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Research Article

Inherited deficiency of the enzyme adenosine deaminase (ADA) results in a syndrome of severe combined immunodeficiency (SCID). Children with ADA- -SCID lack ADA in all cells and tissues. In contrast, a "partial" deficiency of ADA has been described in six immunologically normal children from four different "families." These children lack ADA in their erythrocytes but retain variable amounts of activity in their lymphoid cells. We have examined ADA activity in lymphoid line cells from four of these children, who are unrelated, for evidence of genetic heterogeneity. One child, who is Caucasian, has an enzyme with increased electrophoretic mobility, a diminished isoelectric point (pI 4.8 vs. NI = 4.9) and very low activity (2.3 vs. NI = 82.9 +/- 12.9 nmol/mg protein per min); as a second child has an enzyme with normal electrophoretic mobility but increased isoelectric point (pI = 5.0), markedly diminished heat stability at 56 degrees C (t1/2 = 4.2' vs. NI = 40') and low activity (12.1); a third has an enzyme with only diminished heat stability (t1/2 = 6.5'), no detectable abnormality in charge and almost normal activity (41.9); while the fourth exhibits only diminished ADA activity (25.0) with no striking qualitative abnormalities. Thus, we have found evidence for three different mutations at the structural locus for ADA in three of these individuals, (a) an acidic, […]

Genetic Heterogeneity in Partial Adenosine Deaminase Deficiency

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ABSTRACT Inherited deficiency of the enzyme adenosine deaminase (ADA) results in a syndrome of severe combined immunodeficiency (SCID). Children with ADA$^-$-SCID lack ADA in all cells and tissues. In contrast, a "partial" deficiency of ADA has been described in six immunologically normal children from four different "families." These children lack ADA in their erythrocytes but retain variable amounts of activity in their lymphoid cells.

We have examined ADA activity in lymphoid line cells from four of these children, who are unrelated, for evidence of genetic heterogeneity. One child, who is Caucasian, has an enzyme with increased electrophoretic mobility, a diminished isoelectric point (pI 4.8 vs. Nl = 4.9) and very low activity (2.3 vs. Nl = 82.9±12.9 nmol/mg protein per min); as a second child has an enzyme with normal electrophoretic mobility but increased isoelectric point (pI = 5.0), markedly diminished heat stability at 56°C ($t_{1/2}$ = 4.2′ vs. Nl = 40′) and low activity (12.1); a third has an enzyme with only diminished heat stability ($t_{1/2}$ = 6.5′), no detectable abnormality in charge and almost normal activity (41.9); while the fourth exhibits only diminished ADA activity (25.0) with no striking qualitative abnormalities.

Thus, we have found evidence for three different mutations at the structural locus for ADA in three of these individuals, (*a*) an acidic, low activity heat stable mutation (*b*) a basic, somewhat higher activity, heat labile mutation, and (*c*) a relatively normal activity heat labile mutation. In the fourth, there is as yet no compelling evidence for a mutation at the structural locus for ADA and a mutation at a regulatory locus cannot be excluded.

INTRODUCTION

Inherited deficiency of the enzyme adenosine deaminase (ADA)[1] results in a syndrome of severe combined immunodeficiency (SCID), which, if untreated, is fatal in early childhood (1–3). In children with ADA$^-$-SCID, the enzyme is deficient not only in erythrocytes but in lymphoid cells and in all cells and tissues studied (4, 5). As a result of this total body deficiency of adenosine deaminase, affected children accumulate and/or excrete increased amounts of the substrates of ADA, including adenosine, 2′-deoxyadenosine and 2′-*O*-methyladenosine (reviewed in 6). These children also accumulate massive amounts of the potentially toxic metabolite deoxy ATP in their erythrocytes and lymphocytes (7, 8). In vivo and in vitro evidence indicates that the accumulation of these compounds is responsible for the deleterious effects of the enzyme deficiency on the immune system (7, 9, reviewed in 10).

In contrast, at least six children have been discovered who lack adenosine deaminase in their erythrocytes but who retain adenosine deaminating activity in their lymphoid cells (11–16, and GM 4396 submitted by Dr. H. Meuwissen). These children have all been

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[1] *Abbreviations used in this paper:* ADA, adenosine deaminase; SCID, severe combined immunodeficiency.

immunologically normal to date. Two of these children have been previously shown to accumulate and/or excrete only minimally increased amounts of deoxyadenosine, adenosine, and deoxy ATP (14, 15) consistent with the hypothesis that in these immunologically normal children, total body ADA is sufficient to prevent accumulation of lymphotoxic metabolites.

We have previously reported that in two of these immunologically normal, "partially" ADA-deficient children, adenosine deaminase activity is unstable at 56°C, but otherwise indistinguishable from normal ADA_1 1 with respect to electrophoretic mobility, Michaelis constant (K_m), and molecular weight (15). This diminished heat stability is consistent with a mutation at the ADA structural locus. We now report that lymphoid cells from one of these two previously studied partially ADA-deficient patients contain an enzyme with a more basic isoelectric point, while lymphoid line cells from a third partially ADA-deficient individual contain ADA with an increased anodal electrophoretic mobility and more acidic isoelectric point. Lymphoid cells from this third child contain markedly diminished ADA activity with only minimally decreased heat stability. In contrast, lymphoid cells from a fourth partially deficient child exhibit only somewhat diminished ADA activity with as yet no compelling evidence for a structural mutation.

METHODS

Cell lines. Lymphoid line cells were obtained from four children who lacked ADA activity in their erythrocytes but retained ADA activity in peripheral blood mononuclear cells. These cell lines were GM 2294 (14, 15), GM 3043 (11–13), GM 4396, and KS (16). Normal cell lines assayed in this study were GM 3201 and EBV13. (GM lines were obtained from the Camden National Institutes of Health [NIH] Genetic Mutation Repository). To minimize possible genetic drift in culture, cells of the GM series were frozen in multiple aliquots in liquid nitrogen so that experiments could be repeated either using cell lines regrown from the original stock held in this laboratory or from samples freshly obtained from the NIH Genetic Mutant Repository Cells were cultured in 10% fetal calf serum in RPMI 1640 and harvested 2 d after feeding, which usually represented the end of log phase of growth. Cells were centrifuged over a Hypaque-Ficoll gradient to eliminate nonviable cells. Extracts were prepared by freeze-thawing and sonication as previously described (15) and then centrifuged for 5 min in a Beckman microfuge (Beckman Instruments, Inc., Fullerton, CA). The removal of nonviable cells and the centrifugation of the extract resulted in a somewhat higher enzyme activity when expressed per milligram protein than we have previously reported. Cells were sometimes stored overnight at −70°C before assay.

Enzyme activity. ADA activity was determined essentially as previously described both by a "linked" spectrophotometric assay that measures the conversion of adenosine to uric acid, by addition of exogenous purine nucleoside phosphorylase and xanthine oxidase (15, 17) and by minor modifications of the assay measuring the conversion of [^{14}C]adenosine to inosine described by Coleman (15, 18). The conversion of the adenosine is measured in the presence and absence of EHNA, an inhibitor of the ADA isozymes deficient in ADA$^-$-SCID. The respective substrate concentrations used in the two assays in this study were 0.5 and 0.1 mM.

Enzyme activity and heat stability. Studies of heat stability of adenosine deaminase were performed as previously described (15, 19) except that adenosine deaminase was determined using the "linked" assay.

Electrophoresis and isoelectric focusing. Cell extracts were electrophoresed in starch gel and stained for adenosine deaminase activity as described by Spencer, Hopkinson, and Harris (20). Iodoacetate was added, when specified, to a final concentration of 10 mM and the cell extract incubated at 25°C for 1 h prior to electrophoresis. 2-Mercaptoethanol was added at a final concentration of 140 mM (21). Isoelectric focusing in acrylamide gels was performed essentially as previously described (22) using a gradient of pH 4.0 to 6.0 and enzyme activity visualized as for starch gels. Protein was determined by the method of Lowry (23).

RESULTS

ADA activity. ADA activity was easily detectable in all four cultured lymphoid line cells from the four individuals who lack ADA in their erythrocytes (Table I), but varied greatly in the four different lines. The highest ADA activity was found in lymphoid line cells derived from patient GM 3043 and the lowest in that of GM 4396 (~5% of normal). Cells from KS also showed substantial activity, while GM 2294 exhibited ~10–15% of normal activity. Similar results were obtained when ADA was determined by measuring the conversion of [^{14}C]adenosine to inosine (18), which is inhibitable by EHNA, an inhibitor of the major ADA isozye, ADA_1, lacking in children with ADA$^-$-SCID. All lines had markedly greater activity than that observed in lymphoid line cells from ADA$^-$-SCID.

Heat stability. Two of the lymphoid line cells (GM 3043 and GM 2294) showed marked instability of ADA at 56°C, confirming our previous report (15) (Table I, Fig. 1). ADA from GM 2294 has consistently been slightly less stable than that from GM 3043. The two other lymphoid line cells (GM 4396 and KS) had only somewhat diminished heat stability.

Electrophoretic mobility. Extracts of lymphoid line cells from all four partially ADA deficient individuals were electrophoresed in starch gel and stained for ADA activity. Normal ADA_1 from freshly prepared cell extracts exhibits a major primary band of enzyme activity and two equally spaced secondary isozymes that have increasing anodal mobility and diminishing intensity of activity relative to the primary isozyme (Fig. 2). Additionally, two common allozymes of the ADA_1 locus, ADA_1 1 and ADA_1 2, are seen in extracts from different individuals. The ADA_1 2 allozyme has diminished electrophoretic mobility relative to the ADA_1 1 allozyme (23). Several rare variants have also

TABLE I

Genetic Heterogeneity in Partial ADA Deficients

	ADA* Activity	$t_{1/2}$‡ Heat stability	Electrophoretic mobility§	Isoelectric point
Normals				
GM 3201	82.9±13	40.0	ADA-1	4.9
EBV13	57.7	—	—	—
Partial ADA deficients				
GM 4396	—	20.0	Increased	4.8
GM 2294	5.9	3.5	ADA-1	5.0
KS	25.0	25.0	ADA-1	—
GM 3043	41.9	6.5	ADA-1	4.9
Complete ADA deficients				
$n = 4$	0.22±0.13	—	—	—

* Nanomoles per milligram protein per minute in cultured lymphoid line cells spectrophotometric assay. Values for EHNA inhibitable deamination of [^{14}C]adenosine were: Nl GM 3201 = 57.7; GM 4396 = 2.3; GM 2294 = 4.4; KS = 8.1; and GM 3043 = 51.8.

‡ Time (minutes for 50% loss of activity at 56°C).

§ Anodal mobility compared to the normal ADA_1 allozyme, starch gel electrophoresis pH 6.5.

been described with altered electrophoretic anodal mobility relative to ADA_1 1 (24). Extracts of cells from three of the children (12, 15) showed a major band of enzyme activity with mobility indistinguishable from that of cells from an individual of normal ADA_1 1 phenotype. In contrast, the ADA activity of GM 4396 had an increased anodal mobility such that the major band coincided in mobility with the first of the two secondary bands of normal ADA_1 1 (Fig. 2).

The primary and secondary isozymes of ADA have been reported to contain a free sulfhydryl group available for combination with endogenous and exogenous sulfhydryl reagents (21). Thus, on "storage," normal ADA undergoes an increase in anodal mobility most easily explained by combination with oxidized gluta-

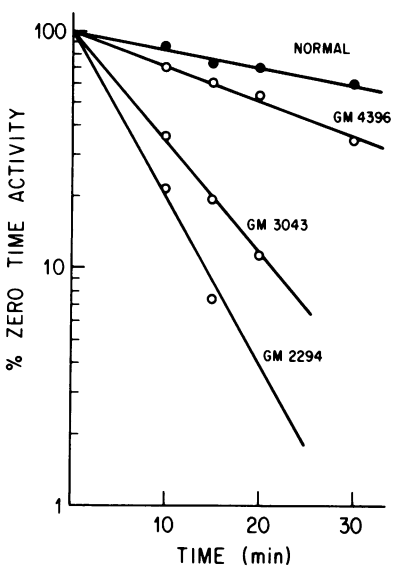

FIGURE 1 Determination of heat stability of ADA in lymphoid line cells from four partially ADA-deficient children vs. normal. Cells were grown and harvested as described in Methods and suspended at a protein concentration of >1 mg/ml in 0.15 M NaCl, 0.05 M Tris, pH 7.5. Equal volumes of cell lysate at equal protein concentrations were incubated for the indicated time intervals at 56°C. After removal to ice, aliquots were assayed by the spectrophotometric assay.

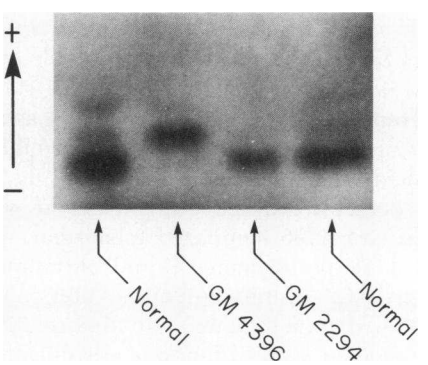

FIGURE 2 Mobility in starch gel of ADA from two partial ADA deficients compared with normal. Cell extracts were electrophoresed at pH 6.5 and then enzyme activity detected as previously described (20). GM 2294 as well as KS and GM 3043 (not shown in this photograph) also had an anodal electrophoretic mobility indistinguishable from the normal ADA_1 1 lymphoid line in channels 1 and 4. GM 4396 shows an increased anodal electrophoretic mobility.

thione present in the cell extract. This increased anodal mobility following storage can be reversed in stored extracts by addition of 2-mercaptoethanol and induced in fresh extracts by addition of iodoacetate (21). When freshly prepared extracts of lymphoid cells were incubated with 2-mercaptoethanol prior to electrophoresis, no change in mobility of the ADA was seen, and the ADA from GM 4396 retained an increased anodal mobility. When extracts were incubated with iodoacetate, both ADA from normals and the ADA from each of the partial deficients showed an increased anodal mobility such that the major band of the ADA activity now migrated with the mobility of the first of the two minor, secondary bands of enzyme activity. Under these conditions, the ADA from GM 4396 retained the same increased anodal mobility relative to normal as it exhibited in the absence of iodoacetate.

Alterations in electrophoretic mobility in starch gel could in theory reflect a difference in molecular weight rather than charge, since, to some extent, starch gel acts as a molecular sieve. Additionally, electrophoretic mobility reflects net charge and may be influenced by factors additional to isoelectric point. We therefore determined the isoelectric point of the enzyme from three of the partial deficients compared with normal by isoelectric focusing in horizontal flat bed acrylamide gels and staining for enzyme activity. The major band of ADA from GM 4396 had a lower more acidic isoelectric point than that from normal (4.8 vs. 4.9), consistent with its more rapid migration to the anode in starch gel electrophoresis at pH 6.5. This isoelectric point of GM 4396 was identical with that of the normal secondary isozyme of normal ADA, as would be expected from its co-migration with the normal secondary isozyme on electrophoresis in starch gel. Unexpectedly, the enzyme from GM 2294 had a higher, more basic than normal isoelectric point (pI = 5.0), despite the fact that it was indistinguishable from normal ADA_1 1 following electrophoresis in starch gel. Enzyme from GM 3403 had a pI as well as an electrophoretic mobility in starch gel that could not be distinguished from that of normal ADA_1 1. We also could not detect a difference in molecular weight of ADA from GM 4396 compared to normal, as determined by high performance liquid chromatography on a Waters I-125 protein column (Waters Associates, Milford, MA, data not shown). No abnormality in K_m could be detected, using adenosine as substrate. These parameters have previously been found to be normal for GM 2294 and GM 3043 (15).

DISCUSSION

We have studied lymphoid line cells derived from four immunologically normal children who, although they lack ADA in their erythrocytes, have easily detectable, albeit variable amounts of ADA in their lymphoid cells. These amounts of ADA activity are at least 10-fold greater than that found in lymphoid line cells of children who are deficient for ADA in both erythrocytes and lymphoid line cells and suffer from ADA^--SCID (Table I).

Several different mechanisms could result in genetic absence of ADA in erythrocytes but presence in lymphoid cells. These include: (*a*) the presence of two different ADA isozymes controlled by separate genetic loci in the two types of cells; (*b*) a mutation at a regulatory locus controlling expression of ADA in erythrocytes of (*c*) mutation(s) at the structural locus resulting in an unstable enzyme. The first possibility is unlikely since several studies have previously documented that a single genetic locus on chromosome 20 codes for the major ADA catalytic activity in all tissues including both erythrocytes and lymphocytes (25, 26). Several pieces of evidence do support the existence of a regulatory locus controlling expression of ADA. Thus, Valentine has reported autosomal dominant inheritance of a 50-fold increase in ADA activity of erythrocytes, but normal activity in lymphocytes and fibroblasts (27, 28). Siciliano et al. (29) have reported re-expression of human ADA in hybrids between a human tumor line not expressing ADA and a murine cell line. A mutation at a structural locus coding for an unstable enzyme could result in absent or markedly diminished activity in erythrocytes. Thus, erythrocytes are incapable of synthesizing new protein and have a relatively long life span, during which time an unstable enzyme could be expected to diminish markedly in activity.

The four partially ADA-deficient children studied here would appear to have four different mutations, at least three of which would appear to be at the structural locus for ADA. Thus, although lymphoid line cells GM 2294 and GM 4396 both have very low ADA activity (10 and 5% of normal), the ADA in the two cell lines differs qualitatively. Thus, GM 4396 has an enzyme with increased anodal electrophoretic mobility, decreased, more acidic isoelectric point and relatively normal heat stability, while GM 2294 has an enzyme with electrophoretic mobility not detectably different from normal but increased, more basic isoelectric point and markedly diminished heat stability. The other two individuals (GM 3043 and KS) have higher ADA activity with GM 3043 having activity that overlaps with normal. These two can be distinguished qualitatively by the fact that GM 3043 has an enzyme with markedly diminished heat stability (albeit consistently slightly more stable than that from GM 2294). Thus, three of these individuals would appear to have three different mutations at a structural

locus for ADA that can be qualitatively differentiated one from the other as having (*a*) diminished, more acidic isoelectric point (GM 4396), (*b*) increased, more basic isoelectric point and diminished heat stability (GM 2294), and (*c*) normal isoelectric point and diminished heat stability (GM 3043). The enzyme from KS did not exhibit any striking qualitative abnormality and therefore a mutation at a regulatory locus must still be considered.

One of the rare variants of ADA (ADA^5) found in screening of normal populations has an increased anodal mobility similar to that observed in GM 4396. Although no individual homozygous for ADA^5 has been described, individuals heterozygous for $ADA^{5\text{-}1}$ would appear to have exhibited normal ADA in erythrocytes and equal expression of the ADA^5 and ADA^1 allele (24). However, we cannot rule out the possibility that GM 4396 is homozygous for ADA^5 and additionally has a mutation at a regulatory locus. However, the rarity of each of these events alone (i.e., homozygosity for ADA^5 and partial ADA deficiency) makes the occurrence of the two together highly unlikely.

In any study using cultured cells, the possibility for "genetic drift" in culture must be considered. In the three cell lines that demonstrated abnormalities of ADA, we have been able to demonstrate the same abnormalities using cultures expanded from multiple independent small aliquots of frozen cells. Such precautions were not possible with cells from KS, but in this case, no qualitative abnormality has been seen. Additionally, we have had multiple ADA normal B cell lines in continuous culture for 2–3-yr periods and have never observed any alterations in ADA electrophoretic mobility or isoelectric point during that period.

Of interest is that at least two of these partially ADA-deficient individuals were black (GM 3043; GM 2294), while a third comes from the Mediterranean basin (KS). It is therefore tempting to speculate that a partial ADA gene might confer some advantage against intraerythrocytic parasites such as malaria and therefore be more prevalent in such areas.

To date, none of these children have had significant immunologic abnormalities. We have also examined three of these individuals and four additional newly discovered partial ADA-deficients for purine metabolites that are markedly elevated in children with the complete enzyme deficiency and immunodeficiency and have found only minimal albeit detectable elevations of these metabolites (4, 14, and unpublished observations). However, the extent of accumulation of abnormal metabolites that can be tolerated over many years by the lymphoid system is at the moment unknown. It is to be expected that genetic heterogeneity will result in a child with a partial enzyme deficiency severe enough to result both in accumulation of metabolites and some degree of immunologic abnormality.

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