Biochemical Characterization of a Leukemia-Associated Inhibitor (LAI) Suppressing Normal Granulopoiesis In Vitro

By Tor Olofsson and Inge Olsson

Low-density (<1.077 g/ml) marrow or blood cells from patients with acute or chronic leukemia release a high molecular weight substance called "leukemia-associated inhibitor" (LAI) that reduces the fraction of normal marrow CFU-c in S-phase as measured with the 3H-TdR suicide technique. LAI from conditioned media or 3M KCl extracts of subcellular fractions behaved homogenously on gel chromatography, showing an apparent molecular weight greater than 500,000. However, ion-exchange chromatography and isoelectric focusing indicated considerable charge heterogeneity for LAI molecules. Results from SDS-polyacrylamide gel electrophoresis indicated that the biologic activity resides in a subunit of 30,000 daltons. The findings of marked affinity for Con-A-Sepharose, marked susceptibility to mild periodate treatment, partial susceptibility to protease digestion, and relative resistance to heating suggest that LAI is a glycoprotein. Data from radiolabeling of cell surface components and sucrose density gradient centrifugation are consistent with LAI being a peripheral cell membrane glycoprotein, which may suppress normal granulopoiesis in leukemia.

The Dysfunction of leukemia cells and the deficient production of normal cells resulting in anemia, thrombocytopenia, and granulocytopenia cause the most serious clinical problems in acute leukemia. The underlying mechanisms may involve a suppression of normal marrow cell proliferation and maturation tentatively exerted by the leukemic cells. Several studies have provided evidence for the inhibition of normal marrow growth in agar cultures,1,2 or diffusion chambers3,4 by leukemia cells, conditioned media, or extracts of such cells.5,6 Inhibition has been shown to be directed against both committed granulopoietic stem cells1,2,3,6 and pluripotent stem cells.3,4 It has been hypothesized that such inhibitory mechanisms are present also in vivo and may explain the suppression of normal hematopoiesis in leukemia and the growth advantage of leukemic cells.7

In a previous study we described the production by low-density cells from patients with leukemia of a high molecular weight substance that reduced the fraction of normal marrow CFU-c in S-phase as measured by the 3H-thymidine suicide technique.8 This effect appeared to be reversible. The production of the inhibitor, called LAI (leukemia-associated inhibitor), correlated to cell concentration and was dependent on an active cell metabolism and protein synthesis. It was readily released into the culture medium during 3–5 hr of incubation by the majority (22/27) of patients with acute or chronic myeloid leukemia and a minority (3/13) of patients with acute or chronic lymphocytic leukemia, whereas normal cells failed to produce detectable LAI. CFU-c in 5/7 cases of chronic myeloid leukemia and 4/4 cases of acute myeloid leukemia were unresponsive to LAI.

In the present study, we describe the biochemical characterization of LAI. The data indicate that it is a high molecular weight glycoprotein most likely located on the cell surface.

MATERIALS AND METHODS

Patients

Blood or bone marrow cells were obtained from 5 patients with chronic myeloid leukemia (CML) in the chronic phase and 7 patients with acute myeloid leukemia (AML). The CML patients all had elevated WBC (>30 x 109/liter), and the AML patients were studied at diagnosis or in relapse. Spleen cells were obtained at splenectomy from one patient with CML before blastic crisis and from one patient with myelofibrosis in a transitional phase, who developed AML a few months later. One patient had chronic lymphocytic leukemia (CLL).

Assay of LAI

Conditioned media, cell extracts, and samples from the various purification steps were assayed for LAI activity by their ability to reduce the fraction of normal CFU-c in S-phase, as described in detail previously.9 Low-density (<1.077 g/ml) normal marrow cells (0.5 ml of 3 x 106/ml) were incubated with an aliquot (0.1–0.5 ml) of the test material for 60 min and immediately subjected to 3H-TdR suicide. Washing the target cells before addition of 3H-TdR did not abolish the effect of LAI.9 Controls were run with McCoy’s medium containing 5% fetal calf serum (FCS) or with the appropriate buffer. After being washed twice, the cells were plated in agar medium with 5% normal marrow colony-stimulating factor.9 Colonies (>40 cells) were counted on day 10 of culture. The decrease of colony counts on 3H-TdR exposure is a measure of the fraction of CFU-c in S-phase. CFU-c subjected to LAI are less sensitive to the suicidal dose of 3H-TdR

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and show a smaller fraction of such cells in S-phase than the controls. When the assay is performed in this manner LAI does not affect colony formation by cells unexposed to \( ^3\)H-Tdr. Consequently, the LAI-induced reduction of CFU-c in S-phase is reversed during growth in agar.

**Conditioned Media**

Heparinized blood or bone marrow from patients with acute myeloid leukemia (AML), chronic myeloid leukemia (CML), or chronic lymphocytic leukemia (CLL) were collected and separated during growth in agar.

**Cell Extracts**

Low-density leukemia cells (<1.077 g/ml) or unseparated spleen cells were homogenized in 0.34 \( M \) sucrose at a concentration of 2-3 \( \times 10^8 \) cells/ml. The homogenates were centrifuged at 8000 \( g \), and the supernatants used for sucrose density gradients or extraction with 3\( M \) KCl. In the latter case, the 8000 \( g \) supernatant was put in dialysis bags and dialyzed against 3\( M \) KCl for 24-30 hr. Material precipitating during dialysis was removed by centrifugation before continuing the dialysis against 0.15 \( M \) NaCl containing 5 \( M \) HEPES (BDH Chemicals Ltd., Poole, England) pH 7.4 (called HEPES-NaCl in the following text). The dialyzed material was concentrated on XM300 Diaflo membranes before assay of LAI.

In some experiments the 10,000 \( g \) supernatants obtained from homogenates of CML blood cells or low-density normal marrow cells were centrifuged at 130,000 \( g \) for 3 hr. The pellet was suspended in 3\( M \) KCl and extracted in the cold overnight. Precipitates were removed and the extract dialyzed against HEPES-NaCl.

**Sucrose Density Gradients**

Ten-milliliter continuous sucrose gradients (density range: 1.10-1.30 g/ml) were overlayered with 1.5-2.0 ml of 8000 \( g \) supernatants of homogenized cells and centrifuged at 65,000 \( g \) for 30 hr (rotor model SW 25.1; LKB-Beckman Instruments, Bromma, Sweden). One-milliliter fractions were collected with a peristaltic pump (Varioperpex 2120, LKB-Beckman Instruments, Bromma, Sweden). Density was read in a refractometer (TS Meter, American Optical, Buffalo, N.Y.) and absorbance at 450 nm in a Hitachi/Model 101 spectrophotometer. The fractions were then dialyzed against 3\( M \) KCl for 24-30 hr, precipitates removed by centrifugation, and dialysate concentrated against HEPES-NaCl. After concentration on XM300 Diaflo membranes to the original volume (1 ml), the fractions were assayed for LAI.

**Radiolabeling of Cell Surface Proteins**

Peroxidase-mediated iodination of cell surface proteins was performed in the following manner: 5 \( \times 10^8 \) low-density AML or CML cells were incubated in 10 ml of Hank's balanced salt solution (HBSS) with 2 \( \mu g \)/ml myeloperoxidase, \( ^{125}I \) 20 \( \mu g \)/ml glucose oxidase (Hughes & Hughes Ltd., Romford, England), 0.5 \( \mu m \)ole NaI, and 2 \( mCi \) \( ^{125}I \) (Amersham, England) at \( 37^\circ C \) for 3 hr. The cell-free supernatants were collected by centrifugation at 8000 \( g \), and 3\( M \) KCl and extracted in the cold overnight. Precipitates removed by centrifugation, and dialysate continued against HEPES-NaCl before assay of LAI. Fractions (5-10 ml) were collected and absorbance read at 280 nm. After determination of the ionic strength, all fractions were dialyzed against HEPES-NaCl before assay of LAI.

**Ion-Exchange Chromatography**

DEAE-Sephacel A50 or DEAE-Sepacel (Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.10 \( M \) NaCl, 10 \( M \) HEPES, pH 7.4, was packed in a 1 \( \times \) 25 cm column at 4\( ^\circ \)C. Conditioned media were dialyzed against the starting buffer before application to the column. The column was eluted with a volume of starting buffer equal to 3-4 times the sample volume before elution continued with a linear gradient of 0.10-0.60 \( M \) NaCl in 10 \( M \) HEPES, pH 7.4. Fractions (5-10 ml) were collected and absorbance read at 280 nm. After determination of the ionic strength, all fractions were dialyzed against HEPES-NaCl before assay of LAI.

**Isoelectric Focusing**

Preparative isoelectric focusing (pH 3.5-10.0) in a sucrose gradient was performed on a CML-cell-conditioned medium. A LKB 8101 electrofocusing column (volume 110 ml; LKB-Beckman Instruments, Bromma, Sweden) was used. The sample was introduced into the 3 middle fractions, electrofocusing started at 0.5 \( W \), and run for 30 hr. The column was slowly emptied, collecting fractions of 3 ml (fractions 1-6 and 21-30) or 1 ml (fractions 7-20). Absorbance at 280 nm and pH was measured before an extensive dialysis against HEPES-NaCl and assay of LAI.

**Gel Chromatography**

Sephacryl S-6B (Pharmacia Fine Chemicals) in HEPES-NaCl was packed in a 2.5 \( \times \) 100 cm column. Conditioned media or cell extracts concentrated on XM300 Diaflo membranes were applied to the column in a volume of 3-4 ml and eluted in 10-15-ml fractions at a flow rate of 10-15 ml/90 min at 4\( ^\circ \)C. Protein concentration was monitored by ultraviolet absorption at 280 nm.

**Affinity Chromatography**

Five to eight milliliters of concanavalin-A-Sepharose (Pharmacia Fine Chemicals) in 0.15 \( M \) NaCl, 1 \( M \) CaCl\(_2\), 1 \( M \) MgCl\(_2\), 1 \( M \) MnCl\(_2\), and 0.01 \( M \) sodium acetate buffer pH 6.4 was packed in a column (1 \( \times \) 10 cm) at 4\( ^\circ \)C. Samples were corrected for pH and supplemented with 1 \( M \) CaCl\(_2\), MgCl\(_2\), and MnCl\(_2\) before application to the column. Sample volume was 2-5 ml. The column was eluted with the sodium acetate buffer until ultraviolet-absorption at 280 nm of the eluate reached a minimum. Thereafter, the elution was continued with 50 \( M \) \( \alpha \)-methyl-D-glucoside in starting buffer. All fractions were dialyzed extensively against HEPES-NaCl before assay of LAI.

**Electrophoresis in SDS-Polyacrylamide Gels**

Electrophoresis in polyacrylamide gels, in the presence of sodium dodecyl sulphate (SDS) and reducing agents, was performed in a discontinuous buffer system as described by Neville.[11] Upper gels were 3.2 % 6.25 (T x C) and lower gels were 11.1 % 0.9 (T x C). Upper buffer was 0.040 M boric acid, 0.041 M Tris, and 0.15% SDS, pH 8.64; lower buffer was 0.0494 M HCl, 0.1716 M Tris, pH 8.47. Samples and standards were pretreated with 1.6% SDS, 0.05 \( M \) Na\(_2\)CO\(_3\), and 10% \( \beta \)-mercaptoethanol, and dialyzed for 2 hr against 0.1% SDS and 0.05% dithioerythritol in HEPES-NaCl before application on the gels. Standards were 10-20 \( \mu l/gel \). Electrophore-
BIOCHEMICAL CHARACTERIZATION OF LAI

sis was run at 1.5 mA/gel until the dye front was 5 mm from the end of the gel. The following proteins (2 mg/ml) were used as standards for molecular weight (mol wt) determination: phosphorylase A, mol wt 92,500 (Sigma Chemicals, St. Louis, Mo.); human albumin, mol wt 68,000 (Kabi, Stockholm, Sweden); and egg albumin, mol wt 40,000 (Armour Lab., Eastbourne, England). Following electrophoresis, the gels, with the exception of the upper ones, were immediately cut into 1-mm sections. All the gel slices, including the upper gels, were immersed in 2 ml of HEPES-NaCl and dialyzed extensively. The gel slices were then removed and the extract assayed for LAI. For localization of marker proteins, gels were stained with 0.25% Coomassie Blue R (Sigma Chemicals, St. Louis, Mo.) in 45% methanol and 9.2% acetic acid.

In one case, 10 µl of concentrated LAI-containing material from Con-A-Sepharose chromatography was labeled with 121I- as described. The labeled material was mixed with the same unlabeled material before treatment with SDS, Na2CO3, and β-mercaptoethanol as described above.

**Periodate Treatment of LAI**

LAI-containing conditioned media or semipurified LAI were incubated with 1 mM sodium metaperiodate in the dark at room temperature for 10 min. The reaction was terminated by the addition of 20 mM glycerol, and the samples ultrafiltrated on XM300 Diaflo membranes with McCoy's medium.

**Enzyme Treatment of LAI**

A CML-cell-conditioned medium with known LAI activity was treated with the following enzymes: 0.5 mg/ml protease (from Streptomyces griseus, type VI; Sigma Chemicals, St. Louis, Mo.); 0.5 mg/ml α-chymotrypsin (from bovine pancreas type II; Sigma Chemicals); 1.0 mg/ml ribonuclease-A (from bovine pancreas type II-A; Sigma Chemicals); 0.3 mg/ml deoxyribonuclease (from bovine pancreas; Sigma Chemicals); and 0.5 IU/ml neuraminidase (from Vibrio comma; Behringwerke AG, Marburg, West Germany). Incubations were at room temperature for 4 hr. The conditioned media were then diluted 10 times with McCoy's medium and concentrated on XM300 Diaflo membranes to their original volume (1.3 ml) before assay of LAI.

**Antiserum Against LAI**

LAI-containing extracts of polyacrylamide gel slices were used to immunize rabbits. Extracts were emulsified with an equal volume of Freund's complete adjuvant (Difco Lab., Detroit, Mich.) and injected into the distal part of the hind legs. A booster dose with an equal amount in Freund's incomplete adjuvant was injected subcutaneously after 4 wk. Blood was collected by bleeding the ears 2 wk later. The immunoglobulin fraction was purified by carboxylic acid precipitation and ion-exchange chromatography on DEAE-cellulose. LAI-containing conditioned media were incubated overnight at +4°C with anti-LAI-immunoglobulin equivalent to 30% untreated antiserum. The samples were centrifuged at 900 g for 10 min and Millipore filtered (pore size 0.45 µm) before assay of LAI.

**RESULTS**

**Sucrose Density Gradient Centrifugation**

Figure 1A shows the distribution in a sucrose density gradient of the 8000 g supernatant obtained from a homogenate of radioiodinated AML cells. The gradient contained two regions with banding material visible to the naked eye, corresponding to fractions 7 and 10. The radioactive material showed a distinct profile with a peak in fraction 10 (density 1.11 g/ml). After extraction with 3M KCl, dialysis and ultrafiltration LAI was assayed on 0.5 ml of each fraction. It showed a distribution almost identical with that of the radioactive cell surface material except for fractions 12–15, corresponding to the cell sap, which contained some solubilized radioactive material but no LAI activity. These results show that light-density membrane material was iodinated and that LAI could be extracted by 3M KCl from material with an identical density. It is therefore suggested that LAI may be present on the cell surface of intact cells, from which it could be shed into the medium.

In another experiment, a homogenate of CML cells was processed in an identical manner. Radioactive cell surface components peaked in fractions 9 and 10 (1.120 and 1.105 g/ml, respectively), with the peak of
was any LAI extracted from a crude granule fraction prepared by differential centrifugation between 300 and 9000 g. In fact, all LAI-containing structures of such a cell homogenate pelleted between 9000 and 130,000 g (results not shown). Extracts (3M KCl) of the 130,000 g pellet of normal marrow cell homogenates (3 cases) did not contain detectable LAI.

**Ion-Exchange Chromatography**

Figure 2A shows an example of ion-exchange chromatography of a conditioned medium prepared from 3 x 10^6 low-density CML cells. A minor part of LAI did not absorb to the column. A major part was eluted in fractions 9–10 immediately on starting the gradient. Another part of LAI was eluted within the major protein peak (fractions 15–18) at approximately 0.22 M NaCl, and in this case, a minor part of LAI was eluted at approximately 0.44 M NaCl (fractions 28–30).

The elution patterns of LAI of CML and AML cells were almost identical, as shown in Fig. 2B, where the chromatograms of 2 CML and 1 AML light-density cell-conditioned media were compared. There are principally three regions for the elution of LAI: (1) 0.10–0.12 M NaCl, (2) 0.20–0.24 M NaCl (included in the major protein peak), and (3) 0.40–0.44 M NaCl in some of the conditioned media analyzed. The conditioned medium of high-density (>1.077 g/ml) CML cells was also chromatographed under identical conditions. It is obvious from Fig. 2B that these cells do not release detectable LAI; instead, material is eluted at approximately 0.22 M NaCl, which increases the fraction of normal marrow cell conditioned medium in S-phase. Chromatography on DEAE-Sephadex did not result in substantial purification of LAI.

**Isoelectric Focusing**

Isoelectric focusing of a CML-cell conditioned medium confirmed the observation of charge heterogeneity of LAI revealed by ion-exchange chromatography. There were three regions of LAI-activity with peaks at pH 3.0, 5.8, and 8.3 (Fig. 3).

**Gel Chromatography**

Gel chromatography on Sepharose 6B was generally performed as the first purification step on LAI after it had been concentrated by ultrafiltration through XM300 Diaflo membranes. The elution pattern of LAI was reproducible within a narrow range and effectively separated LAI from the bulk of protein in the samples chromatographed. Figure 4A shows the elution of LAI of a low-density AML-cell conditioned medium. The peak elution volume of LAI was 272 ml (Ve/Vo = 1.53). Ferritin (mol wt 440,000

![Graph](https://via.placeholder.com/150)

**Fig. 2.** (A) Ion-exchange chromatography on DEAE-Sephadex of a CML-cell conditioned medium. Elution buffer was 0.10 M NaCl, 10 mM HEPES, pH 7.4, and the gradient was 0.10–0.80 M NaCl, 10 mM HEPES, pH 7.4. Conductivity was measured and converted to molarity of NaCl from a standard curve. (- - -) LAI activity; (○-○) absorbance at 280 nm; (-----) M NaCl. Control value for the LAI-assay was 173 colonies/dish without 3H-TdR. (B) Comparison of ion-exchange chromatograms of CML and AML cell conditioned media. Experimental conditions as in (A). Data on absorbance at 280 nm were excluded and changes of CFU-c in S-phase plotted against molarity of NaCl in the gradient. (○-○) CML; (△-△) AML; (Δ-Δ) CML: all conditioned media of low-density cells (<1.077 g/ml). (○-○) CML: conditioned medium of high-density cells (>1.077 g/ml). Control values for the LAI assay were 173, 88, 136, and 196 colonies/dish, without 3H-TdR, respectively.
daltons) when chromatographed under identical conditions had a peak elution volume of 305 ml (Ve/Vo = 1.69). Although no precise molecular size determinations can be made from these chromatograms, they suggest that LAI is greater than 500,000 daltons. A minor part of the LAI activity was eluted earlier than the major peak of LAI and probably represented aggregated material.

In another experiment, the 3M KCl extract of the 130,000 g pellet of homogenized CML cells (see Materials and Methods for details) was subjected to ion-exchange chromatography on DEAE-Sepharose. The LAI-containing fractions (test results not shown) eluted at 0.17–0.20 M NaCl, and at 0.28–0.36 M NaCl were pooled, concentrated on XM100 dialysis membranes, and dialyzed against HEPES-NaCl before application to Sepharose 6B. The chromatogram (Fig. 4B) showed an elution pattern of LAI almost identical to that of conditioned media, with one major peak of activity at 280 ml (Ve/Vo = 1.60) and some activity eluted earlier than the major peak. Sepharose chromatography gave a 15–120-fold purification of LAI, depending on the source of starting material.

The behavior of LAI on gel filtration and ion-exchange chromatography suggests that LAI consists of molecular species with identical size but with considerable charge heterogeneity.

Conditioned medium from the only CLL patient (1/7) expressing LAI activity so far was chromatographed on Sepharose 6B. Fractions from 235 to 290 ml elution volume were assayed. The elution pattern was similar to that observed for LAI of AML or CML, with a small peak of activity at 235 ml (23% reduction of CFU-c in S-phase) and a major peak at 280 ml (45% reduction of CFU-c in S-phase).

Affinity Chromatography

Conditioned media of AML cells (2 cases), CML spleen cells (1 case), and extract of spleen cells from a patient with myelofibrosis were chromatographed on Sepharose 6B; fractions corresponding to the elution volume of LAI were pooled, concentrated on XM300 Diaflo membranes to 2–5 ml, and subjected to affinity chromatography on Con-A–Sepharose. In another case, the conditioned medium of CML cells was chromatographed on DEAE-Sephadex and the peak of LAI activity at 0.10–0.12 M NaCl taken to affinity chromatography. Independent of the source of the starting material, all LAI activity was bound to Con-A–Sepharose and was eluted with α-methyl-D-glucos-
68). There were only three visible bands on the gel, each fraction and assayed for LAI. Approximately 80% of the activity was found within the relative mobility of 0.12-0.22 (Fig. 6A). Insignificant amounts of LAI were also found in fractions of higher mobility. The distribution of radioactive cell surface material. AML and CML cells behaved almost identically. These results are consistent with the localization of LAI to the cell surface.

In another experiment, a conditioned medium of AML cells was chromatographed on Sepharose 6B and Con-A–Sepharose before being subjected to SDS-electrophoresis. The gel slices were pooled three in each fraction and assayed for LAI. Approximately 80% of the activity was found within the relative mobility of 0.12–0.17 and the rest at 0.17–0.23 (Fig. 6B). There were only three visible bands on the gel, none of them corresponding to LAI. Comparison with the marker proteins indicated a molecular weight for LAI of 150,000–170,000.

Periodate and Enzyme Treatment of LAI

Mild periodate treatment of LAI-containing conditioned media (one CML and one AML patient) and α-methyl-d-glucoside eluted fractions from a Con-A–Sepharose chromatography (extract of spleen cells of myelofibrosis) destroyed the LAI activity (Table 1). LAI was resistant to most enzymes tested, except protease, which partially destroyed its activity (Table 1). Heating an LAI-containing conditioned medium of CML cells to 60°C for 10 min did not abolish the activity, whereas 90°C for 10 min partially destroyed it (Table 1).

Antiserum Against LAI

Incubation of AML-cell conditioned media with purified immunoglobulin from an antiserum against LAI totally removed LAI from the conditioned media (Table 1). This finding indicates that the immunoglobulin preparation contained antibodies against LAI.

DISCUSSION

In the present work we describe the biochemical characterization and partial purification of a leukemia-associated inhibitor (LAI) that reduces the fraction of normal CFU-c in S-phase as measured by 3H-TdR suicide technique. The production and action of LAI have been described previously.³ The action of LAI is nontoxic and apparently reversible by washing the target cells before plating in agar. The results were consistent with an effect of LAI on the rate of entry of CFU-c into S-phase rather than interference with cells already in S-phase.

The rapid conditoning of medium with LAI from AML or CML cells suggested that it could be a cell surface component readily released to the medium. Peroxidase-mediated iodination of intact cells was utilized to radiolabel cell surface proteins, followed by homogenization and separation in sucrose density gradients. This procedure allowed us to localize the cell surface fragments in the gradient. The distribution of LAI corresponded precisely with the peak of radioactive cell surface material. AML and CML cells behaved almost identically. These results are consistent with the localization of LAI to the cell surface.

However, it has not yet been possible to prove that the LAI molecules are radiolabeled by this procedure because final purification of substantial amounts of LAI has not been achieved at present. The finding that LAI was extracted from membrane fractions with 3M KCl is consistent with the behavior of peripheral cell...
surface glycoproteins rather than cell surface proteins integrated in the membrane. The density distribution of LAI-containing membrane fragments clearly distinguishes LAI from the inhibitor of K562 cells, which bands in sucrose density gradients at 1.18 g/ml (Olofsson T, unpublished observation).

Ion-exchange chromatography revealed heterogeneity of LAI with regard to charge. This was confirmed with isoelectric focusing, showing peaks of activity at pH 3.0, 5.8, and 8.3. It is emphasized, however, that fractions were pooled before assay of LAI, and therefore, the true pH-optima may not be as widely distributed as indicated by the figures. Nevertheless, only one major peak of LAI was found on gel chromatography. The finding that the pooled fractions of LAI, eluted at different molarities of NaCl on ion-exchange chromatography, gave only one major peak on gel chromatography strongly suggests that molecular species of LAI with different charges are all of the same size. The fact that a major part of LAI was eluted with the bulk of protein on ion-exchange chromatography made this procedure unsuitable for purification purposes.

Gel chromatography on Sepharose 6B proved to be an effective first purification step after XM300 Diaflo membrane filtration. The elution pattern of LAI was highly reproducible and indicated a molecular weight for native LAI greater than 500,000 daltons. Because of the scarcity of molecular size markers in this region, more accurate determinations of the molecular size of LAI have not yet been performed. Gel chromatography resulted in a 15–120-fold purification, depending on the starting material. It is emphasized that true calculations of the degree of purification are difficult,
Table 1. Effects of Periodate, Enzymes, Heat, and Antiserum on LAI

<table>
<thead>
<tr>
<th>Treatment and Conditioned Media</th>
<th>Normal Marrow CFU-c in S-Phase (%)</th>
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<tr>
<td></td>
<td>Untreated Media</td>
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<td>Periodate (1mM)</td>
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<tr>
<td>CML-CM</td>
<td>38.1</td>
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<tr>
<td>AML-CM</td>
<td>38.1</td>
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<tr>
<td>Con-A–Sepharose</td>
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<tr>
<td>Enzymes (CML-CM)</td>
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<td>Protease</td>
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<td>(0.5 mg/ml)</td>
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<tr>
<td>α-chymotrypsin</td>
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<tr>
<td>(0.5 mg/ml)</td>
<td>36.1</td>
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<tr>
<td>RNase (1.0 mg/ml)</td>
<td>36.1</td>
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<tr>
<td>DNase (0.3 mg/ml)</td>
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<td>Neuraminidase</td>
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<tr>
<td>(0.5 IU/ml)</td>
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<tr>
<td>Heat (CML-CM)</td>
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<tr>
<td>60°C 10 min</td>
<td>36.1</td>
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<td>90°C 10 min</td>
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<tr>
<td>Anti-LAI-Ig</td>
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Conditioned media (CM) and a Con-A–Sepharose preparation were treated as indicated in the left column and tested for LAI activity. All “untreated CM” did significantly reduce CFU-c in S-phase (0.001 < p < 0.05). In the right column, “treated CM,” is indicated whether the treatment destroyed the LAI activity (p values from Student’s t test) or not (NS, not significant). Control colony counts were 50 colonies/dish (periodate) and 122 colonies/dish (enzymes, heat, and anti-LAI-Ig).

since there is not yet any simple method to quantify LAI. This also excludes calculation of recovery of LAI. The figures given are the lowest estimates made from the chromatograms.

All LAI is strongly bound to Con-A–Sepharose and eluted with α-methyl-D-glucoside, indicating that it is a glycoprotein. This conclusion was also substantiated by the finding that periodate treatment of LAI abolished its activity. Furthermore, LAI was partially destroyed by protease and heating to 90°C for 10 min, but was resistant to nucleases consistent with its identity as a glycoprotein. SDS-polyacrylamide electrophoresis showed only one region of LAI activity at a molecular weight of 150,000–170,000 daltons. This suggests that native LAI either consists of 4–5 identical subunits or a lower number of the active subunits covalently bound to one or more biologically inactive subunit of unknown size. The fact that biologic activity was repeatedly shown to reside within regions of polyacrylamide gels without any stainable material illustrates the biologic potency of LAI, but it also points to the major problem with purification of LAI, i.e., the difficulty to obtain enough starting material.

LAI was obtained from low-density cells of patients with AML or CML and spleen cells from patients with CML or myelofibrosis. No biochemical differences were discovered in LAI from these different sources. In a previous study, LAI activity was found only in a minority of ALL and CLL patients. It has not yet been possible to establish if the LAI found in ALL is identical to that found in the myeloproliferative disorders mentioned above. Preliminary findings in this work on conditioned medium from one CLL patient expressing LAI activity indicated similar behavior on gel filtration as with LAI of AML and CML cells.

Inhibition of normal marrow growth in agar or diffusion chambers by leukemia cells has been described by several authors. So far, these inhibitory substances have not been biochemically characterized. A few reports describe in part the biochemistry of inhibitors produced by normal mature granulocytes, but both the biochemical characteristics and the mechanism of action distinguish them from LAI. Broxmeyer et al. have described an inhibitor from slowly sedimenting cells of patients with leukemia that suppresses the colony formation of normal marrow cells. No biochemical data are available for comparison with LAI, but its cytotoxic activity against CFU-c in S-phase and its extreme heat lability clearly distinguish it from LAI.

It is possible that LAI is readily released from low-density cells in leukemia leading to suppression of the proliferation rate of normal marrow CFU-c. By suppression of normal granulopoiesis LAI may give a growth advantage to the leukemic cells and explain why normal granulopoiesis eventually fails. Final purification of LAI is now under way and may, together with cell separation studies of the LAI-producing cells, allow us to define the pathophysiological role of LAI in leukemia.

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