

Biochemical Consequences of Adenosine Deaminase Inhibition *in Vivo*

Differential Effects in Acute and Chronic T Cell Leukemia^a

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INTRODUCTION

The biochemical mechanisms whereby an inherited deficiency of adenosine deaminase (ADA; EC 3.5.4.4) activity results in selective toxicity to lymphoid cells has been the subject of considerable interest. Experimental evidence obtained with cultured lymphoid cell lines *in vitro* has indicated that 2'-deoxyadenosine, a substrate for ADA, is selectively phosphorylated to dATP in T lymphoblasts^{1,2} and that dATP accumulation directly correlates with inhibition of DNA synthesis and cell death.^{3,4} An alternative hypothesis for the lymphocyte depletion in ADA deficiency entails the accumulation of *S*-adenosylhomocysteine and consequent inhibition of methylation reactions mediated by *S*-adenosylmethionine.^{5,6} Accumulation of *S*-adenosylhomocysteine could in theory result either from an excess of adenosine generated by ADA inhibition⁵ or from inactivation of the catabolic enzyme *S*-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1) by deoxyadenosine.⁶ A third possibility is that ATP depletion may be a concomitant of dATP accumulation in mature lymphocytes^{7,8} and could directly mediate the toxicity to these cells.

In examining the therapeutic efficacy of 2'-deoxycoformycin (DCF), a tight-binding inhibitor of ADA,⁹ in refractory lymphoid malignancies we were directly concerned with elucidating the biochemical consequences of inhibiting ADA activity *in vivo*. Our goal was to correlate the biochemical sequelae with both the efficacy and the clinical toxicity of deoxycoformycin treatment. We also hoped to shed additional light on the mechanism by which lymphoid cells are lysed by pharmacologic inhibition of ADA activity *in vivo*.

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MATERIALS AND METHODS

Chemicals. Radioactive nucleosides were obtained from Amersham/Searle Corporation (Arlington Heights, IL). Unlabeled nucleosides, nucleotides, and calf intestinal ADA were obtained from Sigma Chemical Company (St. Louis, MO). Deoxycoformycin was obtained from Warner-Lambert/Parke-Davis, Inc. (Ann Arbor, MI).

Assays. Adenosine deaminase was measured by a modification of the method of Van der Weyden *et al.*¹⁰ *S*-adenosylhomocysteine hydrolase was measured as described by Hershfield *et al.*¹¹ Plasma and urine adenosine and deoxyadenosine concentrations were determined by high-pressure liquid chromatography, using the method of Koller *et al.*¹² Using 1 ml of cold 60% aqueous methanol, ATP and dATP were extracted from 10^7 lymphoblasts. Nucleotides were extracted from a diluted RBC suspension by immersion in a boiling water bath for one minute. Samples were then cooled and centrifuged at $200 \times g$ for 3 min, and the supernatant was filtered through a 0.22μ filter. Adenine nucleotides were quantitated either by the DNA polymerase assay or by high-pressure liquid chromatography using a Partisil-10 SAX ion-exchange column (Whatman, Inc., Clifton, NJ) and an isocratic mobile phase of $0.45 \text{ M NH}_4\text{H}_2\text{PO}_4$, pH 3.6, at a flow rate of 3 ml/min. *S*-adenosylmethionine, *S*-adenosylhomocysteine, and total adenine ribonucleotide and deoxyribonucleotide pools were measured according to Hershfield *et al.*¹³

Patient Studies. The protocol for these studies was approved by the Committee to Review Investigation Involving Human Beings, of the University of Michigan Medical Center, and by the Food and Drug Administration. Patients had been heavily pretreated with cytotoxic chemotherapy and were considered refractory to the standard agents used to treat lymphoproliferative malignancies.

Deoxycoformycin was reconstituted in 100 ml of 5% dextrose in water, containing 10 mEq of NaHCO_3 (pH 8.2). Plasma and urine nucleosides, lymphoblast and erythrocyte ADA activity, and nucleotides were measured daily or more frequently.

RESULTS

Data demonstrating the selective toxicity of 2'-deoxyadenosine in the presence of an ADA inhibitor for T (as compared to B) lymphoblasts^{1,2} *in vitro* indicated that acute T lymphoblastic leukemia might be effectively treated by ADA inhibition *in vivo*. Administration of 2'-deoxycoformycin to a patient with refractory T cell acute lymphoblastic leukemia demonstrated that inhibition of both red cell and lymphoblast ADA activity could be achieved (FIGURE 1). The dose of dCF was rapidly escalated because of a rising white cell count that necessitated leukapheresis and a deteriorating clinical condition. The peak plasma level of dCF achieved with these infusions was $8 \mu\text{M}$ on day 5. Inhibition of T lymphoblast ADA activity on day 5 was followed by a rapid and marked elevation of plasma deoxyadenosine levels to $104 \mu\text{M}$ on day 7 (FIGURE 2A) which in turn was associated with a rise in lymphoblast dATP concentration (FIGURE 2B), inactivation of lymphoblast *S*-adenosylhomocysteine hydrolase activity (FIGURE 2C), and a precipitous fall in white cell count (FIGURE 2D) with

complete elimination of marrow lymphoblasts.¹⁴ Synthesis of DNA was completely inhibited by day 6 in these cells, which were initially capable of the rapid incorporation of [³H]thymidine into DNA (FIGURE 3). The subsequent clinical course of the patient was marked by renal failure, pulmonary edema, and death.

Of further note was the profound depletion of ATP documented in the patient's erythrocytes as dATP accumulated in these cells.¹⁵ Depletion of ATP was not documented in peripheral blood lymphoblasts prior to day 6, at which point DNA synthesis had been completely inhibited (FIGURE 3). Treatment of six additional patients with non-T-cell acute lymphoblastic leukemia was associated with peak plasma

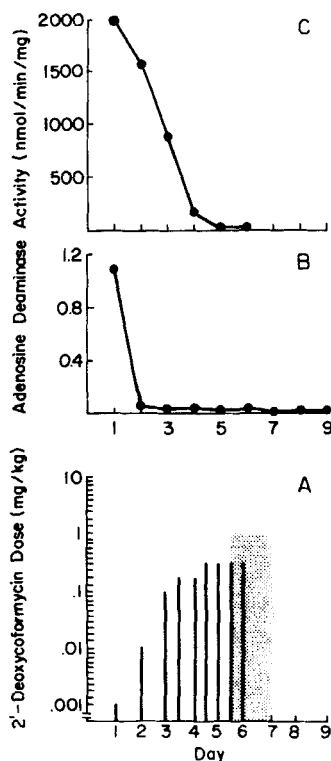


FIGURE 1. Effect of 2'-deoxycoformycin administration on lymphoblast and red cell ADA activity. (A) Dose of 2'-deoxycoformycin. Each bar represents the infusion of drug over 10 minutes. The shaded area corresponds to a continuous intravenous infusion of the drug. (B) Red cell ADA activity. (C) Lymphoblast red cell ADA activity. (From Mitchell *et al.*¹⁴ Reprinted by permission from Blood.)

deoxyadenosine levels ranging from 3.1 to 24 μ M and the accumulation of dATP ranging from 148 to 1,053 pmol/ 10^6 cells in peripheral blasts (TABLE 1). We observed extensive lysis of the circulating blasts (87-100%) in each instance, but the bone marrows remained hypercellular and no complete remissions were documented. Incubations of peripheral blood leukemic cells with dCF *in vitro* in conjunction with deoxyadenosine routinely demonstrated that the leukemic cells from these patients were capable of accumulating dATP.¹⁶

We have subsequently treated a patient with T cell chronic lymphocytic leukemia with dCF and have asked whether the biochemical mechanisms involved in the lysis

of these long-lived cells are analogous to or different from those in more immature cells actively engaged in DNA synthesis. The patient was a 78-year-old female with a six-month history of elevated white count and hepatosplenomegaly. Her white count rose progressively with conventional therapy and was $250 \times 10^9/l$ at the time dCF therapy was initiated. The lymphocytes were characterized as positive for T11 (92%) and T4 (83%) surface antigens and were negative for T8. They appeared to be mature by conventional morphologic criteria.

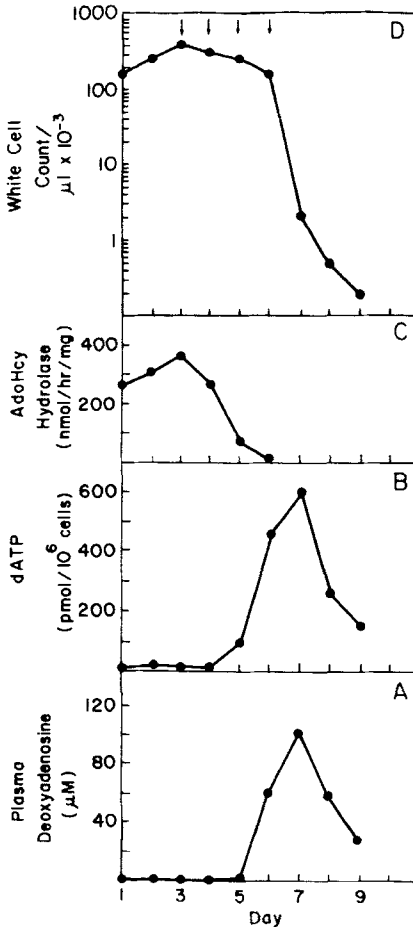
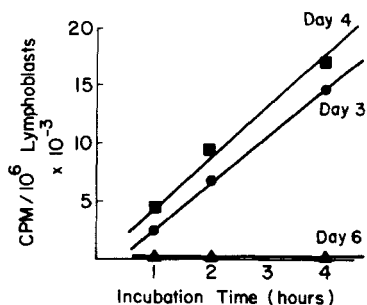


FIGURE 2. Correlation of (A) plasma deoxyadenosine concentration with (B) lymphoblast dATP levels, (C) S-adenosylhomocysteine hydrolase activity, and (D) white blood count. (From Mitchell *et al.*¹⁴ Reprinted by permission from Blood.)

The results of *in vitro* incubations of peripheral blood mononuclear cells from this patient with dCF and 1 μM and 5 μM deoxyadenosine are shown in FIGURE 4. Cell viability, as measured by trypan blue dye exclusion, remained 100% in control cultures containing no additives but dropped by 75% in incubations containing 1 or 5 μM deoxyadenosine. Levels of dATP did not rise significantly in cells incubated with 1 μM deoxyadenosine and rose only two to three times in cells incubated with 5 μM . Nevertheless, intracellular ATP levels dropped by 50% in the cultures containing deoxyadenosine.

FIGURE 3. Synthesis of DNA in T lymphoblasts during treatment with 2'-deoxycoformycin. Cells were incubated at a concentration of 10^6 /ml in RPMI-1640 + 10% horse serum at 37°C in the presence of $1 \mu\text{Ci}/\text{ml}$ [^3H]thymidine (20 Ci/mmol); CPM, counts per minute in a trichloroacetic acid precipitate filtered on Whatman GF/A disks.



Administration of very low doses of dCF (0.05 mg/kg/d × 2-3 d) to the patient on two occasions resulted in measurable increases in the excretion of deoxyadenosine and, to a far lesser extent, adenosine in the urine, but did not cause a measurable rise in plasma deoxyadenosine ($<0.05 \mu\text{M}$) or adenosine ($<0.05 \mu\text{M}$) concentrations (FIGURE 5). Lymphocyte dATP levels remained at the pretreatment level of approximately $5 \text{ pmol}/10^6$ cells throughout both treatments. During the first course, lymphocyte ATP levels remained constant; a transient decline in ATP was observed during the second course. Despite the paucity of these changes, the white cell count fell from $250 \times 10^9/1$ to $6 \times 10^9/1$ during the first course and from $300 \times 10^9/1$ to $2.5 \times 10^9/1$ during the second course.

A comparison of the effects of ADA inhibition with those of *S*-adenosylhomocysteine hydrolase inhibition on lymphocyte metabolites during the second course of treatment is shown in FIGURE 6. Inhibition of ADA activity was readily achieved and was associated with a pronounced increase in free intracellular adenosine to $28 \text{ nmol}/10^9$ cells. Total adenine deoxyribonucleotide (dAXP) pools did not increase, while adenine ribonucleotide (AXP) pools fell slightly on days 2 and 3. Activity of *S*-adenosylhomocysteine hydrolase fell to 28% of control levels by day 4. The rise in the intracellular level of *S*-adenosylhomocysteine on the second day led to an abrupt decline in the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine (the methylation index)^{5,11} from 47 to 6. The patient suffered no toxicity directly attributable to dCF therapy.

TABLE 1. Summary of Metabolic Changes following dCF Therapy in Refractory Acute Lymphoblastic Leukemia

Patient No.	Peak Level in Plasma (μM)		Peak Level of dATP in Leukemic cells ($\text{pmol}/10^6$ Cells)	Reduction in Blast Count (Maximum %)
	Ado ^a	dAdo ^b		
1	2.1	104	502	100
2	9.6	6.8	926	97
3	1.5	6.4	148	98
4	<0.05	3.1	640	98
5	13.0	10.1	1053	100
6	12.7	9.6	723	99
7	11.6	24.0	700	87

^a Adenosine.

^b 2'-Deoxyadenosine.

DISCUSSION

It is clear from our data and from the work of many others¹⁷ that inhibition of ADA activity by dCF results in the lysis of both immature (*i.e.*, leukemic) and mature lymphoid cells *in vivo*. It has also become apparent that the administration of this drug in high doses has been associated with serious toxicities ranging from anemia and hepatic dysfunction to pulmonary edema, renal failure, and death.¹⁷ It therefore becomes critical to determine which biochemical events are essential for the desired

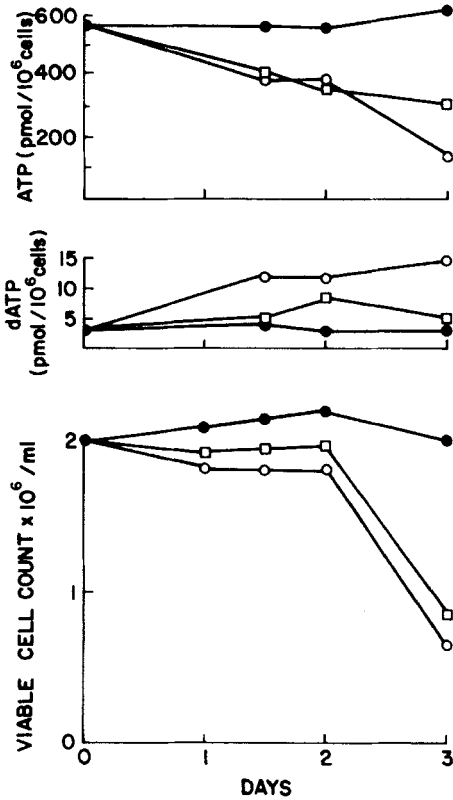


FIGURE 4. Effects of incubations of mature T leukemic cells with 2'-deoxyadenosine and 2'-deoxycoformycin. (●—●) control cultures with no additives; (□—□) 2'-deoxycoformycin (5 μM) plus 2'-deoxyadenosine (1 μM); (○—○) 2'-deoxycoformycin (5 μM) plus 2'-deoxyadenosine (5 μM). Cells were incubated at a concentration of 2×10^7 cells/ml in RPMI-1640 medium plus 10% horse serum for the indicated time periods.

lympholytic effects of the drug and to dissociate these from those more directly associated with clinical toxicity.

The treatment of acute lymphoblastic leukemia with dCF has been associated with increases in both plasma and urinary deoxyadenosine levels and with the accumulation of dATP in leukemic cells. Increased levels of dATP have been directly associated with inhibition of DNA synthesis, although the mechanism by which this occurs has not been completely elucidated.⁴ Our studies on a single patient with T cell acute lymphoblastic leukemia demonstrate a strong temporal relationship between dATP accumulation and lymphoblast lysis that we have also observed in non-T-cell ALL.¹⁶

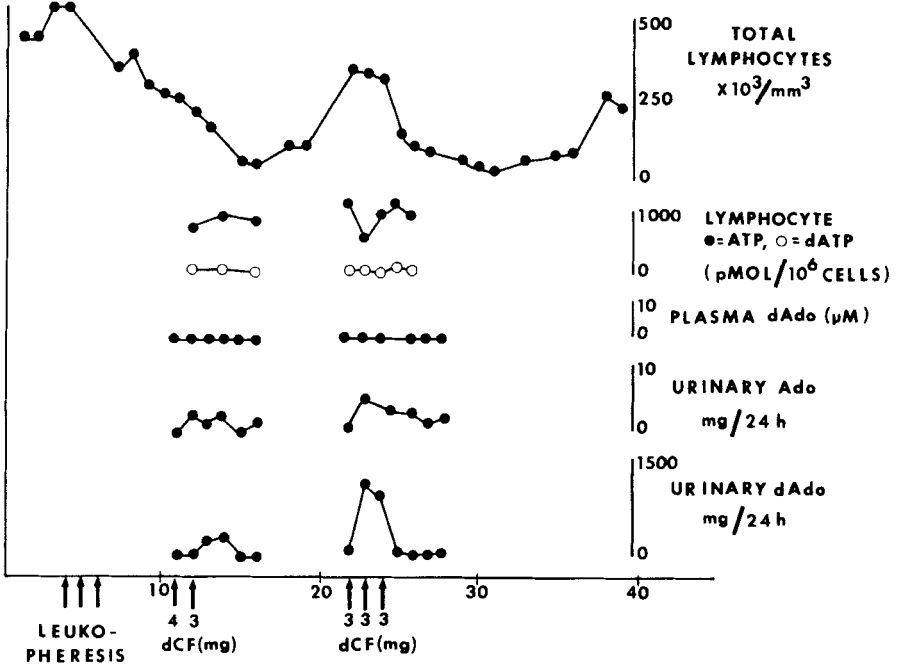
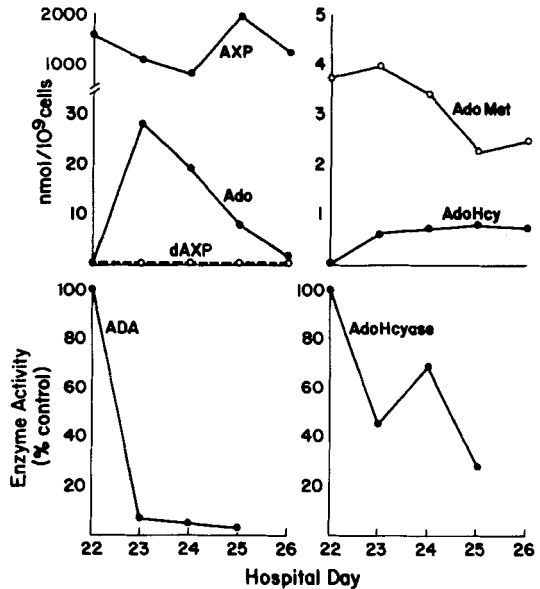


FIGURE 5. Biochemical effects of 2'-deoxycoformycin therapy in T cell chronic lymphocytic leukemia. The 2'-deoxycoformycin was administered over ten minutes at the indicated doses and times. *Ado*, adenosine; *dAdo*, 2'-deoxyadenosine.

FIGURE 6. Effects of ADA and S-adenosylhomocysteine hydrolase inhibition in T cell chronic lymphocytic leukemia. *AXP*, intracellular adenine ribonucleotides (AMP + ADP + ATP); *dAXP*, adenine deoxyribonucleotides; *AdoMet*, S-adenosylmethionine; *AdoHcy*, S-adenosylhomocysteine; *AdoHcyase*, S-adenosylhomocysteine hydrolase.



It would thus appear that deoxynucleotide accumulation plays a major role in the cytotoxic effects of DNA inhibition in these immature cells.

In contrast, the mechanism(s) by which mature T lymphocytes are killed by deoxyadenosine in the presence of an ADA inhibitor is (are) far less clear. Carson *et al.*⁶ described depletion of ATP in conjunction with dATP accumulation in normal peripheral blood lymphocytes incubated with dCF and deoxyadenosine. The prevention of toxicity by deoxycytidine and the absence of lymphocytotoxicity in lymphocytes incubated with adenosine plus homocysteine led to the conclusion that dATP accumulation accompanied by ATP depletion was of primary importance in causing the death of these cells. Others have suggested that impairment of mRNA polyadenylation¹⁸ and decreased overall rates of RNA synthesis¹⁹ are of primary importance.

We have studied the *in vitro* metabolism of dCF and deoxyadenosine in T4⁺ mature cell lines derived from patients with cutaneous T cell lymphomas and in leukemic cells taken directly from these patients.²⁰ These studies showed both that the mature T cell lines were far less sensitive than T lymphoblasts to the cytotoxic effects of deoxyadenosine in the presence of an ADA inhibitor and that they accumulated far less dATP. We postulated that mature T cells have significantly different routes of deoxyadenosine metabolism than do immature T cells and that *in vivo* cytotoxicity might not be directly related to dATP accumulation.

The data presented here indicate that the malignant T4⁺ cells from a patient with T cell chronic lymphocytic leukemia were killed *in vitro* by the combination of 1 μ M or 5 μ M deoxyadenosine plus dCF and that, despite a lack of dATP accumulation, ATP levels fell in culture. The *in vivo* data resulting from dCF administration to this patient are even more striking; despite a complete lack of dATP accumulation *in vivo*, the malignant cells were lysed in impressive numbers by low doses of dCF that did not cause measurable elevations of plasma adenosine or deoxyadenosine, but increased urinary deoxyadenosine excretion. Whether or not the cell lysis was mediated by the accumulation of S-adenosylhomocysteine and the fall in the methylation index, by the slight decrease in ATP levels seen in course 2, or by other mechanisms cannot be determined from these data.

We would conclude that pharmacologic inhibition of ADA activity *in vivo* clearly leads to lysis of T lymphoblasts and of mature T cells, both malignant and nonmalignant. Accumulation of dATP appears to be a major metabolic event in the lysis of T lymphoblasts, but may not be important for the toxicity to more mature cells. Since early studies focused only on differences in 2'-deoxyadenosine toxicity for T and B lymphoblasts,^{1,2} it is not at all clear that the T cell selectivity claimed in those studies pertains to mature lymphocytes. Indeed, the clinical spectrum of ADA deficiency encompasses deficits of both T and B lymphocytes, and the biochemical basis for the inherited disease state remains to be definitively elucidated.

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