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Interleukin 1 as an autocrine growth factor for acute myeloid leukemia cells

(cell proliferation/cytokines/human neoplasia)

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ABSTRACT Production of interleukin 1 (IL-1) by leukemic cells was studied in 13 cases of acute myeloid leukemia. Intracytoplasmic immunofluorescence studies showed that the cells invariably contained the cytokine. Endogenous labeling studies demonstrated that acute myeloid leukemia cells produced either only the 33-kDa propeptide or both the propeptide and the 17-kDa mature form of IL-1 β . The 33-kDa propeptide IL-1 α was always produced but was less frequently released. Involvement of IL-1 in leukemic cell growth was investigated using two antibodies specific for IL-1 subtypes, which inhibited spontaneous cell proliferation in the six cases studied. After acid treatment of the cells, a surface receptor for IL-1 could be demonstrated, which mediated ¹²⁵I-labeled IL-1-specific uptake by leukemic cells. Furthermore, recombinant IL-1 α or IL-1 β induced significant cell proliferation in 10 of 12 cases. The above findings were uncorrelated with the cytologic type (French-American-British classification) of leukemia. Our studies suggest that IL-1 may act as an autocrine growth factor in most cases of acute myeloid leukemia.

Acute myeloid leukemia (AML) is a malignant process characterized by abnormal growth and maturational arrest of myeloid precursor cells. These abnormalities may be related to the escape by leukemic cells from normal genetic control mechanisms. Alternatively, AML cells may become independent from the supply of exogenous growth factors, which are necessary for optimal proliferation and differentiation of their normal counterparts. This second mechanism could be due to the ability of AML cells to synthesize and respond to growth factors. An autocrine secretion of growth factors may be operational in many malignancies (1). Recently leukemic cells from some AML patients have been reported to constitutively produce granulocyte macrophage-colony stimulating factor (2, 3), one hormone that stimulates immature myeloid cell proliferation and differentiation (4).

We have shown previously that cells from some AML patients release large amounts of interleukin 1 (IL-1) *in vitro* (5). This latter feature was related to the presence *in vivo* of coagulation abnormalities, such as the diffuse intravascular coagulation syndrome. Because IL-1 appears to promote the growth of several cell types, we suggested that IL-1 could operate as an autocrine growth factor—at least for some AML clones. In this study, we demonstrate that cells from all AML patients studied can produce IL-1. We also show that anti-IL-1 antibodies can modulate the spontaneous proliferation of leukemic cells and that exogenous human recombinant (r) IL-1 α or IL-1 β interacts specifically with leukemic cells and enhances their growth ability.

MATERIALS AND METHODS

Patients. Thirteen patients with AML were randomly selected; diagnosis of AML was based on clinical, morphological, and cytochemical criteria, according to the French-American-British classification (6). All patients were studied before any treatment, and informed consent was obtained.

Reagents. Purified recombinant IL-1 α and IL-1 β were obtained as described (7, 8). The specific activity of both was 1.3×10^7 half-maximal units per mg in the thymocyte costimulation assay. Recombinant tumor necrosis factor (TNF) and interferon γ (IFN- γ), specific activity 9.6×10^6 units/mg in the L929 cytotoxicity assay and 2.1×10^7 National Institutes of Health reference units per mg, respectively, were gifts from Biogen (Geneva). Neutralizing antisera against rIL-1 α and rIL-1 β were obtained in rabbits. These sera contained 10^7 and 0.5×10^6 neutralizing units per ml, respectively, and did not affect the proliferation of continuous IL-2-dependent normal T-cell lines when used at a final dilution of 1:50.

Separation and Culture of Leukemic Cells. Leukemic cells were isolated by Ficoll/Hypaque (Pharmacia, Prodotti Gianni, Milan) density gradients as described (5). Cells were washed and resuspended in RPMI 1640 (Flow Laboratories, Milan) supplemented with 2 mM L-glutamine, 100 units of penicillin per ml, 100 μ g of streptomycin per ml, 100 μ g of polymixin B per ml (hereafter referred to as complete medium, CM), and 10% fetal calf serum (Flow). After separation, cytocentrifuged smears were stained with May-Grünwald-Giemsa and scored for atypical cells. When necessary, cell suspensions were depleted of normal monocytes by incubation at 37°C for 1 hr in plastic Petri dishes and depleted of T cells by rosetting with neuraminidase-treated sheep erythrocytes. All suspensions always contained >95% malignant cells.

To obtain conditioned medium, cells were cultured in complete medium/fetal calf serum at a concentration of 1×10^6 cells per ml for 48 hr. For the cell proliferation studies, different concentrations of leukemic cells from 1×10^4 cells per ml to 2.5×10^5 cells per ml were incubated in 96-well microtiter plates for 48 hr with or without rIL-1 α or rIL-1 β , rTNF, or rIFN- γ at 5 ng/ml and with neutralizing antibodies anti-IL-1 α , anti-IL-1 β , or preimmune rabbit serum as control. Different dilutions, 1:4–1:32, of IL-1-containing leukemic cell supernatants were tested in the same culture conditions. Cultures were treated with 0.5 μ Ci of [³H]thymidine (³H]Thd) (specific activity 25 Ci/mmol; 1 Ci = 37 GBq; Amersham, Prodotti Gianni) 18 hr before harvesting, and the radioactivity was determined in a liquid scintillation counter (Beckman Analytical, Milan).

Abbreviations: IL-1, interleukin 1; AML, acute myeloid leukemia; TNF, tumor necrosis factor; IFN- γ , interferon γ ; r, recombinant; [³H]Thd, [³H]thymidine.

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Evaluation of IL-1 Production. The IL-1 produced by leukemic cells was assessed by intracytoplasmic immunofluorescence, endogenous labeling and immunoprecipitation experiments, and measurement of biological activity.

Immunofluorescence was performed essentially as described by Bayne *et al.* (9). Briefly, freshly drawn leukemic cells were cytocentrifuged, fixed in 2% (wt/vol) paraformaldehyde, and permeabilized with 0.1% Triton X-100; the cells were then incubated with a 1:50 dilution of anti-IL-1 antiserum or preimmune rabbit serum as control, washed, and stained with fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum (Cappel Laboratories, BCI Human, Milan) at a final dilution of 1:100. Specimens were examined either by conventional microscope or by a confocal scanning laser microscope developed by B. Amos and J. White (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.).

IL-1 activity in culture supernatants was assayed as described (10) by the thymocyte costimulation assay with rIL-1 β as standard.

Endogenous labeling and immunoprecipitation studies were performed as described (11). Freshly drawn leukemic cells were incubated at 10^7 cells per ml for 6 hr in methionine-free RPMI 1640 medium (Flow) supplemented with 5% dialyzed fetal calf serum in the presence of [35 S]methionine at 100 μ Ci/ml (specific activity, 800 Ci/mmol, NEN/DuPont Italia, Firenze). Cells were then centrifuged, washed, and lysed in phosphate-buffered saline with 0.25% Nonidet P-40. Samples of both cell lysates and supernatants were immunoprecipitated with 5 μ l of anti-IL-1 α or anti-IL-1 β antisera or of preimmune rabbit serum as control, followed by 50 μ l of protein A-Sepharose CL-4B (Pharmacia). Immunoprecipitates were extensively washed, eluted by boiling in Laemmli stacking buffer (12) containing 5% (vol/vol) 2-mercaptoethanol, run on 12% NaDodSO $_4$ /PAGE, and autoradiographed.

Radioiodination of rIL-1 α . rIL-1 α was radioiodinated using the method reported by Lowenthal and MacDonald (13). The specific activity was 0.5×10^5 cpm/ng. The material produced a single band of 17 kDa in a NaDodSO $_4$ /PAGE analysis and retained its biological activity.

Surface Binding and Uptake of 125 I-Labeled IL-1 α . Binding experiments were performed at 4°C for 2 hr as described (13, 14). Cells were incubated in Hepes-buffered RPMI 1640 medium at pH 3.0, washed, and resuspended in medium at pH 7.4 supplemented with 0.02% sodium azide and 0.5% bovine serum albumin. Leukemic cells ($3-6 \times 10^6$) were incubated with different concentrations (10 pM–3 nM) of 125 I-labeled IL-1 α , with or without a 100-fold excess of unlabeled IL-1 α . Free radioactivity was separated from bound radioactivity by centrifugation through an oil gradient. The same procedures were also applied to either the murine T-cell line EL4 or purified normal T lymphocytes stimulated for 16 hr with phorbol myristate acetate. Data were analyzed by a scientific data analysis program (Enzfitter-Biosoft, Cambridge, U.K.) to determine the K_d of the reaction and the number of receptor molecules per cell.

For determination of IL-1 uptake, 3×10^6 cells were incubated for 2–4 hr at 37°C with 0.5 or 1 nM 125 I-labeled IL-1 with or without excess unlabeled IL-1. The suspensions were then centrifuged, the supernatants were harvested, and the cells were washed three times with phosphate-buffered saline and lysed in Nonidet P-40 as above. Aliquots of both supernatants and cell lysates were dissolved in the scintillation mixture and directly counted or treated overnight with 10% (vol/vol) trichloroacetic acid at 4°C, neutralized in Laemmli stacking buffer, and analyzed by NaDodSO $_4$ /PAGE as above.

RESULTS

IL-1 Production by AML Cells. Cells from 13 randomly selected AML patients were analyzed for the release of IL-1 activity in culture supernatants by use of the thymocyte costimulation assay and for intracellular IL-1 by cytoplasmic immunofluorescence with anti-IL-1 α or anti-IL-1 β monospecific antibodies. These analyses demonstrated that cells from 10 of the 13 cases studied released IL-1 activity spontaneously (Table 1) after a 48-hr culture period. By contrast, every case showed a high proportion of cells ($\geq 80\%$) containing detectable IL-1 molecules (Fig. 1) either before or after the culture. In particular, two-color immunofluorescence indicated that both α and β molecular forms of IL-1 were present within the same leukemic cell, although differences in staining intensities were often seen (data not shown).

To investigate in more detail the molecular pattern of IL-1 production and release, leukemic cells from eight patients were endogenously labeled with [35 S]methionine for 6 hr. Both cell lysates and supernatants were subsequently immunoprecipitated with antibodies to IL-1 α or IL-1 β , and the immunoprecipitates were analyzed by NaDodSO $_4$ /PAGE. Results of these experiments demonstrated that cells from all patients synthesized the 33-kDa precursor molecule of both IL-1 α and IL-1 β , although the former was less abundant (Fig. 2). The 33-kDa pro-IL-1 β , but not pro-IL-1 α , was consistently released in the culture supernatant. By contrast, the 17-kDa mature IL-1 β was immunoprecipitated from the culture supernatants of five cases of the eight studied in which IL-1 activity was also detected.

Role of IL-1 in AML Cell Proliferation. That cells from all AML patients produced IL-1 suggested that this cytokine could be involved in some functions of the leukemic cells. Because IL-1 has been demonstrated to promote cell growth in several systems (15), the role of IL-1 for AML cell proliferation was evaluated. Cells (1×10^5) from 10 patients were cultured for 48 hr with or without neutralizing anti-IL-1 α or anti-IL-1 β antibodies, and their proliferative activity was evaluated. In all cases the anti-IL-1 antibodies could inhibit the spontaneous [3 H]Thd uptake in a dose-dependent fashion, although to a different extent (Table 2). This phenomenon was independent of the level of spontaneous cell proliferation, which varied greatly in the different AML patients. Interestingly, anti-IL-1 α antibodies were less effective than anti-IL-1 β in inhibiting cell proliferation. This finding is consistent with the poor production of IL-1 α , as determined by the endogenous labeling experiments de-

Table 1. IL-1 activity released by AML cells

Patients (cell type)	IL-1 activity, units/ml
C.A. (M1)	56
B.G. (M4)	140
M.R. (M4)	65
F.I. (M2)	136
D.M. (M2)	47
B.E. (M2)	80
S.I. (M5)	168
P.A. (M5)	21
Z.D. (M2)	24
B.S. (M5)	28
M.M. (M2)	
B.T. (M2)	
C.L. (M1)	

AML cells were cultured at 1×10^6 cells per ml for 48 hr. Supernatants were harvested and tested for IL-1 activity in the thymocyte costimulation assay. The cytological types, according to French-American-British classification (6), appear in parentheses. For comparison, unstimulated or lipopolysaccharide-stimulated monocytes from eight normal donors yielded under the same conditions 20 ± 2 and 75 ± 11 units/ml, respectively.

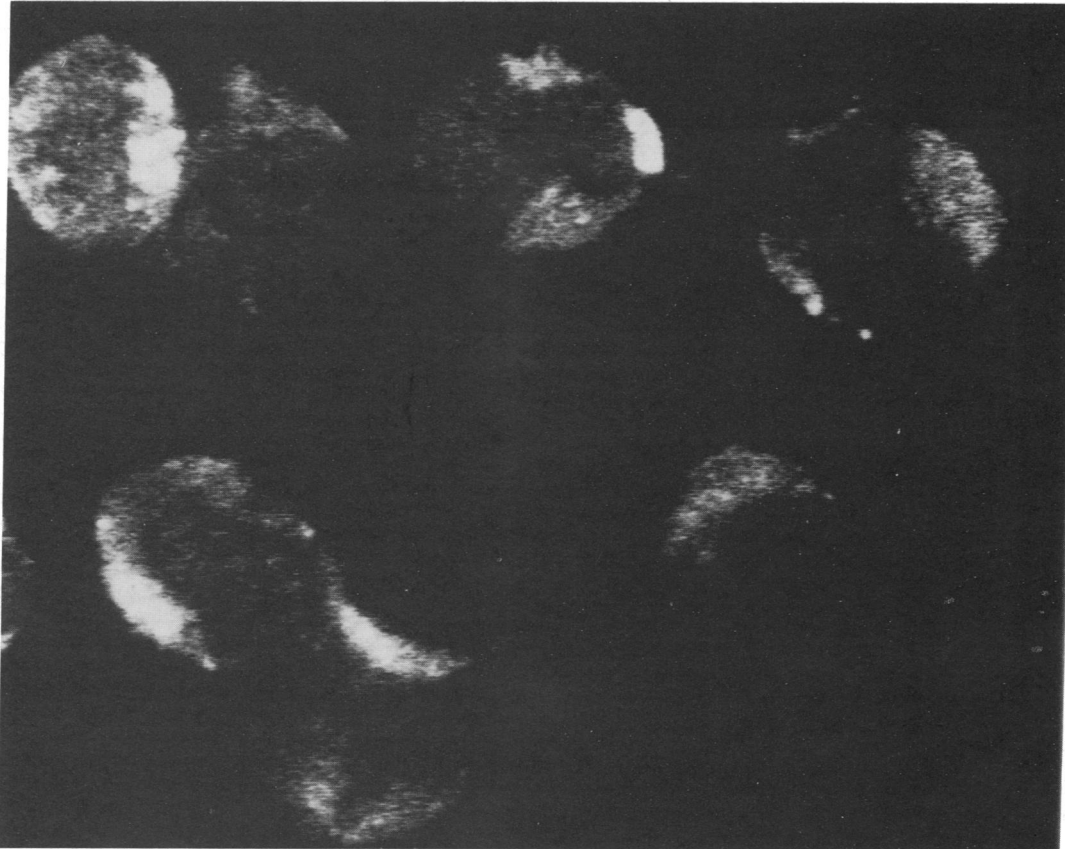


FIG. 1. Immunocytochemical detection of IL-1 in AML cells. Freshly drawn AML cells were fixed in 2% paraformaldehyde, permeabilized by 0.1% Triton X-100, and stained with polyclonal rabbit anti-IL-1 antibodies followed by fluorescein-conjugated goat anti-rabbit antibodies. The cells were examined by a confocal scanning laser microscope.

scribed above. An intriguing observation is that anti-IL-1 antibodies also could inhibit the cell growth in patient M.M., whose cells did not release detectable IL-1 (Table 2).

These results suggested that IL-1 was involved in AML cell proliferation as an autocrine growth factor. Further support for this thesis came from the observation that IL-1-containing supernatants from AML cells cultured at high density could elicit a significant proliferative response of the cells, cultured at lower density, from the same or another AML patient. Table 3 shows the results of two selected experiments.

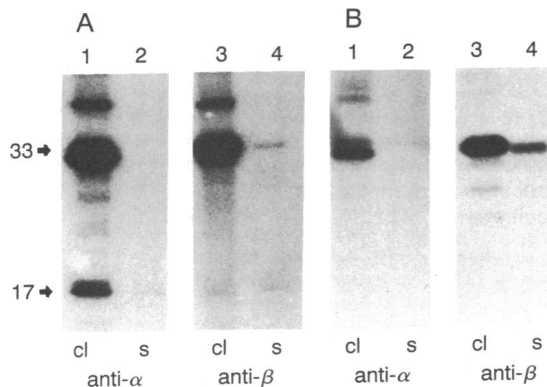


FIG. 2. Different patterns of IL-1 production by cells from AML cases. Leukemic cells were endogenously labeled with [³⁵S]methionine for 6 hr. Cell lysates (cl) and supernatants (s) were immunoprecipitated with anti-IL-1 α (lanes 1 and 2) or anti-IL-1 β (lanes 3 and 4) polyclonal antibodies, and the immunoprecipitates were analyzed by NaDodSO₄/PAGE. Preimmune sera failed to precipitate any detectable material. Two representative experiments (A and B) of eight performed are shown. Figures at left represent kDa.

Supernatants obtained from 48-hr cultures of 1×10^6 cells per ml from patients M.R., B.G., and F.I., tested against 1×10^5 cells per ml from patients M.R. and M.M., induced a significant proliferative response. The table also shows that the [³H]Thd incorporation induced by the supernatants was markedly reduced by anti-IL-1 antibodies.

Proliferative Response of AML Cells to Exogenous IL-1. We further investigated whether or not exogenous IL-1 could substitute for the autocrine growth factor of the supernatant. AML cells were cultured with or without rIL-1 α or rIL-1 β or other recombinant cytokines, such as TNF and IFN- γ as control. Table 4 shows that both rIL-1 α and rIL-1 β could

Table 2. Effect of anti-IL-1 α and anti-IL-1 β antibodies on AML cell proliferation

Added serum	[³ H]Thd incorporation by cells from patient, cpm		
	M.R.	B.G.	M.M.
None	3845	11,474	2040
anti-IL-1 α			
1:50	2004	8,875	1875
1:250	2850	9,436	1920
1:1000	3475	11,314	2100
anti-IL-1 β			
1:50	950	3,796	680
1:250	1250	4,235	920
1:1000	2947	10,384	1820
Control (1:50)	3780	10,990	2129

AML cells were cultured at 5×10^5 cells/ml for 48 hr with or without anti-IL-1 α , anti-IL-1 β , or control serum. Results of three representative experiments of six performed are shown. Data are expressed as mean of triplicate cultures; SD was always <10%.

Table 3. Proliferative activity of AML cells induced by AML cell supernatants

Stimulus	³ H]Thd incorporation, cpm			
	M.R.		M.M.	
	None	anti-IL-1*	None	anti-IL-1*
None	1,450	456	560	298
Supernatant				
M.R.	7,850	2647	4,880	1498
B.G.	8,950	3879	7,540	1579
F.I.	10,480	3900	15,640	2968

AML cells from patients M.R. and M.M. were cultured at 1×10^5 cells per ml for 48 hr with or without AML cell supernatants (1:4 final dilution). Data are shown as the mean of triplicate cultures; SD was $<10\%$.

*Anti-IL-1 α and anti-IL-1 β antisera were added at 1:100 final dilution. Controls with the same amounts of preimmune rabbit serum consistently failed to show any toxic effect.

induce cell growth in 10 of 12 cases, with a stimulation index ranging from 3 to 25. Anti-IL-1 antibodies specifically blocked the phenomenon, and rTNF or rIFN- γ , as controls, failed to promote cell proliferation (data not shown). In most cases the supply of exogenous rIL-1 α or rIL-1 β allowed the establishment of continuous cell lines that grew for >2 mo. Interestingly, different cell concentrations had to be tested in each AML case to detect proliferation in response to the exogenous factor—possibly due to interference of the endogenously available IL-1. The response to exogenous IL-1 was neither related to the presence or absence of IL-1 activity in the cell supernatants nor to the level of spontaneous cell proliferation (see also Table 1 for comparison).

IL-1 Binding and Uptake by AML Cells. To demonstrate directly a specific interaction between IL-1 and its surface receptors, we performed binding experiments with ¹²⁵I-labeled rIL-1 using cells from several AML cases. Widely accepted procedures (13, 14) consistently failed to show specific receptors on this particular cell type. However, we reasoned that the endogenous ligand could be occupying the receptors; we therefore incubated the cells in acidic buffer to remove any such ligand. Fig. 3 shows that after acid treatment specific binding occurred. K_d of the reaction was $\approx 2 \times 10^{-10}$ M and, from the Scatchard analysis of the data, we calculated that an average number of 200 receptor molecules per cell was expressed. We further tried to demonstrate IL-1 internalization by AML cells. Cells were incubated for 4 hr at 37°C with different concentrations of ¹²⁵I-labeled rIL-1

Table 4. IL-1-induced proliferation of AML cells

Patient	³ H]Thd incorporation, cpm		
	None	rIL-1 α	rIL-1 β
M.R.*	1,211	14,530	13,876
B.S.*	2,491	7,889	8,143
F.I.*	12,595	53,888	51,459
B.T.*	2,470	18,300	21,100
M.M.*	912	15,219	14,345
D.M.*	812	4,426	4,915
B.G.†	2,238	20,295	19,896
Z.D.†	3,248	13,044	11,621
C.A.‡	1,456	4,145	4,768
P.A.‡	2,103	13,087	14,001
B.E.*	5,354	5,250	5,987
S.I.*	1,314	1,544	1,654

AML cells were cultured in triplicate at 2.5×10^5 cells per ml (*) or 1×10^5 cells per ml (†), or 1.25×10^4 cells per ml (‡) for 48 hr with or without rIL-1 α (100 units/ml) or rIL-1 β (100 units/ml). Data are shown as mean of triplicate cultures; SD was $<10\%$. Control cultures with other recombinant factors (TNF, IFN- γ) did not show any significant increase in the spontaneous proliferation of AML cells.

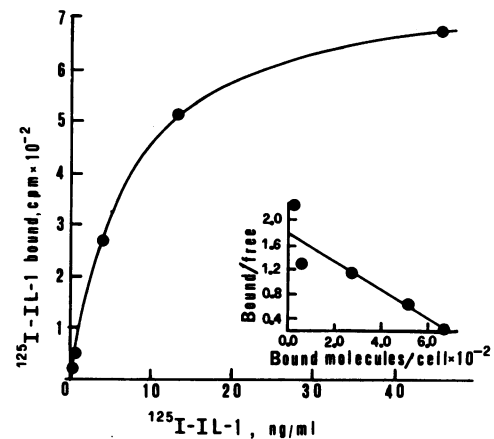


FIG. 3. ¹²⁵I-labeled IL-1 surface binding. Cells (3×10^6) from patient P.A. were incubated at 4°C for 2 hr with different concentrations of ¹²⁵I-labeled IL-1 α . The specific radioactivity of the molecule was 5.1×10^4 cpm/ng. Specific binding was calculated by subtracting the count of samples incubated with 100-fold excess of unlabeled IL-1 α . Nonspecific binding was $\leq 25\%$. Comparable results were obtained in three different experiments with cells from other AML patients.

with or without excess unlabeled rIL-1. At the end of the incubation period, cells were washed, lysed, and aliquots from cell lysates and supernatants were harvested for direct counts, trichloroacetic acid precipitation, and gel analysis. A specific uptake of ¹²⁵I-labeled IL-1 was evident at 37°C (Fig. 4) but not at 4°C (data not shown). Further evidence came from NaDodSO₄/PAGE analysis, which showed that a dose-dependent uptake of ¹²⁵I-labeled IL-1 by the cells, as indicated by a 17-kDa band, occurred when cells were incubated with different amounts of ¹²⁵I-labeled IL-1 without but not with excess unlabeled rIL-1. Supernatants containing both ¹²⁵I-labeled IL-1 and unlabeled IL-1 displayed more intense bands in comparison with supernatants containing labeled IL-1 only. It is noteworthy that no IL-1 degradation was seen (data not shown).

DISCUSSION

We demonstrate that cells from AML patients invariably produce IL-1, which in turn supports the proliferation of the leukemic cells. This contention is supported by the following experimental observations: (i) endogenous labeling studies and immunofluorescence analysis show that either the 33-kDa propeptide form of IL-1 or both the propeptide and the 17-kDa mature forms are synthesized by most cells from the single leukemic clones; (ii) leukemic cells can recognize specifically exogenous IL-1, as shown by binding and uptake of radiolabeled IL-1 molecules; (iii) proliferation of AML cells in culture is affected by anti-IL-1 antibodies, and culture supernatants containing IL-1 specifically increase the leukemic cell growth. The latter phenomenon can be reproduced

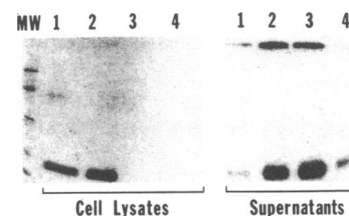


FIG. 4. NaDodSO₄/PAGE analysis of ¹²⁵I-labeled IL-1 α present in cell lysate and supernatant from AML cells incubated at 37°C for 4 hr without (lanes 1 and 2) or with (lanes 3 and 4) 100-fold excess of unlabeled IL-1 α . ¹²⁵I-labeled IL-1 α was used at concentrations of 0.5 nM (lanes 1 and 4) or 1 nM (lanes 2 and 3).

with the use of exogenous rIL-1. Altogether, these data indicate that IL-1 acts as an autocrine growth factor for AML cells. The evidence that cells from all AML cases actively synthesize IL-1 suggests a general role for this cytokine in leukemic cell proliferation. The observation that the cells from all our cases produced IL-1 confirms and extends the data reported by Griffin *et al.* (16), who showed easily detectable IL-1 β mRNA in cultured cells from 10 of 17 cases. In this connection, it has recently been reported that only in one of two cases of AML did cells produce IL-1 and in one of five cases did IL-1 elicit a proliferative response (17). These discrepancies are probably related to the more sensitive experimental procedures used in our studies, such as the endogenous labeling and immunofluorescence analyses for IL-1 detection and the various cell concentrations used in the proliferation assays. In our studies, cells displaying an active spontaneous proliferation failed to respond to exogenous IL-1 at the highest cell densities, and a response was detectable only when cells were cultured at lower densities. At high cell concentrations, the endogenously produced IL-1 may obscure the effects of the exogenous factor.

In our experiments, IL-1 α and IL-1 β were equally effective in promoting cell growth, a finding consistent with the evidence that both molecules compete for the same receptor (18–20). However, in most AML cases, IL-1 β was the molecular form more abundantly produced, a finding that could explain why anti-IL-1 β antibodies were more effective in inhibiting spontaneous cell growth. However, in those cases in which IL-1 α was produced in significant amounts, anti-IL-1 α antibodies profoundly affected cell proliferation.

AML cells were equipped with specific receptors that were occupied by the endogenously produced IL-1. As in other systems in which autocrine factors were involved (21, 22), acid treatment could remove endogenous IL-1 and hence allow analysis of the receptor molecule. Both the affinity and the number of receptors were comparable to those reported for other cell types. In particular, unlike the receptors for other cytokines, a few hundred molecules were expressed on each single cell. The efficient binding and utilization of endogenous IL-1 after production could account for the absence of IL-1 activity in the supernatants of cells from the two cases studied in which anti-IL-1 antibodies did affect cell proliferation. Alternatively, leukemic cells could use the 33-kDa propeptide form of IL-1 β they consistently release, which is devoid of biologic activity when tested on murine T cells (23).

Cells from two of the twelve patients studied, who were high producers of IL-1, failed to respond to the exogenous factor at any cell concentration tested. Again, an explanation could be the production, even at low cell concentrations, of endogenous factor in amounts sufficient to sustain cell proliferation.

In our series, IL-1 involvement in cell proliferation was shown for cells of different cytological types. This suggests that an autocrine pathway is related to the malignant nature of the cells, rather than being reminiscent of the physiological behavior of normal myeloid counterparts. Alternatively, the autocrine mechanisms of proliferation could have a wider significance than that so far suspected.

Previously IL-1 was reportedly able to promote proliferation of several cell types such as, for example, T and B lymphocytes, immature myeloid precursors, epidermal cells, fibroblasts, etc. (15). The precise mechanisms underlying the growth-promoting activity of IL-1 remain unclear. However, data obtained from studies on the T-cell system, in which IL-1 induces IL-2 and IL-2 receptor expression (24–27), suggest that a single basic mechanism—i.e., induction of growth factor or growth factor receptor expression is responsible for the effect of IL-1 on cell proliferation. Support for this hypothesis comes from recent evidence showing that IL-1 synergizes with granulocyte colony-stimulating factor, perhaps upregulating the expression of its receptor on normal

myeloid cell precursors (28), and that IL-1 can induce granulocyte/macrophage colony-stimulating factor expression by endothelial cells (29). Because myeloid leukemic cells produce and proliferate in response to granulocyte/macrophage colony-stimulating factor (2, 3), it seems possible that IL-1 is part of a more complex autocrine loop.

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