

CONCISE REPORT

Purging Murine Leukemic Marrow With Alkyl-Lysophospholipids

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Autologous bone marrow transplantation is potentially curative in the treatment of acute leukemia if residual leukemic cells in the marrow can be eliminated prior to transplantation. We studied the purging effects of a synthetic alkyl-lysophospholipid (ALP) on marrow containing leukemic cells from a transplantable myelomonocytic leukemia (WEHI-3B) in BALB/c mice. Simulated remission bone marrow containing 2% leukemic cells treated in vitro with 20 and 100 $\mu\text{g}/\text{mL}$ of ET-18-OCH₃ (1-octadecyl-2-methyl-sn-glycerol-3-phosphocholine) significantly prolonged survival of lethally irradiated transplanted recip-

ients. At a dose of 100 $\mu\text{g}/\text{mL}$, 88% of the mice survived for the duration of the experiment (approximately five months). Autopsies showed that 25% of these survivors had microscopic evidence of leukemia. Thus, in vitro treatment of marrow eliminated leukemic blasts and spared sufficient normal stem cells to allow hematologic reconstitution. The effect of ET-18-OCH₃ is not entirely selective for leukemic cells. A spleen colony assay showed that ALP has some cytotoxic effect on normal hematopoietic stem cells.

BONE MARROW TRANSPLANTATION for the treatment of acute leukemia is an important therapeutic strategy designed to destroy residual leukemic cells and replace marrow with normal hematopoietic elements. There is evidence to suggest that transplantation is at least as effective as conventional chemotherapy and may be superior¹; however, the exact role of transplantation in the treatment of acute leukemia needs further clarification. Bone marrow is usually transplanted from a genotypical HLA-identical sibling donor. A major complication of the procedure is severe graft-*v*-host disease (GVHD), which occurs in 30% to 50% of recipients.² Autologous transplantation avoids GVHD but marrow harvested during remission usually contains small numbers of residual leukemic cells that must be purged if the transplant is to be curative. In some respects, purging the marrow to be used for transplantation is similar to techniques for eliminating leukemic cells in the whole animal. Ideally, differential toxicity should be achieved with sparing of normal stem cells and eradication of leukemic cells. A promising approach to differential toxicity is the use of an alkyl-lysophospholipid (ALP) as a purging agent.

The ALPs are synthetic analogues of 2-lysophosphatidyl-choline (LPC). The latter is readily susceptible to enzymatic degradation in vivo. An ether bond in the sn-1 position of the glycerol moiety prevents hydrolysis

of the aliphatic side chain by lysophospholipase, and a methoxy group on the sn-2 position prevents acylation by LPC-acyltransferase. We used the synthetic analogue 1-octadecyl-2-methyl-sn-glycerol-3-phosphocholine (ET-18-OCH₃) in our experiments (Fig 1). This analogue is degraded primarily by a 1-0-alkylcleavage enzymes.³ The drug may have selective hematopoietic toxicity, sparing normal cells that contain alkyl-cleavage enzymes capable of degrading ALP, and destroying leukemic cells that lack cleavage enzymes.^{4,5}

In this report, we studied autologous marrow transplantation in mice, and evaluated the purging potential of ALP on a simulated remission bone marrow containing leukemic cells from a transplantable myelomonocytic leukemia.⁶

MATERIALS AND METHODS

A remission bone marrow was simulated by mixing WEHI 3-B leukemic cells, harvested from ascitic fluid, with normal murine marrow cells so that the concentration of leukemic cells was 2%. After mixing, the percentage was confirmed by differential counts of Giemsa-stained smears. Normal bone marrow was obtained from the femurs of female BALB/c mice. Dr Donald Metcalf (Cancer Research Unit, Walter and Eliza Hall Institute of Medical Research, Victoria, Australia) provided the WEHI 3-B myelomonocytic leukemia, which has been maintained by serial subcutaneous or intraperitoneal passage in BALB/c mice since October 1972. Neoplastic cells constituted greater than 80% of the cells in the ascitic fluid. Cytologically, they had immature nuclear chromatin, nucleoli, basophilic cytoplasm, and azurophilic granules in the region of the Golgi. Cytochemical analysis of the leukemic cells showed enzymatic markers similar to human myelomonocytic leukemia: myeloperoxidase and α -naphthyl butyrate esterase.

The simulated remission bone marrow was incubated with varying concentrations of ALP (ET-18-OCH₃), supplied by Dr Paul G. Munder of the Max-Planck-Institut für Immunbiologie (Freiburg, FRG). Storage of stock solutions of 1,000 $\mu\text{g}/\text{mL}$ in RPMI 1640 containing 20% fetal calf serum (FCS) was at -20°C , and they were stable for at least nine months without loss of activity. The cell suspension was incubated with the drug for 24 hours at 37°C , since

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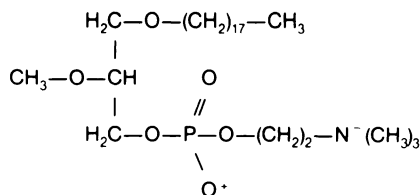


Fig 1. Structural formula of the ALP ET-18-OCH₃. ET, ether bond; 18, number of carbon atoms in the aliphatic side chain; OCH₃, radical in the sn-2 position.

it has been shown that temperature strongly influences the cytotoxicity of ALP.⁷ Incubation volumes were 0.5 mL and the concentration of nucleated cells was 5×10^7 /mL. Final concentrations of ALP in the incubation mixtures were 0, 2.5, 5.0, 10.0, 20.0, and 100.0 $\mu\text{g}/\text{mL}$.

After the incubation period, untreated normal bone marrow and treated and untreated simulated remission bone marrow were injected intravenously into lethally irradiated female BALB/c mice. Each mouse transplanted with the simulated leukemic remission marrow received 5×10^6 normal nucleated marrow cells and 10^5 WEHI 3-B leukemic cells. The dose of normal cells approximated 2×10^8 nucleated cells per kilogram of body weight, which was adequate for successful hematologic reconstitution in our control animals, and is a dose that can be achieved from human donation.⁸ Recipient animals received a total dose of 780 rad of total body irradiation with ¹³⁷Ce (model GC-40, Gammacell Atomic Energy Ltd, Ottawa, Canada) 24 hours before transplantation. As a control, one group was not irradiated and received untreated remission marrow containing the leukemic cells. Each group contained from nine to 16 mice.

The mice were caged individually and received food and water ad libitum. We terminated the experiment after five months. All animals were autopsied and organs were examined histologically for evidence of leukemia.

We used the spleen colony assay to quantitate the effect of ALP on normal hematopoietic stem cells.⁹ Normal marrow was obtained from the femurs of BALB/c female mice. The cells were suspended, at a concentration of 10^6 /mL, in RPMI 1640 containing 10% FCS in the presence of 0, 2.5, 5.0, 10.0, 20.0, or 100.0 $\mu\text{g}/\text{mL}$ of ALP, and incubated in a CO₂ atmosphere at 37 °C for 24 hours. After the incubation period, 0.1 mL of the treated marrow was injected into the tail vein of heavily irradiated (730 rad) syngeneic mice. Spleen colonies were counted ten days after injection. Each group contained ten mice.

Survival curves were plotted by the method of Kaplan and Meier,¹⁰ and log rank and Wilcoxon *P* values were used to compare groups. Standard linear regression techniques were used for evaluating the spleen colony assay. The log fraction of the colony-forming units (CFUs) surviving per spleen was plotted against the log dose. Student's *t* test and Wilcoxon's rank sign test were used to compare groups.

RESULTS

The survival of mice receiving total body irradiation was 11.5 ± 0.96 days (mean \pm SEM). Irradiated mice transplanted with 5×10^6 normal murine marrow cells were successfully rescued. In this experiment, the incidence of radiation deaths in transplanted mice was 9%. Irradiated mice transplanted with the untreated simulated remission leukemic bone marrow survived 20.2 ± 1.0 days, a period beyond which deaths could

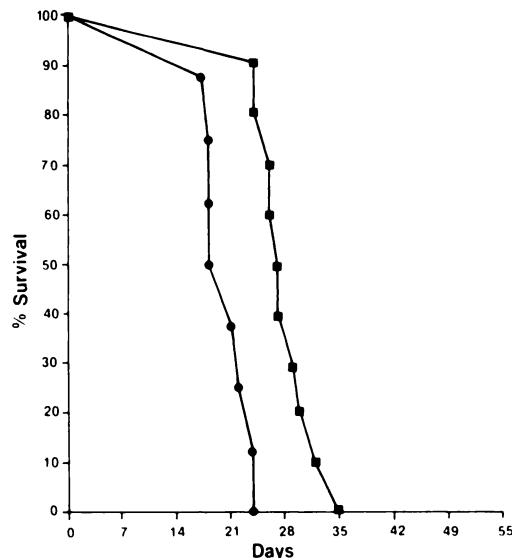


Fig 2. Survival of female BALB/c mice transplanted with a simulated leukemic bone marrow containing 10^5 WEHI 3-B leukemic cells and 5×10^6 normal nucleated hematopoietic cells. ●, irradiated (780 rad) mice ($n = 9$); ■, nonirradiated mice ($n = 10$).

not be attributed to irradiation, suggesting reestablishment of normal hematopoiesis. These mice died of leukemia. Nonirradiated mice transplanted with leukemic marrow were more resistant to the leukemia and died at 28.0 ± 1.1 days (Fig 2).

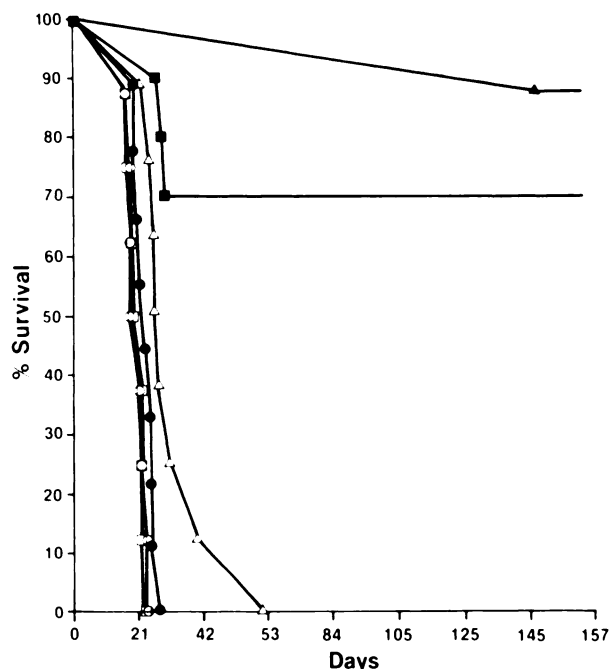


Fig 3. Kaplan-Meier plot of the survival of irradiated female BALB/c mice transplanted with a simulated leukemic bone marrow pretreated in vitro with the ALP ET-18-OCH₃. ○, ALP 0 $\mu\text{g}/\text{mL}$ ($n = 9$); □, ALP 2.5 $\mu\text{g}/\text{mL}$ ($n = 10$); ●, ALP 5.0 $\mu\text{g}/\text{mL}$ ($n = 10$); △, ALP 10.0 $\mu\text{g}/\text{mL}$ ($n = 10$); ■, ALP 20.0 $\mu\text{g}/\text{mL}$ ($n = 10$); ▲, ALP 100.0 $\mu\text{g}/\text{mL}$ ($n = 16$).

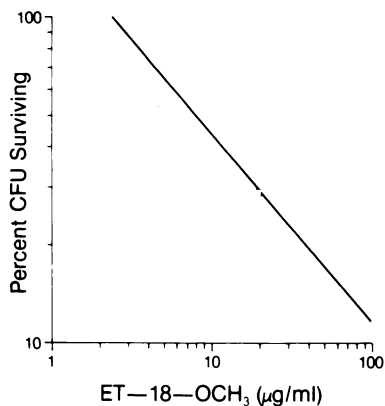


Fig 4. Survival curve for the spleen colony-forming ability of murine (BALB/c) marrow cells treated in vitro at 37 °C for 24 hours with the ALP ET-18-OCH₃.

In vitro pretreatment of the marrow with ALP above a dose of 5 µg/mL impressively improved survival. A significant effect was observed when marrow was pretreated in vitro with 10 µg/mL ($P < .05$); however, all mice died of leukemia. When marrow was treated with doses of 20 and 100 µg/mL, 70% and 88% of the mice survived, respectively (Fig 3). The difference in survival between these groups was not significant. Autopsies showed that 50% and 25% of the long-term survivors, respectively, had microscopic evidence of leukemia, whereas the others had no evidence of neoplastic disease.

The spleen colony assay showed that ALP does have some cytotoxic effect on normal hematopoietic cells (Fig 4). Suppression of spleen colony formation, after exposure to ALP, was significant at all doses tested, compared with the untreated control ($P < .05$); however, approximately 30% survival of normal marrow CFUs was observed with doses of 20 µg/mL.

DISCUSSION

Mice provide an ideal model for the study of autologous bone marrow transplantation in acute leukemia: inbred strains have an identical gene pool for the donor and recipient, the quantity of cells that can be harvested for transfusion is comparable with human marrow transplantation, an in vivo technique exists for quantitating normal and neoplastic hematopoietic stem cells, and transplantable neoplasms are available for evaluating both acute lymphoblastic and myeloblastic leukemias. In this study, we focused our attention on acute myeloid leukemia using a transplantable tumor with the characteristics of acute myelomonocytic leukemia.

Several approaches have been used in animal experiments to purge syngeneic marrow of leukemic cells. These include heterologous cytotoxic complement-mediated antileukemic antibodies,¹¹⁻¹³ antibody-toxin

conjugates,^{14,15} and 4-hydroperoxycyclophosphamide (4HC), which is a chemotherapeutic agent with greater toxicity to cycling bone marrow progenitors.¹⁶ In humans, attempts have been made to purge malignant cells from marrow using 4HC, antithymocyte globulin, and heterologous and monoclonal antibodies.¹⁷⁻¹⁹ Experience is still too limited to evaluate the success of these agents in humans.

We used ALP to eradicate a low percentage of leukemic myelomonoblasts from murine marrow. Long-term survival resulted when marrow was treated with 20 and 100 µg/mL. ALP has tumoricidal activity by two different mechanisms. It enhances macrophage-mediated killing of tumor cells and it also has a direct cytotoxic effect by altering lipid synthesis.²⁰ Although we did not specifically distinguish between these mechanisms, the latter was most likely the cause of cytotoxicity in our experiments. Successful ablation of tumor cells from tissue in vitro does not necessarily correlate with eradication of neoplastic cells in vivo. The differences may be due to the distribution of the drug, local concentrations, drug metabolism, or the total tumor burden requiring eradication. Although we were able to achieve long-term survival in animals inoculated with marrow containing 10⁵ WEHI 3-B cells pretreated in vitro with 20 or 100 µg/mL of ALP, we observed no effect when mice were inoculated intraperitoneally with 10⁵ WEHI-3B cells and treated with daily intraperitoneal doses of 5, 10, and 20 mg/kg of ALP (unpublished observations).

Normal hematopoietic cells can withstand treatment with ALP, as shown by tritiated thymidine incorporation into normal human bone marrow cells, despite treatment by this agent.⁴ Hematopoietic toxicity was not observed in a patient treated with the drug.²¹ In contrast, we did detect a cytotoxic effect on normal hematopoietic stem cells using the spleen colony assay; however, even at 100 µg/mL, a dose that frequently showed no spleen colony formation, complete hematologic reconstitution occurred when the treated marrow was transplanted and only one of 16 mice had a transplant-related death, indicating that the lethally irradiated transplanted animal is a more sensitive means than the spleen colony assay for evaluating the survival of hematopoietic stem cells. We calculate that each mouse received 25 times as many leukemic blasts as normal stem cells. Based on the number of leukemic cells injected and the postulated number of normal hematopoietic stem cells in murine bone marrow,²² one may conclude that ALP has a significant differential toxicity with a relative sparing of normal hematopoietic stem cells.

Our results show that in vitro treatment of murine-simulated remission leukemic marrow with a suffi-

cient concentration of the ALP ET-18-OCH₃ eliminates leukemic blasts, prolongs survival after transplantation, and spares sufficient normal stem cells to allow normal hematologic reconstitution.

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