In Vivo Inactivation of Erythrocyte S-Adenosylhomocysteine Hydrolase by 2′-Deoxyadenosine in Adenosine Deaminase-Deficient Patients

MICHAEL S. HERSHFIELD and NICHOLAS M. KREDICH, *Departments of Medicine and Biochemistry, Division of Rheumatic and Genetic Diseases, Duke University Medical Center, Durham, North Carolina 27710*
DENNIS R. OWNBY, HELEN OWNBY, and REBECCA BUCKLEY, *Department of Pediatrics, Duke University Medical Center, Durham, North Carolina 27710*

ABSTRACT The cytotoxic nucleoside 2′-deoxyadenosine is excreted in excessive amounts by individuals with genetic deficiency of adenosine deaminase, and may be in part responsible for the severe combined immune dysfunction from which they suffer. Earlier studies from this laboratory showed that 2′-deoxyadenosine causes the irreversible inactivation of the enzyme S-adenosylhomocysteine hydrolase by an active site-directed, "suicide-like" process. In this communication we have demonstrated similar inactivation of S-adenosylhomocysteine hydrolase in hemolysate and in intact erythrocytes, as well as a striking deficiency of S-adenosylhomocysteine hydrolase activity in the erythrocytes of three adenosine deaminase-deficient patients. In vivo suicide-like inactivation of S-adenosylhomocysteine hydrolase by 2′-deoxyadenosine may contribute to the cytotoxicity of 2′-deoxyadenosine and to the immune dysfunction in adenosine deaminase deficiency.

INTRODUCTION

The cytotoxicity of the nucleosides adenosine and 2′-deoxyadenosine may explain the severe combined immune dysfunction in genetic deficiency of adenosine deaminase (ADA,[1] EC 3.5.4.4) (1). Adenosine arrests the growth of human lymphoblast mutants unable to convert it to adenine nucleotides (2). A high affinity adenosine binding protein, postulated to mediate this nucleotide-independent toxicity (2), has been identified as the enzyme S-adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1) (3), which catalyzes the reversible reaction, adenosine + L-homocysteine ⇌ AdoHcy. Because the equilibrium constant of this reaction favors AdoHcy formation (4), loss of ADA can lead to accumulation of AdoHcy (5), a product and potent inhibitor of numerous S-adenosylmethionine-dependent methyl transfer reactions. Such inhibition of transmethylation appears to account for nucleotide-independent adenosine toxicity to mouse (5) and human lymphoblasts.[2]

Hershfield (6) has recently proposed that AdoHcy hydrolase may be a "target" for nucleotide-independent effects of 2′-deoxyadenosine, as well as of adenosine, in ADA deficiency. Thus, human lymphoblast AdoHcy hydrolase was found to bind [^{3}H]2′-deoxyadenosine tightly, and binding was associated with irreversible inactivation of catalytic activity (6) by an active site-directed, "suicide-like" (7, 8) process. We now wish to report evidence for the operation of this process in vivo—the striking deficiency of AdoHcy hydrolase activity in the erythrocytes of three ADA-deficient patients.

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[1] *Abbreviations used in this paper*: ADA, adenosine deaminase; AdoHcy, S-adenosylhomocysteine; EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine.

[2] Kredich, N. M., and M. S. Hershfield. 1979. S-Adenosylhomocysteine toxicity in normal and adenosine kinase-deficient lymphoblasts of human origin. *Proc. Natl. Acad. Sci. U. S. A.* In press.

METHODS

Materials. Radioactive chemicals were obtained from Amersham/Searle Corp. (Arlington Heights, Ill.); nonradioactive compounds from Sigma Chemical Co. (St. Louis, Mo.) or P-L Biochemicals, Inc. (Milwaukee, Wis.); cellulose thin-layer plates from Eastman Kodak Co. (Rochester, N. Y.); and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) from Burroughs Wellcome Co. (Research Triangle Park, N. C.).

Preparations of cell extracts. Hemolysates were prepared from heparinized blood by mixing packed erythrocytes (washed twice with phosphate-buffered saline at 4°C) with an equal volume of distilled water followed by freezing, thawing, and centrifuging at 9,000 *g* for 30 min. For high pressure liquid chromatographic analysis, cells and plasma were extracted in $HClO_4$ and neutralized as previously described (5).

Enzyme assays. AdoHcy hydrolase was assayed by measuring conversion of [8-^{14}C]adenosine and L-homocysteine to AdoHcy by a thin-layer chromatographic method (3, 6). Reaction mixtures (0.05 ml) contained 5–20 μl of diluted (0- to 20-fold) hemolysate; 25 mM potassium phosphate, pH 7.0; 1 mM dithiothreitol; 1 mM Na_2 EDTA; 5 μM EHNA; 150 μM [8-^{14}C]adenosine (20–40 cpm/pmol); and 5 mM L-homocysteine. The ADA inhibitor, EHNA (9), was used to prevent rapid catabolism of [^{14}C]adenosine and(or) 2′-deoxyadenosine and did not interfere with AdoHcy hydrolase activity. Enzyme activity was linear both with time for up to 60 min, and with the hemolysate concentration. Assays were performed in duplicate and for two time points. ADA, adenine phosphoribosyltransferase, hypoxanthine-guanine phosphoribosyltransferase, and adenosine kinase were assayed as previously described (2).

Protein was assayed by the method of Lowry et al. (10) and hemoglobin as cyanomethemoglobin. High pressure liquid chromatographic measurements were determined using a Waters model 6000 instrument, (Waters Associates, Inc., Milford, Mass.) with a Whatman Partisil SAX column (Whatman, Inc., Clifton, N. J.) for dATP (11), and a Waters C_{18} uBondapak column (Waters Associates, Inc.) for adenosine and 2′-deoxyadenosine. Separation of nucleosides was achieved with 50 mM Na acetate, pH 5.0, containing 5% methanol.

Description of patient. A severely ill 9-wk-old caucasian male was transferred to Duke University Medical Center (Durham, N. C.) with a presumptive diagnosis of severe combined immunodeficiency disease, based upon a 5-wk history of persistent cough, oral moniliasis, failure to thrive, and laboratory data which included a leukocyte count of 5,300 per mm^3 with 19% lymphocytes, 38% polys, 3% stabs, 8% monocytes, 32% eosinophils, 1% basophils; and diminished immunoglobulin levels (IgG = 110, IgM = 5, and IgA = 5 mg/dl). The diagnosis was confirmed by additional findings of persistent lymphopenia (350–1,000 per mm^3) with E_n rosetting of <2% (normal = 64%); and lack of blastogenic response of peripheral blood mononuclear cells to phytohemagglutinin, pokeweed mitogen, or concanavalin A, using methods previously described (12, 13). ADA deficiency was found in erythrocytes and peripheral mononuclear cells (see Results). *Candida albicans* meningitis was diagnosed and responded to treatment with 5-fluorocytosine and amphotericin B. Transfusion therapy (14) with fresh irradiated whole blood at a dose of 30 ml/kg at weekly intervals for 4 wk, then biweekly, begun at 10 wk of age had not yet resulted in significant alteration in the patient's immune status.

RESULTS

The pretreatment activities of AdoHcy hydrolase, ADA, and adenine phosphoribosyltransferase in hemolysates of three unrelated patients are shown in Table I, and the variation in these activities in the Durham patient during a 2-mo period from diagnosis through the initial period of hypertransfusion are shown in Fig. 1. All three patients had virtually undetectable ADA activity, and each had <2% of mean control AdoHcy hydrolase activity; adenine phosphoribosyltransferase was within the normal range for this laboratory (Table I). Activities of hypoxanthine-guanine phosphoribosyltransferase, adenosine kinase, and purine nucleoside phosphorylase

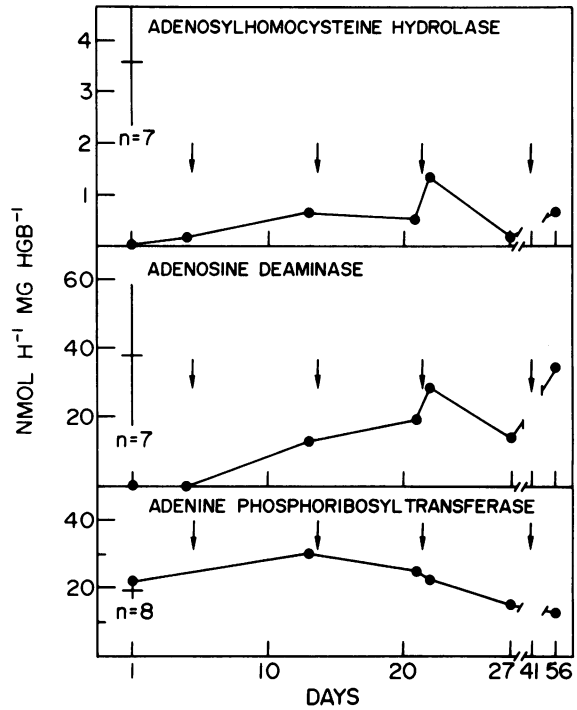

FIGURE 1 Activities of erythrocyte AdoHcy hydrolase, ADA, and adenine phosphoribosyltransferase before and during first 7 wk of transfusion therapy. Arrows indicate transfusions with irradiated blood. Means and standard deviations of controls are indicated at left-hand side of each panel by +.

TABLE I

Pretreatment Erythrocyte Enzyme Specific Activities in Untreated ADA-Deficient Patients

Patient No.	Sex	Location	Adenosylhomocysteine hydrolase	ADA	Adenine phosphoribosyltransferase
				% control*	
1	Male	Durham	1.3	<0.1	146
2	Male	Toronto	0.9	<0.1	71
3	Female	Wisconsin	1.4	<0.1	166

* Control specific activities (n = 9): adenosylhomocysteine hydrolase, 3.65±1.01; ADA, 37.02±19.17; and adenine phosphoribosyltransferase, 20.41±4.04 nmol/h per mg protein (mean±SD).

were also within the normal range (data not shown). With hypertransfusion, both ADA and AdoHcy hydrolase activities increased in the Durham child's erythrocytes, reflecting the contribution of donor cells (Fig. 1). However, AdoHcy hydrolase activity did not increase to the extent that would have been expected from the increase in ADA activity. Furthermore, there appeared to be a greater fall in AdoHcy hydrolase than in ADA activity between transfusions. For example, the last set of data in Fig. 1 were obtained 2 wk after the fifth transfusion, at which time the patient's hemolysate had about 17% of the mean control AdoHcy hydrolase, but ≅90% of mean ADA activity. Similarly, erythrocytes obtained from the Toronto patient 6 d after a transfusion had ≅20% of control AdoHcy hydrolase activity, but ADA activity in the normal range.

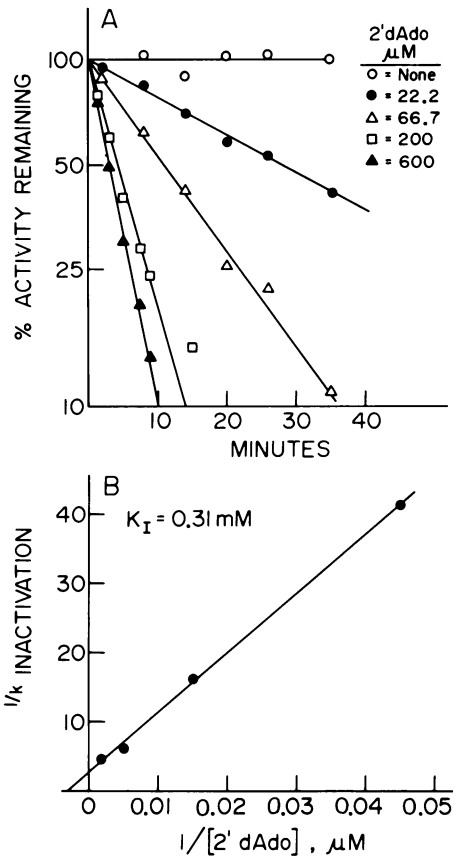

FIGURE 2 (A) Kinetics of inactivation of erythrocyte AdoHcy hydrolase by 2'-deoxyadenosine. Aliquots of normal adult hemolysate were incubated at 37°C with the indicated concentrations of 2'-deoxyadenosine (2'dAdo) in 25 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM dithiothreitol; and 5 μM EHNA. At various times 5-μl aliquots were transferred to AdoHcy hydrolase assay mixtures (see Methods) and incubated for an additional 15 min. (B) Reciprocal plot of first-order rate constants for inactivation ($k_{inactivation}$) calculated from (A) vs. 2'-deoxyadenosine concentration. K_I (7) is an index of the equilibrium constant for the enzyme 2'-deoxyadenosine complex from which inactivation proceeds (see Discussion). The maximal rate of inactivation at saturating 2'-deoxyadenosine, estimated from the y intercept in (B), is ≅0.36/min for the experiment shown.

Inactivation by 2'-deoxyadenosine of AdoHcy hydrolase in a normal adult hemolysate is shown in Fig. 2. When whole blood from a normal individual was incubated with 10 μM EHNA to inhibit ADA, 10 and 50 μM 2'-deoxyadenosine inactivated intracellular AdoHcy hydrolase at initial rates of ≅4.7 and 13.9%/min, with 44 and 83% inactivation occurring by 60 min. Slowing of the initial rate of inactivation in intact cells may have resulted at least partly from depletion of inactivator by conversion to intracellular nucleotides, a process which could not occur in the study shown in Fig. 2. After exposure of whole blood to 10 μM [^{3}H]deoxyadenosine for 60 min, followed by lysis and fractionation of the crude hemolysate by gel filtration (as described [6] using Ultrogel AcA 34 [LKB Instruments, Rockville, Md.], fractionation range 20,000–350,000 mol wt), radioactivity coeluted with the peak of AdoHcy hydrolase activity. Treatment with acid released bound tritium, indicating a noncovalent complex. However, as with the human lymphoblast enzyme (6) prolonged dialysis (72 h) did not restore hydrolase activity, either to the hemolysates from ADA-deficient patients or to enzyme inactivated by 2'-deoxyadenosine in vitro; control enzyme preparations did not lose activity during dialysis. Mixing of undialyzed patient and control hemolysates gave the predicted AdoHcy hydrolase activity (not shown). Although this suggests the absence of a conventional inhibitor, prolonged coincubation of hemolysates, necessary to detect slow inactivation by a low concentration of 2'-deoxyadenosine in the patient's hemolysate, was not done.

Peripheral blood mononuclear cells were not available prior to transfusion, but were obtained by Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals Inc., Piscataway, N. J.; Hypaque, Winthrop Laboratories, New York) gradient centrifugation (12, 13) just before the third transfusion. The sample consisted largely of aggregated platelets and contained roughly equal percentages of lymphocytes and monocytes, and did not show a blastogenic response to phytohemagglutinin or pokeweed mitogen. This preparation had virtually no measurable ADA activity (indicating the presence of patient, and not donor, cells), but did contain measurable AdoHcy hydrolase activity. Unfortunately, we did not have a similarly constituted preparation of control cells for comparison of the amount of activity per cell. However, based upon protein content and adenine phosphoribosyltransferase activity, the patient's mononuclear cells appeared to possess roughly normal AdoHcy hydrolase activity. This suggests that his erythrocyte deficiency did not result from a mutation in a structural gene for AdoHcy hydrolase. Such a defect

would, of course, be highly unlikely to occur in three unrelated ADA-deficient patients.

We found a marked increase in dATP content of the patient's erythrocytes (≅210 μM, compared with a control of <5 μM) obtained before transfusion, at a time when 2′-deoxyadenosine and adenosine concentrations in his plasma were each ≅1 μM. Erythrocyte dATP had fallen to ≅35 μM just before the third weekly transfusion, when plasma adenosine and 2′-deoxyadenosine were undetectable (<0.2 μM).

DISCUSSION

Binding of [^{3}H]2′-deoxyadenosine to human lymphoblast (6) and erythrocyte AdoHcy hydrolase is associated with irreversible inactivation of the enzyme proceeding with first order kinetics, with saturation of the rate of inactivation at high 2′-deoxyadenosine concentration. Inactivation and binding of 2′-deoxyadenosine are both prevented by normal enzyme substrates, adenosine and AdoHcy (6). These findings indicate that inactivation proceeds from an enzyme-2′-deoxyadenosine complex, and suggest an active site directed process which has many characteristics of a suicide process. The latter usually involves covalent attachment of inactivator to enzyme, which does not occur with 2′-deoxyadenosine. Further studies of the mechanism of inactivation are in progress.

We believe that prolonged exposure to low concentrations of 2′-deoxyadenosine has led to the in vivo inactivation of AdoHcy hydrolase in the erythrocytes of the patients we have studied. This conclusion is supported by the finding of measurable amounts of the deoxynucleoside in pretransfusion plasma, and by demonstration of rapid inactivation of the enzyme activity in intact normal erythrocytes treated with an ADA inhibitor and 10 μM 2′-deoxyadenosine. Impaired deamination of 2′-deoxyadenosine by ADA-deficient patients is also responsible for the striking accumulation of dATP we and others (15–18) have observed in their circulating blood cells. Because there is continued overexcretion of 2′-deoxyadenosine by ADA-deficient patients despite transfusion therapy (18), continued inactivation by 2′-deoxyadenosine may explain the inability to maintain as high a level of AdoHcy hydrolase as ADA activity by repeated hypertransfusion (Fig. 2). If this conclusion is correct, then serial measurement of erythrocyte AdoHcy hydrolase activity may be a useful and convenient way to gauge continuing exposure to 2′-deoxyadenosine in ADA deficiency.

We are presently examining factors that determine the degree and effect of AdoHcy hydrolase inactivation in model cell systems. Mature erythrocytes are incapable of new protein synthesis, and therefore are especially susceptible to enzyme inactivation. The relatively slow protein turnover in nondividing, unstimulated lymphocytes might also predispose these cells to develop significant deficiency of AdoHcy hydrolase in ADA-deficient patients. Because AdoHcy hydrolase is the primary AdoHcy catabolizing enzyme in mammalian cells, its nearly complete inactivation should result in accumulation of AdoHcy and inhibition of many transmethylation reactions. Such inhibition might interfere with several essential processes requiring methylation, including, among others, ribosomal RNA maturation, messenger RNA processing, and perhaps membrane function.

The studies reported here and elsewhere (3, 6) establish AdoHcy hydrolase as a target for 2′-deoxyadenosine as well as adenosine (3, 5) toxicity when ADA activity is inhibited or genetically deficient. Further studies will be required to determine the extent to which inactivation of AdoHcy hydrolase contributes to the immume dysfunction in ADA deficiency. However, the disease presents an example of a unique mechanism for production of a secondary enzyme deficiency resulting from a primary inborn metabolic error.

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