Effect of M20 Interleukin-1 Inhibitor on Normal and Leukemic Human Myeloid Progenitors

By Tony Peled, Michal Rigel, David Peritt, Eitan Fibach, Avi J. Treves, and Vivian Barak

This study aimed to assess the effect of the M20 interleukin-1 (IL-1) inhibitor on normal and leukemic hematopoietic cells. The M20-derived IL-1 inhibitor was found to inhibit the growth of various hematopoietic cells. The in vitro proliferation of myeloid cell lines in serum-containing medium or proliferation of these cells induced by IL-1 in serum-free medium (measured by [3H]-TdT) were inhibited by the M20 IL-1 inhibitor. In addition, growth of normal progenitors and fresh leukemic cells stimulated by granulocyte-macrophage colony-stimulating factor (GM-CSF) (as measured by colony and liquid systems) was also inhibited by this factor. After the removal of the IL-1 inhibitor at the peak of growth inhibition, leukemic and normal progenitor cells retain their ability to grow and develop into GM-CSF colonies. These results show that the growth inhibition phenomena were reversible and did not result from a cytotoxic effect. Our data suggest that the M20-derived IL-1 inhibitor might function as a true negative growth regulator of normal and leukemic hematopoietic cells.

The IMMUNOHEMATOPOIETIC system is regulated by a group of glycoprotein growth factors (cytokines). Interleukin-1 (IL-1) is mainly a monocyte-derived cytokine with a broad spectrum of immunostimulatory and inflammatory effects. Recent in vitro, as well as in vivo studies have shown its role in the regulation of hematopoiesis. For example, it synergizes with other cytokines (eg, granulocyte colony-stimulating factor [CSF-G], granulocyte-macrophage CSF [GM-CSF], macrophage-CSF [M-CSF], and IL-3) in stimulating early hematopoietic stem cells in a clonogenic assay of 5-fluorouracil-treated bone marrow cells, enhances the in vitro survival of purified granulocyte and macrophage progenitor cells in the absence of CSF, and sustains the in vitro viability and proliferation of fresh leukemic cells.

The different in vitro and in vivo IL-1-associated effects have been shown to be inhibited by different factors that were identified and isolated from various sources. We have previously reported on the isolation and characterization of an IL-1 inhibitory factor secreted by the M20 human myelomonocytic cell line. The factor was found to be a protein with a molecular weight of 52 ± 5 Kd and an isoelectric point (pI) of 4.2.1,3,5

In vitro experiments indicated that the factor inhibited IL-1-associated proliferative responses such as those of mouse thymocytes, human T cells or fibroblasts. However, it did not affect IL-6-induced thymocyte proliferation, IL-2-dependent cell lines, or background proliferation of unstimulated lymphocytes, suggesting that this inhibitor is specific for IL-1-dependent responses. In vivo, this factor was found very active in reducing specifically IL-1-induced inflammatory parameters. The M20-derived IL-1 inhibitor was recently found to be immunologically distinct from the IL-1 receptor antagonist (IL-1ra) or the IL-1 receptor antagonist protein (IRAP).

Negative growth regulators have been recently recognized as important participants in the regulation of the hematopoietic system. These cytokines include tumor necrosis factor-α (TNF-α), TNF-β, transforming growth factor-β (TGF-β), various species of interferons (IFNs), and some specific peptides that inhibit recruitment of stem cells into cell cycle. In addition, the IL-1ra inhibited to certain extent the growth of some leukemic cell lines as well as freshly explanted cells from some patients with acute myeloid leukemia. However, the in vivo role of negative regulators and their significance in normal and leukemic hematopoiesis, remain less well characterized than those of the stimulating growth factors.

In the present study we assessed the effect of the M20-derived IL-1 inhibitor on normal and leukemic hematopoietic cells. The results showed that this IL-1 inhibitor reversibly prevented cell cycling without affecting cell differentiation. The results suggest that IL-1 inhibitors might have an important regulatory role in normal and leukemic hematopoiesis and suggest the possibility of its application in therapy.

MATERIALS AND METHODS

IL-1 inhibitor preparation. IL-1 inhibitor was obtained from the conditioned medium of the M20 cell line as described. Briefly, serum-free supernatants were concentrated by vacuum ultrafiltration using dialysis tubing and purified on a high performance liquid chromatography-dithyl aminomethyl (HPLC-DEAE) column. Alternatively, the inhibitor was purified by molecular sieving on a Sephacryl S-300 (Pharmacia, Uppsala, Sweden) column, followed by Isoelectric focusing (IEF) in free solution, using the Rotofor Cell (BioRad, Richmond, VA). Fractions were collected, dialyzed, and bioassayed, and the fractions in the active peak, corresponding to a molecular weight of about 52 Kd and pI 4.1 to 4.2, were pooled and stored at 70°C. The inhibitor was routinely tested for its ability to inhibit the proliferation induced in vitro by IL-1 in the mouse thymocyte assay. Additional tests included the D10 and fibroblast proliferation assays. One unit of IL-1 inhibitor was defined as the amount of material causing 50% inhibition of the proliferation mouse thymocytes induced by 1 U of recombinant IL-1 (rIL-1).

Cells and cultures. Mononuclear cells derived from the peripheral blood of normal and leukemic patients or the bone marrow of normal donors were isolated by Ficoll/Hypaque (Pharmacia, Pordotti, Gianni, Milan) density gradients. Cells were washed and resuspended in α-minimal essential medium (GIBCO, Grand

From the Departments of Hematology and Oncology, Hadassah University Hospital, Jerusalem, Israel.

Address reprint requests to Vivian Barak, PhD, Dept of Oncology, Hadassah University Hospital, POB 12000, Jerusalem 91120, Israel.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.
Island, NY). When necessary, cell suspensions were depleted of monocytes by incubation at 37°C for 2 hours in plastic petri dishes containing 2% human serum.

The cell lines studied included the human myelomonocytic cells HL-60 (clones 5, 1-5, D2, 9, and C4), U937,22 M20,24 and K56225 and the murine cell lines WEHI26 and murine erythroleukemia (MEL).27 Cells were cultured in α-medium supplemented with 10% heat-inactivated (56°C for 30 minutes) fetal calf serum (FCS) or in serum-free medium supplemented with 4 mg/mL bovine serum albumin (BSA) and incubated at 37°C in a humidified atmosphere of 5% CO2 in air. The percentage of viable cells was determined by the trypan blue exclusion technique. For morphologic assessment of the cells, cytospin (Shandon, Cheshire, UK) slide preparations were stained with May-Grünewald-Giemsa and examined under a light microscope.

Growth assays. The effect of the IL-1 inhibitor and the human recombinant cytokines GM-CSF, IL-6 (Genetics Institute, Boston, MA), and IL-1 (Genzyme, Boston, MA) on cell growth was assayed by the liquid and colony systems. For the liquid system, cells were plated at 1 x 106/mL for myeloid leukemic cell lines or 1 x 107/mL for freshly obtained leukemic or normal peripheral blood cells. The number of cells was determined by direct counting under a light microscope. DNA synthesis was determined by the incorporation of 3H-thymidine (1 mCi/well, 5 mCi/mmol; ICN Radiochemicals, Irvine, CA), added 16 hours before harvesting onto glass fiber strips (Pied Cell harvester; Cambridge Technologies, Inc, Massachusetts). Scintillating fluid was added and vials counted in quadruplicates in a beta counter (LKB, Bromma, Sweden). For the colony system, 1 x 105/mL myeloid leukemic cells were seeded in semisolid medium composed of 0.85% (wt/vol) methylcellulose (4,000 centipoise; Fisher Scientific Co, Fair Lawn, NJ) in α-medium supplemented with 20% FCS (vol/vol). Normal bone marrow cells were cloned (1 x 105 cells/mL) in methylcellulose-containing α-medium, 30% FCS, 100 U/mL GM-CSF, 1% deionized BSA, 1 x 10-3 mol/L 2-mercaptoethanol, and 1.5 mmol/L glutamine. Cells were added and dispensed in 1 mL aliquots into 35-mm plastic nontissue culture petri dishes (Falcon, Oxnard, CA). The cultures were incubated at 37°C in a sealed chamber in a humidified atmosphere of 7% CO2, 6% O2, and 87% N2. Colony numbers were scored with an inverted microscope.

RESULTS

The effect of the M20-derived IL-1 inhibitor on myeloid leukemic cell lines. GM-CSF, IL-6, and IL-1 were compared for their ability to enhance proliferation of myeloid leukemic cell lines. Under optimal culture conditions, ie, in the presence of serum (10%) and high cell concentration (5 x 107/mL), none of the cytokines significantly altered cell growth. IL-1 was the only one that stimulated growth under suboptimal conditions, ie, serum-free medium and low cell concentration (1 x 107/mL) (Fig 1), while GM-CSF and IL-6 had no effect (data not shown).

This IL-1–stimulated proliferation was suppressed by the M20-derived IL-1 inhibitor in a dose-dependent manner (Fig 2). Although IL-1 did not affect cell growth in serum-supplemented cultures (Fig 1), the inhibitor suppressed the growth of various myeloid and erythroid cell lines in such cultures (Fig 3). It is interesting to note, however, that the M20 cell line, from which the inhibitor was originally derived, was only marginally inhibited under optimal culture conditions.

To analyze the interaction between IL-1 and the inhibitor, we tested the effect of increasing concentrations of IL-1 on the IL-1 inhibitor suppression of growth. As much as 200 U of IL-1 was unable to overcome the growth inhibitory effect of 3 to 12 U of inhibitor in either serum-supplemented or serum-free medium (data not shown).

The inhibitory effect of the IL-1 inhibitor depended on the initial cell density; it exceeded 90% at low density (106/mL), but was reduced at high densities (Fig 4).

Growth inhibition of myeloid cells may be associated with terminal cell differentiation, cytotoxicity, or a reversible arrest of the cells at a particular phase of the cell cycle.28 IL-1 inhibitor did not induce differentiation, as judged by cell morphology, nitroblue tetrazolium (NBT) reduction, and phagocytic activity (data not shown). Viability, assayed by the Trypan Blue exclusion technique, was not reduced after 4 days of incubation with the inhibitor. Moreover, after prolonged incubation (7 days), the viability in control cultures decreased (60% viability at 4 x 109

Fig 1. The effect of IL-1 on myeloid cell proliferation. The indicated cell lines, at a concentration of 1 x 105 cells/mL, were cultured either in serum-free or serum-containing medium (10% FCS) in the presence of 0 (□), 325 (□), and 650 U/mL (■) IL-1. 3H-thymidine incorporation was determined on day 4, and the results were expressed as the proliferative index, calculated as the mean cpm in IL-1–treated cultures divided by the mean cpm in untreated cultures. Each point represents a mean of eight samples and in each case the SEM is less than 10%.

Fig 2. The effect of the M20 IL-1 inhibitor on cell proliferation. The indicated cell lines were cultured at 1 x 105 cells/mL in serum-free medium supplemented with 200 U IL-1 with or without increasing concentrations of IL-1 inhibitor. After 4 days of incubation, cells were counted and the results are expressed as a percentage of growth inhibition. Each point represents a mean of eight samples and in each case the SEM is less than 10%. (●) M20; (□) WEHI; (▲) HL-60.
Fig 3. The effect of M20 IL-1 inhibitor on proliferation of various myeloid and erythroid cell lines. Cultures were initiated at $1 \times 10^5$ cells/mL in the presence of 10% FCS with or without 12 U IL-1 inhibitor. On day 3, the cultures were incubated for 24 hours with [H] thymidine and radioactivity determined. The effect of the inhibitor on cell proliferation is expressed as a percentage of the control. Each point represents a mean of four samples and in each case the SEM is less than 10%.

To determine the reversibility of the inhibitor's effect, cells were cultured in its presence for 3 to 6 days, and then washed and recultured either in liquid (Fig 5) or semi-solid medium (Fig 6). Under both culture conditions, growth inhibition was found to be reversible. In liquid medium (Fig 5), after the removal of the inhibitor, cells reached saturation density of $10^6$ cells/mL within 5 days. In semi-solid medium, cells maintained their high cloning efficiency even after 5 days of pre-exposure to IL-1 inhibitor and after the removal of the inhibitor cloning efficiency was similar to that of untreated cells. In contrast, when the inhibitor was included in the cloning medium, colonies were unable to develop. These results indicate that the growth arrest by the IL-1 inhibitor was not a result of cytotoxicity or terminal differentiation, but rather a reversible block of cell cycling.

Fig 4. The effect of cell concentration on growth inhibition by the IL-1 inhibitor. WEHI cell cultures were initiated at different cell concentrations in the presence (O) or absence (C) of 12 U IL-1 inhibitor. The results are presented as actual number of cells per milliliter (---O-; ---C--) and as a percentage of growth inhibition (---O--; ---C--). Each point represents a mean of four samples and in each case the SEM is less than 10%.

The effect of the M20-derived IL-1 inhibitor on normal myeloid progenitors. The effect of IL-1 inhibitor on normal hematopoiesis was studied in both liquid and semi-solid culture systems. For this purpose, peripheral blood or bone marrow mononuclear cells were cultured in the presence of GM-CSF, with or without the inhibitor. In liquid culture, cells were assessed at various times morphologically and their rate of proliferation was determined by [H]-thymidine incorporation (Table 1). In cultures treated with the IL-1 inhibitor, only few early myeloid or erythroid precursors and no mitotic cells could be observed and thymidine incorporation was reduced by 90% as compared with control cultures. In semi-solid medium (Fig 7), the IL-1 inhibitor produced a dose-dependent inhibition of myeloid colony development of the GM-CSF-stimulated bone marrow cells. As with the leukemic cell lines, the growth inhibition of the normal progenitors was reversible; after 4

Fig 5. The reversibility of the growth inhibition effect induced by M20 IL-1 inhibitor. WEHI cells ($1 \times 10^5$/mL) were cultured in the presence (O) or absence (C) of 12 U/mL IL-1 inhibitor. After 3 days of incubation, aliquots were washed twice and recultured at the initial concentration of $1 \times 10^5$ cells/mL in the absence of IL-1 inhibitor (---O--; ---C--). Each point represents a mean of four samples and in each case the SEM is less than 10%.

Fig 6. The effect of the M20 IL-1 inhibitor on the cloning of WEHI cells. WEHI cells, precultured in the presence of 12 U/mL IL-1 inhibitor for 5 days, were washed and cloned (500 cells/mL) with or without increasing concentrations of IL-1 inhibitor. The number of the colonies was determined 7 days after cloning. Each point represents the mean of four samples and in each case the SEM is less than 10%.
EFFECTS OF M20 IL-1 INHIBITOR

Table 1. IL-1 Inhibitor Effect on Peripheral Blood Light Density Cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>GM-CSF</th>
<th>GM-CSF + Inhibitor</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O.Z.</td>
<td>5,714</td>
<td>439</td>
<td>92</td>
</tr>
<tr>
<td>I.A.</td>
<td>5,846</td>
<td>584</td>
<td>90</td>
</tr>
<tr>
<td>M.R.</td>
<td>5,189</td>
<td>420</td>
<td>92</td>
</tr>
</tbody>
</table>

Six-day liquid cultures of monocyte-depleted peripheral blood light density cells supplemented with GM-CSF (100 U/mL) with or without IL-1 inhibitor (12 U/mL) were tested for their proliferation activities. The results are expressed as cpm of thymidine incorporation and percent of growth inhibition.

days of exposure, removal of the inhibitor restored their capacity to develop large colonies.

The effect of M20-derived IL-1 inhibitor on fresh myeloid leukemic cells. IL-1 inhibitor was also found to effectively suppress the growth of freshly explanted cells derived from patients with myeloid leukemia and stimulated by GM-CSF or GM-CSF plus IL-1 (Fig 8). But, in contrast to normal hematopoietic progenitors and in vitro-established leukemic cell lines, prolonged incubation (6 to 8 days) of freshly explanted leukemic cells with the inhibitor markedly decreased their viability. This effect was not associated with modification of the differentiation status of the cells (data not shown).

DISCUSSION

A protein, purified from the M20 myelomonocytic cell line, has been previously shown to inhibit IL-1-mediated responses, in vitro as well as in vivo. In the present investigation we show that this protein suppresses the proliferation of normal and leukemic myeloid cells as well. In established cell lines, growth inhibition was reversible and was not associated with a loss of viability. The cells were accumulated and arrested in the G1 phase of the cell cycle (data not shown), but upon removal of the M20-derived inhibitor, cell proliferation was resumed. This could be shown in liquid medium by the increase in cell number and in semi-solid medium by the development of large colonies. While the results in liquid medium could be attributed to a selective survival of a minor subpopulation, the high cloning efficiency in semi-solid medium indicated that the majority of the population maintained their self-renewal capacity after removal of the inhibitor.

Similar results were obtained with normal myeloid progenitors. Upon continuous exposure to the inhibitor, myeloid colony formation was prevented, but removal of the inhibitor allowed normal colony development. The effect of the IL-1 inhibitor was also studied by culturing peripheral blood mononuclear cells in liquid medium in the presence of GM-CSF. Control cultures supported the proliferation and differentiation of progenitors, resulting in the appearance, after 6 days, of proliferating (determined by uptake of 3H-thymidine), morphologically identifiable myeloid precursors. These cells did not appear in cultures treated with the IL-1 inhibitor. But, after washing and cloning of the cells in GM-CSF-supplemented semisoluid medium, myeloid colonies developed. These results suggest that the IL-1 inhibitor reversibly arrested the proliferation of these progenitors.

In variance with established cell lines and normal progenitor cells, the viability of freshly explanted leukemic cells was markedly decreased after long incubation with the inhibitor. This effect was observed even when cultures were initiated at high cell density and were supplemented with optimal concentration of GM-CSF or GM-CSF and IL-1.

The apparent specificity of the inhibitor towards IL-1 has been previously shown both in vitro and in vivo. In vitro, the inhibitor suppressed only IL-1-induced thymocyte proliferation. In contrast, IL-2- and IL-6-induced thymocyte proliferation, proliferation of IL-2-dependent cell lines, unstimulated thymocytes, or lymphocytes were all unaffected by the inhibitor. Similarly, in vivo experiments showed an ability of the inhibitor to suppress IL-1-induced,
but not IL-6- or TNF-induced, inflammatory parameters.  
In the present study we showed that, in the myeloid system, the inhibitor suppressed proliferation induced by IL-1 in serum-free medium. But, it also inhibited the proliferation of established leukemic cell lines in FCS-supplemented cultures in the absence of added IL-1, as well as the proliferation of fresh leukemic cells and normal progenitors stimulated by other cytokines (ie, GM-CSF). Although IL-1 was not added to these cultures, its involvement due to its presence in serum, its secretion by accessory cells or due to autocrine production by the target cells, could not be ruled out.  
IL-1 inhibition may be due to downregulation of IL-1 synthesis or release,  
blocking the IL-1 receptor, or interfering with IL-1 signal transduction at the postreceptor level. The M20 IL-1 inhibitor has been recently shown to be immunologically distinct from the IL-1ra. Moreover, the addition of IL-1 (up to 200 U) together with the M20-derived inhibitor did not significantly reverse the inhibitory effect, suggesting that growth arrest by this inhibitor may be caused by interference with intracellular signaling pathways.  
The regulation of hematopoiesis is associated with both positive and negative regulators of growth and differentiation. Among the negative regulators are TGF-α, TGF-β, restrictin, as well as some specific peptides. The function of the negative regulators is to modulate the recruitment of the stem and progenitor cells into cell cycle and differentiation and to prevent exhaustion of the stem cell pool.  
The present study suggests that the IL-1 inhibitor may be involved in such physiologic regulation. IL-1 plays a pivotal role in the regulation of the immunohematopoietic system, both directly and through the induction of other cytokines. The IL-1 inhibitor may, therefore, function as an important negative regulator in normal and leukemic hematopoiesis. This inhibition might have some important clinical applications. Its cytostatic effect on normal hematopoietic progenitors may have a protective value during treatment with phase-specific cytotoxic drugs or irradiation. Also, its cytotoxic effect on leukemic cells may be used for selective elimination of the cells, thus these effects offer the possibility of using IL-1 inhibitor as a new therapeutic modality.

REFERENCES

33. Leo M, Segal GM, Bugby GC: Interleukin-1 induces human bone marrow derived fibroblasts to produce multilineage hematopoietic growth factors. Exp Hematol 15:983, 1987
Effect of M20 interleukin-1 inhibitor on normal and leukemic human myeloid progenitors

T Peled, M Rigel, D Peritt, E Fibach, AJ Treves and V Barak