMM detection, beyond which mixed colony growth is essentially "zero-order," suggests that the proliferative activity of CFU-GEMM occurs independently of Ep. That Ep is required for CFU-GEMM detection may only reflect the operational requirement of this in vitro assay (i.e., recognizable terminal hemoglobinization of the Ep-dependent erythroid component within the mixed colony).

Recognition of megakaryocytic elements within mixed colonies was rendered less difficult by the greater maturity of these cells in the modified assay conditions used here than was observed in mixed colonies grown under original culture conditions. The reported numbers of colonies containing these cells is based on Wright's stain appearance of easily characterized large multinucleate cells fulfilling conventional histologic criterion for megakaryocytes, which can be convincingly demonstrated in a number of mixed colonies. The acid phosphatase staining of these large megakaryocytes after 10% formalin fixation13,16 was used as supporting evidence for the presence of these cells. However, in the absence of a highly specific histochemical marker for cells of human megakaryocyte lineage, such as acetylcholinesterase in rodent megakaryocytes,22,23 other methods of identification have been sought. Preliminary immunofluorescent studies with an antiplatelet glycoprotein antiserum24 have also demonstrated the megakaryocyte content of such colonies (R. Ash, R. Detrick, and E. D. Zanjani: unpublished data), and the use of other probes, such as 3H-serotonin autoradiography and monoclonal anti-platelet antibody labeling, is under investigation here. Such methods may allow adaptation of this culture system to the more detailed study of human megakaryocytopoiesis in vitro.

Together, these data clearly demonstrate the CFU-GEMM to be a distinct multipotential hemopoietic precursor cell class. The availability of functional in vitro assays such as that utilized here now offers the possibility of direct investigations concerning the normal physiology of this human pluripotential stem cell, as well as the study of hematologic disease states involving the stem cell compartment.

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