INTERLEUKIN 1 ENHANCES GROWTH FACTOR-DEPENDENT PROLIFERATION OF THE CLONOGENIC CELLS IN ACUTE MYELOBLASTIC LEUKEMIA AND OF NORMAL HUMAN PRIMITIVE HEMOPOIETIC PRECURSORS

By TRANG HOANG,* ANDRÉ HAMAN,* OTELINDA GONCALVES,
FRANÇOIS LETENDRE,‡ MIREILLE MATHIEU,‡ GORDON G. WONG,§
AND STEVEN C. CLARK§

From the *Clinical Research Institute of Montreal, Quebec, Canada; the ‡Hotel-Dieu Hospital,
Montréal, Quebec, Canada; and the §Genetics Institute, Cambridge, Massachusetts

IL-1 is an important growth regulatory molecule secreted by stimulated macrophages (1) and a variety of other cell types (reviewed by Duff, reference 2). Molecular cloning of the sequences encoding IL-1 has revealed the existence of two distinct but related species of IL-1, designated IL-1α and IL-1β, which act through a common receptor (3) and appear to have very similar if not identical biologic activities (4). IL-1 was first identified as a lymphocyte-activating factor, but more recently has been found to possess many different activities with a wide variety of cell types. IL-1 acts as a cofactor for Con A activation of resting T cells (5); as an inducer of IL-2 production by T cell lines (6); as a growth and differentiation factor for B cells (7); and as a primary mediator of the inflammatory response (8, 9).

The importance of IL-1 in the regulation of hemopoiesis has only recently been widely recognized. Many different investigators have shown that IL-1 induces the production of several different hemopoietic growth factors, including both granulocyte/macrophage CSF (GM-CSF)[1] and granulocyte CSF (G-CSF) by a variety of cell types including lung fibroblasts (10), human mononuclear phagocytes (11), and bone marrow–derived stromal cells (12). In addition, IL-1 has been found to share many of the activities originally ascribed to the cytokine known as hemopoietin 1 (H-1) (13–16). Thus, IL-1 acts synergistically with both macrophage CSF (M-CSF, also known as CSF-1) and G-CSF in the stimulation of murine macrophage progenitors having high proliferative potential (16, 17) and with GM-CSF in the stimulation of proliferation of primitive murine stem cells (15). Finally, incubation of human bone marrow cells with IL-1 results in an increased proportion of hemopoietic stem cells in cycle (18).

We have previously described a growth factor activity called leukemic blast growth factor (LBGF) (19, 20) that supports colony formation by leukemic blast progen-

itors, found in the peripheral blood of patients with acute myeloblastic leukemia. One of the best sources of this activity is the conditioned medium (CM) from a human bladder carcinoma cell line designated HTB9–5637 (19). This cell line has been shown to produce G-CSF (21), GM-CSF (22), and, most recently, IL-1α (15). Analysis of the HTB9-CM has previously demonstrated that both GM-CSF and G-CSF contribute to the LBGF activity elaborated by the carcinoma cells. Here, we report that IL-1α also acts synergistically with GM-CSF and to a lesser degree with G-CSF in supporting acute myeloblastic leukemia (AML) blast cell colony formation. Reconstitution experiments with all three cytokines suggest that the combination of factors can account for all of the HTB9-CM LBGF activity. Furthermore, when tested with normal bone marrow–derived human progenitors, IL-1α also acted synergistically with GM-CSF in the stimulation of colony formation by primitive GM-CFC, erythroid (BFU-E), and multipotential (CFU-GEM) progenitors. These studies indicate that IL-1α functions in the human system, as has been demonstrated previously in the murine system (15), as a regulator of early stem cell proliferation.

Materials and Methods

Source of Cells and Growth Factors.

Blasts were separated from the peripheral blood of AML patients at presentation (Hotel-Dieu Hospital, Montreal, Canada) by centrifugation on a Ficoll Hypaque gradient (Pharmacia Fine Chemicals, Laval, Quebec, Canada). Cells were stored at −70°C or in liquid nitrogen until use. Normal bone marrows came from marrow aspirates done for diagnosis, which subsequently appeared to have a normal cellularity and normal differential cell count.

IL-1α (Genzyme, Boston, MA), purified from *Escherichia coli* extracts to a final specific activity of 10^8 U/mg, was stored in aliquots at 4°C. GM-CSF was purified to a specific activity of 8×10^6 U/mg, from culture supernatants of a CHO cell line transformed with the human GM-CSF cDNA (23). rG-CSF was the crude conditioned medium from another CHO cell line engineered to express human G-CSF. 1 U/ml of activity corresponds to a concentration resulting in half maximal stimulation proliferation of primary CML blasts. The antibody to GM-CSF was a polyclonal rabbit antiserum raised against rhGM-CSF. At a dilution of 1:500, it neutralized 50 U/ml of GM-CSF.

HTB9-conditioned medium was harvested 1 wk after confluency, filtered, and stored at 4°C as described elsewhere (19). It had an optimal activity at 10% (vol/vol) in culture and was included in all experiments as a positive control.

Assays of Growth Factors.

Blast Assay. AML blasts were plated at concentrations chosen so that no colony growth occurred in absence of an exogenous source of growth factor (7,000 cells per 100 μl for all five patients included in the study). Cells were plated in Iscove's modified Dulbecco's medium (IMDM, Gibco, Grand Island, NY), supplemented with FCS (10%, Gibco), and viscosified with methylcellulose (1% wt/vol, Fluka, Switzerland). The cultures were done in quadruplates in 96-well dishes (Linbro, Flow Laboratories, McLean, VA) at 100 μl per well.

Normal Bone Marrow Cultures. Mononuclear cells were separated on a Ficoll Hypaque gradient, depleted of adherent cells by an overnight incubation in IMDM supplemented with FCS (10%). Nonadherent cells were plated at 4×10^4 cells/ml in IMDM supplemented with FCS (10%), deionised and delipidated BSA (10 mg/ml, electrophoretically pure, Hoechst, Behring Diagnostics, La Jolla, CA), iron saturated transferrin (300 μg/ml, Hoechst), and α-thioglycerol (7.5×10^{-5} M, Sigma Chemical Co., St. Louis, MO) and viscosified with methyl-cellulose (1%, Fluka), as described previously (24). Step 1 eythropoietin (Terry Fox Cancer research laboratories, University of British Columbia, BC) was added at a final concentration of 1 U/ml. Cultures were done in duplicate, in 35-mm Petri dishes (Lux, Miles Scientific, Napierville, Quebec, Canada) at 1 ml per dish. Colonies were enumerated 14 d

after initiating the cultures and were scored as erythroid (BFU-E), granulocytic and macrophagic (GM-CFC), or granulocytic/erythroid/macrophagic (CFU-GEM), based on colony morphology.

Northern Blots and Hybridisation Conditions.   RNA was extracted from the HTB9 cells by the guanidine isothiocyanate technique (25), denatured in the presence of glyoxal (BDH, Toronto, Ontario, Canada) subjected to electrophoresis in 1% agarose (Pharmacia Fine Chemicals, Uppsala, Sweden), and transferred to nitrocellulose as described by Thomas (26). The IL-1α probe was the Pst1 fragment of the human IL-1α cDNA (3). It was labeled by random priming with the Klenow to a sp act of 2×10^8 cpm/μg (27). The filter was prehybridized for 4 h at 65°C in presence of 120 mM Tris/8 mM EDTA/600 mM NaCl pH, 7.4, (4× SET), 0.1% sodium pyrophosphate, 0.2% SDS (BioRad, Richmond, CA), and Heparin (100 μg/ml, Sigma Chemical Co.). The filter was hybridized overnight in the same buffers as above except that the heparin was increased to 500 μg/ml, dextran sulphate (Pharmacia Fine Chemicals) added at 1 g/9.6 ml and the probe added at 10^6 cpm/ml. The filter was washed under stringent conditions (final wash at 0.1× SET, 0.2% SDS for 30 min at 65°C) and exposed. The relative mobilities of molecular weight standards were obtained using ^{32}P-labeled denatured fragments of phage λ DNA (Hind III) and φ χ (Hae III), which were analyzed in parallel.

Results

Blasts from five patients with AML (Table I) were plated in the presence of increasing concentrations of rIL-1α either alone or in the presence of an optimal concentration of rGM-CSF, determined by a separate titration (Fig. 1 *A*). IL-1α had no effect by itself (Fig. 1 *B*) but synergized with rGM-CSF in the stimulation of blast colonies in three out of five AML samples. The effect was striking, with a 6–10-fold increase in colony count over that induced by rGM-CSF alone at the highest concentration of rIL-1α (Fig. 1 *C*). No effect, neither additive nor inhibitory, was seen with AML-4 (Fig. 1) and -5 (Fig. 4).

While GM-CSF showed little stimulation of blast colonies by itself, rG-CSF under the same culture conditions appeared to be a more potent stimulator (Fig. 2 *B*). For that reason, the synergistic effect of IL-1α with G-CSF was less dramatic than that observed with rGM-CSF. It was, however, significant with AML-1 and -3 and, to a lesser degree, with AML-4 (Fig. 2 *A*). No effect was observed with AML-2 and -5.

Previous studies have indicated that GM-CSF and G-CSF have a synergistic effect on AML blast progenitors (28, 29). The combination of the two growth factors, however, does not reach the level of stimulation observed with HTB9 CM (Fig. 3, *ordinate*). We therefore tested whether or not IL-1α would exhibit a further synergistic

TABLE I

Clinical Data on the AML Patients Included in the Study

Patient	FAB classification	Percent blasts
AML-1	M2	68
AML-2	M4	60
AML-3	M5b	38
AML-4	M4	>95
AML-5	M3	90
AML-6	M1	40

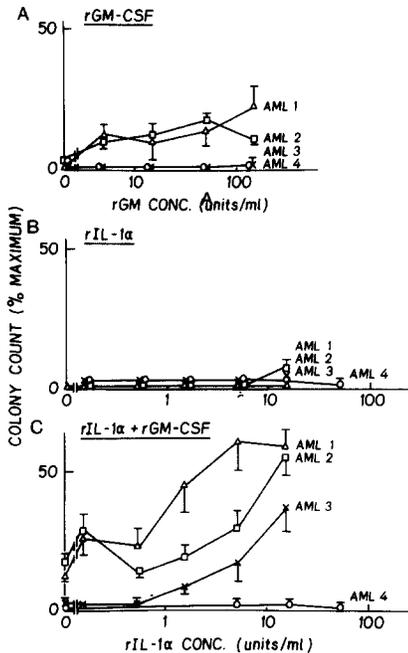The proportion of blasts was expressed as a percentage of total nucleated cells in the peripheral blood.

FIGURE 1. Effects of rIL-1α on the proliferation of blast clonogenic cells in the presence of rGM-CSF. (A and B) Individual titration curves for rGM-CSF and rIL-1α. (C) Titration of rIL-1α in the presence of rGM-CSF (50 U/ml). Values shown were normalized to the number of colonies obtained with HTB9 CM. Maximum colony counts were: 87 ± 18 (AML-1); 185 ± 23 (AML-2); 123 ± 11 (AML-3); 52 ± 7 (AML-4). Error bars shown are SD from quadruplate cultures normalized in the same way as colony counts.

activity in the presence of the two CSFs. The dose-response curves shown in Fig. 3 indicated that 10–15 U/ml IL-1 reconstituted the level of stimulation observed with HTB9 CM in the presence of both rGM-CSF and rG-CSF. Because the cell line HTB9 produced IL-1α (Fig. 5) and both GM-CSF and G-CSF (21, 22), the synergistic effects of the three growth factors could account for most of the stimulatory activity observed in the medium conditioned by the cells. No additional effect of IL-1α was seen with AML-5, whereby the synergistic effects of GM-CSF and G-CSF reconstituted the stimulation induced by HTB9-CM indicating that these cells were not responsive to IL-1α (Fig. 4). The same growth pattern was observed with AML-6 (Fig. 4). There was however a discrepancy with the other AML blasts in that rIL-1α alone was capable of supporting colony formation up to 22% of that obtained with our standard HTB9-CM.
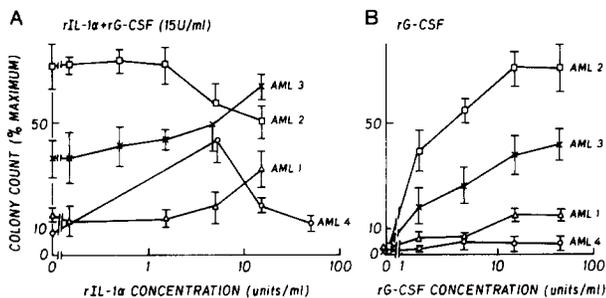

FIGURE 2. Effects of rIL-1α on the proliferation of blast clonogenic cells in the presence of rG-CSF. (A) Titration of rIL-1α in the presence of rG-CSF (15 U/ml). Normalization and maximum colony counts as in Fig. 1. (B) Individual titration curves for rG-CSF.
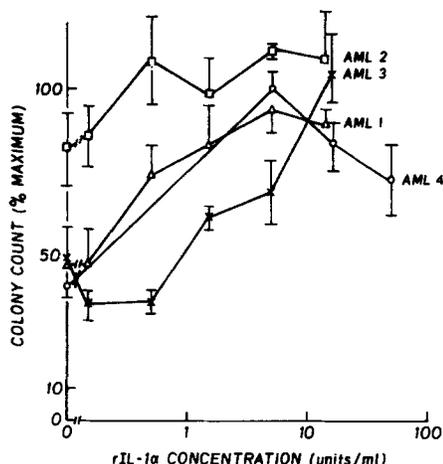
FIGURE 3. Synergistic effects of rIL-1α on the proliferation of blast progenitors in the presence of both rGM-CSF and rG-CSF. Values shown on the ordinate were obtained with both rGM-CSF (50 U/ml) and rG-CSF (15 U/ml) in the absence of rIL-1α. Normalization and maximum colony counts as in Fig. 1.

GM-CSF has been reported to act as an autocrine stimulator of AML blast proliferation (30). Because IL-1α showed a synergistic activity with an exogenous source of GM-CSF, we tested the possibility that it might also synergize with the autocrine GM-CSF. Blasts from AML-6 were therefore plated at higher cell concentration (10^4 per well) in order to increase the chance of cell interaction. Data shown in Table II indicated again that rIL-1α supported colony formation in absence of any other growth factor and the effect was dose dependent. However, when the same titration with rIL-1 was done in parallel in presence of the neutralizing antibody directed against GM-CSF, no significant colony formation was observed. The inhibition was
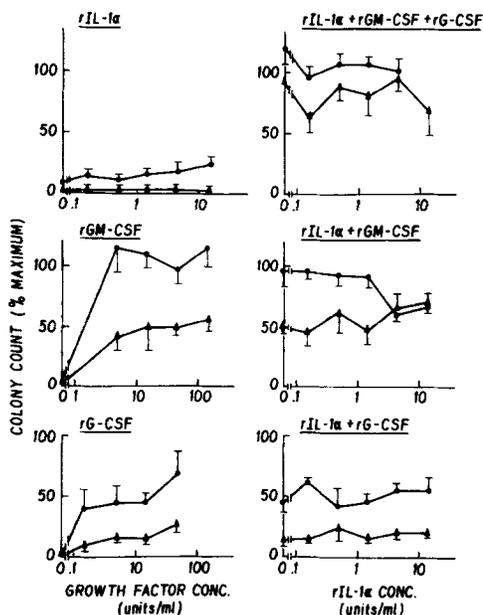

FIGURE 4. Absence of synergy between rIL-1α and rGM-CSF or G-CSF in the stimulation of two patient samples. (Left panels) Individual titration curves for each growth factor alone. (Right panels) Titration of rIL-1α in the presence of rGM-CSF (50 U/ml) and/or G-CSF (15 U/ml). Values shown were normalized to the number of colonies obtained with HTB9-CM. Maximum colony counts were: 65 ± 13 for AML-5 (▲) and 96 ± 5 for AML-6 (●).
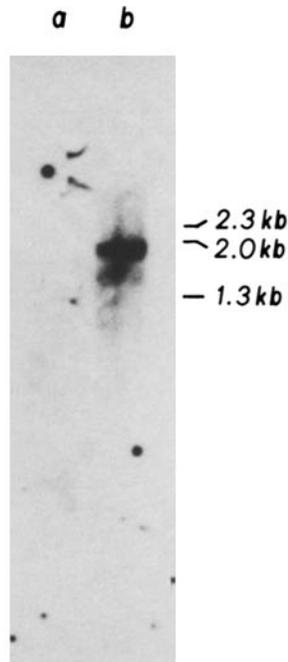
a   b

— 2.3 kb
— 2.0 kb

— 1.3 kb

FIGURE 5.   The HTB9 cells express the IL-1α mRNA. Total RNA
(10 μg) from HTB9 cells was analyzed by RNA blotting using the la-
beled IL-1α cDNA as probe (lane b). Total RNA from AML blasts
(lane a) were denatured and analyzed in parallel as negative control.

not due to a nonspecific toxicity of the antibody, because it did not affect colony
formation supported by rIL-3 (Table III). The results indicated that the stimulatory
effect observed with IL-1α alone at higher cell concentration was due to a syner-
gistic activity of the growth factor with the endogenous GM-CSF produced by the cells.

IL-3 has been shown to support blast colony formation (31). We therefore tested
the possibility whether IL-1α may also enhance the IL-3-dependent colony forma-
tion from AML blasts. Data shown in Fig. 6 indicated that addition of IL-1α to op-
timal concentrations of IL-3 resulted in a twofold increase in colony formation. En-
hancement of colony growth was dependent on the concentration of IL-1α added
and was optimal at 8 U/ml.

TABLE II

Synergistic Effects of rIL-1 with the Autocrine GM-CSF Produced by AML Blasts

rIL-1α	Colonies per 10^4 cells	
	− Anti-GM-CSF	+ Anti-GM-CSF
(U/ml)		
0	6 ± 5	−
0.15	5 ± 4	1 ± 1
1.5	9 ± 1	3 ± 2
5	17 ± 6	4 ± 3
15	35 ± 9	4 ± 3

Blasts were from AML-6. The polyclonal anti-GM-CSF was added at a dilution of 1:500,
sufficient to neutralize 50 U of GM-CSF. Results shown are mean of quadruplate cultures.

TABLE III

Specificity of the Neutralizing Antibody against GM-CSF

Growth Factor	Antibody	Colonies per 10^5 bone marrow cells				
		CFU-E	GM-CFC	M-CFC	BFU-E	CFU-GEMM
None	–	660 ± 120	2 ± 2	4 ± 2	44 ± 0	0
rGM-CSF	–	560 ± 120	20 ± 8	28 ± 6	130 ± 6	20 ± 2
rGM-CSF	Anti-GM-CSF	504 ± 93	0	0	64 ± 30	0
rIL-3	–	558 ± 9	46 ± 2	24 ± 2	270 ± 40	36 ± 4
rIL-3	Anti-GM-CSF	540 ± 6	88 ± 2	14 ± 2	270 ± 14	32 ± 2

Growth factor concentrations were 10 U/ml. Erythropoietin was added to the cultures at 1 U/ml. The concentration of anti-GM-CSF was the same as in Table II. Duplicate cultures were established with nonadherent mononuclear cells from normal donors as described in Materials and Methods.

When tested on nonadherent normal bone marrow cells, rIL-1α supported erythroid colony formation (BFU-E) in the absence of any other growth factor (Fig. 7). No other type of colony was observed. Because IL-1 has been shown to induce GM-CSF release from monocytes, we investigated the possibility that the stimulation of BFU-E might be due to a low level of GM-CSF production by monocytes that either were generated during the course of the culture or were not totally removed by the overnight adherence. In the presence of the polyclonal antibody to GM-CSF, the number of erythroid colonies induced by rIL-1α was reduced by half. The results suggest that part of the effect of IL-1α on normal bone marrow cells could be explained by an endogenous release of GM-CSF.

At low concentrations of nonadherent normal bone marrow cells, rIL-1 synergized with rGM-CSF in the stimulation of multipotential colonies (Fig. 8). While there was a sixfold increase in multipotential colonies, the increases in granulocyte/macrophage and erythroid colony formation were only two fold (Table IV). In the presence of rG-CSF, there was no significant effect of rIL-1 in the stimulation of either CFU-GEM, BFU-E, or GM-CFC over the level attained with rG-CSF alone (Table IV).
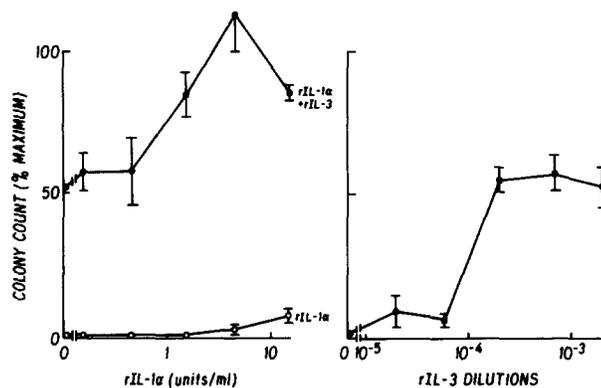

FIGURE 6. IL-1α acts synergistically with IL-3 in the stimulation of AML blast clonogenic cells. Blasts from AML-3 were plated at 5,000 cells per well. Maximum colony count was 113 ± 18.
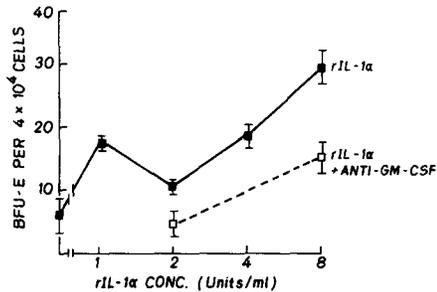
FIGURE 7. Inhibition of rIL-1α-supported BFU-E colony formation by anti-GM-CSF-serum. The polyclonal antibody to GM-CSF was added at a final dilution of 1:1,000, sufficient to neutralize 25 U of GM-CSF per ml (data not shown). Culture conditions as in Table IV.

Discussion

IL-1, long recognized as an important immune mediator, has also proved to be capable of affecting hemopoietic progenitor cell proliferation through at least two different mechanisms. First, IL-1 has been shown by many investigators to indirectly support hemopoietic colony formation by activating different cell types to release a variety of growth factors including GM-CSF and G-CSF (10–12). Second, although IL-1 by itself is incapable of supporting hemopoietic colony formation, in the murine system it has been found to greatly enhance the abilities of the different CSFs to support colony formation by primitive pluripotent stem cells (13–16). This synergistic activity, originally associated with a cytokine designated H-1, is believed to arise from the interaction of IL-1 with quiescent progenitors, which causes them to enter the cell cycle and become CSF responsive (13–16). In our cultures of AML blasts and of normal human bone marrow progenitors, we have observed similar effects providing evidence that IL-1 functions analogously in the human system.

We have found previously that the conditioned medium from the human bladder carcinoma cell line (HTB9-5637) provided a good source of growth factor activity for colony formation by primary AML blast cells, an activity designated LBGF (19). In our efforts to identify the factor(s) responsible for this activity, we have both tested the responsiveness of AML blasts to different known growth factors and purified the activity from the cell line–conditioned medium (HTB9-CM). This conditioned medium has proved to be an excellent source of G-CSF (21), GM-CSF (22), and IL-1α (15). The synergistic effects of G-CSF and GM-CSF in the stimulation of AML blast progenitors have been well documented (18, 19); our observations here suggest
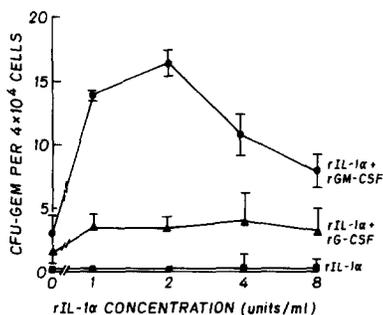

FIGURE 8. Augmentation of rGM-GSF-dependent multipotential (CFU-GEM) colony formation of normal marrow precursors by rIL-1α. rGM-CSF was added at a concentration of 50 U/ml and rG-CSF at 15 U/ml. Values shown on the ordinate were obtained with each growth factor in the absence of IL-1α. Culture conditions as in Table IV.

Table IV

*Effects of rGM-CSF and rG-CSF on the Proliferation of GM-CFC and
BFU-E from Normal Donors*

Growth factor added	Colonies per 4×10^4 cells	
	GM-CFC	BFU-E
None	0	6 ± 4
rIL-1α	2 ± 0	30 ± 2
rGM-CSF	22 ± 1	36 ± 6
rGM-CSF + rIL-1α	47 ± 8	67 ± 1
rG-CSF	50 ± 2	32 ± 1
rG-CSF + rIL-1α	44 ± 1	35 ± 1

Results shown were the highest on a dose-response curve for each growth factor
(rGM-CSF, 50 U/ml; rG-CSF, 15 U/ml; rIL-1α, 8 U/ml). Duplicate cultures
were established at 4×10^4 nonadherent mononuclear cells per ml.

that IL-1α may also contribute to the LBGF activity of HTB9-CM. In fact, the combination of G-CSF, GM-CSF, and IL-1α supports AML blast colony formation as effectively as does the HTB9-CM. Further experiments with appropriate antibodies are underway to determine if these three factors together account for all of the activity originally called LBGF.

The analysis of the ability of the blast cells from five different AML patients to form colonies revealed a significant heterogeneity of responsiveness to the different growth factors. IL-1α by itself was incapable of supporting blast colony formation in cultures of primary blast cells plated at low density. However, with four out of five patient samples tested, IL-1α acted synergistically with the CSFs in support of AML blast colony formation. These five AML blast samples each had a distinct pattern of growth factor responsiveness: one sample (AML-1) yielded a small number of colonies when plated with either G- or GM-CSF, and the colony count was amplified 2–5-fold by addition of IL-1α; one sample (AML-2), which revealed strong synergy in responsiveness to the combination of GM-CSF and IL-1α, plated with comparable efficiency in the presence of G-CSF alone and this efficiency was not increased by IL-1 stimulation; a third patient sample (AML-3) was responsive to G- but not GM-CSF, and the plating efficiency with either CSF was significantly increased by IL-1α; the fourth sample (AML-4) was refractory to GM-CSF even in combination with IL-1α and to G-CSF alone but revealed significant colony formation with G-CSF in combination with IL-1α; and the fifth sample (AML-5), which displayed no IL-1α-responsiveness, yielded colonies in the presence of either G- or GM-CSF alone and gave optimal colony formation with the combination of the two CSFs. Although in these studies we have not distinguished between direct and indirect effects of IL-1α, the observed synergy with GM-CSF and G-CSF is not likely to be due to enhanced production of these factors in culture, because the synergistic effects were clear even when the CSFs were added at concentrations at least threefold in excess of those required for maximal colony formation in the absence of IL-1α. We have not, however, eliminated the possibility that IL-1α induced the production of other growth factors (IL-3 or IL-6, for example) in culture that resulted in enhanced colony formation. In at least one case, this possibility is not likely because the GM-CSF-autocrine blasts from patient AML-6 also demonstrated synergy when cultured

in the presence of IL-1α alone, and this colony formation was substantially blocked by a neutralizing antibody against GM-CSF.

Our observations of the synergy between the different factors in support of the growth of AML blasts prompted us to test for similar effects on the colony formation of normal human hemopoietic progenitors. When tested with nonadherent bone marrow cells, IL-1α alone was found to support the formation of a few erythroid (BFU-E) colonies, although occasional granulocyte and macrophage colonies were also observed (data not shown). Inclusion of a neutralizing antiserum against GM-CSF in these cultures resulted in a 50% reduction in the colony counts, indicating that at least part of the IL-1α-supported colony formation was mediated by enhanced production of GM-CSF by cells in the cultures. However, in the presence of exogenous rGM-CSF, IL-1α resulted in a sixfold increase in the number of multipotential (CFU-GEM) colonies when compared with cultures maintained in GM-CSF alone. A smaller enhancement of BFU-E and GM-CFC colony formation was also observed. Although we again have not distinguished between direct and indirect effects of IL-1α, the selective stimulation of multipotential precursors is consistent with results from studies in the murine system, which suggest that IL-1 acts synergistically with various CSFs in supporting colony formation by increasing the proportion of stem cells in cycle (13–16). Nonetheless, it will be important to test IL-1 in combination with all of the different CSFs using more highly purified human progenitor cells and appropriate antisera to directly distinguish between direct and indirect effects of IL-1.

Analysis of normal hemopoietic progenitor populations is greatly complicated by the extreme heterogeneity of the cells within any purified cell fraction. Although the clonogenic cells present in circulation in any AML patient are also likely to be biologically heterogeneous, surface antigen phenotypic analysis indicates that they may be much less so than normal cells: roughly one third of patients have clonogenic blast cells with surface markers characteristic of the CFU-GEM or earlier, and the remaining two thirds appear to be related to the later, committed progenitors designated CFU-GM (reviewed in reference 32). Although the clonogenic blast cells of AML possess growth characteristics that clearly distinguish them from their normal counterparts, the availability of large numbers of growth factor–responsive cells from different patients provides a unique opportunity for detailed analysis of the interactions of hemopoietic cells with the CSFs and IL-1. With these cell populations, it should be possible to determine if IL-1 stimulation of human cells results in enhanced proliferation through activation of CSF receptor expression (14), through shortening of the Go time of the quiescent cells (18), or through a combination of both mechanisms. It will similarly be of interest to use this model to study the mechanism of synergy between GM-CSF and G-CSF with a leukemic cell population whose normal counterpart has yet to be described.

Summary

IL-1 is released by activated monocytes and is thought to be a key mediator of the host immune response. The availability of the purified and, more recently, recombinant IL-1 has allowed the characterization of other biological properties of this molecule. Thus, IL-1 is thought to have the same properties as hemopoietin 1, a growth factor that has been shown to act on primitive murine hemopoietic cells.

Here we report that rIL-1 acts synergistically with granulocyte/macrophage CSF (GM-CSF) or granulocyte CSF in the stimulation of clonogenic cells from many patients with acute myeloblastic leukemia (AML). Although IL-1 by itself has no effect on AML blasts, it can support colony formation under conditions where there is detectable production of endogenous GM-CSF. IL-1 also promotes the growth of multipotential progenitors from normal human bone marrow cells in the presence of GM-CSF. These observations support the hypothesis that in the hemopoietic system, IL-1 has a selective effect on primitive precursors.

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