Selective Killing of Malignant Cells from Leukemic Patients by Alkyl-Lysophospholipid

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ABSTRACT

We studied the effects of the alkyl-lysophospholipid, 1-octadecyl-2-methyl-sn-glycerol-3-phosphocholine (ALP), on human leukemia cells from 56 patients with various leukemias and on normal bone marrow progenitors in order to assess the application of ALP as an in vitro marrow-purging agent. The tumoricidal activity was analyzed by the elimination of clonogenic leukemia cells (leukemic colony-forming cells), by the inhibition of the proliferative capacity ([3H]thymidine incorporation) of leukemia cells, and by the elimination of viable leukemia cells measured with flow cytometry. The tumoricidal activity of ALP was dose and incubation time related, as, although to a lesser extent, held true for normal marrow progenitors. For some leukemias the ALP dose necessary for the elimination of 100% of the leukemic colony-forming cells is probably too toxic for normal marrow cells. The results of this study strongly support the possibility that ALP is a promising purging agent in the majority of patients with leukemias.

INTRODUCTION

Intensive cytotoxic therapy followed by bone marrow grafts from HLA-identical donors provides the best chance of cure for patients with acute leukemias (1-4). Unfortunately, this approach is limited to younger patients who have HLA-matched donors and, as a consequence, only a few patients may benefit from this procedure. The application of BMT5 can increase substantially by using autologous marrow grafts. However, autologous marrow from patients with acute leukemias in remission may contain residual viable leukemia cells. Attempts to eliminate residual leukemia cells from marrow grafts are generally confined to reagents which recognize membrane antigens expressed on malignant cells (immunopurging) and to chemotherapeutic drugs (chemopurging) (5-10). Unfortunately, with respect to membrane antigens, no specific antigens on leukemia cells are known and leukemia cells are heterogeneous (11-16) and probably are also different from their progenitors (15, 17-19). Furthermore, malignant cells can escape from immunopurging by, for example, antigen modulation or decreased antigen expression (20). Chemopurging has the disadvantage of being toxic to normal hematopoietic cells as well (21-23).

A promising approach for purging residual leukemia cells from the marrow may involve the use of the alkyl-lyso phospholipid ALP (24-26). ALP is a synthetic analogue of 2-lysophatidylcholine and has shown selective toxicity to established leukemia cell lines with sparing of normal hematopoietic progenitors (27, 28). The mechanisms by which ALP exerts its differential toxicity are essentially unknown, although most data from in vitro studies point to preferential accumulation of ALP in tumor cells wherein it might disturb normal phospholipid metabolism and, as a consequence, cause injury to the cell membrane (24, 29-31).

In this report we describe the results of the cytotoxic effect of ALP for leukemia cells from 56 patients with leukemias. The cytotoxic effects were analyzed with leukemic cell colony growth, [3H]thymidine incorporation, and a double immunofluorescence technique using monoclonal antibodies for leukemia cell markers combined with a vital stain which was carried out with flow cytometry. Furthermore, the effects of ALP to normal bone marrow progenitors were examined with colony assays. The results demonstrate that ALP is an efficacious agent for leukemia cell killing and a promising purging agent. However, the optimal dose of ALP for leukemia cell killing varied among the patients, and in some patients the effective doses for leukemia cell killing may be too toxic for normal bone marrow progenitors as well.

MATERIALS AND METHODS

Patients. The characteristics of the 56 patients studied are given in Table 1. The diagnosis of the leukemias was established by morphology, cytochemical staining, and surface marker analysis using a panel of monoclonal antibodies. Fifty-two patients were studied at the time of diagnosis, including 4 patients who could be studied again during their first relapse, and 4 patients were studied only during their first relapse. All, except 2 patients, had more than 85% blasts in the peripheral blood and all had an elevated leukocyte count >10,000 cells/μl. Of 56 patients 26 had (primary) AML, including the FAB classification type M1-M5; 10 had secondary AML (FAB class M1: 3 patients, M2: 5 patients, M4: 2 patients), 8 after preceding myelodysplastic syndrome FAB class refractory anemia with an excess of blasts and 2 treated previously with chemotherapy and radiotherapy for testicular carcinoma; 15 had ALL, with immunological phenotyping CALLA (8 patients), T-cell (5 patients), B-cell (1 patient), and null-cell (1 patient); and 5 had chronic myeloid leukemia in blast transformation (CML-BT), 3 with myeloid and 2 with lymphoid transformation. The described study was approved by the local investigational review board and informed consent was obtained from the patients.

Preparation of Leukemic Cells. Peripheral blood samples (and bone marrow cells for some experiments) of untreated patients were collected in tubes containing preservative-free heparin. Leukemic cells were obtained by Ficoll-Isoopaque density gradient centrifugation, interphase mononuclear cells were recovered, and the cells were washed twice with phosphate-buffered saline and then resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 20% AB serum. Peripheral blood samples from two patients with <85% blasts in the peripheral blood (53 and 69%) were further purified by T-cell depletion (with 2-aminoethylisothiouronium bromide-treated sheep RBCs). The samples always contained >95% blasts. Some experiments (see below) were performed with fresh (unrefrigerated) cells; however, in most instances cells were cryopreserved until use.

Leukemic Cell Lines. The cell lines HL60 (promyelocytic cell line) and K562 (erythroleukemic cell line) were continuously cultured at

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3 The abbreviations used are: BMT, bone marrow transplantation; ABMT,
autologous bone marrow transplantation; L-CFC, leukemic colony-forming cell;
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Table 1 Patient population

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (yr), median (range)</th>
<th>Male/Female</th>
<th>AML</th>
<th>M1+</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>Secondary AML</th>
<th>After myelodysplastic syndrome</th>
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<td>56</td>
<td>43 (6-75)</td>
<td>34/22</td>
<td>26</td>
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<td>3</td>
<td>5</td>
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</table>

*FAB class.
2 Immunological phenotype.

37°C in a 5% CO₂ humidified atmosphere in RPMI 1640 medium containing 20% AB serum and passaged twice weekly. Studies were performed in the log-phase growth.

Cryopreservation and Thawing of the Cells. Leukemic cells resuspended in RPMI 1640 medium containing 20% AB serum and 10% dimethyl sulfoxide (Sigma Chemical Company, St. Louis, MO) were cryopreserved in the vapor phase of liquid nitrogen. Freezing experiments were performed in freezing vials containing a 1.8-ml final volume and a final cell concentration of 1-10 × 10⁶ cells/ml. For the experiments, leukemic cells were thawed rapidly by placing the vials in a 37°C bath, and after thawing the dimethyl sulfoxide concentration was lowered stepwise by dilution with cold (4°C) RPMI 1640 medium containing 20% AB serum. Cells were washed at 4°C, dead cells were removed by Ficoll-Isopaque density gradient centrifugation, and the interphase mononuclear cells were diluted in RPMI 1640 medium containing 20% AB serum and viability was determined by trypan blue dye exclusion. For the studies described below, the number of cells were adjusted after incubation with ALP to achieve comparable cell concentrations.

Normal Bone Marrow Cells. Normal bone marrow cells were obtained by aspiration from the sternum during thoracic surgery. All donors gave informed consent for research purposes. Marrow cells were collected in RPMI 1640 medium containing preservative-free heparin, placed over Ficoll-Isopaque, and separated by centrifugation. The interphase mononuclear cells were diluted in RPMI 1640 medium containing 20% AB serum and washed twice in RPMI 1640 medium containing 1% bovine serum albumin (Organon Teknika), and 10 M of the monoclonal antibody (anti-HLA-DR or anti-CALLA; Becton Dickinson), showing 70% reactivity with the surface antigen of the whole leukemic cell population (>95% leukemic blasts in all samples), was added and incubated for 30 min at 4°C. Cells were washed and incubated with rabbit anti-mouse Ig, biotin conjugated (Dakopatts, Copenhagen, Denmark) for 30 min on ice, washed again, and incubated with avidin-phycoerythrin (Becton Dickinson) for 30 min on ice. After two additional wash steps, cells were incubated for 10 min at room temperature with 0.02 µg FDA/ml, and the cells were analyzed in the cytofluorograph at a maximal rate of 2000 cells/s. A control specimen containing FDA and rabbit anti-mouse Ig, biotin conjugated, and with avidin-phycoerythrin but without the monoclonal antibody specific for the leukemic cells served as the negative control.

RESULTS

Effect of ALP on the Leukemic Cell Lines HL60 and K562. The cytotoxic effect of ALP on ALP-sensitive HL60 cells and on ALP-resistant (relative) K562 cells is shown in Fig. 1. The clonogenic leukemic cells have a clear dose-related (and incubation time-related) response to ALP, e.g., 20 µg ALP/ml/24
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10^5 cells plated after 24 h of incubation with 0 µg ALP/ml (controls) were 636 (range, 8–5714) for patients with AML, 77 (range, 20–1737) for patients with ALL, and 390 (range, 6–714) for patients with CML-BT. Cells in surviving colonies (100 µg ALP/ml) were morphologically blast cells.

Correlation between the Effect of ALP on Clonogenic Leukemic Cells and Other Patient Characteristics. Four patients (3 with AML, 1 with ALL) were studied at the time of presentation as well as during their first relapse. In these patients no differences were observed for the cytotoxic effect of ALP on the L-CFCs at presentation or at relapse. Ten patients (7 had L-CFCs) had secondary AML, and no differences for the cytotoxic effects of ALP on L-CFCs could be observed between secondary and primary AMLs. The same data of no differences for cytotoxic effects of ALP on L-CFCs held true for FAB subclasses (patients with AML) and for C-ALL versus T-ALL. From some patients fresh samples and frozen/thawed samples were studied, and although generally more L-CFCs were observed for fresh samples, the percentage of survival of the L-CFCs seemed to be equal. In addition, L-CFCs from 3 patients with AML were set up from the bone marrow as well as from the peripheral blood, and no differences in cytotoxic effect of ALP for both L-CFCs were found.

Effect of ALP on [3H]Thymidine Incorporation. The inhibition of [3H]thymidine in the blasts of 36 patients with AML and of 15 patients with ALL by ALP (different concentrations and incubation times) is shown in Fig. 3 and Table 3. Median (and range) values for control cpm (0 µg ALP/ml) after 24 and 48 h were 6,600 (800–89,000) and 4,300 (500–104,300), respectively.

Effect of ALP on Clonogenic Leukemic Cells. L-CFCs were found in 28 of 36 (78%) patients with AML, including 7 of 10 (70%) patients with secondary AML, in 8 of 15 (53%) patients with ALL, and in 4 of 5 patients with CML-BT. The cytotoxic effect of ALP with different concentrations (20, 50, and 100 µg ALP/ml) and incubation times (24, 48 h) on L-CFCs is shown in Fig. 2 and in Table 2. The median numbers of L-CFCs/2 × 10^5 cells plated after 24 h of incubation with 0 µg ALP/ml (controls) were 636 (range, 8–5714) for patients with AML, 77 (range, 20–1737) for patients with ALL, and 390 (range, 6–714) for patients with CML-BT. Cells in surviving colonies (100 µg ALP/ml) were morphologically blast cells.

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Table 3 Inhibition of [3H]thymidine incorporation in the leukemic cells from patients with AML and ALL

<table>
<thead>
<tr>
<th>ALP (µg)/ml</th>
<th>Treatment duration (h)</th>
<th>AML</th>
<th>ALL</th>
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<tbody>
<tr>
<td>20</td>
<td>24</td>
<td>63 (60–100)*</td>
<td>76 (34–95)</td>
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<td>48</td>
<td>96 (10–100)</td>
<td>99 (79–100)</td>
<td>50 (25–95)</td>
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<td>50</td>
<td>96 (35–100)</td>
<td>96 (71–100)</td>
<td>82 (45–90)</td>
</tr>
<tr>
<td>48</td>
<td>100 (44–100)</td>
<td>100 (98–100)</td>
<td>92 (78–95)</td>
</tr>
<tr>
<td>100</td>
<td>100 (80–100)</td>
<td>100 (100–100)</td>
<td>100 (80–100)</td>
</tr>
</tbody>
</table>

* Values are expressed as % inhibition of control.

Fig. 3. The effect of varying concentrations of ALP for 24 h on [3H]thymidine incorporation in AML cells (36 patients) and in ALL cells (15 patients).
Cells of the marrow from 11 normal marrow donors were incubated with 100 μg ALP/ml for 24 h. Fig. 4 shows the results of 3 patients (patient A: AML, HLA-DR+; patient B: ALL, CALLA+; patient C: ALL, HLA-DR+); the leukemic cells of 2 of these 3 patients failed for colony growth in vitro. The left upper and lower quadrants represent nonvital cells; the right lower quadrants represent vital marker-negative cells. The right upper quadrants of A1, B1, and C1 represent the vital marker-positive cells after ALP treatment. For patients A, B, and C a cytotoxicity (elimination of vital marker-positive cells) of 98, 97.5, and 98.5%, respectively, was found. The mean (and range) of cytotoxicity for all 8 patients with HLA-DR+ cells was 98% (95–98.5%) and for 2 patients with CALLA+ cells a cytotoxicity of 97.5% and 99% was observed.

Effect of ALP on Normal Bone Marrow Cells. Pluripotent (CFU-GEMM) and lineage-restricted (CFU-GM) progenitor cells of the marrow from 11 normal marrow donors were incubated with 0, 20, 50, and 100 μg ALP/ml for 24 h. Fig. 5 shows the effect of ALP on these normal progenitors. The median numbers of CFU-GEMM and of CFU-GM/2 × 10³ cells plated after 24 h of incubation with 0 μg ALP/ml (controls) were 37 (range, 12–62) and 269 (range, 189–392), respectively. After 24 h of incubation 50 and 100 μg ALP/ml resulted in a (mean) decrease of CFU-GEMMs of 40 and 74%, respectively, and a decrease of CFU-GMs of 70 and 83%, respectively. From 6 donors long-term marrow cultures were set up after incubation with 100 μg ALP/ml for 24 h and succeeded in 5 patients, and generally more CFU-GMs were generated after 21 days of culture (median increase of 11%; range, 0–40%) than the number found after 24 hours of culture, and stromal marrow cells were observed as well (data not shown).

DISCUSSION

Intensive cytotoxic therapy followed by ABMT is an attractive treatment approach for patients with leukemias who are not candidates for allogeneic BMT. A major obstacle of autologous marrow infusion may be the presence of residual leukemia cells in the remission marrow and that outgrowth of these leukemia cells do contribute to the high relapse rate observed after ABMT. The contribution of residual leukemia cells in the graft to the relapse rate can be measured only by comparing the results of autologous versus syngeneic marrow infusion because syngeneic BMT provides the yardstick to measure the success of intensive cytotoxic therapy followed by “normal” marrow infusion. Although the number of syngeneic BMT recipients is small and treatment modalities are heterogeneous, the relapse rate after syngeneic BMT for patients with AML in their first CR remission is rather high (34), indicating the need for more intensive cytotherapeutic treatments. Nevertheless, the use of autologous marrow grafts selectively purged from leukemia cells in vitro will be the ultimate goal for the performance of ABMT.

In the current study the cytotoxicity of ALP for leukemia cells from 56 patients was analyzed in vitro by the elimination of clonogenic leukemia cells (L-CFCs), by the inhibition of the proliferative capacity ([3H]thymidine incorporation) of leukemia cells, and by the elimination of viable leukemia cells in cell suspension, using a double immunofluorescence technique and carried out with flow cytometry. It is generally assumed that L-CFCs are the progenitor cells that may act as leukemic stem cells in vivo; thus this will be the preferable assay to evaluate the efficacy of purging agents. However, at the moment L-CFCs cannot be generated in vitro from all patients with leukemias (35, and this report), indicating that complementary assays are necessary, as we performed with the inhibition of [3H]thymidine incorporation as well as with flow cytometry analysis.

Using L-CFCs we could demonstrate the efficacy of ALP as an in vitro cytotoxic agent in the majority of patients with leukemias. After treatment with 100 μg ALP/ml for 24 h L-CFCs could not be generated anymore in 19 of 28 (68%) patients with AML and in 6 of 8 (75%) patients with ALL. Although few patients with CML-BT were studied, it seemed that leukemic cells from patients with CML-BT were more...
resistant to ALP. Under identical ALP incubation conditions \([^{3}H]\)thymidine incorporation in leukemia cells was inhibited in 27 of 36 (75%) patients with AML and in 8 of 15 (53%) patients with ALL. Using \([^{3}H]\)thymidine incorporation, we observed comparable results for patients with CML-BT as stated above for L-CFCs. Flow cytometry analysis was performed in 10 patients and with this assay we found that a median of 98% of the viable leukemia cells were killed with 100 \(\mu\)g ALP/ml/24 h. It should be noted, however, that in 8 of 10 patients leukemia cells were phenotyped with a leukemia cell nonspecific monoclonal antibody (anti-HLA-DR), indicating that, despite the fact that the samples contained >95% leukemic blasts, the few marker-positive cells still viable after ALP treatment can be nonleukemic. Although for the individual patient the elimination of L-CFCs, inhibition of \([^{3}H]\)thymidine incorporation, and flow cytometry analysis were correlated, leukemic cells from some patients had, under identical ALP incubation conditions, no \([^{3}H]\)thymidine incorporation but still some growth of L-CFCs and vice versa.

Ideally, a purging agent should be selectively toxic for leukemia cells. We observed with increasing ALP concentrations (and incubation times) a decrease of CFU-GMs and of CFU-GEMMs. With ALP incubation conditions (100 \(\mu\)g ALP/ml/24 h) that killed L-CFCs in the majority of the patients, still 26% of the CFU-GEMMs retained their viability and (more) CFU-GMs could be generated from long-term cultures, indicating that ALP will be a promising purging agent. Other rather relevant data from this study are that leukemia cells from patients at the time of diagnosis had the same sensitivity for ALP as at the first relapse and that no differences in sensitivity were observed for the subclasses of acute leukemias or for primary AML versus secondary AML.

Previous studies of the tumoricidal activity of ALP were carried out with highly sensitive (e.g., HL60; see Fig. 1) established leukemic cell lines (27, 28, 30, 36) and with \([^{3}H]\)thymidine incorporation assays with leukemia cells from patients (24, 26, 37). As such, these results cannot be translated directly to clinical application. In addition, the (low) ALP dose used in these studies had no effect on normal marrow progenitors, which does not hold true for ALP doses necessary for the elimination of clonogenic leukemia cells (this study). Furthermore, human leukemias have different sensitivities for ALP. Indeed, few clonogenic leukemia cells remained viable after high doses of ALP in some patients, indicating that ALP cannot be considered as a selective purging agent for all patients with leukemias and has to be tested on an individual base.

The mechanisms by which ALP exerts the tumoricidal activity are generally unknown. Previous studies have pointed to the selective deficiency of alkyl cleavage enzymes, which are capable of degrading ALP, in malignant cells (24, 27); however, recent studies (and own observations) have shown that such correlation does not exist (30, 38). Recent data have pointed to other mechanisms, including inhibition of protein kinase C by ALP (39, 40), reduction of the binding capacity of (epidermal) growth factor receptors on malignant cells by ALP (41), and (higher) levels of ether lipids in tumor cells which increase the sensitivity to ALP (42). Regardless of the mechanisms, ALP probably causes cell killing by the preferential accumulation in tumor cells (30, 31).

Preclinical studies of the use of purging agents are ongoing but are generally confined to immunopurging (monoclonal antibodies) and to chemopurging (chemotherapeutic drugs). Major drawbacks of immunopurging are the heterogeneity of leukemia cells (11–16), and probably, they also differ from their progenitors (15, 17–19). Chemotherapeutic agents are toxic to normal marrow progenitors as well (21–23). Therefore, the results of clinical studies with the use of immuno- or chemopurged marrow grafts are difficult to interpret (9, 43–45).

In conclusion, ALP is a promising purging agent for human leukemias. However, human leukemias have different sensitivities for ALP, indicating that the optimal dose of ALP varied from patient to patient, and in some patients the optimal dose can be too toxic for normal bone marrow progenitors as well.

REFERENCES


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