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Modulation of Cell Proliferation and Cytokine Production in Acute Myeloblastic Leukemia by Interleukin-1 Receptor Antagonist and Lack of its Expression by Leukemic Cells

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Interleukin-1 (IL-1) is spontaneously produced by acute myeloblastic leukemia (AML) cells. IL-1 also induces synthesis of colony-stimulating factors (CSFs) and sustains leukemic growth. An IL-1–specific inhibitor has been recently purified and cloned; this molecule binds to IL-1 receptors but has no IL-1 activity, fulfilling the characteristics of an IL-1 receptor antagonist (IL-1ra). Because high-affinity binding sites for IL-1ra were shown on AML cells by radioligand binding studies, we studied the effect of IL-1ra on the proliferative activity of blast cells isolated from 16 cases of AML. In each case, spontaneous proliferation was inhibited by addition of the IL-1ra in a dose-dependent manner (1 to 100 ng/mL). Culture supernatants of unstimulated leukemic cells contained IL-1β and granulocyte-macrophage CSF (GM-CSF), but when incubated with the IL-1ra, a reduction or disappearance of GM-CSF was observed in 8 of 10 cases, whereas spontaneous IL-1 production was reduced in four of seven cases. By Northern hybridization, IL-1β gene transcripts were shown in 20 of 23 AML cases, whereas IL-1ra–specific messenger RNA was present in only two of the patients studied. These data show a role for IL-1 in the spontaneous proliferation and cytokine production of AML cells and suggest that an imbalanced synthesis of IL-1 and of its natural receptor antagonist may contribute to the unrestricted growth of AML cells.

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THE AUTONOMOUS GROWTH of cancer cells is frequently driven by the same factors that stimulate normal cell proliferation. Cancer cell proliferation may take place in the context of autocrine circuits, whereby neoplastic cells produce their own growth factors and use them to proliferate. Growth can also occur via paracrine circuits, whereby they divide in response to growth factors released by the surrounding cells.[1,2] Common to both modalities is the interaction of ligands with specific surface receptors that convey the growth signal to the nuclear compartment. In acute myelogenous leukemia (AML), autocrine and paracrine loops supply the malignant cells with potent stimulants, such as the colony-stimulating factors (CSFs).[3-6] We and others have reported that in AML the autocrine production of interleukin-1 (IL-1) supports neoplastic growth in vitro[7,8] and maintains the autocrine synthesis of granulocyte-macrophage CSF (GM-CSF) and granulocyte CSF (G-CSF).[9] Studies have also shown spontaneous expression of the IL-1β gene in freshly obtained AML cells, whereas blood leukocytes from healthy donors do not express this gene unless stimulated.[4] Antibodies to IL-1α and β suppress the spontaneous proliferation of AML cells.[7-12]

A 23-Kd glycoprotein isolated from the urine of a patient with monocytic leukemia[13] was shown to inhibit a variety of biologic activities of IL-1. This IL-1 "inhibitor" specifically blocked the binding of IL-1 to its cell surface receptors.[14] Recently, this IL-1 inhibitor has been cloned and its amino acid sequence showed significant structural homology with both IL-1α and β.[15] The nonglycosylated form of the IL-1 inhibitor competes with IL-1 for the binding to specific receptors, but is devoid of IL-1 biologic activity; accordingly, it has been renamed the IL-1 receptor antagonist (IL-1ra).

We therefore sought to investigate the effects of IL-1ra on leukemic cell growth and on functional parameters of AML cells. In addition, because some neoplastic cells have been characterized by the absence of factors that reduce growth, we studied IL-1ra gene expression in freshly obtained leukemic cells.

MATERIALS AND METHODS

Leukemic cells. Leukemic cells of 28 randomly selected AML patients were obtained from bone marrow aspirate or peripheral blood samples. Approval was obtained from the Institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki. The diagnosis of AML was established by morphology, cytochemical staining, and surface marker analysis, using a panel of antimyeloid monoclonal antibodies (MoAbs), as detailed elsewhere.[3-5] Leukemic cells were purified by Ficoll-Hypaque gradient centrifugation (> 90% pure blasts) and total cellular RNA was immediately extracted as described.[3-5] Cell viability was confirmed by flow cytofluorimetry of cells stained with propidium iodide, 10 μg/mL, for 1 hour.[16] Vials of leukemic cells of each patient were also cryopreserved in 10% dimethyl sulfoxide (DMSO) in the vapor phase of liquid nitrogen until use.

Cell culture. For proliferation studies, leukemic cells were cultured in 96-well round-bottom microtiter plates in the presence or absence of different reagents at 2.5×10^5/mL in RPMI 1640 medium, supplemented with 2 mmol/L L-glutamine and 10% fetal calf serum (FCS; HyClone, Logan, UT), hereafter referred to as complete medium. Cells were pulsed with 0.5 μCi of ^{3}H-thymidine (Amersham, Little Chalfont, Buckinghamshire, UK; specific activity, 25 Ci/mmol) 12 hours before harvesting.

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Human recombinant IL-1α (IL-1α) had a specific activity of 1.3 × 10^7 half-maximal units/mg in the thymocyte costimulation assay; this cytokine was obtained through the courtesy of Dr A. Galazka (Glaxo Institute for Molecular Biology, Geneva, Switzerland). Human recombinant IL-1β (generously provided by Dr D. Boraschi, Sclavo, Siena, Italy) had a specific activity of 1 × 10^7 U/mg of protein. Human recombinant IL-1ra was obtained from Dr R.C. Thompson (Synergen Inc, Boulder, CO); this cytokine was inactivated by heating at 90°C for 15 minutes. The IgG fractions of neutralizing sheep antisera against IL-1α and β, a generous gift from Dr S. Poole (National Institute for Biological Standards and Controls, Potters Bar, UK), were obtained by affinity chromatography on Protein A-Sepharose columns and used at a final concentration of 5 μg/mL. Recombinant human GM-CSF, purchased from Genzyme, Inc (Boston, MA), had a specific activity of 5 × 10^7 proliferation units/mg.

Binding studies. IL-1α was radioiodinated using the method reported by Lowenthal and MacDonald.[17] The labeled protein had a specific activity of 0.5 mCi/mg and produced a single band of 17 Kd in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For binding studies, leukemic cells, 10^6 per tube, were pretreated at pH 3.0 (RPMI 1640 medium brought to pH 3.0 with phosphoric acid 1 mmol/L) for 1 minute at 4°C, washed, and resuspended in medium at pH 7.4 supplemented with 0.02% sodium azide. Cells were then treated with various concentrations of IL-1α or of IL-1ra for 30 minutes at room temperature, and finally incubated with 0.2 nmol/L ^{125}I-IL-1α for 2 hours at 4°C. Specific binding and percent of inhibition of specific binding to various concentrations of IL-1ra were calculated relative to the control samples.

Measurement of cytokine production. Leukemic cells (10^6/mL) were cultured in complete medium in the presence or absence of IL-1ra for 48 hours. Supernatants were then collected and cells lysed through three cycles of freezing and thawing. IL-1β,[18] GM-CSF,[19] and IL-6[20] were measured by specific, non-cross-reacting radioimmunoassays (RIA). Each RIA has been validated using the cytokine in their respective biologic assay.

Northern blots. Total cellular RNA was electrophoresed in agarose gels, and blotted as previously described.[3-5] Membranes were hybridized with cDNA probes labeled with ^{32}P-dCTP by the method of Feinberg and Vogelstein.[21] The cDNA for the IL-1ra was obtained by polymerase chain reaction (PCR) using messenger RNA (mRNA) from AML-193 cells as substrate.[22] The cells were treated with 4 nmol/L phorbol myristate acetate (PMA) and 1 ng/mL GM-CSF for 24 hours before lysis and extraction of poly-A$^+$ RNA. PCR primers were synthesized to correspond to the 5′ and 3′ ends of the mature IL-1ra coding sequence as published by Eisenberg et al.[15] The IL-1β cDNA probe was a gift of Dr Steven Clark (Genetics Institute, Cambridge, MA). The chicken actin cDNA was obtained from the American Type Culture Collection (ATCC; Rockville, MD).

RESULTS

IL-1ra and IL-1α compete for binding to surface receptors. To show the existence of specific binding sites for IL-1ra on myeloid cells, radioligand binding studies were performed. The percentage of binding inhibition of iodinated IL-1α in the presence of increasing concentration of cold IL-1α and IL-1ra was evaluated. As shown in Fig 1, unlabeled IL-1ra was only slightly less efficient than unlabeled IL-1α in exerting a dose-dependent inhibition of ^{125}I–IL-1α binding to surface receptors, indicating the existence of high-affinity binding sites for IL-1ra on AML cells. It should be noted that these data represent the binding to high-affinity IL-1 receptors on leukemic cells, which are uncovered after removal of the endogenous cytokine with an acid wash.[8]

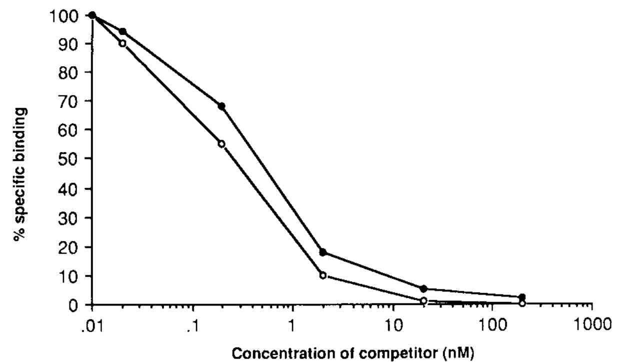

Fig 1. Effect of IL-1ra on binding of ^{125}I-labeled rIL-1α to AML blasts. Binding experiments were performed incubating 10^6 AML cells for each point with increasing concentrations of either unlabeled (○) rIL-1α or (●) rIL-1ra in the presence of 0.2 nmol/L ^{125}I-labeled rIL-1α.

IL-1ra inhibits spontaneous AML cell proliferation in vitro. On the basis of this observation, in a preliminary experiment we evaluated the effect of different concentration of IL-1ra on the in vitro growth of blasts from three AML patients characterized by different levels of spontaneous proliferation (Fig 2). In these cases, regardless of the spontaneous baseline proliferation, we observed a significant inhibitory effect at concentrations as low as 1 ng/mL and up to 50 ng/mL. There was no further reduction in proliferation using higher doses of IL-1ra. For this reason, 13 additional AML cases were studied using a fixed concentration of 100 ng/mL of IL-1ra. In each case, we could measure some inhibition of spontaneous AML cell proliferation ranging from 27% to 82% (Table 1). In these conditions, the ex vivo proliferation of AML cells depends

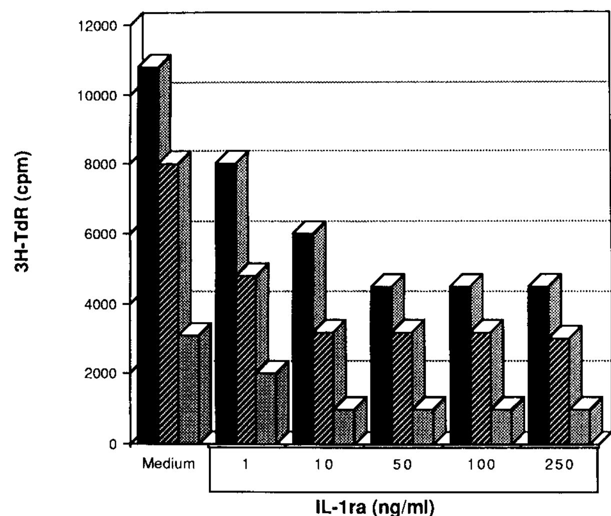

Fig 2. Dose-dependent effect of IL-1ra on leukemic cell proliferation from three different AML patients. Cells were cultured for 48 hours at the concentration of 2.5 × 10^5/mL, in the presence or absence of the indicated stimuli. ^{3}H-TdR was added during the last 12 hours of culture. Results are expressed as mean cpm of triplicate cultures. SD was ≤10%. (■) Patient 1; (▨) patient 2; (▤) patient 3.

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Table 1. Effect of IL-1ra on Spontaneous Proliferation of AML Blasts

Case No.	^{3}H-TdR Incorporation (cpm) Medium Alone	rIL-1ra*	% decrease	Anti–IL-1α +Anti–IL-1β†	% decrease	rIL-1β†‡
1	10,786	3,990	63	6,540	39	25,348
2	7,847	2,893	63	4,001	49	28,345
3	3,180	750	76	2,110	34	8,530
4	31,752	7,375	77	19,367	39	61,459
5	1,201	590	51	804	34	3,980
6	15,134	8,386	45	10,345	32	21,476
7	20,797	12,687	39	15,890	24	28,789
8	1,414	275	81	789	45	4,873
9	4,413	1,659	62	2,234	49	16,578
11	4,824	2,003	58	3,201	34	37,658
12	3,262	922	72	1,756	46	7,916
13	50,392	8,897	82	28,547	43	51,458
14	1,048	204	80	260	75	4,520
15	1,549	1,004	33	ND	ND	ND
16	14,429	10,521	27	13,824	4	28,643

Cells were cultured for 48 hours at the concentration 2.5 × 10^5/mL in the presence or absence of the indicated stimuli. ^{3}H-TdR was added during the last 12 hours of cultures. Results are expressed as mean cpm of triplicate cultures. SD was ≤10%. Control IgG from pre-immune sheep serum showed no toxicity in the proliferation assay.

Abbreviation: ND, not determined.

*IL-1ra was used at the final concentration of 100 ng/mL.

†Purified IgG from sheep anti–IL-1α and anti-IL-1β antisera were used at the final concentration of 1 μg/mL.

‡rIL-1β was used at the final concentration of 10 ng/mL.

on the competitive interaction with the same receptor of two ligands: the endogenously produced IL-1 and the exogenously added IL-1ra. The concentration of IL-1 produced in culture is dependent on the cell number and on the rate of synthesis and uptake, which is particular for each leukemic clone.[8] Experiments performed with lower numbers of cells producing high amounts of IL-1 and GM-CSF showed inhibition of proliferation in excess of 80% (data not shown).

A time course analysis showed that the inhibitory activity of the IL-1ra on AML cell proliferation was detectable after 12 hours of culture and reached a plateau at 48 to 60 hours (Fig 3). To investigate whether the effect of the IL-1ra was reversible, AML cell proliferation was assessed in cultures exposed to IL-1ra for 48 hours. At the end of this period, cells were washed and replated. At this time, cell viability, assessed by flow cytofluorimetry, was not significantly different between untreated and treated cultures. However, as shown in Table 2, AML blasts exposed to IL-1ra had a strongly reduced proliferation rate, either spontaneous or induced by exogenous IL-1β. The exposure to GM-CSF was followed by a partial proliferative response.

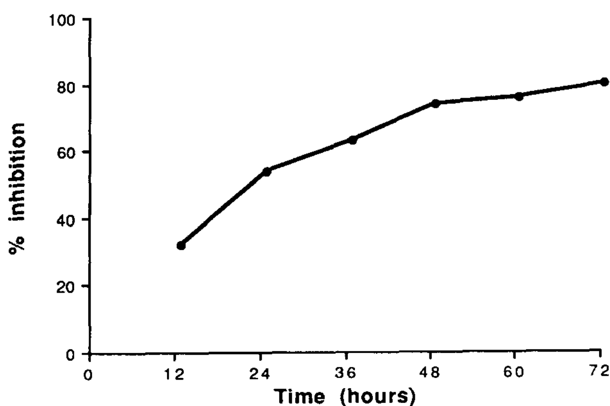

Fig 3. Time-dependent effect of IL-1ra on spontaneous AML cell proliferation. Leukemic blasts from patient no. 4 were cultured at 2.5 × 10^5/mL with 100 ng/mL of IL-1ra for the indicated times. Results are expressed as percentage of inhibition of ^{3}H-TdR incorporation.

IL-1ra regulates IL-1β and GM-CSF production by AML blasts. To investigate further the molecular mechanisms of action of IL-1ra on leukemic cells, the spontaneous production of IL-1β, GM-CSF, and IL-6, in basal conditions and in the presence of IL-1ra, was assessed. As expected from previously published data,[6,8] constitutive production of cytokines was variable among the patients ranging for GM-CSF from a minimum of 140 pg/mL in

Table 2. Proliferation of Leukemic Cells After Removal of IL-1ra

Stimulus	^{3}H-TdR Incorporation (cpm) Control*	IL-1ra*
None	5,855	690
IL-1β†	10,002	524
GM-CSF†	36,199	2,014

*Leukemic cells from patient no. 4 were cultured for 48 hours in the presence of 100 ng/mL of IL-1ra or of heat-inactivated IL-1ra as control. Cells were washed and spontaneous or cytokine induced proliferation was assessed after additional 48 hours by ^{3}H-TdR incorporation. Results are expressed as mean cpm of triplicate culture. SD was always less than 10%. Data from one representative experiment of three performed are shown.

†Cytokines were used at the final concentration of 10 ng/mL.

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patient no. 4 to a maximum of 3.6 ng/mL in the case of patient no. 7. Similar results were obtained with IL-1β where as low as 72 pg/mL was detected in the culture supernatants of patient no. 8. In other patients, such as nos. 7 and 9, we could detect up to 5 ng/mL of IL-1β secreted. Figure 4A shows that the spontaneous release of GM-CSF was reduced by IL-1ra in 8 of 10 cases. A similar inhibition of spontaneous IL-1β production by AML cells was observed (Fig 4B) in four of seven cases studied. In both instances, the effect was dose-dependent (data not shown). A similar reduction in spontaneous IL-6 production was also observed (data not shown).

IL-1ra and IL-1β gene expression in AML blasts. In the light of the clear-cut biologic activity of IL-1ra on AML cells, it was important to establish whether leukemic cells constitutively expressed the IL-1ra gene. As indicated in the representative Northern analysis shown in Fig 5, no IL-1ra–specific mRNA could be detected in the majority of the cases studied (21 of 23), even after prolonged autoradiographic exposures. Only in two cases, of the M4 and M5 cytologic French-American-British (FAB) subtype, could small but detectable amounts of IL-1ra mRNA be found. By contrast, IL-1β gene expression was readily observed in 20 of the 23 cases studied. These experiments were performed with RNAs purified from freshly isolated uncultured leukemic cells. We also noted that GM-CSF–stimulated monocytes of a healthy donor induced large amounts of mRNA specific for both IL-1ra and IL-1β (Fig 5). The induction of IL-1 inhibitory activity by GM-CSF has been observed previously.[22]

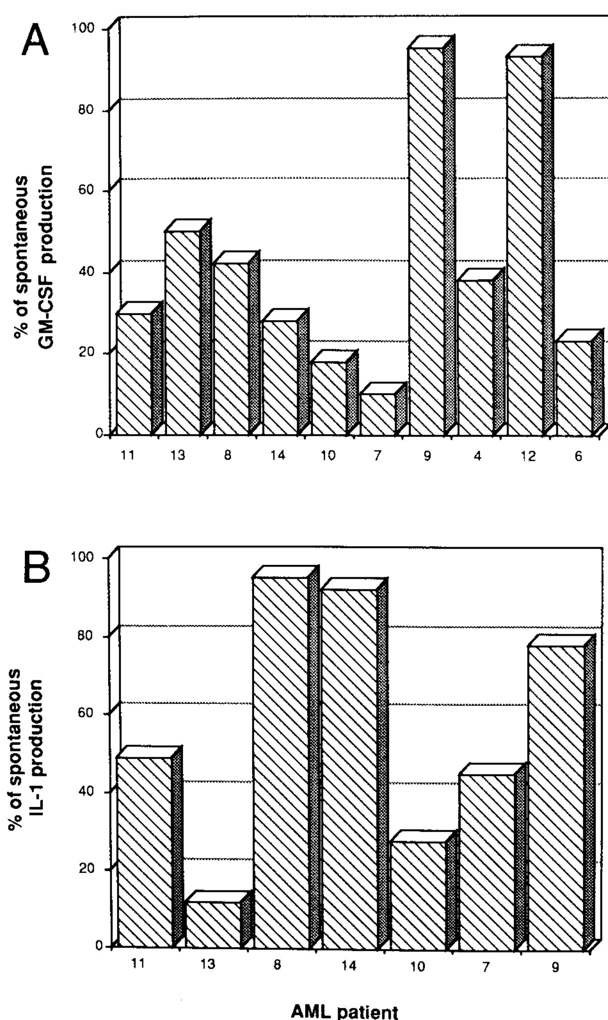

Fig 4. Effect of IL-1ra on GM-CSF (A) and IL-1β (B) production by AML blasts. AML cells (10^6/mL) from the indicated patients were cultured with 200 ng/mL of IL-1ra for 48 hours. GM-CSF and IL-1β were measured in culture supernatants by specific radioimmunoassays. Results are expressed as percent of the cytokine production by untreated AML cells.

DISCUSSION

The proliferation of normal hematopoietic precursors is controlled by several growth factors that act in an orderly manner.[23] It is believed that in leukemic cells coordination between proliferation and differentiation is lost, mostly because of an uncontrolled supply of growth factors. In particular, IL-1 and GM-CSF take part in autocrine and paracrine circuits that may support leukemic growth in vivo.[3-12] IL-1 exerts pleiotropic and potentially harmful activities and its biologic effects are therefore carefully controlled. One such control mechanism is likely exerted by the IL-1ra that prevents cytokine interaction with its surface receptors on target cells.

In this study we found that IL-1ra could inhibit spontaneous AML cell proliferation and that, in the majority of the cases studied, the inhibition was profound. The basis of this inhibition appears to be the blockade of endogenous IL-1 binding to surface receptors. Competition experiments showed nearly the same affinities of both IL-1α and IL-1ra for the receptors in AML cells. As the rate of synthesis of IL-1 varies from patient to patient, the amounts of IL-1ra required to obtain a significant reduction in cell growth will be different.

Occupancy of IL-1 receptors as low as 10% is sufficient to deliver a biologic signal[24,25]; it is therefore necessary to completely saturate the binding sites on the target cells to observe growth suppression in vitro. In view of this, the residual proliferative activity of IL-1ra–treated leukemic cell cultures is probably due to the ongoing production of different autocrine growth factors; in fact, leukemic cells exposed to IL-1ra partially responded to exogenous GM-CSF. However, the observation that they failed to proliferate to recombinant IL-1 (rIL-1) suggests that an additional effect of IL-1ra on blast cells may be the internalization and recirculation of IL-1 receptor molecules on the cell surface.

A possible mechanism of action of IL-1ra on AML cell growth is suggested by the experiments showing decreased release of GM-CSF and IL-1 by IL-1ra–treated cells. Although spontaneous GM-CSF production is not a universal feature in AML, it is known that other factors, such as G-CSF[9] or IL-6,[26] can be produced by hematopoietic cells in an IL-1–dependent fashion, and evidence indicates that multiple autocrine circuits operate within a single malignant clone.[27] IL-1 appears to play a key, hierarchic role in each of these circuits. It is not surprising, therefore, that

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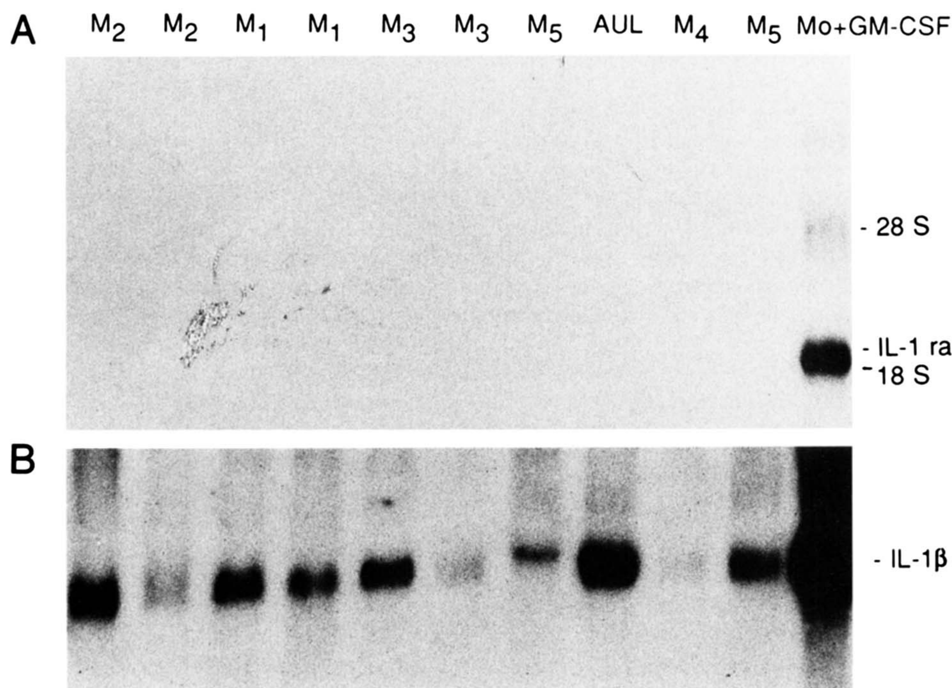

Fig 5. Expression of IL-1ra and IL-1β genes in AML cells. Total cellular RNA was purified from uncultured AML blasts of the indicated patients, blotted, and hybridized to the IL-1ra–specific probe. The same blot was later washed and rehybridized with a cDNA probe for IL-1β. As a positive control, RNA from normal monocytes stimulated with human rGM-CSF (100 ng/mL) was used.

blocking the interaction between IL-1 and leukemic cells, either through neutralization of IL-1 activity or through prevention of IL-1 binding to receptors, results in the interruption of such circuits and eventually in growth arrest. Inhibition of growth was apparently irreversible, as assessed up to 5 days after removal of IL-1ra, suggesting that interruption of autocrine loops operating in leukemic cells may lead to lethal consequences for malignant blasts. In this respect, preliminary experiments indicate that in some, though not all AML cases, a prolonged incubation (120 hours) with IL-1ra is associated with a reduced leukemic blasts viability (data not shown).

As previously shown by us and others, we confirmed that the majority of resting unstimulated AML populations show IL-1 gene expression.[4,28] However, under the same experimental conditions, we could not observe significant IL-1ra gene expression, with the possible exception of two cases. The failure of AML cells to express IL-1ra mRNA was not an absolute one in that in two cases we found low but detectable levels of transcription and, after in vitro culture in the absence of deliberate stimulation, some leukemic blasts from different AML patients expressed the IL-1ra gene, even though simultaneous production of IL-1–specific mRNA was simultaneously increased (data not shown). In this respect, it has been shown that myeloid cell lines produce IL-1 inhibitors after in vitro stimulation with phorbol esters or GM-CSF.[22] However, in agreement with our results, no significant inhibitory activity could be detected in resting conditions. The discrepancy between IL-1 and IL-1ra secretion by leukemic cells is somehow surprising, because the kinetics of gene expression of these cytokines in monocytes is comparable.[15]

As suggested by recent experiments performed by Bot et al,[29] secretion of growth factors by leukemic cells probably reflects the amplification of a physiologic event rather than being leukemia-specific, because normal bone marrow cells, under appropriate conditions of stimulation, present a similar activity. The molecular dissection of autocrine and paracrine circuits influencing growth of normal as well as leukemic progenitors should provide important tools for manipulating hematopoiesis for therapeutic purposes.

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