ATP depletion as a consequence of adenosine deaminase inhibition in man
(adenosine/deoxyadenosine/deoxycoformycin/dATP)

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ABSTRACT Hereditary deficiency of the enzyme adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) results in an immunodeficiency syndrome characterized by a marked reduction in circulating lymphocytes. We have administered 2′-deoxycoformycin, a potent inhibitor of adenosine deaminase, to a patient with a lymphoproliferative malignancy. The clinical consequences of pharmacologic inhibition of adenosine deaminase activity included an abrupt decrease in the lymphocyte count, abnormalities of renal and hepatic function, and hemolytic anemia. The plasma concentrations of adenosine and deoxyadenosine rose to peak values of 13 μM and 5 μM, respectively, and erythrocyte dATP levels increased to 110 pmol/10⁶ cells over 9 days. There was a corresponding decrease in erythrocyte ATP levels from 128 to <6 pmol/10⁶ cells. A similar profound reduction in ATP occurred in the erythrocytes of a second patient. The rapid and unexpected depletion of ATP associated with dATP accumulation may account, at least in part, for the toxicity associated with 2′-deoxycoformycin administration. The inverse relationship of ATP and dATP raises major questions about the control of energy metabolism in erythrocytes.

Inherited deficiency of adenosine deaminase (ADA; adenosine aminohydrolase, EC 3.5.4.4) results in a severe immunodeficiency disease characterized by marked lymphopenia and loss of both T and B lymphocyte functions (1, 2). ADA catalyzes the deamination of adenosine and deoxyadenosine in the purine salvage pathway. Numerous studies have implicated the accumulation of metabolites derived from one or both of these substrates in the pathogenesis of the immune disorder (3–7).

In the absence of ADA activity, deoxyadenosine is phosphorylated to dATP. Accumulation of this metabolite has been demonstrated in the erythrocytes from adenosine deaminase-deficient children (4, 5) and correlates with both inhibition of DNA synthesis and cytotoxicity in cultured lymphoid cells (8–11). Deoxyadenosine is also known to inactivate S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1) (12) and, by this mechanism, may result in an increase in S-adenosylhomocysteine, a compound also associated with lymphocytotoxicity in vitro (13).

We have administered 2′-deoxycoformycin (dCF), a potent ADA inhibitor, to a patient with a lymphoproliferative malignancy. Exogenous inhibition of ADA activity resulted in an increase in plasma deoxyadenosine, an increase in erythrocyte dATP, and an inhibition of S-adenosylhomocysteine hydrolase activity. In addition, we observed unexpected and profound depletion of ATP as dATP accumulated in the erythrocytes of patients treated with this drug.

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MATERIALS AND METHODS

[14C]Adenosine (50.4 mCi/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) and [3H]dTTP (15 Ci/mmol) were obtained from New England Nuclear. Unlabeled deoxyribonucleotides, calf intestinal adenosine deaminase, and beef liver alkaline phosphatase (type IX) were purchased from Sigma. Deoxyribonucleotides were further purified by chromatography on QAQ Sephadex (14). DNA polymerase I from Escherichia coli was obtained from Boehringer Mannheim. All other chemicals were reagent grade and obtained from standard commercial sources.

Enzyme Determinations. Heparinized whole blood was centrifuged at 500 X g and the plasma was removed. The cells were resuspended in an equal volume of Seligman's balanced salt solution, and dextran was added to a final concentration of 0.4%. The cells were allowed to stand in a 20-ml syringe placed upright in a 37°C incubator for 1 hr. The plasma containing leukocytes was removed from the top of the syringe and centrifuged at 500 X g for 10 min. The pelleted leukocytes were collected and washed once in 10 mM Tris/140 mM NaCl, pH 7.4, to lyse contaminating erythrocytes. Erythrocytes sedimented by dextran were collected by centrifugation. Leukocytes or erythrocytes were then washed three times in 10 mM Tris/150 mM NaCl, pH 7.4 (buffer A), and lysed by three freeze-thaw cycles. The lysates were centrifuged at 20,000 X g for 30 min, and the supernatants were dialyzed overnight at 4°C against buffer A. All assays for ADA activity were carried out by the method of Van der Weyden et al. (15), measuring the conversion of [14C]adenosine to [14C]inosine. Specific activity is expressed as nmol/min per mg of protein. S-Adenosylhomocysteine hydrolase activity was measured by the method of Hershfield (12).

Plasma Adenosine and Deoxyadenosine Levels. Plasma obtained from heparinized whole blood was deproteinized by the method of Somogyi (16). Adenosine and deoxyadenosine were quantitated by high-pressure liquid chromatography on a μBondapak C18 column (Waters Associates). The nucleosides were separated by isocratic elution using 50 mM KH₂PO₄/10% methanol, pH 4.5, and detected at 254 nm. Peaks were identified on the basis of retention times. The identities of adenosine and deoxyadenosine peaks were confirmed by treating the sample with excess exogenous calf intestinal ADA and monitoring the formation of inosine and deoxyinosine, respectively.

ATP and dATP Determinations. Ribonucleotides and deoxyribonucleotides were extracted from 1 X 10⁶ erythrocytes with 1 ml of cold 60% aqueous methanol at −20°C overnight (6). The recovery of known quantities of ATP and dATP from

Abbreviations: ADA, adenosine deaminase; dCF, 2′-deoxycoformycin.
RESULTS

Clinical Effects. The absolute lymphocyte count (Fig. 1) was within the normal range until day 4, when it fell abruptly from 1000 to 200/μl. Between days 5 and 8, the serum glutamicoxalacetic transaminase value increased 4-fold as did the blood urea nitrogen value. After the drug was discontinued, on day 8, the transaminase value returned toward the normal range. On day 10, the patient became hypotensive and confused. The blood pressure returned to normal after administration of isotonic saline. Subsequently, the blood urea nitrogen increased to 80 mg/dl, creatinine increased to 2 mg/dl, and urine output fell to <400 ml/24 hr. The hemoglobin value decreased from 13.7 to 9.9 g/dl, serum lactate dehydrogenase increased from 487 to 1790 units/liter (normal <220 units/liter), and bilirubin increased to 6.3 mg/dl. The peripheral blood smear showed marked anisocytosis with erythrocyte fragmentation. The prothrombin time, partial thromboplastin time, and fibrinogen level remained normal; the direct antithrombin test was negative. The patient was thought to have a hemolytic anemia and was given several units of packed erythrocytes. Hypotension recurred and the patient died 18 days after initiation of treatment with dCF. At postmortem examination, dermal infiltration with lymphoid cells was noted. Pulmonary edema, pneumonia, and bilateral pleural effusions were present. The liver, kidneys, and heart were unremarkable by both gross and microscopic examinations.

Biochemical Effects. Activity of ADA in erythrocytes was completely inhibited after the initial dose of dCF at 0.01 mg/kg and remained inhibited until erythrocytes were transfused on day 12 (Fig. 2). ADA activity in peripheral leukocytes was totally inhibited by day 3. The concentration of adenosine in plasma increased from <0.1 μM to 13.7 μM on the first day after initiation of therapy and remained at 3–10 μM for 9 days (Fig. 3). Plasma deoxyadenosine levels increased more slowly and reached a maximum of 5 μM on day 6. Erythrocyte S-adenosylhomocysteine hydrolase activity had been inactivated by day 2. The increase in circulating deoxyadenosine was coincident with an increase in the level of erythrocyte dATP as determined by high-pressure liquid chromatography (see Fig. 4) and by the DNA polymerase assay (see Fig. 5). dATP levels in erythrocytes reached 110 pmol/10^6 cells on day 9 and then declined.

Despite the marked increase in erythrocyte dATP, the total adenine nucleotide content in each erythrocyte extract, as measured by absorbance at 260 nm, was constant. Therefore, the adenine ribonucleotides and deoxyribonucleotides were separated and quantitated by high-pressure liquid chromatography (Fig. 4). Serial chromatograms of the adenine nucleotides showed a progressive increase in the size of the dATP peak and diminution of the ATP peak. The results of duplicate nucleotide determinations on erythrocytes from this patient are shown in Fig. 5A. There was a gradual replacement of erythrocyte ATP by dATP on days 4–9 and a profound depletion of erythrocyte ATP by day 11. A second patient with T-cell acute
lymphoid malignancies and as immunosuppressive agents. Early clinical trials in England demonstrated that the administration of dCF was associated with profound lymphopenia in patients with solid tumors, and no adverse clinical effects were observed (20). In contrast, preliminary results of the use of dCF in the treatment of lymphoid malignancies in this country have aroused considerable concern about possible drug-related toxicity. Our patient with refractory T-cell acute lymphoblastic leukemia had a complete lysis of the leukemic cells (19), but acute tubular necrosis developed and the patient died several days later of pulmonary edema. Nephrotoxicity, altered mental status, hemolytic anemia, and pulmonary infiltrates have also been observed in other patients (21). In the present case, the administration of dCF resulted in a marked decrease in the number of normal circulating lymphocytes but did not resolve the dermal infiltration by malignant lymphocytes. Of major concern, however, was whether the hypotensive episode and subsequent clinical deterioration of our patient were directly related to the drug. The postmortem examination yielded no specific information in answer to this question.

The effects of dCF administration were more evident at the biochemical level. Erythrocyte ADA activity was completely inhibited by the low initial dose of dCF, whereas leukocyte ADA activity was still half its original level. Despite the residual enzyme activity in these cells, and presumably in other organs, plasma adenine levels increased after the initial dose and remained increased throughout the course of therapy. A measurable increase in plasma deoxyadenosine occurred only after all ADA activity in peripheral blood cells had been inhibited (day 3) and was actually preceded by inactivation of erythrocyte S-adenosylhomocysteine hydrolase activity. The increase in erythrocyte dATP paralleled the increase in plasma deoxyadenosine concentration. Both events correlated temporally with the decrease in the patient's lymphocyte count as well as with the progressive abnormalities in hepatic and renal function.

Increased levels of dATP have been thought to result in cytotoxicity by inhibiting ribonucleotide reductase and depleting cells of other deoxynucleoside triphosphates required for DNA synthesis (22–24). There is evidence, however, that this mechanism may not suffice to explain the toxicity of deoxyadenosine, in the presence of an ADA inhibitor, for cultured T lymphoblasts (11). In addition, it is unclear whether this mechanism would account for cytotoxicity in cells such as normal peripheral lymphocytes which are not actively synthesizing DNA. One alternative mechanism of deoxyadenosine toxicity that is independent of alterations in the nucleotide pools has been proposed (7). Deoxyadenosine is a potent inactivator of S-adenosylhomocysteine hydrolase, which catalyzes the synthesis and hydrolysis of S-adenosylhomocysteine (12), and S-adenosylhomocysteine accumulation has been demonstrated to inhibit methylation reactions mediated by S-adenosylmethionine (13). This mechanism could account for deoxyadenosine-mediated toxicity to nondividing cells of both lymphoid and nonlymphoid origins. Although we have confirmed that inactivation of S-adenosylhomocysteine hydrolase is a sensitive indicator of plasma deoxyadenosine levels, the role of such inactivation in promoting lymphotoxicity in vivo remains to be determined.

We have demonstrated that the pharmacologic inhibition of ADA activity in man causes a profound depletion of erythrocyte ATP as dATP accumulates, a finding that has been confirmed in the erythrocytes of additional patients. This inverse relationship of ATP and dATP in erythrocytes was completely unexpected. In vitro incubations of human erythrocytes with adenosine analogues such as 2'-fluoroadenosine
have revealed progressive synthesis of large amounts of the corresponding nucleoside triphosphate without affecting ATP concentrations (25). In addition, there is direct evidence that the glycolytic pathway is capable of generating adequate ATP to support marked increases in phosphorylating activity (26). It therefore seems unlikely that simple overutilization of ATP

in the process of dATP synthesis provides an adequate explanation for our observation.

Several alternative hypotheses must be considered. First, increased levels of deoxyadenosine resulting from ADA inhibition may compete directly with adenosine for transport or phosphorylation. The dependence of erythrocyte adenine nucleotide pools on adenosine phosphorylation has been clearly demonstrated in individuals with a hereditary 50- to 70-fold increase in erythrocyte ADA activity (27). The rapid deamination of adenosine to inosine in these cells decreases the adenosine available for phosphorylation and decreases ATP pools by at least 50%, resulting in hemolytic anemia. It appears unlikely, however, that the concentrations of deoxyadenosine resulting from dCF treatment in this patient (3-10 μM) could substantially decrease the phosphorylation of adenosine, which was also increased in concentration. Estimates of the $K_m$ of adenosine kinase for adenosine have ranged from 0.4 to 6.0 μM (28, 29). This enzyme may also play a major role in the phosphorylation of deoxyadenosine (30) but has a far lower affinity for this substrate (28, 30). Deoxyadenosine at high concentrations (100 μM) substantially decreases the conversion of adenosine to AMP by the human placental enzyme (29), but $K_i$ values for the human erythrocyte enzyme have not been reported. The complete resolution of this issue must await clarification of the relative roles and kinetics of adenosine and deoxyadenosine phosphorylating enzymes in erythrocytes.

Another possibility is that dCF itself might interfere with ATP generation. In vitro studies with Ehrlich ascites tumor cells and mouse lymphoma cells have suggested that dCF impairs the synthesis of adenine nucleotides from $^{14}$C-adenosine in these cells (31). Although the mechanism of this effect was not determined, dCF does not appear to inhibit adenosine kinase directly or to affect either de novo purine biosynthesis or the purine salvage pathway. It is of note, however, that dCF administration does not decrease erythrocyte ATP levels in the absence of deoxyadenosine generation and dATP synthesis.

Finally, dATP might act as a direct feedback inhibitor of ATP synthesis during glycolysis. The potential role of dATP as a phosphate donor in energy transfer reactions (32) makes such a regulatory role plausible in the overall conservation of cellular energy. It is apparent, however, that this mechanism does not
apply in the presence of chronic dATP increases. ATP levels in the erythrocytes of children with inherited ADA deficiency, when accurately measured, have been within or slightly below the normal range (4, 5, 33), and erythrocyte dATP/ATP ratios have ranged from 0.2 to 1.7. In our patients, the peak values of erythrocyte dATP roughly approximated the levels found in cells from ADA-deficient individuals, but the dATP/ATP ratios were 9.5 and 39.5, respectively. Because erythrocyte dATP levels may already be increased at birth in inherited ADA deficiency (34), it is not possible to investigate the potentially comparable metabolic alterations associated with the initial accumulation of dATP. It would be of interest, however, to follow ATP levels as dATP reaccumulates in the erythrocytes of these children when transfusion therapy (35) is discontinued.

It is readily apparent that the biochemical consequences of pharmacologic inhibition of ADA activity are more extensive than first envisaged and may account for some of the toxicity of this drug. Elucidation of the mechanism of ATP depletion may provide more general insights into the factors regulating energy metabolism in erythrocytes.

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