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Research Article

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Adenosine Deaminase Deficiency with Normal Immune Function

AN ACIDIC ENZYME MUTATION

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ABSTRACT In most instances, marked deficiency of the purine catabolic enzyme adenosine deaminase results in lymphopenia and severe combined immunodeficiency disease. Over a 2-yr period, we studied a white male child with markedly deficient erythrocyte and lymphocyte adenosine deaminase activity and normal immune function. We have documented that (*a*) adenosine deaminase activity and immunoreactive protein are undetectable in erythrocytes, 0.9% of normal in lymphocytes, 4% in cultured lymphoblasts, and 14% in skin fibroblasts; (*b*) plasma adenosine and deoxyadenosine levels are undetectable and deoxy ATP levels are only slightly elevated in lymphocytes and in erythrocytes; (*c*) no defect in deoxyadenosine metabolism is present in the proband's cultured lymphoblasts; (*d*) lymphoblast adenosine deaminase has normal enzyme kinetics, absolute specific activity, $S_{20,w}$, pH optimum, and heat stability; and (*e*) the proband's adenosine deaminase exhibits a normal apparent subunit molecular weight but an abnormal isoelectric pH. In contrast to the three other adenosine deaminase-deficient healthy subjects who have been described, the proband is unique in demonstrating an acidic, heat-stable protein mutation of the enzyme that is associated with <1% lymphocyte adenosine deaminase activity. Residual adenosine deaminase activity in tissues other than lymphocytes may suffice to metabolize the otherwise lymphotoxic enzyme substrate(s) and account for the preservation of normal immune function.

INTRODUCTION

Adenosine deaminase (EC 3.5.4.4, ADA),[1] an enzyme in the purine catabolic pathway, catalyzes the conversion of deoxyadenosine to deoxyinosine and adenosine to inosine. A marked genetic deficiency of ADA has been causally associated with an autosomal recessive form of severe combined immunodeficiency disease (SCID) (1). A review of the clinical features of 34 ADA-deficient patients in 26 families has substantiated the severe T cell depletion and variable humoral immunosuppression associated with this disorder (2–5). In the absence of ADA activity, deoxyadenosine is converted to deoxy ATP, as first demonstrated in the erythrocytes of affected patients (6–8). The selective accumulation of deoxy ATP in cultured T cells is associated with inhibition of DNA synthesis and cell death. B cells and other tissues having high deoxynucleotide-degrading enzyme activity (9–11) may be protected from deoxy ATP accumulation and may thus be spared in this disorder.

Substantial genetic heterogeneity of ADA has been described. Whereas ADA activity is totally absent in erythrocytes and lymphocytes from SCID patients, adenosine deaminating activity of 1 to 30% of normal has been reported in selected tissues of some of these individuals (12). In particular, fibroblast cell lines from nine different ADA-deficient patients showed enzyme activity ranging from 0.2 to 3.8% of normal, whereas the level of ADA immunoreactive protein varied by

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[1] *Abbreviations used in this paper:* ADA, adenosine deaminase; CRM, cross immunoreactive protein; EHNA, *erythro*-9-[3-(2-hydroxynonyl)] adenine; SCID, severe combined immunodeficiency disease.

0.8 to 22% of normal, strongly suggesting variable genetic expressions of this enzyme (13).

Recently three ADA-deficient subjects have been described with no detectable ADA in erythrocytes, but with residual ADA activity ranging from 15 to 24% of normal in their immunologically normal lymphocytes (14–17). In two of these cases, the enzyme was demonstrated to be more heat labile than normal, suggesting a reason for its absence in senescent erythrocytes (16). The residual lymphocyte ADA activity in these cases was reported to be sufficient to protect these individuals from toxic accumulation of deoxy ATP and subsequent immune dysfunction. In this study we describe a unique ADA-deficient healthy subject with a new acidic protein mutation of ADA associated with <1% lymphocyte ADA activity but with normal immune function.

METHODS

Radioisotopes and chemicals

^{125}I-Protein A (89 μCi/μg) was obtained from New England Nuclear (Boston, MA). [8-^{14}C]Adenosine and [U-^{14}C]deoxyadenosine were supplied by Amersham Corp. (Arlington Heights, IL). Gibco Laboratories (Grand Island, NY) provided RPMI 1640 medium and horse serum; Flow Laboratories (McLean, VA) provided fetal bovine serum. Eastman Kodak Co. (Rochester, NY) supplied the X-Omat R x-ray film. Ampholites (pH 3.5–10.0) were obtained from LKB Instruments, Inc. (Rockville, MD) and the nitrocellulose paper (GS type 0.22 μm) was purchased from Millipore Corp. (Bedford, MA). 2′-Deoxycoformycin (Pentostatin) was obtained from the Parke-Davis division of Warner-Lambert Co. (Ann Arbor, MI), and *erythro*-9-[3-(2-hydroxynonyl)]-adenine (EHNA) was provided by Burroughs Wellcome and Co. (Research Triangle Park, NC). All other reagents and supplies were of the highest quality commercially available.

Patient

Patient E.M. is a 2-yr-old white male diagnosed with erythrocyte ADA deficiency during the New York State mandatory neonatal screening program. E.M. was born in April 1980, after an uneventful pregnancy. He had a transient diaper rash and oral thrush in the first several months of life. Since that time, he has had no illnesses with the exception of a few colds and several episodes of otitis media, which have responded to antibiotic therapy. He has developed normally, with height and weight at the 90th percentile. Physical examination was consistently normal; tonsils were observed and lymph nodes were palpated at 4 mo.

Laboratory data showed a normal hemoglobin, leukocyte count, and platelet count. Total lymphocyte counts ranged from 3,575 to 5,952/mm^3 (normal) with 70% T cells on repeated determinations. Delayed hypersensitivity to *Candida albicans* and mumps was present and the responses of peripheral blood lymphocytes to phytohemagglutinin and allogeneic cells were normal. Immunoglobulin levels rose appropriately with age; at 18 mo, he had an IgG of 802 mg/100 ml, IgM 86 mg/100 ml, IgA 96 mg/100 ml, IgD 1.2 mg/100 ml, and IgE 11 U/ml. EAC cells were 10% and SIg-bearing cells 16.5%.

Titers of isohemagglutinin A and B were 1:256 and 1:32, respectively (high normal), and the Schick test showed no induration. Titers of antibodies to diphtheria and tetanus were 6.0 and 2.8 units respectively (high normal), and to poliomyelitis I, II, and III were 1:128, 1:128, and 1:4, respectively (normal), following a usual immunization program with DPT and Salk-killed polio vaccine.

Radiographs of the chest, skull, and pelvis, karyotype analysis, and a bone-marrow study were normal. He remained healthy through his most recent evaluation in March 1983.

Origin and maintenance of lymphoblast and fibroblast cell lines

Normal B lymphoblastoid cell lines (GM 130, GM 131, GM 333, GM 1078, GM 558 and MGL-8), severely ADA-deficient B lymphoblastoid cell lines (GM 2471, GM 2606, GM 2445, and GM 2756), and the B lymphoblastoid cell line derived from the proband (GM 4396) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). All lymphoblastoid cell lines were cultured in RPMI 1640 medium containing 10% horse serum at 37°C in the presence of 5% CO_2. All medium was supplemented with fresh glutamine (2 mM final concentration) immediately before use.

Normal child skin fibroblast cell lines (GM 969, GM 38, and GM 498) have been previously characterized (13). The skin fibroblast cell line derived from the proband E.M. (GM 4395) was obtained from the Human Genetic Mutant Cell Repository. All fibroblast cell strains were cultured in minimum essential medium supplemented with 10% ADA-deficient fetal calf serum as previously described (13).

Enzyme analyses

Lymphoblasts and fibroblasts were harvested during late log-phase growth and all cell samples were prepared for enzyme analyses as previously described (13, 18).

ADA enzyme activity was assayed by a previously described radiochemical technique (18). Assay conditions utilized either the substrate [8-^{14}C]adenosine or [U-^{14}C]2′-deoxyadenosine at sp act 2 mCi/mmol and a final concentration of 4 mM (high substrate) or 0.4 mM (low substrate). The specific enzyme activity was expressed as the number of nanomoles of inosine or deoxyinosine formed per minute per milligram of cell protein. The concentration of total cell extract protein was determined by the method of Lowry et al. (19), with bovine serum albumin as standard.

The concentration and relative affinity of immunoreactive ADA was quantitated by radioimmunoassay as previously described (13, 20). Procedures for the determination of the Michaelis constant (*K*m) for adenosine and 2′-deoxyadenosine, enzyme inhibition with 2′-deoxycoformycin and EHNA, pH optimum, $S_{20,w}$ and heat stability of ADA have been described (18).

Gel electrophoresis

Starch gel electrophoresis and staining for ADA activity were performed as described by Spencer et al. (21) with 0.1 M Tris/maleate, pH 7.4, used as the gel and running buffer.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed in 10 × 12-mm slab gels by the method of Laemmli (22). Gels were then subjected to protein blot analysis as described in the following section.

Urea isoelectric focusing was performed in 7.8% poly-

acrylamide slab gels containing 8.5 M urea and 5% ampholites (LKB Instruments, pH 3.5–10.0). This procedure has been previously described for ADA (23).

Protein blot analysis

After SDS or isoelectric focusing, proteins were electrophoretically transferred to nitrocellulose sheets according to Towbin et al. (24). The immunological identification of ADA on the nitrocellulose paper (the blot) was carried out as previously described (23). The blot was incubated with 20 ml of buffer A (10 mM Tris/HCl, pH 7.4, containing 154 mM NaCl, 3% bovine serum albumin, and 0.02% sodium azide) at room temperature overnight. The protein blot was then incubated for 4 h with anti-human erythrocyte ADA serum (50 μl in 20 ml buffer A) followed by four washes with buffer B (10 mM Tris/HCl and 154 mM NaCl). The blot was then incubated with 20 ml of buffer A containing ^{125}I-labeled protein A (20,000 cpm/ml) for 4 h, followed by extensive washing with buffer B. The blot was then air dried and the labeled immunocomplex was visualized by autoradiography after exposure of X-Omat-R x-ray film to the blot at −70°C for 24 to 72 h.

Adenosine, deoxyadenosine, and deoxy ATP determinations

Plasma, obtained from heparinized whole blood, and urine were assayed for adenosine and 2′-deoxyadenosine by high-pressure liquid chromatography according to the method of Koller et al. (25).

Ribonucleotides and deoxyribonucleotides were extracted from cell pellets containing 1×10^6 lymphocytes, using 1 ml of cold 60% aqueous methanol at −20°C for 12 h. The methanol extract was then evaporated and the residue resuspended in distilled water for assay (26). Nucleotides were extracted from erythrocytes by boiling and were quantitated by high-pressure liquid chromatography on a Partisil −10 SAX anion exchange column (Whatman, Inc., Clifton, NJ). Deoxy- and ribonucleotides were separated by isocratic elution with 0.45 M ammonium phosphate at pH 4.2 and detected at 254 nM, as previously described (27). Deoxy ATP levels in extracted lymphocytes were quantitated by the DNA polymerase assay with [^{3}H]deoxy TTP and calf thymus DNA, as previously reported (26).

Deoxyadenosine metabolism in B lymphoblasts

Deoxyadenosine phosphorylation. B lymphoblast lysates were dialyzed overnight against 5 mM Hepes, 0.15 M NaCl, 1 mM EDTA, pH 7.4 and diluted for assay in this buffer containing 50 μM 2′-deoxycoformycin and 2 mg/ml bovine serum albumin. The extract was assayed for deoxyadenosine phosphorylating capability in the presence of 50 mM Tris/HCl, pH 7.4, 1.5 mM $MgCl_2$, 50 mM KCl, 50 μM 2′-deoxycoformycin, 1.25 mM GTP, 1.25 mM mercaptoethanol, and 500 μM [^{14}C]deoxyadenosine (sp act 1.5 μCi/μmol). The reaction was stopped by heating at 85°C for 2 min. Aliquots were spotted onto DE 81 disks, which were washed twice in ammonium formate, pH 7.0, twice in distilled water, and once in 95% ethanol. The dried disks were counted in toluene-based scintillation fluid in a Tracor model 6892 counter (Tracor Analytic Inc., Elk Village, IL). The reaction was linear over 90 min and counts obtained by the disk method were virtually identical to those obtained by adding counts in deoxy AMP, deoxy ADP, and deoxy ATP separated on paper by high-voltage electrophoresis in 0.025 M borate buffer, pH 9.0. The *Km* of crude, dialyzed lymphoblast extracts for 2′-deoxyadenosine under these conditions was 130 μM.

Deoxy ATP accumulation in intact cells. B lymphoblasts were incubated at a concentration of 1×10^6 cells/ml in RPMI 1640 medium containing 10% heat-inactivated horse serum and 50 μM 2′-deoxycoformycin alone and with 50 μM 2′-deoxyadenosine for 4 h. Cells were extracted as previously described and deoxy ATP levels quantitated by the DNA polymerase assay (26).

2′-Deoxyadenosine toxicity. B lymphoblasts were cultured at an initial concentration at 2×10^5 cells/ml in RPMI 1640 medium and 10% horse serum in the presence of 50 μM 2′-deoxycoformycin and 2′-deoxyadenosine concentrations ranging from 0 to 200 μM. The ID_{50}, defined as the dose of 2′-deoxyadenosine that inhibited growth by 50% of the control, was determined for each cell line at 72 h.

RESULTS

ADA enzyme activity. The proband E.M. is a 2-yr-old white male with apparently normal cellular and humoral immunity. The levels of ADA enzyme activity and immunoreactive protein in erythrocytes, lymphocytes, B lymphoblastoid cell lines, and skin fibroblast cell strains from normal subjects and the proband, as well as ADA levels in erythrocytes of the proband's family, are given in Table I. ADA enzyme activity is undetectable in the proband's erythrocytes, whereas both parents and one sibling are heterozygous for the ADA deficiency. These data are consistent with an autosomal recessive mode of inheritance, as has been established for patients with ADA deficiency and SCID (1–3). The level of ADA in the proband's peripheral lymphocytes is only 0.9% of control values; however, enzyme activity is detectable at 4% of control in an Epstein-Barr virus transformed B lymphoblastoid cell line (GM 4396) and 14% of control in a cultured skin fibroblast cell strain derived from the proband (GM 4395). Kinetic analysis of GM 4396 and GM 4395 proband cell extracts demonstrates that the ADA activity in both cases exhibits a normal *Km* for adenosine and deoxyadenosine (48–52 μM) and is completely inhibited by 2′-deoxycoformycin. When the lymphoblast cell extract, GM 4396, is assayed at a high adenosine substrate concentration (4.0 mM final) with and without 200 μM of the ADA inhibitor, EHNA, >90% of the total adenosine deaminating activity is inhibited by EHNA. Under similar assay conditions, the ADA activity in the GM 4395 fibroblast cell extract is completely inhibited by EHNA. By radioimmunoassay, the level of proband ADA immunoreactive protein (termed CRM) appears to be proportional to enzyme activity and the CRM has a normal affinity for the ADA antibody. As a result, the enzyme has a calculated normal

TABLE I
ADA Activity and Protein Levels

Cell type	Specific activity		CRM level*		Absolute specific activity
	nmol/min/mg	*% control*	*ng CRM/mg*	*% control*	*nmol/min/ng CRM*
Erythrocytes					
normal (5)‡	0.9±0.3§	100	1.5±0.5	100	553
E.M. (proband)	$<0.8 \times 10^{-3}$	<0.09	<0.001	<0.07	—
C.M. (father)	0.29±.01	32	0.54±.06	36	537
P.M. (mother)	0.44±.02	49	0.67±.02	55	656
L.M. (sister)	1.02±.04	113	1.83±.21	122	557
C.M. (brother)	0.54±.01	60	0.79±.05	52.6	683
Lymphocytes					
normal (3)	80.9±4.9	100	158.5±15.5	100	510
E.M.	0.7±0.11	0.9	2.62±0.2	1.6	278
B Lymphoblast lines					
normal (5)	49.0±7.4	100	92.9±12.5	100	527
E.M. (GM 4396)	2.1±0.2	4.3	4.3±0.2	4.6	495
ADA⁻SCID					
GM 2756	0.04±0.01	0.08	0.63±0.15	0.68	63
GM 2471	0.08±0.02	0.16	0.54±0.10	0.58	148
GM 2606	0.01±0.01	0.02	0.33±0.05	0.35	30
GM 2445	0.06±0.01	0.12	0.41±0.03	0.44	146
Fibroblast lines					
normal (3)	16.23±1.9	100	29.6±1.5	100	548
E.M. (GM 4395)	2.22±0.4	14	4.2±0.3	14	528
ADA⁻SCID (9)	—	(0.2–3.8)‖		(0.8–22)‖	(15–608)

* CRM indicates immunoreactive ADA protein.
‡ Number of subject samples tested given in parenthesis.
§ Mean±1 SD based on at least three determinations of each separate sample.
‖ Range of values for percentage of control of specific activity and CRM level and values for absolute specific activity from reference 13.

absolute specific enzyme activity in the different cell samples studied. The only exception is that found in the proband's lymphocytes, which show a twofold decrease in absolute specific enzyme activity (Table I).

Properties of ADA from proband and normal B lymphoblasts. The $S_{20,w}$ determined for normal B lymphoblast ADA (MGL-8) is 3.7 and corresponds to the previously published values for the normal lymphoblast enzyme (18). From this value, a molecular weight of 36,500 is calculated for the native ADA using the method of Martin and Ames (28) with catalase, yeast alcohol dehydrogenase, bovine serum albumin, and cytochrome *C* used as standards. The average sedimentation coefficient calculated from sucrose gradients for proband lymphoblast ADA was also 3.7. The pH optimum of proband B lymphoblast ADA is broad, with a range between pH 6 and 8 and is indistinguishable from that of normal lymphoblast enzyme.

The heat stability of lymphoblast ADA was determined as previously described (18). Lymphoblast cell lysates of proband GM 4396 and normal MGL-8 (protein concentration 2 mg/ml) were separately incubated at 64°C in buffer A. Under the conditions of this assay, GM 4396 ADA exhibits a logarithmic decay of enzyme activity with an average $t_{1/2}$ of 28 min, while the $t_{1/2}$ of normal ADA (MGL-8) extract is 32 min.

Starch gel electrophoresis of ADA. ADA in erythrocytes and lymphoblasts exists as a single polypeptide with primary electrophoretic forms determined by two autosomal allelic genes ADA^1 and ADA^2, which are reflected in the homozygous (1-1 and 2-2) and the heterozygous (1-2) forms of ADA (29). The characteristic secondary isozyme pattern presented by each of these three ADA types arises from posttranslational modification of the primary gene product and may be due in part to sulfhydryl group modification on the enzyme (30–32).

Normal and GM 4396 lymphoblast cell extracts and normal hemolysate extracts were electrophoresed in a starch gel with Tris/maleate buffer and stained for

ADA enzymatic activity as described in Methods. Fig. 1 shows a representative experiment. When samples are treated with iodoacetamide to alkylate ADA sulfhydryl groups before gel electrophoresis, normal hemolysate (RBC, 1-2, lane 1) exhibits the two primary ADA 1 and ADA 2 isozymes, whereas normal lymphoblast extract (MGL-8, 1-1, lane 3) shows only the ADA 1 isozyme without any evidence of secondary isozyme processing. Under this condition, the ADA in the lymphoblast extract from the proband (GM 4396, lane 2), clearly migrates faster than both the 1-1 and 1-2 phenotypes, suggesting that it is more acidic. To test whether the proband acidic ADA isozyme represented a normal ADA secondary isozyme or was a unique acidic variant, the sample was electrophoresed in buffer containing the alkylating ion, maleate, which should shift all normal primary and secondary ADA isozymes to the anode (29). As shown in Fig. 1, samples in lanes 5 and 7, the normal hemolysate (RBC, 1-1) and lymphoblast extract (MGL-8, 1-1) exhibit a shift in isozyme pattern with major conversion to a prominent anodal form. Under this condition, the proband ADA (lane 6) demonstrates a major spot of ADA activity which is again faster migrating or more acidic than the normal ADA isozymes. Preincubation of proband GM 4396 extract with 50 mM maleate before electrophoresis enhances the more acidic isozyme, but does not completely eliminate the presence of the less acidic isozyme (lane 4).

SDS-gel electrophoresis of ADA. Comparison of the apparent subunit molecular weight of proband GM 4396 and normal MGL-8 lymphoblast ADA was made using SDS-gel electrophoresis and protein blot analysis. Normal lymphoblast ADA exhibits an apparent subunit molecular weight of 40,000 (Fig. 2, lane MGL-8) comparable to that previously reported for the purified normal erythrocyte and B lymphoblast ADA (23, 32). The apparent subunit molecular weight of ADA from the proband (shown at two concentrations in Fig. 2, lanes GM 4396) is indistinguishable from that of the normal lymphoblast enzyme. These data also demonstrate the absence of any significant quantities of immunoreactive ADA degradation products in this cell

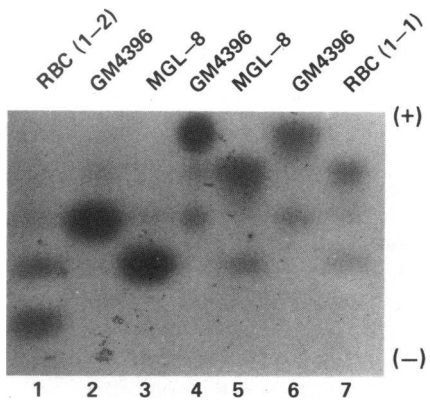

FIGURE 1 Starch gel electrophoresis of proband and normal ADA. Crude cell lysates were electrophoresed in a starch slab gel with Tris/maleate buffer, pH 7.4, and stained for ADA enzyme activity as described in Methods. This figure shows the area of the gel containing all detectable spots of ADA enzyme activity. All lanes contained samples diluted or concentrated to approximately equal ADA activity (100 nmol/min per ml). Lane *1*, normal erythrocyte lysate, 1-2 phenotype (RBC 1-2); lanes *2* and *6*, proband B lymphoblast extract (GM 4396); lanes *3* and *5*, normal B lymphoblast extract, 1-1 phenotype (MGL-8); lane *7*, normal erythrocyte lysate, 1-1 phenotype (RBC 1-1). Samples in lanes *1–3* were treated with 10 mM iodoacetamide and the sample in lane *4* was treated with 50 mM maleate before electrophoresis. Samples in lanes *5*, *6*, and *7* were untreated. The direction of electrophoresis was toward the anode (+).

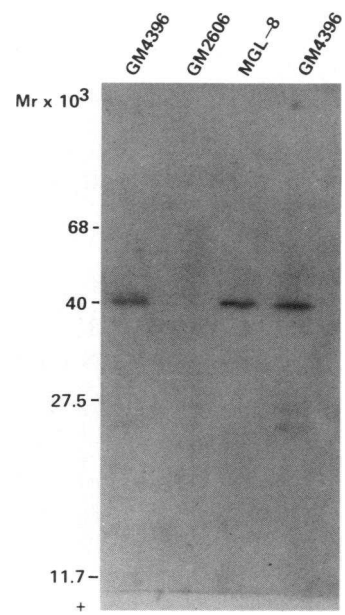

FIGURE 2 SDS-polyacrylamide gel electrophoresis of B lymphoblast ADA. Crude B lymphoblast cell extracts were electrophoresed in a 10% SDS-polyacrylamide slab gel and ADA immunoreactive protein was detected with the protein blot technique described in Methods. The direction of gel electrophoreses was toward the anode (+). Protein blots were exposed for 48 h. Lane GM 4396, proband B lymphoblast extract (samples left to right are 580 μg protein, 2.5 ng ADA CRM, and 1,160 μg protein, 5.0 ng ADA CRM); lane GM 2606, severe ADA deficient B lymphoblast extract (1,160 μg protein, <0.4 ng ADA CRM); lane MGL-8, normal B lymphoblast extract (57 μg protein, 5 ng ADA CRM). Subunit molecular weight standards are indicated. Bovine serum albumin (68,000), calf ADA (40,000), carbonic anhydrase (29,000), and cytochrome *C* (11,700) were electrophoresed in an adjacent lane, transferred to the nitrocellulose paper and stained with Coomassie Brilliant Blue.

extract. The faint bands in lane GM 4396 at ~25,000 molecular weight are observed only occasionally and their presence does not appear to correlate with any loss in intensity of the major ADA band. These bands may represent nonspecific antibody binding in this experimental system. As a control, lane GM 2606 shows the analysis of a lymphoblast extract from the severely ADA-deficient cell line (GM 2606 with <0.04% of control immunoreactive protein). No detectable ADA band is evident with this cell extract.

Urea isoelectric focusing of ADA. Before isoelectric focusing of ADA in lymphoblast cell extracts, the enzyme was partially purified by Sephadex G-75 gel filtration (32) and recovered in 85–90% yield. Pooled enzyme was dialyzed in distilled water 1/1,000 vol/vol overnight and lyophilized. This partial enzyme purification was found to be necessary to ensure the transfer and binding of ADA protein to the nitrocellulose paper after the isoelectric focusing step. As shown in Fig. 3 panel A, purified human erythrocyte ADA (lane E-ADA) exhibits three prominent immunoreactive bands detectable by autoradiography after binding anti-ADA IgG and ^{125}I-protein A. The most basic band is the primary ADA isozyme, while the more acidic bands represent secondary isozymes of ADA produced by posttranslational modification (21, 29). Normal lymphoblast ADA (lane MGL-8) from an extract of cells harvested at mid-log phase is present as only one immunoreactive band and shows no evidence of the secondary acidic ADA isozymes as observed for the purified erythrocyte enzyme. On occasion, however, an additional acidic secondary isozyme of ADA has been observed, suggesting enzyme processing similar to erythrocyte ADA (data not shown). Proband GM 4396 ADA reveals two immunoreactive ADA bands (Fig. 3 *A*, lanes GM 4396) corresponding in position to the two most acidic erythrocyte ADA isozymes. Fig. 3 *B*, lane GM 4396 shows the proband ADA analyzed from a sample of GM 4396 lymphoblasts harvested during early log phase of growth. There is an apparent absence of the most acidic isozyme suggesting a diminution in the posttranslational modification of the parent (more basic) proband isozyme. A lymphoblast extract of the ADA-deficient cell line GM 2606 was partially purified and used as a negative control (lane GM 2606).

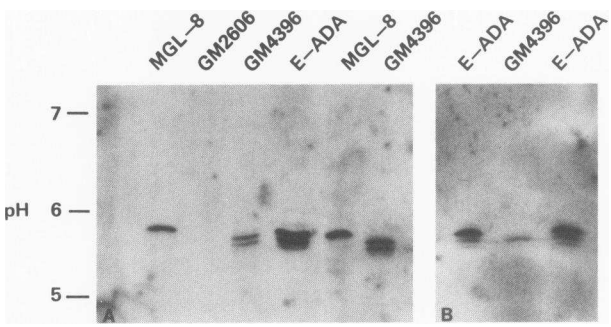

FIGURE 3 Urea isoelectric focusing gel of ADA. B lymphoblast cell extracts were prepared from cells harvested during mid-log-phase of growth. Each cell extract was partially purified by Sephadex G-75 gel filtration before analysis. Samples were focused in a polyacrylamide slab gel and ADA immunoreactive protein was detected with the protein blot technique described in Methods. This figure shows the area of the autoradiograph containing all detectable immunoreactive bands. The protein blot was autoradiographed for 72 h. (*A*) MGL-8, normal B lymphoblast extract; GM 2606, severe ADA-deficient B lymphoblast extract; GM 4396, proband B lymphoblast extract; E-ADA, purified erythrocyte ADA. (*B*) lanes E-ADA and GM 4396 are the same as panel *A*, except GM 4396 B lymphoblasts were harvested during early log-phase of growth. All samples contained 10 ng ADA CRM, with the exception of GM 2606, which was used at a total protein concentration equal to that of the GM 4396 sample.

Deoxyadenosine metabolism. Adenosine and 2′-deoxyadenosine levels were measured in the proband's plasma and urine over a 2-yr period. As shown in Table II, levels of adenosine and deoxyadenosine are undetectable in the plasma at values less than 0.5 μM and 0.1 μM, respectively. In contrast, both nucleosides are present at low concentrations in spot urine samples, but were not detected in control samples. Deoxy ATP levels are elevated in erythrocytes (18.2±1.5 nmol/ml), but to values far less than those reported in ADA-deficient, immunodeficient children (105–1,400 nmol/ml) (5). Lymphocyte deoxy ATP levels are mildly elevated in two of three determinations.

To investigate the possibility that the proband's lymphocytes were resistant to 2′-deoxyadenosine toxicity, we compared deoxyadenosine metabolism in Epstein-Barr virus transformed B cell lines from E.M. with that in lines from two normal individuals. Table III demonstrates that the phosphorylation of 2′-deoxyadenosine by cytoplasmic extracts and in intact cells and the ID_{50} for deoxyadenosine do not differ from those in normal B cell lines in which ADA is inhibited by 2′-deoxycoformycin.

DISCUSSION

A marked deficiency of the purine catabolic enzyme ADA has been causally associated with an autosomal recessive form of SCID in well over 30 patients to date (2–5). In all of these patients, ADA enzyme activity is undetectable in erythrocytes, but low amounts of putative ADA activity have been reported in other tissues (12). However, the nature of this adenosine deaminating activity has not been carefully evaluated in all cases.

TABLE II
Adenosine, Deoxyadenosine, and Deoxy ATP Levels

	Adenosine		Deoxyadenosine		Deoxy ATP	
	Plasma	Urine	Plasma	Urine	Erythrocytes	Lymphocytes
		μM			*nmol/ml*	*pmol/10⁶ cells*
Control (4)*	<0.5	<0.5‡	<0.1	<0.5	<1.0	<2.5
Proband E.M.						
8/80§	<0.5	<0.5	<0.1	8.8	18.6	<2.5
10/80	<0.5	3.5	<0.1	16.8	16.0	5,6, 6.0
2/81	0.7	1.8	0.4	7.3	18.8	4.6
5/81	<0.5	2.0	<0.1	19.8	19.3	—
1/82	<0.5	<0.5	<0.1	25.0	—	—

* Number of determinations on separate samples processed simultaneously with the proband's samples.
‡ Urinary adenosine levels in freshly extracted urines from normal individuals are 2.9±1.6 μM (means±SD; n = 10). No adenosine was detected in control urines processed with the proband samples.
§ Dates samples were obtained.

The lack of sufficient ADA activity in these patients has been shown to lead to an increase in deoxyadenosine and the selective accumulation of deoxy ATP in lymphocytes of affected children (8). The accumulation of deoxy ATP in cultured T cells in which ADA activity has been pharmacologically inhibited results in inhibition of DNA synthesis and cell death, although the precise mechanism of cytotoxicity has not been determined (3). Tissues and cells that do not phosphorylate low concentrations of deoxyadenosine and/or that have high deoxynucleotidase activity do not accumulate high levels of deoxy ATP and are apparently spared in this disorder (9–11).

As an exception to the classical patient with ADA deficiency and SCID, three healthy subjects with severe erythrocyte ADA deficiency have been described

TABLE III
Comparison of Deoxyadenosine Metabolism by B Lymphoblasts from Proband and Normal Controls

	ADA activity	Deoxyadenosine* phosphorylation	Deoxyadenosine ID_{50}‡	Deoxy ATP accumulation§
	nmol/min/mg		*μM*	*pmol/4h/10⁶ cells*
Normal				
MGL-8	41.2	0.16	52	51
GM 558	46.7	0.23	24	194
Proband				
GM4396	4.1	0.23	42	122

* Cells extracts were incubated in the presence of 50 μM 2′-deoxycoformycin and 500 μM [^{14}C]2′-deoxyadenosine. Counts incorporated into deoxynucleotides were measured on DEI disks as described in Methods.
‡ Cell growth experiments were performed in the presence of 50 μM 2′-deoxycoformycin. Values represent the concentrations of 2′-deoxyadenosine resulting in a 50% reduction in cell growth as compared with control cultures containing 2′-deoxycoformycin alone at 72 h.
§ Intact lymphoblasts were incubated at a concentration of 10^6 cells/ml in the presence of 50 μM deoxyadenosine and 50 μM 2′-deoxycoformycin. Deoxy ATP values were measured by the DNA polymerase assay as described in Methods. Values are expressed as the increment in deoxy ATP in cultures containing 2′-deoxyadenosine and 2′-deoxycoformycin over those containing 2′deoxycoformycin alone.

(14–17). These subjects have in common ADA activity ranging from 15 to 24% of normal in their quantitatively and functionally normal peripheral blood lymphocytes. In two of these cases, both of African descent, the residual enzyme activity has a normal apparent molecular weight, electrophoretic migration, and *Km* for substrates adenosine and deoxyadenosine; however, in both subjects, the enzyme is found to be more heat labile than normal. The residual lymphocyte ADA activity in these cases is apparently sufficient to prevent the toxic accumulation of deoxy ATP and to preserve normal immune function.

Over a 2-yr period, we have studied a white male child with severe erythrocyte ADA deficiency discovered during the New York State mandatory neonatal screening program. This ADA-deficient healthy subject has normal cellular and humoral immunity but, in contrast to the other ADA-deficient healthy subjects, has <1% of normal lymphocyte ADA activity. ADA enzymatic activity and protein measured by radioimmunoassay were found to be undetectable in the proband's erythrocytes. However, his lymphocytes contained residual enzyme activity equivalent to 0.9% of control values with an immunoreactive ADA protein present at 1.6% of normal CRM. From these data, we conclude that the lymphocyte enzyme has one-half of normal calculated absolute specific activity, which, in conjunction with its normal enzyme kinetics, suggests the presence of catalytically inactive enzyme.

The nature of the proband ADA was further characterized from an actively growing cultured B lymphoblastoid cell line and a skin fibroblast cell line derived from the subject. Data obtained from the proband lymphoblastoid cell line, GM 4396, indicated that the level of ADA activity and immunoreactive protein were equivalent at 4% of normal (2.1 nmol/min per mg). We and others have previously shown that lymphoblasts also contain a low level of a distinct aminohydrolase, which is distinguishable from ADA on the basis of its high *Km* for adenosine, insensitivity to the ADA inhibitor EHNA, and lack of cross-reactivity with ADA antibody (18). In the present study, >90% of the ADA activity in the proband lymphoblast extract was inhibitable with EHNA and the ratio of enzyme activity to CRM gave a calculated normal absolute specific activity, suggesting that nonspecific aminohydrolase activity was not interfering with our enzyme analyses.

The values for proband lymphoblast ADA activity and CRM are both significantly higher than those determined in four unrelated lymphoblastoid cell lines derived from patients with severe ADA deficiency and SCID (0.02–0.16%, see Table I). However, these values for proband ADA are substantially lower than those previously reported for two lymphoblastoid cell lines derived from ADA-deficient healthy subjects (18 to 57% of normal) (16, 33).

ADA was also analyzed from a fibroblast cell line derived from the proband. Fibroblasts were cultured in medium containing ADA-deficient fetal calf serum to prevent possible uptake of exogeneous ADA into the cultured cells and were harvested in late log-phase growth. Enzyme and CRM analyses showed that the level of ADA in the cultured fibroblasts was 14% of normal. Previous analysis in our laboratory of lines from nine ADA-deficient SCID patients demonstrated a mean ADA activity of only 1% of normal with a range of 0.2 to 3.8% (13). Although Hirschhorn et al. (34) have reported 20% of normal ADA activity in fibroblast cell lines of four patients with severe ADA deficiency and SCID, these data were obtained from cells cultured to very late confluence (2–3 wk after plating), a condition that results in a rise in the specific activity of the enzyme relative to total cell protein. Also at the time of their study, the residual ADA activity measured was not confirmed by inhibition with a specific ADA inhibitor or with the use of radioimmunochemical assay. Under similar conditions, Chen et al. (35) also described one fibroblast cell strain from another patient with ADA deficiency and SCID with 10% of normal ADA activity. However, under our uniform assay conditions on cells harvested in log-phase growth, the data indicate that 14% of normal ADA (2.2 nmol/min per mg) in the proband's fibroblasts represents a significant increase over the activity present in fibroblasts from other ADA-deficient individuals.

The presence of multiple molecular and electrophoretic forms of normal ADA in different tissues has complicated the analysis of putative mutant forms of ADA. The small form of the enzyme (M_r = 38,000) is a single polypeptide exhibiting electrophoretic forms determined by two autosomal allelic genes ADA^1 and ADA^2 (16, 29, 31, 32). The large form of the enzyme (M_r = 298,000) is composed of two molecules of small form ADA and one molecule of an ADA-binding protein (M_r = 200,000–213,000) (36). The large form of the enzyme can exist as tissue-specific electrophoretic variants generated by charge heterogeneity of the ADA-binding protein associated with the enzyme (37, 38). In our studies, cultured B lymphocytes were chosen as a source for the further biochemical characterization of normal and proband ADA because the enzyme is present as the small form and the properties of the ADA polypeptide can be determined in the absence of the ADA-binding protein (18). Furthermore, B lymphoblasts can be cultured indefinitely and in large quantity to provide adequate amounts of enzyme for biochemical studies.

The molecular weight of the ADA activity in normal

and proband lymphoblast extracts was assessed by sucrose gradient ultracentrifugation analysis. Proband lymphoblast ADA appears to have a normal native molecular weight of 36,500 based on $S_{20,w}$ measurements without evidence of any large molecular weight forms of the enzyme. Other properties such as *K*m for adenosine and deoxyadenosine. EHNA-sensitivity, absolute specific activity, and pH optimum are similar to those of the normal enzyme.

In contrast to other ADA-deficient healthy subjects who show an increased heat lability of ADA (five times greater than normal) in their cultured lymphoblasts (16), the proband lymphoblast ADA has normal heat stability. In addition, whereas the two other ADA-deficient healthy subjects exhibit a normal ADA (1-1) phenotype (16), lymphoblast ADA from the proband clearly migrates faster than either a 1-2 or 1-1 normal phenotype. This finding suggested that there was either a size change (smaller) and/or a charge change (more negative) in the proband ADA molecule. Further investigations of the structural properties of lymphoblast ADA were facilitated by the use of an immunoprotein blot analysis. Using this technique, we were able to study the isoelectric properties and the subunit molecular weight of normal and proband ADA from either partially purified or crude lymphoblast cell lysates. The immunochemical detection of ADA was found to be specific and sensitive enough to allow the detection of 2 ng of ADA CRM. In this immunoblot assay for ADA, the proband ADA protein has a normal apparent subunit molecular weight (M_r = 40,000) by SDS-gel electrophoresis and appears as an acidic variant by denaturing isoelectric focusing gel electrophoresis. We conclude from these studies that the proband has a structural mutation in the ADA molecule resulting in a more acidic but heat-stabile enzyme with normal molecular weight and kinetic properties.

The ADA substrates, adenosine and deoxyadenosine, were detected at low concentrations in spot urine samples and deoxy ATP levels were slightly elevated in erythrocytes and lymphocytes from this subject. However, these values are 10% or less of those reported for other ADA-deficient SCID patients (5). The low concentrations of adenosine, deoxyadenosine, and deoxy ATP in proband samples in conjunction with his normal immunologic status suggest either that residual ADA activity in other body tissues is metabolizing the potentially lymphocytotoxic ADA substrates or that there are concomitant abnormalities of other lymphocyte enzymes concerned with deoxyadenosine phosphorylation or deoxynucleotide metabolism. The latter hypothesis appears less likely, since the B lymphoblastoid cell line from the proband (GM 4396) is as sensitive to the toxicity of deoxyadenosine and accumulates deoxy ATP at a similar rate to those of normal B lymphoblastoid lines in which ADA is inhibited by 2′-deoxycoformycin. These results do not support a defect in deoxyadenosine phosphorylation or deoxynucleotide degradation in this subject's cells, at least at the higher deoxyadenosine concentrations required by the relative insensitivity of B cells to purine deoxyribonucleosides (26). On the other hand, the relatively high ADA activity in the proband's cultured fibroblasts provides some evidence for the presence of higher ADA activity in nonhematopoietic tissues in vivo. Such activity could explain the relatively low levels of adenosine, 2′-deoxyadenosine, and deoxy ATP in this individual, and hence the preservation of normal immune function. It remains possible that immunodeficiency may develop at a later date because of fluctuations in the levels of the ADA substrates, although these have remained relatively constant over a long period, or to prolonged exposure of lymphocytes to very low 2′-deoxyadenosine concentrations. At the present time, however, this individual represents a unique form of ADA deficiency in a healthy subject and represents another example of genetic heterogeneity in partial ADA deficiency.

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