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Granulocyte colony-stimulating factor enhances interleukin 3-dependent proliferation of multipotential hemopoietic progenitors

(G-CSF/hemopoietic stem cells)

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ABSTRACT **In cultures of spleen cells from normal mice, recombinant human granulocyte colony-stimulating factor (G-CSF) supported the formation of multipotential blast cell colonies. Serial replating of the blast cell colonies in the presence of G-CSF, however, failed to demonstrate any direct effect of G-CSF on murine multipotential progenitors. We therefore examined the effects of G-CSF in combination with murine interleukin 3 on proliferation of murine blast cell colony-forming cells. The time course of total colony formation and multilineage colony formation by spleen cells harvested from mice 4 days after injection of 5-fluorouracil at 150 mg/kg was significantly shortened in cultures containing both factors in contrast with cultures supported by either factor alone. Serial observations of individual multipotential blast cell colonies (mapping) revealed that blast cell colonies emerged at random time intervals in the presence of interleukin 3 or G-CSF. The appearance of blast cell colonies, however, was significantly hastened in cultures containing both factors relative to cultures grown with either factor. In cultures of day-2 post-5-fluorouracil bone marrow cells, G-CSF in concentrations as low as 1 unit/ml revealed synergism with interleukin 3 in supporting the proliferation of multipotential progenitors. This synergistic activity may explain the previous *in vivo* studies suggesting the effects of G-CSF on apparent multipotential stem cells.**

Granulocyte colony-stimulating factor (G-CSF) was first identified as a factor that can induce differentiation in murine myeloid and myelomonocytic leukemia cell lines. When this factor was purified from medium conditioned by lung tissues, its identity with the factor that supports granulocyte colony formation from normal marrow was established (1). Since then, the genes for murine (2) and human G-CSF (3, 4) have been cloned, and the recombinant human protein has been tested *in vivo* for stimulation of neutrophil production in mice (5), hamsters (6), and monkeys (7). These studies have shown that G-CSF elicits a rapid dose-dependent elevation of neutrophil counts when injected into animals.

The targets of G-CSF are generally considered to be at the late-maturational stages of the neutrophil lineage (8, 9). Results from several laboratories, however, do not corroborate the rigid lineage specificity proposed for G-CSF. For example, Mizoguchi *et al.* (10) saw a significant increase in the colony-forming units in spleen (CFU-S) in nude mice transplanted with a human lung carcinoma known to produce G-CSF. In addition, a tritiated thymidine "suicide" study revealed that in the tumor-bearing animals a larger fraction of CFU-S are active in the cell cycle. By use of a clonal-transfer technique, Metcalf and Nicola (11) demonstrated that murine G-CSF directly supports the survival and proliferation of some fetal-liver multipotential progenitors. Together, these observations indicated that the targets of G-CSF may include multipotential hemopoietic progenitors. To reconcile these discrepancies, we studied the effects of human G-CSF on murine hemopoietic progenitors, including multipotential blast-cell colony-forming cells. Our observations suggest that although G-CSF as a single agent does not support the proliferation of multipotential progenitors, G-CSF acts synergistically with mouse interleukin 3 (IL-3) to shorten the G_0 period of stem cells, resulting in an earlier appearance of multilineage colonies in culture.

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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 at 150 mg/kg of body weight (12).

Factors. Two different human G-CSF preparations were used. Conditioned medium from COS-1 cells transfected with cDNA coding for human G-CSF was used as a source of mammalian cell-derived factor (8). This conditioned medium supported half-maximal colony formation in a murine bone marrow colony assay at a final dilution of 1:10,000. In addition, G-CSF expressed in *Escherichia coli* was purified as described (4). Murine IL-3 ($\approx 5 \times 10^6$ units/mg of protein) was purified to homogeneity from medium conditioned by WEHI-3 cells as described (13). Chinese hamster ovary cell-derived recombinant human erythropoietin (Epo) with specific activity of 300,000 units/mg was a gift from the Genetics Institute Pilot Development Program. For preparation of WEHI-3-conditioned medium (CM), the supernatants of cultures of WEHI-3 cells were concentrated 20× by a Pellicon cassette system (Millipore) equipped with a polysulfone cassette with a M_r 10,000 pore size. The concentrated CM was added to cultures to a final concentration of 1% (vol/vol).

Clonal Cell Culture. Methylcellulose cell culture was done in 35 mm Lux suspension culture dishes (5221R; Miles). 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), 30% fetal calf serum (Flow Laboratories), 1% deionized bovine serum albumin (Sigma), 1×10^{-4} M

Abbreviations: IL, interleukin; Epo, erythropoietin; 5-FUra, 5-fluorouracil; G-CSF, granulocyte colony-stimulating factor; GM, granulocyte/macrophage; M, megakaryocyte; GEM, granulocyte/erythrocyte/macrophage; GMM, granulocyte/macrophage/megakaryocyte; GEMM, granulocyte/erythrocyte/macrophage/megakaryocyte; Bl, blast cell; CM, conditioned medium.

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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 through an inverted microscope according to the criteria described (14, 15). Abbreviations for colony types are as follows: granulocyte/macrophage (GM) colonies; megakaryocyte (M) colonies; granulocyte/erythrocyte/macrophage (GEM) colonies; granulocyte/macrophage/megakaryocyte (GMM) colonies; granulocyte/erythrocyte/macrophage/megakaryocyte (GEMM) colonies; and blast cell (Bl) colonies (15).

RESULTS

Colony Formation by Spleen Cells from Normal Mice Supported by Various Dilutions of G-CSF. Conditioned medium from the G-CSF-expressing COS cells at various dilutions actively supported the formation of GM colonies (Table 1). In addition, a few GMM and M colonies appeared in the cultures. Cytological analysis of the GM colonies revealed not only neutrophil colonies but also neutrophil/macrophage colonies. This observation agrees with earlier reports (11, 16) demonstrating the ability of G-CSF to support formation of neutrophil/macrophage colonies in serum-containing culture. Unexpectedly, the G-CSF-containing supernatant supported the formation of Bl colonies on day 16 of culture. These were relatively large Bl colonies, occasionally reaching sizes of >1000 cells. In contrast, in the four dishes containing IL-3, there was a total of two Bl colonies at the same date of incubation. Some Bl colonies in G-CSF cultures were multipotential in nature, as demonstrated by replating of individual Bl colonies. A total of 26 Bl colonies, harvested between days 12 and 16 of incubation, were replated. All 26 colonies revealed secondary colony formation; 11 colonies yielded secondary GM colonies only, whereas 15 colonies expressed multilineage capabilities in secondary colonies. The variable replating efficiencies and the types of secondary colonies derived from the 15 multipotential blast cell colonies were similar to those we had reported (17).

Replating of Pooled Blast Cells Supported by G-CSF. To test whether G-CSF alone can directly support the proliferation of multipotential progenitors, 70 cells pooled from Bl colonies grown originally in either WEHI-3 CM or G-CSF were replated in the presence of G-CSF in quadruplicate cultures. None of the resultant secondary colonies from either the WEHI-3 CM or the G-CSF-supported blast cells revealed multiple lineages; only GM colonies appeared in the secondary cultures (Table 2). In the presence of WEHI-3 CM and Epo, however, 280 cells pooled from WEHI-3 CM-supported Bl colonies yielded 19 multilineage (GEM, GMM, and GEMM) colonies. The G-CSF-supported blast cells, when replated in WEHI-3 CM and Epo, also yielded several multilineage colonies.

Table 2. Colony formation by pooled blast cells derived from cultures with WEHI-3 CM or G-CSF

Stimulus in secondary culture	Secondary colonies, no.						
	GM	GEM	GMM	GEMM	Mast	Bl	Total
WEHI-3 CM Bl colonies							
G-CSF	60	0	0	0	0	0	60
WEHI-3 CM + Epo	167	4	4	11	4	1	191
No factor	0	0	0	0	0	0	0
G-CSF-supported Bl colonies							
G-CSF	98	0	0	0	0	0	98
WEHI-3 CM + Epo	232	5	0	4	0	0	241
Mock CM	0	0	0	0	0	0	0

Data represent the total number of colonies in quadruplicate cultures each containing 70 blast cells. On day 12 of cultures with WEHI-3 CM, 23 Bl colonies each containing 15 to 499 cells (mean 286 cells per colony) were picked, pooled, washed, and replated. For the other primary culture blast cells were derived from 30 Bl colonies each containing 10–500 cells (mean, 232 cells per colony) supported by G-CSF. Mast, mast cell colonies.

Although blast cells did not yield multilineage colonies in the presence of G-CSF alone, G-CSF may have supported proliferation of a few cell divisions of multipotential progenitors during the early days of incubation. To test this possibility, we serially replated pooled blast cells in experiments using the method we described previously for murine GM-CSF (18). Approximately 5×10^5 spleen cells from normal mice were cultured in the presence of a 1:1000 dilution of G-CSF. On day 12, 30 Bl colonies each containing 10–500 cells (mean, 232 cells per colony) were pooled, washed, and then replated in G-CSF. On day 2 of culture, a total of 189 clusters of cells ranging from 6 to 26 cells per cluster were picked, pooled, washed, and then cultured in the presence of WEHI-3 CM plus Epo. Only GM colonies developed. From these observations, we concluded that G-CSF alone does not directly support proliferation of multipotential progenitors.

Time Course of Colony Formation Supported by G-CSF, IL-3, or a Combination of G-CSF and IL-3. We have previously shown that murine (17) and human (19) IL-3 support formation of multipotential Bl colonies. Here we have shown that G-CSF either indirectly or in combination with other factors will also support Bl colony formation. To clarify the relationship between G-CSF and IL-3 with respect to their effects on primitive progenitors, we examined colony formation by murine spleen cells enriched for multipotential progenitors. The spleen cells from mice treated 4 days previously with 5-FUra were plated in culture in the presence of 2.0 units/ml of recombinant human Epo and a 1:1000 dilution of

Table 1. Effects of various dilutions of G-CSF on colony formation by spleen cells from normal mice

Stimulus	GM	B	M	GEM	GMM	GEMM	Mast	Bl	Total
G-CSF (1:100)	21 ± 4	0	1 ± 1	0	1 ± 1	0	0	1 ± 1	24 ± 5
G-CSF (1:1000)	18 ± 2	0	1 ± 1	0	1 ± 1	0	0	3 ± 1	23 ± 2
G-CSF (1:10,000)	17 ± 5	0	0	0	2 ± 1	0	0	2 ± 1	20 ± 4
G-CSF (1:100,000)	12 ± 1	0	0	0	0	0	0	2 ± 1	14 ± 2
G-CSF (1:1,000,000)	5 ± 1	0	0	0	0	0	0	0	5 ± 1
IL-3	27 ± 5	0	1 ± 1	0	5 ± 1	0	19 ± 4	1 ± 1*	51 ± 6
IL-3 + Epo	23 ± 2	16 ± 3	2 ± 1	2 ± 1	4 ± 2	5 ± 2	15 ± 4	1 ± 1†	67 ± 6
Mock CM	1 ± 1	0	0	0	0	0	0	0	1 ± 1
Control	1 ± 1	0	0	0	0	0	0	0	1 ± 1

Data represent mean ± SD of the number of colonies in quadruplicate cultures each containing 5×10^5 cells. Control, no factor. B, erythroid bursts. Mast, mast cell colonies.

*A total of two Bl colonies were identified.

†A total of three Bl colonies were identified.

G-CSF and/or IL-3 at 200 units/ml. The plates were examined on an inverted microscope, and the numbers and types of colonies were recorded daily. The total number of colonies supported by the factors are presented in Fig. 1*A*. IL-3 supported the formation of a larger number of colonies than did G-CSF, and the maximal colony count in the IL-3-containing cultures was reached earlier (day 15) than in the G-CSF-containing cultures (day 22). However, the combination of the two factors significantly hastened the appearance of colonies; within 10 days the maximal number of colonies were seen. Fig. 1*B* shows the data for GEMM colonies. Because the combination of the two factors significantly hastened the appearance of GEMM colonies, IL-3 and G-CSF appear to act synergistically in support of the proliferation of early hemopoietic progenitors.

Serial Observations of Bl-Colony Formation by Day-4 Post-5-FUra Spleen Cells. To examine further the mechanisms of synergism between IL-3 and G-CSF, we mapped the formation of individual multipotential Bl colonies supported by IL-3, G-CSF, or the combination of the two factors. In this experiment, 1×10^6 day-4 post-5-FUra spleen cells were plated per dish. The emergence of new Bl colonies and their subsequent proliferation and differentiation *in situ* were recorded daily as described (17, 20). In Fig. 2, the analysis of the growth of Bl colonies that later revealed multilineage expression is presented. The total numbers of multipotential Bl colonies supported by IL-3, G-CSF, or the combination of the factors were 18, 14, and 17, respectively. Multipotential Bl colonies appeared earlier in IL-3-containing than in the G-CSF-containing cultures. The average time at which individual colonies reached 100 cells was calculated to be 10.3 ± 2.0 days in IL-3 and 13.2 ± 2.4 days in G-CSF. The combination of the two factors significantly hastened the appearance of Bl colonies as shown in Fig. 2*C*. The average time needed by the Bl colonies to reach the size of 100 cells per colony was estimated to be 7.4 ± 0.8 days in the presence of the two factors. When the doubling times of individual multipotential Bl colonies were calculated from the most linear portion of the individual colony growth curves, progenitor cells from the IL-3, G-CSF, or the combination groups were estimated to be 14.3 ± 4.8, 14.9 ± 3.7, and 14.6 ± 3.2 hr, respectively. Because the cell growth rates did not statistically differ among the different cultures, these results

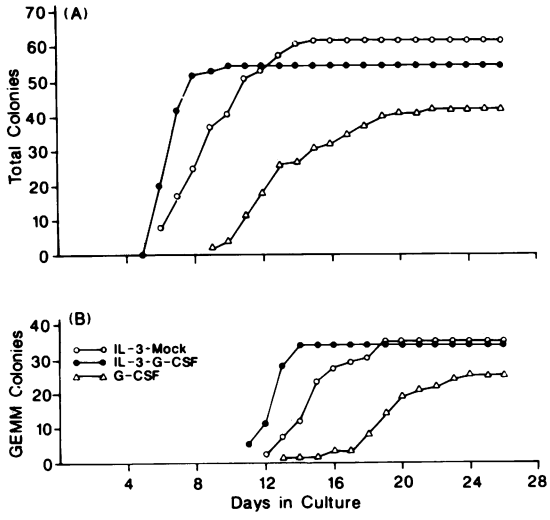

FIG. 1. Time course of colony formation in cultures of day-4 post-5-FUra spleen cells. The daily colony counts represent the total numbers of colonies from four dishes, each containing 1×10^6 spleen cells. The cultures contained Epo at 2 units/ml and IL-3 at 200 units/ml and/or a 1:1000 dilution of G-CSF as indicated. (*A*) Total colonies. (*B*) GEMM colonies.

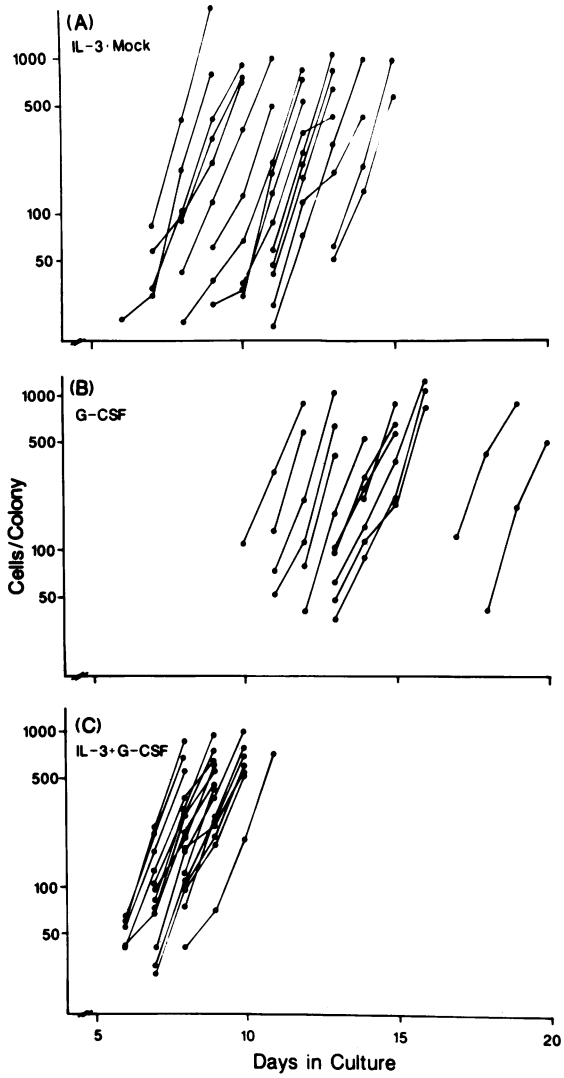

FIG. 2. Growth rates of individual blast cell colonies that later revealed GEMM expression. The data are from colonies identified in each of two plates seeded with 1×10^6 day-4 post-5-FUra spleen cells in the presence of IL-3 plus mock conditioned medium (*A*); G-CSF (*B*); and IL-3 plus G-CSF (*C*).

indicated that G-CSF acts synergistically with IL-3 by shortening the average time that multipotential progenitors remain in G_0.

Colony Formation by Day-2 Post-5-FUra Bone Marrow Cells. To confirm the synergism between G-CSF and IL-3 in support of early stem cell proliferation, we tested the two cytokines in cultures of day-2 post-5-FUra bone marrow cells. With comparable cell preparations, Stanley and his colleagues (21) saw dramatic synergistic effects of hemopoietin-1 with IL-3. We have confirmed, in BDF1 mice, that the nadir of Bl colony- and GEMM colony-forming cells in the marrow occurs 2 days after injection of 5-FUra at 150 mg/kg and that the significant recovery of the progenitor pools is evident by day 4 (data not shown). We therefore tested purified recombinant human G-CSF in concentrations ranging from 10^6 units/ml to 1 unit/ml for its synergism with IL-3. To allow the expression of erythroid potential by the multipotential progenitors, cells were cultured in the presence of Epo at 2 units/ml. The time courses of colony formation in the different cultures are presented in Fig. 3. In cultures supported by IL-3, colony formation reached its maximal plateau level on day 22, and a total of 72 colonies including 15 GEMM colonies grew. In contrast, in cultures stimulated by a combination of G-CSF (10^3–10^6 units/ml) and IL-3,

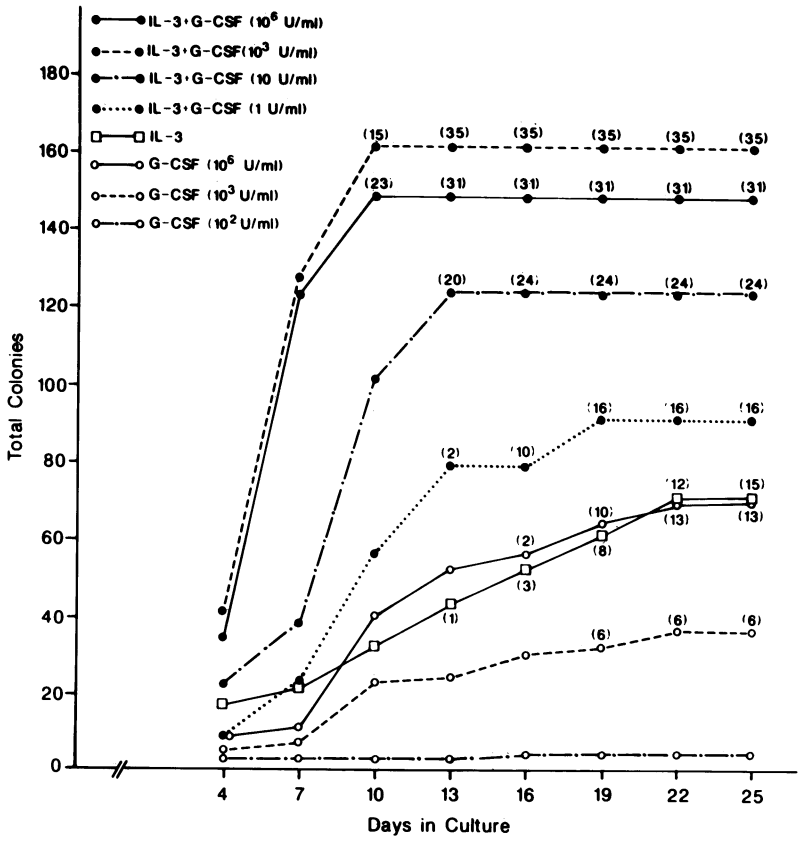

FIG. 3. Time course of colony formation in cultures of day-2 post-5-FUra bone marrow cells. Each culture contained 5 × 10^4 day-2 post-5-FUra bone marrow cells, Epo at 2 units/ml, IL-3 at 200 units/ml and/or varying concentrations of G-CSF as indicated. The bracketed numbers indicate the numbers of GEMM colonies. G-CSF at 1 unit/ml and 10 units/ml failed to support colony formation.

colony formation reached its maximal plateau level on day 10, and a total of 145–162 colonies including 31–35 GEMM colonies were identified. G-CSF at low concentrations yielded no colonies, but in combination with IL-3 G-CSF increased the total number of colonies (including GEMM colonies) and hastened their appearance. Only at very high concentrations (10^3–10^6 units/ml) did G-CSF alone support the formation of a few multilineage colonies (6–13 GEMM colonies). These data clearly evinced the synergism between G-CSF and IL-3 in supporting proliferation of primitive hemopoietic progenitors.

DISCUSSION

The prevailing concept concerning the steady state of the cell cycle states that most hemopoietic stem cells are dormant and begin cycling after varying time intervals. The long G_0 period has been proposed to protect the genetic integrity of these stem cells by providing time for DNA-damage repair (22). The proliferation of primitive hemopoietic progenitors appears to be regulated by a number of factors. Foremost, IL-3 is necessary for proliferation of multipotential progenitors. Investigators in many laboratories, including ours (17, 19), have shown that IL-3 supports multilineage colony formation in culture. We proposed that IL-3 provides a permissive milieu for proliferation of multipotential progenitors but does not trigger stem cells in G_0 to begin cell cycling (17). Earlier, Metcalf *et al.* (23) proposed that murine GM colony-stimulating factor supports a few cell divisions of multipotential progenitors. Investigations in our laboratory suggested that GM colony-stimulating factor supports proliferation of a subpopulation of multipotential progenitors that are responsive to IL-3 (18). Stanley and his co-workers (21) presented evidence that hemopoietin-1, which was purified from culture supernatant from a bladder carcinoma cell line, 5637 (24), possesses synergistic activity with IL-3 in support of proliferation of hemopoietic progenitors. Most recently, Mochizuki *et al.* (25) provided evidence that IL-1 accounts for at least some of the hemopoietin-1 activity of the 5637 supernatant. In this paper, we have shown that G-CSF also acts synergistically with IL-3 in support of Bl colony formation and that the synergism results from apparent shortening of the average period of G_0 residence of hemopoietic stem cells. Because the bladder carcinoma cell line, 5637, was one of the original sources for human G-CSF (26), G-CSF may also account for some of the hemopoietin-1 activity of the culture supernatant of the carcinoma cell line.

The ability of G-CSF to work synergistically with IL-3 in the proliferation of primitive hemopoietic stem cells may account for various observations *in vitro* and *in vivo*. Although G-CSF supports the formation of multipotential Bl colonies by spleen cells from normal mice, G-CSF as a single agent appears not to support the proliferation of multipotential progenitors. Suzuki *et al.* (27) reported that IL-3 synthesis is induced during syngeneic mixed lymphocyte reaction. The Bl colonies in G-CSF-containing cultures most likely were supported by a combination of G-CSF and endogenously produced IL-3 and/or other factors. Synergism between G-CSF and IL-3 may account for some of the pharmacologic effects of G-CSF. Welte *et al.* (7) observed that injections of human G-CSF significantly shortens the period of cyclosphosphamide-induced neutropenia in primates. If the function of G-CSF were only to support the terminal proliferation of committed neutrophil precursors, shortening of the neutropenic period by G-CSF could not be adequately explained. In these animals, the injected G-CSF may have interacted with the endogenously-produced IL-3 in shortening of the G_0

period of stem cells—thus leading to the early recovery from the neutropenic phase.

In a separate report (28), we have shown that IL-6 [also known as B-cell-stimulatory factor 2 (29) or interferon $\beta 2$ (30)] acts synergistically with IL-3 in shortening the G_0 period of stem cells. Both the genomic structures and the amino acid sequences of IL-6 reveal similarity with G-CSF (31), indicating that the two cytokines are distantly related and may have evolved to assume related but distinct functions in host defense. After infection, the host organism responds both by activation of the immune system and also by an increased rate of neutrophil production. In addition to its role in stem cell proliferation, IL-6 has been found to support terminal steps in B-cell differentiation, thereby enhancing immunoglobulin secretion by B cells (31) and also to serve as a second signal in the mitogen-dependent activation of T cells (32). Similarly, G-CSF has been shown to actively support the growth and differentiation of neutrophil progenitors, as well as to prime the mature neutrophils for responding to invading organisms. Perhaps it is not coincidental that these two important regulators of different aspects of host defense are also capable of stem cell interactions that should result in an enhanced rate of neutrophil (and possibly lymphocyte) proliferation and thus a more effective response to infection.

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