Effects of Recombinant Human Granulocyte Colony-Stimulating Factor (CSF), Human Granulocyte Macrophage-CSF, and Gibbon Interleukin-3 on Hematopoiesis in Human Long-Term Bone Marrow Culture

by Lucia H. Coutinho, Andrew Will, John Radford, Raffaella Schiró, Nydia G. Testa, and T. Michael Dexter

We have studied the effects of recombinant human granulocyte colony-stimulating factor (rH-G-CSF), hG macrophage-CSF (hGM-CSF), and gibbon interleukin-3 (gIL-3) on cell proliferation and differentiation in human long-term bone marrow culture (LTBMC). hG-CSF induced a maximal increase of 2.3-fold in both total nonadherent cells and GM cluster-forming cells, but only an increase of 1.7-fold in GM-colony-forming cell (GM-CFC) numbers, influencing mainly neutrophil differentiation. Cultures treated with hGM-CSF demonstrated a peak of 12.8-, 21- and 3.2-fold elevations in total nonadherent cells, cluster, and GM-CFC, respectively, and influenced differentiation of neutrophils, monocytes, eosinophils, and lymphocytes. Cultures treated with gIL-3 demonstrated the largest expansion in the GM-CFC population, reaching a maximum of 5.3-fold in relation to that of unstimulated controls. IL-3 treatment also increased the numbers of GM clusters and mature cells (including all myeloid cells and lymphocytes) 7.8- and 4.8-fold, respectively. Similar quantitative and qualitative changes were induced by G-CSF, GM-CSF, and IL-3 in LTBMCs of patients in remission after treatment for acute lymphoblastic leukemia or Hodgkin’s lymphoma. Overall, the expansion of GM progenitor cells in cultures treated with growth factors was larger in the adherent cell layer than in the nonadherent cell fraction. In addition, hGM-CSF, gIL-3, and hG-CSF to a less extent, increased the cycling rates of GM-CFC progenitors located in the adherent layer. This results indicate that hG-CSF is a much less potent stimulus of hematopoiesis in LTBMC than the other CSFs assayed, and that the increases in cell production after treatment with G-CSF, GM-CSF, or IL-3 may be achieved by primary expansion of different cell populations within the hierarchy of the hematopoietic system. The effects of the growth factors were transient and the longevity of hematopoiesis in the cultures was not altered, suggesting that treatment with IL-3, GM-CSF, or G-CSF had not compromised the ability of primitive cells to give rise to mature cells. This indicates that the stromal microenvironment in LTBMC can override potential differentiation-inducing activities of the CSFs.

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HEMATOPOIETIC growth factors are a family of glycoproteins that are defined and characterized by their ability to support the clonogenic growth of hematopoietic progenitor cells in vitro.1 With the use of DNA technology, purified recombinant forms of four human growth factors, generally known as colony-stimulating factors (CSFs), became available: macrophage CSF (M-CSF), granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), and interleukin-3 (IL-3).2,5

Studies using purified populations of progenitor cells and serum-free culture conditions have demonstrated that, despite some overlap in the responding cell population, each CSF has a unique spectrum of biologic activities when used alone. For instance, M-CSF acts mainly on progenitors restricted to the monocyte/macroage lineage,4 while G-CSF stimulates only granulocyte precursors.2 In contrast, IL-3 and GM-CSF affect the growth of various progenitors, including those committed to granulocyte and macrophage production, erythropoiesis, megakaryocytopoiesis, and also influence the growth and development of multipotential precursors.3,8-10 However, the capacity of murine IL-3 to induce stem cell self-renewal and to stimulate several cell lineages1 suggests that IL-3 is active at a more primitive level than the other CSFs. The discovery of a primate hematopoietin (gibbon IL-3 [gIL-3]), which is structurally related to murine IL-3, represented an important step in identifying the equivalent human factor and analyzing the functional relationship between the different molecules.3 In fact, the growth of human blast cell colonies, which express multilineage differentiation on replating (a characteristic in common with pluripotent self-renewing stem cells), was shown to be primarily supported by either gibbon or human IL-3, but not by GM-CSF.12,13

Other mechanisms controlling hematopoiesis, in particular the behavior of stem cell populations, appear to involve cellular interactions with the narrow stromal microenvironment, as shown in long-term bone marrow culture (LTBMC) of murine cells, where maintenance of stem cell self-renewal resulted in hematopoietic production for several months.14 Subsequently, the culture conditions were adapted for the growth of human marrow cells.15 The key feature of these cultures is the formation of an adherent cell layer that shows structural and functional similarities to the stromal microenvironment in vivo.16 Radioimmunoassay techniques and molecular probes for the analysis of CSF RNA have shown that the stromal cell layer can produce some CSFs, including M-CSF and GM-CSF, although production of IL-3 has not yet been detected.17,18 However, close cellular interactions are an important phenomenon accounting for stroma-induced hematopoiesis.19

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Recently, clinical trials of recombinant (r)G-CSF and hGM-CSF have been performed in various centers, and future trials with IL-3 are imminent. The initial reports on the efficacy of GM-CSF and G-CSF in enhancing the circulating levels of leukocytes have established the potential therapeutic role of hematopoietic growth factors in various clinical settings, although much still remains to be learned regarding the target cells and interactions between the various CSFs. For this reason, we compared the spectrum of biologic activities of G-CSF, GM-CSF, and IL-3 on hematopoiesis in LTBMC. The cultures were treated for as long as 10 weeks to test whether prolonged stimulation of hematopoiesis would lead to exhaustion of progenitor cells (particularly in view of the potential role that IL-3 plays in stem cell proliferation).

MATERIALS AND METHODS

Patient Population and Cell Separation

Aliquots of normal bone marrow cells for allogeneic transplants were obtained with informed consent from the donors. Additionally, marrow cells from patients who had been treated for Hodgkin's disease (HD) or acute lymphoblastic leukemia (ALL) were collected by aspiration from the posterior superior iliac crest. Cells were taken after receiving the appropriate informed consent.

The nine children with ALL had been treated with UKALL VIII (Medical Research Council trial for ALL), and the two Hodgkin's disease patients presenting with no marrow involvement had received 7 or 8 cycles of MOPP. At the time of the study all patients were off treatment and with normal blood counts. The time since the last course of chemotherapy varied from 12 to 34 months.

The specimens collected in Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO, Paisley, Scotland) with 200 U of preservative-free heparin (sodium heparin, Weddel Pharmaceutics Ltd, Rixon, Wales) were separated from red blood cells (RBCs) by gravity sedimentation in 0.1% methylcellulose over 30 minutes at room temperature. Light density cell fractions were prepared by density centrifugation (400 x g for 20 minutes) in lymphocyte separation medium (LSM) (1.077 g/mL) (Flow, Ayerst, Scotland).

Growth Factors

Three purified recombinant growth factors were used: G-CSF, GM-CSF, and IL-3. Both hG-CSF and gIL-3 were provided by Amgen Biologics (Thousand Oaks, CA), while hGM-CSF was a gift from Biogen Research Corp (Cambridge, MA). Serial dilutions of each factor were prepared under sterile conditions in phosphate-buffered saline (PBS) and 0.1% serum albumin to avoid nonspecific binding to the plastic vials. Stock and dilution vials of hG-CSF and gIL-3 were maintained at 4°C until used. The stock of hGM-CSF was stored at -70°C, but dilution vials were kept at -20°C for a maximum of 3 months without any apparent loss in biologic activity.

Clonal Assays for Hematopoietic Progenitor Cells

Cultures in methylcellulose. Cells were plated at a density of 10^6/mL in 30% fetal calf serum (FCS, Flow), 10% conditioned medium from 5637 tumor cell line (as a source of CSFs) 1% deionized serum albumin (wt/vol) (Sigma, Dorset, UK), 2 U of partially purified human urinary erythropoietin (Terry Fox Lab, Vancouver, Canada), and 1.3% methylcellulose in IMDM (wt/vol) (Dow Chemical Co) supplemented with extra glutamine (4 x 10^{-4} mol/L/mL), sodium selenite (10^{-7} mol/L/mL) (BDH, Dorset, UK), and a thyroglycerol (2.5 x 10^{-4} mol/L/mL) (Sigma). Aliquots of 1 mL were plated in duplicates in Multiwells tissue culture plates (Falcon, Oxford, UK) for 14 days under fully humidified conditions in an atmosphere of 5% CO_2 and 5% O_2 in nitrogen at 37°C.

Under an inverted microscope, colonies of ≥50 translucent cells were scored as GM-colony-forming cells (GM-CFC). Bursts of ≥3 clusters of hemoglobinized RBCs were counted as burst-forming units-erythroid (BFU-E), whereas colonies containing both translucent and RBCs were classified as colony-forming unit granulocyte erythroid megakaryocyte macrophage (CFU-GEMM). The nature of the cells was confirmed on cytospin preparations after double-staining with May-Grünwald Giemsa and 3-3'dimethoxybenzidine.

Cultures in agar. Cells at a concentration of ≥10^3/mL were inoculated in Iscove's medium supplemented with 20% FCS, 10% 5637 conditioned medium, and 0.33% final concentration of agar (Bacto). The culture mixture was plated in volumes of 1 mL in 35-mm Petri dishes (Falcon) in triplicates. After an 11-day incubation at 37°C in a fully humidified atmosphere of 5% CO_2 in air, GM colonies (≥50 cells per aggregate) and clusters (less than 50 cells per aggregate) were scored using a dissecting microscope (40x magnification). Agar was used in preference to methylcellulose for scoring clusters because of the better cell immobilization achieved.

Dose-Response Studies With Growth Factors

CSF dose titration experiments were performed in methylcellulose cultures with light density normal bone marrow cells after a two-step procedure of cell adherence to plastic. This consisted of overnight incubation of cells at a concentration of 2 x 10^6/mL with 20% FCS at 37°C, followed by a further 1-hour incubation in a new plastic recipient to achieve satisfactory depletion of adherent accessory cells. Plates without exogenous growth factors were also established to exclude endogenous colony formation. The plating efficiencies of cultures supplemented with rG-CSF, rGM-CSF, or rIL-3 at different doses were compared with positive control cultures containing 10% of 5637 conditioned medium. The doses of the factors that yielded maximum GM colony numbers corresponding to ≥100% of that seen in 5637 CM-stimulated cultures were 50 ng/mL, 10 ng/mL, and 2 ng/mL of G-CSF, GM-CSF, and IL-3, respectively.

As others observed, G-CSF could only stimulate progenitors of the GM lineage, while GM-CSF could support maximum GM colony formation but only one third or even lower proportions of the plateau number of erythroid and mixed colonies stimulated by 5637 CM or IL-3.

LTBMC

RBC depleted bone marrow aspirates at concentrations of 1.5 x 10^6 cells/mL were inoculated in 25-cm^2 tissue culture flasks (NUNC) in a 10 mL total volume of 10% FCS (pretreated batch, Flow), 10% horse serum (pretreated batch, Gibco), 5 x 10^{-7} mol/L hydrocortisone sodium succinate (Sigma), and single strength Iscove's medium (350 mOsM/kg) supplemented with penicillin and streptomycin. The cultures were gassed with 5% CO_2 in air and incubated at 33°C. After 7 days, and subsequently at weekly intervals, the cultures were demidepopulated and half of the supernatant medium was replaced by fresh growth medium. The suspension cells recovered after feeding were counted with trypan blue (to exclude the nonviable cells) in a Neubauer hemocytometer. The cells were then plated in clonal assays and differential morphologic counts were performed on cytospin preparations stained with May-Grünwald Giemsa. The establishment and appearance of a stromal cell layer in the cultures were monitored weekly by examination under an inverted microscope.
Groups of paired cultures were inoculated with hG-CSF, hGM-CSF, and gIL-3 alone. The factors were added at the beginning of the cultures and during weekly feeding at doses similar or twofold higher than those required for maximal colony formation in a mixed colony assay. Such doses were 2 ng/mL of gIL-3, 50 or 100 ng/mL of hG-CSF, and 10 ng/mL of hGM-CSF.

After 4, 6, and 10 weeks, stromal cell layers from control and CSF-treated LTBMCs were detached by a 10-minute exposure to 0.25% trypsin. The single cell suspensions resulting were washed twice in Iscove's medium with 20% FCS for abrogation of further trypsin action. The cells were plated in agar as described after being counted.

Stability of Growth Factors in LTBMC

To determine the endogenous level of factor production as well as the stability of factors added to LTBMC, conditioned media from control and CSF-treated cultures, harvested 7 days after feeding,
were tested for colony-stimulating activity by the in vitro bioassay. A volume of 0.1 to 0.2 mL of supernatant media was added to $10^5$ light density and nonadherent normal bone marrow cells in 0.33% agar cultures. Triplicates were prepared for the assay of each sample. Control cultures were stimulated by freshly prepared growth factors at doses corresponding to 1/3 or 1/6 of the plateau levels for maximum colony formation (10 ng/mL of G-CSF, 1 ng/mL of GM-CSF, and 0.4 ng/mL of IL-3), and calculated to be equivalent to the nominal concentrations of CSF present in the volume of added supernatant. To check that colony growth was dependent on exogenous factor, replicate cultures were set up without exogenous CSF.

*Tritiated Thymidine Suicide*

The $^3$H-TdR suicide test, performed with minor modifications from the method described by Iscove et al., was used to determine the proportion of cycling hematopoietic progenitor cells present within the stromal cell layer. Paired 1-mL aliquots of 0.5 to 1 x $10^6$ trypsinized adherent cells were incubated for 30 minutes at 37°C in the presence of either 100 µCi/mL of $^3$H-TdR, of specific activity 740 GBq/mmol (New England Nuclear, Boston, MA), or of an equal volume of Iscove’s medium. Incorporation of $^3$H-TdR was stopped by placing the cells in ice for 5 minutes and subsequently by washing them twice in Iscove’s medium containing 100 µg/mL of unlabeled thymidine (Sigma). Thereafter, 0.5 to 1 x $10^6$ cells were plated in 1 mL of agar culture medium containing a final concentration of 10 µg/mL of cold thymidine. The difference in numbers of colonies between cells previously exposed to $^3$H-TdR and the control groups was expressed as percentage kill, and as the proportion of hematopoietic progenitors undergoing DNA synthesis.

**Statistical Analysis**

The results are expressed as the mean ± SEM for the data obtained from separate experiments. The probability of significant differences between groups of LTBMCs was determined by comparing the logarithm of the values from CSF-treated cultures with those of the matched controls using a paired t-test. Statistical significance was taken at the 5% level.

**RESULTS**

**Growth Characteristics in LTBMC in Response to Recombinant Growth Factors**

*Kinetics of nonadherent cells.* Figure 1 shows the absolute control values for total nonadherent cells (A) and for GM progenitors (B and C) of CSF untreated cultures of normal bone marrows and of marrows from patients successfully treated for ALL or HD. In normal cultures, after the initial decline in total cell numbers for the first 5 weeks and in progenitor levels for the first 3 weeks, steady-state productions of nonadherent cells and of cluster-forming cells were maintained throughout the culture period. A plateau phase for GM-CFC was sustained up to week 6, and although the numbers gradually fell after 7 weeks, the cultures remain hematopoietically active for at least 10 weeks. Cultures from patients successfully treated for lymphoproliferative disorders and who were in unmaintained remission showed a defect in hematopoietic cell production, where GM-CFC numbers were 5 to 10 times lower than in normal controls. This has been described elsewhere.

The effects of optimal concentrations of G-CSF, GM-CSF, and IL-3 on the nonadherent cells of LTBMCs from five normal individuals are shown in Fig 2. It was only after 2 weeks that dramatic changes in the cellularity of the cultures could be appreciated even at first glance under an inverted microscope. Significant increases ($P < .01$) in total number of nonadherent cells were achieved with GM-CSF over the period between 3 and 8 weeks, culminating in a maximum of 12.8-fold increase at 5 weeks (Fig 2A). IL-3 also induced significant increases up to 4.8 times the cellularity in control cultures at similar times ($P < .01$ and $P \leq .05$). However, in cultures with G-CSF the increase in cell production was much less remarkable, but still reached statistical significance at 5 weeks ($P = .01$). GM-CSF was the most effective in stimulating the generation of cluster forming cells between the period of 2 and 4 weeks ($P \leq .01$), causing a maximum average fold enhancement of 21 times (Fig 2B). IL-3 also increased the production of cluster-forming cells during a similar period of time, but less than did GM-CSF. The changes in GM clusters preceded the increase in cellularity by 1 week and were more transient than those observed in total number of nonadherent cells. The effect of G-CSF was erratic, causing no consistent increase or decrease in numbers of cluster-forming cells.

The growth factor that enhanced the GM-CFC population more markedly was IL-3, causing an average increase of 1.8-

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*Fig 3.* The effects of optimal doses of hG-CSF (C) (50 ng/mL), hGM-CSF (A) (10 ng/mL), and gIL-3 (B) (2 ng/mL) on production of non-adherent cells (A) and GM-CFC (B) in cultures from patients with lymphoid malignancies in unmaintained remission. Results are shown as the percentage mean ± SEM of values measured in untreated cultures of 11 experiments.
to 5.3-fold from week 2 to week 7, when all values reached levels of significance \((P < .02)\) (Fig 2C). The addition of GM-CSF also increased significantly \((P < .01)\) the production of GM-CFC, but less intensively and for a shorter period than did IL-3; ie, from week 3 to week 5. The stimulating effect of G-CSF was modest but still showed a significant difference at week 4 \((P = .05)\).

To evaluate whether the observed abnormal low levels of progenitor cells in cultures of the patients treated for ALL or HD could be corrected by growth factors, G-CSF and GM-CSF were added to marrow cultures from eight patients and IL-3 to cultures from three patients at doses equivalent to those used for normal cultures. As illustrated in Fig 3A, the trend and magnitude of increase in total cell number as a result of continuous treatment with G-CSF, GM-CSF, and IL-3 were remarkably similar to those produced in normal LTBMCs. At week 3 an average significant increase of 4.6-fold with GM-CSF could be demonstrated, and by 5 weeks the difference was even more pronounced with a 15.8-fold increase. Although IL-3 exhibited a less efficient stimulus than GM-CSF, the cellularity in IL-3-treated cultures increased significantly from week 3 to week 6, and reached a peak of an 11.5-fold increase at week 5. However, at 6 weeks the counts started to decrease at a similar rate to that in normal cultures. Relatively little change was induced by G-CSF, except for a small but still significant increase \((P = .05)\) at 5 weeks. GM-CSF also markedly increased the levels of GM-CFC, up to an average of 4.3 times the control unstimulated population. The output of progenitor cells in the patients' cultures was greatest with IL-3 after a sixfold increase. However, in both groups of cultures GM progenitors were brought to levels within the range for normal controls, at least at one time during the period of GM-CSF or IL-3 maximum stimulation (eg, at 3 weeks an average of 715 and 1,595 GM colonies in GM-CSF- and IL-3-treated cultures, respectively, were above the average of 505 colonies for normal controls). If anything, there was a decline in GM-CFC numbers in cultures treated with G-CSF.

Overall, the effects of growth factors in LTBMC were transient. The levels of mature and progenitor cells returned to baseline control values after 5 to 8 weeks despite the continuous administration of the stimulating molecules; but most importantly, the longevity of the cultures was not altered by treatment with the CSFs.

**Addition of growth factors to long-established LTBMCs.**

To determine whether proliferative exhaustion of the system alone could explain the transient effects of the factors added continuously on a weekly basis from the time of culture inception, GM-CSF and IL-3 were inoculated into established cultures beginning on week 6. The results in Fig 4 show that although the magnitude of response was less pronounced, the increase in the target cell populations was still substantial. The pattern of cell proliferation in response to the factors was comparable with that of cultures treated from the time of establishment; ie, maximum fold changes of total nonadherent cells and clusters were obtained with GM-CSF, while the largest expansion of GM colonies was seen with IL-3.

**Morphologic analysis of nonadherent cells.**

Differential cell counts of normal cultures during the period of increased cell proliferation (Table 1) showed that G-CSF generated an apparent expansion of total granulocytes and monocytes, whereas GM-CSF led to a striking rise in early and late granulocytes, lymphocytes, eosinophils, and also increased levels of monocytes (at later stages). IL-3 produced a dramatic amplification of all myeloid cells and lymphocytes.
In cultures from ALL patients, the differentiation effects of the factors were very similar to those described above. The elevations in the number of nonadherent cells induced by GM-CSF and IL-3 were accompanied by an absolute increase in early and late granulocytes, lymphocytes, eosinophils, and less obviously of monocytes (at least at this stage in culture). Relatively little effect was seen on production of neutrophils in cultures treated with G-CSF.

Stability of growth factors in LTBMC. To determine if optimal concentrations of growth factors were maintained during the intervals of weekly feeding and whether or not fluctuations in their levels related to the degree of ongoing hematopoiesis, media containing G-CSF, GM-CSF, or IL-3 harvested from LTBMCs (7 days after their last feeding) at 2, 4, 5, 6, or 8 weeks were tested in GM-CFC assays of normal bone marrow cells. Supernatant media from all five normal LTBMCs and from three separate ALL cultures were biologically assayed for CSF activity at the above time points. Virtually all the activity of the added factors could be recovered, indicating that the effects obtained on hematopoiesis in cultures of normal and ALL marrows were comparable, and that even at times of maximum hematopoiesis only minimal amounts of the added growth factors were being used (Table 2).

Influence of growth factors on the adherent cell layer. Normally after 1 week in LTBMC, small islands of adherent spindle-shaped fibroblastoid cells and large macrophages are visible. By week 3, a confluent layer of adherent cells is seen, containing increasing numbers of adipocytes and closely associated foci of hematopoietic cells. G-CSF had no effect on the rate of development or the phenotypic organization of the adherent stromal cell layer. However, GM-CSF– and

### Table 1. Effects of G-CSF, GM-CSF, and IL-3 on the Differential of Supernatant Cells From LTBMC

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>LTBMC Group</th>
<th>Absolute Counts (× 10⁴) at Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BI + Pro</td>
<td>LG</td>
</tr>
<tr>
<td>Normal LTBMC</td>
<td>8.7 ± 4.5</td>
<td>140 ± 90</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>25 ± 17</td>
<td>210 ± 90</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>190 ± 110</td>
<td>1,200 ± 90</td>
</tr>
<tr>
<td>IL-3</td>
<td>200 ± 130</td>
<td>630 ± 440</td>
</tr>
<tr>
<td>LTBMC from ALL patients</td>
<td>23.7 ± 9.8</td>
<td>80 ± 21</td>
</tr>
<tr>
<td>G-CSF</td>
<td>19.1 ± 13</td>
<td>48 ± 21</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>146.6 ± 41</td>
<td>291 ± 65</td>
</tr>
<tr>
<td>IL-3</td>
<td>203 ± 77</td>
<td>476 ± 201</td>
</tr>
</tbody>
</table>

The means ± SEM of differential cell counts from separate experiments of normal (n = 3) and ALL cultures (n = 7) are shown. Abbreviations: BI + Pro, blasts + promyelocytes; LG, late granulocytes including myelocytes, meta, and polymorphs; Mo, monocytes/macrophages; Eos, eosinophils; LTBMC, long-term bone marrow cultures.

### Table 2. Stability of rCSFs in the Supernatant Media of LTBMC

<table>
<thead>
<tr>
<th>Culture Period</th>
<th>Supernatant Media (final concentration of GFs/plate)</th>
<th>No. GM-CFC (per 10⁵ bone marrow cells)</th>
<th>CSF Activity (% recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>G–LTBMC (10 ng/mL)</td>
<td>29.0 ± 5.0 (4)</td>
<td>83.5 ± 8.0</td>
</tr>
<tr>
<td></td>
<td>GM–LTBMC (1 ng/mL)</td>
<td>37.0 ± 8.0 (6)</td>
<td>98.6 ± 6.0</td>
</tr>
<tr>
<td></td>
<td>IL-3–LTBMC (0.4 ng/mL)</td>
<td>13.0 (2)</td>
<td>76.0</td>
</tr>
<tr>
<td></td>
<td>Control–LTBMC</td>
<td>0.2 ± 0.2 (6)</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>G–LTBMC (10 ng/mL)</td>
<td>46.0 ± 14.0 (3)</td>
<td>79.0 ± 7.5</td>
</tr>
<tr>
<td></td>
<td>GM–LTBMC (1 ng/mL)</td>
<td>71.0 ± 13.5 (3)</td>
<td>100.0 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>IL-3–LTBMC (0.4 ng/mL)</td>
<td>ND (3)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Control–LTBMC</td>
<td>0 (3)</td>
<td>0</td>
</tr>
<tr>
<td>5/6</td>
<td>G–LTBMC (10 ng/mL)</td>
<td>29.0 ± 2.0 (3)</td>
<td>101.0 ± 9.0</td>
</tr>
<tr>
<td></td>
<td>GM–LTBMC (1 ng/mL)</td>
<td>33.0 ± 9.0 (5)</td>
<td>102.0 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>IL-3–LTBMC (0.4 ng/mL)</td>
<td>19.0 ± 3.0 (3)</td>
<td>99.0 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>Control–LTBMC</td>
<td>0 (5)</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>G–LTBMC (10 ng/mL)</td>
<td>16.0 (1)</td>
<td>76.0</td>
</tr>
<tr>
<td></td>
<td>GM–LTBMC (1 ng/mL)</td>
<td>45.0 (1)</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>IL-3–LTBMC (0.4 ng/mL)</td>
<td>17.0 (1)</td>
<td>68.0</td>
</tr>
<tr>
<td></td>
<td>Control–LTBMC</td>
<td>0 (1)</td>
<td>0</td>
</tr>
</tbody>
</table>

Numbers of colonies stimulated by supernatant media of control and CSF-treated LTBMCs are shown. The CSF activity in the media recovered 7 days after feeding is expressed as the percentage of colony numbers in cultures stimulated by equivalent concentrations of freshly prepared growth factors. Spontaneous colony growth in the absence of exogenous CSF was never observed. Number of individual supernatants assayed shown in parentheses. Abbreviation: ND, not done.
IL-3–treated cultures showed, even after the first week, distinctly more populated stromal layers; however, by week 6 such differences were no longer noticeable. Thus, increases in the cellularity of the stromal layers in those cultures might have been primarily a consequence of the hematopoietic amplification within the stroma. Furthermore, adipogenesis was greatly reduced in cultures treated with GM-CSF or IL-3 (Fig 5, A through D). In addition, if GM-CSF or IL-3 was applied after the adherent cell layer had been fully established, involution of mature adipocytes occurred. This may be related to the increased hematopoietic stimulation, since neither GM-CSF nor IL-3 produced this effect on the adipocytic cells of the stroma when added to cultures where hematopoiesis had been ablated by irradiation (data not shown). However, these observations do not, per se, exclude a direct effect of any of the factors on the growth of stromal cells. Preliminary data on the immunocytochemical characterization of the adherent cell layer in control and CSF–treated cultures showed that GM-CSF treatment induced at least a twofold increase in the proportion of endothelial cells.77

Hematopoietic cells within the adherent cell layer. At two time points during the period of increased cell production and later, the adherent cell layers were assessed for their
hematopoietic content. As shown in Table 3, at 4 weeks there was a marginal and insignificant increase in the cellularity of the adherent layers in cultures with G-CSF, while the numbers of adherent cells in cultures containing either GM-CSF or IL-3 (except for one experiment) were 1.7- to 3.6-fold higher than those in controls and reached levels of significance ($P < .02$). More interestingly, the increase in numbers of hematopoietic progenitor cells within the adherent layer of cultures treated with growth factors was even greater than their increase in total adherent cells. For example, G-CSF cultures showed 2.0- to 8.4-fold increase in progenitor cells ($P = .04$), while GM-CSF and IL-3 cultures had as many as 1.6 to 18 times the number of progenitor cells of control cultures ($P = .01$). However, the increased hematopoietic production provoked by exogenous growth factors was not sustained for more than 4 weeks. By 6 weeks the counts of adherent cells in those cultures had returned to the levels of controls, and the numbers of progenitor cells were then relatively lower than those in unstimulated cultures. However, this decline in progenitor cells within the stromal cell layer is compensated for by an increase in the migration of progenitor cells to the supernatant medium, as the kinetics of the GM-CFC population in that fraction of the cultures suggest (see Figs 2 and 3). In addition, total numbers of
Table 3. Influence of hG-CSF, hGM-CSF, and gIL-3 on the Cellularity and Hematopoietic Progenitor Cell Content Within the Adherent Cell Layer of LTBMCs

<table>
<thead>
<tr>
<th>Weeks in Culture</th>
<th>Exp No.</th>
<th>Total Adherent Cells (x 10⁶)</th>
<th>Culture Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CSF</td>
<td>G-CSF</td>
</tr>
<tr>
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<td>1</td>
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<tr>
<td></td>
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<td>12</td>
</tr>
<tr>
<td></td>
<td>6</td>
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<td>24</td>
</tr>
<tr>
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</tbody>
</table>

Results are shown as single values from 8 separate experiments, 5 with normal bone marrow samples (1 through 5) and 3 with ALL samples (6 through 8).

GM-CFC (adherent plus nonadherent averages of 687 and 539 GM progenitors, respectively), but still slightly increased in cultures treated with GM-CSF or IL-3 (with an average of either 869 or 839 GM-CFC). The fact that there was no deleterious effect by the addition of CSFs is also supported by the normal longevity of the cultures, as indicated in two representative experiments where the numbers of adherent progenitor cells from CSF-treated cultures at week 10 remained similar to those in the untreated controls (Table 3).

Proliferative Status of Adherent Cell Layer-Derived GM-CFC.

The percentage of cycling GM progenitors within the adherent layer, as shown in Table 4, was determined in 4- and 6-week-old LTBMCs, 7 days after the previous feeding, when most of the earliest progenitors are found to be out of cycle.28 A relatively low proportion of progenitors was found to be in S phase in unstimulated cultures (17.6% ± 5.8% 3HtdR kill). G-CSF caused a marked increase in the proportion of cycling progenitor cells at week 4, but it seemed to lose its stimulative effect by week 6. In contrast, GM-CSF- and IL-3-supplemented cultures showed consistently throughout this period highly significant increases (P < .001) in the number of progenitor cells undergoing DNA synthesis.

Table 4. Effects of hG-CSF, hGM-CSF, and gIL-3 on the Proliferative State of GM Progenitors Within the Stromal Cell Layer in LTBMCs

<table>
<thead>
<tr>
<th>Weeks in Culture</th>
<th>Exp No.</th>
<th>Experiment Group (% cells in S phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CSF</td>
</tr>
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<tr>
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<td>4</td>
<td>9</td>
</tr>
</tbody>
</table>

The percent of cells in S phase reflects the reduction in colony numbers with 3HtdR as compared with control medium. The values are the calculated means of three replicates; SD was ±5% for all tests. Cultures from one ALL and from four normal separate experiments were analyzed on weeks 4 and/or 6.

DISCUSSION

Now that the efficacy of recombinant G-CSF, GM-CSF, and IL-3, alone or in combination, to elicit dramatic increases in blood cell production in vivo has been established,20,29 and given the rate at which trials using hematopoietic hormone therapy are being applied to various clinical conditions, a number of important questions are raised. These include the spectrum of progenitor cell types responsive to G-CSF, GM-CSF, or IL-3 within the environment of the bone marrow; the effects of these agents on primitive (stem) cells; and whether or not long-term treatment with growth factors might disturb the balance between self-renewal and differentiation, resulting in exhaustion of the stem cell pool. While unequivocal assays do not exist presently for human stem cells, we have monitored the production of progenitor cells and the longevity of hematopoiesis in LTBMC as a marker for the persistence of primitive cells able to produce lineage-restricted progenitors and mature cell progeny.

When recombinant growth factors were added to LTBMC on a weekly basis, marked enhancement of hematopoiesis could be achieved with hGM-CSF or gIL-3, while hG-CSF appeared to be a much less potent stimulator. In normal cultures and cultures from patients in remission of lymphoid malignancies, G-CSF had relatively little stimulatory effect on the production of nonadherent mature and progenitor cells. Others using similar doses of recombinant hG-CSF in LTBMC reported comparable changes in GM-CFC levels in the supernatant phase (an increase of less than twofold for only 2 weeks), although in contrast to our findings, a persistent and marked increase in neutrophil production was observed.30 It is possible that unknown factors in our culture media could inhibit the differentiation-inducing activity of the CSFs.
G-CSF on neutrophil precursors, and this could explain the difference in our results from those of the previous study. However, we found that G-CSF could increase the production of GM-CFC considerably within the stromal layer. G-CSF acts primarily on late progenitors restricted to granulocyte differentiation; yet, in combination with other factors such as M-CSF and GM-CSF, it can recruit a population of early stem cells into development. Therefore, it is possible that the substantial increase detected in the numbers of GM-CFC within the stromal layer of two normal and two ALL cultures treated with G-CSF might have resulted from the synergistic interactions of exogenous G-CSF with locally produced M-CSF or GM-CSF on recruiting cells from a pre–GM-CFC compartment. Accordingly, others have reported that hG-CSF added to LTBMC facilitates mainly an increase in GM-CFC content of the stromal cell layer. Furthermore, in those studies, stromal layer-associated nonadherent cells derived from G-CSF–treated LTBMC were found to have a higher proliferative activity in suspension culture than that of cells explanted from control cultures. However, by using 3HTdR suicide test, we analyzed directly the effect of G-CSF on the proliferative activity of progenitor cells located within the adherent layer. Our results showed that the cycling responsiveness of GM progenitors to G-CSF in LTBMC was ephemeral. Although a definite conclusion cannot be drawn in view of the small sample size assessed at each time point, studies in vivo demonstrated only a small increase in the cycling of GM progenitors in the marrow of patients with small cell lung carcinoma after administration of G-CSF.

In contrast, both hGM-CSF and gIL-3 exhibited stronger effects on hematopoiesis in LTBMC, enhancing significantly the production of both nonadherent mature and progenitor cells for a period of 3 to 6 weeks. The changes in these cultures in the degree of fat cell formation may well be relevant to the reduction of fat marrow, which occurs in vivo under conditions of extreme hematopoietic need or exogenous stimulation. Furthermore, the increase in GM-CFC activity in both supernatant and adherent layer fractions suggests that GM-CFC and IL-3 act on an earlier progenitor cell population to generate GM-CFC. However, as previously demonstrated in clonal studies, there might have been a subpopulation of pre–GM-CFC cells that responded only to IL-3 stimulus, since a greater increase in GM-CFC numbers was obtained in cultures with IL-3 than that seen in cultures with GM-CSF. This study has also shown that both GM-CSF and IL-3 can influence the proliferative capacity of GM progenitors within the stromal layer by markedly increasing their cycling rates. This is in agreement with the findings of high numbers of actively cycling GM-CFC in the marrow of patients being treated with GM-CSF.

In addition, the growth factors affected the production of myeloid cells in a hierarchical mode. The greatest expansion in the GM-CFC population was seen in cultures with IL-3, while higher numbers of total nonadherent cells and of progenitors more mature than GM-CFC (including the cluster-forming cells) were obtained with GM-CSF stimulation. Perhaps it is surprising that G-CSF treatment led to comparatively small increases in the production of mature and progenitor cells. These results indicate that IL-3, GM-CSF, and G-CSF increase production of myeloid cells, at least in vitro, by primarily expanding different populations of progenitor cells within the hierarchy of the hematopoietic system. However, we cannot determine whether the increase in myelopoiesis is due to the direct effects of these growth factors on their target cells or to stimulation of micronenvironmental cells to produce endogenous CSFs or other factors, which in turn stimulate myelopoiesis.

In vivo studies showed that G-CSF and GM-CSF can elicit dramatic changes in circulating leukocytes, while IL-3 failed to elevate peripheral counts. Such results contrast with the ones in LTBMC, where G-CSF was the factor that stimulated hematopoiesis to a lesser extent. This might reflect additional effects of the growth factors in vivo that cannot be accounted for in the LTBMC system, such as those influencing the marrow release of mature cells, the demargination of intravascular neutrophils, or the inhibition of their extravascular migration. However, the significance of the results reported here lies on the information regarding the proliferative changes of cells in response to G-CSF, GM-CSF, and IL-3 in a bone marrow-like environment. In addition, the data suggest that overlapping but distinct spectra of target cells respond to GM-CSF and IL-3, which conform with the synergy of their actions reported in vivo.

In untreated cultures, adherent GM progenitors exhibited an average 3HTdR value of 17.6% at a time in culture when the earliest progenitors are found to be in a steady-state level of minimal proliferation. The most likely explanation for the relatively high percentage of cells in S phase found in this study (0% to 36%) in relation to the findings of others (0% to 13%) is that no distinction was made between progenitors of low proliferative capacity (colonies of less than 500 cells), which are continuously cycling, and of those of high proliferative capacity (colonies of greater than 500 cells), which are cut out of cycle 7 days postmedia change. However, it should be noted that in the presence of growth factors, GM-CSF or IL-3 in particular, the cycling rate of adherent progenitors at a time of expectedly low indices of proliferation increases up to 93%. Hence, our data show that those factors can bypass the negative control of the stromal layer by triggering quiescent hematopoietic progenitors into S phase. The fact that the percentage of cycling cells is often higher than 50%, the level of maximal proliferation achieved by unstimulated cultures 2 to 3 days postmedia change, indicates that further proliferative response is obtained by adding growth factors to this culture system. Nevertheless, although profound increases in progenitor cell proliferation occurred, the longevity of the cultures did not change. This suggests that the primitive cells, located in the adherent cell layer (putative stem cells), are not being lost as a consequence of induced differentiation and proliferative stress. This observation supports the concept that the mechanisms which regulate the numbers of these primitive cells are under strict control of the stromal cell layer, probably involving cellular interactions.

Finally, and perhaps most intriguing is the fact that despite continuous administration, growth factors had transient effects on hematopoiesis in LTBMC. The expansion of hematopoiesis observed when factors were added to long-
established cultures suggests that progenitor cells still present after 6 weeks in culture can be induced to undergo a proliferative response. Recent studies focusing on the interactions of CSFs with other cytokines in the regulation of hematopoiesis have shown that GM-CSF can stimulate production of tumor necrosis factor (TNF) by monocytes, and that TNF is a potent inhibitor of GM-CFC production and of granulopoiesis in LTBMC. In addition, it has been reported that GM-CFC progenitors from patients receiving rhGM-CSF acquire greater sensitivity to inhibitory molecules, such as acidic ferritin and TNF. Whether or not down-modulation of CSF receptors or the acquisition of greater sensitivity to these or other inhibitory molecules (which may be produced by macrophages) are part of a mechanism by which the stromal cell layer regulates the turnover of primitive hematopoietic cells in response to added growth factors needs further investigation.

ACKNOWLEDGMENT

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Effects of recombinant human granulocyte colony-stimulating factor (CSF), human granulocyte macrophage-CSF, and gibbon interleukin-3 on hematopoiesis in human long-term bone marrow culture

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