

# Erythrocyte Adenosine Deaminase Deficiency without Immunodeficiency

## EVIDENCE FOR AN UNSTABLE MUTANT ENZYME

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**ABSTRACT** Inherited deficiency of the purine salvage enzyme adenosine deaminase (ADA) gives rise to a syndrome of severe combined immunodeficiency (SCID). We have studied a 2.5-yr-old immunologically normal child who had been found to lack ADA in his erythrocytes during New York State screening of normal newborns. His erythrocytes were not detectably less deficient in ADA than erythrocytes of ADA<sup>-</sup>SCID patients. In contrast, his lymphocytes and cultured long-term lymphoid cells contained appreciably greater ADA activity than those from patients with ADA<sup>-</sup>SCID. This residual ADA activity had a normal molecular weight and  $K_m$  but was markedly unstable at 56°C. His residual erythrocytes-ADA activity also appeared to have diminished stability *in vivo*. ADA activity in lymphoid line cells of a previously reported erythrocyte-ADA-deficient !Kung tribesman was found to contain 50% of normal activity and to exhibit diminished stability at 56°C. ATP content of erythrocytes from both partially ADA-deficient individuals was detectably greater than normal (12.3 and 6.1 vs. normal of 2.6 nmol/ml packed erythrocytes). However, the dATP content was insignificant compared to that found in erythrocytes of ADA<sup>-</sup>SCID patients (400–1,000 nmol/ml packed erythrocytes). The New York patient, in contrast to normals, excreted detectable amounts of deoxyadenosine, but this was <2% of deoxyadenosine excreted by ADA<sup>-</sup>SCID patients. Thus, the residual enzyme in cells other than erythrocytes appears to be sufficient to almost totally prevent accumulation of toxic metabolites.

## INTRODUCTION

Inherited deficiency of adenosine deaminase (ADA),<sup>1</sup> an enzyme of purine salvage, usually results in the syndrome of severe combined immunodeficiency disease (SCID) (1–3). Approximately 85% of ADA-deficient infants present with full blown, classic SCID. In these patients, cellular and humoral immune function are both profoundly impaired, their illness begins during the first few months of life, and they succumb rapidly to their multiple infections. Approximately 15% of ADA-deficient infants become ill at a later age and have a more prolonged course, although they too eventually succumb to their disease within the first few years of life. *In vitro* tests of immune function in these latter patients reveal a more pronounced defect of cellular than of humoral immunity (2, 3). This clinical heterogeneity could result from either genetic heterogeneity or random differences in exposure to infectious agents. Genetic heterogeneity alone would appear to account for the one clinically healthy, immunologically normal individual who was inadvertently discovered during population studies of the !Kung tribe of the Kalahari Desert (4). This !Kung tribesman lacks ADA in his erythrocytes but his lymphocytes contain appreciable ADA activity (5). Extensive prior studies of many different populations, directed at determining frequencies of the genetically polymorphic forms of ADA, had not detected any other healthy individual deficient for ADA (6). As part of the New York State mandatory screening of newborns, a second individual who totally lacks ADA in his erythro-

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<sup>1</sup>Abbreviations used in this paper: ADA, adenosine deaminase; EHNA, erythro-9-(2-hydroxy-3-nonyl-) adenine; LTL, long-term lymphoid RBC, erythrocyte(s); SCID, severe combined immunodeficiency disease.

cytes but who has remained clinically healthy and immunologically normal at 2.5 yr of age (7)<sup>2</sup> has been ascertained. We have quantitatively and qualitatively examined ADA activity in this child's erythrocytes and lymphoid cells, both cultured and uncultured. We have compared this child's ADA activity with that found in cells of patients with ADA deficiency and SCID and in cells of the previously reported healthy !Kung tribesman. We have also compared adenosine and deoxyadenosine metabolites in cells and body fluids. The lymphocytes and cultured lymphoid line cells of our patient, similar to the !Kung tribesman, have greater residual activity than do those of other ADA-deficient patients who are immunodeficient, but this adenosine deaminating activity is unstable in vitro, as is the ADA of the !Kung tribesman. However, this ADA activity is sufficient in both our patient and the !Kung tribesman to almost totally prevent the accumulation of substrates of ADA and their potentially toxic metabolites in vivo.

## METHODS

Nucleosides and nucleotides were obtained from Sigma Chemical Company, (St. Louis, Mo.) and/or P-L Biochemicals, Inc. (Milwaukee, Wis.). [8-<sup>14</sup>C]Adenosine ( $\approx 50 \mu\text{Ci}/\mu\text{M}$ ) was purchased from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.) and [8-<sup>14</sup>C]deoxyadenosine ( $\approx 44 \mu\text{Ci}/\mu\text{M}$ ) from New England Nuclear (Boston, Mass.), nucleoside phosphorylase and xanthine oxidase from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) Erythro-9-(2-hydroxy-3-nonyl) (EHNA) was obtained from Burroughs Wellcome Co. (Research Triangle Park, N. C.), *Escherichia coli* DNA polymerase (catalogue L500-06316) from Worthington Biochemicals (Freehold, N. J.), and calf thymus DNA from Sigma Chemical Co.

### *Adenosine and deoxyadenosine deaminating activity.*

*Method 1 ("high substrate").* Adenosine and deoxyadenosine deaminating activity were assayed as previously described (8) by a modification of the method of Hopkinson (9) in which the substrate concentration is approximately four times that originally used. This method measures the increase in absorbance at 293 nm resulting from the conversion of adenosine to inosine by endogenous ADA and then to uric acid by the sequential action of exogenous nucleoside phosphorylase and xanthine oxidase at 37°C. In brief, a reaction mixture was made with 1.3 mM adenosine or deoxyadenosine in 0.05 M Na<sub>2</sub>PO<sub>4</sub> buffer, pH 7.5, 1  $\mu\text{g}/\text{ml}$  nucleoside phosphorylase, and 50  $\mu\text{g}/\text{ml}$  xanthine oxidase. Aliquots of cell suspension (0.1 ml of a 1:41 dilution of washed erythrocyte(s) (RBC) or from 25 to 500  $\mu\text{g}$  of lymphocyte or lymphoid line cell lysate protein) were added per milliliter of reaction mix and the rate of increase in absorbance at 293 nm determined for a linear portion of the curve. The rate of reaction was linear with varying protein concentration for the aliquots used. For assay of RBC the endogenous nucleoside phosphorylase

is not rate limiting, and exogenous nucleoside phosphorylase was therefore omitted. The activity of a reagent blank, minus cell extract, was determined simultaneously, to correct for the ADA contaminating the commercial exogenous enzyme preparations.

*Method 2 ("low substrate").* To increase the specificity of the ADA assay, enzyme activity was determined at low substrate concentration and as that activity which could be inhibited by 50  $\mu\text{M}$  EHNA, an inhibitor of ADA. Such assay conditions should eliminate measurement of the activity of a second isozyme of ADA (ADA<sub>2</sub>) which is present in normal amounts in ADA<sub>1</sub>-SCID patients, is active at high substrate concentrations, and is not inhibitable by EHNA (10). Additionally, the assays were performed in tris EDTA buffer to inhibit possible formation of inosine by sequential phosphorylation, deamination, and dephosphorylation of adenosine through alternative pathways. On a kinetic basis alone, enzyme activity determined at 0.09 mM substrate concentration should be  $\approx 70\%$  of that seen at 1.3 mM substrate, calculating from a  $K_m$  of 40  $\mu\text{M}$ . The conversion of [<sup>14</sup>C]adenosine or deoxyadenosine to inosine or deoxyinosine (and hypoxanthine) was measured essentially by minor modifications of the method of Coleman (11). In brief, lysates were incubated at 37°C with 0.09 mM [<sup>14</sup>C]adenosine (Schwarz/Mann,  $\approx 50 \mu\text{Ci}/\mu\text{mol}$ ) or deoxyadenosine (New England Nuclear,  $\approx 44 \mu\text{Ci}/\mu\text{mol}$ ) in 24 mM Tris, 2.4 mM EDTA buffer, pH 7.5, in the presence and absence of 50  $\mu\text{M}$  EHNA. The reaction was terminated at intervals between 30 and 120 min by addition of 10  $\mu\text{l}$  of 2.1 N perchloric acid to 50- $\mu\text{l}$  aliquots of the reaction mixture. After centrifugation and subsequent neutralization of the supernate with 10  $\mu\text{l}$  of 2.21 N KOH, 20- $\mu\text{l}$  aliquots were cochromatographed with internal standards on DE 81 paper in 1 mM ammonium formate. The relevant areas were identified under UV light, the entire chromatogram appropriately sectioned, and radioactivity of all sections determined. Adenosine and deoxyadenosine were widely separated from hypoxanthine, inosine, and deoxyinosine. Virtually no counts (<0.1%) were found as nucleotides under these conditions of assay. Activity was calculated by determining the percentage of the total counts recovered which were present as hypoxanthine and inosine. Lysates were appropriately diluted to result in 5–30% conversion of substrate. Assays were performed at two or more time points and at two protein concentrations. Results reported are for values that are linear for protein concentration and time of assay.

*Determination of ADA phenotype.* The normal inherited polymorphic forms of ADA (ADA<sub>1</sub>, ADA<sub>2</sub>, and ADA<sub>2-1</sub>) were determined by electrophoresis in starch gel as described by Spencer et al. (12).

*Heat stability.* Cell pellets were resuspended in 0.15 M NaCl, 0.05 M Tris, pH 7.5, at a protein concentration of >1 mg/ml, to avoid effects of ionic strength and protein on heat stability (13, 14). The ADA phenotype of all normal lymphoid cells used in heat stability experiments, unless otherwise noted, was ADA<sub>1</sub>, as normal ADA<sub>2-1</sub> is slightly less stable than ADA<sub>1</sub> (13, 14). Equal volumes of cell lysate at equal protein concentrations were incubated for the indicated time intervals at 56°C. After removal to ice, the lysates were appropriately diluted so that assay of the zero time sample would result in  $\approx 10$ –30% conversion of the [<sup>14</sup>C]adenosine and would be equivalent in the lysates to be compared. Two dilutions were assayed at each time point in the presence and absence of EHNA.

*Sephadex G-200 chromatography.* Chromatography of cell lysates was performed on 0.6  $\times$  30-cm Sephadex G-200 columns (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) which had been calibrated with nucleoside phosphorylase

<sup>2</sup> Borkowsky, W., A. A. Gershon, L. Shenkman, and R. Hirschhorn. Adenosine deaminase deficiency without immunodeficiency: clinical and metabolic studies. Manuscript submitted for publication.

(89,000), peptidase B (54,000) and ADA (32,000). Lysates of  $20 \times 10^6$  cells were centrifuged in a Beckman microfuge (Beckman Instruments, Inc., Fullerton, Calif.) for 3 min, applied to the column, and eluted with 0.025 M Tris, 2.25 mM EDTA, pH 7.5, at a flow of 1.7 ml/h. Fractions of 0.25 ml were collected and assayed as described under "low substrate."

$K_m$ . ADA activity of two concentrations of lysates at various substrate concentrations was measured and  $K_m$  determined by standard Lineweaver-Burk plots.  $K_m$ s were determined after assay both by the high substrate method and by appropriate dilutions of [ $^{14}$ C]adenosine with or without added unlabeled adenosine.

*Source of long-term lymphoid cells.* Long-term lymphoid lines were initiated from peripheral blood by the National Institutes of Health Human Genetic Mutant Cell Repository (Camden, N. J.), from patients of Dr. R. Hong (GM 2606) (15), Dr. A. Rubinstein (GM 2756) (15, 16), Dr. S. Polmar (GM 2471) (8, 15, 17), Dr. T. Jenkins (GM 3043) (4, 5, 18), Dr. J. Lightbody (GM 1715) (19), Dr. P. Papageorgiou (GM 2825), and from the patient reported here (GM 2294) (7, 15). Cells from patients of Dr. R. Hong (15) and Dr. C. Griscelli were also studied but long-term lymphoids (LTL) are not available. A series of genetically polymorphic enzyme markers was determined at intervals to confirm the identity of the lines under study.

The clinical and immunological studies of the 2.5-yr-old immunologically normal child studied here have been previously reported (7) and other than transient hypogammaglobulinemia at 6 mo, no abnormalities of cellular and humoral immunity *in vivo* or *in vitro* have been detected.

*Preparation of cell extracts.* RBC were washed three times with the removal of the buffy coat and packed cells diluted 1:41 in 0.001 M  $\text{PO}_4$  or in 0.001 M Tris for some assays at low substrate. Deficient RBC were diluted 1:5 or 1:10 for assays with radiolabeled compounds. RBC of the RBC-ADA-deficient healthy patient and the !Kung tribesman were separated from lymphocytes on Hypaque-Ficoll (Radiopaque Media, Winthrop Laboratories, New York, N. Y.) and then washed three times with further removal of the remaining buffy coat.

Lymphoid cells were suspended in 0.001 M Tris, pH 7.5, at such concentrations that the Tris did not affect the Lowry determinations, frozen and thawed five times, and briefly sonicated. Aliquots were then appropriately diluted for assay.

*Deoxy and adenosine kinase.* These activities were assayed as described by Carson (20), except that the final concentration of deoxyadenosine was 0.246 mM, adenosine 103 mM, ATP 4.4 mM, and  $\text{MgCl}_2$  2.2 mM.

*dATP.* dATP in RBC was determined by high-pressure liquid chromatography on a Laboratory Data Control System with a variable UV monitor using a Whatman Partisil 10 SAX column (Whatman, Inc., Clifton, N. J.) as described by Cohen (15) and by the more sensitive DNA polymerase assay adapted from Solter et al. (21). Essentially, 10- $\mu$ l aliquots of perchloric acid precipitated, KOH-neutralized extracts were incubated in the presence of saturating concentrations of unlabeled dCTP, dTTP and dGTP, and [ $^{14}$ C]dCTP (Amersham Corp., Arlington Heights, Ill., 414 mCi/mmol) using a template of calf thymus DNA and *E. coli* DNA polymerase (Worthington Biochemicals). The assay was linear between 25 and 1,000 pmol/10- $\mu$ l sample.

*Deoxyadenosine and adenosine.* Deoxyadenosine and adenosine in urine were determined both with and without prior separation on anion exchange columns (AG 1-X2, Bio-Rad Laboratories, Richmond, Calif.) essentially as described by Kuttesch et al. (22), and fractions lyophilized. The urinary nucleosides were separated by high-pressure liquid chroma-

tography on a Waters  $^{18}\text{C}$  Bondapak reverse phase column (Waters Associates, Milford, Mass.) by a modification of the method of Brown (23) using a flow rate of 1.5 ml/min and a 30-min linear gradient starting with 10 mM ammonium phosphate buffer, pH 5.5, and ending with 20% methanol in the initial buffer. Adenosine and deoxyadenosine were identified by coelution with standards, 250:260 ratios, and peak shift to the elution volume of inosine and deoxyinosine following incubation with exogenous calf-intestinal ADA.

*Separation of RBC on discontinuous density gradients.* Before centrifugation blood was either passed through filter paper or, in later experiments, through  $\alpha$ -cellulose-microcrystalline cellulose columns as described by Beutler (24) for removal of leukocytes. RBC of differing densities were then separated by centrifugation on arabinogalactan gradients as described (25). Gradient fractions were washed, and aliquots lysed for determination of pyruvate kinase activity and hemoglobin content. Aliquots for assay of ADA were lysed as above but were also lysed in 0.01 M  $\text{PO}_4$ , 0.001 M EDTA, pH 7.5, in which lymphocytes remained intact, centrifuged and the supernate assayed for ADA.

## RESULTS

*ADA activity in RBC, lymphocytes, and LTL line cells.* ADA activity of the child's RBC, determined at high substrate concentrations (1.3 mM), was <1% of normal, and indistinguishable from ADA activity in RBC of four ADA-deficient patients with SCID (ADA $_1$ -SCID) (Table I). (RBC-ADA activity of both his parents was below 2 SD from the normal.) ADA activity of his mononuclear cells, determined at high substrate concentrations, was  $214 \pm 43$  U (17% of normal) compared with an average of  $136 \pm 66$  U (10% of normal) in mononuclear cells from five ADA $_1$ -SCID. Although average ADA activity in the RBC-ADA $^-$  healthy child's mononuclear cells appeared to be slightly higher than in mononuclear cells of the immunodeficient children (214 vs. 136 U), there was overlap of individual values between the two groups and the difference was not significant ( $P > 0.2$ ). No such overlap was observed upon determination of ADA activity of cultured lymphoid line cells. ADA activity of LTL line cells derived from this patient was markedly greater than that observed in LTL of ADA $^-$ -SCID patients (590 vs. 13 U). Normal lymphoid line cells contained an average of 2,003 U (range, 1,200–2,700).

The relatively minor difference in ADA activity of peripheral blood mononuclear cells, but marked difference in ADA activity of LTL cells from our ADA $^-$  healthy patient, as compared with that of patients with ADA $^-$ -SCID, led us to further investigate this adenosine deaminating activity. Schrader et al. (10) have described an additional ADA activity in some normal tissues which differs from the major ADA in that it is active only at high substrate concentrations, is not inhibitable by EHNA (an inhibitor of the usual form of ADA), and is found in normal amounts in tissues of an ADA-deficient child. In accordance with isozyme nomenclature we have tentatively denoted the high-

**TABLE I**  
*ADA Activity\* in RBC, Mononuclear Cells, and Lymphoid Line Cells at High (1.3 mM) Substrate*

Patient	RBC	Mononuclear cells	Lymphoid line cells
ADA <sub>1</sub> <sup>-</sup> -healthy (n)‡	0.4±0.7 (3)	214±43 (5)	590.0±115.0 (6)
ADA <sub>1</sub> <sup>-</sup> -SCID (n)‡	1 —§	119±11 (3)	17.0 (2)
	2 —§	156 (1)	7.4 (2)
	3 0.0 (1)	—	—
	4 1.8 (1)	100 (2)	—
	5 —	67 (2)	6.7 (2)
	6 0.9 (1)	240 (1)	22.0±0.16 (4)
	7 0.91 (1)	—	—
Average	9.9±0.1	136±66	13.3±7.5
Normals	85.0±22.0 (77) <sup>  </sup>	1,291±315 (17) <sup>  </sup>	2,003.0±641.0 (5) <sup>  </sup>

Patient 1 = GM 2756; 2 = GM 2606; 3 = GM 2825; 4 = patient (R.J.) of Dr. R. Hong; 5 = GM 2471; 6 = GM 1715; 7 = patient S of Dr. C. Griscelli (see Methods for further identification).

\* Measured in nanomoles per milligram protein or hemoglobin per hour at 37°C.

‡ Number of determinations.

§ Patient transfused.

<sup>||</sup> Number of individuals tested.

affinity ADA which is deficient in ADA<sup>-</sup>-SCID and can be inhibited by EHNA as ADA<sub>1</sub> and the low-affinity ADA which is present in normal amounts in ADA<sup>-</sup>-SCID as ADA<sub>2</sub>. Because the residual ADA activity we had measured in cells from ADA<sup>-</sup> patients at high substrate could represent in whole or in part this additional ADA<sub>2</sub> activity, we determined ADA activity under conditions designed to measure only the activity of ADA<sub>1</sub>. We therefore have determined activity at low substrate concentration and as EHNA-inhibitable activity. RBC both from our healthy patient and from three ADA<sup>-</sup> patients with SCID contained <0.1 U EHNA-inhibitable ADA activity (Table II) compared to normal activity of 61 U. Under these conditions of assay, lymphocytes from six immunodeficient patients were also markedly deficient and contained an average of 12.4 U EHNA-inhibitable ADA activity compared with normal activity of 665 U. In contrast, lymphocytes from the healthy patient still exhibited substantial EHNA-inhibitable ADA activity (157 U). Lymphocytes from the !Kung tribesman also contained more ADA activity (45 U) than those of ADA<sup>-</sup>-SCID. (However, the activity of the !Kung lymphocytes and/or RBC may be factitiously low because the cells were in transit for almost 1 wk before separation and assay.) ADA activity of cultured LTL line cells derived from five ADA<sup>-</sup>-SCID patients showed a pattern similar to that of peripheral blood lymphocytes. LTL from ADA<sup>-</sup>-SCID were profoundly deficient in activity, with an average ADA activity of 5.4 U compared with normal activity of 1,558. LTL line cells from our RBC-ADA<sup>-</sup> patient again

exhibited substantial ADA activity (227±150 U; range, 90–564). Lymphoid line cells derived from the !Kung tribesman contained an average of 887±291 U (range, 524–1,306). ADA activity of lymphoid line cells from our ADA<sup>-</sup> healthy patient and from the !Kung tribesman showed much greater variability at different times than did that from individual normal lymphoid lines. It is of interest that the activity of lymphoid line cell extracts from patient 6 (GM 1715) was virtually undetectable only when determined at low substrate and as EHNA-inhibitable activity (Table I vs. Table II).

In theory, a mutation of the ADA enzyme molecule could affect adenosine deaminating activity relative to deoxyadenosine deaminating activity. Because accumulation of deoxyadenosine compounds appears to be implicated in the pathophysiology (14, 15, 17, 24) we determined deoxyadenosine deaminating activity. The ratio of deoxyadenosine to adenosine deaminating activity was essentially the same in our patient's lymphoid cells as in normals (Table III). In three of the four ADA<sup>-</sup>-SCID patients' cells tested, deoxyadenosine deaminating activity had a ratio of adenosine to deoxyadenosine deaminating activity similar to normal. In the cells of a fourth patient (GM 1715), the ratio of adenosine:deoxyadenosine deaminating activity appeared to be low. However, the absolute activities in these cells were extremely low and would require further investigation. Deoxyadenosine and adenosine phosphorylating activity was also similar in lymphoid cell extracts from normals, ADA<sup>-</sup>-SCID patients, and our partially ADA<sup>-</sup> patient (data not shown).

TABLE II  
ADA Activity,\* in RBC, Mononuclear Cells, and Lymphoid Line Cells  
at Low (0.09 mM) Substrate

Patient	RBC	Mononuclear cells	Lymphoid line cells
ADA <sub>1</sub> <sup>-</sup> -healthy (n)‡	0.05±0.02 (3)	156.9 (2)	277.0±150.0 (12)
ADA <sub>1</sub> <sup>-</sup> !Kung (n)‡	0.13§ (1)	44.9§ (1)	887.0±291.0 (6)
ADA <sub>1</sub> <sup>-</sup> -SCID (n)‡			
1	— <sup>  </sup>	24.9 (2)	9.24±3.2 (4)
2	— <sup>  </sup>	16.2 (1)	4.03±2.2 (6)
3	<0.01 (2)	6.2 (2)	3.70 (1)
4	0.05 (2)	1.3 (1)	—
5	—	6.0 (1)	9.20±2.6 (4)
6	—	—	0.95±0.55 (4)
7	0.05 (1)	19.8 (1)	—
Average	0.035±0.02	12.4±9.25	5.42±3.66
Normals	60.9±21.0 (14) <sup>¶</sup>	665.0±154.0 (14) <sup>¶</sup>	1,558.0±472.0 (5) <sup>¶</sup>

RBC-ADA activity for the !Kung tribesman and the ADA<sup>-</sup>-healthy individual was determined on Hypaque-Ficoll-separated RBC (see text) in order to reduce possible contribution of lymphocyte ADA activity.

Patients are the same as in Table I. Total ADA activity in five ADA<sup>-</sup>-SCID lymphoid line cells: 1 = 15.5±4.5; 2 = 4.7±2.2; 3 = 5.0; 5 = 12.9±3.8; 6 = 2.7±1.2.

\* Measured in nanomoles per milligram protein or hemoglobin per hour at 37°C, inhibitable by 50 μM EHNA±SD. Total ADA activity of erythrocytes = 0.19 for ADA<sup>-</sup>-healthy; 0.26 for !Kung and 0.103±0.02 (range = 0.08–0.12) for ADA<sup>-</sup>-SCID.

‡ Number of independent determinations on same individual.

§ Activity may be factitiously low because the cells were in transit for almost 1 wk before separation and assay.

<sup>||</sup> Patient transfused.

<sup>¶</sup> Number of individuals tested.

The existence of substantial ADA activity in peripheral blood lymphocytes and lymphoid line cells (cells actively synthesizing protein) and virtually absent ADA in the nonprotein synthesizing RBC of our patient suggested that he might have an unstable enzyme. We

TABLE III  
Deoxyadenosine Deaminating Activity\* of Lymphoid Line Cells (Low Substrate)

Patient	Lymphoid line cells	Ratio Ar:dAr
ADA <sub>1</sub> <sup>-</sup> -healthy (n)‡	297.0±63.0 (3)	1.3±0.1
ADA <sub>1</sub> <sup>-</sup> -SCID (n)‡		
1	7.7 (2)	1.1
2	3.4 (1)	1.2
5	10.2 (2)	1.0
6	1.4 (2)	0.6
Average	5.68±4.0	1.0±0.3
Normals	1,276.0±361.0 (7) <sup>§</sup>	1.3±0.2

\* Measured in nanomoles per milligram protein per hour at 37°C.

‡ Number of independent determinations on same individual.

§ Number of individuals tested.

therefore determined EHNA-inhibitable activity in RBC of different ages, separated by centrifugation on arabinogalactan gradients (25). The expected age dependency of the cell fractionation was verified by the progressive decline of pyruvate kinase activity in the gradient fractions (25). ADA in normal RBC essentially showed no age-dependent decrease in activity ( $t_{1/2}$  = 231 d) (Fig. 1). Initial determinations of ADA activity in gradient-separated RBC of an ADA<sup>-</sup> healthy child revealed a two-component curve of decay with a very short half-life (3.2 d) of the first component (not shown). However, the presence of small but detectable numbers of lymphocytes (containing substantial ADA activity) in the reticulocyte rich fraction could have been responsible for this result. To further reduce lymphocyte contamination we column-purified RBC, lysed them in a buffer in which lymphocytes remained microscopically intact, and assayed the supernate. Determination of ADA activity of such gradient-separated RBC of our ADA<sup>-</sup> healthy child revealed that all fractions contained <1% of normal ADA activity (Fig. 1). Fractions rich in reticulocytes exhibited greater EHNA-inhibitable ADA activity than did those containing older RBC (0.14 vs. 0.03 U). These results were

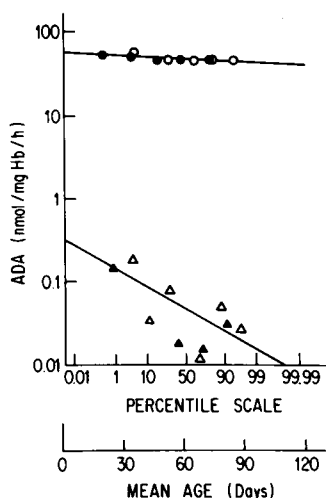


FIGURE 1 ADA activity of RBC separated according to age on an arabinogalactan gradient (25).  $\circ$ , Normal;  $\Delta$ , ADA-deficient healthy child. Solid and open symbols denote two separate experiments (see text for details).

consistent with the existence of an unstable enzyme with a  $t_{1/2}$  of 22.5 d ( $r = 0.751$ ,  $P = 0.0124$ ). However, because of the greater difference in enzyme content between RBC and lymphocytes of the patient than that of normals, we could not definitively rule out the possibility that undetected contamination of the upper layers of reticulocytes by small numbers of lymphocytes was responsible for the apparent increased activity in these fractions.

We next determined the stability of the enzyme to heating. ADA of normal lymphoid line cells (ADA<sub>1</sub> 1 phenotype) exhibited a logarithmic decay of enzyme activity at 56°C with a  $t_{1/2}$  of  $\approx 50$  min, as previously reported for ADA<sub>1</sub> 1 (13). (Fig. 2). ADA of the patient's lymphoid line cells was very unstable and had lost 50% of activity in <10 min. ADA activity of lymphocytes from the !Kung tribesman was also unstable with a  $t_{1/2}$  of 10 min. Residual EHNA-inhibitable ADA activity of lymphoid line cells from one ADA<sub>1</sub><sup>-</sup> patient (GM 2606) with SCID showed normal stability (Fig. 2). ADA activity of a second patient with ADA<sub>1</sub><sup>-</sup>-SCID (GM 2756) appeared to have intermediate stability with a suggestion of an unstable component and a component with normal stability.

To further characterize the ADA activity in normals, ADA<sub>1</sub><sup>-</sup>-healthy and ADA<sub>1</sub><sup>-</sup>-SCID individuals, lymphoid line extracts were chromatographed on Sephadex G-200. ADA<sub>1</sub> activity can exist as a low molecular weight ( $\approx 32,000$  daltons) catalytic unit or combined with an enzymatically inactive subunit of  $\approx 100,000$  mol wt which has varying degrees of glycosylation (26-30). The several forms of the enzyme thus contain the same catalytic subunit and are inhibitable by EHNA. In contrast, the ADA<sub>2</sub> isozyme has been reported as a 100,000

mol wt species and is essentially not inhibitable by EHNA (10). Various tissues and fibroblasts can contain both high and low molecular weight forms of ADA<sub>1</sub> (26, 31). In contrast, normal LTL, similar to RBC, upon electrophoresis on starch gel and staining, exhibit detectable amounts of only the anodally migrating, low molecular weight form of ADA<sub>1</sub>. On column chromatography >97% of normal lymphoid line ADA eluted with a molecular weight of  $\approx 32,000$  (Fig. 3). The remainder of the activity was found as a high molecular weight species which was also inhibitable by EHNA. Under these conditions, EHNA noninhibitable activity is not detected in normal lymphoid line cells. ADA of lymphoid line cells derived from our patient also eluted primarily as a 32,000-mol wt species. About 8% of the activity eluted as a high molecular weight species which was also inhibitable by EHNA. ADA of lymphocytes and lymphoid line cells of the !Kung tribesman also demonstrated a major peak of ADA activity at 32,000 daltons. In contrast, chromatography of enzyme activity from lymphoid line cells of an ADA<sup>-</sup>-SCID patient (GM 1715), in which >50% of ADA activity was not EHNA-inhibitable (Table II), showed only a minor EHNA-inhibitable peak at 32,000 mol wt with the remainder of the ADA activity eluting as high molecular weight activity (>200,000; not shown) which was essentially not inhibitable by EHNA. This ADA activity could represent a dimer of the ADA<sub>2</sub> isozyme described by Schrader (22).

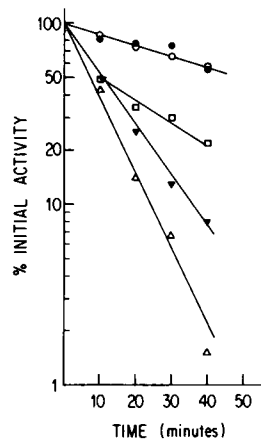


FIGURE 2 Stability of ADA at 56°C. Lysates of lymphoid line cells were aliquoted, incubated at 56°C for various time intervals, and ADA activity determined (see text for details).  $\circ$ , Normal ADA<sub>1</sub> 1;  $\Delta$ , ADA<sup>-</sup> healthy child;  $\blacktriangle$ , ADA<sup>-</sup>!Kung tribesman;  $\square$ , ADA<sup>-</sup>-SCID (GM 2756);  $\bullet$ , ADA<sup>-</sup>-SCID (GM 2606). Results of a single representative experiment are shown. Average stability of ADA activity after incubation for 30 min at 56°C was determined in separate independent experiments: normal =  $61.5 \pm 8.4$  ( $n = 4$ ); ADA<sup>-</sup>-healthy =  $6.8 \pm 4.3$  ( $n = 4$ ); ADA<sup>-</sup>!Kung =  $20.0 \pm 6.6$  ( $n = 3$ ); ADA<sup>-</sup>-SCID (GM 2606) =  $76.0 \pm 1.7$  ( $n = 3$ ); ADA<sup>-</sup>-SCID (GM 2756) =  $30.2 \pm 3.0$  ( $n = 3$ ).

The apparent  $K_m$  of ADA in lymphoid line cells of our RBC-ADA<sub>1</sub><sup>-</sup>-healthy patient and of the lymphocytes from the !Kung tribesman was like that of ADA in normal lymphocytes, lymphoid line cells, and fibroblasts (30–70  $\mu$ M) (Fig. 4A). In contrast, lymphoid line ADA activity of GM 1715 demonstrated a Lineweaver-Burk plot with two components, one with a normal  $K_m$  and a second component with a  $K_m$  that ranged between 350–1,000  $\mu$ M (Fig. 4B). GM 1715 was also slightly different from the other ADA<sup>-</sup>-SCID LTL on gel filtration and ratio of activity at high and low substrate (see previous results). However, we have found this second  $K_m$ , in addition to ADA with a normal  $K_m$  in cultured cells from each of the three additional ADA<sub>1</sub><sup>-</sup>-SCID patients we have examined (GM 2578, 2606, and 2756). The finding of the same abnormality in all four unrelated patients, in light of the prior de-

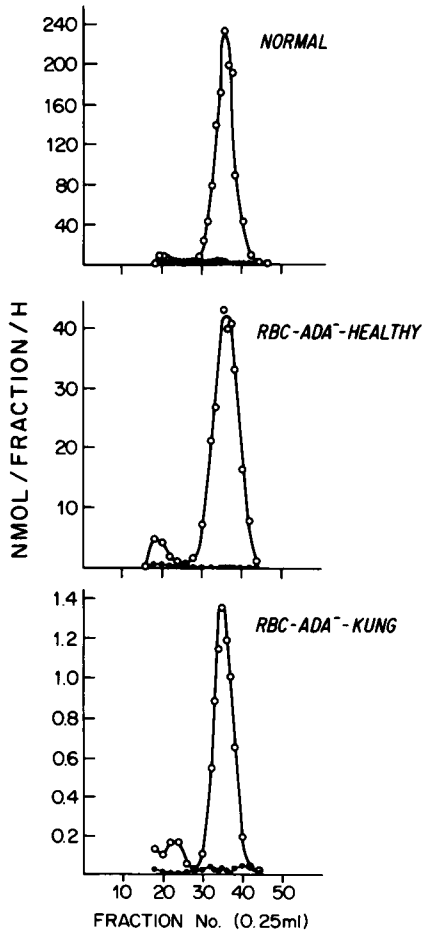


FIGURE 3 Sephadex G-200 gel chromatography of ADA activity of lymphoid line cells. The major peak of ADA activity occurred at an apparent molecular weight of 32,000 and was inhibitable by EHNA in lymphoid line cells from normals, the partially ADA<sup>-</sup>-healthy child, and lymphocytes of the partially ADA<sup>-</sup>-!Kung tribesman (see text for details).

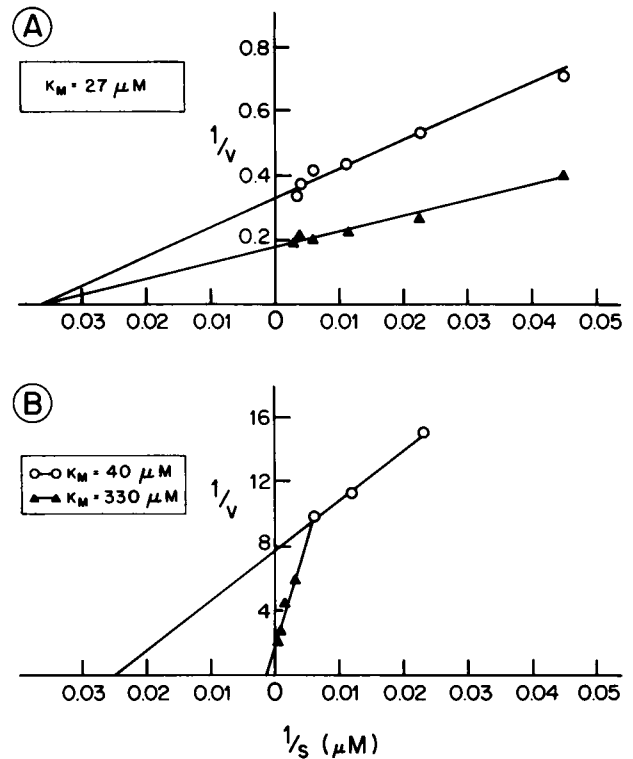


FIGURE 4  $K_m$  of ADA of lymphoid cells. (A) Lineweaver-Burk plot of ADA activity in lymphoid cells of two individuals who have marked deficiency of ADA in RBC but only partial deficiency in lymphoid cells. The  $K_m$  is like that observed in normal lymphoid cells.  $\blacktriangle$ , !Kung tribesman (GM 3043);  $\square$ , RBC-ADA<sup>-</sup> healthy child (GM 2294). (B) ADA of lymphoid line cells derived from an ADA<sub>1</sub><sup>-</sup>-SCID patient (GM 1715). The Lineweaver-Burk plot suggests that total residual ADA activity reflects two species of ADA; the  $K_m$  of 40  $\mu$ M is like that of the normal ADA<sub>1</sub> isozyme (affected in ADA<sup>-</sup>-SCID), whereas the  $K_m$  of 330  $\mu$ M is like that of the normal ADA<sub>2</sub> isozyme (10). Over 90% of the ADA activity determined at high substrate eluted as a non-EHNA inhibitable high molecular weight species, properties which are characteristic of the ADA<sub>2</sub> isozyme (not shown). The  $K_m$  of ADA<sub>2</sub> isozymes is not detected in normal lymphoid cells under these conditions because in normal cells it represents a minor percentage of total ADA activity.

scription of an additional form of ADA (ADA<sub>2</sub>) with a similar low affinity for substrate, would suggest that this second  $K_m$  represents the detection of the low amounts of the normal ADA<sub>2</sub> isozyme unmasked by the absence of the major high affinity ADA<sub>1</sub>. Although highly unlikely, it cannot be ruled out that the double  $K_m$  represents the expression of two different mutant alleles for ADA in each of the four patients.

**Electrophoretic mobility.** The ADA of both our patient and that previously reported (5) of the !Kung tribesman was indistinguishable from that of normal ADA<sub>1</sub> of a 1 phenotype.

**Metabolites.** To assess whether the residual en-

zyme activity was sufficient to prevent accumulation of metabolites *in vivo*, we determined dATP in RBC and deoxyadenosine and adenosine in urine. Normal RBC were found to contain 2–4 nmol of dATP/ml packed RBC ( $2.6 \pm 1.1$ ). RBC of two ADA-deficient patients with SCID contained between 400 and 1,000 nmol/ml packed RBC, similar to previously reported values (15, 32). Our patient's RBC were originally reported to contain normal amounts of dATP (15). More sensitive determinations revealed slightly, but detectably increased RBC dATP (12.3 nmol/ml packed RBC; normal,  $2.6 \pm 1.1$ ). He also excreted detectable amounts of deoxyadenosine ( $1.1 \mu\text{M}$ ) but this was  $\approx 1\%$  of that excreted by two ADA<sup>-</sup> immunodeficient patients ( $50\text{--}100 \mu\text{M}$  deoxyadenosine; normal,  $<0.5 \mu\text{M}$ ). Adenosine excretion of  $2.5 \mu\text{M}$  was within the normal range (up to  $3.6 \mu\text{M}$ ). RBC from the !Kung tribesman also contained detectable dATP (6.1 nmol/ml packed RBC). Urine was not available for examination.

## DISCUSSION

Inherited deficiency of the purine salvage enzyme ADA (EC 3.5.4.4) is responsible for approximately one-third of unselected cases of SCID (including X-linked and autosomal recessive), or almost 50% of autosomal-recessive SCID (2, 33). As in any rare autosomal-recessive disorder in a heterogeneous population, it can be expected that different cases will represent different mutations at the same locus as part of an allelic series (34). In a nonconsanguineous family, a given affected individual may even be a "compound heterozygote" carrying two different "deficiency" alleles. Evidence for some degree of genetic heterogeneity has been provided by the demonstration of both cross reacting material positive and cross reacting material negative patients (35). In addition, aberrations in electrophoretic mobility and heat stability have been found (36, 37). Perhaps the clearest example of genetic heterogeneity in this disorder has been the description of an immunologically normal !Kung tribesman who has virtually absent ADA activity in RBC but 20% of normal activity in lymphocytes (5).

We have studied ADA activity in various cells of a second individual who was detected by virtue of absent RBC ADA during New York State-mandated screening of newborns. This black child is immunologically normal and healthy at 2.5 yr of age (7). He has RBC-ADA activity not detectably different from that of RBC of ADA<sup>-</sup> individuals with SCID both at high and low substrate concentrations.

Initially, mononuclear cell ADA activity was determined under conditions which could measure activity of the ADA<sub>2</sub> isozyme in addition to the activity of ADA<sub>1</sub>, which is deficient in ADA<sup>-</sup>SCID. Under these conditions, our patient's mononuclear cells contained de-

tectably but not strikingly greater ADA activity than did mononuclear cells from patients with ADA<sub>1</sub><sup>-</sup>SCID. In contrast, when assayed under conditions more specific for measurement of the major human form of ADA (ADA<sub>1</sub>), mononuclear cells from ADA<sup>-</sup>SCID patients contained very little residual activity (average = 2% of normal) although mononuclear cells of our ADA<sup>-</sup> healthy patient still exhibited substantial activity (24% of normal). The !Kung tribesman has previously been reported to have 20% of normal lymphocyte ADA activity (5). The values reported here for ADA activity of lymphocytes from the !Kung tribesman, although higher than mononuclear cell ADA of ADA<sup>-</sup>SCID, are relatively lower than previously reported. However, the values for the !Kung cells may be factitiously low as both the lymphocytes and RBC were isolated from whole blood in ACD solution which had been in transit for over 4 d and arrived at room temperature.

LTl line cells from both our patient and the !Kung tribesman also exhibited substantial ADA activity, in contrast to lymphoid line cells from patients with ADA<sup>-</sup>SCID. Lymphoid line cells derived from the !Kung tribesman exhibited activity similar to lymphoid lines from obligate heterozygotes for ADA deficiency and individual values overlapped with those of normal lymphoid line cells. ADA activity in lymphoid line cells from our patient showed marked variation. We have previously reported that ADA activity of normal and deficient fibroblasts shows significant (approximately twofold) variation with differing growth conditions (31). Such alterations may also occur in lymphoid line cells and be magnified by the presence of an unstable enzyme.

ADA activity in lymphoid cells of both the !Kung tribesman and our patient was markedly less stable *in vitro* than normal. Because both may share a common racial background, it is tempting to speculate that they carry the same mutations. In addition, an ADA<sup>-</sup>SCID patient whose lymphoid line ADA suggested a two-component heat stability shares the same ethnic background as does the !Kung, our patient, and another patient whose ADA was reported to be less stable when exposed to heat (26). Determination of the amino acid and base substitutions involved will be required to determine whether these patients do indeed carry the same mutation.

We have also attempted to examine *in vivo* stability of the ADA by determining activity in RBC of different ages. The results are compatible with a diminished half-life of ADA *in vivo*. However, because the ratio of activity in lymphocytes to that in RBC is  $\approx 100:1$  in the patient (but only about 10:1 in normals), any preferential contamination of the reticulocyte-rich layers by lymphocytes could give the appearance of an age-related decrease of the patient's ADA activity. There-



fore, despite the precautions taken to eliminate such lymphocyte contamination, complete confirmation of the *in vivo* instability would require sensitive histochemical techniques to identify reticulocytes as the cells containing greater ADA activity. More important, the observed rate of decline in ADA activity in RBC extrapolates to  $\approx 1\%$  of normal RBC activity in the patient's reticulocytes. Therefore, unless ADA synthesis is "turned off" before the reticulocyte stage, the degree of *in vivo* instability alone cannot account for the amount of enzyme activity observed in lymphocytes. It is possible that this offspring of unrelated parents carries two different deficiency alleles for ADA<sub>1</sub>. Unfortunately, neither parent is currently available for further study.

Whatever the mechanism, it is clear that *in vivo* there is sufficient ADA in tissues other than RBC of this patient to prevent marked accumulations of either dATP in RBC, ATP, or cyclic AMP in lymphocytes (7)<sup>2</sup> or marked excretion of deoxyadenosine in urine. Looking at the finding in a reverse fashion, excretion of up to 0.48  $\mu\text{mol}$  deoxyadenosine/d and 12 nmol/ml RBC dATP is compatible with normal immune function.

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