Deoxyribonucleoside Triphosphate Accumulation by Leukemic Cells

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The toxicity of the deoxyribonucleosides, 2′-deoxyadenosine, 2′-deoxyguanosine, and thymidine, for human T lymphoblasts is mediated by the accumulation of the corresponding deoxyribonucleoside triphosphate (dATP, dGTP, or dTTP, respectively). We have examined whether leukemic cells of non-T-cell origin are capable of accumulating deoxyribonucleotides in culture and whether this capability correlates with the activities of purine metabolizing enzymes in these cells. We have found that non-T, non-B acute lymphoblastic leukemia cells with low ecto-5′-nucleotidase and high adenosine deaminase activities increase their dATP pools by greater than tenfold when exposed to deoxyadenosine and an inhibitor of adenosine deaminase in culture. Cells from 2 of 9 patients with chronic lymphocytic leukemia and 4 of 11 patients with acute nonlymphoblastic leukemia achieved similar elevations in dATP, but there was no relationship between dATP accumulation and adenosine deaminase, purine nucleoside phosphorylase, or ecto-5′-nucleotidase activities. Treatment of four individuals with acute lymphoblastic leukemia with the adenosine deaminase inhibitor, 2′-deoxycoformycin, resulted in elevations in plasma deoxyadenosine concentrations and in increments in lymphoblast dATP levels that were similar to those measured in lymphoblasts cultured with deoxyadenosine and deoxycoformycin prior to treatment. In vitro incubations of leukemic cells with deoxyribonucleosides may provide a rational basis for the use of these compounds as chemotherapeutic agents.

INHERITED DEFICIENCIES of two enzymes in the purine metabolic pathway have been associated with the selective depletion of lymphoid cells in man. Adenosine deaminase (ADA; EC 3.5.4.4) deficiency results in severe combined immunodeficiency disease characterized by loss of both T and B lymphocytes,[1] while purine nucleoside phosphorylase (PNP; EC 2.4.2.1) deficiency results in a selective T-cell deficit.[2] Lymphocytotoxicity appears to result from the accumulation of the 2′-deoxyribonucleoside substrates for these two enzymes; deoxyadenosine in the case of ADA deficiency and deoxyguanosine in the case of PNP deficiency.[3-5] Deoxyadenosine, deoxyguanosine, and the pyrimidine deoxyribonucleoside, thymidine, are all markedly and selectively cytotoxic to cultured human T lymphoblasts,[6-8] and the toxicity appears to be mediated by the metabolism of these compounds to their corresponding deoxyribonucleoside triphosphates (Fig. 1).

Inhibitors of ADA and of PNP thus have the potential to act as selective chemotherapeutic agents for T-cell malignancies.[9-12] We have attempted to define which other leukemia cell populations might respond to such therapy by examining the ability of intact leukemic cells to synthesize and accumulate deoxyribonucleoside triphosphates from the corresponding deoxyribonucleosides in culture. In addition, we have asked whether deoxyribonucleotide accumulation correlates with the activities of the purine metabolizing enzymes ADA, PNP, and ecto-5′-nucleotidase (EC 3.1.3.5), and whether accumulation by cells in vitro predicts responsiveness to circulating deoxyribonucleosides in vivo.

MATERIALS AND METHODS

In Vitro Incubations

Leukemic cells were obtained from heparinized peripheral blood samples from patients with a recent diagnosis of or relapse of acute leukemia or with a chronic leukemia. No patient had received chemotherapy within 3 wk of the time of study, and verbal informed consent was obtained from each individual. The types of leukemia were determined by standard morphological and histochemical techniques. All acute lymphoblastic leukemic cells were shown not to form rosettes with sheep erythrocytes or to have surface immunoglobulin, and are thus referred to as non-T, non-B cell adult lymphocytic leukemia (ALL). Leukemic cells constituted at least 95% of the white cell population in all patients with acute leukemia and chronic lymphocytic leukemia.

The mononuclear cell fraction containing the leukemic cells was isolated on a Ficoll-Hypaque gradient, washed once in RPMI 1640 tissue culture medium, and suspended in RPMI 1640 plus 10% heat-inactivated horse serum. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 at a final concentration of 10^6 cells/ml. Incubations were performed in duplicate in the presence of (A) no additives, (B) deoxyadenosine (50 μM) alone, (C) 2′-deoxycoformycin (5 μM or 50 μM) alone, (D) deoxyadenosine (50 μM) and 2′-deoxycoformycin (5 μM or 50 μM), (E) deoxyguanosine (50 μM), or (F) thymidine (1mM). After 4 hr, 10^6 cell aliquots were sedimented at 400 g for 10 min, resuspended in 1 ml cold 60% methanol, and left overnight at −20°C. Following evaporation of the methanol, the cell extract was resuspended in distilled H_2O and assayed for concentrations of dATP, dGTP, or dTTP by the DNA polymerase assay, as previously described.[6] Results are expressed as the increment in deoxyribonucleoside triphosphate levels in cells incubated with deoxyribonucleosides over values obtained in the control cultures containing no additives.

Enzyme Assays

Intact leukemic cells freshly isolated on Ficoll-Hypaque were suspended in Hanks' balanced salt solution free of Ca^{++} and Mg^{++},

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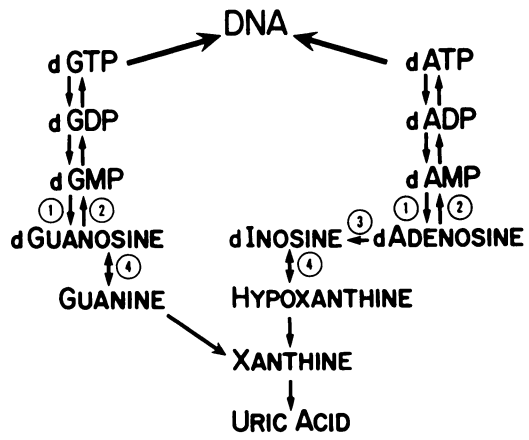

Fig. 1. Purine deoxyribonucleoside metabolism. (1) 5'-Nucleotidase; (2) deoxyribonucleoside kinases; (3) adenosine deaminase; (4) purine nucleoside phosphorylase.

and assayed for ecto-5'-nucleotidase activity using adenosine monophosphate (AMP) as a substrate, by methods previously reported.[13] Assays were performed in triplicate and activity was demonstrated to be linear with time and cell number. The remaining leukemic cells were washed twice in 10 m*M* Tris–0.9% NaCl, pH 7.4, and frozen at −70°C in 2 vol of buffer until assay for ADA and PNP activity. Cell pellets were lysed by three freeze-thaw cycles and were centrifuged at 30,000 *g* for 30 min. The supernatants were dialyzed overnight against Tris-NaCl buffer and assayed immediately for ADA[14] and PNP[15] activities. Protein concentrations were determined by the Lowry method, and the specific activities expressed as nmole/min/mg protein.

Patient Studies with 2'-Deoxycoformycin

Four patients with non-T, non-B cell acute lymphoblastic leukemias, which were completely refractory to conventional therapy, were treated with 2'-deoxycoformycin infusions of 1 mg/kg/day until leukemic cell ADA activity was completely inhibited. Levels of dATP in lymphoblasts were determined following in vitro incubations prior to treatment and were determined daily during therapy. Plasma levels of adenosine and deoxyadenosine were also monitored daily by high-pressure liquid chromatography.[16] The protocol for 2'-deoxycoformycin administration was approved by the Human Use Committee of the University of Michigan and by the Food and Drug Administration. Written informed consent was obtained from each patient prior to treatment.

RESULTS

Leukemic cells from 12 patients with non-T, non-B cell ALL were examined for levels of dATP, dGTP, or dTTP after 4 hr of exposure to the corresponding deoxyribonucleoside. In the case of deoxyadenosine, 2'-deoxycoformycin (dCF) was added to the incubations at a concentration of 5 μM or 50 μM to inhibit cellular ADA activity. In the absence of deoxycoformycin, deoxyadenosine was rapidly deaminated to deoxyinosine and did not result in increments in dATP levels. Two concentrations of deoxycoformycin were used in each experiment to control for incomplete inhibition of ADA activity. DeoxyATP values in the presence of deoxyadenosine and 50 μM deoxycoformycin were slightly higher than those obtained with deoxyadenosine and 5 μM deoxycoformycin in those cells with high ADA activity. Neither concentration of deoxycoformycin alone had an effect on dATP pool size. Consequently, we are reporting the data obtained uniformly with 50 μM deoxycoformycin.

Figure 2 demonstrates an inverse hyperbolic relationship between deoxyribonucleotide accumulation after 4 hr of incubation and ecto-5'-nucleotidase activity in these cells. The line of best fit was generated from a family of 8 regression curves by a Hewlett-Packard Model 9825 computer and is described by the equation $y = ax^b$. This relationship was statistically

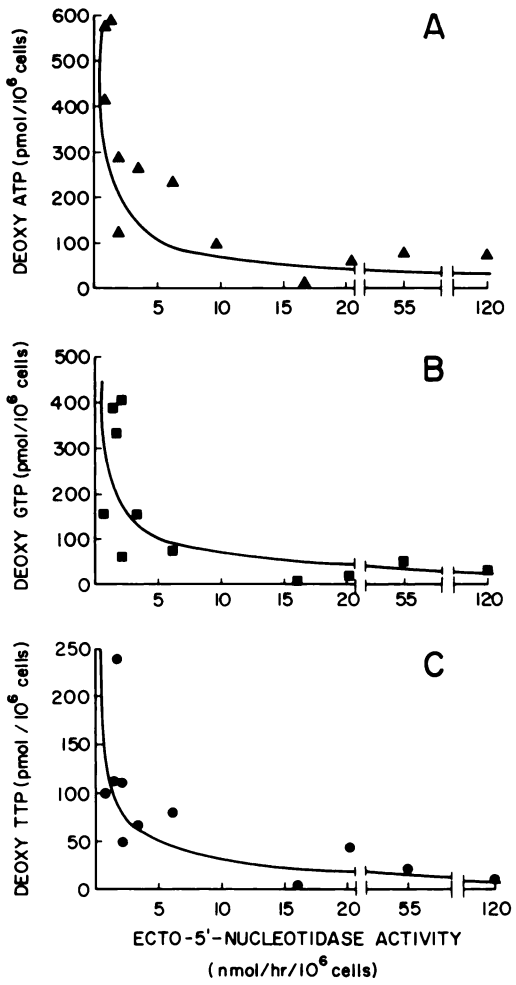

Fig. 2. Correlation of ecto-5'-nucleotidase activity with deoxyribonucleoside triphosphate accumulation in non-T, non-B cell acute lymphoblastic leukemia. (A) DeoxyATP increment in cells incubated with deoxyadenosine (50 μM) and the ADA inhibitor, 2'-deoxycoformycin. Control dATP levels in the absence of additives or the presence of deoxycoformycin alone ranged from <5 pmole/10^6 cells (undetectable) to 20 pmole/10^6 cells. (B) DeoxyGTP increment in cells incubated with deoxyguanosine (50 μM). (C) DeoxyTTP increment in cells incubated with thymidine (1m*M*).

significant only for dATP accumulation, with a correlation coefficient of 0.70 and a p value of 0.01. However, the same cells with low ecto-5'-nucleotidase activity, which accumulated dATP when exposed to deoxyadenosine, demonstrated substantial increases in dGTP when exposed to deoxyguanosine. Smaller increments in dTTP were measurable in cells with low ecto-5'-nucleotidase activity when exposed to thymidine concentrations 20-fold higher than those of deoxyadenosine and deoxyguanosine.

We also examined the relationship between ecto-5'-nucleotidase activity and deoxyribonucleoside triphosphate accumulation in mononuclear cells from normal individuals and from patients with other types of hematologic malignancies (Fig. 3). Normal mononuclear cells had a mean ecto-5'-nucleotidase activity of 22 nmole/hr/10^6 cells. Cells from 8 normal individuals accumulated 81.8 ± 70 pmole dATP/10^6 cells, but less than 10 pmole dGTP or dTTP under these culture conditions. Cells from 2 patients with chronic lymphocytic leukemia and 3 with acute nonlymphoblastic leukemia accumulated more than 200 pmole dATP/10^6 cells, but none of these cells accumulated dGTP or dTTP, and there was no correlation of dATP accumulation with low ecto-5'-nucleotidase activity.

Figure 4 shows the relationship of ADA activity to dATP accumulation. Normal mononuclear cells had mean ADA activity of 46 nmole/min/mg protein. Acute lymphoblastic leukemic cells, with activity ranging from 480 to 2,000 nmole/min/mg protein accumulated more than 200 pmole dATP/10^6 cells, whereas cells with lower ADA activity did not. There was no relationship between dATP accumulation and ADA activity in nonlymphoblastic leukemias. Purine

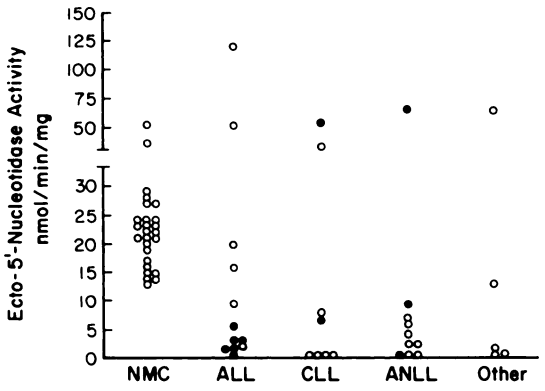

Fig. 3. Relationship of ecto-5'-nucleotidase activity to dATP accumulation in normal and leukemic cells. Open circles (O) represent dATP levels less than 200 pmole/10^6 cells. Closed circles (●) represent dATP levels greater than 200 pmole/10^6 cells. NMC, normal mononuclear cells; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; ANLL, acute nonlymphoblastic leukemia; Other, Sézary's syndrome, hairy cell leukemia, blast crisis chronic granulocytic leukemia.

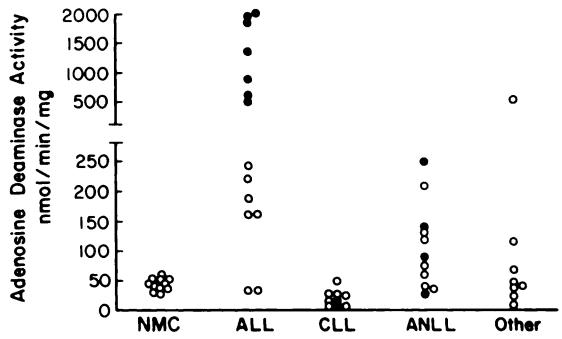

Fig. 4. Relationship of adenosine deaminase activity to dATP accumulation in normal and leukemic cells. See legend to Fig. 3.

nucleoside phosphorylase activity bore no relationship to dATP accumulation (Fig. 5) or to dGTP or dTTP accumulation (data not shown) in acute lymphoblastic leukemia, chronic lymphocytic leukemia, acute nonlymphoblastic leukemia, or the miscellaneous leukemia cell category.

2'-Deoxycoformycin was administered to 4 patients with non-T, non-B cell acute lymphoblastic leukemia whose cells had previously been incubated with dCF and 2'-deoxyadenosine in vitro. Complete data on the biochemical and therapeutic responses of 11 patients treated with this drug are being reported separately.[17] Figure 6 demonstrates the effects of a continuous infusion of dCF at a rate of 1 mg/kg/day on plasma 2'-deoxyadenosine concentrations and lymphoblast dATP levels in one patient. Lymphoblast ADA activity had been completely inhibited 24 hr following the initiation of treatment. The plasma deoxyadenosine concentration rose to 3.6 μM on day 3, paralleled by an increase in lymphoblast dATP to 140 pmole/10^6 cells, and followed by a marked fall in the peripheral lymphoblast count. Retreatment on days 13–16 produced

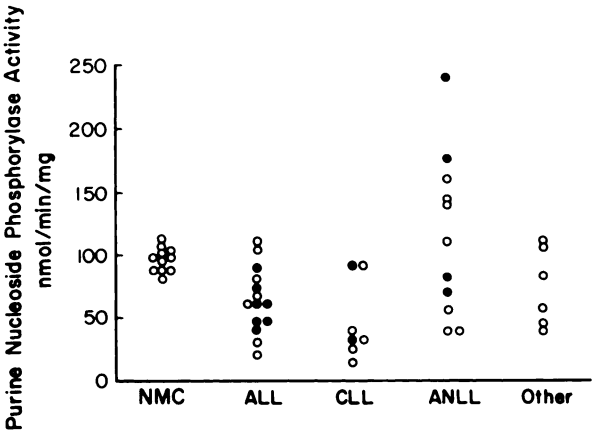

Fig. 5. Relationship of purine nucleoside phosphorylase activity to dATP accumulation in normal and leukemic cells. See legend to Fig. 3.

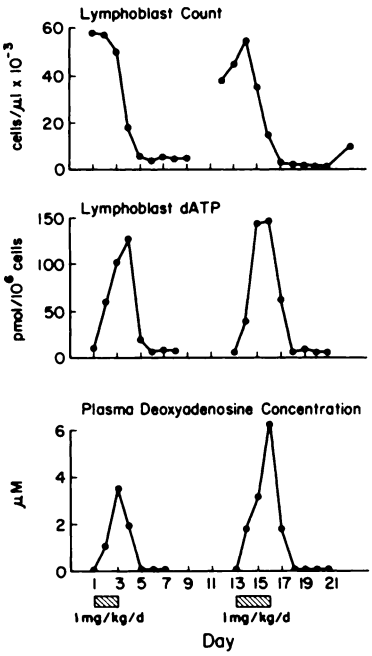

Fig. 6. Effect of ADA inhibition on plasma deoxyadenosine concentration, lymphoblast dATP levels, and lymphoblast count in a patient with non-T, non-B cell acute lymphoblastic leukemia. 2'-Deoxycoformycin was administered as a continuous infusion at a dose of 1 mg/kg/day on the days indicated by the shaded areas.

a rise in deoxyadenosine to 6.4 μM, an increase in lymphoblast dATP to 148 pmole/10^6 cells, and recurrent lysis of peripheral lymphoblasts, although the number of blasts in the marrow decreased only transiently.

Table 1 demonstrates the similarity in in vitro dATP levels accumulated by acute lymphoblastic leukemic cell levels to the maximal intracellular dATP levels achieved following ADA inhibition in vivo in four patients with non-T, non-B ALL. Maximal plasma deoxyadenosine levels, resulting from deoxycoformycin treatment in these individuals, ranged from 6.4 to 10.1 μM and were temporally coincident with the peak lymphoblast dATP values shown. Lysis of the majority of peripheral lymphoblasts occurred within 1–3 days after maximal intracellular dATP levels were reached.

Table 1. Comparison of dATP Levels in Lymphoblasts Incubated With 2'-Deoxycoformycin and Deoxyadenosine In Vitro With Levels Achieved Following Inhibition of ADA Activity In Vivo

Lymphoblast dATP (pmole/10^6 cells)		
Post-incubation	Maximal Level After 2'-Deoxycoformycin Treatment	Percent Lysis of Peripheral Blood Lymphoblasts
100	148	98.0
420	723	99.7
1,075	926	97.6
595	1,053	100

DISCUSSION

The activities of purine metabolizing enzymes have been measured in a large number of hematologic malignancies in an attempt to further refine the morphological classifications of these disorders.[18-20] We have extended these observations to see whether any relationship exists between these enzyme activities and the ability of leukemic cells to accumulate deoxyribonucleoside triphosphates. We have shown that cells from certain patients with non-T, non-B cell acute lymphoblastic leukemia will accumulate deoxyribonucleoside triphosphates when exposed to deoxyribonucleosides in culture. These cells are characterized by low ecto-5'-nucleotidase activity and by high adenosine deaminase activity, a phenotype that also characterizes lymphoblasts of T-cell origin, but which does not clearly differentiate T from non-T-cell acute lymphoblastic leukemia. In contrast, cells from 2 of 9 patients with chronic lymphocytic leukemia and 4 of 11 patients with acute nonlymphoblastic leukemia increased their dATP pools by greater than tenfold in the presence of deoxyadenosine and deoxycoformycin, but there was no relationship between dATP accumulation and any of the enzyme markers examined.

We and others have previously demonstrated that deoxyadenosine and deoxyguanosine are selectively cytotoxic to T-lymphoblast cell lines in vitro and that sensitivity to these deoxyribonucleosides correlates with and is apparently mediated by the accumulation of dATP and dGTP, respectively.[6,7] Such cytotoxicity is not observed with B lymphoblasts, nor do these cells accumulate deoxyribonucleotides. The mechanisms whereby deoxyribonucleoside triphosphates rapidly and selectively accumulate in T lymphoblasts remain under investigation. Studies with mutants of the T-lymphoblast CCRF–CEM line have indicated that deoxycytidine kinase activity is important in the phosphorylation of deoxyadenosine and deoxyguanosine in these cells.[21] In addition, it is clear that decreased catabolism of deoxyribonucleotides contributes to their accumulation in T cells. Low activities of the degradatory enzyme ecto-5'-nucleotidase and of cytoplasmic nucleotidase(s) have both been associated with increased sensitivity of cultured lymphoblasts to deoxyadenosine.[22-24] Although decreased activity of the ecto enzyme, located on the external surface of the cell membrane, is not etiologically related to intracellular deoxyribonucleotide accumulation,[24-26] it may serve as an indirect marker of deoxyribonucleoside sensitivity. Our study suggests such a relationship in the non-T,

non-B acute lymphoblastic leukemic cells, but not in the more differentiated chronic lymphocytic leukemia cells, which are frequently characterized by low ecto-5'-nucleotidase activity.[27]

2'-Deoxycoformycin is a potent inhibitor of adenosine deaminase that has been demonstrated to be markedly lympholytic, but that also causes significant nonlymphoid toxicity.[9,28-30] Administration of this drug to patients in sufficient doses invariably results in elevated levels of plasma and urinary deoxyadenosine.[17] Although T cells have been particularly sensitive to the cytotoxic effects of deoxyadenosine in vivo, some patients with non-T, non-B cell acute lymphoblastic leukemia and with chronic lymphocytic leukemia have also responded with a fall in the peripheral leukemic cell count.[29,30] We have postulated that in vitro incubation studies with deoxycoformycin and deoxyadenosine might predict, on the basis of dATP accumulation, which cells would be sensitive to increases in plasma deoxyadenosine in vivo. Since a very limited number of patients with refractory lymphoproliferative diseases have been candidates for this drug, we have been unable to test this hypothesis fully. However, the preliminary data presented in Table 1 indicate that there may be a relationship between the dATP levels reached after a short-term incubation with a relatively high deoxyadenosine concentration (50 μM) and in the in vivo levels reached after more prolonged exposure to lower and more fluctuating deoxyadenosine concentrations (1–10 μM). Although based on limited data, this observation might suggest that dATP levels reach an equilibrium point that is more dependent on the balance of kinase and nucleotidase activities within the cell, as well as on the rate of DNA synthesis, than on exogenous deoxyadenosine concentrations.

It remains unclear what levels of dATP must be achieved to result in cytotoxicity. We have used a somewhat arbitrary value of 200 pmole dATP/10^6 cells, i.e., at least a tenfold increase in pool size, as indicative of substantial accumulation. However, the cells from one of our patients treated with deoxycoformycin lysed after accumulating a maximum of 148 pmole/10^6 cells. In addition, deoxyadenosine is known to inhibit the enzyme S-adenosylhomocysteine hydrolase[31] and may have effects on cell growth that are independent of its ability to promote dATP accumulation. Thus, we are unable to conclude that in vitro incubations of leukemic cells would necessarily be predictive of in vivo responsiveness to ADA or PNP inhibitors in all instances. Nevertheless, the relationship among deoxynucleoside triphosphate accumulation, low ecto-5'-nucleotidase activity, and high adenosine deaminase activity in non-T-cell acute lymphoblastic leukemia should serve as a basis for analyzing responsiveness to deoxyribonucleosides as chemotherapeutic agents.

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