Effect of Recombinant Hematopoietic Growth Factors on Proliferation of Human Marrow Progenitor Cells in Serum-Deprived Liquid Culture

By Anna Rita Migliaccio, Giovanni Migliaccio, and John W. Adamson

We investigated the effects of recombinant interleukin-3 (IL-3), granulocyte-macrophage and granulocyte colony-stimulating factors (GM-CSF and G-CSF), and erythropoietin (Ep) on the number of human hematopoietic progenitors after two to ten days of incubation in liquid cultures deprived of fetal bovine serum (FBS). The source of progenitor cells was normal human marrow depleted of T lymphocytes and/or adherent cells. When adherent cell-depleted marrow was cultured without growth factors, the number of progenitor cells was relatively constant for periods up to eight days. In contrast, a progressive decline in the number of progenitor cells was detected in cultures of nonadherent, T-cell-depleted marrow cells. In both cases, the addition of IL-3 increased by two- to fourfold over input the number of erythroid burst-forming cells (BFU-E) per culture. The number of BFU-E peaked either at day 4 or 8. G-CSF had no effect on the number of progenitor cells per culture. GM-CSF and Ep had no effect in cultures of nonadherent marrow cells but maintained the number of BFU-E in cultures of nonadherent, T-cell-depleted marrow cells. The addition of a neutralizing anti-GM-CSF monoclonal antibody, but not anti-IL-3 neutralizing antiserum, decreased the number of BFU-E in cultures of nonadherent marrow cells. None of the growth factors investigated enhanced the number of GM progenitors to the same degree as the number of BFU-E. However, in cultures of nonadherent, T-cell-depleted marrow cells, IL-3 and GM-CSF maintained the number of GM progenitors up to eight days. These results indicate that IL-3 alone is capable of increasing the number of BFU-E and of maintaining the number of GM progenitors in liquid culture, whereas GM-CSF and Ep are capable of maintaining, but not increasing, BFU-E in this system.

MATERIALS AND METHODS

Cell preparation. Marrow cells, obtained from normal volunteers with their informed consent and under a protocol approved by the institution's human subjects review committee, were aspirated from the posterior iliac crest into heparinized syringes. Specimens were then diluted 1:1 with Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY), and the marrow cells were suspended with a pipette. The light-density cell fraction was separated by Ficoll (Organon Teknika, Durham, NC) gradient centrifugation (400 g for 20 minutes). The adherent cells were removed by a double incubation of the cell suspension in plastic flasks containing 10^6 cells/mL IMDM supplemented with 10% (vol/vol) FBS (HyClone, Logan, UT) at 37°C and 5% CO2 in air. The nucleated cell recovery was 40% to 60%. The final monocyte concentration was evaluated by AD acetate esterase staining and was 6%. The cells were then washed twice with IMDM containing 1% bovine serum albumin (BSA, Sigma, St Louis) and either incubated in liquid culture or plated in semisolid assay to determine the number of progenitor cells. In some experiments, T lymphocytes were also depleted by soybean agglutination as described previously. In this case, the nucleated cell recovery was 15% to 25% and the final concentration of AD acetate-positive or CD2- and CD3-positive cells was 1%. CD2- and CD3-positive cells were evaluated by indirect

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immunofluorescence as described\textsuperscript{11} using the murine monoclonal antibodies 35.1 and 64.1, which recognize epitopes present on CD2 and CD3, respectively, on human T lymphocytes.\textsuperscript{12}

**Hematopoietic growth factors.** The purified recombinant human hematopoietic growth factors used in liquid culture included Ep (1.5 U/mL = $6 \times 10^{-10}$ mol/L),\textsuperscript{13} G-CSF (1,000 U/mL = $2.3 \times 10^{-10}$ mol/L)\textsuperscript{3} (from AMGen, Thousand Oaks, CA) and GM-CSF (100 U/mL = $4.5 \times 10^{-10}$ mol/L).\textsuperscript{14} IL-3 (10 U/mL = $2 \times 10^{-11}$ mol/L) was in the form of conditioned medium from COS-I cells transfected with a plasmid designed for expression of human IL-3\textsuperscript{1} (from Genetics Institute, Boston). The concentration of IL-3 was 10 U/mL conditioned medium, and the specific activity was 10 U/µg protein (S. Clark, personal communication, 1987). In control experiments, a volume of sham-transfected COS cell supernatant, corresponding to the maximum IL-3 concentration used, was added to the cultures. Each growth factor was used at the concentration that gave the maximum number of erythroid bursts or GM-colonies in semisolid assay under FBS-deprived conditions.\textsuperscript{11}

**Neutralizing antibodies.** The anti-GM-CSF neutralizing monoclonal antibody was affinity purified from ascites fluid derived from mice injected intraperitoneally with hybridoma cells. The hybridoma cells were obtained from spleen cells of mice immunized against purified human GM-CSF. One microliter of purified monoclonal antibody contained 1 µg immunoglobulin and completely neutralized 100 U GM-CSF as determined by colony-forming assay (K. Kaushansky, unpublished observations). The IL-3 neutralizing antisera (a gift of Genetics Institute) was obtained from a rabbit immunized with human IL-3, and one-hundred microliters of antisera completely neutralized 10 U IL-3 as determined by colony-forming assay (S. Clark, personal communication). For neutralization experiments, GM-CSF or IL-3 was incubated for one hour at room temperature with amounts of anti-GM-CSF or anti-II-3, respectively, which were predetermined to neutralize completely the biologic activity of the growth factors. The solution was then added to the marrow cell liquid culture.

**Incubation in liquid culture.** Nonadherent marrow cells (10\textsuperscript{6} cells/mL) or nonadherent, T-cell–depleted marrow cells (10\textsuperscript{6} cells/mL) were incubated for two to ten days in 1 mL IMDM containing β-mercaptoethanol (7.5 x 10\textsuperscript{-3} mol/L), antibiotics (100 µg penicillin, 250 ng amphotericin B, and 100 µg streptomycin), deionized BSA (2 x 10\textsuperscript{-5} mol/L), BSA-adsorbed cholesterol (4 µg/mL) and soybean lecithin (12 µg/mL), iron-saturated human transferrin (5 x 10\textsuperscript{-7} mol/L), insulin (1.7 x 10\textsuperscript{-6} mol/L), nucleosides (10 µg/ml each), inorganic salts, sodium pyruvate (5 x 10\textsuperscript{-4} mol/L), and L-glutamine (2 x 10\textsuperscript{-3} mol/L) as previously described.\textsuperscript{15,16} All chemicals were obtained from Sigma. The batch of BSA used was electrophoretically pure and lacked detectable GM-CSF (limit of detection 10\textsuperscript{-10} mol/L) or G-CSF (limit of detection 6 x 10\textsuperscript{-10} mol/L), as determined by radiinmuonassay (RIA), and IL-6, as determined by a specific bioassay.

Identical aliquots of 1 mL each were established in separate 12-mL loosely capped polystyrene tubes ( Falcon, Becton Dickinson, Oxnard, CA) and incubated at 37°C in a fully humidified tissue culture incubator at 5% CO\textsubscript{2} in air. At days 2, 4, 8, and 10 of culture, a separate set of tubes was removed from the incubator and the number of progenitors in each tube was determined by culturing the cells in methylcellulose, as previously described (ref 17 and below). In some experiments, aliquots of marrow cells in liquid culture were stained with trypan blue and the number of cells was counted with a microcentrifuge, or were cytocentrifuged and stained with May-Grunwald/Giems.

**Assay of hematopoietic progenitors.** Each 1-mL dish contained the following components in 1MDM:\textsuperscript{17} methicellulose (0.8% wt/vol, final concentration), β-mercaptoethanol (7.5 x 10\textsuperscript{-3} mol/L), antibiotics (100 U penicillin, 250 ng amphotericin B, and 100 µg streptomycin), a selected heat-inactivated lot of FBS (40%, vol/vol) and 3 x 10\textsuperscript{5} nonadherent or 3 to 10 x 10\textsuperscript{5} nonadherent, T-cell–depleted marrow cells. Ep (1.5 U/mL) and/or PHA-LCM (3%, vol/vol) was added to stimulate maximum colony growth. Assays were performed for the numbers of progenitor cells contained in 40-µL aliquots obtained at various times from the liquid cultures. The possibility that stimulatory or inhibitory activities could be present in 40 µL liquid culture and affect the assay for progenitor cells was excluded in preliminary experiments in which the cells were plated before or after washing.

Cultures were established in duplicate. Plates were incubated at 37°C in a fully humidified incubator with 5% CO\textsubscript{2} in air and scored with an inverted microscope at 12 to 14 days for the presence of erythroid bursts and GM colonies.

**Scoring criteria.** Erythroid bursts (containing ≥200 cells) were identified on the basis of their color (orange-red). Colonies composed of >50 nonerythroid cells were scored as GM colonies.

**RESULTS**

**Time course of the number of marrow cells in liquid culture.** The number of cells and their morphology were investigated at various times of liquid culture. In cultures of nonadherent marrow cells, the cell numbers varied virtually constant since variations of only ±20% were observed per tube on the different days (Table 1). Although the cultures of Ep, G-CSF, GM-CSF, or IL-3 did not affect the number of cells per tube (Table 1). Neither was morphology of the cells in culture changed, nor was it influenced in any consistent way by the presence of the different growth factors (Table 2). In cultures of nonadherent, T-cell–depleted marrow cells, the cell number per tube increased twofold over time (Table 1). The addition of IL-3 did not further increase cell numbers.

**Survival and proliferation of hematopoietic progenitors in cultures of nonadherent marrow cells.** The survival of BFU-E and CFU-GM in serum-deprived cultures of nonadherent marrow cells and in the absence of exogenous growth

<table>
<thead>
<tr>
<th>Table 1. Changes in No. of Cells Over Time in Liquid Cultures of Nonadherent or Nonadherent, T-Cell–Depleted Marrow Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Stimulus</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>IL-3</td>
</tr>
<tr>
<td>GM-CSF</td>
</tr>
<tr>
<td>G-CSF</td>
</tr>
<tr>
<td>Ep</td>
</tr>
</tbody>
</table>

ND, not done.

*Results expressed as the number of cells × 10\textsuperscript{6} and × 10\textsuperscript{5} for nonadherent or nonadherent, T-cell–depleted marrow cells, respectively per milliliter and as the mean (± SD) of three experiments performed in duplicate.
LIQUID CULTURE OF HUMAN BFU-E AND CFU-GM

Table 2. Morphology of Nonadherent Marrow Cells After Eight Days in Liquid Culture

<table>
<thead>
<tr>
<th>Day in Culture</th>
<th>Stimulus</th>
<th>Polys + Bands</th>
<th>Myelocytes + Blasts</th>
<th>Macrophages</th>
<th>Erythroblasts</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>49/25</td>
<td>17/21</td>
<td>6/2</td>
<td>10/21</td>
<td>18/31</td>
</tr>
<tr>
<td>8</td>
<td>None</td>
<td>53/31</td>
<td>23/15</td>
<td>3/8</td>
<td>12/13</td>
<td>9/33</td>
</tr>
<tr>
<td>8</td>
<td>Ep</td>
<td>71/23</td>
<td>15/12</td>
<td>2/10</td>
<td>6/16</td>
<td>6/39</td>
</tr>
<tr>
<td>8</td>
<td>GM-CSF</td>
<td>68/29</td>
<td>16/17</td>
<td>2/13</td>
<td>3/15</td>
<td>10/26</td>
</tr>
<tr>
<td>8</td>
<td>G-CSF</td>
<td>54/25</td>
<td>31/22</td>
<td>1/9</td>
<td>6/14</td>
<td>8/30</td>
</tr>
<tr>
<td>8</td>
<td>IL-3</td>
<td>33/32</td>
<td>26/14</td>
<td>18/15</td>
<td>7/10</td>
<td>16/29</td>
</tr>
</tbody>
</table>

Results obtained in two separate experiments are shown and are expressed as the percentage of a given cell type obtained by differential counting of 200 cells.

factors was variable. Figure 1 shows the BFU-E survival from six different marrow donors. In general, the number of BFU-E per culture declined at day 2 and 4. In three of the subjects, this decrement was followed by an increase to input levels at day 8. In one case, a twofold increase in BFU-E numbers was observed at day 2, followed by a decline to 68% of the input value at day 8. In general, however, the number of BFU-E was maintained for at least 8 days (Fig 2A), whereas the number of CFU-GM declined to 48% of the input at day 2 (Fig 2B).

In the presence of IL-3 (2 x 10^{-11} mol/L), a two- to threefold increase in the number of BFU-E was observed (Fig 2A). The peak increase generally was observed at day 8, although in two experiments the peak number of BFU-E was observed at day 4. The presence of GM-CSF, Ep, or G-CSF did not affect the survival of BFU-E in cultures of nonadherent marrow cells (Fig 2A). In the presence of IL-3 or GM-CSF, the number of CFU-GM remained constant until day 4 and decreased to 50% of input at day 8 (Fig 2B). The presence of Ep or G-CSF did not affect the survival of CFU-GM in these cultures (described in legend to Fig 2).

The addition of anti-GM-CSF decreased the number of BFU-E in cultures not supplemented with growth factors but had no effect in cultures supplemented with IL-3 or when preincubated with GM-CSF (Table 3). The addition of
Table 3. Survival of BFU-E in Liquid Cultures of Nonadherent or Nonadherent, T-Cell–Depleted Marrow Cells: Effect of Neutralizing Antibodies (α) Against Human GM-CSF or IL-3

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>None</th>
<th>GM-CSF</th>
<th>IL-3</th>
<th>αGM-CSF</th>
<th>αGM-CSF + GM-CSF</th>
<th>αGM-CSF + IL-3</th>
<th>αIL-3</th>
<th>αIL-3 + GM-CSF</th>
<th>αIL-3 + IL-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFU-E/Culture* (% of Input) Nonadherent Marrow Cells</td>
<td>63</td>
<td>105</td>
<td>257</td>
<td>14</td>
<td>51</td>
<td>214</td>
<td>89</td>
<td>123</td>
<td>119</td>
</tr>
<tr>
<td>BFU-E/Culture* (% of Input) Nonadherent, T-Cell–Depleted Marrow Cells</td>
<td>69</td>
<td>99</td>
<td>120</td>
<td>33</td>
<td>61</td>
<td>ND</td>
<td>73</td>
<td>138</td>
<td>ND</td>
</tr>
<tr>
<td>BFU-E/Culture* (% of Input) Day 4 8</td>
<td>23</td>
<td>112</td>
<td>273</td>
<td>5</td>
<td>75</td>
<td>250</td>
<td>32</td>
<td>91</td>
<td>104</td>
</tr>
<tr>
<td>BFU-E/Culture* (% of Input) Day 4 8</td>
<td>8</td>
<td>128</td>
<td>755</td>
<td>8</td>
<td>65</td>
<td>750</td>
<td>8</td>
<td>151</td>
<td>178</td>
</tr>
</tbody>
</table>

ND, not done. Further details are given in the Materials and Methods section.

*Two representative experiments are shown. Numbers of BFU-E at day 0 of culture of nonadherent or nonadherent, T-cell–depleted marrow cells were 2,430 and 500, respectively. Similar results were obtained in two additional experiments.

anti–IL-3 did not affect the number of BFU-E in cultures not stimulated with growth factors or supplemented with GM-CSF (Table 3). Preincubation of IL-3 with this antiserum partially reduced the increase in the number of BFU-E observed in cultures supplemented with IL-3 (Table 3).

Survival and proliferation of human hematopoietic progenitors in cultures of nonadherent, T-cell–depleted marrow cells. A progressive decline in the number of both BFU-E and CFU-GM was observed with time in cultures of nonadherent, T-cell–depleted marrow cells in the absence of exogenous growth factors (Fig 3). Only 10% of BFU-E and 40% of CFU-GM survived eight days of culture. These results were observed for all seven marrow specimens studied.

In the presence of IL-3, the number of BFU-E per culture increased two- to fourfold with time (Fig 3A). In contrast, the number of CFU-GM only was maintained in cultures supplemented with IL-3 (Fig 3B). The increment in the number of BFU-E detected in cultures supplemented with IL-3 was reduced to one-third or one-fourth by preincubation of the IL-3 preparation with anti–IL-3 (Table 3). The number of BFU-E was not affected by preincubation of IL-3 with anti–GM-CSF neutralizing antibody (Table 3).

Under the same conditions, GM-CSF maintained the number of BFU-E but not the number of CFU-GM (Fig 3). The GM-CSF maintenance of BFU-E was abolished by preincubation of the GM-CSF with anti–GM-CSF (Table 3). The number of BFU-E was not affected by incubation of the GM-CSF with anti–IL-3 (Table 3).

DISCUSSION

In steady-state conditions, the size of the compartments of hematopoietic progenitors in the marrow is kept constant by a balance between differentiation and recruitment of new elements from more primitive cells. In vitro models have been established to simulate these two processes: differentiation of progenitor cells into colonies composed of mature cells can be observed in semisolid cultures supplemented with appropriate growth factors, and a constant number of progenitor cells may be maintained over a period of months by stromal layers in long-term marrow cultures. However, under the same circumstances, the number of progenitor cells can actually increase. This is the case during the massive expansion of the compartment of erythroid progenitors which occurs in the embryonic liver and, in the adult, during marrow recovery from treatment with cytotoxic drugs or reconstitution after transplantation. Experimental models of such an expansion have been represented until now by in vivo animal models.

We used the recently described culture conditions that support formation of erythroid bursts in the absence of FBS to investigate the effect of recombinant human Ep, G-CSF, GM-CSF, and IL-3 on the in vitro survival and proliferation of human hematopoietic progenitors. The growth factors were used at concentrations that induced the maximum number of erythroid bursts and/or GM-colonies.
in semisolid cultures of human bone marrow.\textsuperscript{11} The rationale for this is provided by the observation that with mouse cells the IL-3 concentration required for maximum formation of blast-cell derived colonies\textsuperscript{25} or the maximum CFU-S increment in liquid culture (A.R. Migliaccio, unpublished observations) is lower than the IL-3 concentration required for optimum erythroid burst growth in semisolid medium.

When nonadherent marrow cells are placed into serum-deprived suspension culture, the progenitor cells survive for at least eight days without added growth factors. This effect is likely mediated by factors produced by accessory cells since it is abrogated by removal of T lymphocytes. One of the factors produced responsible for maintenance of progenitor cells is GM-CSF, since addition of an anti–GM-CSF neutralizing monoclonal antibody blocked survival of BFU-E in cultures of nonadherent marrow cells and addition of GM-CSF restored the survival of BFU-E in cultures of nonadherent, T-cell–depleted marrow cells.

Similar to results with liquid cultures of murine cells,\textsuperscript{6,7} the addition of IL-3 increased by two- to fourfold the number of human BFU-E in FBS-deprived cultures either of nonadherent or nonadherent, T-cell–depleted marrow cells. This effect was not due to contaminants present in the COS cell supernatant because the addition of medium conditioned by mock-transfected cells had no effect on the survival of BFU-E and preincubation of the IL-3 preparation with an anti–IL-3 antiserum reduced substantially the increase in BFU-E numbers. The anti–IL-3 antiserum had no effect on the survival of BFU-E in cultures of nonadherent marrow cells not supplemented with growth factors, suggesting that detectable amounts of IL-3 are not released by accessory cells under these conditions. Furthermore, addition of anti–IL-3 antiserum did not affect the maintenance of BFU-E supported by GM-CSF, indicating that if the action of GM-CSF on the erythroid lineage is indirect, it is not mediated by stimulation of IL-3 production.

We have not yet been able to increase the number of GM progenitors in these cultures to the same extent as BFU-E. This may be due to the culture conditions selected for the erythroid lineage.\textsuperscript{11-26} However, a still-unidentified growth factor may be necessary to increase the number of GM progenitors. Consistent with this is the fact that a synergistic activity that is distinct from IL-3 and GM-CSF\textsuperscript{27} has been identified in long-term marrow cultures and, under the conditions described, erythroid progenitors declined in numbers more rapidly than did GM progenitors.\textsuperscript{28} In cultures of nonadherent, T-cell–depleted marrow cells, Ep, but not G-CSF, was able to maintain the number of BFU-E (Fig 3A), confirming the role exerted by Ep on early erythroid progenitors.\textsuperscript{16,28}

The different activities of GM-CSF and IL-3 in this assay may reflect their different biologic roles in vivo. GM-CSF is produced by many different cell types potentially present in the marrow microenvironment,\textsuperscript{29} whereas IL-3 is produced only by activated T lymphocytes.\textsuperscript{30} Furthermore, GM-CSF has been found adsorbed to the aminoglycans in the extracellular matrix of long-term marrow cultures,\textsuperscript{31} but IL-3 has not yet been detected in any normal tissues of either adult or fetal origin.\textsuperscript{32} Under steady-state conditions, GM-CSF alone may be sufficient to maintain early erythroid progenitors and to start the erythroid and myeloid differentiation process. IL-3, or other growth factors with related activity, may be produced under circumstances in which amplification of the compartment of hematopoietic progenitors would be necessary.

One of the critical variables in the transplantation process is the number of progenitor cells transplanted.\textsuperscript{33} Recently, pretreatment of marrow cells with IL-3 was reported to enhance the 30-day radioprotection in mice undergoing transplantation\textsuperscript{34} because of the better capacity of IL-3–treated cells to reconstitute the marrow and spleen.\textsuperscript{35} However, earlier studies showed that murine stem cells cultured with IL-3 lose their capacity to repopulate the thymus.\textsuperscript{36} Thus, any conclusion as to the usefulness to the transplant process of incubating marrow cells with growth factors such as IL-3 is premature.

The increase in the number of BFU-E which we observed in liquid culture may result either from induction of self-replication of a subset of BFU-E or from recruitment of BFU-E from more primitive cells driven to differentiate. At present, we cannot discriminate between these two possibilities. The recent results of Leary et al\textsuperscript{37} appear to favor the latter interpretation. These researchers found that if IL-3 or GM-CSF was added on day 14 to cultures of My-10\textsuperscript{+} marrow cells in the presence of a low concentration of serum, multipotent blast cell colonies with high replating capability would appear by day 21. However, the cell giving rise to these multipotent colonies may not be the same cell that gives rise to the increased number of BFU-E in our culture system. First, the observed frequency of cells that gave rise to blast colony cells is low (1 cell/10\textsuperscript{5} My-10\textsuperscript{+} marrow cells); second, the resulting colonies are composed mainly of macrophages. Consequently, if the same cell is being induced to proliferate in both systems, a significant proportion of the progenitor cells being studied by Leary et al may actually have died during the first 14 days of culture without growth factors.\textsuperscript{37} Nevertheless, since we actually observe at least a threefold increase in the number of early erythroid progenitors in our experiments, the culture system we describe may provide the opportunity to investigate the conditions that regulate the size of the compartments of various progenitor cells under normal or neoplastic conditions.

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