CONCISE REPORT

Acidic Isoferritins (Leukemia-Associated Inhibitory Activity) Fail to Inhibit Blast Proliferation in Acute Myelogenous Leukemia

By Raymond Taetle

Cell-free extracts of bone marrow and blood cells from patients with leukemia contain an inhibitor of normal granulocyte/macrophage progenitor (CFU-GM) proliferation (leukemia-associated inhibitory activity, LIA) identified as acidic isoferritins. A comparison was made of the action of crude LIA prepared from frozen-thawed leukemic blood cells and purified spleen ferritin from a patient with chronic myelogenous leukemia, on the proliferation of blast progenitors from patients with acute myelogenous leukemia (AML), and on the promyelocytic leukemia cell line, HL-60. Crude LIA showed no inhibition of blast progenitor or HL-60 proliferation at low concentrations, but inhibited the proliferation of CFU-GM. At higher concentrations, crude LIA inhibited both blast cells and CFU-GM. Purified spleen ferritin failed to inhibit blast progenitors or HL-60 cells at any concentration tested, but inhibited both 7-day and 14-day CFU-GM. Using the thymidine "suicide" technique, the action of LIA was confirmed as being on CFU-GM in S-phase, but it failed to affect the proliferation of blast cells in S-phase. It is concluded that acidic isoferritins inhibit normal CFU-GM but not blast cells from patients with AML. Acidic isoferritins could confer a proliferative advantage of the leukemic clone over its normal counterparts.

DURING THE COURSE of acute and chronic leukemia, the leukemic clone comes to dominate its normal counterparts and to suppress production of normal blood elements. The means by which abnormal clones gain dominance over normal blood cells is unknown, but previous in vitro studies have suggested that the blast cells may suppress normal myeloid progenitor proliferation.1,2

Broxmeyer and coworkers have shown that leukemic blasts and cells from patients with acute and chronic myeloid and lymphoid leukemias contain a non-species-specific, high molecular weight material that suppresses in vitro proliferation of normal granulocyte/macrophage progenitors (CFU-GM).3-9 This compound has been termed leukemia-associated inhibitory activity (LIA). LIA can be detected early in the induction of experimental murine leukemia with Abelson and Friend viruses and appears to preferentially inhibit CFU-GM in the process of synthesizing DNA (S-phase).4,7,8 It fails to inhibit the proliferation of CFU-GM from patients with nonremission acute leukemia and CFU-GM from some patients with chronic leukemia and acute leukemia in remission.3,5 LIA has recently been identified by Broxmeyer et al. as acidic isoferritins.9

In order for acid isoferritins to confer a proliferative advantage on acute leukemia blasts, they should inhibit normal cellular proliferation, but not that of the blast cell population. The action of acidic isoferritins on blast cells from patients with acute myelogenous leukemia (AML) was examined, and its effects on clonogenic blast cells contrasted to its effects on normal CFU-GM. We find that acidic isoferritins fail to inhibit the proliferation of blast cells from patients with AML.

MATERIALS AND METHODS

Patient Material and Processing of Specimens

Blood was obtained by venipuncture from 8 patients with acute nonlymphocytic leukemia with WBC counts of greater than 20,000/μl and 80% or greater blast forms. Heparin was used as an anticoagulant. Marrow was collected in heparin from the posterior iliac crest of patients without hematologic malignancies undergoing evaluation for anemia. Marrow specimens used for study were histologically normal.

Peripheral blood or bone marrow specimens were separated on Ficoll/Hypaque gradients (Ficoll/Paque, Pharmacia Fine Chemicals, Piscataway, N.J.) at 1200 g for 20 min. The interface cells were collected by aspiration and washed 3 times in alpha medium (Grand Island Biologicals, Grand Island, N.Y.). Leukemic blood cells were used fresh or frozen in 10% DMSO at -80°C.

Culture of Normal Granulocyte/Macrophage Progenitors (CFU-GM)

CFU-GM were grown as previously described in 0.3% agar.10 The final culture mixture contained 2 x 10^3/ml bone marrow mononuclear cells with alpha medium with nucleosides, 15% fetal bovine serum (FCS, Flow Laboratories, Rockville, Md.), and 20% placental conditioned medium prepared as described by Schlunk11 or 10% GCT cell line conditioned medium (Gibco).12 Triplicate or quadruplicate 1-ml aliquots were incubated in 35 mm culture plates at 37°C in 7.5% CO2. Aggregates of >40 cells were scored as colonies, and aggregates of 10-40 cells as clusters using an inverted micro-

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653
clusters. Fourteen-day cultures grew 82 ± 5 colonies (mean ± SD, n = 5) and 269 ± 189 clusters. Seventeen-day cultures grew 82 ± 65 colonies and 184 ± 112 clusters.

**Blast Progenitor Culture**

Blast progenitors from AML peripheral blood were grown as previously described using the technique of Buick et al. as modified by Minden et al. Leukemia peripheral blood cells were incubated with AET-treated sheep red blood cells (SRBC) for at least 1 hr and subjected to a second Ficoll-Hypaque separation. The T-lymphocyte-depleted (SRBC negative) cells were recovered from the interface and washed twice in alpha medium. T-cell-depleted cells at 4 × 10⁷/ml were plated in quintuplicate 0.1-ml aliquots in microtiter plates as previously described. The final culture mixture contained alpha medium with nucleosides, 15% FCS, and 10% media conditioned by PHA-stimulated, normal mononuclear cells prepared as described by Aye. Cultures were incubated at 37°C in 5% CO₂ for 5–7 days and aggregates of >20 cells scored as colonies with an inverted microscope. Blast progenitor cultures grew 78 ± 40 colonies/4 × 10⁵ cells (mean ± SD, n = 10).

**Culture of HL-60 Cells**

The human promyelocytic leukemia cell line developed by Collins and coworkers was maintained in continuous culture in alpha medium with FCS. Liquid cultures for study were initiated at 2 × 10⁵ cells/ml and viable cell counts assessed by trypan blue exclusion every 2 days. HL-60 colony-forming cultures were performed in 0.3% agar. The final culture mixture contained 20,000 HL-60 cells/ml in 15% FCS and alpha medium. Aggregates of >50 cells were scored as colonies with an inverted microscope after 14 days incubation at 37°C in 5% CO₂. The plating efficiency of HL-60 cells in colony-forming culture for these studies was 6% ± 3.3% (mean ± SD, n = 12).

**Leukemia-Associated Inhibitory Activity (LIA)**

Crude LIA was prepared according to the method originally described by Broxmeyer et al. First, 2 × 10⁸/ml leukemia peripheral blood cells in alpha medium were subjected to several cycles of rapid freezing and thawing. The mixture containing the disrupted cells was then immediately Millipore-filtered and stored in aliquots at -80°C. Each frozen aliquot of LIA was used only once and tested at concentrations from 5% to 20% for its effects on blast progenitors, on HL-60 cells, and on normal CFU-GM by addition to culture plates just prior to plating. Each batch of crude LIA was tested against autologous blast cells and cells from at least 5 other patients with AML.

Acidic isoferritin (pH < 5.0 after isoelectric focusing) from the spleen of a patient with chronic myelogenous leukemia was prepared as previously described and was a gift from Dr. H. Broxmeyer, Sloan Kettering Institute for Cancer Research, New York, N.Y. The purified spleen ferritin contained at least 94% acidic isoferritins, as determined by radioimmunoassay. This “pure” LIA was tested for its effects on blast colony-forming cells from 5 patients with AML, on colony growth of HL-60 cells, and on the growth of CFU-GM by addition to culture plates just prior to plating. Pure LIA was tested over a concentration range of 10⁻⁶ to 10⁻³ M.

**Assessment of the Specificity of LIA for Cell Cycle Status**

The action of LIA on CFU-GM or blast progenitors synthesizing DNA was examined using the thymidine “suicide” technique. Leukemic blood cells or normal bone marrow cells were incubated in media, with 10% crude LIA, or with 10⁻³ M purified acidic isoferritins (pure LIA). Cells were then washed and again incubated in media or exposed to high specific activity (100 μCi/ml) ³H-TdR. All cells were washed in media containing cold thymidine and plated in the appropriate colony-forming assay. The percent of colony-forming cells synthesizing DNA (S-phase) was determined by the reduction in colony-forming capacity after incubation with ³H-TdR.

The colony growth of the following groups was thus assessed in these experiments: (A) control cells incubated in media; (B) cells treated for 2 hr with purified LIA; (C) cells treated for 2 hr with 10% crude LIA; (D) cells treated with ³H-TdR; (E) cells treated with pure LIA and subsequently exposed to ³H-TdR; and (F) cells treated with crude LIA and subsequently exposed to ³H-TdR.

**Characterization of Cells in Colonies**

Colonies grown from blast progenitors were picked with fine capillary pipettes, washed, and prepared as cytopsins or assessed for SRBC rosette-forming capacity. Cells contained in the colonies were morphologically blasts and promyelocytes when stained with Wright-Giemsa stain. Less than 10% of pooled cells from blast colonies formed SRBC rosettes.

In order to assess whether the inhibitory action of LIA was specific for either granulocyte or macrophage progenitor cells, cytosin preparations of pooled colonies grown in the CFU-GM assay were also examined. In addition, whole agar plates were fixed, mounted, and stained with hematoxylin, as described by Wu and examined microscopically.

**Statistical Methods**

Significant differences between experimental groups were assessed using the Student's t test for paired or unpaired observations.

**RESULTS**

The effect of crude LIA on the proliferation of leukemia blast colonies or HL-60 cells in colony-forming culture and on 7- and 14-day CFU-GM and clusters is shown in Fig. 1. Data points shown represent the mean ± SE of 3–8 experiments. At concentrations from 10% to 20%, 20%–37% inhibition of both 7- and 14-day CFU-GM proliferation was observed. In contrast, leukemia blast cells were inhibited only at

![Fig. 1. The effect of various concentrations of cell-free extracts from AML peripheral blood cells (crude LIA) on colony growth. Seven-day CFU-GM (○—○); 14-day CFU-GM (□—□); HL-60 promyelocytic leukemia cell line (■—■); and AML peripheral blood blast colony-forming cells (○—●). Points are means ± SE of 5–8 experiments.](www.bloodjournal.org)
the highest concentration of LIA tested (20%), and at lower LIA concentrations were resistant to LIA inhibition. Variability in the magnitude of inhibition noted from specimen to specimen was seen in both CFU-GM and blast cell cultures. Significant inhibition ($p < 0.05$) of CFU-GM growth was seen at concentrations of 10%–20% crude LIA prepared from all 4 patients. At concentrations of 5%–15%, significant inhibition was not seen in blast cultures with any crude LIA preparation. At a concentration of 20%, crude LIA from patient 1 showed significant inhibition of blast colony growth, and crude LIA from patient 3 showed significant inhibition of HL-60 colony growth.

The disparity between effect of LIA on normal progenitors and leukemic blasts was even more apparent when the effect of purified acidic isoferritins (LIA) on these cells was examined (Fig. 2). Again, results represent means ± SE of 5 or more experiments. Both 7- and 14-day CFU-GM and clusters showed highly consistent inhibition by purified acidic isoferritins ranging from 16% to 32%. Inhibition by acidic isoferritins was significant ($p < 0.05$ or less) at all concentrations listed for both 7- and 14-day colonies and clusters. Maximum colony and cluster inhibition by purified LIA approximated closely the percent of CFU-GM expected to be in S-phase. In two studies in which continuous exposure of these cells to LIA was compared to their thymidine suicide rate at the time they were plated, the percent of CFU-GM inhibited by the $^{3}$H-TdR (30% and 37%) and by LIA (33% and 42%) was nearly identical. Cells contained in CFU-GM colonies from LIA-containing plates were examined as cytopsin preparations and in whole fixed agar plates. The inhibitory activity of LIA affected both granulocyte and macrophage progenitors equally. Blast progenitors from patients with AML, and HL-60 cells grown in colony-forming culture, were completely resistant to the effects of purified LIA (Fig. 2). This difference between the effects of acidic isoferritins on blast colony-forming units ($p < 0.05$ or less) and HL-60 cells ($p < 0.01$ or less) was significant at all pure concentrations. HL-60 cells grown in liquid culture were also resistant to pure LIA (data not shown).

In order to examine the specificity of LIA for colony-forming cells in S-phase, experiments were conducted to examine the effects of crude LIA or $10^{-3}M$ acidic isoferritins on CFU-GM and on leukemic blast progenitors on S-phase. In Table 1, the effect of a 2-hr incubation with crude or purified LIA on the proliferation of CFU-GM is shown. Under these conditions, LIA inhibited the proliferation of normal CFU-GM, and the magnitude of this inhibition in both experiments approximated the inhibition seen with $^{3}$H-TdR. Further, when normal bone

Table 1. Effect of 2-hr Incubation With Crude LIA or Purified Acidic Isoferritins on Colony Proliferation and Cell Cycle Status of CFU-GM and Blast Progenitors

<table>
<thead>
<tr>
<th>Condition</th>
<th>CFU-GM</th>
<th>Acute Isoferritin (10^{-3}M) (%)</th>
<th>Crude LIA (10%)</th>
<th>Acute Isoferritin (10^{-3}M) (%)</th>
<th>Crude LIA (10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Mean ± SE)</td>
<td>174 ± 4</td>
<td>121 ± 6 (70)§</td>
<td>142 ± 2 (82)</td>
<td>136 ± 5 (78)§</td>
<td>115 ± 3 (66)§</td>
</tr>
<tr>
<td>(per 2 x 10^5 cells)</td>
<td>123 ± 5</td>
<td>84 ± 5 (68)§</td>
<td>78 ± 4 (63)§</td>
<td>85 ± 5 (69)§</td>
<td>78 ± 4 (63)§</td>
</tr>
<tr>
<td>Blast colonies (per 4 x 10^5 cells)</td>
<td>32 ± 1</td>
<td>21 ± 1 (66)§</td>
<td>39 ± 1 (122)</td>
<td>27 ± 4 (85)§</td>
<td>21 ± 1 (66)§</td>
</tr>
<tr>
<td></td>
<td>96 ± 7</td>
<td>63 ± 3 (66)§</td>
<td>101 ± 6 (105)</td>
<td>99 ± 8 (103)</td>
<td>65 ± 3 (66)§</td>
</tr>
</tbody>
</table>

*Incubation with $^{3}$H-TdR (100 µCi/ml) for 20 min.
†Percent control.
‡Incubated for 2 hr with LIA then exposed to $^{3}$H-TdR.
§Significantly different from control ($p < 0.05$).
marrow cells were incubated with LIA, washed, and subsequently exposed to $^3$H-TdR, no further inhibition resulted. These data are similar to those previously reported by Broxmeyer et al. and suggest that the primary activity of LIA is to inhibit CFU-GM in S-phase.

In contrast, a 2-hr exposure of leukemic blast cells to LIA did not result in inhibition of blast progenitor growth, and the percent of blast progenitors inhibited by subsequent exposure to high specific activity $^3$H-TdR did not change after incubation with LIA. These data suggest strongly that LIA does not inhibit blast progenitors in S-phase, and that the cell cycle status of blast progenitors is not significantly altered by a 2-hr exposure to LIA.

**DISCUSSION**

Leukemia-associated inhibitory activity (LIA) was originally described by Broxmeyer and colleagues as a soluble factor released by freezing and thawing leukemic bone marrow and blood cells. This material and its cell of origin have since been well characterized. The cells producing LIA are non-T, non-B, lymphoid-like cells or promonocytes with receptors for the Fe portion of immunoglobulin.  

It inhibits the proliferation of normal granulocyte/macrophage progenitor cells while they are synthesizing DNA and is present in greater concentrations in acute untreated leukemia than chronic myeloproliferative syndromes. CFU-GM grown from acute leukemia patients in partial remission and from some patients in remission are not inhibited by LIA. As noted above, this material has been now characterized as acidic isoferritins and has been found in some normal cells.

To this extensive data base is now added the observation that purified LIA (acidic isoferritins) fails to inhibit the proliferation of blast cells from patients with acute nonlymphocytic leukemia. Using the blast progenitor culture system, we have established that this lack of inhibitory activity occurs across a broad patient base, as well as in the continuous leukemia cell line HL-60. These data are thus consistent with the hypothesis that LIA confers a proliferative advantage on the leukemic clone over its normal myeloid counterparts.

The inhibitory activity of crude and purified acidic isoferritins for normal progenitors noted in these studies is more modest than that reported in prior investigations. The maximum LIA-induced inhibition with continuous exposure in culture, the thymidine suicide rate of the CFU-GM, and inhibition observed after a 2-hr incubation with LIA were all very similar. This suggests that, in these studies, the action of LIA in the culture plates persisted only for a short period of time. Acidic isoferritins have also been identified in some lots of placental conditioned medium, and it is possible that differences in observed degrees of inhibition relate to the presence of these materials in our reagents. Subsequent studies using GCT cell line conditioned medium, which does not contain inhibitory acidic isoferritins (Broxmeyer, personal communication), have shown greater degrees of CFU-GM inhibition with LIA (Taetle, R., unpublished observations). In these studies, inhibition of both 7- and 14-day CFU-GM was also noted; in previous studies, only 7-day CFU-GM were affected. The reasons for these differences are not apparent. The data presented here do confirm the specificity of LIA for CFU-GM synthesizing DNA.

The physiology of bone marrow suppression in malignant myelo- and lymphoproliferative disorders is obviously complex, and little concrete data is available on this subject. Previous studies in chronic myelogenous leukemia (CML) have suggested that feedback inhibition from soluble granulocyte inhibitors is deficient and that PGE inhibition in CML and other myeloproliferative disorders is also deficient. It is likely that, in acute leukemia, multiple factors also operate to produce excess blast proliferation and inhibition of normal cell growth. It appears that LIA represents one of these mechanisms.

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