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

By Alessandro Rambaldi, Maria Torcia, Stefania Bettoni, Edouard Vannier, Tiziano Barbui, Alan R. Shaw, Charles A. Dinarello, and Federico Cozzolino

Interleukin-1 (IL-1) is spontaneously produced by acute myeloblastic leukemia (AML) cells. IL-1 also inducibly synthesizes 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.

© 1991 by The American Society of Hematology.
Human recombinant IL-1α (IL-1α) had a specific activity of 1.3 × 10^10 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 × 10^10 U/mg of protein. Human recombinant IL-1ra was obtained from Dr. R.C. Thompson (Synergem 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 × 10^10 proliferation units/mg.

Binding studies. IL-1α was radioiodinated using the method reported by Lowenthal and MacDonald. 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 ^125I-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 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 for its respective biologic assay.

Northern blots. Total cellular RNA was electrophoresed in agarose gels, and blotted as previously described. Membranes were hybridized with cDNA probes labeled with ^32P-dCTP by the method of Feinberg and Vogelstein. The cDNA for the IL-1ra was obtained by polymerase chain reaction (PCR) using messenger RNA (mRNA) from AML-193 cells as substrate. 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 mRNA. 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. 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 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 ^125I-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.

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

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Medium Alone</th>
<th>rIL-1ra*</th>
<th>% decrease</th>
<th>Anti-IL-1a</th>
<th>rAnti-IL-1β†</th>
<th>% decrease</th>
<th>rIL-1β†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10,786</td>
<td>3,990</td>
<td>63</td>
<td>6,540</td>
<td>39</td>
<td>25,348</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7,847</td>
<td>2,935</td>
<td>63</td>
<td>4,001</td>
<td>49</td>
<td>28,345</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3,180</td>
<td>750</td>
<td>76</td>
<td>2,110</td>
<td>34</td>
<td>8,530</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>31,752</td>
<td>7,375</td>
<td>77</td>
<td>19,387</td>
<td>39</td>
<td>61,459</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1,201</td>
<td>590</td>
<td>51</td>
<td>804</td>
<td>34</td>
<td>3,980</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>15,134</td>
<td>8,386</td>
<td>45</td>
<td>10,345</td>
<td>32</td>
<td>21,476</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>20,797</td>
<td>12,687</td>
<td>39</td>
<td>15,890</td>
<td>24</td>
<td>28,789</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1,414</td>
<td>275</td>
<td>81</td>
<td>789</td>
<td>45</td>
<td>4,873</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>4,413</td>
<td>1,659</td>
<td>62</td>
<td>2,234</td>
<td>49</td>
<td>16,578</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1,549</td>
<td>1,004</td>
<td>33</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>14,429</td>
<td>10,521</td>
<td>27</td>
<td>13,824</td>
<td>4</td>
<td>28,643</td>
<td></td>
</tr>
</tbody>
</table>

Cells were cultured for 48 hours at the concentration 2.5 x 10^5/mL in the presence or absence of the indicated stimuli. ^I-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.

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

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>H-TdR incorporation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Control*</td>
</tr>
<tr>
<td>IL-1β†</td>
<td>10,002</td>
</tr>
<tr>
<td>GM-CSF†</td>
<td>36,199</td>
</tr>
</tbody>
</table>

*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 ^I-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.

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

**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.22 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; 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-CSF25 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
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. 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. 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.

As suggested by recent experiments performed by Bot et al., 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.

ACKNOWLEDGMENT

We are grateful to Dr R.C. Thompson and Synergen for generously providing the IL-1ra.

REFERENCES


Modulation of cell proliferation and cytokine production in acute myeloblastic leukemia by interleukin-1 receptor antagonist and lack of its expression by leukemic cells

A Rambaldi, M Torcia, S Bettoni, E Vannier, T Barbui, AR Shaw, CA Dinarello and F Cozzolino