The Effects of Acetaldehydephosphamide, a Novel Stable Aldophosphamide Analogue, on Normal Human and Leukemic Progenitor Cells in Vitro: Implications for Use in Bone Marrow Purging

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ABSTRACT

Acetaldehydephosphamide (A-ALD), a novel in vitro active and stable derivative of aldophosphamide, kills human bone marrow-derived granulocytic-macrophage colony-forming cells (GM-CFC) independent of the cell cycle. The surviving fraction of GM-CFC is an exponential function of the drug concentration and time of exposure. Variation of marrow light-density cell concentration between 2 x 10^4 and 10 x 10^4/ml does not significantly influence its GM-CFC toxicity.

Marrow depleted of GM-CFC by A-ALD subsequently generates GM-CFC when grown in suspension cultures. During the early period after treatment with A-ALD the number of surviving GM-CFC (size of surviving GM-CFC compartment) does influence the speed of the GM-CFC repopulation in suspension cultures. The importance of the number of surviving GM-CFCs for the growth and maintenance of GM-CFC population in such suspension cultures diminishes with time. No significant differences are observed after 2 wk, indicating that the ancestor stem cell population and its regenerative potential responsible for in vitro hematopoiesis have not been significantly affected by the drug treatment. A-ALD-treated progenitor cells retain their ability to integrate with the previously established marrow stromal cell layer and generate GM-CFC within this layer to an extent comparable to that of untreated marrow cells.

The effect of A-ALD on human hematopoiesis is comparable to that of 4-hydroperoxycyclophosphamide. Its advantage over 4-hydroperoxycyclophosphamide is a greater stability in vitro. It has sparing effect on myeloid leukemia cell line (KBM-3) and -resistant (KBM-3/D0X) leukemic cells. Thus, A-ALD appears to be a promising drug for in vitro purging of bone marrow cells.

INTRODUCTION

Hematological malignancies are drug-sensitive neoplasias characterized by a steep dose-response relationship between the cell kill and the dose of the drug. Consequently, an increase in the dose of the chemotherapeutic agent should potentially improve the cure rate (1). High-dose chemotherapy with autologous bone marrow transplantation is a treatment for acute leukemias for which bone marrow is collected while the disease is in remission when the potential for leukemic cells to be present in the bone marrow is minimal.

Due to our poor ability to detect minimal residual disease in the bone marrow (2, 3), contamination of such marrow with leukemic cells is one of the major obstacles for successful autologous bone marrow transplantation. Hence, along with effective conditioning regimens aimed to eradicate residual leukemia in vivo, removal of leukemic cells from marrow in vitro is crucial for successful use of this treatment procedure. In acute myelogenous leukemia, the use of chemotherapy in vitro seems, at present, to be the most practical and promising approach to marrow purging.

Of all of the drugs tested to date, the in vitro active cyclophosphamide derivative 4-HC showed most promise as a chemotherapeutic agent in preclinical models (4) and therefore was introduced in clinical trials (5–8). 4-HC appears to have a unique potential for being more toxic to leukemic cells and normal marrow clonogenic progenitor cells than to primitive hematopoietic stem cells responsible for marrow transplantation potential (9–12). The degree of toxicity of 4-HC to a particular cell type is influenced by the intracellular level of aldohexose dehydrogenase (12), an enzyme that converts aldophosphamide, the open chain tautomer of 4-HC, to an inactive metabolite. This modulation of 4-HC metabolism seems to be responsible for its selective toxicity within the hematopoietic system (12).

A disadvantage of the use of 4-HC is its low level of stability in vitro (7, 13) and the variability of its activity with the in vitro concentration of both nucleated cells and erythrocytes (14, 15). Nevertheless, its potential for selectively eliminating leukemic cells, while sparing multipotent hematopoietic stem cells, encouraged the search for other cyclophosphamide derivatives that have greater stability, more selective cell action, and less dependence on cell concentration.

Recently, Wang and Farquhar reported the synthesis of a new series of aldophosphamide analogue precursors that are stable under neutral aqueous conditions but convert facilely to the corresponding free aldehydes in biological media (16). In this report, we present our findings on the in vitro effects of one of these compounds, A-ALD, on human normal and leukemic progenitor cells in vitro.

MATERIALS AND METHODS

Drugs. The A-ALD used in this study was designed and synthesized at our institution by Y. Wang and D. Farquhar. Its synthesis, characterization, and chemical properties will be reported elsewhere (16). It has a molecular weight of 379. A-ALD is very stable in neutral aqueous solutions; its half-life in 0.05 M phosphate buffer (pH 7.4) at 37°C is 52 h, contrasting with a half-life of 1.5 h for 4-HC (7). Unlike 4-HC, A-ALD has to be activated (Fig. 1), the first activation step being hydrolysis by carboxylate hydrolases (esterases) to give the corresponding hemiacetal. The hemiacetal then undergoes further ester- and diol mediated hydrolyses to the aldophosphamide which exists in equilibrium with 4-hydroxyphosphamide. The major endproducts of the activation pathway are phosphorodiamic acid and acrolein (16). Aldophos-

1 The abbreviations used are: 4-HC, 4-hydroperoxycyclophosphamide; GM-CFC, granulocyte-macrophage colony-forming cells; A-ALD, acetaldehydephosphamide; PBS, phosphate buffered saline; FCS, fetal calf serum; IMDM, Iscove’s modified Dulbecco’s medium; S-IMDM, IMDM supplemented with 12.5% FCS, 12.5% horse serum, and 5 x 10^-m hydrocortisone sodium succinate; KBM-3, myeloid leukemia cell line; KBM-3/D0X, myeloid leukemia cell line rendered resistant in vitro to doxorubicin (Adriamycin); RBC, red blood cells; ASCL, autologous feeder layers of bone marrow stromal cells; IC50, 50% inhibitory concentration; IC90, 90% inhibitory concentration; LDBM, light-density bone marrow fraction.

ACETALDOPHOSPHAMIDE: A NEW DRUG FOR MARROW PURGING

**Fig. 1.** Activation pathway of A-ALD. The first activation step for A-ALD (J) is hydrolysis by esterases to give the corresponding hemiacetal (2) which then undergoes further esterase-mediated hydrolyses to give aldehyde hydrate (3) which spontaneously loses water to form aldophosphamide (4). Aldophosphamide exists in equilibrium with 4-hydroxyphosphamide (5) and by E2 elimination converts to phosphorodiamidic mustard (6) and acrolic (7).

Phosphamide is detoxified by biotransformation to carboxyphosphamide by aldehyde dehydrogenases.

After dilution in PBS at pH 7.2, A-ALD was either used immediately or kept frozen at −20°C and used after thawing. The 4-HC was a generous gift of ASTA-Werke (Bielefeld, West Germany). It was diluted in PBS and used immediately.

Normal Bone Marrow. Bone marrow was obtained from normal volunteers or patients with solid tumors undergoing diagnostic workup. All bone marrow donors gave informed consent and were advised about the risks of the procedure. Bone marrow was subjected to fractionation on a one-step Ficoll-Hypaque density gradient (1.080 g/cm³). A LDBM containing all immature cells (including progenitor cells) and free of erythrocytes was washed twice in PBS and used throughout.

Studies on in vivo Hematopoietic Toxicity of A-ALD. In all experiments, bone marrow cells were suspended in warm (37°C) PBS containing Ca²⁺, glucose (300 mg/liter), 5% FCS, and various concentrations of A-ALD. To assay the effect of cell density, the cell concentrations were adjusted to 2 x 10⁶/ml and 10 x 10⁶/ml, respectively. Cells were then incubated at 37°C in a water bath at ambient atmosphere. At the end of the incubation, the cells were diluted with 10 volumes of ice-cold PBS and washed twice to remove excess drug. Thereafter, the treated cells were assayed for GM-CFC or fibroblast colony-forming cells. Surviving fractions were calculated.

Assay for GM-CFC. Standard, double-layer semisolid agar technique was used with human placenta-conditioned medium as a source of colony-stimulating factor in the underlayer (17). Control and A-ALD-treated cells (1 x 10⁶/ml) were plated in 1 ml of IMDM supplemented with 0.3% agar (Bacto; Difco, Detroit, MI) and 20% FCS in triplicate Petri dishes (35 mm diameter). On Day 7, clones containing 8 or more cells were scored. The numbers on treated plates were compared with numbers in nontreated cultures and surviving fractions were calculated. Survival curves were constructed on a semilog plot. Results are presented as the mean ± SD of results from at least triplicate cultures. Concentrations of A-ALD required to inactive 50 and 90% of GM-CFC (IC₅₀ and IC₉₀, respectively) were calculated.

Assay for Fibroblast Colony-Forming Units. Control and A-ALD-treated bone marrow cells were resuspended in S-IMDM. Ten ml of cell suspension containing 9 x 10⁶ bone marrow cells were introduced into tissue culture flasks (25-cm²; Corning Glass Works, Corning, NY) and cultured at 37°C, 5% CO₂ in air. Three flasks were used as control cultures and for each drug concentration. The medium was changed every 4 days. After 10 days flasks were washed with PBS, fixed with methanol, and stained with Wright-Giemsa stain. Fibroblastoid colonies containing more than 50 cells were scored as fibroblast colony-forming cells. Surviving fractions, survival curves, IC₅₀, and IC₉₀ were obtained.

Long-Term Suspension Cultures of Bone Marrow. A two-step procedure was used to set up long-term suspension cultures of bone marrow as reported previously (9, 11). Briefly, in the first step ASCL supplying a hematopoietic microenvironment were established in tissue culture flasks with 1 x 10⁶ LDBM cells suspended in S-IMDM. After incubation for 4 days in a fully humidified atmosphere of 5% CO₂ in air, all nonadherent cells were removed with careful and repeated washing of adherent cells. The nonadherent cells or fresh LDBM cells were then treated with various doses of A-ALD at cell concentrations of 2 x 10⁶/ml, washed, and seeded on ASCL (10⁶ cells in 10 ml S-IMDM/flask). Flasks were then incubated at 37°C and 5% CO₂ atmosphere in air for a period of up to 28 days; 90% of the medium was changed every 3 to 4 days and the cultures were assayed at intervals to determine the total number of GM-CFC.

The total number of GM-CFC per flasks was calculated at each interval for each drug concentration and compared with the total number of GM-CFC initially inoculated into each flask culture. The flasks containing nontreated cells served as indicators of the quality of the culture system used; cultures containing only ASCL were used to estimate the background numbers of GM-CFC generated from the nontreated ASCL. At the end of the culture period, all nonadherent cells were carefully retrieved by repeated washing of the adherent cell layer and then assayed as described above. Adherent cell layers were trypsinized with a mixture of 0.2% trypsin and EDTA solution (GIBCO, Grand Island, NY) for approximately 2 min at room temperature; cells were resuspended and assayed for the presence of GM-CFC. Duplicate cultures were used for each experimental point. Results were expressed as the mean ± SD from duplicate flask cultures.

Studies on Early GM-CFC Recovery in Suspension Cultures. Bone marrow cells (10⁶/ml) were treated with A-ALD at concentrations ranging from 6.0 µg to 10.0 µg/ml for 60 min as described above, washed, and resuspended in S-IMDM. After assaying the suspension for surviving GM-CFC, 9 x 10⁶ cells in 10 ml S-IMDM media were introduced into tissue culture flasks for liquid cultures at 37°C and 5% CO₂ in air. At regular intervals of up to 8 days, 0.8 ml of cell suspension was removed and assayed for GM-CFC present in the flasks. Duplicate flask cultures were used for control and treated cells.

Sensitivity of Myelogenous Leukemia Cell Lines to A-ALD. Myelogenous leukemia cell line KBM-3 and its doxorubicin-resistant subline KMB-3/DOX were developed in our laboratory from a patient with monocytic leukemia and propagated in IMDM supplemented with 10% FCS. They were seeded at 3 x 10⁶/ml and split every 3 to 4 days. When used for drug studies, they were growing at an exponential rate. Cells (1 x 10⁶) were added to 1 ml of PBS containing various drug concentrations and incubated for 60 min at 37°C. After that, the excess drug was removed by repeated washing in ice-cold PBS, and a fraction of cells was assayed to determine the number of surviving clonogenic cells using a single-layer system of semisolid agar medium (IMDM, 15% FCS, and 0.3% agar) without an external source of colony-stimulating activity.

Leukemic cells (1 x 10⁶/ml/dish) were plated in each of three 35-mm dishes. After 7 days at 37°C in a fully humidified atmosphere of 5% CO₂ in air, the clones containing 8 or more cells were scored under an inverted microscope. The surviving fractions were calculated, dose-response curves constructed, and the IC₅₀ and IC₉₀ doses determined for treated cultures. Results are expressed as the mean ± SD of at least triplicate cultures. The cloning efficiency of untreated leukemic cells varied between 20 and 30%.

To study the effect of the admixture of RBC, washed human RBC were added to a hematocrit of 0.8% along with various concentrations of the drug.
RESULTS

Survival of Human Bone Marrow-derived GM-CFC as a Function of Pulse Exposure to Increasing Doses of A-ALD: Comparison with 4-HC. Exposure of bone marrow cells in vitro to A-ALD resulted in the killing of GM-CFC in a dose-dependent manner, the surviving fraction essentially being an exponential function of the dose (Fig. 2). The results demonstrated in vitro cytotoxic activity and a mode of action independent of the cell cycle, features that are compatible with those of an alkylating agent. When compared with the effect of 4-HC under identical experimental conditions, including identical marrow (Fig. 2), a very similar response of GM-CFC to both agents was seen. The slopes of the dose-response curves were almost identical for both drugs. When exposed at $2 \times 10^4$ cells/ml/60 min, the $IC_{50}$ and $IC_{90}$ for A-ALD were 1.40 and 4.35 µg/ml, respectively, at $10 \times 10^6$ cells/ml/60 min, the corresponding values were 1.45 and 4.45 µg/ml. In the same cell population, the $IC_{50}$ and $IC_{90}$ for 4-HC at $2 \times 10^6$ cells/ml/60 min were 3.90 and 11.30 µg/ml and at $10 \times 10^6$ cells/ml/60 min, 4.93 and 15.30 µg/ml, respectively. The ratio of $IC_{50}$ for A-ALD and that for 4-HC was between 2.5 and 3, based on drug weight, and it was approximately 4 based on molarity indicating higher potency of A-ALD in vitro. Under stringent conditions, when RBC-free LDBM cells were exposed to A-ALD, there was little evidence that cell density was a major factor influencing the extent of GM-CFC killing within the range tested ($2 \times 10^6$ and $10 \times 10^6$/ml).

The GM-CFC from bone marrow cultured for 4 days in vitro were more sensitive to A-ALD than were GM-CFC from fresh bone marrow. The former had an $IC_{50}$ of 0.45 µg/22 $\times 10^6$ cells/ml/60 min while the latter had an $IC_{50}$ of 1.10 µg/22 $\times 10^6$ cells/ml/60 min (Fig. 3). Because both populations were treated at the same density, this factor alone cannot account for this discrepancy.

Effect of A-ALD on Marrow CFC-F. The dose response of CFC-F after incubation of the bone marrow ($1 \times 10^7$/ml/60 min at 37°C) with progressively larger concentrations of A-ALD is shown in Fig. 4. The survival of CFC-F in treated marrows is an exponential function of the exposure dose of A-ALD. The $IC_{50}$ for the CFC-F was 2.2 µg/ml and the $IC_{90}$ dose was 5.9 µg/ml. A decrease in the size of the colonies was noted after exposure to A-ALD concentrations above 6.0 µg/ml.

Regeneration of GM-CFCs from Bone Marrow Depleted of GM-CFC by Exposure to A-ALD. Autologous nonadherent LDBM cells and fresh autologous LDBM cells were exposed to progressively higher doses of A-ALD (selected to eliminate various fractions of GM-CFC) and seeded on ASCL. The highest dose was selected to eliminate all GM-CFC as assayed by colony formation in agar cultures containing $5 \times 10^5$ treated bone marrow cells/ml/dish. The results with fresh LDBM cells are summarized in Table 1. Results with nonadherent marrow cells grown in vitro are shown in Table 2. Although the numbers of GM-CFC in control cultures were maintained at their initial level for 1 wk before they declined, different GM-CFC kinetic values were seen in A-ALD-treated cultures. In cultures of freshly explanted and treated LDBM cells there was an increase in the size of the GM-CFC pool during the first week that was inversely related to the initial number of GM-CFC; e.g., the GM-CFC numbers increased by factors of 9 and 35 in cultures treated with 6 and 10 µg/ml, respectively. Two wk after treatment, the GM-CFC compartment in all of the treated cultures was larger than that in the controls ($P < 0.01$) and remained so throughout the culture period (Table 1). Similar GM-CFC kinetic values were observed in cultures of nonadherent marrow cells grown for 4 days in vitro prior to treatment (Table 2).
ACETALDOPHOSPHAMIDE: A NEW DRUG FOR MARROW PURGING

Fig. 3. Survival of GM-CFC from fresh and in vitro grown human marrow as a function of increasing concentrations of A-ALD. Each point, mean ± SD of results in triplicate cultures.

Fig. 4. Survival of human marrow fibroblast colony-forming cells (F-CFC) as a function of 60-min exposure to progressively higher concentrations of A-ALD. Each point, weighed mean ± SD of 2 experiments. IC_{50} = 2.20 µg; IC_{95} = 5.87 µg/10^7 cells/60 min.

Table 1: Regrowth of GM-CFC from bone marrow initially depleted of GM-CFC by A-ALD and subsequently grown in vitro on autologous feeder layers

<table>
<thead>
<tr>
<th>A-ALD (µg/22 × 10^6 in 1 ml/60 min)</th>
<th>No. of GM-CFC/flask (1 × 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Day 6</td>
</tr>
<tr>
<td>0</td>
<td>29.5 ± 4.8</td>
</tr>
<tr>
<td>6</td>
<td>3.6 ± 0.6^*</td>
</tr>
<tr>
<td>10</td>
<td>0.5 ± 0.1^*</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Significantly (P < 0.01, Student's t test) different from controls.
* Significantly (P < 0.05, Student's t test) different from controls.
* Flasks containing feeder layer only for control of endogenous background production of GM-CFC by cells entrapped in the adherent cell layer. At all intervals values significantly different (P < 0.0001, Student's t test) from cultures to which either untreated or treated marrow cells were added.
* ND, not done.

Table 2: Regrowth of GM-CFC in Long-term bone marrow cultures after treatment of nonadherent cells with A-ALD and subsequently reseeded on autologous feeder layers

<table>
<thead>
<tr>
<th>A-ALD (µg/22 × 10^6/60 min)</th>
<th>No. of GM-CFC/flask (1 × 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Day 6</td>
</tr>
<tr>
<td>0</td>
<td>24.5 ± 2.80</td>
</tr>
<tr>
<td>6</td>
<td>0.80 ± 0.48^*</td>
</tr>
<tr>
<td>10</td>
<td>0.10 ± 0.09^*</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>0^d</td>
<td>0</td>
</tr>
</tbody>
</table>

^d ND, not done.
^e Significantly different (P < 0.01) from controls.
^d Significantly different (P < 0.05) from controls.
^d Flasks containing feeder layer only for control of endogenous background production of GM-CFC by cells entrapped in the adherent cell layer. At all intervals values significantly different (P < 0.0001, Student's t test) from cultures to which either treated or untreated nonadherent marrow cells were added.

342
flasks containing the feeder layer only, small numbers of GM-CFC were generated from the adherent cell layer. It never exceeded 5% of the lowest GM-CFC counts detected in experimental cultures.

Analysis of the presence of GM-CFC within the adherent stromal cell layer after 4 wk of culture revealed that approximately half of all GM-CFC in the flasks were contained within this layer. Similar results were obtained with fresh (2 experiments) and in vitro cultured (1 experiment) autologous bone marrow cells treated with A-ALD (Table 3). The total number of GM-CFC within the adherent cell layer was higher in flasks seeded with A-ALD-treated cells than it was in control cultures seeded with untreated cells. Background GM-CFC counts in flasks with adherent cell layers alone were less than 10% of those in cultures seeded with control or A-ALD-treated marrow.

Early Regrowth Kinetics of GM-CFC in Suspension Cultures of Untreated and A-ALD-treated Human Bone Marrow. In control cultures, all GM-CFC were maintained at almost identical levels during an 8-day period. In A-ALD-treated cultures, the kinetics of the increase in the whole GM-CFC compartment was a function of the concentration of A-ALD to which the cells were initially exposed and, consequently, was related to the size of the surviving GM-CFC pool. The more the pool size was reduced, the more rapid relative "regrowth" of GM-CFC in relation to the seeded GM-CFC numbers occurred (Fig. 5).

The kinetic values of recovery of GM-CFC in cultures treated with 8 µg/ml A-ALD and depleted of 99.4% GM-CFC indicate a doubling time of approximately 8 h. In cultures treated with 10 µg/ml, there was a lag period of 36 h; during the subsequent 24 h the GM-CFC number increased 10 times. If such an increase was due to GM-CFC self-renewal, the required doubling time would be less than 6 h, assuming no loss from their compartment because of differentiation.

Effect of A-ALD on the Survival of L-CFC of Myelogenous Leukemia Cell Lines KBM-3 and KBM-3/ADR. The sensitivity of L-CFC to progressively higher concentrations of A-ALD was compared with that of human GM-CFC exposed to progressively higher doses of A-ALD (Fig. 6). The computed survival curves show that, like normal GM-CFC, the killing of L-CFC is an exponential function of the drug concentration at a fixed time. Both KBM-3 and its doxorubicin-resistant subline KBM-3/DOX were 2–3 times more sensitive to A-ALD than was GM-CFC (Fig. 6). Comparison of inhibitory concentration values for KBM-3 and KBM-3/DOX revealed resistance indices of only 1.36 at IC50 and 1.11 at IC90, indicating negligible cross-resistance between doxorubicin and A-ALD in this system.

Addition of erythrocytes to KBM-3 cells along with A-ALD significantly decreased the toxicity of A-ALD to clonogenic cells (Fig. 7).

**DISCUSSION**

A-ALD, a new aldophosphamide derivative, killed human marrow GM-CFC as well as leukemic clonogenic cells in vitro in an exponential fashion, suggesting that it has a cell cycle-independent mode of action. The kinetic values of its cell killing were comparable to those of 4-HC. Because various progenitor cells, including GM-CFC and multipotent progenitors, appear equally sensitive to 4-HC (8, 10, 14, 18, 19), we have used GM-CFC as indicators of the in vitro toxicity of A-ALD for comparative purposes.

One disadvantage of in vitro use of 4-HC is the modulation of its toxicity by cell concentration (14, 15, 20), particularly by the admixture of RBC (14, 20). We have, therefore, used two different cell concentrations in otherwise stringent assay con-

**Table 3 Recovery of GM-CFC from adherent cell feeder layers seeded with bone marrow cells treated with increasing concentrations of A-ALD**

<table>
<thead>
<tr>
<th>A-ALD, (µg/l x 10³/m³/60 min)</th>
<th>Fresh bone marrow cells</th>
<th>Bone marrow cells cultured in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cells per feeder layer (x 10⁶)</td>
<td>GM-CFC per feeder layer (x 10⁶)</td>
</tr>
<tr>
<td>0</td>
<td>21.6 ± 4.0</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>6</td>
<td>22.3 ± 1.4</td>
<td>3.3 ± 0.8</td>
</tr>
<tr>
<td>10</td>
<td>20.4 ± 1.3</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>12</td>
<td>33.6 ± 4.2*</td>
<td>4.7 ± 2.1</td>
</tr>
<tr>
<td>0*</td>
<td>17.4 ± 0.3*</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

* Bone marrow cells grown in suspension cultures for 5 days before exposure to A-ALD.

* Significantly different (P ≤ 0.05, Student's t test) from controls.

* Significantly different (P ≤ 0.01, Student's t test) from controls.

**Fig. 5. Kinetics of the recovery of human marrow GM-CFC from marrow depleted of GM-CFC by exposure to progressively higher concentrations of A-ALD. Each point, mean ± SD of results from triplicate cultures from 2 separate experiments. Percentage numbers at Time 0 indicate the size of the GM-CFC pool immediately after exposure to A-ALD as % of the nontreated control cultures.**
ACETALDOPHOSPHAMIDE: A NEW DRUG FOR MARROW PURGING

Fig. 6. Comparison of the survival of leukemic clonogenic cells from 2 human myeloid leukemia cell lines with survival of human marrow GM-CFC exposed to progressively higher concentrations of A-ALD. Mean ± SD of quadruplicate cultures. GM-CFC, KBM-3 cell line; KBM-3/DOX (ADR/ID) cell line.

Fig. 7. Effect of human erythrocytes on the sensitivity of clonogenic human myeloid leukemia cells exposed for 60 min to progressively higher concentrations of A-ALD in the absence (O) and presence (•) of washed erythrocytes (hematocrit = 0.8%). Each point, mean ± SD of results from triplicate cultures.

In our studies, variations in the cell concentrations between 2 × 10⁶ and 10 × 10⁶ cells/ml did not have a significant effect on the slopes of dose-response curves for either A-ALD or 4-HC, although at 10 × 10⁶ the curves tended to diverge with a shift to the right and less steep slope for the 4-HC curve, indicating a slight decrease in toxicity. The presence of RBC does, however, significantly modify the A-ALD toxicity to leukemic clonogenic cells in vitro; this aspect of A-ALD action is no different from that of 4-HC and calls for stringent conditions in further preclinical evaluation of the action of A-ALD in vitro. Similarly, our preliminary results indicate that the toxicity of A-ALD on GM-CFC is decreased by the presence of RBC. We are currently studying this effect in greater detail with regard to interindividual variations. The absence of cross-resistance with the commonly used antileukemic drug doxorubicin is of importance because some leukemic clonogenic cells surviving in remission bone marrow that was collected for autologous bone marrow transplantation might have been selected for doxorubicin resistance.

Data from long-term suspension cultures of GM-CFC-depleted bone marrow unequivocally show the sparing effect of A-ALD on the hematopoietic in vitro transplantation potential of such marrow. The kinetic values of in vitro regrowth of GM-CFC in suspension cultures of A-ALD-treated bone marrow indicate repopulation of the GM-CFC pool from a more primitive pre-GM-CFC pool, possibly multipotent stem cells. An alternative explanation for the regrowth of GM-CFC from the minute surviving GM-CFC pool presumes a very high capacity for self-renewal of GM-CFC and an extremely short doubling time as indicated by our kinetic analysis during the first 8 days in suspension cultures after A-ALD treatment. Our findings make this explanation unlikely. Finally, the absence of a shoulder region on A-ALD dose-response curves makes intracellular repair in GM-CFC an unlikely explanation as well. Thus, the data strongly suggest that a primitive (pluripotent?) stem cell is affected by A-ALD to a much lesser degree than are more differentiated committed progenitors. Using bone marrow grown in vitro prior to treatment with A-ALD we could show that these cells survive at least for 4 days in vitro and retain their GM-CFC-generating potential. A similar in vitro sparing effect of 4-HC on putative human ancestors of lineage-committed progenitors has been described (9-11) and is consistent with experimental (21) and clinical (22) observations of the differential effect of cyclophosphamide on hematopoietic cells in vivo. Bone marrow depleted to various degrees of GM-CFC by ionizing radiation failed to regrow GM-CFC in suspension cultures, a finding also supporting the idea of a selective effect of A-ALD on various hematopoietic precursors.

Even when ASCLs were depleted of nonadherent cells at an early stage (before the development of a multilayered cell system), a few GM-CFC or their ancestors were entrapped in such a layer. Such small background of endogenously generated GM-CFC does not represent any obstacle in the interpretation of the data.

The relative ease in preparing A-ALD in bulk quantities and its stability in vitro (16) represents a distinct advantage over 4-HC. The active metabolite in both drugs is the same and our results indicate a similar effect on normal marrow progenitors; on a molar basis, A-ALD is approximately 4 times more potent.

* M. Beran, manuscript in preparation.
than 4-HC, however, enzymatic activation of A-ALD by esterases is necessary (Fig. 1) to yield aldoophosphamide/4-OH cyclophosphamide.

Esterases are ubiquitous in all mammalian tissues and, at least in vitro, hog liver carboxylate esterase converts A-ALD rapidly to aldoophosphamide and thereafter into phosphoramidate mustard and acrolein, with a half-life of only a few minutes (16). This enzyme-directed activation does not cause any higher interindividual variation at least in terms of GM-CFC toxicity. Whether it might translate into some tumor tissue-selective action because of different content and/or activities of esterase isoenzymes is unknown and currently under investigation.

In summary, A-ALD is a new stable aldoophosphamide analogue with a biological effect on GM-CFC and leukemic clonogeneic cells comparable to that of 4-HC. It has a remarkable sparing effect on pre-GM-CFC (stem cells) and higher in vitro potency than 4-HC in terms of GM-CFC toxicity. Our results indicate that it has potential for in vitro purging of bone marrow for use in autologous bone marrow transplantation as an alternative to 4-HC.

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