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Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors

(hemopoietic stem cells/clonal cell culture/growth factors)

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ABSTRACT **Interleukin-6 (IL-6, also known as B-cell stimulatory factor 2/interferon β2) was previously shown to support the proliferation of granulocyte/macrophage progenitors and indirectly support the formation of multilineage and blast cell colonies in cultures of spleen cells from normal mice. We report here that IL-3 and IL-6 act synergistically in support of the proliferation of murine multipotential progenitors in culture. The time course of total colony formation by spleen cells isolated from mice 4 days after injection of 5-fluorouracil (150 mg/kg) was significantly shortened in cultures containing both lymphokines relative to cultures supported by either of the two factors. Serial observations (mapping) of individual blast cell colonies in culture revealed that blast cell colonies emerged after random time intervals in the presence of IL-3. The average time of appearance in IL-6 alone was somewhat delayed, and in cultures containing both factors the appearance of multilineage blast cell colonies was significantly hastened relative to cultures grown in the presence of the individual lymphokines. In cultures of day-2 post-5-fluorouracil bone marrow cells, IL-6 failed to support colony formation; IL-3 alone supported the formation of a few granulocyte/macrophage colonies, but the combination of factors acted synergistically to yield multilineage and a variety of other types of colonies. In this system, IL-1α also acted synergistically with IL-3, but the effect was smaller, and no multilineage colonies were seen. Together these results indicate that IL-3 and IL-6 act synergistically to support the proliferation of hemopoietic progenitors and that at least part of the effect results from a decrease in the G_0 period of the individual stem cells.**

Hemopoiesis is a complex developmental process that is regulated in part by the interactions of many different polypeptide hormones with a variety of different hemopoietic progenitor and stem cells. The first of these hormones to be identified, erythropoietin (Epo), was shown to be the physiological regulator of erythropoiesis (1, 2). Subsequently, a family of hemopoietic growth factors known as colony-stimulating factors (CSFs) was identified through clonal culture of progenitor cells (for reviews, see refs. 3 and 4). During the past two decades, the biochemical and biological properties of the CSFs have been the subject of active investigation, recently culminating in the molecular cloning of the genes encoding each of the CSFs and thereby enabling a detailed analysis of the properties of the respective recombinant proteins (3, 4). These studies have revealed that interleukin 3 (IL-3) supports the proliferation of multipotential hemopoietic progenitors, whereas macrophage CSF and granulocyte CSF appear to be limited to supporting proliferation of progenitors committed to the macrophage and granulocyte lineages, respectively (3). The target cells of granulocyte/macrophage CSF (GM-CSF) include bipotent progenitors capable of developing into either macrophages or granulocytes and a population of multipotent progenitors as well (5, 6). The potential therapeutic importance of several hemopoietic factors is already under investigation: intermittent injections of Epo have successfully corrected the intractable anemia of patients with chronic renal failure (7), and periodic injections or continuous infusions of GM-CSF (8, 9) or granulocyte CSF (10, 11) into animals or patients have significantly elevated the blood leukocyte counts, thereby demonstrating potential for the treatment of patients with various forms of bone marrow suppression.

In addition to the CSFs and Epo, several other cytokines originally identified because of their activities on lymphoid targets have proven to have important effects on myeloid cells as well. IL-4, originally identified by its ability to enhance proliferation of B lymphocytes (12), is a potent mast cell growth factor (13, 14). IL-5 was originally thought to be a specific B-cell growth factor (15) but has subsequently been found to be identical to eosinophil-differentiation factor (16). IL-6 was identified independently as an interferon β2 (17), B-cell stimulatory factor 2 (18), a 26-kDa inducible protein in fibroblasts (19), and as a growth factor for murine GM progenitors (G.G.W., S.C.C., K.I., M.O., J. S. Witek-Giannotti, P. A. Temple, R. Kriz, C. Ferenz, R. M. Hewick, unpublished data). The effects of IL-6 on the proliferation of murine GM progenitors were shown to be direct, but IL-6 was also found to indirectly support the formation of several types of multilineage hemopoietic colonies, including those derived from early blast cells. We now report that IL-6 acts synergistically with IL-3 in supporting the proliferation of multipotential progenitors in culture. These results provide further evidence that IL-6 plays an important role in the production of hemopoietic cells, possibly at the level of the primitive pluripotent stem cell.

MATERIALS AND METHODS

Cell Preparation. Ten- to 15-week-old female BDF1 mice were obtained from Simonsen Laboratories (Gilroy, CA). Single-cell suspensions were prepared from pooled spleens or femurs of three mice. 5-Fluorouracil (5-FUra) (Adria Laboratories, Columbus, OH) was administered i.v. through the tail vein of the mice in a dose of 150 mg/kg of body weight (20, 21).

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Abbreviations: IL, interleukin; Epo, erythropoietin; 5-FUra, 5-fluorouracil; CSF, colony-stimulating factor; H-1, hemopoietin-1; GM, granulocyte/macrophage colonies; GMM, granulocyte/macrophage/megakaryocyte colonies; GEMM, granulocyte/erythrocyte/macrophage/megakaryocyte colonies; U, units.

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Factors. The source of IL-6 was a medium conditioned by COS-1 cells transfected with cDNA coding for human IL-6 (18) as described elsewhere (22). Murine IL-3 ($\approx 5 \times 10^6$ units (U)/mg of protein) was purified to homogeneity from medium conditioned by WEHI-3 cells as described previously (23). Recombinant human IL-1α was produced in *Escherichia coli* harboring expression plasmid ptrpIL-1α, which will be described in detail elsewhere. IL-1α was purified to homogeneity by sequential fractionation with high-performance liquid chromatography on columns of TSK gels DEAE-5PW, SP-5PW, and G2000 SWG (Toyo Soda Manufacturing). The purified preparation showed a molecular size of 18,300 daltons and an isoelectric point of 5.0. The specific activity of IL-1α was 2×10^7 U/mg determined by the lymphocyte activation assay (24). Partially purified human urinary Epo with 370 units of activity per mg was provided by M. Kawakita (Kumamoto University Medical School, Kumamoto, Japan).

Clonal Cell Culture. Methylcellulose cell culture was done in 35-mm Lux suspension culture dishes (5221R; Miles Laboratories). One milliliter of culture consisted of 5×10^5 spleen cells from normal mice, 1×10^6 spleen cells from 5-FUra-treated mice, or 5×10^4 bone marrow cells from 5-FUra-treated mice; α medium (Flow Laboratories); 1.2% 1500 centipoise methylcellulose (Fisher Scientific); 30% fetal calf serum (Flow Laboratories); 1% deionized bovine serum albumin (Sigma); 1×10^{-4} M mercaptoethanol (Eastman); and hemopoietic growth factors. Dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2.

In routine experiments, colony types were determined on day 16 of incubation by *in situ* observation on an inverted microscope according to the criteria described previously (25). Abbreviations for colony types include the following: granulocyte/macrophage colonies (GM); granulocyte/erythrocyte/macrophage colonies (GEM); granulocyte/macrophage/megakaryocyte colonies (GMM); and granulocyte/erythrocyte/macrophage/megakaryocyte colonies (GEMM) (26).

RESULTS

Analysis of IL-6-Dependent Hemopoietic Colony Formation. IL-6 by itself actively supports colony formation by hemopoietic progenitors derived from the spleens of normal mice as summarized in Table 1. The colonies found in the IL-6-containing cultures were predominantly of the GM type, although significant numbers of blast cell and multilineage colonies were also routinely observed. Analysis of several different dilutions of the IL-6-containing COS-1 cell supernatant revealed that a 1:1000 dilution yielded optimal colony formation, whereas a significant diminution in colony number was seen at a 1:10,000 dilution. We therefore routinely used the recombinant IL-6 at a final dilution of 1:1000 for subsequent experiments. Under these conditions, IL-6 proved better than optimal concentrations of IL-3 in supporting blast cell colonies, but IL-3 was more effective as a stimulus of many other colony types including GM, GMM, and mast cell colonies.

Enhancement of the Rate of Colony Formation by the Combination of IL-3 and IL-6. Earlier, we have shown that IL-3 supports formation of multipotential blast cell colonies by spleen cells of 5-FUra-treated mice (27). Because IL-6 also supported the proliferation of early primitive progenitors (Table 1), we analyzed in detail the time course of appearance of multilineage colonies supported by either factor alone or by a combination of the two factors at saturating concentrations. To enrich for the blast cell colony progenitors, the mice were injected with 5-FUra (150 mg/kg) 4 days before sacrifice. The spleen cells were plated in culture in the presence of Epo (2.0 U/ml) and either IL-3, IL-6, or a combination of both factors. Daily examination of the plates revealed that the colonies grown in the presence of IL-3 appeared earlier than those grown in the presence of IL-6. The number of colonies in IL-3-containing cultures attained a plateau level of 62 colonies per four plates on day 14, whereas the number in IL-6-containing cultures did not reach an apparent plateau value of 50 until day 23 (Fig. 1*A*). Surprisingly, the combination of both factors resulted in a significant enhancement of the rate of appearance of colonies; the apparent plateau level of colony number, equivalent to that achieved with IL-3 alone, was attained by day 9. Very similar results were obtained for the kinetics of appearance of GEMM colonies in these cultures (Fig. 1*B*). GEMM colonies appeared earlier in the presence of IL-3 than in the presence of IL-6, but the combination of both factors significantly hastened the rate of colony appearance relative to that observed with either factor alone.

Serial Observations on Blast Cell Colony Formation by Day-4 Post-5-FUra Spleen Cells. We have previously described methods for serially mapping the development of individual blast cell colonies supported by pokeweed mitogen spleen-conditioned medium (21) or IL-3 (27) from spleen cells isolated 4 days post-5-FUra injection. These mapping studies strongly suggested that the multipotential cells that yield blast cell colonies are normally dormant in culture and that they enter the cell cycle randomly, concomitantly with the acquisition of growth factor responsiveness. We have used this method to analyze the effects of IL-3 and IL-6 on the time of appearance of blast cell colonies in culture. In these experiments, 1×10^6 day-4 post-5-FUra spleen cells were plated per dish, and the emergence of new blast cell colonies and the subsequent proliferation and differentiation of these colonies were recorded daily. The results are presented in Fig. 2. In total, 18, 15, and 16 blast cell colonies were identified, which later revealed GEMM lineages in the cultures containing IL-3, IL-6, or the combination of the two factors, respectively. The serial observations of these colonies demonstrated that the development of multipotential blast cell colonies was earlier in the presence of IL-3 than in IL-6. The average number of days required for colonies to reach 100 cells was calculated to be 10.3 ± 2.0 days in the IL-3-containing cultures and 13.5 ± 3.3 days in IL-6-containing cultures. In

Table 1. Colony formation by spleen cells of normal mice

Growth factor	GM	B	M	EM	GEM	GMM	GEMM	Mast	Bl	Total
IL-6, 1:100	12 ± 3	0	3 ± 1	0	0	2 ± 1	0	0	3 ± 1	19 ± 2
1:1000	12 ± 2	0	2 ± 1	0	0	1 ± 1	0	0	2 ± 2	18 ± 5
1:10,000	4 ± 1	0	0	0	0	0	0	0	0	4 ± 1
IL-3	22 ± 3	0	4 ± 1	0	0	6 ± 2	0	16 ± 5	0	47 ± 7
IL-3 + Epo	20 ± 3	27 ± 3	2 ± 1	1 ± 1	1 ± 1	3 ± 1	3 ± 1	14 ± 4	0*	70 ± 4
Mock CM	3 ± 1	0	0	0	0	0	0	0	0	3 ± 1
None	2 ± 2	0	0	0	0	0	0	0	0	2 ± 2

Data represent mean ± SD of the number of colonies in quadruplicate cultures each plated with 5×10^5 cells. B, erythroid bursts; M, megakaryocyte colonies; EM, erythrocyte/megakaryocyte colonies; Mast, mast cell colonies; Bl, blast cell colonies; CM, conditioned medium.
*One blast cell colony was present in a total of four dishes.

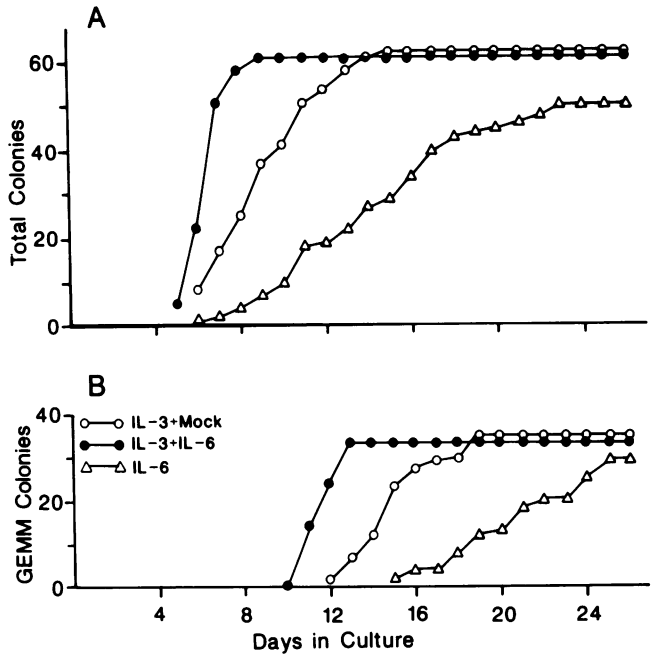

FIG. 1. Time course of cumulative numbers of colonies identified in four plates each containing 1×10^6 spleen cells of 5-FUra-treated mice. Culture contained IL-3 (200 U/ml) and/or 1:1000 dilution of IL-6. Concentration of Epo was 2.0 U/ml. (*A*) Total colonies. (*B*) GEMM colonies.

cultures containing both factors, this time was reduced to 7.3 ± 0.7 days (Fig. 2*C*). The doubling times of individual blast cell colonies, based on the most linear portions of the growth curves, were estimated to be 14.3 ± 4.8, 13.3 ± 3.0, and 15.2 ± 3.9 hr in culture supported by IL-3, IL-6, and a combination of IL-3 and IL-6, respectively. Because the growth rates were not statistically different in the three culture systems, these data strongly suggested that the combination of IL-3 and IL-6 significantly shortens the time that the multipotential stem cell remains in G_0 phase of the cell cycle.

Colony Formation by Day-2 Post-5-FUra Bone Marrow Cells. Stanley and his colleagues (28, 29) have demonstrated that hemopoietin-1 (H-1) acts synergistically with either macrophage CSF or IL-3 to support the proliferation of early murine hemopoietic progenitors and that these effects were especially dramatic using bone marrow cells harvested 2 days after injection of 5-FUra. We have confirmed in BDF1 mice that the nadir of blast cell colony and GEMM colony-forming cells in the marrow occurs 2 days after injection of 5-FUra (150 mg/kg) and that significant recovery of the progenitor pools is evident by day 4 (data not shown). We therefore used day-2 post-5-FUra bone marrow cells to analyze the synergism between IL-3 and IL-6 in supporting colony formation by very early stem cells. As shown in Fig. 3*A*, IL-6 alone did not support colony formation from the day-2 post-5-FUra marrow, whereas 24 GM colonies were identified in the IL-3-supported cultures. In contrast, the combination of both factors supported the formation of 76 colonies from a total of 2×10^5 marrow cells by day 16, and among these were significant numbers of multilineage (GMM) colonies. Several groups have recently demonstrated that IL-1α acts synergistically with different CSFs in supporting the proliferation of early stem cells and can account for most, if not all, of the H-1 activity present in cell line sources of this factor (30, 31). We have, therefore, directly compared the ability of IL-1α with that of IL-6 to act synergistically with IL-3 in supporting colony formation by 2-day post-5-FUra bone marrow cells. Although IL-1α at final concentrations of 2 and 20 ng/ml

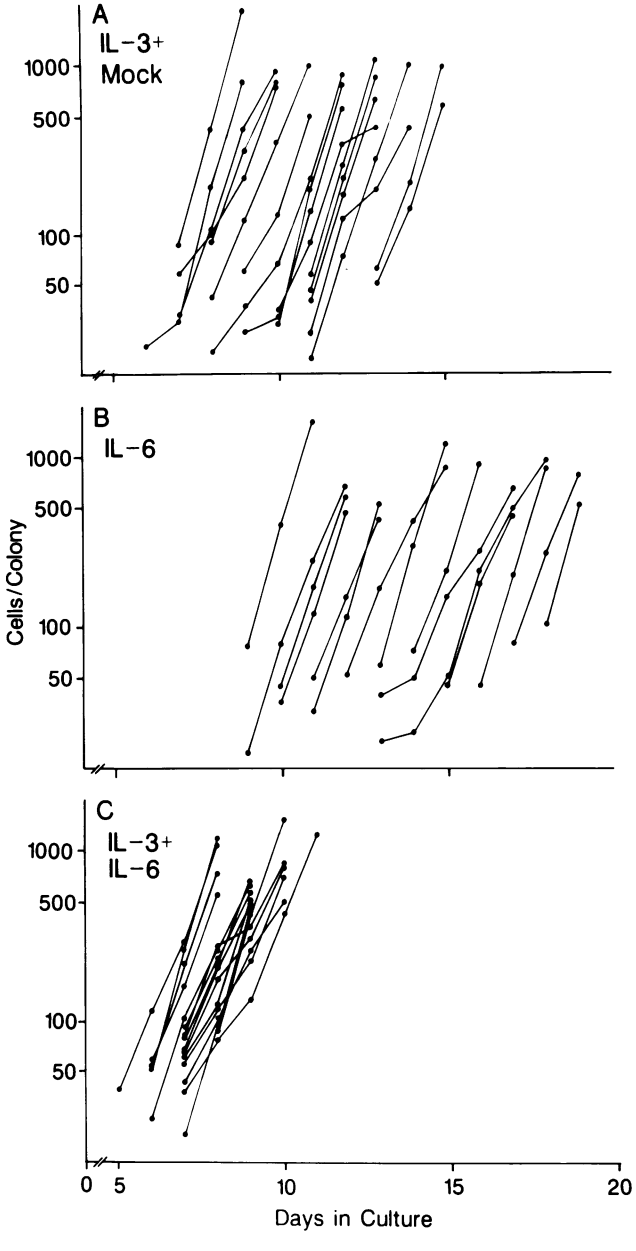

FIG. 2. Graphic presentation of cell number changes in individual blast cell colonies that later revealed GEMM expression. Data represent colonies in two plates each containing 1×10^6 spleen cells of 5-FUra-treated mice. Cultures were stimulated by (*A*) IL-3 plus mock conditioned-medium; (*B*) IL-6; and (*C*) IL-3 plus IL-6.

acted synergistically with IL-3 in these cultures, the total number of colonies was less than that supported by the combination of IL-6 and IL-3 (Fig. 3*A*), and none of the colonies were recognizable as GMM.

Similar results were obtained in comparable cultures supplemented with Epo (2.0 U/ml) (Fig. 3*B*). IL-6 did not support colony formation in the presence of Epo, whereas ≈20 GM colonies were identified in the cultures containing IL-3 and Epo. In contrast, the cultures containing IL-3, IL-6, and Epo yielded 53 colonies, including many recognizable as GEMM colonies. Again, the combination of IL-1α, IL-3, and Epo was less effective than the mixture that included IL-6, and no GEMM colonies were evident among the colonies that grew in the IL-1α-containing cultures. These data clearly evinced the synergism between the action of IL-6 and IL-3 in supporting the proliferation of primitive hemopoietic progenitors.

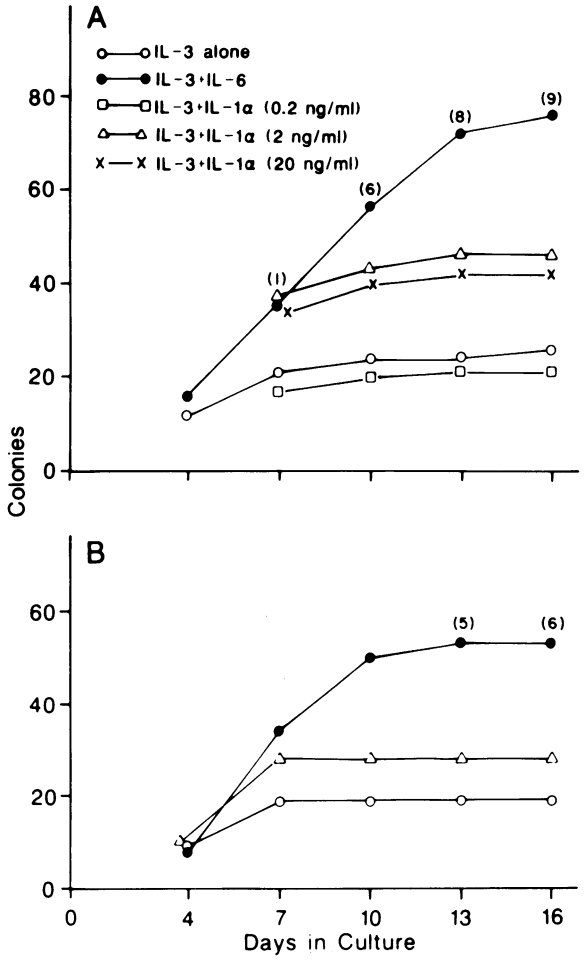

FIG. 3. Time course of cumulative numbers of colonies identified in four plates each containing 5×10^4 marrow cells of 5-FUra-treated mice. Culture was done without Epo (*A*) or with Epo (*B*). Numbers inside brackets indicate the number of GMM colonies (*A*) or GEMM colonies (*B*). Control cultures stimulated by IL-6 or IL-1α alone either in the absence (*A*) or presence (*B*) of Epo did not show colony formation.

DISCUSSION

In steady-state hemopoiesis there is considerable experimental evidence that most hemopoietic stem cells reside in a nonproliferating state of cell cycle known as G_0 (32). This model is based on results derived from many different systems including analysis of the death of spleen colony-forming units after incorporation of tritiated thymidine into DNA (33), analysis by similar thymidine suicide studies of multilineage colony formation in culture (34), analysis of the effects of high doses of 5-FUra on spleen colony formation (20), and our mapping studies of the growth of individual blast cell colonies in culture (21). It has been proposed that residence in G_0 phase confers time for the stem cells to repair DNA damage, thereby allowing the long-term maintenance of the genetic integrity of stem cell populations (32).

Previously we have shown that IL-3 supports the formation of single and multilineage colonies, including blast cell colonies in methylcellulose cultures of spleen cells from 5-FUra-treated mice (27). Serial observations of the development of individual colonies indicated that blast cell colony-forming cells begin active proliferation after spending apparently varying periods of time in a dormant state of the cell cycle. Withholding the IL-3 from the cultures until the seventh day after plating resulted in a decrease by 50% in the numbers of colonies compared with cultures initiated in the presence of IL-3 without affecting the proliferation or differentiation characteristics of the late-emerging multipotential blast cell colonies. Based on these results, we proposed that IL-3 does not trigger stem cells into active cell proliferation but rather provides an appropriate milieu for progenitor proliferation after the dormant cells have begun to divide. Our results here show that the combination of IL-6 with IL-3 does not augment the rate of proliferation of the progenitor cells but does shorten the time course of appearance of colonies, including blast cell and multilineage colonies in culture of day-4 post-5-FUra spleen cells. Although we have not distinguished between indirect and direct mechanisms of action, the end result of the IL-3 and IL-6 interaction appears to be a significant decrease in the G_0 residence time of the individual hemopoietic progenitors found in this cell population.

Other factors have been identified that interact with the CSFs in supporting hemopoietic colony formation. Quesenberry *et al.* (35) have recently described an activity in the medium conditioned by TC-1 cells, a cell line derived from adherent marrow cells, which reveals synergistic effects on multilineage colony formation in the presence of IL-3. Stanley and coworkers (28, 29) have purified a factor, designated H-1, which acts synergistically with macrophage CSF (CSF-1) or IL-3 in supporting hemopoietic colony formation. Several investigators have recently demonstrated that IL-1α, a factor that is abundantly expressed by the cellular source of the purified H-1 (human bladder carcinoma cell line, 5637), displays similar synergism with various CSFs and may be identical to H-1 (30, 31). Here we have demonstrated that IL-6 acts synergistically with IL-3 in support of the proliferation of multipotential progenitors derived from day-2 post-5-FUra bone marrow. The combination of IL-6 and IL-3 was more effective than the combination of IL-1α and IL-3 in this system, both in terms of the absolute number of colonies formed and in the number of multilineage colonies. Because IL-6 is expressed by a variety of stimulated cells (19, 36), including IL-1-treated bone marrow-derived fibroblasts (39), it is possible that the H-1 activity of IL-1α results from the induction of IL-6 production by accessory cells in the culture. Because we have used crude populations of post-5-FUra spleen and marrow cells, we have not distinguished between direct and indirect effects of either IL-1α or IL-6 in these culture systems. It will be of great interest to determine the relative contribution of IL-1α, IL-6, and possibly other factors to the H-1-like activities present in the medium conditioned by 5637 cells, TC-1 cells, and other cellular sources of synergistic factors.

Analysis of the interactions of growth factors with target cell populations in other systems has recently led to a two-signal model in which a competence factor and a progression factor interact to mediate the transition of cells from G_0 to a cycling state (37, 38). The analogy with the synergistic interactions of IL-1α, or IL-6, with IL-3 in supporting hemopoietic cell proliferation leads us to speculate that IL-6 or IL-1α may act directly or indirectly as competence factors, whereas IL-3 functions as a progression factor for the initiation of proliferation of hemopoietic stem cells in G_0. In testing this model, we hope to further elucidate the sequence of events leading to the active proliferation of stem cells following perturbation of steady-state hemopoiesis.

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