Synergistic Factors for Stem Cell Proliferation: Further Studies of the Target Stem Cells and the Mechanism of Stimulation by Interleukin-1, Interleukin-6, and Granulocyte Colony-Stimulating Factor

By Kenji Ikebuchi, James N. Ihle, Yoshikatsu Hirai, Gordon G. Wong, Steven C. Clark, and Makio Ogawa

Serial observations of blast cell colony development from spleen cells of mice treated with 5-fluorouracil (5-FU) four days earlier revealed that either form of human interleukin-1 (IL-1α or IL-1β) hastens the emergence of interleukin-3 (IL-3)–dependent blast cell colonies. This activity was essentially indistinguishable from the effect of interleukin-6 (IL-6) or granulocyte colony-stimulating factor (G-CSF) in the same system, an effect that we have ascribed previously to a shortening of the G₀ period of the dormant stem cells. We also analyzed the time courses of colony formation from cultures of day-2 post-5-FU marrow cells supported by IL-1α, IL-6, or G-CSF alone or in combination with IL-3. In the presence of IL-3, G-CSF and IL-6 but not IL-1α hastened the development of colonies and increased the numbers of multilineage colonies relative to cultures of IL-3 alone. This observation, together with our previous data from the human system, suggests that the synergistic effect of IL-1 is likely due to induction of secondary growth factors, including IL-6 and G-CSF, by accessory cells in culture. The effect of IL-6 on G₀ was confirmed by analysis of the cycling status of progenitor cells in short-term culture. While neither IL-3 nor IL-6 alone had any effect on the cycling status, the combination of factors resulted in a rapid recruitment of quiescent cells into cell cycle (within 48 hours) as represented by a twofold increase in the numbers of multipotential progenitors and a significant increase in the sensitivity of these cells to H-thymidine with high specific activity. Combinational testing of all of these synergistic factors revealed that the target cell populations for the IL-1, IL-6, and G-CSF overlap considerably, suggesting that they all may act through a common mechanism. This is further supported by our finding that cells from blast cell colonies grown in the presence of a combination of any one of the synergistic factors with IL-3 replate with higher efficiency and yield more multilineage secondary colonies than those from colonies grown in IL-3 alone. These findings provide further evidence that IL-1, IL-6, and G-CSF serve to integrate the immediate host responses to infection through augmentation of effector cells and antibody production as well as the longer term host responses by recruitment of dormant hemopoietic stem cells into active cell cycling.

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overlap considerably with those of IL-6 and G-CSF. We also present results from our analysis of the effects of the synergistic factors on the replating efficiencies of cells from blast cell colonies and on the cell cycle state of the cells incubated for two days in suspension cultures. These studies provide further insight into the mechanisms of action of the synergistic factors in controlling the cycling status of stem cells in culture.

MATERIALS AND METHODS

Factors. Murine IL-3 (approximately 5 \times 10^6 U/mg protein) was purified to homogeneity from medium conditioned by WEHI-3 cells as described previously. Chinese hamster ovary (CHO) cell-derived recombinant human erythropoietin (Ep) was a generous gift from the Pilot Development Program of the Genetics Institute, Cambridge, MA. Recombinant human IL-1a was produced in Escherichia coli harboring expression plasmid pTR IL-1a and was purified to homogeneity by sequential fractionation with high-performance liquid chromatography on columns of TSK DEAE-5PW, TSK SP-5PW, and TSK G2000SW (Toyo Soda Manufacturing Co, Ltd, Japan). The purified preparation showed a molecular weight (mol wt) of 18,300 daltons and an isoelectric point of 5.0. The specific activity of IL-1a was 2 \times 10^8 U/mg determined by the lymphocyte activation assay. Recombinant human IL-18 was purified from E coli transfected with a plasmid containing cDNA encoding human IL-1b as described previously. Recombinant human IL-6 was purified from the conditioned media (CM) of cos-1 cells transfected with the plasmid p CSF-309 as described previously. Its specific activity was 8.8 \times 10^7 U/mg protein according to the murine bone marrow assay. Purified recombinant human G-CSF expressed in E coli with specific activity of 10^8 U/mg was a generous gift from Dr Lawrence M. Souza, AMGen, Thousand Oaks, CA. For preparation of WEHI-3 CM, the supernatants of cultures of WEHI-3 cells were concentrated 20x by a Pellicon cassette system (Millipore Corp, Bedford, MA) equipped with a polysulfone cassette with a 10,000-mol wt pore size. The concentrated CM was added to cultures at a final concentration of 1% (vol/vol).

In addition, several other lymphohemopoietic factors were tested for their ability to work synergistically with IL-3. Cos-derived human recombinant granulocyte-macrophage-CSF (GM-CSF), macrophage-CSF (M-CSF), and IL-5 were generous gifts from the Drug Development Program, Genetics Institute. 1:1,000 dilutions of GM-CSF, M-CSF, and IL-5 produced maximal colony formation in culture (data not shown). Cos-recombinant murine IL-4 and yeast-derived recombinant murine GM-CSF with a specific activity of 10^8 U/mg were gifts from Dr K. Arai, DNA Research Institute of Molecular and Cellular Biology, Palo Alto, CA. Murine CSF-1 (M-CSF) (approximately 8 \times 10^6 U/mg protein) purified to homogeneity from serum-free mouse L-cell CM was a gift from Dr F. R. Stanley, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY. Recombinant human and murine IL-2 were purchased from the Genzyme Corp, Boston.

Clonal cell culture. Ten- to 15-week-old female BDF, mice were obtained from Simonsen's Laboratories, Gilroy, CA. Single-cell suspensions were prepared from pooled spleens of three mice. S-FU (Adria Laboratories, Inc, Columbus, OH) was administered intravenously (IV) through the tail vein of the mice in a dosage of 150 mg/kg body weight. Spleen cells were harvested four days later, and bone marrow cells were harvested two days after S-FU injection.

Methylcellulose cell culture was carried out in 35-mm Lux suspension culture dishes (#5221R, Miles Laboratories, Inc, Naperville, IL). One milliliter of culture consisted of 5 \times 10^9 spleen cells from normal mice, 1 \times 10^9 day-4 post-S-FU spleen cells or 5 \times 10^6 day-2 post-S-FU marrow cells, α-medium (Flow Laboratories, Inc), 1.2% 1,500 centipoise methylcellulose (Fisher Scientific Co, Ncorss, GA), 30% fetal calf serum (Flow), 1% deionized bovine serum albumin (Sigma Chemical Co, St Louis), 1 \times 10^{-4} mol/L mercaptoethanol (Eastman Organic Chemicals, Rochester, NY), and hemopoietic factors. The standard concentrations of hemopoietic factors used in our culture were as follows: murine IL-3, 200 U/mL; human Ep, 2 U/mL; human IL-1a, 2 ng/mL; human IL-1b, 2 ng/mL; human IL-6, 10 ng/mL, and human G-CSF, 1,000 U/mL. Dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO2.

In routine experiments, colony types were determined by in situ observation on an inverted microscope according to the criteria described previously. Abbreviations for colony types are GM, granulocyte-macrophage colonies; B, erythroid bursts; M, megakaryocyte colonies; EM, erythocyte-megakaryocyte colonies; GEM, granulocyte-erythrocyte-macrophage colonies; GMM, granulocyte-macrophage-megakaryocyte colonies; GMG, granulocyte-erythrocyte-megakaryocyte colonies; Mast, mast cell colonies; and Bl, blast cell colonies.

1H-thymidine suicide study. Spleen cells from 5-FU-treated mice in 5 \times 10^6 cells/mL concentration were incubated in 35-mm Lux suspension culture dishes for two days in the presence of nucleoside-free α-medium containing 10^{-4} mol/L mercaptoethanol, 1% deionized bovine serum albumin, 10^{-7} mol/L sodium selenite (Sigma), 300 μg/mL fully iron-saturated human transferrin (approximately 98% pure, Sigma), 16 μg/mL soybean lecithin (Sigma), 9.6 μg/mL cholesterol (Calbiochem, La Jolla, CA), and growth factors. One hour before the end of incubation, 20 μCi methyl-3H-thymidine (42 Ci/mmol, Amersham Corp, Arlington Heights, IL) or an equivalent concentration of unlabeled thymidine (Sigma) was added to cultures. At the conclusion of incubation, cells were harvested, washed twice with α-medium containing 100 μg/mL unlabeled thymidine, and plated in methylcellulose culture containing 30% fetal calf serum, eight nucleosides at 10 mg/L concentration, 200 U/mL IL-3, 10 ng/mL IL-6, 1,000 U/mL G-CSF, 2 ng/mL IL-1b, and 2 U/mL Ep for measurement of the surviving fraction of progenitors. Cells were plated at a concentration of 1 \times 10^5 cells per dish based on cell counting before exposure to thymidine, and incubation was carried out for 14 days.

RESULTS

Colony formation supported by IL-1α and IL-1β. Spleen cells from normal mice cultured in the presence of either IL-1α or IL-1β consistently yielded low numbers of hemopoietic colonies, typically at about 20% of the level seen in IL-3-supported cultures (Table 1). Optimal colony formation was achieved in the presence of 0.2 ng/mL of either IL-1α or IL-1β, and no increase in the colony number was observed, even at concentrations of 200 ng/mL (2,000 ng/mL in the case of IL-1β). Although the IL-3–supported cultures yielded GMM, mast cell, and blast cell colonies in addition to GM colonies, in the presence of either form of IL-1 alone, only GM colonies were obtained. The frequency of GM colony formation in the IL-1-containing cultures was approximately 50% of that obtained in the presence of IL-3.

To distinguish between direct and indirect effects of the IL-1 on GM colony formation, we replated blast cells from primary blast cell colonies grown in the presence of WEHI-3 CM into secondary cultures containing either IL-1 (2 to 200 ng/mL) alone or IL-3 (200 U/mL). The blast cells used in
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**Table 1. Effects of Differing Concentrations of IL-1α or IL-1β on Colony Formation by Spleen Cells From Normal Mice**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>GM</th>
<th>M</th>
<th>GMM</th>
<th>Mast</th>
<th>B1</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>IL-1α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>200 ng/mL</td>
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<td>0</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10±3</td>
</tr>
<tr>
<td>0.02 ng/mL</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2±1</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>2,000 ng/mL</td>
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<td>0</td>
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<td>10±2</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
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<td>10±3</td>
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<tr>
<td>2 ng/mL</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9±1</td>
</tr>
<tr>
<td>0.2 ng/mL</td>
<td>8±3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8±3</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 U/mL</td>
<td>23±9</td>
<td>2±2</td>
<td>4±2</td>
<td>18±4</td>
<td>1±1</td>
<td>48±6</td>
</tr>
<tr>
<td>No factor</td>
<td>1±1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1±1</td>
</tr>
</tbody>
</table>

Data represent mean ± SD of the number of colonies in quadruplicate cultures, each containing 5 x 10^6 spleen cells from normal mice.

This experiment were isolated on day 16 from cultures seeded with day-4 post-5-FU spleen cells. A total of 11 colonies, each containing 42 to 500 cells (mean, 247 cells/colony) were harvested, pooled, and plated in the presence of growth factors at 70 cells/mL, a low cell density that greatly diminishes the possibility that accessory cell-factor production might influence the progenitor cell growth in culture. IL-1 did not support colony formation; only a few GM clusters containing fewer than 50 cells were observed (data not shown). In contrast, IL-3-supported cultures yielded significant numbers of GM and GMM colonies, demonstrating that the target cell population contained significant numbers of colony-forming cells. These results indicate that although IL-1 may have some limited capacity to support several divisions by a few progenitors, most of the GM colony formation in the IL-1-supported culture is likely to result from IL-1 stimulation of growth factor production by accessory cells in culture.

**Serial observation of formation of multipotential blast cell colonies supported by IL-3 and combinations of IL-3 and IL-1.** We have performed serial observations (mapping studies) on the formation of individual multipotential blast cell colonies in culture to examine in detail the effects of IL-1 on the kinetics of colony formation supported by IL-3. For these studies, day-4 post-5-FU spleen cells were plated in the presence of either IL-3 or IL-1 alone or a combination of these factors, and the emergence and subsequent proliferation and development of the individual blast cell colonies were recorded daily as described previously. Similar analysis has shown that both IL-6 and G-CSF act synergistically with IL-3 through shortening the G0 period of the quiescent stem cells. Earlier, Stanley et al demonstrated the synergistic effect of H-1 using murine marrow cells harvested two days after injection of 5-FU. We have confirmed, using BDF1 mice, that the nadir of the multipotential progenitors in bone marrow occurs two days after injection of 5-FU. Therefore, we compared the synergistic ability of IL-1α with that of IL-6 and G-CSF on colony formation from day-2 post-5-FU murine marrow cells. Marrow cells harvested two days after 5-FU injection were plated at 5 x 10^6 cells/mL in methylcellulose culture containing 2 U/mL Ep and 200 U/mL IL-3, with or without one of the synergistic factors. The time course of colony formation in the different cultures is presented in Fig 2. No colony formation was seen in cultures stimulated by IL-1α alone. IL-6 alone supported the formation of only one GM colony. In cultures supported by IL-3 or G-CSF alone, colony formation reached its maximal
plateau level on day 19. In the presence of IL-3 and one of the synergistic factors, colony formation was significantly hastened and the maximal plateau levels were reached by day 13. The synergistic ability of G-CSF and IL-6 on multipotential progenitors were similar, as manifested by 18 and 17 GEMM colonies. The synergistic ability of IL-1α was significantly weaker than that of IL-6 or G-CSF, and the combination of IL-1α and IL-3 supported the same number of GEMM colonies as IL-3 alone. One interpretation of this result is that the synergistic activity of IL-1α on multipotential cells is indirect and that the secondary production of growth factors in situ by accessory cells in the bone marrow is significantly less than that in the spleen cells.

**Effects of IL-6 on the sensitivity of stem cells to 3H-thymidine.** Our mapping studies of the blast cell colony development (Fig 1) and time course analysis of colony formation from day-2 post-5-FU marrow cells (Fig 2) indicated that IL-1, IL-6, and G-CSF in the presence of IL-3 shorten the G0 period of the dormant hemopoietic stem cells. To further test this conclusion, we used 3H-thymidine "suicide" experiments to examine the cycling status of progenitor cells that have been exposed to IL-3 alone, IL-6 alone, or a combination of IL-3 and IL-6. In these studies, day-4 post-5-FU spleen cells were incubated at a concentration of 5 x 10⁶ cells/mL in serum-free culture for 47 hours in the presence of the respective factors and were then exposed to 3H-thymidine with high specific activity for one hour. The numbers of surviving colony-forming cells were finally determined by plating in a secondary methylcellulose culture in the presence of an optimal combination of hemopoietic growth factors. As summarized in Fig 3, neither IL-3 alone nor IL-6 alone affected the final yield of CFU-GEMM in the secondary cultures nor did they affect the sensitivity of these progenitor cells to 3H-thymidine. These results demonstrated that the CFU-GEMM isolated from 5-FU-treated animals remained in G0, even after exposure for two days to the individual growth factors. However, the progenitor cells incubated for two days in the presence of both IL-3 and IL-6 yielded almost twice as many CFU-GEMM when plated in secondary culture, and this increase was abrogated by brief exposure of the primary culture to 3H-thymidine with high specific activity (Fig 3). These data indicate that many of the quiescent CFU-GEMM leave G0 and enter a proliferative, 3H-thymidine-sensitive phase of the cell cycle during a two-day exposure to a combination of IL-3 and IL-6. Similar
effects were noted with other types of colony-forming cells, most of which yielded GM colonies. This observation was consistent with previous studies that have demonstrated that effects were noted with other types of colony-forming cells, each derived from suspension culture in a petri dish seeded with \(5 \times 10^6\) cells. "No incubation" group indicates an additional control group in which cells were harvested for analysis of progenitor numbers immediately after plating in suspension culture without factors.

**Effects of combinations of synergistic factors.** To analyze the target cell populations that respond to the synergistic effects of the different cytokines, we examined the cloning efficiency of progenitor cells obtained from day-4 post-5-FU spleens in the presence of IL-3 in all possible combinations with IL-1β, IL-6, and G-CSF. As summarized in Table 2, none of the combinations of synergistic factors yielded more colonies than that obtained with IL-3 alone. These data suggest that the target cell populations for each of the synergistic factors overlap significantly and may be essentially identical.

**Effects of synergistic factors on the replating potentials of blast cell colonies.** To further characterize the effects of the synergistic factors on blast cell colony formation, we have compared the replating efficiencies of blast cell colonies grown in the presence of IL-3 alone with those grown in the presence of the individual synergistic factors in combination with IL-3. In each case, \(1 \times 10^6\) spleen cells from 5-FU–treated mice were cultured in the various growth factor combinations, and newly appearing, small blast cell colonies consisting of 40 to 100 cells were picked and replated in secondary culture containing WEHI-3 CM and Ep. In cultures containing IL-3 alone, the small blast cells colonies were identified between days 5 and 15, while in the cultures containing one of the synergistic factors, colony formation was hastened, and the blast cell colonies were identified between days 5 and 8 of incubation. On days 10 to 12 of secondary culture, the number and type of colonies was estimated in situ using criteria described previously. In these studies, approximately 70% of the total blast cell colonies in each group yielded at least one GEMM colony in secondary culture. The majority of the remaining blast cell colonies were restricted to GM lineages. The data from the blast cell colonies that revealed GEMM expression in secondary culture are presented in Table 3. Of the blast cell colonies derived from the cultures containing IL-3 alone, the average replating efficiency of the individual colonies was approximately 30%. In comparison, the average replating efficiency of the colonies picked from the cultures supplemented with any one of the three synergistic factors in addition to IL-3 was approximately 50%. Although there was considerable variation between the replating efficiencies of individual colonies within each group, the difference between the blast cell colonies picked from the cultures containing IL-3 alone and those with IL-3 in combination with a synergistic factor was statistically significant (\(P < .05\) by unpaired \(t\) test). The relative incidence of GEMM colonies

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**Table 2. Effects of Combinations of Synergistic Factors on Colony Formation by Spleen Cells From 5-FU–Treated Mice**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>GM</th>
<th>M</th>
<th>GEM</th>
<th>GMM</th>
<th>GEMM</th>
<th>Mast</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3 (200 U/mL)</td>
<td>4 ± 1</td>
<td>2 ± 1</td>
<td>1 ± 1</td>
<td>0 ± 1</td>
<td>11 ± 2</td>
<td>2 ± 1</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>G-CSF (1,000 U/mL)</td>
<td>6 ± 1</td>
<td>0 ± 1</td>
<td>0 ± 1</td>
<td>0 ± 1</td>
<td>5 ± 2</td>
<td>0 ± 1</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>IL-6 (10 ng/mL)</td>
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<td>1 ± 1</td>
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<td>5 ± 2</td>
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<td>11 ± 2</td>
</tr>
<tr>
<td>IL-1β (2 ng/mL)</td>
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<td>0 ± 1</td>
<td>0 ± 1</td>
<td>1 ± 1</td>
<td>0 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>IL-3 + G-CSF</td>
<td>7 ± 1</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>0 ± 1</td>
<td>9 ± 2</td>
<td>2 ± 1</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>IL-3 + IL-6</td>
<td>7 ± 3</td>
<td>2 ± 1</td>
<td>1 ± 1</td>
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<td>9 ± 2</td>
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<td>19 ± 5</td>
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<td>2 ± 1</td>
<td>2 ± 1</td>
<td>11 ± 2</td>
<td>1 ± 1</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>IL-3 + G-CSF + IL-6</td>
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<td>2 ± 1</td>
<td>1 ± 1</td>
<td>0 ± 1</td>
<td>10 ± 1</td>
<td>1 ± 1</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>IL-3 + G-CSF + IL-1β</td>
<td>8 ± 2</td>
<td>1 ± 1</td>
<td>2 ± 1</td>
<td>0 ± 1</td>
<td>8 ± 3</td>
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<td>19 ± 5</td>
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<td>IL-3 + IL-6 + IL-1β</td>
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<td>1 ± 1</td>
<td>2 ± 1</td>
<td>1 ± 1</td>
<td>9 ± 3</td>
<td>0 ± 1</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>IL-3 + G-CSF + IL-6 + IL-1β</td>
<td>7 ± 2</td>
<td>1 ± 1</td>
<td>3 ± 2</td>
<td>1 ± 1</td>
<td>11 ± 1</td>
<td>0 ± 1</td>
<td>21 ± 2</td>
</tr>
</tbody>
</table>

Data represent mean ± SD of the number of colonies in quadruplicate cultures, each containing \(1 \times 10^6\) spleen cells from 5-FU–treated mice. Experiments were carried out in the presence of 2 U/mL Ep in order to allow erythroid expression.
was not affected by inclusion of the synergistic factors in the primary culture. However, the average yield of GEMM colonies obtained per blast cell colony was significantly higher when blast cell colonies were grown in the presence of IL-3 and any one of the synergistic factors than in colonies from cultures containing only IL-3 ($P < .05$ by $t$ test). These results demonstrated that the presence of a synergistic factor in culture increases the frequency of cells within the blast cell colony that are capable of yielding either multipotential or monopotential colonies in secondary culture.

**Examination of other lymphohemopoietic cytokines for synergism with IL-3.** None of the other recombinant lymphohemopoietic cytokines revealed any synergy with IL-3 in support of colony formation by spleen cells from post-5-FU-treated mice. The factors tested included human Ep (2 U/mL), GM-CSF (1:1,000), M-CSF (1:1,000), IL-2 (100 U/mL), IL-5 (1:1,000), IL-6 (1:1,000), and murine GM-CSF (100 U/mL), M-CSF (200 U/mL), IL-2 (100 U/mL), and IL-4 (100 U/mL). None of these factors hastened the time course of IL-3-supported colony formation.

**DISCUSSION**

The growth and development of early multipotential progenitors in culture is absolutely dependent on the presence of a multilineage CSF such as IL-3. However, both in normal mice as well as in mice four days after treatment with 5-FU, most of the hemopoietic stem cells are in the $G_0$ phase of the cell cycle, and IL-3 is incapable of altering this dormant state. In cultures with IL-3 alone, early blast cell colonies emerge randomly, presumably indicating that the clonogenic cells in culture leave $G_0$ after random time intervals and grow in response to IL-3. We have found, however, that four cytokines, IL-1α, IL-1β, IL-6, and G-CSF all act in synergy with IL-3 in supporting the growth of these early progenitors and that each of these factors, in combination with IL-3, hastens the emergence of blast cell colonies by shortening the $G_0$ period of the cell cycle. None of the other cytokines tested including IL-2, IL-4, IL-5, GM-CSF, M-CSF, and Ep revealed similar effects in combination with IL-3.

Although IL-1, IL-6, and G-CSF all behave identically as synergistic factors in the blast cell colony development from day-4 post-5-FU spleen cells, differences have emerged in comparisons of the factors in cultures of day-2 post-5-FU bone marrow cells. In the presence of IL-3, both G-CSF and IL-6 but not IL-1α significantly hastened the development of all colonies and increased the numbers of GEMM colonies relative to cultures with IL-3 alone. We have reported previously that human IL-6 and G-CSF but not IL-1α display synergy in support of IL-3-dependent human blast cell colony formation from enriched human progenitors (nonadherent CD34-positive marrow cells). Because the IL-1 is a potent inducer of IL-6 and G-CSF expression by fibroblasts, endothelial cells, and other cells, and because CD34-positive cells are virtually free of these cells, we concluded that the synergistic effects of IL-1 are indirectly mediated by secondary production of cytokines by accessory cells present in culture. Further proof of the indirect nature of the synergistic activity of IL-1 will require use of appropriate immunologic reagents.

Our mapping studies on the appearance of blast cell colony formation have led to the conclusion that at least part of the interactions between IL-3 and the synergistic factors IL-1, G-CSF, or IL-6 result from a shortening of the $G_0$ period of dormant stem cells. We have provided further support for this conclusion by examining the effects of IL-3 and IL-6 on hemopoietic colony-forming cells in serum-free suspension culture for two days. In this study, IL-3, and to a lesser degree, IL-6, as single agents promoted the survival but not proliferation of the cultured progenitor cells. However, when tested in combination, IL-3 and IL-6 allowed a twofold expansion of both multipotential (CFU-GEMM) and committed (CFU-GM) progenitors during the two-day culture period. Exposure of the cells to $^3$H-thymidine with high specific activity at the end of the culture period confirmed this finding; cells cultured for two days in either factor alone or in the absence of factors were resistant to the exposure to $^3$H-thymidine, while the progenitors grown in the presence of both IL-3 and IL-6 were not. The finding that IL-6 alone does not render the cultured cells sensitive to the radioactive nucleotide is consistent with the concept that this molecule serves as a competence factor in the proliferation of progeni-
tor cells, perhaps promoting the transition out of G0 but not all the way to the S phase.21,24,27 The later, proliferative stages of the cell cycle may require the presence of a progression factor, IL-3, which by itself is capable of supporting proliferation of cells once they leave G0. Although we have not yet tested G-CSF in this system, our mapping studies suggest that it behaves identically to IL-6 in shortening the G0 period of the quiescent stem cells, and it is likely also to act as a competence factor in support of IL-3-dependent blast cell proliferation.

In addition to their effects on the cycling status of quiescent stem cells, our replating experiments have indicated that the synergistic factors in combination with IL-3 result in increased numbers of both multipotential and monopotential colony-forming cells within the developing blast cell colony. Previously, we and others have presented evidence for the stochastic nature of the decision-making process of the stem cell in choosing between self-renewal and terminal differentiation.28,29 Similarly, micromanipulation studies of individual blast cells have provided evidence that the decision process of the developing progenitor in choosing between the different lineages is also stochastic.30,31 The synergistic factor enhancement of the replating efficiencies of cells within the developing blast cell colony that we have observed could represent an alteration in the distributional parameters (p) of the individual cells such that the possibilities of self-renewal and lineage commitment are no longer equally probable. However, this effect might also be explained by the alteration in the kinetics of cell growth within the blast cell colony that would result from the shortening of the G0 period of the early stem cells. Because repetitive replating of blast cell colonies is possible, self-renewal of the blast cell colony-forming cell must occur to some extent during blast cell colony formation.13 By shortening the time the individual blast cell colony-forming cells remain in G0, the synergistic factors in combination with IL-3 should favor multiplication of early colony-forming cells within the developing blast cell colony without affecting the rate at which the maturing cells no longer yield colonies on secondary replating. This effect would yield more divisions of the primitive progenitors in the developing colony, thereby yielding a higher secondary replating efficiency and an increase in the total number of multipotential and monopotential colonies. In addition, both IL-627 and G-CSF in the presence of serum support the proliferation of the late committed murine GM progenitors, an effect that could also contribute to the increase in absolute numbers of CFU-GM in the blast cell colonies.

Although IL-6 and G-CSF are distinct molecules with separate receptors,42 they appear to be distantly related in evolution.29,30 This relationship is evident in the two proteins and also in the conservation of the overall structures of their genes.31 It is tempting to speculate that these genes have evolved to assume related but different functions in host defense that serve to integrate the immune response with the primary defense against infection mediated largely by the neutrophil. Following infection, the host responds with a higher level of immunoglobulin synthesis, a response that may in large part be due to IL-6 production. At the same time, the rate of neutrophil production increases dramatically, presumably due to the release of G-CSF by various cell types at the site of infection. The common ability of IL-6 and G-CSF to interact with IL-3 in support of stem cell proliferation would result in enhanced production of hemopoietic and possibly "lymphopoietic" progenitors and thereby render the overall response more effective. The ability to study the effects of these different cytokines in animal models of infection should provide new insights into these interactions.

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REFERENCES

13. Messner HA, Yamasaki K, Jamal N, Minden MM, Yang...


Synergistic factors for stem cell proliferation: further studies of the target stem cells and the mechanism of stimulation by interleukin-1, interleukin-6, and granulocyte colony-stimulating factor

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