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EFFECTS OF ALDEHYDE DEHYDROGENASE INHIBITORS ON THE *EX VIVO* SENSITIVITY OF MURINE LATE SPLEEN COLONY-FORMING CELLS (DAY-12 CFU-S) AND HEMATOPOIETIC REPOPULATING CELLS TO MAFOSFAMIDE (ASTA Z 7557)*

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Abstract—The effects of inhibitors of aldehyde dehydrogenase activity on the sensitivity of murine pluripotent hematopoietic stem cells to oxazaphosphorine anticancer agents, e.g. mafosfamide, were examined using two different assay procedures. In the first part of the investigation, the *ex vivo* sensitivity of murine day-12 spleen colony-forming cells (CFU-S) to mafosfamide was determined in the absence and presence of known inhibitors of aldehyde dehydrogenase activity, viz. diethyldithiocarbamate and cyanamide. These results were compared to those generated for day-8 CFU-S. Day-12 CFU-S were less sensitive to mafosfamide, and to phosphoramide mustard, although the difference in sensitivity to the latter was less marked. Diethyldithiocarbamate and cyanamide each potentiated the cytotoxic action of mafosfamide toward both day-12 and day-8 CFU-S; they did not potentiate the cytotoxic action of phosphoramide mustard toward these cells. Since cellular aldehyde dehydrogenases are known to catalyze the oxidation of 4-hydroxycyclophosphamide/aldophosphamide, the major transport form of mafosfamide, to the relatively nontoxic acid, carboxyphosphamide, the results suggest that intracellular aldehyde dehydrogenase activity is a determinant of the sensitivity of day-12 CFU-S, as well as of day-8 CFU-S, to mafosfamide and other oxazaphosphorines, e.g. cyclophosphamide. In the second part of this investigation, a murine syngeneic bone marrow transplantation model was used to determine the *ex vivo* sensitivity of murine hematopoietic repopulating cells to mafosfamide in the absence and presence of diethyldithiocarbamate. Specifically, the ability of treated marrow grafts to repopulate the hematopoietic system, and thereby save recipients from the otherwise lethal effect of total body irradiation, was determined. Diethyldithiocarbamate potentiated the cytotoxic action of mafosfamide, but not that of phosphoramide mustard, toward hematopoietic repopulating cells. These observations support our previous contention that aldehyde dehydrogenase activity is an operative determinant with regard to the sensitivity of murine pluripotent hematopoietic stem cells to oxazaphosphorines.

Although the basis for the relatively favorable margin of safety exhibited by oxazaphosphorine nitrogen mustard anticancer and immunosuppressive agents, e.g. cyclophosphamide, 4-hydroperoxycyclophosphamide and mafosfamide, has not been fully elucidated, it may be due in large part to a relative insensitivity of hematopoietic stem cells to these agents [1, 2]. We reported recently [2] that concurrent exposure of murine bone marrow cells to cytotoxic agents and inhibitors of aldehyde dehydrogenase activity results in a potentiation of the cytotoxic action of oxazaphosphorines toward spleen colony-forming cells (CFU-S‡); these cells display characteristics of pluripotent hematopoietic stem cells (reviewed in Ref. 3). Cellular aldehyde dehydrogenases are known to catalyze the oxidation of 4-hydroxycyclophosphamide/aldophosphamide, an intermediate in cyclophosphamide, 4-hydroperoxycyclophosphamide and mafosfamide activation, to the relatively nontoxic acid, carboxyphosphamide [4–10]. Thus, we concluded that the basis for the relative insensitivity of murine pluripotent hematopoietic stem cells to oxazaphosphorines is the aldehyde dehydrogenase activity contained in these cells.

In the experiments referred to above, we assayed for CFU-S by counting macroscopic spleen colonies on day 8 following transplantation. However, CFU-S are apparently a heterogeneous population of cells [11–18], and it has been suggested [15] that cells that give rise to late (day 11–14) spleen colonies, as compared to those that give rise to early (day 6–8) spleen colonies, may be more representative of "true" pluripotent stem cells because of their propensity for self-renewal and for the generation of mixed lineage colonies. For this reason, we have repeated our previous experiments, this time using day-12 CFU-S as our model.

In the second part of this investigation, we utilized a murine syngeneic bone marrow transplantation

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‡ Abbreviations: CFU-S, colony-forming unit—spleen; TBI, total body irradiation; DDTC, diethyldithiocarbamate; and IC_{50}, concentration of drug required to effect a 50% inhibition of colony formation.

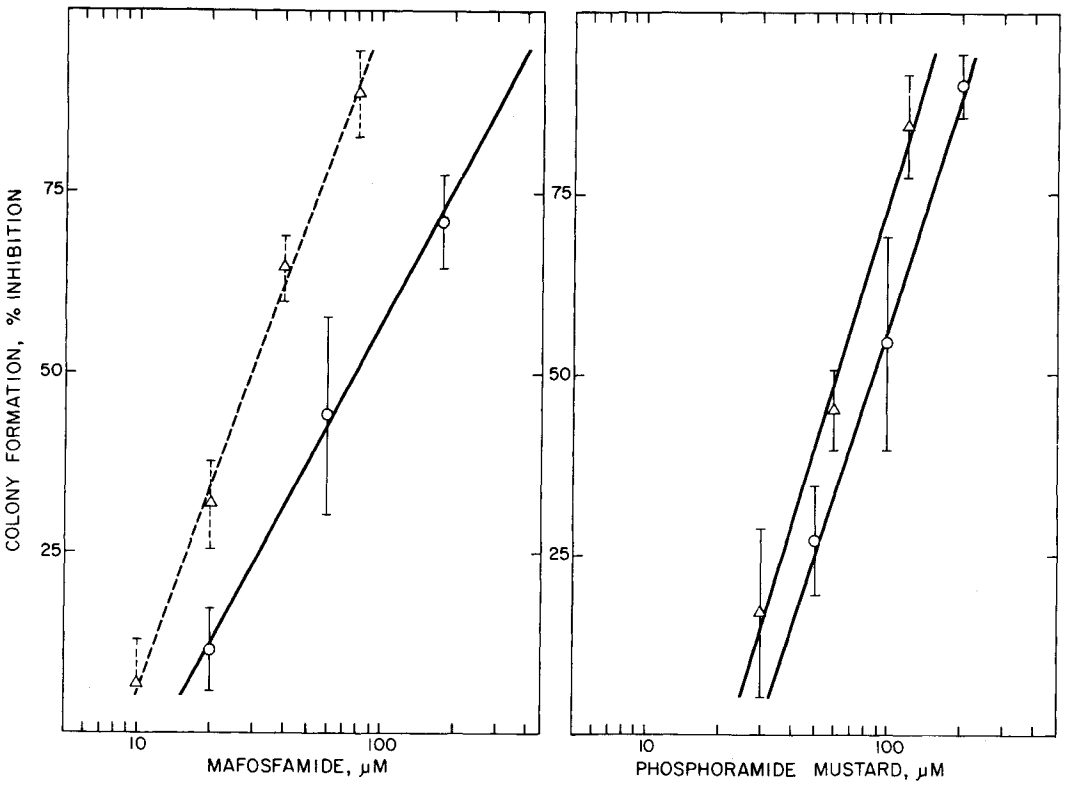

Fig. 1. Sensitivity of murine day-8 and day-12 CFU-S to mafosfamide and to phosphoramide mustard *ex vivo*. BALB/c bone marrow cells were incubated for 60 min at 37°, vehicle or cytotoxic agent was added, and incubation was continued at 37° for an additional 30 min. The cells were then harvested, resuspended in drug-free medium, and assayed for day-8 (△) or day-12 (○) CFU-S as described in Materials and Methods. Control colony formation per 8×10^4 nucleated cells injected: vehicle, 19–23 day-8 colonies and 21–29 day-12 colonies. Points: mean ± SD of observations made in three to five mice. Dashed line: data previously reported [2] and shown here for comparative purposes. IC_{50} Values and 95% confidence intervals (parentheses): mafosfamide/day-8 CFU-S, 30 (28–32) μM; mafosfamide/day-12 CFU-S, 79 (61–100) μM; phosphoramide mustard/day-8 CFU-S, 61 (54–70) μM; phosphoramide mustard/day-12 CFU-S, 85 (74–98) μM.

model as an alternative method of determining the proliferative capability of murine pluripotent hematopoietic stem cells following *ex vivo* drug treatment, and as an additional method in which to ascertain whether aldehyde dehydrogenase activity is an operational determinant with regard to the sensitivity of pluripotent hematopoietic stem cells to oxazaphosphorines. Specifically, the effect of an aldehyde dehydrogenase inhibitor on the *ex vivo* sensitivity of hematopoietic repopulating cells to mafosfamide was determined by measuring the ability of treated, transplanted, syngeneic bone marrow cells to save mice from the otherwise lethal effect of total body irradiation (TBI).

* 2-[bis-(2-Chloroethyl)-amino]-4-(2-sulfoethylthio)-tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide cyclohexylamine salt. Mafosfamide is a relatively stable precursor of 4-hydroxycyclophosphamide/aldophosphamide. It rapidly and spontaneously (without benefit of enzymatic involvement) gives rise to 4-hydroxycyclophosphamide/aldophosphamide under physiologic conditions.

MATERIALS AND METHODS

Materials. Mafosfamide* and phosphoramide mustard·cyclohexylamine were supplied by Dr. P. Hilgard (Asta-Werke AG, Bielefeld, Federal Republic of Germany) and Mr. L. H. Kedda (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) respectively. Diethyldithiocarbamate (DDTC) and cyanamide were purchased from the Sigma Chemical Co., St. Louis, MO. Fetal bovine serum was purchased from the GIBCO Laboratories, Grand Island, NY.

All drugs were dissolved in serum-free drug-exposure medium, *vide infra*. Drug solutions were sterilized by passage through 0.22 μm Millipore filters; all were used within 30 min of preparation and were kept on ice prior to their use. Drug-exposure medium was a phosphate-buffered saline-based solution, pH 7.4, prepared as previously described [2].

Animals. BALB/c mice were obtained from the University of Minnesota Mouse Colony. Male mice, aged 12–16 weeks, were used. Animals were housed

in plastic cages fitted with filtered lids and were given standard laboratory food and water *ad lib*. The drinking water of irradiated mice was supplemented with oxytetracycline and neomycin during the entire course of the experiment.

Preparation of murine bone marrow cell suspensions, and drug exposure. Murine bone marrow cell suspensions were prepared in drug-exposure medium (supplemented with 10% fetal bovine serum for use in the repopulation experiments) as previously described [2]. Portions of the cell suspension were transferred to 15-ml centrifuge tubes, and vehicle or aldehyde dehydrogenase inhibitor (0.5 ml) was added. The cell suspensions were then incubated for 60 min at 37°. Vehicle or cytotoxic agent (0.1 ml) was added, and the cell suspensions were incubated at 37° for an additional 30 min. The nucleated cell concentration during drug exposure was 4×10^5 cells/ml in a total volume of 5 ml, and 5×10^5 cells/ml in a total volume of 6 ml, for the CFU-S and repopulation assays respectively. Immediately following drug exposure, cells were pelleted by centrifugation (300 *g*) and resuspended in fresh, serum-free medium.

CFU-S assay. The assay of Till and McCulloch [19] was used as previously described [2] with the exception that macroscopic spleen colonies were counted on day 8 or day 12. Control colony formation ranged from 18 to 28 day-8 colonies and 18 to 29 day-12 colonies formed per 8×10^4 nucleated cells injected.

Repopulation assay. Drug- or vehicle-treated cells were injected into the tail veins of lethally-irradiated syngeneic mice. Recipient mice had been conditioned with 1100 rad TBI (Mark I ^{137}Cs irradiator, 150 rad/min) on the day before transplantation. Mice were injected with 1×10^6 nucleated cells in a volume of 0.5 ml; 5×10^4 viable cells are required to save all lethally-irradiated animals (see Results). Following transplantation, animal survival was monitored for 30 days. All mice (N = 28) receiving TBI but no cells, i.e. medium only, died between day 9 and day 14 following sham transplantation. All mice (N = 22) receiving TBI plus 1×10^6 cells treated with vehicle, or aldehyde dehydrogenase inhibitor only, survived for more than 30 days.

Data analysis. Computer-assisted regression analysis was used to generate all straight line functions. The method of Tallarida and Murray [20] was used to generate IC_{50} values and 95% confidence intervals.

RESULTS

Others have reported that murine day-12 CFU-S are less sensitive to oxazaphosphorines than are day-8 CFU-S [21–23]. Such observations could be explained if CFU-S are heterogeneous and the various cell populations differ in their sensitivity to oxazaphosphorines. Initial experiments (Fig. 1) confirmed that day-12, as compared to day-8, CFU-S are indeed less sensitive to mafosfamide. Moreover, day-12 CFU-S were also less sensitive to phosphoramide mustard; the difference in sensitivity was less marked, but statistically significant ($P < 0.05$). This latter observation suggests that the observed difference in sensitivity to oxazaphosphorines between day-8 and day-12 CFU-S is not due to a factor that influences the sensitivity of cells to oxazaphosphorines specifically, e.g. aldehyde dehydrogenase activity.

The observed difference in sensitivity to mafosfamide between early and late spleen colony-forming cells was examined more closely in a 14-day time course experiment (Fig. 2). The experimental period was limited to 14 days for technical reasons, viz it was not possible to accurately count individual colonies after 14 days because of crowding and because of the appearance of a large number of minute, but macroscopically visible, nodules. Spleen colony formation reached a maximum 6 days after the injection of vehicle-treated marrow cells; thereafter, the number of colonies remained constant. In contrast, a plateau level of colony formation was not reached until 10 days after the injection of marrow cells treated with 50 μM mafosfamide and may not have been reached at all following the injection of marrow cells treated with 150 μM mafosfamide. Moreover, at all time points, spleen colony size was noticeably smaller when marrow cells were treated with mafosfamide. While not inconsistent with a multiple population scenario, these observations are also consistent with the possibility that the same cell population is being assayed at all time points and that the basis for the apparent differential sensitivity

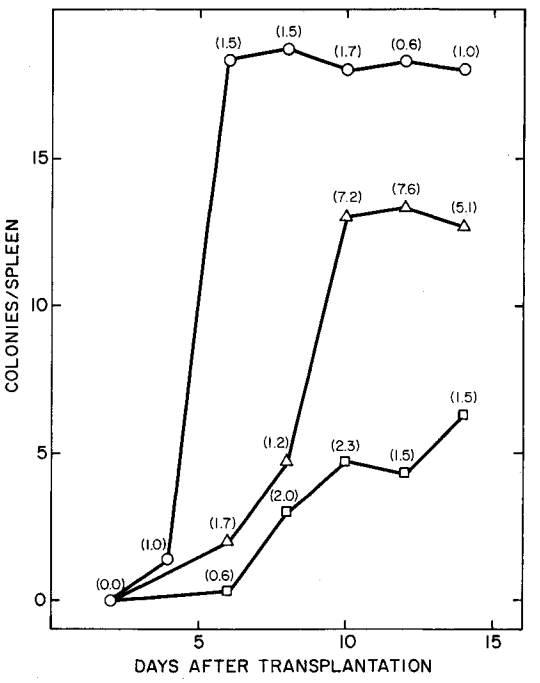

Fig. 2. Time course of murine spleen colony formation from control and mafosfamide-treated bone marrow cells. BALB/c bone marrow cells were incubated for 60 min at 37°, vehicle (○) or mafosfamide (△, 50 μM; □, 150 μM) was added, and incubation was continued at 37° for an additional 30 min. Following incubation, the cells were harvested, resuspended in drug-free medium, and assayed for CFU-S as described in Materials and Methods. Macroscopic spleen colonies were counted at various times following transplantation. Points: mean of observations made in three mice. Parentheses, SD.

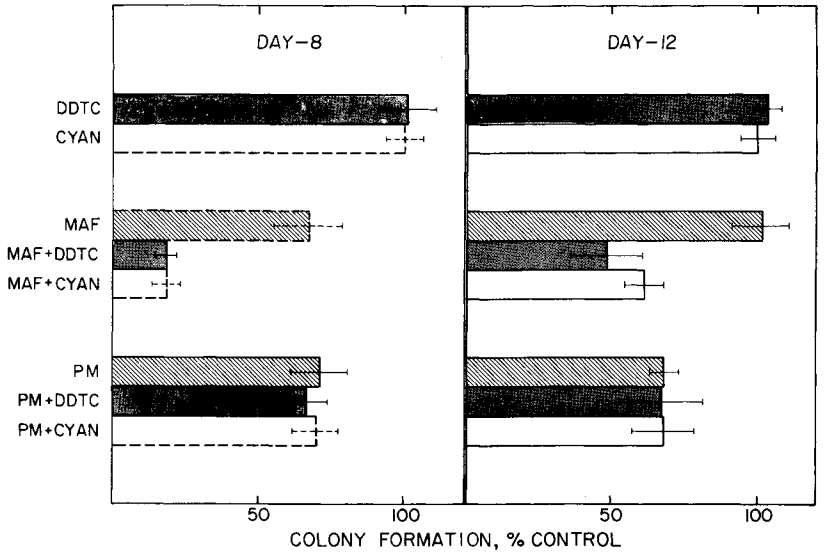

Fig. 3. Sensitivity of murine day-8 and day-12 CFU-S to fixed concentrations of mafosfamide and phosphoramide mustard in the absence and presence of inhibitors of aldehyde dehydrogenase activity. BALB/c bone marrow cells were incubated for 60 min at 37° in the absence or presence of DDTC (300 μM) or cyanamide (60 μM). Vehicle or cytotoxic agent was then added, and incubation was continued at 37° for an additional 30 min. Concentrations of cytotoxic agents used in the day-8 and day-12 CFU-S experiments, respectively, were mafosfamide (20 μM, 20 μM) and phosphoramide mustard (30 μM, 60 μM). Following incubation, the cells were harvested, resuspended in drug-free medium, and assayed for day-8 or day-12 CFU-S as described in Materials and Methods. Results are expressed as percent of the relevant control. Control colony formation per 8×10^4 nucleated cells injected: vehicle, 20–28 day-8 colonies and 18–25 day-12 colonies; DDTC, 22–28 day-8 colonies and 22–24 day-12 colonies; and cyanamide, 20–27 day-8 colonies and 21–24 day-12 colonies. Bars: mean ± SD for observations made in three to five mice. Dashed bars: data previously reported [2] and shown here for comparative purposes.

is a sublethal effect of the drug that alters the proliferation kinetics of the surviving colony-forming cells, i.e. a cytostatic effect. If such is indeed the case, the differential sensitivity to mafosfamide (and other agents) exhibited by day-8 and day-12 CFU-S would, in fact, be artifactual with regard to cytocidal action, and would be the result of an inappropriate experimental design.

The effects of aldehyde dehydrogenase inhibitors on the sensitivity of day-12 CFU-S to mafosfamide, and to phosphoramide mustard, were examined in the next series of experiments (Fig. 3). DDTC and cyanamide, both known inhibitors of aldehyde dehydrogenase activity [24, 25], were not cytotoxic at the concentrations used and potentiated the cytotoxic action of mafosfamide toward day-12 CFU-S. They did not potentiate the cytotoxic action of phosphoramide mustard toward these cells. These results are essentially identical to those obtained when day-8 CFU-S were assayed. The ability of DDTC to potentiate the cytotoxic action of mafosfamide toward day-12 CFU-S was examined more closely (Fig. 4). Day-12 CFU-S were 2.4 times more sensitive to mafosfamide in the presence of DDTC.

In the next series of experiments, an alternative method of determining murine pluripotent hematopoietic stem cell proliferative function following *ex vivo* drug treatment, viz. a murine syngeneic bone marrow transplantation model, was utilized to ascertain whether aldehyde dehydrogenase activity is an operational determinant with regard to the sensitivity of murine hematopoietic repopulating cells to mafosfamide. A preliminary experiment established that, in the absence of any drug pretreatment, injection of at least 5×10^4 viable nucleated bone marrow cells was required to save all mice from the otherwise lethal effects of TBI (Fig. 5). DDTC potentiated the cytotoxic action of mafosfamide, but not that of phosphoramide mustard, toward hematopoietic repopulating cells as measured by the ability of treated marrow cells to save mice from lethal TBI (Fig. 6).

DISCUSSION

The first part of this investigation was predicated on the possibility that day-12 CFU-S, as compared to day-8 CFU-S, may more accurately measure the "true" pluripotent hematopoietic stem cell population [14–18]. The most persuasive evidence in this regard was provided by Magli *et al.* [15]. These investigators reported that many early (day 6–8) spleen colonies are predominantly monolineage in nature, lack cells capable of forming secondary colonies, and do not develop into the multilineage colonies, seen at later time points (day 11–14), that do contain cells capable of secondary colony formation. Thus, they concluded that pluripotent stem cells are only measured in assays in which spleen colonies are counted at later time points, i.e. after day 11.

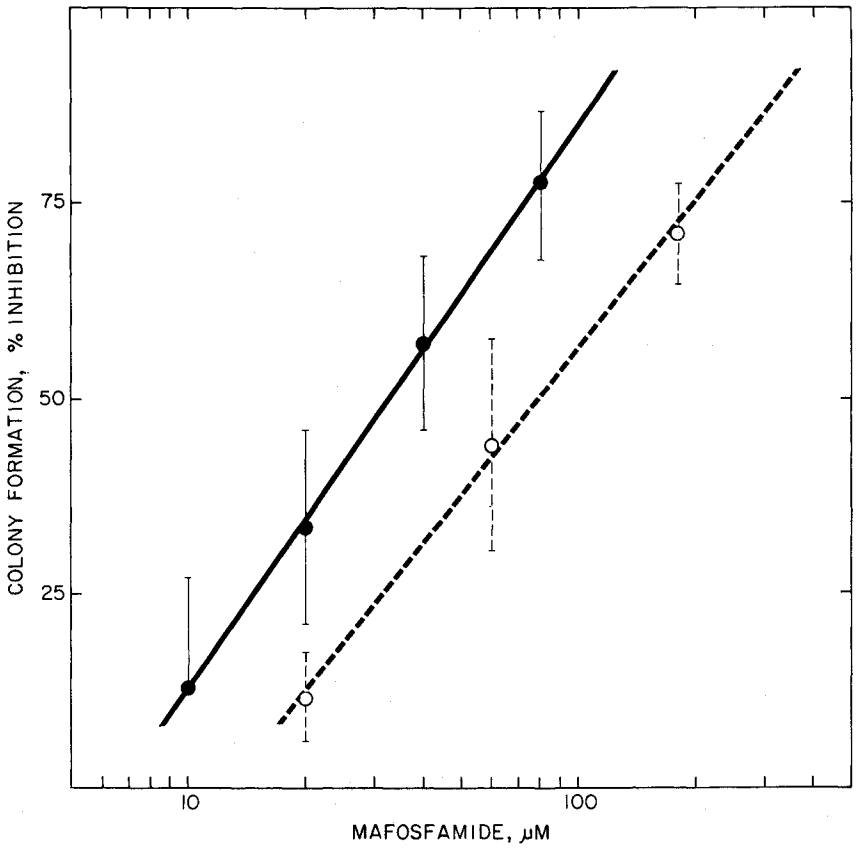

Fig. 4. Sensitivity of murine day-12 CFU-S to mafosfamide in the absence and presence of a fixed concentration of DDTC. BALB/c bone marrow cells were incubated for 60 min at 37° in the absence (○) or presence (●) of 300 μM DDTC. Vehicle or mafosfamide was then added, and incubation was continued at 37° for an additional 30 min. Following incubation, the cells were harvested, resuspended in drug-free medium, and assayed for day-12 CFU-S as described in Materials and Methods. Results are expressed as percent inhibition of the relevant control. Control colony formation per 8×10^4 nucleated cells injected: vehicle, 23–29 colonies; and DDTC, 22–25 colonies. Points: mean ± SD of observations made in four mice. Dashed line: data previously presented in Fig. 1 and shown here for comparative purposes. IC_{50} Values and 95% confidence intervals (parentheses): mafosfamide, 79 (61–100) μM: mafosfamide + DDTC, 33 (27–40) μM.

Inhibitors of aldehyde dehydrogenase activity potentiated the cytotoxic action of mafosfamide toward both day-8 and day-12 CFU-S. Thus, regardless of whether day-8 spleen colony formation is an accurate measure of the "true" pluripotent hematopoietic stem cell population, aldehyde dehydrogenase activity appears to be an operative determinant with regard to the sensitivity of pluripotent hematopoietic stem cells to oxazaphosphorines.

This conclusion is supported by the results obtained in the second part of this investigation, in which the effect of DDTC on the sensitivity of murine hematopoietic repopulating cells to mafosfamide, and to phosphoramide mustard, was determined using a murine syngeneic bone marrow transplantation model. When mice are exposed to high doses of total body irradiation, they die in approximately 9–18 days from infection and hemorrhage resulting from blood cell aplasia [26]. Death of these animals can be prevented by the infusion of syngeneic donor bone marrow cells that are capable of repopulating the recipients' hematopoietic and immune systems [27]. The capability of a given marrow sample to save a lethally-irradiated recipient is proportional to the quantity and quality of hematopoietic precursors present in the sample. Thus, by measuring the ability of a drug-treated marrow sample to save animals from the otherwise lethal effect of TBI, a syngeneic bone marrow transplantation model of the type described above can be used to determine the sensitivity of repopulating cells in the marrow graft to cytotoxic agents. The assumption is that cytotoxicity toward precursor cells that are directly responsible for the hematopoietic reconstitution of lethally-irradiated recipients is being measured. However, the possibility that an indirect effect on repopulating cells due to cytotoxicity toward accessory cells that may be required for marrow engraftment cannot be ruled out.

DDTC potentiated the cytotoxic action of mafos-

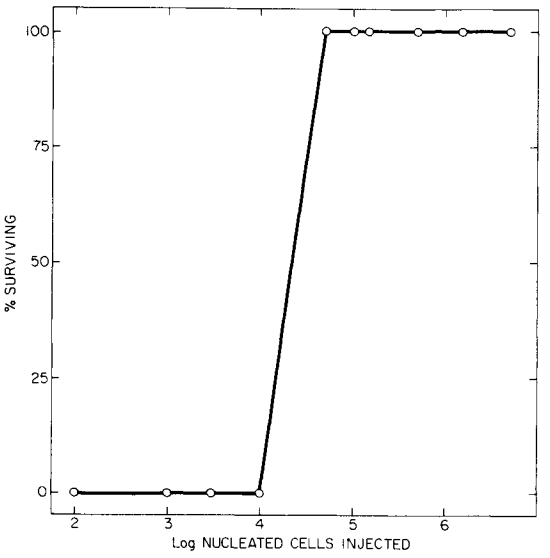

Fig. 5. Number of bone marrow cells required to save mice from the effects of lethal total body irradiation. Increasing numbers of nucleated syngeneic bone marrow cells were injected into lethally-irradiated (1100 rad TBI) BALB/c mice (N = 4 or 6 mice for each point). Animal survival was monitored for 30 days following transplantation.

famide, but not that of phosphoramide mustard, toward hematopoietic repopulating cells, providing further support for the contention that pluripotent hematopoietic stem cells contain the relevant aldehyde dehydrogenase activity.

The significance of our findings with regard to oxazaphosphorine-specific selective cytotoxicity has been discussed previously [2]. An additional point is brought out by the results of the present investigation. Recently, much attention has been focused on the use of autologous bone marrow transplantation in the treatment of various hematopoietic neoplasias [28]. Successful outcome requires selective *ex vivo* elimination of residual tumor cells that are present in the marrow aspirates. Oxazaphosphorines have shown promise in that regard [29, 30], presumably as a consequence of a relative insensitivity of hematopoietic repopulating cells to these agents. Our finding that hematopoietic repopulating cells contain significant amounts of the relavant aldehyde dehydrogenase activity, in conjunction with studies indicating that certain oxazaphosphorine-sensitive leukemia cells lack, or contain small amounts of, this activity [10, 31, 32], may explain the presumed relative insensitivity of hematopoietic repopulating cells to the oxazaphosphorines.

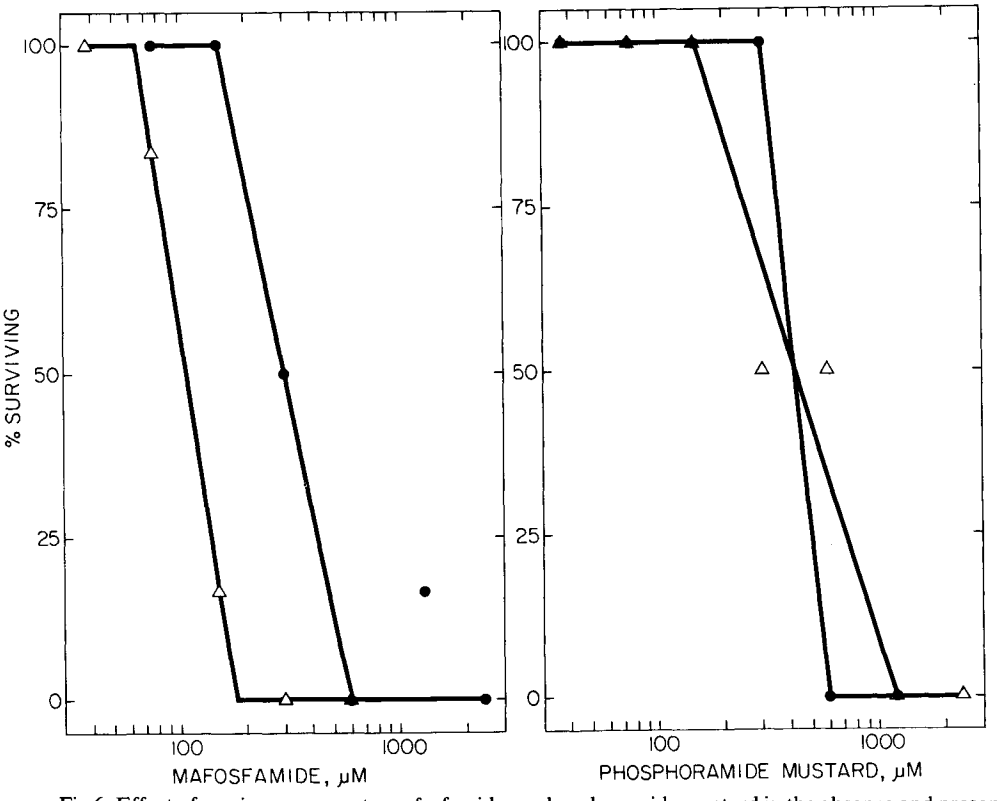

Fig 6. Effect of *ex vivo* exposure to mafosfamide or phosphoramide mustard in the absence and presence of DDTC on the ability of transplanted syngeneic bone marrow cells to repopulate and save lethally-irradiated recipient mice. BALB/c bone marrow cells were incubated for 60 min at 37° in the absence (●) or presence (△) of 300 μM DDTC. Vehicle or cytotoxic agent was then added, and incubation was continued at 37° for an additional 30 min. Following incubation, the cells were harvested, resuspended in drug-free medium, and injected into lethally-irradiated (1100 rad TBI) BALB/c recipients (N = 4 or 6 mice for each point) as described in Materials and Methods. Animal survival was monitored for 30 days following transplantation. Fifty percent animal survival: mafosfamide, 300 μM; mafosfamide + DDTC, 107 μM; phosphoramide mustard, 425 μM; phosphoramide mustard + DDTC, 425 μM.

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