Proc. Natl. Acad. Sci. USA
Vol. 75, No. 1, pp. 472–476, January 1978
Medical Sciences

Deoxyadenosine triphosphate as a potentially toxic metabolite in adenosine deaminase deficiency

(enzyme deficiency/immunodeficiency)

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Communicated by James B. Wyngaarden, October 20, 1977

ABSTRACT **The inherited deficiency of adenosine deaminase (adenosine aminohydrolase; EC 3.5.4.4) activity in humans is associated with an immunodeficiency. Some of the immunodeficient and enzyme-deficient patients respond immunologically to periodic infusions of irradiated erythrocytes containing adenosine deaminase. It has been previously reported that erythrocytes and lymphocytes from immunodeficient and enzyme-deficient children contained increased concentrations of ATP, and in the one child studied after erythrocyte infusion therapy, the intracellular level of ATP diminished. Using high-pressure liquid chromatography that resolves ATP and 2′-dATP, we have observed greater than 50-fold elevations of dATP in the erythrocytes of immunodeficient, adenosine deaminase-deficient patients but not in the erythrocytes of an immunocompetent adenosine deaminase-deficient patient. The erythrocyte dATP in two unrelated adenosine deaminase-deficient, immunodeficient patients disappeared after infusion of normal erythrocytes. We propose that deoxyadenosine, a substrate of adenosine deaminase, is the potentially toxic substrate in adenosine deaminase deficiency, and that the mediator of the toxic effect is dATP, a recognized potent inhibitor of ribonucleotide reductase.**

The recently recognized associations of inherited immunodeficiency diseases with deficiencies of the enzymatic activity of adenosine deaminase (adenosine aminohydrolase; EC 3.5.4.4) (1, 2) or of purine nucleoside phosphorylase (3) have stimulated much study of the relationship between purine metabolism and immune function. To date perhaps 40 children with concomitant deficiencies of adenosine deaminase and immunity have been recognized. However, three children are known to be deficient in adenosine deaminase activity without loss of immune competence (4, g). Several authors have described biochemical abnormalities in materials obtained from immunodeficient, adenosine deaminase-deficient patients, including erythrocytes (5, 6), lymphocytes (7, 8), plasma (6, 8), urine (6), and cultured fibroblasts (9), and others have described relevant observations in model systems, particularly cultured cells of another mammals (10–14). Several modes of therapeutic intervention have been tried in these patients, including transplantation of fetal liver (15, 16), bone marrow (17), or thymic cells (18); interestingly, infusions of normal erythrocytes, containing adenosine deaminase, have demonstrable efficacy in some patients (8).

Several hypotheses have been proposed to explain the mechanism by which the lack of adenosine deaminase activity results in loss of immune function. Nearly all of these hypotheses are based on the assumption that adenosine deaminase normally detoxifies adenosine by converting it to inosine (10). The hypotheses then differ in their proposed mechanisms by which adenosine exerts its cytotoxic effects in cells incapable of eliminating this purine nucleoside. It has been proposed that cyclic AMP mediates the cytotoxic effects of adenosine (11, 19), that the accumulated nucleotides of adenosine induce a pyrimidine nucleotide starvation (10, 12) or inhibit glycolysis (5), and that adenosine combines intracellularly with homocysteine to form *S*-adenosylhomocysteine, which in turn acts as a potent inhibitor of methylation reactions, including the methylation of newly synthesized DNA (20).

There are specific objections to most of these proposals. In a model system, Ullman *et al.* (12) have demonstrated that cyclic AMP is not a necessary mediator of the cytotoxic effect of accumulated adenosine, but the applicability of that model system to the human disease is not clear. Elevated intracellular cyclic AMP concentrations have been observed in adenosine deaminase-deficient lymphocytes. Schmalstieg *et al.* (7) have described slightly elevated levels of intracellular pyrimidine nucleoside triphosphates and cyclic AMP in lymphocytes isolated from an adenosine deaminase-deficient patient, suggesting that there is no general pyrimidine starvation in those cells. Furthermore, the addition of the pyrimidine nucleoside, uridine, to adenosine deaminase-deficient peripheral human lymphocytes did not restore lectin-stimulated DNA synthesis, while the presence of exogenous, pure adenosine deaminase did (21).

A more general objection to all of the above hypotheses is the fact that *in vitro* adenosine deaminase-deficient fibroblasts (9) and lymphocytes (24, g) exhibit little if any increased sensitivity to adenosine. However, the impressive clinical, biochemical, and immunologic responses of certain patients and their lymphocytes to infusion of adenosine deaminase-containing erythrocytes have strongly suggested that an adenosine deaminase substrate is exerting a cytotoxic effect on the immune and skeletal systems in the enzyme-deficient patients (8, 21, 25, 26).

We have observed very abnormal levels of 2′-deoxyadenosine triphosphate (dATP) in the erythrocytes of immunodeficient, adenosine deaminase-deficient patients but not in the erythrocytes of an immunocompetent, adenosine deaminase-deficient patient. Furthermore, we have followed the loss of erythrocyte dATP in two unrelated adenosine deaminase-deficient, immunodeficient patients after the infusion of erythrocytes containing the missing enzyme activity.

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[g] W. Borkowsky, A. Gershon, S. Bajaj, and R. Hirschhorn, unpublished data.

Table 1. Adenosine deaminase activities in erythrocytes and mononuclear cells from patients A, B, C, and D

Subjects	Adenosine deaminase specific catalytic activity, nmol/hr per mg protein	
	Erythrocytes	Mononuclear cells and platelets
Normal*	85 ± 7.5	1360 ± 250
Patient A	2.2	112
Patient B	<2.0	156
Patient C†	3.3	120
Patient D	<2.0	200

Adenosine deaminase specific catalytic activities in crude extracts of erythrocytes and in peripheral blood mononuclear cells and platelets prepared by the Ficoll–Hypaque procedure (22) were determined at New York University Medical Center by described spectrophotometric methods (23).

* Values are ± SEM.

† Values obtained 77 days after infusion.

We propose that deoxyadenosine, the other naturally occurring substrate of adenosine deaminase (27), is the potentially toxic substrate in adenosine deaminase deficiency and that the mediator of the toxic effect is dATP, a recognized potent inhibitor of ribonucleotide reductase.

EXPERIMENTAL PROCEDURES

Materials. Standards of purines, nucleosides, and nucleotides were purchased from Sigma Chemical Co. DNA-dependent DNA polymerase and calf thymus DNA were purchased from Miles Laboratories and [^{3}H]dTTP (60 Ci/mmol) from New England Nuclear Corp. All other reagents were of the highest grades commercially available.

Subjects. The two unrelated adenosine deaminase-deficient children (patients A and B, 5 and 8 months old, respectively) with severe combined immunodeficiencies were each infused with 15 ml of washed fresh frozen irradiated erythrocytes per kg (8). Patient B showed some improvement, as determined by *in vitro* assays of immune function (8, 21), whereas patient A did not respond. Patient C (20 months old) received an erythrocyte infusion 77 days before collection of the available blood and urine samples, and his response was minimal. The adenosine deaminase-deficient, immunocompetent patient (patient D, 10 months old) was detected by screening newborns in the State of New York; his immune functions will be described.[g] Normal children and adults were used as sources of normal erythrocytes. A summary of the patients' residual adenosine deaminase activities is presented in Table 1. At the times of study none of the subjects were receiving any drugs except prophylactic trimethoprim-sulfamethoxazole and dicloxacillin.

Preparation of Samples. Whole blood was withdrawn from the patients into heparinized syringes and promptly centrifuged (3000–10,000 × *g*) at ambient temperature for 1–2 min. The plasma was removed, and an equal volume of cold 2 M perchloric acid was added to the erythrocyte pellet and an equal volume to the plasma. The chilled materials were again centrifuged (3000–10,000 × *g*) for 1–2 min to remove the precipitated protein, and the supernatants of the erythrocytes and of the plasma were neutralized to pH 6–8 with 5 M KOH in order to precipitate potassium perchlorate. After centrifugation the supernatant solutions were frozen and shipped to San Francisco for analysis. Urine samples were shipped frozen or at ambient conditions in the presence of 0.1% sodium azide.

High-Pressure Liquid Chromatography. Chromatography was performed with an Altex metering system with a 20-μl UV analyzer which monitored eluates at 254 and 280 nm. Nucleotide analyses were performed on a Whatman Partisil SAX column with an elution solvent of 0.45 M potassium phosphate buffer, pH 3.6. A Waters μBondapak C_{18} reverse phase column was used for the analysis of purine bases and nucleosides. The eluting solvent was 10 mM potassium acetate, pH 5.5/3% methanol or 10 mM *n*-heptane sulfonate, pH 3.3/7% methanol. Flow rates for both systems were 2.0 ml/min.

Identification of Peaks. The chromatographed ribonucleotides, ribonucleosides, deoxyribonucleosides, and purines were identified by comparison of their retention volumes and relative absorbances at 254 and 280 nm with those of known standards. For identification of the unknown nucleoside triphosphate, the material in the relevant peak (peak 2) from the preinfusion erythrocytes of patient A was collected after elution from the Partisil SAX column. A UV spectrum of that fractionated material was determined, and the fractionated material was subjected to hydrolysis by boiling in 1 M perchloric acid for 30 min (28), neutralization with KOH, and chromatography on the μBondapak C_{18} reverse phase column. The fractionated material of peak 2 and known standards were also treated with sodium periodate by the method of Keith and Gilham (29) and then rechromatographed on the Partisil SAX column.

Enzymatic Determinations of dATP. The concentrations of the dATP in erythrocyte extracts were determined by the method of Solter and Handschumacher (30) from the dependence of the DNA polymerase reaction upon the presence of the triphosphates of all four deoxyribonucleosides. The reactions were for 60 min in the presence of saturating concentrations of dCTP (2 μM), dGTP (2 μM), and [^{3}H]dTTP (2 μM). The extent of reaction was dependent upon and linear with the volume of added erythrocyte extract.

RESULTS

Upon high-pressure chromatographic analysis of the erythrocytic nucleotides we noted the presence of a significant quantity (about 340 nmol/ml of packed erythrocytes) of an unusual nucleoside triphosphate in the extracts from patient C. Subsequent analysis of similarly prepared erythrocyte extracts from patients A and B before their treatment by erythrocyte infusion revealed the presence of an even greater quantity of that abnormal nucleoside triphosphate (peak 2, Fig. 1) which is not present in extracts of normal erythrocytes. In the extracts of erythrocytes obtained from patients A and B after they had been infused with normal erythrocytes, there was a first-order decay constant in patient A of about 0.3 day^{-1} (Figs. 1 and 2).

To identify the unusual nucleotide we collected the material present in peak 2 of Fig. 1 and analyzed it. It possessed a UV spectrum indistinguishable from that of ATP. The material, when mixed with known 2′-dATP, cochromatographed with that standard on the Partisil SAX column. After the UV-absorbing material was boiled in 1 M $HClO_4$ for 30 min, it chromatographed on the μBondapak C_{18} column as adenine. After treatment with sodium periodate (29), the unknown material of peak 2 and the known dATP still chromatographed on the Partisil SAX column as untreated, known dATP, while identically treated ATP eluted in the void volume of that column, as do adenine and adenosine. The concentration of ATP in the initial erythrocytes of patient A was independently determined to be 1100 nmol/ml of packed cells by the DNA-dependent DNA polymerase reaction (30). dATP in normal erythrocytes

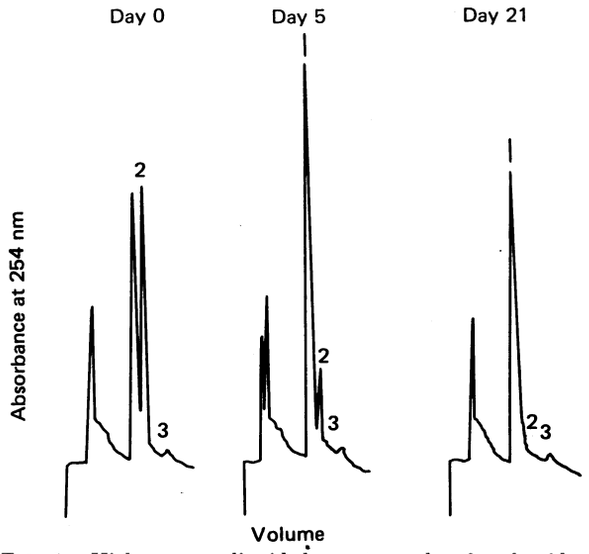

FIG. 1. High-pressure liquid chromatography of nucleoside triphosphates in erythrocytes of patient A. Blood was drawn from patient A before (day 0) and 5 and 21 days after an infusion of erythrocytes containing adenosine deaminase. The soluble nucleotides of the erythrocytes were extracted with perchloric acid and neutralized, and equal volumes of each sample were subsequently analyzed on a Partisil SAX column. The elution buffer was 0.45 M potassium phosphate, pH 3.6; flow rate was 2.0 ml/min. The profiles depict the elution of ATP, dATP, and GTP as peaks 1, 2, and 3, respectively. The points of sample injection are indicated by vertical lines below the baseline; the retention times of ATP, dATP, and GTP were 9, 10, and 13 min, respectively.

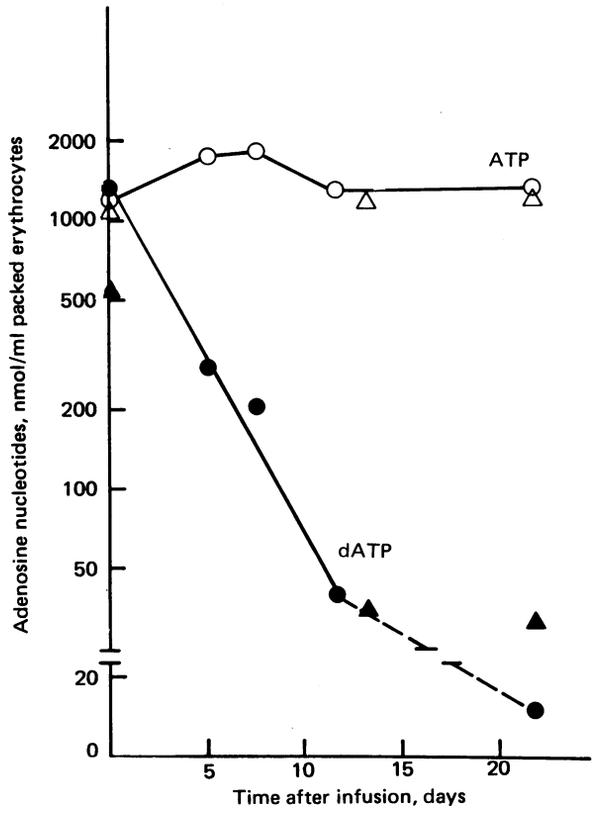

FIG. 2. Concentrations of ATP and dATP in the erythrocytes of patients A and B after erythrocyte infusion. From peak height analyses such as those depicted in Fig. 1 and of known concentrations of nucleotide standards, the concentrations of erythrocytic ATP (○, △) and dATP (●, ▲) were determined in samples drawn from patient A (○, ●) and from patient B (△, ▲) at the indicated times after erythrocyte infusion. The half-life of dATP after transfusion in patient A is approximately 2.5 days. In both patients the dATP levels were less than 40 μM at day 21. The concentration of ATP in the erythrocytes of five normal subjects is 1250 ± 100 (SEM) nmol/ml of packed cells, and dATP is not detectable (less than 20 nmol/ml of packed cells).

was 8 nmol/ml of packed cells by the same assay procedure. Thus, we conclude that the material in peak 2 is 2′-dATP.

High-pressure chromatographic analysis of the erythrocytes from the immunocompetent, adenosine deaminase-deficient patient (D) did not reveal any (<20 μM) dATP. Furthermore, dATP was not detected in similarly prepared erythrocytes from two untreated, unrelated immunodeficient patients lacking purine nucleoside phosphorylase nor in the erythrocytes from patients with Lesch–Nyhan disease (lacking hypoxanthine phosphoribosyltransferase) (data not shown).

Table 2. Adenine and adenosine concentrations in plasma and urine of patients A, B, C, and D

Subjects	Plasma		Urine
	Adenine, μM	Adenosine, μM	adenine, μM
Normal	<1	<1	<2
Patient A			
Day 0	1.8	4.6	16
Day 5	<1	<1	—
Day 7	<1	<1	<4
Day 10	<1	3.4	—
Day 21	<1	3.2	<4
Patient B			
Day 0	<1	<1	—
Day 14	<1	<1	—
Day 22	<1	<1	—
Patient C	<1	6.7	18
Patient D	<1	2.0	<1

Plasma and urine samples were analyzed for adenine, adenosine, and deoxyadenosine on a Waters μBondapak C_{18} column. The elution solvent for analysis of urine contained 10 mM *n*-heptane sulfonate, pH 3.3/7% methanol; that for analysis of plasma extracts contained 10 mM potassium acetate, pH 5.5/3% methanol. Quantitation was based on analyses of known concentrations of standards. Deoxyadenosine was not detectable (<2 μM) in urine or plasma, and adenosine was not detectable (<2 μM) in urine.

The plasma and urine samples from the enzyme-deficient patients contain less than 2 μM deoxyadenosine, but in three of the four patients they contained detectable quantities of adenosine and/or adenine, both of which disappeared after the infusion of erythrocytes (Table 2). The plasma from the adenosine deaminase-deficient but immunocompetent patient (D) contained significant quantities of adenosine but no adenine. His urine was also free of adenine. When 2′-deoxyadenosine (12 μM) was added to normal plasma, and the mixture extracted by the perchloric acid method, no free adenine was generated and the deoxyadenosine could be detected by high-pressure liquid chromatography at 12 μM levels.

Other abnormalities of nucleotide concentrations in adenosine deaminase-deficient erythrocytes (patients A, B, C, and D) included reduced-to-absent CDP-choline and somewhat increased UDP-glucose and UDP-galactose concentrations. However, these specific abnormalities were not unique to the enzyme-deficient erythrocytes; we observed similar abnormalities in erythrocytes from two purine nucleoside phosphorylase-deficient patients (data not shown), and we and others have observed the same in those from Lesch–Nyhan patients

(31), who are not immunodeficient. Although we do not understand the basis for these abnormalities, they do not appear to be related to immune dysfunction.

DISCUSSION

It seems evident that the deficiency of adenosine deaminase activity causes immune dysfunction in humans. This evidence has been strengthened by the observations that the introduction of the missing enzyme can in some cases promote the *in vivo* (8) and *in vitro* (21) restoration of immune function. However, the lack of enhanced sensitivity of adenosine deaminase-deficient cells to adenosine cytotoxicity (9, 24) suggested that this adenosine deaminase substrate was not potentially toxic. The only major metabolic abnormality noted in cells from the enzyme-deficient patients has been increased ATP (6–9), but the assays used by Polmar and his coworkers (5, 8), Mills *et al.* (6), and Schmalsteig *et al.* (7) do not distinguish between ATP and dATP (32). Our observation that the erythrocytes of the immunodeficient, adenosine deaminase-deficient patients contain a greater than 50-fold increase in dATP, which disappears upon *in vivo* infusion of normal erythrocytes and is not present in the immunocompetent, enzyme-deficient child, lends strong support to the hypothesis that dATP is the toxic metabolite responsible for the immune deficiency. It seems likely that the reported 5- to 20-fold increase in lymphocyte ATP in the adenosine deaminase-deficient patients (7, 8) is at least partially due to increased dATP. Unfortunately we did not have available for study any adenosine deaminase-deficient lymphocytes from an immunodeficient child prior to treatment to determine whether this supposition is correct.

Since only dividing cells contain ribonucleotide reductase activity (33), the erythrocytic dATP must have been derived from an extracellular deoxyribonucleoside even though our current techniques are not sufficiently sensitive to detect plasma deoxyadenosine. It is clear from the reconstruction experiment described above that plasma deoxyadenosine was not hydrolyzed to adenine during the sample preparation. Most likely, the uptake of deoxyadenosine is so rapid as to deplete the extracellular fluids of that metabolite even in the absence of adenosine deaminase. The daily urinary excretion of 1.8 mmol of deoxyinosine by a purine nucleoside phosphorylase-deficient patient (34) provides clear evidence for a comparable *in vivo* rate of deoxyadenosine production.

The correlation between the dATP levels in the erythrocytes of the subjects examined and their lack of immune function suggests that this reduced nucleotide is the mediator responsible for loss of immune function in adenosine deaminase deficiency. There are several mechanisms by which dATP might be toxic to cells: (*i*) It can serve as a substrate for adenylate cyclase (35, 36), generating a 2′-deoxy analog of cyclic AMP. (*ii*) It is a known potent inhibitor of the ribonucleotide reductase responsible for the reduction of all of the purine and pyrimidine ribonucleotides to their respective 2′-deoxyribonucleotides (37), necessary precursors for DNA synthesis. (*iii*) It most likely exerts its cytotoxic effects by causing the cellular depletion of the other reduced purine and pyrimidine nucleotides (37–42), that is, dCTP, dTTP, and dGTP. This last hypothesis is testable both *in vitro* and *in vivo* since all of the deoxynucleosides appear to have specific kinases in mammalian tissues (43, 44).

It is also possible that the deoxyadenosine could itself, as does adenosine (11, 19, 45, 46), serve as an agonist at the cell membrane to activate adenylate cyclase and promote the accumulation of cyclic AMP, a known immunosuppressive agent (47). This latter hypothesis seems unlikely from studies in a model system of mouse T-cell lymphoma cells[h] and from the observation in at least one system that 2′-deoxyadenosine is not a particularly effective agonist (48).

The adenosine deaminase-deficient cells from patients must now be reexamined, but for enhanced sensitivity to deoxyadenosine rather than to adenosine (9, 24). If they exhibit increased sensitivity to this adenosine deaminase substrate, then the ability of exogenous reduced ribonucleosides and/or an inhibitor of adenosine transport, such as dipyridamole (49), to reverse the cytotoxicity can be evaluated *in vitro*. In S49 mouse T-cell lymphoma cells the reduced ribonucleosides are capable of reversing the cytotoxic effects of micromolar concentrations of deoxyadenosine in the presence of an inhibitor of adenosine deaminase.[h]

Although the enzyme-deficient, immunocompetent children in the !Kung tribe have detectable residual adenosine deaminase activity in their cells (4), there may be other, unrecognized reasons that they and patient D described herein have retained immune function. Perhaps their mutant adenosine deaminases have retained the ability to deaminate deoxyadenosine and thereby can protect their immune systems. Contrariwise, some immunodeficient patients may possess mutant enzymes incapable of deaminating deoxyadenosine but that have retained catalytic activity towards adenosine. The latter would likely be missed by current screening procedures.

Since the erythrocytes of patient D do not contain detectable dATP, it would be interesting to known whether the same is true in materials from the !Kung children (4). If so, these observations may in the future allow the prediction of which adenosine deaminase-deficient patients will be immunodeficient. We have not obtained sufficient data from adenosine deaminase-deficient patients to determine whether there exists a biochemical basis from which to predict which individuals will respond to erythrocyte infusions. Responsiveness may depend upon whether immune precursor cells have survived the disease to the time of therapy.

One might expect common mechanisms or mediators of the immune deficiencies in adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency. As noted above, dATP is not detectable in the erythrocytes of purine nucleoside phosphorylase-deficient patients. However, patients deficient in purine nucleoside phosphorylase do accumulate 2′-deoxyguanosine (34), which, in at least some cells, can be phosphorylated to dGTP. dGTP, like dATP, can inhibit ribonucleotide reductase activity, but unlike dATP, dGTP inhibits only the enzyme activities that reduce GDP to dGDP and CDP to dCDP (37) and thus leads to the depletion of intracellular dCTP (42). In the model system previously described (12) the addition of micromolar concentrations of deoxyguanosine increases the intracellular concentration of dGTP and kills the T-lymphoma cells.[i] This toxic effect of exogenous deoxyguanosine can be reversed when deoxycytidine is simply added to the culture medium.[i] Interestingly, the human tissue with greatest deoxyguanosine phosphorylating activity is the thymus (50, 51). These observations may explain the similar immunologic phenotypes of adenosine deaminase and purine nucleoside phosphorylase deficiencies and the unique vulnerability of T lymphocytes to the loss of the latter enzyme activity.

This work was supported by a Center for Medical Genetics Grant GM-19527, National Institutes of Health Grants HD-07778, AI-10404, AI-11576, and AI-10343, and the National Foundation. R.H. is a re-

[h] B. Ullman, L. J. Gudas, A. Cohen, and D. W. Martin, Jr., unpublished data.

[i] L. J. Gudas, B. Ullman, and D. W. Martin, Jr., unpublished data.

cipient of a Research Career Development Award (AI-70254). D.W.M. is an Investigator, Howard Hughes Medical Institute. A.C. is supported by a National Institutes of Health Program Project Grant, Clinical Pharmacology–Pharmacokinetics.

Note Added in Proof. Recently Carson *et al.* (52) have found that thymus and spleen exhibit the highest deoxyadenosine phosphorylating activity of all human fetal tissue examined.

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