Differential Protection of Normal and Malignant Human Myeloid Progenitors (CFU-GM) From Ara-C Toxicity Using Cycloheximide

By Christopher A. Slapak, Robert L. Fine, and Carol M. Richman

Cycloheximide, a reversible protein synthesis inhibitor, is thought to block DNA replication in normal cells by preventing synthesis of a labile protein. In animal systems, cycloheximide protects normal cells from cytotoxic S-phase specific agents, such as cytosine arabinoside (Ara-C). Malignant cells appear not to be susceptible to cycloheximide-induced cycle arrest and, subsequently, may not be protected from Ara-C cytotoxicity. The effect of cycloheximide on granulocyte/macrophage progenitors (CFU-GM) after in vitro Ara-C exposure was examined using normal human bone marrow, malignant progenitors from patients with chronic myelogenous leukemia (CML), and clonogenic cells from the human acute nonlymphocytic leukemia cell lines HL-60 and KG-1. Mononuclear or clonogenic cells were incubated for one hour with cycloheximide, followed by the addition, for three or 17 hours, of Ara-C before being plated in a methylcellulose culture system. CFU-GM survival was significantly increased if normal cells were treated with cycloheximide before Ara-C exposure. Similar cycloheximide pretreatment of CML progenitors and clonogenic HL-60 and KG-1 cells failed to protect CFU-GM from Ara-C-induced cytotoxicity.

EFFECTIVE TREATMENT of nonlymphocytic leukemia is limited by the lack of cytotoxic drug specificity for neoplastic hematopoietic cells. Chemotherapy destroys normal as well as malignant progenitors, leading to severe myelosuppression. Attempts to improve the therapeutic index of drugs used in the treatment of acute leukemia by manipulating cell cycle kinetics (e.g., cell synchronization or recruitment) have not been consistently more effective than empirically designed treatment protocols.

Cycloheximide and other reversible protein synthesis inhibitors have been shown in animal systems to protect normal epithelial and bone marrow cells from S-phase specific cytotoxic agents, such as cytosine arabinoside (Ara-C). In normal cells, synthesis of a labile protein is necessary for DNA synthesis to proceed. Inhibition of protein synthesis prevents DNA synthesis and therefore diminishes Ara-C toxicity. In transformed cells, DNA replication appears to be independent of protein synthesis. Thus, cycloheximide could potentially affect a differential protection of normal vs. malignant cells from phase-specific cytotoxic drugs. To evaluate this hypothesis in a human myelopoietic system, we examined the effect of cycloheximide on the in vitro cytotoxicity of Ara-C on normal granulocyte/macrophage progenitors (CFU-GM), on malignant progenitors from patients with chronic myelogenous leukemia (CML), and on clonogenic cells from human acute nonlymphocytic leukemia cell lines HL-60 and KG-1.

MATERIALS AND METHODS

Normal bone marrow samples were collected from paid adult volunteers after obtaining written informed consent. Peripheral blood samples from patients with Philadelphia chromosome-positive CML in the stable chronic phase were obtained during routine clinical evaluations. All patients were untreated at the time of study. Heparinized bone marrow aspirates were separated on Ficoll-Hypaque density gradients (S.G. 1.077) to obtain mononuclear cells for study. In patients with CML, fresh (two patients) or cryopreserved (seven patients) mononuclear cells obtained by leukapheresis were used instead of Ficoll-Hypaque-separated cells. The cryopreserved cells had been frozen in 10% dimethylsulfoxide and 20% autologous plasma using a controlled rate of -1°C/minute and were stored in the low vapor phase of liquid nitrogen (approximately -150°C). Vials of cryopreserved cells were thawed rapidly at 37°C and diluted tenfold over ten minutes and then rapidly diluted to the appropriate cell concentration before drug incubation. HL-60 and KG-1 cells were maintained in suspension culture using RPMI 1640 medium containing 15% heat-inactivated fetal calf serum (Flow Laboratories, Bethesda, Md) and were routinely subcultured every seven days. To ensure log phase growth, cells were also subcultured 72 hours before each study.

Cycloheximide (Sigma Chemical Co, St Louis) was incubated with mononuclear cell suspensions at a final concentration of 1 or 10 μg/mL. Ara-C (Upjohn Co, Kalamazoo, Mich) was added to the cell suspensions one hour after the addition of cycloheximide in final Ara-C concentrations of 1 or 10 μg/mL. Fresh solutions of cycloheximide and Ara-C were prepared for each experiment.

Mononuclear cells (10^6/mL) from normals, patients with CML, HL-60, or KG-1 cells were incubated at 37°C, 10% CO2/air for four to 18 hours in Alpha medium (Flow) containing 20% fetal calf serum. Four sets of cell suspensions were prepared for each incubation time: medium alone, cycloheximide alone, Ara-C alone (three or 17 hours), and one hour of cycloheximide, followed by incubation with Ara-C for three or 17 hours. In all experiments, cycloheximide remained in the suspension culture during Ara-C exposure. In three experiments, cycloheximide and Ara-C were added simultaneously for a three-hour incubation. After incubation, each cell suspension was washed three times and plated in quadruplicate using a methylcellulose culture system at final concentrations of 1 x 10^5 cells per milliliter for normal bone marrow, 1 to 2 x 10^5/mL for CML samples, 2 x 10^5/mL for HL-60, and 2 x 10^5/mL for KG-1 cells. Phytohemagglutinin-stimulated leukocyte conditioned medium was the source of colony-stimulating activity. After 14 days of incubation at 37°C in a 10% humidified CO2 atmosphere, colonies of more than 20 cells (representing CFU-GM) were counted using an inverted microscope at 50× magnification. Wright-stained cytocentrifuge preparations of individual colonies from normals reveal maturing granulocytes or macrophages or both using this culture technique. All results were expressed as a percentage of controls incubated without Ara-C or cycloheximide. The mean ± SE of the
Cycloheximide Protects CFU-GM from Ara-C

RESULTS

Normal bone marrow. Exposure of normal bone marrow mononuclear cells to cycloheximide for one hour before treatment with Ara-C significantly increased the percentage of surviving CFU-GM (Table 1). Exposure to 1 µg/mL of Ara-C for three hours resulted in less than 50% colony survival, which increased if cells were pretreated with cycloheximide at 1 µg/mL and significantly increased with 10 µg/mL (P < .02). Similarly, toxicity from a higher-dose Ara-C exposure (10 µg/mL) could beameliorated by one hour of pretreatment with cycloheximide at 1 µg/mL (P < .01) or 10 µg/mL (P < .001). Cycloheximide itself was only minimally toxic to CFU-GM at this duration of exposure.

When Ara-C exposure was increased from three hours to 17 hours, significant protection was still afforded if cells were treated with cycloheximide for one hour before addition of Ara-C. However, with the increased exposure time, cycloheximide itself was toxic at the higher concentration. Ara-C cytotoxicity at 1 µg/mL was reduced with significantly increased colony survival when cells were preexposed to cycloheximide at 1 µg/mL (P < .005). Colony survival after a 17-hour, 10 µg/mL Ara-C incubation could be increased with cycloheximide at both 1 µg/mL (P < .01) or 10 µg/mL (P < .05).

In three experiments, increasing cycloheximide preexposure from one hour to six hours resulted in no additional protective effect. Simultaneous addition of cycloheximide and Ara-C failed to yield any protective effect. In cultures with 1 or 10 µg/mL Ara-C and no cycloheximide, the percentage of CFU-GM survivals were 45% ± 7% and 12% ± 6%, respectively. In three experiments, the simultaneous addition of 1 µg/mL cycloheximide to the Ara-C containing cell suspensions resulted in CFU-GM survival for 1 and 10 µg/mL Ara-C of 35% ± 5% and 9% ± 6%. Using 10 µg/mL cycloheximide, the mean CFU-GM survivals were 32% ± 6% (P < .05) and 6% ± 4%, respectively.

Chronic myelogenous leukemia. Cycloheximide pretreatment of mononuclear cells from nine patients with CML failed to protect CFU-GM from Ara-C–induced cytotoxicity (Table 2). A three-hour Ara-C exposure at 1 µg/mL yielded 26% ± 50% colony survival, which was not increased, but was, on the contrary, significantly decreased when cells were pretreated for one hour with cycloheximide at 1 or 10 µg/mL. With a higher Ara-C dose (10 µg/mL), preexposure to cycloheximide was minimally toxic at 1 µg/mL but was toxic at 10 µg/mL.

Acute leukemia cell lines HL-60 and KG-1. Pretreatment of HL-60 cells for one hour with cycloheximide failed to protect clonogenic cells from toxicity induced by a three-hour exposure to Ara-C (Table 3). In five experiments, cycloheximide alone at 1 µg/mL was nontoxic (mean CFU-GM survival 91% ± 11%). As in the experiments using CML cells, cycloheximide decreased colony survival at both 1 and 10 µg/mL Ara-C.

Increasing exposure to Ara-C to 17 hours decreased the percentage of CFU-GM survival. Again, however, no protec-

### Table 1. Effect of Preincubation in Cycloheximide on Survival of Normal Bone Marrow CFU-GM Exposed to Ara-C

<table>
<thead>
<tr>
<th></th>
<th>Cycloheximide (µg/mL)</th>
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<tbody>
<tr>
<td>Ara-C (µg/mL)</td>
<td>0</td>
</tr>
<tr>
<td>3-hour Ara-C</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>10</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>17-hour Ara-C</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>10</td>
<td>4 ± 1</td>
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</tbody>
</table>

*Mean CFU-GM survival expressed as percentage of the CFU-GM observed in the absence of Ara-C or cycloheximide (± SE of the mean). Six normal bone marrow samples were studied for three-hour and for 17-hour Ara-C exposure times.

†CFU-GM survival with cycloheximide and Ara-C significantly increased compared to Ara-C alone (P < .02).

‡See †, P < .01.

§See †, P < .001.

¶See †, P < .005.

‖See †, P < .05.

### Table 2. Effect of Preincubation in Cycloheximide on Survival of CFU-GM from CML Peripheral Blood Exposed to Ara-C for Three Hours

<table>
<thead>
<tr>
<th>Ara-C (µg/mL)</th>
<th>Cycloheximide (µg/mL)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>10</td>
<td>12 ± 3</td>
</tr>
</tbody>
</table>

Samples from seven of the nine patients studied had been cryopreserved.

*Mean CFU-GM survival expressed as percentage of the CFU-GM observed in the absence of Ara-C or cycloheximide (± SE of the mean).

†CFU-GM survival with cycloheximide and Ara-C significantly decreased compared with Ara-C alone (P < .02).

‡See †, P < .001.

### Table 3. Effect of Preincubation in Cycloheximide on Survival of HL-60 CFU-GM Exposed to Ara-C

<table>
<thead>
<tr>
<th>Ara-C (µg/mL)</th>
<th>Cycloheximide (µg/mL)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>3-hour Ara-C</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>62 ± 4</td>
</tr>
<tr>
<td>10</td>
<td>57 ± 11</td>
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<tr>
<td>17-hour Ara-C</td>
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<td>1</td>
<td>47 ± 3</td>
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<tr>
<td>10</td>
<td>29 ± 2</td>
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</tbody>
</table>

*Mean CFU-GM survival for five experiments expressed as percentage of the CFU-GM observed in the absence of Ara-C or cycloheximide (± SE of the mean).

†CFU-GM survival with cycloheximide and Ara-C significantly decreased compared with Ara-C alone (P < .05).

‡See †, P < .002.

§See †, P < .02.
tive effect of cycloheximide was observed, and cycloheximide cytotoxicity appeared additive with Ara-C. A comparison of CFU-GM survival results with three-hour Ara-C incubation for normal bone marrow, CML blood, and HL-60 cells is shown in Fig 1.

In two experiments with KG-1 cells using a three-hour Ara-C exposure, cycloheximide at 1 μg/mL (nontoxic, CFU-C survival 105%) did not protect CFU-GM. Survival of CFU-GM was 20% with 1 μg/mL Ara-C alone and 17% with preexposure to cycloheximide; 17% with 10 μg/mL Ara-C alone and 13% with cycloheximide preexposure.

**DISCUSSION**

The data presented here demonstrate that cycloheximide pretreatment reduces Ara-C toxicity on normal human bone marrow CFU-GM, in some instances by twofold to threefold. Similar cycloheximide treatment of CML or acute nonlymphocytic leukemia cell lines failed to increase colony survival and in fact enhanced the toxicity of Ara-C. These results suggest that cycloheximide might improve the therapeutic index of Ara-C. While previous studies have shown that protein synthesis inhibitors protect rat intestinal crypt cells, rat bone marrow, and Chinese hamster ovary cells from cytotoxic agents, this study is, to our knowledge, the first to demonstrate such an effect on human hematopoietic cells and the first to show cycloheximide’s differential protection of normal vs. malignant cells from a cytotoxic agent in a human cell culture system.

The mechanism of action of cycloheximide in affecting Ara-C cytotoxicity has not been specifically evaluated in our study but could be ascribed to its cell cycle effect. In normal cells, initiation of DNA synthesis is preceded by synthesis of a labile protein. Treatment with cycloheximide arrests cells, initiation of DNA synthesis is preceded by synthesis of this labile protein, perhaps because the labile protein is not necessary, acquires increased stability, or is synthesized at an increased rate. Baseline differences between normal and malignant myeloid progenitors in the kinetics of cell proliferation or in drug metabolism may play a role in the differential protection of normal cells observed. However, it is likely that pretreatment of normal cells with cycloheximide arrests the cells in G1, protecting them from S-phase specific agents, while malignant cells may not be arrested and proceed from G1 to S with subsequent susceptibility to Ara-C–induced destruction.

L-Asparaginase, a protein synthesis-inhibiting enzyme that is useful in treating acute lymphoblastic leukemia, has been shown to exhibit schedule-dependent synergy with Ara-C on L5178Y murine leukemia when Ara-C treatment is followed by asparaginase. Pretreatment of cells with asparaginase antagonizes the antileukemic effect of Ara-C. Our results, with a different protein synthesis inhibitor, using an in vitro human system failed to show this type of antagonism. Whether Ara-C treatment followed by cycloheximide exhibits schedule-dependent synergy in our system is unknown, although our results indicate that simultaneous addition of Ara-C and cycloheximide is no different than Ara-C alone.

In animal systems, cycloheximide protection of normal cells has also been described with other S-phase specific agents, as well as drugs that do not work exclusively in the S-phase, suggesting that cycloheximide may be useful in combination with several active antineoplastic drugs as well as with irradiation. If cycloheximide preferentially protected normal hematopoietic cells from alkylating agents, such as cyclophosphamide, it might prove useful in preparing remission bone marrow for autologous transfusion in acute leukemia. At present, the options for “purging” residual malignant cells from harvested autologous marrow in vitro include monoclonal antibody treatment and exposure to cytotoxic drugs, such as the cyclophosphamide analog 4-hydroperoxycyclophosphamide (4-HC), which has been shown (in an animal leukemia model) to have some specificity for malignant progenitors. Cycloheximide might improve the selective removal of malignant cells with 4-HC, Ara-C, or other agents.

The results of this study may have direct clinical application, since both cycloheximide and Ara-C have been used clinically in doses expected or documented to produce plasma levels approximating the 1 and 10 μg/mL used in vitro. In addition, intravenous cycloheximide had limited toxicity when evaluated as an antipyretic agent in Hodgkin’s disease. Our data show that short-term exposure to cycloheximide is minimally toxic to normal CFU-GM and protects these normal progenitors from the cytotoxicity of Ara-C, one of the most active drugs used in the treatment of myeloid leukemias. Whether cycloheximide can also protect the
pluripotent stem cell cannot be fully determined with current techniques. However, in autologous bone marrow transplantation, a direct correlation has been documented between total CFU-GM infused and rapidity of granulocyte recovery, suggesting that protection of CFU-GM could at least enhance granulocyte recovery. Successful treatment of leukemia in vivo requires complete eradication of malignant clones with survival of sufficient normal stem cells to repopulate the hematopoietic system. The present study suggests that combination therapy with cycloheximide and Ara-C might preferentially destroy the abnormal clones and decrease the degree of myelosuppression associated with intensive antileukemia therapy.

ACKNOWLEDGMENT

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