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Research Article

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Biochemical and Functional Abnormalities in Lymphocytes from an Adenosine Deaminase-deficient Patient during Enzyme Replacement Therapy

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ABSTRACT Biochemical and immunological properties of lymphocytes were measured repetitively over a period of 40 mo during enzyme replacement by transfusion in a child with adenosine deaminase (ADA) deficiency and severe combined immunodeficiency disease. Catalytically defective ADA protein is present in the child's cells. ADA activity in his lymphocytes is 7 nmol/min per 10^8 cells with 51 ng of ADA protein/10^8 cells by radioimmunoassay. ADA activities in normal cord and adult lymphocytes average 193 and 92 nmol/min per 10^8 cells, respectively, with 429 and 223 ng of ADA protein/10^8 cells. Deoxy(d)ATP accumulates in the patient's erythrocytes and lymphocytes. Transfusion of irradiated packed erythrocytes partially corrects the metabolic defects. Frank metabolic relapse occurs if transfusions are discontinued for several months. The amounts of dATP in erythrocytes and lymphocytes averaged 13 and 2 times normal, respectively, during periods when transfusions were administered every 2–4 wk. Deoxyguanosine triphosphate and deoxycytidine triphosphate in lymphocytes were normal on 11 occasions, but deoxyribosylthymine triphosphate was ninefold increased. On 11 occasions dATP was measured in lymphocytes and erythrocytes isolated simultaneously. There was a positive, but statistically insignificant, correlation between amounts of dATP in the two types of cells ($r = 0.25, P > 0.1$). The absolute peripheral lymphocyte count was correlated with the activity of ADA in circulating erythrocytes and with the response of lymphocytes to phytohemagglutinin ($r = 0.64$, $P < 0.01$; $r = 0.49, P < 0.05$). Response of lymphocytes to stimulation by phytohemagglutinin in vitro and absolute peripheral lymphocyte counts were not significantly correlated with levels of dATP in the erythrocyte or lymphocyte during periods of intensive therapy. Although there was objective improvement during enzyme replacement, the child remained immunodeficient and biochemically abnormal.

INTRODUCTION

Adenosine deaminase catalyzes the deamination of both adenosine and deoxyadenosine. Hereditary lack of adenosine deaminase (ADA)[1] activity causes severe impairment of cellular and humoral immunity (1). The primary metabolic abnormality appears to be decreased catabolism of deoxyadenosine with increased plasma levels of this substance (2, 3). Several secondary metabolic disturbances lead to death or malfunction of lymphocytes. dATP accumulates to extraordinarily high levels in lymphocytes and erythrocytes of most affected children (4–6), possibly because of the relatively high activity of deoxynucleoside kinases and low activity of 5′-nucleotidases in lymphoid precursors (2, 3, 7–9).

Normal erythrocytes contain ADA. Transfusion of

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[1] *Abbreviations used in this paper*: ADA, adenosine deaminase; ADA_1, the major isozyme of ADA; ADA_2, the minor isozyme of ADA; dCF, deoxycoformycin; dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate; dNTP, deoxynucleotide triphosphate; dTTP, deoxyribosylthymine triphosphate.

these cells to ADA-deficient children partially corrects the metabolic abnormalities observed in plasma, urine, and blood cells (4–6, 10–14), although evaluation of data is difficult because of the failure of some laboratories to distinguish ribo- and deoxyribonucleotides. Improvement in immunological status occurs in approximately one-half of treated patients (2). The reasons for differences in response are not known. Because ADA-deficient children who are immunodeficient are profoundly lymphopenic, most biochemical measurements have been made on erythrocytes, plasma, and urine rather than lymphocytes. Biochemical characteristics of the patient's lymphocytes are not well described, particularly with regard to changes during long periods of therapy. Although we have reported elevated dATP in lymphocytes from the peripheral blood and bone marrow of our patient (6), others have not found elevations of dATP in lymphocytes from a different patient who is both ADA- and immunodeficient (15). In this paper we present information about the mutant enzyme in lymphocytes, deoxynucleotide concentrations in both lymphocytes and erythrocytes, and measurements of lymphocyte function. Repetitive studies were carried out over a 40-mo period of enzyme replacement by transfusion of an ADA-deficient child.

METHODS

Patient. A Black male infant appeared normal at birth and weighed 8 lb (75th percentile). He grew very poorly during the first few months of life and by 3 mo was well below the 5th percentile in weight. At 4 mo of age he was diagnosed as ADA deficient with severe combined immunodeficiency (4). Less than 3% of the normal activity of ADA was present in erythrocytes, lymphocytes, and granulocytes. The parents are heterozygous for ADA deficiency. Although they are from the same geographic area, consanguinity has not been proved. Since diagnosis, the patient has been treated by transfusion of irradiated frozen erythrocytes. On several occasions irradiated cryoprecipitate-removed fresh frozen plasma has also been administered. His growth rate increased after transfusion, until at 30 mo he was above the 10th percentile in weight. Since then he has grown less well. Clinical and immunological details of his illness have been presented (12). The patient's bone marrow has remained megaloblastic despite enzyme replacement coupled with the parenteral administration of vitamin B_{12} and folinic acid.

Cell separations and preparation of extracts. Samples of peripheral blood were removed immediately before transfusion and were anticoagulated with EDTA. Purified erythrocytes were prepared by repeated dextran sedimentations, lymphocytes by sedimentation on density gradients (4). Preparations of lymphocytes contained 5–20% monocytes. In some cases, highly purified lymphocytes from cord and peripheral blood were prepared by passing the anticoagulated specimen over columns of Sephadex G-10 to remove monocytes and granulocytes (16). Lymphocytes were then separated from erythrocytes by density gradient centrifugation. Cells were counted and pelleted in conical centrifuge tubes to contain ~5×10^6 mononuclear or 10^9 erythrocytes per tube. Supernatant fluid was removed, and the cells were frozen at −80°C until used in experiments. Deoxynucleotides are stable indefinitely under these conditions. Lymphocytes prepared by the Sephadex method contained <5% monocytes. We could not demonstrate significant differences in deoxynucleotide pool sizes of lymphocytes prepared by the two methods, despite differences in contamination by monocytes.

Urine was collected and frozen immediately after voiding. Greater than 99% of radiolabeled adenosine and deoxyadenosine added to urine was recovered without degradation after 120-min incubation at 37°C. Plasma was separated from cells after several hours at ambient temperature. If transfusions were to be administered, blood was drawn for laboratory studies before transfusion. Nucleosides were extracted from urine with cold 60% methanol (12) and from plasma with barium hydroxide-zinc sulfate (17). Pellets of frozen cells ($2–7 \times 10^6$ nucleated cells or $1–2 \times 10^9$ erythrocytes) were extracted with 1 ml of cold 60% methanol (12). After extraction, samples were centrifuged. The supernatant fraction was removed, dried quickly at 30°C using a Buchler Vortex evaporator, reconstituted with a small volume of water, and centrifuged before assay. Extracts of cells for assay of ADA activity and antigen were prepared by suspending the frozen cell pellets in 250 mM potassium phosphate pH 7.2 plus 1 mM 2-mercaptoethanol and then sonicating. The cell extract was centrifuged for 10 min at 12,000 *g*, and the supernate was used for assays.

Biochemical measurements. ADA activity was assayed by measuring the conversion of [^{14}C]adenosine to [^{14}C]inosine in the presence and absence of 100 μM deoxycoformycin (dCF) supplied as pentostatin by Warner Lambert Co., Detroit, Mich. In several experiments [^{14}C]deoxyadenosine replaced [^{14}C]adenosine as substrate so that the rates of deamination of the ribo- and deoxyribonucleosides could be compared. dCF-sensitive activity (ADA_1) is calculated as total ADA activity in the absence of dCF minus ADA activity in the presence of dCF. Specific activity is expressed in units, where 1 U is 1 nmol of [^{14}C]inosine/min/10^8 cells. dCF-sensitive ADA_1 activity was converted to amount of enzyme protein using a specific activity of 486 μmol/min per mg protein. This is the specific activity of homogeneous human ADA_1 prepared from granulocytes in our laboratory.

Adenosine, deoxyadenosine, S-adenosylhomocysteine, and S-adenosylmethionine in plasma and urine were quantitated by high performance liquid chromatography on a Bondapak C18 column (Waters Associates, Inc., Milford, Mass.). All except S-adenosylmethionine were separated by isocratic elution using a solvent of 50 mM ammonium dihydrogen phosphate-10% methanol, pH 4.5, and detected at 254 nm. S-Adenosylmethionine was separated using ion pair reagents (18). Minimum level of detection was 0.2 μM in the original samples of plasma or urine, as determined by adding nucleosides to normal specimens. The identities of adenosine and deoxyadenosine were confirmed by treating the samples with calf-intestinal ADA and monitoring conversion to inosine and deoxyinosine, respectively (4, 6).

Deoxynucleotides were measured using several important modifications of the DNA polymerase assay we used previously (4, 6). The present method has improved sensitivity and reproducibility. The reaction mixture contained (final concentrations): 50 mM Tris HCl, pH 7.8, 5 mM $MgCl_2$, 1 mM-β-mercaptoethanol, 50 μg/ml bovine serum albumin, 0.5 A_{260} U/ml (20 μg/ml) of primer-template, 1.5 U/ml of *Escherichia coli* DNA polymerase I, and 10 μM of the deoxynucleotide triphosphate (dNTP) in excess. The final reaction volume was 125 μl. For assay of dATP and deoxyribosylthymine triphosphate (dTTP), poly d(A–T) was the primer-template, and either [^{3}H]dTTP or [^{3}H]dATP, as appropriate, was the dNTP in

excess. For assay of deoxyguanosine triphosphate (dGTP), poly d(I-C) was the primer-template and [^{3}H]deoxycytidine triphosphate (dCTP) was the dNTP in excess. For assay of dCTP, DNAse treated DNA was the primer-template, all dNTP except dCTP were in excess, and [^{3}H]dGTP was the labeled dNTP. [^{3}H]dNTP were used at specific radioactivities between 150 and 300 cpm/pmol. Reactions were carried out at 35°C for 60 min. They were terminated by spotting portions in duplicate on Whatman GF/C squares (Whatman, Inc., Clifton, N. J.) and dropping them into cold 5% trichloracetic acid with 1% sodium pyrophosphate. The squares were processed for scintillation counting. Under these conditions dNTP can be assayed in a linear range from 1.0 to 40 pmol. For each extract, deoxynucleotides were assayed using three different amounts of extract. The quantity of dNTP in each extract was calculated from the slope of the line relating the amount of extract to the amount of dNTP, rather than from the kinetics of incorporation of radiolabel at a fixed amount of extract. Extracts of lymphocytes do not contain high activities at enzymes that interfere with the enzymatic assay of deoxyribonucleoside 5'-triphosphates. These have been reported in extracts of HeLa cells (19). In the presence of lymphocyte extracts dADP stimulates ~10% and dAMP ~1% of the incorporation of radiolabel that an equimolar amount of dATP stimulates.

Radioimmunoassay for detecting ADA protein made use of the competition between ^{125}I-labeled human ADA_1 purified from granulocytes (20) and unlabeled ADA in the specimen for binding by ADA_1-specified goat IgG (20, 21). The minimum specific activity for ^{125}I-labeled ADA_1 was 25 μCi/μg. The radioimmunoassay was carried out at 4°C in a 1.5-ml polypropylene microcentrifuge tube. All components of the assay were diluted with 50 mM potassium phosphate, pH 7.2, containing 1 mg/ml bovine serum albumin and 1 mM β-mercaptoethanol (buffer A). IgG anti-ADA was diluted (60-80 μg/ml) to precipitate 30-40% of the ^{125}I-labeled ADA_1 in the assay in the absence of unlabeled ADA. In the normal assay, 25 μl of diluted IgG was mixed with 25 μl of buffer A containing 0.03-100 ng of unlabeled purified human ADA_1 (for the standard curve), an appropriate dilution of cell extract, or buffer A with no addition. The samples were mixed and incubated at 4°C for 16 h. ^{125}I-labeled ADA_1 (25 μl, 15,000 cpm) was added to each tube, and samples were further incubated at 4°C for 24 h. Then, 100 μl of a 10% suspension of IgGsorb, (heat-killed *Staphylococcus aureus* containing protein A; The Enzyme Center, Boston, Mass.) was added to each sample. After 15 min at room temperature, the mixtures were centrifuged at 8,000 *g* for 2 min. The resulting pellets were washed twice with buffer A and then counted to determine ^{125}I radioactivity. IgGsorb bound 2-3% of the ^{125}I radioactivity in the assay in the absence of IgG. The standard curve for binding competition was determined between unlabeled purified human ADA_1 and ^{125}I-labeled ADA. Data for both the standard curve and cell extracts were analyzed by the modified logit-log transformation (22). Cell extracts were assayed at several dilutions.

Our methods of measurement of immunoglobulins, T and B lymphocyte function have been described (12).

RESULTS

Assay and characterization of adenosine deaminase. We used dCF to distinguish ADA_1 and ADA_2 activities in lymphocyte extracts (Table I). The existence of two distinct adenosine deaminating activities has been well documented in man (23). The major isozyme of ADA (ADA_1) is inhibited by erythro-9-(2-hydroxy-3-nonyl)-adenine and dCF (23-25). A minor deaminating activity (ADA_2) appears to be the product of a genetic locus separate from ADA_1, is not as readily inhibited by erythro-9-(2-hydroxy-3-nonyl)adenine as ADA_1 (23-25), and does not cross-react with antiserum to ADA_1 (23, 24). The effect of dCF on human ADA_2 has not been reported. We examined ADA_2 in an extract of human leukemic granulocytes in which ADA_2 comprised ~2% of total ADA activity. ADA_2 was isolated from this extract by Sephadex G-200 chromatography (24). ADA_2 had a high estimated Michaelis constant (K_m) (2-5 mM) for both adenosine and deoxyadenosine and utilized deoxyadenosine at 27% of the rate of utilization of adenosine. ADA_1 purified in our laboratory has K_m values of 0.048 and 0.034 mM for adenosine

TABLE I

Enzymatic and Radioimmunochemical Assay of Adenosine Deaminase in Lymphocytes

Source of lymphocytes	ADA activity*		ADA protein		Ratio of dCF-sensitive activity to RIA protein
	dCF insensitive	dCF sensitive	Calculated from dCF sensitive	Radioimmuno-assay	
	nmol/min/10^8 cells		*ng/10^8 cells*		*nmol/min/ng*
Adult blood	3.7 (2-5)‡	92 (80-100)	189 (165-206)	223 (203-235)	0.41 (0.39-0.43)
Cord blood	5.0 (3-8)	193 (157-215)	397 (323-442)	429 (350-496)	0.45 (0.43-0.47)
Patient	9	7	14	51	0.14
Mother	12	54	111	129	0.42
Father	11	58	119	168	0.35

* When deoxyadenosine was used as substrate, total adenosine deaminase activity in lymphocytes was 70% of the activity observed with adenosine as substrate. On two occasions total adenosine deaminase activity in the patient's lymphocytes was measured with both adenosine and deoxyadenosine as substrates. Average activity with adenosine was 21 nmol/min per 10^8 cells, and with deoxyadenosine was 18 nmol/min per 10^8 cells.

‡ Figures in parentheses are the ranges of three determinations. Lymphocytes from the patient and his parents were only sufficient for a single assay of each.

and deoxyadenosine, respectively, and utilizes deoxyadenosine at 75% of the rate of adenosine utilization (20). ADA_2 did not cross-react with antiserum raised against ADA_1 in our laboratory so that radioimmunoassay of ADA_1 is specific. ADA_2 is less sensitive to inhibition by dCF than ADA_1, although at high concentrations of dCF both ADA_1 and ADA_2 are inhibited. Differential inhibition by dCF can distinguish between ADA_1 and ADA_2 activities in crude tissue extracts. Erythro-9-(2-hydroxy-3-nonyl)adenine has generally been used by other investigators to make this distinction (26).

Normal adult lymphocytes contain 92 U of dCF-sensitive ADA_1 and 3.7 U of dCF-insensitive ADA_2 activity (Table I). This amount of dCF-sensitive activity is equivalent to 189 ng of ADA enzyme protein per 10^8 cells, as calculated from the specific activity of the purified protein prepared in our laboratory. Direct radioimmunoassay detects 223 ng of enzyme protein per 10^8 normal cells, which is similar to the 189 ng predicted from measurements of ADA_1 activity. Since our patient with ADA deficiency was first studied as an infant, we compared ADA in lymphocytes from cord blood with the enzyme in lymphocytes from adult peripheral blood. We were not certain whether the type and amount of ADA in lymphocytes changed during growth and development. Cord blood lymphocytes were readily available, whereas lymphocytes from normal infants were not generally available. Both dCF-sensitive ADA_1 activity and ADA protein were twice as high in cord as in adult lymphocytes (Table I). The range of dCF-insensitive ADA_2 activity overlapped in the two types of cells.

Lymphocytes from the patient contained 9 U of dCF-insensitive ADA_2 and 7 U of dCF-sensitive ADA_1 activity. There was as much dCF-insensitive ADA_2 activity in lymphocytes from the patient as in normal adults, but only 7.6% as much dCF-sensitive ADA_1 activity. ADA_1 protein was easily detected in ADA-deficient cells by radioimmunoassay and amounted to 51 ng protein/10^8 lymphocytes. This is 23% of the amount present in normal adult lymphocytes and 12% of the amount present in cord blood lymphocytes. Deaminase activity was also present in both normal and ADA deficient lymphocytes when deoxyadenosine was used as substrate (Table I). The mother and father of the patient are obligate heterozygotes or carriers of ADA deficiency. Lymphocytes from each parent were deficient in ADA with 59–63% of normal dCF-sensitive ADA_1 activity.

Hirschhorn et al. (26) measured erythro-9-(2-hydroxy-3-nonyl)adenine-sensitive ADA_1 activity in mononuclear cells from six patients with ADA deficiency and severe combined immunodeficiency. Values ranged from 0.1 to 3.7% of normal, with an average of 1.9%. The apparent increase in ADA_1 activity in our patient compared with others may not be statistically significant. We have been unable to make repeated measurements to ascertain error. The increase is worth noting, however, in view of the unusually large amount of immunoreactive protein found in our patient.

Biochemical response to transfusion therapy. Two major biochemical abnormalities were noted in our ADA-deficient patient at the time of initial diagnosis (4). Total ADA activity in erythrocytes was <5% of normal, and dATP had increased to concentrations higher than those of ATP. When restudied some months after diagnosis, we also reported increased amounts of dATP in lymphocytes and increased excretion of deoxyadenosine in the urine (6). We have now completed serial biochemical and immunological studies of the patient over a 40-mo period (Fig. 1). Transfusions of erythrocytes, sometimes with plasma, have been administered during this time to replace partially the missing ADA.

The child was first transfused with 12 ml/kg of packed erythrocytes and 10 ml/kg of frozen plasma. Erythrocytes removed from the patient just before the first transfusion contained 157 pmol dATP/10^6 cells (the normal range is 0.1–0.2 pmol/10^6) and <0.2 U of ADA activity. Lymphocytes, plasma, and urine were not available for study. 3 wk after tranfusion, the amount of dATP in erythrocytes had decreased to 11.6 pmol/10^6 cells, and ADA activity had increased to 0.9 U. By 4 mo after transfusions had begun, the amount of dATP in erythrocytes had stabilized in the range 2–5 pmol/10^6 cells, ADA had increased to 2–5 U (normal is 1–4 U), the number of lymphocytes and concentration of IgG in peripheral blood had begun to rise, and the lymphocyte response in vitro to phytohemagglutinin was nearly normal on several occasions (Fig. 1; reference 12). Despite the appearance of high levels of immunoglobulins, specific antibody production remained very poor. dGTP, dCTP, and dTTP were not detected in erythrocytes at any time and, if present, must have been <0.1 pmol/10^6 cells. After 8 mo of transfusions, their frequency was reduced and restricted to erythrocytes only. Over the following 10 mo ADA activity in erythrocytes remained below 2 U and dATP increased to a maximum of 12 pmol/10^6 erythrocytes. The lymphocyte count fell to 110/μl and mitogen responsiveness was generally less than 10% of normal (Fig. 1; reference 12). During this time the response of the patient's lymphocytes to phytohemagglutinin ranged from 982 to 6,975 cpm [^{3}H]thymidine/10^5 cells, whereas the response of normal lymphocytes ranged from 24,850 to 67,020 cpm. IgG levels remained in the normal range, but specific antibodies were rarely detected. On one occasion an antibody titer of 1:2,040 to cytomegalovirus was demonstrated. Antibodies to common respiratory viruses and fungi could not be

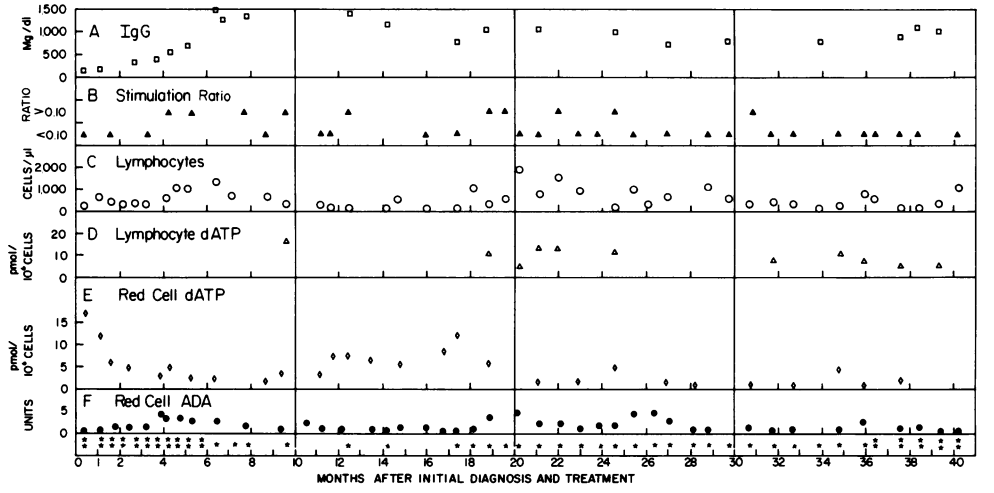

FIGURE 1 Response of an adenosine deaminase-deficient child to transfusion therapy as a form of enzyme replacement. Transfusions were given as marked. One star indicates transfusion of packed erythrocytes only, two stars indicate transfusion of both plasma and erythrocytes. (A) Serum IgG, milligrams per deciliter. (B) Response of peripheral lymphocytes to phytohemagglutinin (12). Stimulation ratio is defined in this paper as the ratio of [^{3}H]thymidine incorporated into the patient's lymphocytes after stimulation to [^{3}H]thymidine incorporated into the same number of normal peripheral lymphocytes in the same experiment. For ease of presentation, values are divided into two classes only: >0.1 and <0.1. Examples of our original data have been presented (12). (C) Peripheral lymphocyte count, cells per microliter. (D) Amount of dATP in lymphocytes, picomoles per 10^6 cells. (E) Amount of dATP in erythrocytes, picomoles per 10^6 cells. At the time of initial diagnosis the value was 157 pmol/10^6 cells. (F) Activity of ADA in erythrocytes, 1 U of activity equals 1 nmol/min per 10^8 cells.

demonstrated. The child responded poorly and transiently to immunization with typhoid vaccine. Intensive transfusion therapy was resumed 18 mo after initial diagnosis with improvement in biochemical abnormalities, but very little improvement in immunological function. Specimens of plasma were analyzed on nine occasions between 1 and 40 mo of diagnosis for the presence of adenosine, inosine, deoxyadenosine, deoxyinosine, and *S*-adenosylhomocysteine. None could be found under conditions that would readily have detected 0.2 μM nucleoside, although the measurements may not have been reliable because of failure to separate plasma from erythrocytes promptly. 11 specimens of urine were examined for the presence of deoxyadenosine, adenosine, *S*-adenosylhomocysteine, and *S*-adenosylmethionine. On two occasions ~13 and 17 mo after transfusions were begun, deoxyadenosine was detected, as we have previously reported (6). Deoxyadenosine was not detected in nine other specimens of urine collected over the 40-mo period. The urines were obtained ~8, 18, 19, 26, 29, 30, 32, 34, and 41 mo after treatment began. Most were collected on days when transfusions were to be given. Adenosine, *S*-adenosylhomocysteine, and *S*-adenosylmethionine were not detected under conditions that would detect 0.2 μM. The specimens of urine were not dilute, but had creatinine concentrations ranging from 26 to 56 mg/dl.

Deoxynucleotides in lymphocytes. Because the patient was both lymphopenic and a young child, it was difficult to obtain sufficient numbers of lymphocytes for biochemical and immunological assays. We have reported that transfusion therapy with erythrocytes does not increase ADA activity in the patient's lymphocytes (6). Using our present method of assaying deoxynucleotides, the concentration of dATP was measured in lymphocytes on 12 occasions over a 40-mo period (Table II). Cells were not obtained at equally spaced intervals of time. Transfusions had been administered 2–4 wk before each specimen of blood was removed for isolation of lymphocytes. Blood was obtained just before transfusion, if drawn on a day when transfusion was scheduled. Cells could not be obtained earlier than 10 mo after diagnosis. At that time the amount of dATP was 16.5 pmol/10^6 cells. This is approximately three times the normal values of 5.3±1.3 and 4.8±2.0 pmol/10^6 cells found in cord and adult peripheral blood lymphocytes, respectively. In this series of measurements dATP in the patient's lymphocytes was >10 pmol/10^6 cells on 7 of 12 occasions over a 40-mo period. Generally the number of lymphocytes was sufficient for a single assay of each of the deoxynucleotides at three dilutions. On the average during periods of transfusion every 2–4 wk, the level of dATP in lymphocytes (10.0±3.9 pmol/10^6 cells) was twice normal (Table II). At 24 mo after diagnosis a particularly high

TABLE II
Deoxynucleotide Levels in Erythrocytes and Lymphocytes

Months after transfusions began	Deoxynucleotides				
	Erythrocytes	Lymphocytes			
	dATP	dATP	dGTP	dTTP	dCTP
	pmol/10⁶ cells				
0	157*	—	—	—	—
10	3.5	16.5	17.2	—	—
20	5.5	11.0	2.5	15.8	5.8
21	—	5.1	3.4	16.7	9.8
22	1.6	13.4	1.4	11.4	11.4
23	1.2	13.8	2.8	—	—
24	1.7	30.5*	3.1	9.2	13.1
26	4.8	11.8	2.9	12.9	8.8
33	1.1	7.9	—	22.1	5.5
36	4.4	11.5	12.4	9.7	4.3
37	0.3	8.9	9.3	12.6	—
39	2.1	5.2	7.2	4.4	—
40	2.4	5.3	14.2	12.5	6.1
42	—	—	—	—	9.7
Patient mean±SD	2.6±1.7	10.0±3.9	6.9±5.5	12.7±4.8	8.3±3.0
n	11	11	11	10	9
Cord blood mean±SD	0.2±0.1	5.3±1.3	5.2±2.3	1.4±1.1	5.2±2.5
n	5	8	8	8	4
Adult blood mean±SD	0.2±0.1	4.8±2.0	6.1±3.3	1.4±0.4	4.2±3.4
n	6	7	7	6	4

* Omitted from statistical calculations.

value of 30.5 pmol/10^6 lymphocytes was observed, but so few cells were available (4×10^6) that the assay could not be repeated.

On 11 occasions the amounts of dATP in erythrocytes and lymphocytes were measured in cells isolated from the same specimen of peripheral blood. Values in the two types of cells were positively, but not significantly, correlated ($r = 0.25, P > 0.1$). Because of the possibility that perturbation of dATP affected the amounts of other deoxynucleotides in lymphocytes, as by feedback inhibition of ribonucleotide reductase, dGTP, dTTP, and dCTP were measured. On the average, the levels of dGTP and dCTP were not significantly different from normal, although the range of values seen in the patient's cells was wider than in lymphocytes from normal donors (Table II). The amount of dTTP in the patient's lymphocytes was higher than normal (12.7±4.8 vs. a normal of 1.4±0.4 pmol/10^6 cells). Identification of the substance as dTTP is based upon the enzymatic assay using DNA polymerase and poly d(A–T). Insufficient material was available to confirm the amount of dTTP by other means such as high-performance liquid chromatography.

DISCUSSION

Since the original description by Giblett et al. (1) >30 ADA-deficient patients with severe combined immunodeficiency have been described (2, 3, 27). The amounts of residual ADA activity have differed from patient to patient, and the nature of this activity is not always clear. Distinctions among the possible sources of ADA activity can be made using combinations of specific enzyme inhibitors and quantitative immunoassays for protein reacting with antiserum to ADA_1 (24, 26, 28, 29). Protein antigenically related to ADA_1 is easily detected in both normal and deficient lymphocytes by radioimmunoassay (Table I). The ratio of dCF-sensitive ADA_1 activity to enzyme protein by radioimmunoassay in adult and cord blood lymphocytes is 0.41–0.45 nmol/min per ng (Table I). This specific catalytic activity of ADA_1 in cell extracts is similar to the activity of pure homogeneous ADA_1 prepared in our laboratory (0.49 nmol/min per ng). Measurements of dCF-sensitive ADA_1 activity and radioimmunoassay of ADA_1 protein do, therefore, permit an estimate of the catalytic activity of ADA_1 protein in lymphocytes. The

ratio of dCF-sensitive ADA_1 activity to the amount of protein detected by radioimmunoassay is 0.14 nmol/min per ng in our child, compared with the normal value of 0.41–0.45 (Table I). Lymphocytes from the ADA-deficient child contain catalytically defective, antigenically reactive ADA_1 protein. The ratio of dCF-sensitive activity to protein detected by radioimmunoassay was within the normal range in the mother, but lower than expected in the father (Table I). The father's lymphocytes may contain catalytically defective ADA_1 protein, but insufficient amounts of blood are available for additional studies. There is no history of consanguinity in the parents, and they may be carriers of different mutations in ADA_1. The ADA-deficient child may be doubly heterozygous for two different ADA_1 deficiency alleles.

Metabolic abnormalities in ADA deficiency and major damage to the immunological system probably occur before birth (3), although only one newborn infant has been studied biochemically. The catabolism of deoxyadenosine is decreased (2, 3), dATP accumulates in lymphocytes and erythrocytes (4–6), and *S*-adenosylhomocysteine hydrolase may be inhibited (30).

In our patient, abnormalities in the concentration of dATP in lymphocytes were quantitatively less severe relative to normal than in erythrocytes. Transfusion of irradiated packed erythrocytes reduced dATP in erythrocytes from 157 pmol/10^6 cells at the time of diagnosis to a plateau value of 1–4 pmol/10^6 cells while transfusions were being administered every 2–4 wk (Table II). Because the normal amount of dATP in erythrocytes is 0.2±0.1 pmol/10^6 cells, dATP in the patient's cells is 5–20-fold increased during treatment. Hirschhorn et al. (31) reported similar elevations of dATP in erythrocytes from one of their patients who was receiving biweekly transfusions. Lymphocytes were not available from our patient until after transfusion therapy had been instituted. dATP in lymphocytes averaged 5–17 pmol/10^6 cells while transfusions were being administered every 2–4 wk (Table II). The normal amount of dATP in lymphocytes is 4.8±2.0 pmol/10^6 cells so the patient's dATP is one- to four-fold increased.

On two occasions ~13 and 17 mo after transfusions began, we had previously measured levels of dATP in the patient's lymphocytes and obtained values 1,760 and 770 pmol/10^6 cells (6). Each time the extraordinarily high values were found the patient was excreting ~30 mg/d of deoxyadenosine in his urine (6), and transfusions for enzyme replacement had not been administered for 10 wk. The simplest interpretation of these data is that frank metabolic relapse occurs if transfusion of erythrocytes is infrequent, i.e., every 10 wk rather than every 2–4 wk. Severe metabolic relapse with accumulation of high levels of dATP in erythrocytes has also been observed by Nelson et al. (32), when transfusions were withheld 4–6 mo in their ADA-deficient child.

Administration of ADA by transfusion of erythrocytes leads to obvious and persistent increases in serum immunoglobulins, although specific antibody production is very poor in our patient (Fig. 1, reference 12). Activity of ADA in erythrocytes was correlated with the absolute lymphocyte count ($r = 0.64$, $P < 0.01$). Activity of ADA and number of lymphocytes were measured in specimens of blood removed before each transfusion. Therefore, ADA represents the lowest activity reached during the interval since the preceding transfusion. Activity of ADA in erythrocytes was not significantly correlated statistically ($P > 0.1$) with: level of dATP in erythrocytes ($r = -0.23$), level of dATP in lymphocytes ($r = -0.21$), or response of lymphocytes to phytohemagglutinin ($r = 0.10$). There were no significant correlations between the level of dATP in lymphocytes and either the lymphocyte count ($r = 0.02$) or their response to phytohemagglutinin ($r = 0.17$). In each statistical calculation related to mitogen responsiveness we used the actual ratio of [^{3}H]-thymidine incorporated into the patient's lymphocytes after stimulation to [^{3}H]thymidine incorporated into the same number of normal peripheral lymphocytes, rather than the two classes used for convenience in Fig. 1. There was a positive, but statistically insignificant, correlation between the level of dATP in erythrocytes and lymphocytes ($r = 0.25$, $P > 0.1$), based upon 11 pairs of measurements. Measurements of dATP in lymphocytes were relatively few in number and were not evenly spaced over the 40 mo of observation. It was particularly difficult to obtain lymphocytes when the peripheral lymphocyte count was low and at times other than when blood was removed before transfusion.

The effect of varying the frequency of erythrocyte transfusions could be examined quantitatively by comparing biochemical and immunological status over the time interval 8–17 mo with the interval 18–27 mo (Fig. 1). Three transfusions of erythrocytes were administered during the first and 11 during the second period. Average ADA activity (±SD) during period one was 0.95±0.64 U compared with period two, 3.2±1.9 U ($P < 0.003$). Significant ($P < 0.05$) differences between the periods were noted in peripheral lymphocyte count (208±159 vs. 817±604) and erythrocyte dATP (6.1±3.4 vs. 2.9±21.0). Insufficient data were available to analyze lymphocyte dATP. Response of lymphocytes to phytohemagglutinin did not differ significantly. Although it is easy to demonstrate increases in the number of circulating lymphocytes as a result of ADA replacement by transfusion, these lymphocytes do not function normally (12). We cannot estimate whether the

increased number of lymphocytes benefits the patient.

The biochemical consequences of hereditary ADA deficiency are complex, as are the functional changes in the immunological system. Significant impairment of most patients has occurred by the time of birth or shortly thereafter. An understanding of the sequence of biochemical changes caused by ADA deficiency may best be defined by studies of patients with pharmacologic inhibition of ADA where measurements can be made repetitively and under well-controlled circumstances (33). Sequential assessments of the functional capacity of lymphocytes and lymphocyte subpopulations can be made in these patients and cannot be made in cells grown in vitro. Alternatively, intensive study of patients in whom the diagnosis is established in the prenatal or perinatal period where damage is not yet severe will be helpful, although patients are rare and appropriate tissue is extremely difficult to obtain.

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REFERENCES

1. Giblett, E. R., J. E. Anderson, F. Cohen, B. Pollara, and H. J. Meuwissen. 1972. Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet.* **II:** 1067–1069.
2. Polmar, S. H. 1980. Metabolic aspects of immunodeficiency disease. *Semin. Hematol.* **17:** 30–43.
3. Mitchell, B. S., and W. N. Kelley. 1980. Purinogenic immunodeficiency disease: clinical features and molecular mechanisms. *Ann. Intern. Med.* **92:** 826–831.
4. Coleman, M. S., J. Donofrio, J. J. Hutton, L. Hahn, A. Daoud, B. Lampkin, and J. Dyminski. 1978. Identification and quantitation of adenine deoxynucleotides in erythrocytes of a patient with adenosine deaminase deficiency and severe combined immunodeficiency. *J. Biol. Chem.* **253:** 1619–1626.
5. Cohen, A., R. Hirschhorn, S. D. Horowitz, A. Rubinstein, S. H. Polmar, R. Hong, and D. W. Martin. 1978. Deoxyadenosine triphosphate as a potentially toxic metabolite in adenosine deaminase deficiency. *Proc. Natl. Acad. Sci. U. S. A.* **75:** 472–476.
6. Donofrio J., M. S. Coleman, J. J. Hutton, A. Daoud, B. Lampkin, and J. Dyminski. 1978. Overproduction of adenine deoxynucleosides and deoxynucleotides in adenosine deaminase deficiency with severe combined immunodeficiency disease. *J. Clin. Invest.* **62:** 884–887.
7. Carson, D. A., J. Kaye, and J. E. Seegmiller. 1977. Lymphospecific toxicity in adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency: possible role of nucleoside kinase(s). *Proc. Natl. Acad. Sci. U. S. A.* **74:** 5677–5681.
8. Carson, D. A., J. Kaye, S. Matsumoto, J. E. Seegmiller, and L. Thompson. 1979. Biochemical basis for the enhanced toxicity of deoxyribonucleosides toward malignant human T cell lines. *Proc. Natl. Acad. Sci. U. S. A.* **76:** 2430–2433.
9. Edwards, N. L., E. W. Gelfand, L. Burk, H. M. Dosch, and I. H. Fox. 1979. Distribution of 5′-nucleotidase in human lymphoid tissues. *Proc. Natl. Acad. Sci. U. S. A.* **76:** 3474–3476.
10. Polmar, S. H., R. C. Stern, A. L. Schwartz, E. M. Wetzler, P. A. Chase, and R. Hirschhorn. 1976. Enzyme replacement therapy for adenosine deaminase deficiency and severe combined immunodeficiency. *N. Engl. J. Med.* **295:** 1337–1342.
11. Schmalstieg, F. C., G. C. Mills, J. A. Nelson, L. T. May, A. S. Goldman, and R. M. Goldblum. 1978. Limited effect of erythrocyte and plasma infusions in adenosine deaminase deficiency. *J. Pediatr.* **93:** 597–603.
12. Dyminski, J. W., A. Daoud, B. C. Lampkin, S. Limouze, J. Donofrio, M. S. Coleman, and J. J. Hutton. 1979. Immunological and biochemical profiles in response to transfusion therapy in an adenosine deaminase deficient patient with severe combined immunodeficiency disease. *Clin. Immunol. Immunopathol.* **14:** 307–326.
13. Hirschhorn, R., P. S. Papageorgiou, H. H. Kesarwala, and L. T. Taft. 1980. Amelioration of neurologic abnormalities after "enzyme replacement" in adenosine deaminase deficiency. *N. Engl. J. Med.* **303:** 377–380.
14. Ziegler, J. B., C. H. Lee, M. B. van der Weyden, A. S. Bagnara, and J. Beveridge. 1980. Severe combined immunodeficiency and adenosine deaminase deficiency: failure of enzyme replacement therapy. *Arch. Dis. Child.* **55:** 452–457.
15. Schmalstieg, F. C., J. A. Nelson, G. C. Mills, T. M. Monahan, A. S. Goldman, and R. M. Goldblum. 1980. Increased purine nucleotides in adenosine deaminase-deficient lymphocytes. *J. Pediatr.* **91:** 48–51.
16. Jerrells, T. R., J. H. Dean, G. L. Richardson, and R. B. Herberman. 1980. Depletion of monocytes from human peripheral blood mononuclear leukocytes: comparison of the Sephadex G-10 column method with other commonly used techniques. *J. Immunol. Methods.* **32:** 11–29.
17. Somogyi, M. 1945. Determination of blood sugar. *J. Biol. Chem.* **160:** 69–73.
18. Kredich, N. M., and D. W. Martin. 1977. Role of S-adenosylhomocysteine in adenosine mediated toxicity in cultured mouse T-lymphoma cells. *Cell.* **12:** 931–938.
19. North, T. W., R. Bestwick, and C. Mathews. 1980. Detection of activities that interfere with the enzymatic assay of deoxyribonucleoside 5′-triphosphates. *J. Biol. Chem.* **255:** 6640–6645.
20. Wiginton, D. A., M. S. Coleman, and J. J. Hutton. 1981. Purification, characterization and radioimmunoassay of adenosine deaminase from human leukaemic granulocytes. *Biochem. J.* **195:** 389–397.
21. Daddona, P. E., M. A. Frohman, and W. N. Kelley. 1979. Radioimmunochemical quantitation of human adenosine deaminase. *J. Clin. Invest.* **64:** 798–803.
22. Rodbard, D., W. Bridson, and P. L. Rayford. 1969. Rapid calculation of radioimmunoassay results. *J. Lab. Clin. Med.* **74:** 770–781.
23. Hirschhorn, R., and H. Ratech. 1980. Isozymes of adenosine deaminase. *Isozymes: Curr. Top. Biol. Med. Res.* **4:** 131–157.

24. Schrader, W. P., B. Pollara, and H. J. Meuwissen. 1978. Characterization of the residual adenosine deaminating activity in the spleen of a patient with combined immunodeficiency disease and adenosine deaminase deficiency. *Proc. Natl. Acad. Sci. U. S. A.* **75:** 446–450.
25. Parks, R. E., K. C. Agarwal, R. P. Agarwal, G. W. Crabtree, T. Rogler-Brown, T. M. Savarese, and J. D. Stoeckler. 1978. Biochemical factors that influence the therapeutic potential of purine analogs. *Inserm (Inst. Natl. Sante Rech. Med.) Colloq.* **81:** 209–238.
26. Hirschhorn, R., V. Roegner, T. Jenkins, C. Seaman, S. Piomelli, and W. Borkowsky. 1979. Erythrocyte adenosine deaminase deficiency without immunodeficiency. *J. Clin. Invest.* **64:** 1130–1139.
27. Meuwissen, H. J., and B. Pollara. 1978. Combined immunodeficiency and inborn errors of purine metabolism. *Blut.* **37:** 173–181.
28. Carson, D. A., R. Goldblum, and J. E. Seegmiller. 1977. Quantitative immunoassay of adenosine deaminase in combined immunodeficiency disease. *J. Immunol.* **118:** 270–273.
29. Daddona, P. E., M. A. Frohman, and W. N. Kelley. 1980. Human adenosine deaminase and its binding protein in normal and adenosine deaminase-deficient fibroblast cell strains. *J. Biol. Chem.* **255:** 5681–5687.
30. Hershfield, M. S., N. M. Kredich, D. R. Ownby, H. Ownby, and R. Buckley. 1979. In vivo inactivation of erythrocyte *S*-adenosylhomocysteine hydrolase by 2′-deoxyadenosine in adenosine deaminase deficient patients. *J. Clin. Invest.* **63:** 807–811.
31. Hirschhorn, R., V. Roegner, A. Rubinstein, and P. Papageorgiou. 1980. Plasma deoxyadenosine, adenosine, and erythrocyte deoxy ATP are elevated at birth in an adenosine deaminase-deficient child. *J. Clin. Invest.* **65:** 768–771.
32. Nelson, J. A., J. F. Kuttesch, R. M. Goldblum, A. S. Goldman, and F. C. Schmalstieg. 1979. Analysis of adenosine and adenine nucleotides in severe combined immunodeficiency disease. *In* Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides. H. P. Baer and G. I. Drummond, editors. Raven Press, New York. 417–427.
33. Siaw, M. F., B. S. Mitchell, C. A. Koller, M. S. Coleman, and J. J. Hutton. 1980. ATP depletion as a consequence of adenosine deaminase inhibition in man. *Proc. Natl. Acad. Sci. U. S. A.* **77:** 6157–6161.