The Regulation of Hemopoiesis in Long-Term Bone Marrow Cultures. II. Stimulation and Inhibition of Stem Cell Proliferation

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The isolation of a DNA synthesis inhibitor (NBME fraction IV) and stimulator (RBME fraction III) specific for the hemopoietic stem cell (CFU-s) from freshly isolated normal adult and regenerating murine bone marrow, respectively, has been well documented. We have utilized long-term liquid bone marrow cultures in a further analysis of the role of these factors in the regulation of CFU-s proliferation. Our results show that shortly after feeding, at a time when the cultured CFU-s are actively proliferating, high levels of the hemopoietic stem cell proliferation stimulator fraction III can be isolated from the culture medium. In contrast, the presence of essentially noncycling CFU-s found in cultures fed 8-10 days previously correlates with high levels of the hemopoietic stem cell inhibitor fraction IV. These results suggest that a certain balance between these factors determines CFU-s proliferation in the long-term cultures. In support of this, DNA synthesis in actively cycling CFU-s in the long-term cultures is inhibited for at least 3 days by the addition of excess NBME fraction IV (inhibitor). Furthermore, DNA synthesis in noncycling cultured CFU-s is stimulated for at least 5 days by the addition of RBME fraction III (stimulator).

Several in vivo studies suggest that the regulation of hemopoietic stem cell proliferation occurs at a local level, i.e., involving short-range interactions. Such studies include the shielded leg experiments, where the majority of the marrow CFU-s in the lead-shielded femur of a mouse can be found in the steady-state “Go phase" of the cell cycle at a time when the CFU-s in the rest of the animal exposed to the irradiation are actively proliferating. Phenylhydrazine (PHZ) treatment of mice results in a similar dichotomy where marrow CFU-s are rapidly proliferating while spleen CFU-s remain quiescent. (Here, “hemopoietic stem cell proliferation" refers to the uptake of thymidine by CFU-s in the S-phase of the cell cycle.) In recent analyses, it has been found that regulation of CFU-s proliferation may be mediated by the balance between opposing activities: a factor that acts as a stimulator of stem cell proliferation and another that inhibits stem cell proliferation.

Such factors that have been isolated by several workers include those obtained by Lord et al. from murine bone marrow. In these experiments, fractionating the incubated cell-free supernatant of a hemopoietic population containing actively proliferating CFU-s such as the marrow of PHZ-treated mice yields a CFU-s proliferation stimulating factor in the molecular weight range of 30,000-50,000 daltons (regenerating bone marrow extract [RBME] fraction III) which rapidly triggers NBM “resting" CFU-s (<10% 3H-TdR kill) into DNA synthesis (30%-50% 3H-TdR kill). Adult murine marrow containing minimally proliferating CFU-s yields a CFU-s proliferation inhibitor in the 50,000-100,000 dalton range (normal bone marrow extract [NBME] fraction IV) which restores the proportion of CFU-s undergoing DNA synthesis in a regenerating hemopoietic population (30%-50% 3H-TdR kill) to steady-state levels (<10% 3H-TdR kill). This effect is specific to the CFU-s population: it does not inhibit the proliferation of the CFU-c (direct descendants of CFU-s) or of the recognizable mature granulocytic or erythrocytic precursors. In addition, the reversibility of the effects of both stimulator and inhibitor has been demonstrated. Further analysis showed that while the relative concentrations of inhibitor and stimulator differ according to the proliferative state of the CFU-s in a given population, both factors are present in that population.

Investigations into the putative role of these molecules have been facilitated by the development of long-term liquid bone marrow cultures in which hemopoietic stem cell proliferation can be maintained for several months. Cultured CFU-s maintenance and differentiation into a variety of hemopoietic precursor cells is dependent on the establishment of a foundation of adherent cells. Several lines of evidence strongly support the interpretation of this adherent layer as the in vitro parallel to the in vivo marrow stromal microenvironment. Upon the establishment of the cultures proper, weekly feeding is carried out by removal and replenishment of the growth medium. This feeding, which depopulates the nonadherent cells (including CFU-s) suspended in the growth medium, seems to be recognized by the adherent “microenvironment" such that 1 day after feeding, the CFU-s lodged within the adherent layer have been triggered into DNA synthesis (30%-50% 3H-TdR...
This proliferative activity is maintained for 3–4 days postfeeding, after which a decline is seen such that 4–5 days after feeding, the $^3$H-TdR kill value has fallen to <10%, and remains at this steady-state "resting" value until the next feeding (which again triggers cultured CFU-s to proliferate actively). While cultured CFU-s undergo this cyclical proliferative response to feeding, the normally high cycling characteristics of the CFU-c population remain unaffected, indicating that in the cultures, as in vivo, regulation specific for CFU-s proliferation occurs.

We report the results of attempts at the isolation of stem cell proliferation stimulatory/inhibitory material from the long-term cultures, and the effects of inhibitor and stimulator obtained, respectively, from normal steady-state or regenerating bone marrow on long-term cultured CFU-s.

**MATERIALS AND METHODS**

**Mice**

Mice used were 8–10-wk-old (C57B1/6 × DBA/2)F1 hybrids (BDF1).

**Long-Term Liquid Bone Marrow Cultures**

Cultures were set up as described by Dexter et al. Briefly, the contents of a single femur were flushed into a plastic flask (25 cm, Falcon) containing 10 ml of growth medium composed of 25% horse serum (Flow Labs) in Fischer's medium supplemented with antibiotics. No attempt was made to obtain single-cell suspensions. Cultures were gassed with 5% CO₂, incubated at 33°C, and fed weekly by removal of half of the growth medium (5 ml) and replenishment with the same amount of fresh medium. By the third week, an adherent layer of cells had formed on the bottom of the flask and a further 10³ freshly isolated syngeneic femoral marrow cells were added. This is week 0 in terms of culture age. Weekly feeding was continued until the cultures were sacrificed. Three to four randomly chosen cultures were used per group. All experiments were performed using 2–6-wk-old cultures.

**Cell Suspensions**

Following gentle agitation, the growth medium containing nonadherent cells (2–5 x 10⁶/culture) was removed from the cultures. The remaining adherent layer cells were first washed in 5 ml of Fischer's medium. Due to the residual cell numbers (on average 10⁴/culture), this first wash was discarded. After addition of a further 3 ml of medium, adherent cells (3–7 x 10⁴/culture) were removed by means of a rubber policeman. After centrifugation (800 g for 10 min), the cells were suspended in a known volume of Fischer's medium.

**Tritiated Thymidine Suicide Assay**

$^3$H-TdR suicide assay technique of Becker et al. was used for measuring the percentage of the CFU-s and CFU-c populations in the S-phase of the cell cycle. Paired 1-ml aliquots of 5–30 x 10⁶ cells were incubated for 30 min at 37°C in the presence of either 200 μCi/ml $^3$H-TdR, of specific activity 15 Ci/m mole, or an equal volume of medium. Upon being placed on ice, a sample of cells was removed to be assayed for CFU-s. The remaining cells were washed twice in Fischer's medium containing 10% horse serum and 100 μg/ml thymidine, then assayed for CFU-c in Fischer's medium containing 10 μg/ml thymidine.

**CFU-C Assay**

Progenitor cells committed to the granulocytic/monocytic pathway, the CFU-c, were assayed by the in vitro semisolid agar technique of Bradley and Metcalf as modified by Testa. Mouse lung-conditioned medium was used as a source of colony-stimulating activity (CSA).

**Irradiation**

Mice were irradiated at a dose of 800 rad (8 Gy) on a linear accelerator producing 12 MeV electrons (dose rate 167 rad/sec).

**CFU-S Assay**

The in vivo spleen-colony forming assay of Till and McCulloch was used to detect colony-forming units in the spleen. Each potentially lethally irradiated mouse was injected in the lateral tail vein with 0.2 ml of cell suspension within 6 hr of irradiation. Ten recipients/group were used. Recipients were killed 8–10 days later, their spleen removed, and fixed in Bouin's solution. Individual spleen nodules were counted and their means calculated.

**Preparation of CFU-s Proliferation Factors**

The procedure of Lord et al. was followed. Two sources of fractions are described here: (1) freshly isolated bone marrow, (2) long-term cultured murine bone marrow. Briefly, freshly isolated normal adult porcine or murine bone marrow cells (NBME) or regenerating murine bone marrow cells (RBME from marrow of mice 5 days after irradiation with 450 rad x-rays) were incubated in saline (2 hr) and the cell-free supernatant filtered through Amicon Diaflo Ultrafiltration membranes into 5 fractions of increasing molecular weight. These fractions were then freeze-dried and stored at −20°C. NBME fraction IV, which constitutes the 50,000–100,000 dalton range, inhibits actively proliferating CFU-s. Thus, it is species-specific and CFU-s-specific. RBME fraction III (30,000–50,000 daltons) rapidly triggers resting CFU-s into DNA synthesis. Both fractions have no toxic effect on CFU-s, CFU-c, or total nucleated cells in the short-term in vitro assay. Secondly, cell-free supernatant from long-term culture growth medium was collected (cultured bone marrow extract—CBME) and fractionated similarly. Growth medium from cultures fed at least 7 days previously and known to contain "resting" CFU-s was assayed for inhibitory and stimulatory material by the short-term in vitro system. Growth medium from long-term cultures fed 1 day previously and known to contain proliferating CFU-s was collected and the cell-free supernatant fractionated. Fractions were assayed for CFU-s proliferation stimulatory and inhibitory material by the short-term in vitro assay system.

**Short-Term In Vitro Assay**

Procedure of Lord et al. was followed. Bone marrow cells from mice treated 7, 6, 4 days previously with phenylhydrazine (PHZ) or spleen cells from mice irradiated with 800 rad and reconstituted with 10⁶ normal marrow cells 8–10 days before were used as the source of actively proliferating CFU-s (30%–50% $^3$H-TdR kill) for assaying for potential stimulators. Paired aliquots of 5 x 10⁶/ml of the appropriate freshly isolated test cells were incubated at 37°C in the presence of the fraction being assayed; the period of incubation being 1.5 hr for fraction III and 4.5 hr for fraction IV. These periods of incubation have previously been shown to be optimal for the effect of the two factors to be manifest.

For assaying in long-term cultures, the desired concentration of freeze-dried NBME or RBME fraction was prepared in Fischer's medium, the solution filtered through a 0.45-μm Millipore filter and added to culture flasks within 10 min. The solutions were added
drop-wise, with the culture flask in a horizontal position, thus giving only minimal physical disturbance. Doses of NBME IV and RBME III were chosen on the basis of the minimum amount required to produce maximum inhibition and stimulation, respectively. These have previously been found to be 20–40 μg/ml/5 x 10⁶ cells NBME IV and 10 μg/ml/5 x 10⁶ cells of RBME III. Bearing in mind that inevitable preparative losses occur, it is seen from Table 1 that these doses compare favorably with apparent physiologic concentrations. The cultures were gassed with 5% CO₂, incubated at 33°C for the desired period, and sacrificed.

RESULTS

Long-Term Culture CFU-s Cycling Characteristics

Figure 1 shows that 1 day after feeding, control long-term culture nonadherent and adherent layer CFU-s are actively proliferating (30%–50% ³HThdR kill). This high proliferative level is maintained for a further 1–2 days, but then declines such that 5 days after feeding, the CFU-s exhibit a ³HThdR kill value of <10%. This low proliferative state is maintained until refeeding occurs. These results are in agreement with previous findings.²¹

Isolation of CFU-s Proliferation Factors From Long-Term Cultures

Culture growth medium was fractionated as described in Materials and Methods. Fractions from the cell-free media of 7-day-old long-term cultures were assayed for putative CFU-s proliferation inhibitory material by the short-term in vitro assay. Dose-

Table 1. Comparison of NBME IV and RBME III Efficiencies In Vitro Versus Physiologic Concentrations

<table>
<thead>
<tr>
<th>Minimum Effective</th>
<th>Yield* μg/10⁶ BM Cells</th>
<th>Minimum Effective</th>
<th>Dose μg/10⁶ BM Cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBME IV 3</td>
<td>4–8</td>
<td>RBME III 1.25</td>
<td>2</td>
</tr>
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</table>

*Quantity of freeze-dried material derived from the supernatant of a bone marrow suspension incubated for 4 hr at 37°C and fractionated on Amicon Diaflo filters.
†Dose of NBME IV or RBME III required to modify the proliferative response of CFU-s in 1-mL suspensions containing 10⁶ bone marrow cells.

response studies for these fractions are shown in Fig. 2. Clearly, 100 μg/ml CBME IV (from day-7 supernatant) inhibits the active proliferation of PHZ BM CFU-s to nonsignificant ³HThdR kill values of <10% (2% ± 4%), while 200 μg/ml of fraction III from day-7 supernatant lacks CFU-s proliferation inhibitory activity. Table 2 shows that the normally high level of CFU-c proliferation remains unaltered by various doses of either fraction III or IV. Thus, fraction IV from day-7 long-term culture growth medium contains CFU-s-specific proliferation inhibitory material. Furthermore, this material is not detectable in fraction III from the same source.

Similarly, fractions obtained from the supernatant of cultures fed 1 day previously were assayed for possible CFU-s proliferation stimulators. Figure 3 shows that 50 μg/ml of fraction III from this source triggers minimally proliferating NBM CFU-s into DNA synthesis (20%–30% ³HThdR kill), while up to 100 μg/ml of fraction IV from the same source lacks stimulator activity. Thus, stimulatory material from this source falls exclusively into the same fraction as does stimulator from regenerating marrow (at least in the dose range tested).

To ascertain whether on day 1 postrefeeding, cultures contained fraction IV inhibitory material and on day 7 postrefeeding, cultures contained fraction III stimulatory material, the respective fractions were tested for activity against regenerating and normal

Table 2. Lack of Effect of CBME III and IV From 7-Day Long-Term Culture Supernatant on Proportion of PHZ BM CFU-c in DNA Synthesis

<table>
<thead>
<tr>
<th>Dose/ml</th>
<th>Percent ³HThdR CFU-c Kill</th>
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<tbody>
<tr>
<td>No addition</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>100 μg CBME IV</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>200 μg CBME IV</td>
<td>41 ± 8</td>
</tr>
<tr>
<td>200 μg CBME III</td>
<td>43 ± 6</td>
</tr>
</tbody>
</table>

Fig. 1. CFU-s proliferation in control BDF1 long-term bone marrow cultures. (—) Nonadherent CFU-s; (······) CFU-s in adherent layer. Results represent the means ± standard error of at least five experiments.

Fig. 2. Effect of various doses of CBME III and IV from 7-day-old long-term culture supernatant on PHZ BM CFU-s proliferation. CBME, long-term cultured bone marrow extract; PHZ BM CFU-s, phenylhydrazine-treated mouse bone marrow CFU-s (i.e., cycling). Results represent the means ± standard error of at least two experiments.

Fig. 3. Dose-response relationship of CBME III or IV to PHZ BM CFU-c proliferation in vitro. Cultures were assayed for ³HThdR kill 50 hr after the last addition of dose. Results represent the means ± standard error of at least eight experiments.
bone marrow cells, respectively. The results (Table 3) show that 100 μg/ml of fraction III from day-7 cultures stimulate NBM CFU-s proliferation (28% ± 7% ³HtdR kill) and that 200 μg/ml of fraction IV from day-1 cultures inhibits PHZ BM CFU-S proliferation (9% ± 5% ³HtdR kill). It should be noted, however, that both these fractions have a lower specific activity compared with the corresponding stimulation and inhibition obtained from 1 and 7 day cultures, respectively.

**Effect of RBME III and NBME IV on Long-Term Cultures**

RBME III and NBME IV activity remain stable for at least 7 days under long-term culture conditions (in the absence of cellular elements) (unpublished data). Upon treatment of long-term cultures (fed 1 day previously) with 40 μg/ml of NBME IV, CFU-s proliferation in both the adherent layer and the suspension was reduced to <10% kill by 12-18 hr (Fig. 4) and maintained at this level for the next 3 days. Table 4 confirms the specificity of NBME IV for CFU-s by showing that during this period CFU-c proliferation was unaffected, remaining at its normally high level (30%-50% ³HtdR kill).

**DISCUSSION**

The information available from in vivo work suggests that control of both stem cell differentiation

![Graph](image-url)
induced CFU-s proliferative states are subsequently maintained for several days may be accounted for by the relatively long-term stability of the two factors under culture conditions. Hence, these studies indicate that the level of stem cell proliferation in vitro (as well as in vivo) may be modulated by the relative balance between the inhibitor and stimulator. This is a complicated problem, however, and may depend on the relative local concentrations present in the adherent layer–suspension interphase. Closer analyses of the concentration of factors produced, their purification, and the level at which their counter-balancing effect becomes apparent are in progress. Future experiments are aimed at characterizing the stimulus for production of these factors and the producing cell(s) themselves. Furthermore, possible alterations in the proposed regulatory pathway in “older” cultures undergoing hemopoietic decline are now being investigated.

REFERENCES

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