Suppression of Normal Granulopoiesis In Vitro by a Leukemia-Associated Inhibitor (LAI) of Acute and Chronic Leukemia

By Tor Olofsson and Inge Olsson

To determine if normal granulopoiesis can be suppressed by substances produced by cells from patients with leukemia, conditioned media from such cells were assayed for their ability to reduce the fraction of normal CFU-c in S-phase or inhibit cluster/colony formation in agar cultures. Low-density (<1.077 g/ml) marrow or blood cells from patients with leukemia, but not from normals, were shown to release a high molecular weight substance (>300,000 daltons) called "leukemia-associated inhibitor" (LAI) that reduces the fraction of normal marrow CFU-c in S-phase. This effect appeared to be reversible. In 10/12 patients with CML, 1/7 with CLL, and 2/6 with ALL, significant amounts of LAI were detected. LAI also reduces the labeling index of normal myeloblasts. LAI is resistant to heat at 60°C for 10 min. Conditioned media also contain a substance that inhibits cluster/colony formation, which is distinguished from LAI by its heat lability. The production of LAI is dependent on an active cell metabolism and is abolished at +4°C or by cycloheximide. LAI is also released in the absence of fetal calf serum and after trypsinization of the cells. Its production reaches a plateau after 3–5 hr of incubation at an optimal cell concentration of 5 x 10^6 cells/ml. When CML or AML cells were used as a target, 9/11 patients were found to be resistant to the action of LAI. LAI may be responsible for suppression of normal granulopoiesis in leukemia and provide a growth advantage for the leukemic cells.

A MOST CHARACTERISTIC feature of acute myeloid leukemia (AML) and other variants of nonlymphocytic leukemia is the development of profound suppression of normal hemopoiesis resulting in serious clinical problems from neutropenia, thrombocytopenia, and anemia. Similarly, in chronic myeloid leukemia (CML), normal hemopoiesis seems to be switched off as a result of the action of the Ph^-containing cell population. However, normal Ph^-negative stem cells are still present although suppressed. The suppressive mechanism in leukemia, if affecting the multipotent stem cell (CFU-s), should obviously be of a reversible noncytotoxic nature, as normal hemopoiesis reappears in the remission phase of acute myeloid leukemia (AML). Alternatively, a noncytotoxic or a cytotoxic effect could be directed against committed stem cells, e.g., stem cells committed for granulopoiesis (CFU-c), without affecting CFU-s. The imbalance of the normal negative and positive feedback mechanisms regulating granulopoiesis is unlikely to be of primary importance for suppression of normal hemopoiesis in leukemia.

The suppressive effect on normal hemopoiesis could be mediated through direct cell-to-cell interaction or humoral factors produced in leukemia. In vitro agar coculture of cells from patients with leukemia and normal marrow cells has demonstrated inhibition of normal CFU-c in some studies, but not in others. More recently, activity was obtained from medium conditioned by leukemia cells or cell extracts, which inhibited normal CFU-c. None of the putative leukemic-cell-derived inhibitors have been biochemically characterized, and their role in the pathophysiology of human leukemia is not clarified.

We report on the noncytotoxic reversible action of a leukemia-associated inhibitor (LAI) on normal granulopoietic stem cells (CFU-c). The biochemical characterization of LAI is described in a separate communication.

MATERIALS AND METHODS

Patients

Blood and bone marrow were collected from patients with chronic myeloid leukemia (CML), chronic myelomonocytic leukemia (CMML), acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia (CLL). Patients with AML or ALL were studied at the time of diagnosis before any treatment was given (with the exceptions indicated in Table 5), and patients with CML, CMML, or CLL had been off treatment for at least 3 wk at the time of blood or bone marrow sampling. Normal bone marrow and blood were obtained from healthy volunteers or patients without any known hematologic disorder.

Collection of Blood and Bone Marrow

Heparinized venous blood, 20 IU/ml heparin, was mixed with an equal volume of 2% dextran in 0.15 M NaCl and left at room temperature for 30–45 min. The dextran-plasma was taken to separation in density gradients. Bone marrow, 4–10 ml, was obtained by sternal puncture and collected in 3 ml McCoy's medium containing 100 IU heparin and 75 U Varidase (Lederle Laboratories, Pearl River, N.Y.). The marrow cells were suspended by repeated suction through a needle (0.8 x 80 mm) before they were applied to density gradients.

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Cell Separation

Dextran-plasma of peripheral blood or bone marrow suspensions were layered on Isopaque-Ficoll gradients\(^6\) (Lymphoprep, Nyegaard & Co., Oslo, Norway, density 1.077 g/ml) and spun for 10 min at 70 g followed by 15 min at 700 g. The cells at the interphase were collected and washed twice in McCoy’s medium and suspended in medium containing 5% fetal calf serum (FCS).

Bone Marrow Culture Techniques

Normal bone marrow cells were cultured in 1 ml of 0.3% agar in McCoy’s medium supplemented with 15% FCS and 10% human placenta-conditioned medium\(^8\) on top of 1 ml of 0.5% agar in McCoy’s medium with 15% FCS in 35-mm Petri dishes (Falcon Plastics, Los Angeles, Calif.). The cells were incubated at 37°C in 7.5% CO\(_2\) in a fully humidified atmosphere. Clusters (3-40 cells) were counted on day 7 and colonies (more than 40 cells) on day 10.

Conditioned media of cells from leukemia patients (100 or 300 µl) were included in the overlayers (1.5 × 10\(^5\) cells/dish) to test the effect on cluster and colony formation.

Cells were also cultured in methylcellulose to study total cell production; 2–3 × 10\(^6\) normal marrow cells (density less than 1.077 g/ml) were mixed with 1.5 ml 0.8% methylcellulose (final concentration) (Dow Methocel A4M; Dow Chemicals, Midland, Mich.) in McCoy’s medium containing 15% FCS and 7% human placenta-conditioned medium in Petri dishes. Conditioned media (100–150 µl) were added, and the cells counted on day 7; 1.5 ml of 0.9% NaCl were added to dilute the methylcellulose and the cells thoroughly suspended with a Pasteur pipette before counting in a hemocytometer.

Conditioning of Media by Cells From Patients With Leukemia

Cells with a density of less than 1.077 g/ml were incubated in McCoy’s medium with 5% FCS at a concentration of 5 × 10\(^5\) cells/ml for 3–5 hr at 37°C. After centrifugation at 700 g for 10 min, the cell-free supernatant was used for assay of LAI activity. Conditioned media were stored frozen at −20°C. The optimal cell concentration for production of LAI was determined by varying the cell concentration over a range of 0.5–10 × 10\(^5\)/ml. Likewise, different incubation times were used to determine the time course for production of LAI.

In some experiments, the cells were incubated at +4°C or in the absence of FCS. Cells were also pretreated with 0.1% trypsin for 20 min (Kabi, Stockholm, Sweden) in the absence of FCS, washed, and then used to condition medium. In one experiment, cells from a patient with CML in blastic crisis were pretreated with cycloheximide (20 µg/ml) for 45 min at 37°C; the cells were washed twice in McCoy’s medium to remove cycloheximide and then incubated for 5 hr to obtain conditioned medium.

Unless otherwise stated, conditioned media were filtered on XM300 Diaflo filters (Amicon Corp., Lexington, Mass.) and the retentates diluted to the original volume with McCoy’s medium containing 5% FCS before assay for LAI. This procedure was adopted because we have found the LAI is a compound of high molecular weight that is totally retained by XM300 filters, whereas material interfering with the assay for LAI is removed by ultrafiltration.

Assay for Leukemia-Associated Inhibitor (LAI)

Modified Marbrook chambers were used to study the effects of soluble products of cells from patients with leukemia on normal bone marrow cells in a system that prevents contact between normal and leukemia cells. The chambers are made up of two compartments separated by a Nuclepore filter (diameter 25 mm, pore size 0.4 µm) (Nuclepore Corp., Pleasanton, Calif.). A quantity of 3 × 10\(^6\) normal bone marrow cells (density less than 1.077 g/ml) in 1.5 ml McCoy’s medium with 5% FCS were incubated in the inner compartment and 10–15 × 10\(^6\) cells from leukemia patients (density less than 1.077 g/ml) in 3 ml McCoy’s medium with 5% FCS in the outer compartment. Controls were run with medium only in the outer compartment. The chambers were incubated at 37°C in 7.5% CO\(_2\) for 70 min to allow soluble leukemia cell products to diffuse over the membrane and interact with the normal cells. The normal marrow cells were then recovered from the inner chamber and divided in 2 equal parts in test tubes for \(^3\)H-thymidine (\(^3\)H-TdR) suicide\(^1\). To one tube, 25 µCi/ml of \(^3\)H-TdR (15–21 Ci/m mole Amersham, England) was added and the cells incubated for 30 min. After washing once in 5 ml McCoy’s medium containing 100 µg/ml cold thymidine and once in 5 ml McCoy’s medium with 5% FCS they were cultured in agar. Colonies were counted on day 10. The decrease of colony counts on \(^3\)H-TdR exposure was taken as a measure of the fraction of CFU-c in S-phase.

Media conditioned by cells from patients with leukemia were assayed for LAI by mixing 0.5 ml with 0.5 ml of low-density normal marrow cells (3 × 10\(^6\)/ml) followed by incubation at 37°C in 7.5% CO\(_2\). Each set consisted of two identically treated tubes. After 60 min of incubation, 25 µCi/ml of \(^3\)H-TdR was added to one tube followed by an additional incubation period of 30 min. The cells were then washed as described above and cultured in agar for determination of the fraction of CFU-c in S-phase. Controls were run with McCoy’s medium containing 5% FCS. Cells subjected to LAI are found to be less sensitive to the suicidal dose of \(^3\)H-TdR and show a smaller fraction of CFU-c in S-phase than the controls. It is emphasized that under these circumstances (cells washed twice before plating), the conditioned media do not inhibit colony or cluster formation by cells unexposed to \(^3\)H-TdR. This is true regardless of the day of counting (day 7 or day 10); e.g., 8 LAI-containing conditioned media were tested against 2 different marrows, the cluster counts were 100% ± 4% (mean ± SD) of the unexposed controls, and colony counts were 103% ± 4% of the controls. The reduction of CFU-c in S-phase is measurable already on day 7 of incubation and remains unchanged on day 10; in 5 experiments, the mean change of CFU-c in S-phase from day 7 to day 10 was 2.8%. Control experiments also showed that \(^3\)H-TdR had no killing effect on CFU-c in the presence of cold thymidine, 50 µg/ml; in 4 experiments with 4 different marrows, the colony counts were 99% ± 6% (mean ± SD) of the controls (without \(^3\)H-TdR but with cold thymidine added).

\(^3\)H-TdR Labeling Indices

A quantity of 5 × 10\(^6\) normal marrow cells in 0.5 ml McCoy’s medium with 5% FCS were mixed with 0.5 ml of LAI-containing conditioned media. After incubation for 60 min at 37°C, \(^3\)H-TdR (1 µCi/ml) was added, and incubation carried out for an additional period of 45 min. The cells were washed twice and smears prepared by use of a cytocentrifuge. The smears were treated with emulsion K2 (Ilford Ltd., Ilford, England) and stained with May-Grünwald-Giemsa. Labeling indices were calculated for myeloblasts, promyelocytes, and myelocytes; the latter two cell types were counted together. Two-hundred myeloblasts and 300 promyelocytes and myelocytes were counted.

Assay of Thymidine Degradation

\(^3\)H-thymidine was chromatographed on Dowex 50W-X8 in 0.1 M ammonium formate buffer, pH 3.2, as described.\(^1\) A quantity of 0.5 µCi of \(^3\)H-TdR was
incubated with 0.5 ml of different LAI-conditioned media for 60 min before ion-exchange chromatography. Fractions of 2 ml were collected and the radioactivity measured on 0.5-ml aliquots of each fraction after addition of 5 ml of Insta-Gel (Packard Instruments Co., Downers Grove, Ill.).

RESULTS

Reduction of CFU-c in S-Phase

In the initial experiments using the modified Marbrook chambers, we observed that low-density cells of CML or AML during a short-term incubation produce substances that inhibit the DNA synthesis of human marrow CFU-c. These results are summarized in Table 1. In 10/12 cases, the fraction of CFU-c in S-phase was reduced in the presence of low-density CML or AML cells. A paired t test showed statistically significant differences from the controls (p < 0.05 for CML cells and p < 0.01 for AML cells). If the Nuclepore membrane in the Marbrook chamber was replaced by a dialysis membrane, it was repeatedly shown that the leukemic cells did not affect the DNA synthesis of the normal marrow CFU-c (data not shown). This observation suggests that the inhibitory substance(s) produced by the CML or AML cells is of high molecular weight. Subsequently, conditioned media were filtered through Diaflo membranes of different pore sizes. These experiments showed that all inhibitory activity was retained by XM300 filters, suggesting a molecular weight of the inhibitory substance(s) of at least 300,000 daltons. We also found that the filtration procedure in some cases unmasked inhibitory activity not detectable in the original conditioned media. Therefore, all subsequent studies were performed with XM300-filtered conditioned media.

The conditioned media of low-density CML, AML, CLL, or ALL cells or low-density normal marrow (n = 5) or blood (n = 5) cells (NMB) were incubated with normal marrow target cells. Controls were with McCoy's medium with 5% FCS. Mean colony counts per dish without 3H-TdR were 88 ± 40 in the 10 normal marrows used as target cells. The values were statistically analyzed by paired t test. Individual results were analyzed by Student's t test, indicated by the number of conditioned media significantly reducing CFU-c in S-phase per number of media tested (0.001 < p < 0.05). NS, not significant.

Table 1. Reduction of CFU-c in S-Phase by CML or AML Cells

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Normal CFU-c in S-Phase (%)</th>
<th>Control</th>
<th>CML</th>
<th>Control</th>
<th>AML</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40.4</td>
<td>25.7</td>
<td>47.0</td>
<td>25.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>41.4</td>
<td>10.0</td>
<td>46.8</td>
<td>34.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>41.7</td>
<td>27.8</td>
<td>56.4</td>
<td>33.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>41.4</td>
<td>50.8</td>
<td>43.6</td>
<td>26.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.4</td>
<td>25.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>28.3</td>
<td>28.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>36.5</td>
<td>17.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>43.6</td>
<td>28.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>39.2 ± 26.6 ± 48.5 ± 29.7</td>
<td>± 4.5 ± 10.9 ± 4.8 ± 4.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p &lt; 0.05</td>
<td>± p &lt; 0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Normal marrow cells (inner compartment) were exposed to intact CML or AML cells (outer compartment) in Marbrook chambers. Controls contained McCoy's medium with 5% FCS in the outer compartment. Mean colony counts per dish without 3H-TdR were 88 ± 40 in the 10 normal marrows used as target cells. The values were statistically analyzed by paired t test.

Table 2. Reduction of CFU-c in S-Phase by Conditioned Media

<table>
<thead>
<tr>
<th>Patients</th>
<th>CFU-c in S-Phase (%)</th>
<th>Paired t Test</th>
<th>Student's t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>(n = 14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CML</td>
<td>(n = 12)</td>
<td>36.5 ± 4.4 (31.2-44.6)</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>(n = 15)</td>
<td>20.9 ± 7.7 (9.0-31.4)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>CLL</td>
<td>(n = 7)</td>
<td>20.9 ± 4.7 (12.8-28.5)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>ALL</td>
<td>(n = 6)</td>
<td>30.4 ± 9.8 (8.3-42.5)</td>
<td>NS</td>
</tr>
<tr>
<td>NMB</td>
<td>(n = 10)</td>
<td>36.5 ± 6.8 (29.4-47.9)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Conditioned media of low-density CML, AML, CLL, or ALL cells or low-density normal marrow (n = 5) or blood (n = 5) cells (NMB) were incubated with normal marrow target cells. Controls were with McCoy's medium with 5% FCS. Mean colony counts per dish without 3H-TdR were 39.2 ± 26.6 ± 48.5 ± 29.7 (Table 1). Ten of twelve CML patients, 12/15 AML patients, 1/7 CLL patients, and 2/6 ALL patients produced inhibitory activity that significantly reduced the fraction of normal CFU-c in S-phase (0.001 < p < 0.05). Exceptional cases (1 CLL and 1 ALL) showed a stimulatory effect with an increased fraction of CFU-c in S-phase.

Since conditioned media from low-density (<1.077 g/ml) cells of normal marrow (n = 5) or normal peripheral blood (n = 5) totally failed to reduce the fraction of normal CFU-c in S-phase (Table 2), we will refer to the inhibitory substance produced by cells from patients with leukemia as "leukemia-associated inhibitor" (LAI) in the following text.

Conditioned media from high-density (>1.077 g/ml) CML cells did not show any LAI activity in three experiments; mean percent CFU-c in S-phase for the controls were 38.4 ± 1.8 (SD) and 50.4 ± 8.6 in the presence of conditioned media.

Experiments were also performed to show that washing the target cells free from conditioned medium before addition of 3H-TdR does not abolish the reduction of CFU-c in S-phase. In two sets of experiments with two different marrow samples, six different
Reduction of Cluster and Colony Growth

When normal marrow cells were exposed to conditioned media throughout the culture period, moderate inhibitory effects were noted on cluster formation in agar and total cell production in methylcellulose (Table 3). It was, however, noted that several conditioned media, although expressing LAI activity (reduction of CFU-c in S-phase), failed to inhibit cluster formation or cell production and vice versa. Increasing the dose of added conditioned media to 300 μl did not significantly increase the inhibition of cluster formation in 14 cases studied.

The discrepancy between reduction of CFU-c in S-phase and inhibition of cluster formation led us to investigate whether these two inhibitory actions were due to the same or different substances. In preliminary experiments, we had observed that LAI was heat stable. Therefore, we tested the heat stability of the cluster inhibitory activity. Seven conditioned media were heated to 60°C for 10 min and tested for inhibition of cluster/colony formation and reduction of CFU-c in S-phase. Untreated conditioned media were tested simultaneously. Results are shown in Table 4. The capacity to reduce CFU-c in S-phase was unaltered after heating to 60°C, whereas cluster/colony inhibition was abolished. We conclude that the cluster/colony inhibitory activity is not identical with LAI. The term LAI should be restricted to the activity reducing CFU-c in S-phase.

Time Course for Production of LAI

Low-density cells from 1 CML patient and 4 AML patients were incubated at a concentration of 5 x 10⁶/ml for 1–20 hr and the conditioned media tested for LAI after XM300 filtration (Fig. 1). LAI was present after 1 hr of incubation, reached its maximum at 3–5 hr, and decreased thereafter. In other experiments (data not shown) we found that the production of LAI could be maintained at its maximum level for about 30 hr if the medium was changed every 4–10 hr.
LEUKEMIA-ASSOCIATED INHIBITOR (LAI)

Fig. 2. Dose-dependent reduction of CFU-c in S-phase by conditioned media of low-density cells from patients with leukemia obtained by 5-hr incubation at different cell concentrations (abscissa). [●●●] AML, [○○○] CML, and [△△△] ALL (control without 3H-TdR had 184 colonies/dish).

Effect of Cell Concentration on Production of LAI

Low-density cells (1 patient each with CML, AML, or ALL) were incubated at a concentration of 0.6-10 × 10⁶ cells/ml for 3-5 hr and the conditioned media were assayed for LAI. Suppression of normal CFU-c in S-phase increased with cell concentration of the conditioned media from 0.6 × 10⁶ cells/ml to 5 × 10⁶ cells/ml but showed little or no increment between 5 and 10 × 10⁶ cells/ml (Fig. 2).

Perturbation of LAI Production

Cells from patients with leukemia failed to produce LAI at +4°C; the untreated controls showed 38.5% ± 8% (SD) of CFU-c in S-phase (n = 3), conditioned media obtained at 37°C 23.8% ± 3.9% (n = 3, p < 0.01), and conditioned media obtained at 4°C 43.9% ± 12.2% (n = 3, not significant). Cells pretreated with cycloheximide, an inhibitor of protein synthesis, also failed to produce LAI in one experiment; untreated control showed 45.7% of CFU-c in S-phase, conditioned medium from leukemia cells 37.1% (p < 0.01), and conditioned medium from cycloheximide pre-treated leukemia cells 43.4% (not significant). These experiments suggest that the production of LAI is dependent on an active metabolism of the cells. Addition of fetal calf serum was not necessary for the production of LAI; in three experiments, the mean control value for CFU-c in S-phase was 34.6% ± 1.2% (SD), and with conditioned media obtained in the absence of FCS 23.9% ± 2.5% (p < 0.01). We also repeatedly observed that trypsinization of the cells did not render them incapable of producing LAI. In fact, trypsinization unmasked LAI production in one case of AML. Trypsinization of normal bone marrow cells (4 cases) did not induce LAI production (control: 31.5% S-phase; conditioned media: 35.0% ± 6.6% S-phase, mean ± SD). These experiments suggest that LAI is actively produced by the cells and not a substance absorbed from the culture medium.

3H-TdR Labeling Indices (LI)

The effects of LAI-containing conditioned media (4 CML and 6 AML) on DNA synthesis of normal granulopoietic precursor cells were estimated from radioautographic smears. The control showed 45% LI for myeloblasts and 26% LI for promyelocytes and myelocytes. Conditioned media significantly reduced LI of myeloblasts to a mean value of 34% (range 28%-40%), p < 0.01 by paired t test) but failed to reduce LI of promyelocytes and myelocytes (mean value 28%, range 16%-34%, not significant). Mean grain counts were not altered by conditioned media; the control showed a mean of 21 ± 8 (SD) grains/cell (n = 43) and conditioned media 24 ± 9 grains/cell (n = 78).

Degradation of 3H-TdR

If the conditioned media used to assay LAI contained thymidine-degrading enzymes, these could in fact reduce the amount of 3H-TdR available to kill cells in S-phase. This would give a false reduction of CFU-c in S-phase. After incubation of 3H-TdR with different LAI-containing conditioned media, no degradation products of 3H-TdR were found on ion-exchange chromatography (Fig. 3). We conclude that the effect of LAI on normal marrow CFU-c is not due to degradation of 3H-TdR. This is also supported by the observation that LAI-containing conditioned media did not reduce the mean grain count of normal marrow cells used for radioautographic smears (see above) and that washing the target cells free from LAI before addition of 3H-TdR did not abolish the reduction of CFU-c in S-phase.

Effects of LAI on Clonogenic Cells in CML and AML

It is an important question whether the clonogenic cells from patients with leukemia are sensitive to LAI. Therefore, we used CML or AML cells as target for conditioned media with known LAI activity against normal marrow cells. Results are shown in Table 5. Three of seven CML patients responded to LAI with a reduced fraction of CFU-c in S-phase, whereas 4/7 patients showed an increase. One patient (no. 3) was tested on 2 different occasions with an interval of 7 mo. On the first occasion, the patient was off treat-
ment for 4 mo and showed 54.0% of CFU-c in S-phase with a significant \((p < 0.01)\) reduction to 30.0% on exposure to LAI-containing conditioned medium. On the second occasion, the patient had been treated with Busulphan for 5 mo, and this time only 11.3% of her CFU-c were in S-phase and responded with an increase to 30.4% \((p < 0.01)\) and 20.9% \((p < 0.05)\) on exposure to LAI. None of the AML patients in remission or early relapse responded to LAI. All conditioned media used in these tests were prepared from patients other than the patients used as target cell donors.

**DISCUSSION**

In the present work we demonstrate that low-density cells from patients with leukemia, but not low-density cells from normal marrow or blood, release a high molecular weight substance, called LAI, that reduces the fraction of normal marrow CFU-c in S-phase.

Several investigators have previously made attempts to test the hypothesis that leukemic cells somehow suppress normal hemopoiesis. Coculture of normal cells and cells from patients with leukemia has not given consistent results. However, normal mouse marrow growth was suppressed by murine leukemia cells in diffusion chambers, and a diffusible substance was shown to be released by the leukemia cells. Conditioned media of rat leukemia cells also provided inhibition of normal CFU-c. In an extensive study, Broxmeyer et al. have shown that extracts or conditioned media of cells from patients with many types of acute and chronic leukemia inhibit colony and cluster formation by normal marrow cells. The inhibitory activity is produced from cells that are nonadherent, of light density, and that have a slow sedimentation rate. Inhibitory cells did not, however, seem to be identical with blast cells. Recently, a human leukemia cell line (K562) has been found to produce inhibition of human CFU-c and to some extent CFU-e also.

The assay system used in the present work for demonstration of LAI includes preincubation of normal marrow cells with conditioned medium followed directly by addition of a suiciding dose of \(^{3}H\)-TdR to determine the fraction of CFU-c in S-phase. The finding that marrow cells exposed to LAI and \(^{3}H\)-TdR grow more colonies than cells exposed to \(^{3}H\)-TdR alone clearly indicates that LAI reduces the fraction of CFU-c in S-phase. Cells treated with conditioned medium alone, washed, and plated in agar grow the same number of clusters/colonies on day 7 and day 10 as untreated cells, suggesting that the effect of LAI on CFU-c is reversible and noncytotoxic. This finding is in contrast to the effect of leukemia-cell conditioned media demonstrated by Broxmeyer et al., although their results strongly suggested that inhibitory activity was against CFU-c during S-phase.

That our assay system utilizing \(^{3}H\)-TdR should be invalidated by thymidine-degrading enzymes known to be present in both normal and leukemia cells could...
be ruled out. Thus, we were unable to demonstrate any thymidine phosphorylase activity in LAI-containing conditioned media. Furthermore, washing the target cells before addition of $^3$H-TdR did not abolish the reduction of CFU-c in S-phase. The fact that no reduction was seen of $^3$H-TdR mean grain counts of the radioautographic smears of cells exposed to LAI also excludes the presence of thymidine-degrading activity.

Initial experiments employing Marbrook chambers clearly indicated that cells from patients with CML or AML released a diffusible substance that inhibited the DNA synthesis of normal marrow CFU-c. Subsequently, we could show that conditioned media of such cells had the same capacity and that the active component is of high molecular weight retained by XM300 Diaflo membranes. The release or production of LAI seems to be a metabolically active process as it is abolished at $+4^\circ$C. It is also dependent on active protein synthesis, as pretreatment of the cells with cycloheximide abolished LAI production. The finding that it is possible to induce production of LAI after trypsinization of the cells and in the absence of serum proteins demonstrates that LAI is not a substance absorbed from the culture medium. All the evidence indicates that LAI is actively synthesized by cells from patients with leukemia.

Studies of the time course for the production of LAI showed that it reached its maximum within 5 hr of incubation and then slowly decreased. This is probably due to loss of activity, since we could show maintenance of LAI production for at least 30 hr by changing the medium every 4–10 hr. These observations suggest that the production of LAI reaches an equilibrium within 3–5 hr but can be induced again by changing the medium. Cell concentration affected LAI production within a range of 0.6–10 x $10^6$ cells/ml. The fact that increasing the cell concentration from 5 to 10 x $10^6$ cells/ml did not result in greater inhibitory capacity most likely reflects a limited responsiveness of the target cells. It should be noted that it was impossible to achieve a total reduction of CFU-c in S-phase during the 90-min LAI was allowed to interact with the target cells. Subclasses of CFU-c could also respond differently to LAI, as described for another inhibitor derived from cells from patients with leukemia. However, the fact that we obtained identical results when cultures were scored on day 7 and day 10 speaks against this possibility. Therefore, we interpret our results to be consistent with an effect of LAI on the rate of entry into S-phase rather than interference with cells already in S-phase.

Continuous presence of conditioned media in the cultures resulted in some reduction of cluster/colony growth in agar and a reduction of total cell production in methylcellulose. This inhibitory activity was distinguished from LAI by its heat lability and may be identical with the inhibitory activity described by Broxmeyer et al. LAI is, however, resistant to heating to $60^\circ$C for 10 min.

With few exceptions, the production of LAI was a consistent phenomenon in myeloid leukemias. In lymphocytic leukemias significant LAI activity was found only in 1/7 CLL patients and 2/6 adult ALL patients. Whether the inhibitory activity discovered in some patients with lymphocytic leukemia is identical with LAI of myeloid leukemia has to be confirmed by biochemical methods. We already know that LAI of CML and AML are behaving identically through all steps of biochemical characterization. Detailed future studies of density, size, and morphology of the LAI-producing cells will help to determine whether the production of LAI is a phenomenon characteristic of both myeloid and lymphocytic leukemias or restricted to the myeloid leukemias.

An important finding was that CFU-c of chronic-phase CML and AML in remission or early relapse in most cases were resistant to the action of LAI. Interestingly, one patient with CML studied on two occasions with an interval of 7 mo switched from responding to nonresponding. Previous studies have indicated that growth regulatory mechanisms do exist during the early stages of CML but are progressively deranged through later stages of the disease. Hypothetically LAI could take part in the retardation of growth on the Ph'-positive CFU-c as long as they are susceptible to its action before development of nonresponding cell populations. The more important action of LAI in CML and AML might be the suppression of normal stem cell proliferation, which in combination with unresponsiveness of leukemic stem cells would leave the leukemic cells to dominate granulopoiesis. The inhibitory activity described by Broxmeyer et al. did not inhibit CFU-c from most patients with leukemia, suggesting a growth advantage of leukemic progenitor cells. Serial studies of CML and AML are now being performed to gain further information as to whether the cells in leukemia change their responsiveness to LAI during the course of the disease.

An important question is the specificity of LAI: Are other hemopoietic stem cells or nonhemopoietic cells affected by LAI? Due to the complex composition of conditioned media, in which we found two inhibitory activities directed against CFU-c, tests for specificity with such media are difficult to interpret and may be misleading. We, therefore, have put priority on purification of LAI, and in an accompanying article we report on the biochemical characterization and purifi-
cation of LAI. Future studies on purified LAI may allow us to study its target cell specificity and define its possible pathogenetic role in leukemia.

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REFERENCES

Suppression of normal granulopoiesis in vitro by a leukemia-associated inhibitor (LAI) of acute and chronic leukemia

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