Comparative in Vitro Effects of Cyclophosphamide Derivatives on Murine Bone Marrow-derived Stromal and Hemopoietic Progenitor Cell Classes

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ABSTRACT

We investigated the in vitro effects of ASTA-Z-7595, ASTA-Z-7557, ASTA-Z-7654, and 4-hydroperoxycyclophosphamide (4HC) on murine stromal fibroblastoid colony-forming units, committed hemopoietic progenitors (erythroid burst-forming units and granulocyte/macrophage colony-forming units), and pluripotent hemopoietic stem cells assayed by the spleen colony-forming unit (CFU-s) assay. In general, the drugs showed a time-and dose-dependent effect on colony-forming unit survival, and the relative toxicities were in the order in which the drugs are listed above. We found a relative sparing of day 12 CFU-s compared with day 7 CFU-s and committed hemopoietic and stromal progenitors, although colony size of day 12 CFU-s was reduced. Our results support two possible mechanisms for delayed or inadequate hemopoietic reconstitution in clinical studies using bone marrow purged with 4-hydroperoxycyclophosphamide or ASTA-Z-7557, i.e., damage to (a) transplantable stromal cells or (b) the hemopoietic stem cells.

INTRODUCTION

In the last few years, high-dose chemotherapy followed by autologous bone marrow transplantation has become an important therapy in the treatment of leukemia (8) and lymphoma (20). Some advantages of autologous bone marrow transplantation over allogeneic marrow transplantation are the avoidance of graft-versus-host disease and post transplantation immunodeficiency, while it overcomes the limited availability of HLA identical sibling donors (11). The main disadvantage of the method is the possible survival of morphologically undetectable tumor cells in the bone marrow graft. To overcome this problem, techniques have been developed to purge tumor cells from the bone marrow grafts.

Using a LBN hybrid rat model of acute myelogenous leukemia Sharkis et al. (19) demonstrated that in vitro incubation of acute myelogenous leukemia cells and bone marrow with 4HC resulted in a high kill of acute myelogenous leukemia cells and a relative sparing of pluripotent stem cells. Since this observation, 4HC and another cyclophosphamide derivative, named ASTA-Z-7557, have been used in several preclinical and clinical studies (6-9, 11, 12). Hemopoietic recovery was observed at drug doses which produced a total inhibition of colony formation by bipotent myeloid precursor cells (CFU-C), which suggests that these drugs caused less damage to the pluripotent hemopoietic stem cell population. Preliminary results supporting this hypothesis have been reported using rat bone marrow (5, 6).

Recent studies have demonstrated the presence of donor stem cells in both human (10) and murine bone marrow transplant recipients (15). Keating et al. (10) using long-term bone marrow cultures showed that, progressively after transplantation, practically all stromal cells in the host bone marrow became of donor origin. This suggests that host stromal cells retain a limited proliferative capacity following irradiation and chemotherapy and are gradually replaced by donor stromal cells. Thus, donor-derived stromal cells may play an important role in reconstitution of the bone marrow after bone marrow transplantation. The chemical purging of tumor cells from bone marrow grafts may also result in stromal precursor cell damage and thereby hamper reconstitution.

In this manuscript, we report on the effects of 4HC, ASTA-Z-7557, and the new cyclophosphamide derivative ASTA-Z-7595 on committed hemopoietic progenitors (BFU-E and CFU-C) and pluripotent hemopoietic stem cells assayed by the CFU-s assay (21) on days 7 and 12. Furthermore, the effect of the drugs on CFU-F, an in vitro representative of the hemopoietic microenvironment (2, 3, 14), was determined.

MATERIALS AND METHODS

Cyclophosphamide Derivatives. 4HC (molecular weight, 291.1), ASTA-Z-7557 (molecular weight, 500.5), ASTA-Z-7595 (molecular weight, 492.4), and ASTA-Z-7654 (molecular weight, 548.0) are all derivatives of cyclophosphamide which spontaneously decompose in aqueous solutions to generate products with the same alkylating and cytotoxic effects as the microsomal activated parent compound (1, 16). The drugs used in these experiments were a gift from Asta Werke (Germany). Solutions were prepared in BSS containing 20% FCS at concentrations of 1, 10, and 100 μg/ml.

Murine Bone Marrow Cells. Adult male (CBA x C57BL)F1 mice (10-20 weeks old) were used. The mice were killed by cervical dislocation prior to removal of both femurs using a sterile technique. Bone marrow was flushed from the femurs with a 23-gauge needle in 6 ml of BSS containing 20% FCS after amputation of the epiphyses. The cells were centrifuged and resuspended in BSS containing 20% FCS.

Drug Treatment. Mouse bone marrow cells (7 x 10⁶ nucleated cells/ml) were incubated with the cyclophosphamide derivatives at drug concentrations of 1, 10, and 100 μg/ml plus 20% FCS after amputation of the ephipense. The cells were then centrifuged and resuspended in BSS containing 20% FCS.

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2 To whom requests for reprints should be addressed.

The abbreviations used are: 4HC, 4-hydroperoxycyclophosphamide; CFU-C, granulocyte/macrophage colony-forming units; BFU-E, erythroid burst-forming units; CFU-s, spleen colony-forming units; CFU-F, fibroblastoid colony-forming units; BSS, Hank's buffered salt solution; FCS, fetal calf serum.

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units. Nucleated cell counts were performed with a Coulter particle counter.

**Spleen Colony Assay.** The CFU-s assay was performed as described by Till and McCulloch (21). Groups of 8 recipient mice received a lethal dose of 9 Gy total body γ-irradiation using a 137 Cs-γ-source (Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of 1.23 Gy/min and were given injections of 5 × 10⁶ treated or untreated control bone marrow cells. After 7 and 12 days spleens were removed and fixed in Telyeysničsky's solution. The number of macroscopically visible spleen colonies was determined. The diameter of the spleen colonies was measured in mm using an inverted microscope and a fine scale transparent matrix.

**Fibroblastoid Colony-forming Units.** Cells were cultured in α-medium (α-modification of Dulbecco's minimal essential medium) containing 0.8% methylcellulose and 20% FCS. One-ml aliquots of culture medium, containing 5 × 10⁶ cells, were plated in 35-mm Costar culture dishes and incubated at 37°C in a humidified atmosphere consisting of 10% CO₂ in air. All determinations were performed in triplicate. On day 10 of culture the dishes were washed with phosphate-buffered saline (8090 μM Na₂HPO₄·2H₂O, 2204 μM KM₂PO₄, 2682 μM KCI and 137 mM NaCl in aqua trident), fixed in methanol, and stained with 10% Giemsa. Fibroblastoid stromal cell colonies containing at least 50 fibroblastic cells were counted with an inverted microscope.

**Granulocyte/Macrophage Colony-forming Units.** Cells were cultured in α-medium (5 × 10⁶ nucleated cells/ml medium) containing 0.8% methylcellulose, 10% FCS, 1% bovine serum albumin, and 10% concanavalin A-stimulated mouse spleen conditioned medium. Cells were cultured for 7 days under the same conditions described for the fibroblastoid cell culture. All determinations were performed in duplicate. Granulocyte/macrophage colony-forming units, containing at least 50 cells, were counted with an inverted microscope.

**Erythroid Burst-forming Units.** Cells (5 × 10⁶ nucleated cells/ml medium) were cultured in the medium described for the CFU-C assay supplemented with erythropoietin (0.5 lU/ml) and mercaptoethanol (10⁻⁴ M) under conditions described earlier. All determinations were performed in triplicate. Bursts containing over 49 cells were counted on day 12. Macroscopic bursts frequently contained hemopoietic cells of other lineages.

## RESULTS

**Effect of Control Incubation on Progenitor Survival.** Since the long incubation times used could affect survival of progenitor cells even in the absence of drugs, incubation controls were performed in all experiments. The experimental cell data are expressed as a percentage of surviving control progenitor cells after the same period of incubation. Table 1 shows that decreased survival of all colony-forming cells was observed with increasing incubation time.

**Effect of Cyclophosphamide Derivatives on Survival.** CFU-s, BFU-E, and 4HC exhibited a dose-dependent effect on CFU-C and CFU-F survival after 1, 6, and 24 h of incubation with different concentrations of ASTA-Z-7557. Data are expressed as a percentage of surviving progenitor cells incubated for 1, 6, or 24 h under control incubation conditions. Each point represents the mean of five replicate experiments, except for the 24-h CFU-F points, which are based on three replicate experiments. Bars, SE.

### Table 1

<table>
<thead>
<tr>
<th>Drug dose (μg/ml)</th>
<th>CFU-C</th>
<th>CFU-F</th>
<th>BFU-E</th>
<th>CFU-s</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
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<td>1</td>
<td>56 ± 10</td>
<td>98 ± 15</td>
<td>97 ± 13</td>
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<tr>
<td>24</td>
<td>51 ± 12</td>
<td>37 ± 10</td>
<td>35 ± 10</td>
<td>76 ± 15</td>
</tr>
</tbody>
</table>

* Arithmetic mean ± 1 SE of two to five separate experiments per progenitor class.

ND, not determined.

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**IN VITRO EFFECTS OF CYCLOPHOSPHAMIDE DERIVATIVES**

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Chart 2. Toxicity of ASTA-Z-7595 on mouse progenitor cells. Bone marrow cells were assayed for CFU-C (○) and CFU-F (■) after 1, 6, or 24 h of incubation with different concentrations of ASTA-Z-7595. Data are presented as indicated in Chart 1.

Effects on Day 12 Spleen Colony Size. Analysis of colony size distribution within control incubated groups revealed no change in size distribution after 0, 1, and 6 h of incubation (Table 3). Approximately 30% of the colonies formed were less than 1 mm, 50% were between 1–2 mm, and around 20% were larger than 2 mm. From Chart 5, it is apparent that both a decrease in size and number of day 12 colonies was observed with increasing incubation time and drug doses.

DISCUSSION

In this comparative study we report the effects of in vitro active cyclophosphamide metabolites on murine bone marrow-derived hemopoietic progenitor cells and on stromal fibroblastoid progenitor cells. Our results clearly demonstrate that ASTA-Z-7557, ASTA-Z-7595, ASTA-Z-7654, and 4HC differed in their toxicity for progenitor cells. 4HC had the greatest toxicity and ASTA-Z-7595 had the lowest, while ASTA-Z-7557 and ASTA-Z-7654 showed an intermediate toxicity for all hemopoietic progenitor types.

Published data comparing the toxicity of different cyclophosphamide derivatives are not uniform. Hervé et al. (7) demonstrated that 4HC had a higher toxicity for human bone marrow CFU-C than did ASTA-Z-7557, while Gorin et al. (4) found that these drugs had a similar toxicity for human progenitor cells. Hagenbeek and Martens (6), using a rat model, compared the toxicity of ASTA-Z-7557, ASTA-Z-7595, ASTA-Z-7654, and 4HC on CFU-C and CFU-s. They found that 4HC had the highest toxicity for these progenitor cell types, followed by ASTA-Z-
IN VITRO EFFECTS OF CYCLOPHOSPHAMIDE DERIVATIVES

Chart 4. Toxicity of 4HC on mouse progenitor cells. Bone marrow cells were assayed for CFU-C (○) and CFU-F (□) after 1, 6, or 24 h of incubation with different concentrations of 4HC. Data are presented as indicated in Chart 1.

7557. In general, the drugs showed a lower toxicity for CFU-s than for CFU-C.

In addition we found a relative sparing of murine day 12 CFU-s (25% of control survival with the 10-μg 6-h dose) which has been proposed to represent the true pluripotent stem cell (13), when compared with day 7 CFU-s, which were strongly affected, as judged by the 2% of control survival with the 10-μg 6-h dose. Similarly, the sparing of human pluripotent hematopoietic stem cells has been implied by successful bone marrow reconstitution in recipients of 4HC-treated grafts in which no CFU-C were detectable (9). Since the in vitro representative of the human pluripotent stem cell has essentially the same sensitivity to 4HC as CFU-C and BFU-E (17), it is likely that a more immature stem cell than the human pluripotent stem cell, as yet undetectable in vitro, survives the purging regimen and reconstitutes the graft recipient.

Chart 5. The effects of ASTA-Z-7557 (A), ASTA-Z-7595 (B), ASTA-Z-7854 (C), and 4HC (D) incubation on the size of colonies generated by day 12 CFU-s. Shaded columns indicate colonies >2 mm, open columns indicate colonies between 1 and 2 mm, and hatched columns indicate colonies <1 mm. Data are expressed as a percentage of surviving day 12 CFU-s colonies under control incubation conditions and represent the mean of two replicate experiments. Bars, SE.

Clinical trials indicate that purging with 4HC (80 μg/ml) (18) or ASTA-Z-7557 (60–80 μg/ml) (8) results in delayed or inadequate bone marrow engraftment. We suggest two possible explanations for these clinical observations. First, delayed engraftment may be due to damage to transplantable stromal cells (Chart 1–4). In both human (10) and murine (15) studies, stromal cells have been indicated to be transplantable via the i.v. route. However, there is no indication that hemopoietic reconstitution requires the presence of transplanted stromal cells. Alternatively, delayed hemopoietic recovery may be due to stem cell damage. Although a relatively large number of day 12 CFU-s survived incubation with the higher drug doses used in our study, the colony size data (Chart 5) may infer an effect on the quality of these cells. Further study is required to determine whether this colony size reduction was the result of delayed colony formation or migration into the spleen due to repairable drug-
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Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (µg/ml)</th>
<th>1 h incubation</th>
<th>6 h incubation</th>
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<tr>
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<td>Day 7 CFUs</td>
<td>Day 12 CFUs</td>
<td>BFUE</td>
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<tr>
<td>ASTA-Z-7551</td>
<td>10</td>
<td>100</td>
<td>810.0 ± 10.7a</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>74.3 ± 9.2</td>
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<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>1.3 ± 0.8</td>
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<td>ASTA-Z-7595</td>
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<td>100</td>
<td>113.0 ± 18.2</td>
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<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>101.0 ± 15.9</td>
</tr>
<tr>
<td>ASTA-Z-7654</td>
<td>10</td>
<td>100</td>
<td>106.0 ± 22.0</td>
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<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>91.7 ± 17.8</td>
</tr>
<tr>
<td>4HC</td>
<td>10</td>
<td>100</td>
<td>27.5 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>111.6 ± 15.8</td>
</tr>
</tbody>
</table>

*p, ND, not determined.

Table 3

Effect of control incubation conditions on the size distribution of day 12 spleen colonies

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Total day 12 CFUs/tube</th>
<th>Colonies</th>
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<tbody>
<tr>
<td></td>
<td>&lt;1 mm</td>
<td>1-2 mm</td>
</tr>
<tr>
<td>0</td>
<td>2991.0 ± 171.1</td>
<td>938.5 ± 161.9</td>
</tr>
<tr>
<td>1</td>
<td>2643.8 ± 17.2</td>
<td>860.9 ± 63.5</td>
</tr>
<tr>
<td>6</td>
<td>2286.7 ± 15.9</td>
<td>780.5 ± 33.2</td>
</tr>
</tbody>
</table>

*Mean ± SE.

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REFERENCES

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