Inhibitor of Hematopoietic Cell Proliferation Derived From a Human Leukemic Cell Line

By Tor Olofsson and Martin J. Cline

A continuously growing human myeloid leukemia cell line (K562) produced a potent high-molecular-weight inhibitor of hematopoietic cell proliferation. It was most active against myeloid stem cells (CFU-C) and proliferating T lymphocytes; it was less active against erythroid precursors (CFU-E) and did not inhibit fibroblasts or established lines of epitheloid cells or B lymphocytes. Inhibition of CFU-C was by direct interaction rather than by modulation of production of colony-stimulating activity and probably occurred at restricted points in the cell cycle. Inhibition could, within limits, be reversed by washing the target cells. Production of inhibitors of hematopoiesis is not a general property of established cell lines, and only two have thus far been identified in screening of 30 such lines.

It is a prevailing concept that granulopoiesis is regulated by positive and negative feedback loops. Colony-stimulating factors (CSF) derived from various sources have been shown to play a central role in granulopoietic growth and differentiation, at least when studied in vitro. With the recently reported purification of one CSF, the opportunities for ascertaining the role of these putative hormones in the controls of granulopoiesis in vivo have substantially increased.

All of the CSF preparations so far described are proteins or glycoproteins covering a wide range of molecular weights. The reported diversity of the potential inhibitors of granulopoiesis is even greater. Serum lipoproteins have been shown to inhibit colony formation in vitro and may possibly bear a relationship to granulopoiesis in vivo. Products of mature granulocytes are reported to inhibit growth in vitro of granulocytic progenitor cells, and low molecular weight (LMW) products from granulocytes have been claimed to fulfill the criteria for a granulocytic chalone, although the role of these chalone-like substances has recently been questioned. Neutrophil products may also inhibit colony-stimulating cells and thereby have a modulatory effect on granulopoiesis in vitro. These different inhibitors have been studied primarily for their relationship to normal granulopoiesis, and their possible role in growth regulation in leukemia is relatively unexplored.

Some reports in the literature have described leukemic cells as producing inhibitors of normal marrow growth in vitro. It is quite possible that some of these as yet poorly defined substances may be related to the inhibition of normal hematopoiesis seen in myeloid leukemia. We reasoned that established leukemic
cell lines might provide sources for this type of inhibitor and prove useful in attempts to confirm or deny a pathogenetic role for leukemic inhibitors. We describe here the biologic effects on hematopoietic cells of a potent high molecular weight (HMW) inhibitor produced by a Ph1 chromosome-positive cell line (K562) established from a patient with chronic myeloid leukemia (CML) in blastic crisis.14

MATERIALS AND METHODS

Leukemic cell line K562 was grown in suspension culture in modified McCoy's medium containing 20% fetal calf serum (FCS) and was subcultured every fifth day. For some experiments the cells were grown in McCoy's medium supplemented with 0.3% bovine serum albumin (BSA) (Sigma Chemical, Saint Louis, Mo.). The cells appeared as undifferentiated blast cells of varying size and showed no maturation products with conventional cytochemical stains. Karyotypic analysis showed a single Ph1 chromosome and a marked aneuploidy with 61-70 chromosomes, with losses and gains of normal chromosomes as well as several marker chromosomes. The cells were shown to be free of mycoplasma. Under the culture conditions used the final cell concentration was 5-6 x 10^6/ml after 5-6 days in culture. The cell-free supernatant fluid (K562-sup) from 5-day-old cultures was collected by centrifugation (1,000 g, 10 mm) and stored at -20°C until used in the inhibition assays described below.

CFU-C assay. Human marrow myeloid progenitors (CFU-C) from bone marrow were cultured in agar essentially as described previously.15 Peripheral blood leukocytes (WBC) at a concentration of 1 x 10^6/ml in 0.5% agar in McCoy's medium (20% FCS) were used to stimulate CFU-C growth. Normal human bone marrow was obtained from the posterior iliac crest of healthy volunteers, processed into a single cell suspension, and separated on Hypaque-Ficoll16 to remove mature granulocytes and erythrocytes. The mononuclear cell fraction was plated in 0.3% agar medium at a concentration of 1-2 x 10^5/ml on top of WBC underlayers. Colonies of more than 40 cells were counted on the tenth day of incubation. For some experiments, CSF in conditioned medium from human blood mononuclear cells15 was used instead of WBC underlayers.

Inhibitor was tested by mixing 100 μl of K562-sup at different dilutions to bone marrow overlayers and scoring CFU-C at 10 days. Agar plates were also scanned on days 7 and 14. Tests were run with three or four replicates.

The fraction of CFU-C in S phase was measured by the 3H-thymidine (3H-TdR, 17 Ci/mmole; New England Nuclear, Boston, Mass.) suicide technique as previously described.17 To assay the effects of K562-sup on this kinetic parameter, the cells were preincubated for 30 min with K562-sup before the isotope was added for the next 30 min.

Mouse CFU-C was cultured as described,18 and the effects of K562-sup were tested as described for human CFU-C. Colonies were scored on day 7.

CFU-E assay. Erythropoietin-responsive erythroid precursor cells (CFU-E) of human marrow were cultured in methylcellulose containing alpha medium (20% FCS) with 1.0 IU of human urinary erythropoietin (from the National Heart, Lung and Blood Institute) per ml.19 Erythroid colonies of eight or more cells were counted on day 7. Murine CFU-E were similarly assayed and colonies counted on day 2. In both cases inhibition by K562-sup was assayed as described for CFU-C.

1H-TdR incorporation in marrow cells. The mononuclear cell fraction of human marrow cells (2 x 10^6/ml) was incubated with K562-sup (5%-10%) for 60 and 180 min at 37°C before 5 μCi 1H-TdR/ml (6.7 Ci/mmole) was added. After another 60 min the incubation was stopped by adding cold phosphate-buffered saline (PBS); the cells were collected by centrifugation and precipitated with ice-cold 7.5% trichloroacetic acid (TCA). The precipitate was washed once in TCA and dissolved in tissue solubilizer (NCS, Amersham/Searle, Arlington Heights, Ill.) and diluted with scintillation fluid (4% Spectrafluor in toluene, Amersham/Searle), and the radioactivity was counted in a Beckman scintillation spectrometer.

Lymphocyte cultures. Heparinized peripheral blood was separated on Hypaque-Ficoll. The mononuclear cells (more than 75% lymphocytes) were used for phytohemagglutinin (PHA)-stimulated lymphocyte cultures. 3 x 10^6 lymphocytes in 3 ml McCoy's medium 20% FCS were incubated with 100 μl PHA-P (Difco, Detroit, Mich.) diluted 1:40. At 72 hr of incubation 100
μl $^3$H-TdR was added (10 μCi/ml, 6.7 Ci/m mole), and the cells were harvested 16 hr later and processed as described for marrow cells. K562-sup was added to the cultures at different times during the incubation period. Tests were run with three to five replicates. In some experiments only the nonadherent cells were used. The mononuclear cells (1 x 10$^7$/ml) suspended in McCoy's medium and 20% FCS were left to adhere to plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.) at 37°C for 4-5 hr before we collected the nonadherent cells.

In other experiments with PHA-stimulated lymphocytes, the incorporation of $^3$H-uridine (New England Nuclear, 20 Ci/m mole) or $^3$H-amino acids (NEN, 1.0 mCi/ml) was studied. $^3$H-Uridine was added at 72 hr (100 μl, 10 μCi/ml) and $^3$H-amino acids were present from the beginning of the culture (100 μl, 10 μCi/ml). Cells were harvested and processed as described above.

Other target cells. K562-sup was tested for cytotoxic activity against several different established human hematopoietic and nonhematopoietic cell lines as well as freshly isolated human skin fibroblasts. The cell lines were 253J, T24, and J82 (bladder carcinoma), LA109 (B lymphocytic), and an acute myeloid leukemia line established within our laboratory. K562-sup was added at a concentration of 10% and viable cell counts established by trypan blue exclusion at 24, 48, and 72 hr of incubation.

RESULTS

Culture conditions for K562. Although the growth of K562 cells was not totally dependent on the presence of FCS in the medium, the production or stability of inhibitory activity was related to the concentration of FCS. When the concentration was decreased from 20% to 10%, 5%, or 2.5%, inhibitory activity decreased slightly in a dose-dependent fashion, whereas cell growth was almost unaffected. The percentage inhibition of colony formation by 3 μl of K562-sup grown under various conditions was as follows: 20% FCS, 89% ± 6%; 10% FCS, 77% ± 4%; 5% FCS, 69% ± 7%; 2.5% FCS, 62% ± 8%. Cells were also cultured in serum-free medium in the presence of 0.3% bovine serum albumin. Under these circumstances, however, both cell growth and the amount of inhibitor produced were diminished (Fig. 1). In addition, inhibitory activity was unstable in this medium and was lost during storage.

Inhibition of CFU-C. When increasing amounts of K562-sup were added to CFU-C cultures, colony formation was inhibited in a dose-dependent fashion (Fig. 1). Although data are supplied only for colonies incubated for 10 days, similar results were obtained at days 7 and 14. To exclude the possibility that K562-sup inhibited the formation of colony-stimulating activity from the underlayers, experiments were performed using preformed CSF in monocyte-con...
conditioned medium. Under these circumstances colony formation was inhibited in the same fashion as when leukocyte underlayers were used as the source of the stimulus. Furthermore, in two experiments it was shown that conditioned medium from monocytes cultured in the presence of K562-sup had the same intrinsic stimulatory activity as medium from monocytes cultured without inhibitor. Thus monocyte-conditioned medium alone gave rise to 214 ± 13 (SE) colonies/10^5 cells, and medium from monocytes grown with K562-sup resulted in 126 ± 7/10^3 cells. This latter activity was no different from that obtained with monocyte-conditioned medium supplemented at the termination of culture with the same volume of K562-sup (131 ± 6 colonies/10^5 cells).

To further characterize the inhibitory action of K562-sup, human marrow CFU-C were stimulated by increasing amounts of monocyte-conditioned medium with the addition of a constant amount of K562-sup (Fig. 2A). It was found that the curves followed the same kinetics as some enzymatic reactions and formed straight lines in reciprocal plots (Fig. 2B). The apparent $K_m$ value for stimulation by CSF was not altered by the addition of inhibitor.

When increasing amounts of bone marrow cells were plated in the presence of a constant amount of K562-sup, the fraction of CFU-C inhibited was the same for all cell concentrations (data not shown). Maximal inhibitory action was reached within 30–60 min of exposure to K562-sup (Fig. 3). Cell viability estimated by trypan blue dye exclusion was unaffected even if marrow cells were incubated with K562-sup for 24 hr. During that period of exposure to K562-sup the CFU-C were completely inhibited, whereas the controls showed 70%–80% recovery of CFU-C.

In two experiments using the ³H-thymidine suicide technique it was shown
Fig. 3. Inhibition of CFU-C by K562-sup after different preincubation time. Bone marrow cells were incubated with 5% K562-sup for 30-120 min at 37°C, washed twice, and then plated in agar. Control cells without inhibitor were treated identically. Means ± SE of four individual experiments.

that K562-sup did not alter the fraction of CFU-C in S phase. Marrow cells were incubated with or without K562-sup and then briefly exposed to lethal doses of 3H-TdR. A concentration of K562-sup was selected that produced partial suppression of CFU-C (Fig. 1). High specific activity 3H-TdR reduced CFU-C in both control and K562-treated cells. The proportional reduction in CFU-C was the same for both treated and control groups. The values for CFU-C in S phase were as follows: exp. 1—control, 51%; K562, 51%; exp. 2—control, 43%; K562, 38%. In partial confirmation of these observations, 3H-thymidine incorporation in human marrow cells was unaffected during short-term exposure to K562-sup (60 min: 5.5% ± 5.5% inhibition, n = 3) but showed variable inhibition if the cells were exposed for longer time periods (180 min: 21.7% ± 13.8%, n = 3). These observations and the use of high molecular weight preparations of K562-sup (>100,000) eliminated the possibility that the previously observed effects were due to cold thymidine in the K562-sup.

The effect of K562-sup showed some degree of species and cell line specificity. K562-sup was less inhibitory against mouse CFU-C than against human CFU-C (Table 1). K562-sup also inhibited erythropoietin-stimulated cloning of human CFU-E in methylcellulose, but the inhibition was less pronounced than for CFU-C and did not reach total inhibition (Table 1). The cloning of mouse CFU-E was only slightly affected (Table 1).

**Inhibition of PHA-stimulated lymphocytes.** During studies of target cell specificity for K562-sup it was found that PHA-stimulated 3H-thymidine incorporation in lymphocytes was markedly decreased with K562-sup. As with CFU-C

<table>
<thead>
<tr>
<th>Final K562-sup Dilution</th>
<th>Human Inhibition (%)</th>
<th>Mouse Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU-C</td>
<td>CFU-E</td>
</tr>
<tr>
<td>1:10</td>
<td>100</td>
<td>64</td>
</tr>
<tr>
<td>1:20</td>
<td>100</td>
<td>58</td>
</tr>
<tr>
<td>1:40</td>
<td>—</td>
<td>15</td>
</tr>
<tr>
<td>1:100</td>
<td>98</td>
<td>6</td>
</tr>
<tr>
<td>1:200</td>
<td>94</td>
<td>—</td>
</tr>
</tbody>
</table>
and CFU-E, inhibition was dose dependent. Figure 4 shows that inhibition was total if the inhibitor was present during the first 24 hr of incubation, and inhibitory effect decreased with later additions to the lymphocyte cultures. If the inhibitor was added together with the isotope at 72 hr of culture, there was almost no inhibition. If lymphocytes were preincubated with K562-sup for 3 hr and then washed before addition of PHA, no significant inhibition of thymidine incorporation was detected. Cell viability was unaltered by K562-sup throughout the culture period and the agglutination seemed unaffected, whereas a reduction of the number of blast-transformed cells could be identified microscopically.

If a monocyte-depleted population was used instead of a mixture of lymphocytes and monocytes, the results were virtually the same. K562-sup also inhibited RNA and protein synthesis in a time-dependent fashion similar to the effect on $^3$H-TdR incorporation (data not shown).

Preliminary experiments were also performed to see if K562-sup inhibited the entry of cells into mitosis. PHA-stimulated lymphocytes of 72-hr cultures were incubated with colcemid 0.1 $\mu$g/ml ± K562-sup, and the frequency of mitoses

Table 2. Effects of K562-sup on Cell Viability of Established Human Cell Lines and Fibroblasts (Control/+ K562-sup)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Viable Cell Counts (x 10^{-6}/ml)</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>253J</td>
<td></td>
<td>2.34/3.33</td>
<td>4.26/3.97</td>
<td>4.77/5.36</td>
</tr>
<tr>
<td>T24</td>
<td></td>
<td>3.59/3.31</td>
<td>2.93/2.62</td>
<td>2.78/2.32</td>
</tr>
<tr>
<td>JB2</td>
<td></td>
<td>1.42/1.39</td>
<td>1.28/1.36</td>
<td>2.20/3.17</td>
</tr>
<tr>
<td>KG</td>
<td></td>
<td>11.38/10.84</td>
<td>13.81/14.40</td>
<td>16.50/14.44</td>
</tr>
<tr>
<td>LA109</td>
<td></td>
<td>5.10/4.12</td>
<td>5.84/4.12</td>
<td>6.40/6.40</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td></td>
<td>0.55/0.55</td>
<td>0.61/0.62</td>
<td>0.71/0.76</td>
</tr>
</tbody>
</table>

253J, T24, and JB2 are established epithelioid cell lines; LA109 is a B cell line; KG is an acute myeloid leukemia cell line.
was followed over the next 8 hr. No differences were detected between controls and inhibitor-treated cells in two experiments.

Tests of other target cells. When K562-sup was added to different established cell lines at a concentration that completely blocked CFU-C growth and lymphocyte proliferation, no inhibition of viable cell counts over 3 days were found among the cell lines tested (Table 2). This was also true for normal human skin fibroblasts in the second passage and for a myeloid leukemia line (KG) growing as colonies in agar (data not shown).

DISCUSSION

Current concepts of the regulation of granulopoiesis suggest a role for inhibitors in the modulation of cell growth within the marrow to achieve the finely tuned balance between production and destruction of cells and to meet the demands when the system is perturbed by disease or artificial means. A number of inhibitors of granulopoiesis have been suggested as candidates for normal physiologic regulators.4'5'7'8 It is not within the scope of this report to discuss these tentative regulators in any detail; suffice it to say that their role in normal granulopoiesis in vivo remains to be established.

The suggested role of inhibitors in the myeloid leukemias is even more complex. For instance, the question of how the suppression of normal hematopoietic cell growth is mediated in leukemia is still enigmatic, and the common belief that leukemic cell products play a role has not been substantiated. Cellular interaction between normal and leukemic marrow cells sometimes gives rise to inhibition of colony growth when tested in agar culture11'13 but at other times does not.5,20

A major obstacle in the studies of inhibitors derived from leukemic cells is the difficulty in obtaining active material in sufficient amount to allow biochemical characterization. We therefore investigated the possibility that established human hematopoietic cell lines produce inhibitors active against normal hematopoietic cell proliferation. Such cell lines might provide rich sources for the study and definition of inhibitory materials. We found it a rare phenomenon that established cell lines produced inhibitors of granulopoiesis in vitro. Only 2 of 30 tested hematopoietic and nonhematopoietic cell lines produced inhibitory activity, one of which was K562 and the other a human lung carcinoma cell line.21 K562 was originally isolated from a pleural effusion from a patient with chronic myeloid leukemia in blastic crisis14 and has been thoroughly characterized. Morphologically it consists of undifferentiated blastlike cells devoid of granules and other signs of granulocytic maturation. Nevertheless, there is substantial evidence that it is myeloid in origin. It retains the Ph1 chromosome and shares surface antigens with mature human granulocytes.22 Furthermore, the cells do not produce immunoglobulins, are free of Epstein-Barr virus and herpes like virus particles,14 and show no terminal deoxynucleotidyl transferase or reverse transcriptase activities.23 Thus there is no evidence that it is lymphoid in origin. This is of importance when judging the possible biologic significance of the K562 inhibitor.

Early during the investigation the question was raised as to whether the inhibitor present in K562-sup was a product released from the cells or an altered FCS protein. The latter possibility was ruled out by the fact that cells growing
in FCS-free medium still produced the inhibitor. The inhibitor concentrations in FCS-free medium were lower than in regular medium, but that can be ascribed to the lower cellular density obtained in medium devoid of FCS. In addition, the inhibitory activity is less stable in the absence of FCS. The inhibitory substance present in K562-sup was stable at \(-20^\circ\text{C}\) for more than 6 mo and could be repeatedly frozen and thawed without loss of activity as long as FCS was present. It was heat labile at 56°C for 30 min. Inhibitory activity was lost if K562-sup was subjected to ether or chloroform extraction and could not be recovered in the solvent residue. It was also lost on Millipore filtration. The active material had an affinity for Sephadex and could not be readily eluted. Ultrafiltration experiments showed retention of the inhibitor by Amicon XM300 filters, indicating an apparent molecular weight greater than 300,000 daltons. It did not adsorb to the Amicon filters to any great extent.

The inhibition of CFU-C by K562-sup is dose dependent and detectable at less than 10 \(\mu\)l/culture plate. When the cell concentration in the K562 cultures was analyzed at the time of harvest of the supernatant, this inhibitory activity corresponded to that derived from less than 3000 cells.

It is important to question whether the inhibition of CFU-C growth by K562-sup is a direct effect on CFU-C or is mediated via interaction with other cells. The possibility that K562-sup simply exerts its effects by inhibiting CSF production was ruled out by experiments showing unaltered CSF production from blood mononuclear cells in the presence of K562-sup. Using preformed CSF instead of WBC underlayers resulted in equal inhibition by K562-sup. These experiments clearly indicate that K562-sup inhibits the effects of CSF rather than its production. The limited kinetic approach to the interaction between CSA and inhibitor on the target cell (CFU-C) that can be achieved with the crude K562-sup available thus far shows that the apparent \(K_m\) value for the interaction between CSF and CFU-C is unaffected by K562-sup. Inasmuch as the \(K_m\) value reveals anything about the affinity of CSF/CFU-C interaction, the data are consistent with the interpretation that the inhibitor probably acts on the CFU-C directly via a mechanism or site different from that mediating the effect of CSF. However, the actual interplay between inhibitor and target cells cannot be conclusively studied until the inhibitor has been sufficiently purified.

The specific phase of the cell cycle affected by K562-sup is unknown, but failure to alter the fraction of CFU-C in S phase rules out that inhibitor specifically blocks DNA synthesis in these cells. Other studies suggest that the inhibitory effect is not mediated by inhibiting entry of cells into mitosis.

The question of target cell specificity is central in judging the biologic significance of inhibitors but is sometimes misinterpreted when it is suggested that only inhibitors with a single cell lineage specificity are of potential biologic significance. That may be true for physiologic inhibitors engaged in the control of
normal cell production but is probably irrelevant as an argument in judging inhibitors of neoplastic origin. It is also important in this context to compare dose-response relationships. K562-sup inhibits cloning not only of CFU-C but also of CFU-E; however, the dose responses are different, so that at low concentrations, where inhibition of CFU-E is minimal, inhibition of CFU-C is profound. The murine counterparts of these stem cells are also slightly inhibited. When K562-sup was tested against human fibroblasts and some established cell lines, no inhibitory activity against these cells could be detected. Therefore we provisionally conclude that the inhibitor is restricted to hematopoietic cells, including lymphocytes.

The inhibition of PHA-stimulated lymphocytes by K562 is an intriguing observation that may prove valuable in defining the site or mechanism of interaction for the inhibitor. So far, experiments have shown inhibition of DNA, RNA, and protein synthesis, but it cannot yet be determined which is the primary event, since all three parameters are interrelated. It is of interest to note that the inhibitor must be present during the earliest stages of culture to show full effect. K562-sup does not directly interact with DNA synthesis, as shown by the failure of late addition of the inhibitor to block 3H-thymidine incorporation. If the lymphocytes are preincubated with K562-sup for 3 hr, washed, and then incubated with PHA, hardly any inhibition of 3H-thymidine incorporation is noted. This suggests that unlike the case of CFU-C the inhibitor is not sufficiently bound to the target cells to allow effective inhibition once the inhibitor is removed from the medium. Preliminary experiments to adsorb the inhibitor to lymphocytes accordingly failed.

ACKNOWLEDGMENT
We are indebted to Carol Le Fèvre for outstanding technical assistance and to Noelle Bersch for performing the CFU-E assays, and to Dr. David Golde for his many helpful suggestions.

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