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Heterogeneity for Adenosine Deaminase Deficiency: Expression of the Enzyme in Cultured Skin Fibroblasts and Amniotic Fluid Cells

SHI-HAN CHEN,[1] C. RONALD SCOTT,[1] AND KATHRYN R. SWEDBERG[1]

INTRODUCTION

Combined immunodeficiency disease is a serious and usually fatal genetic disease of childhood. Affected children lack both cellular and humoral immunity and normally die prior to 1 year of age from viral or bacterial infections [1]. Autosomal recessive and X-linked modes of inheritance have each been implicated in the pathogenesis of the disease. The first biochemical clue that the disorder was caused by an enzyme deficiency was the report by Giblett et al. [2] of a deficiency of adenosine deaminase (ADA) in the red cells of two children with combined immunodeficiency disease. Studies have confirmed that some, but not all, children with the disease have ADA deficiency [3], that the allele for ADA is transmitted on an autosome [4–6], and that gene carriers for the enzyme deficiency are detectable [7].

In this communication we (1) report the quantitative and qualitative expression of ADA in cultured skin fibroblasts from normal individuals as well as heterozygotes and homozygotes for the ADA deficiency allele; (2) present evidence for genetic heterogeniety of ADA deficiency; and (3) offer data on the expression of ADA in cultured amniotic fluid cells.

MATERIALS AND METHODS

Skin biopsies were obtained from 26 controls and two children with ADA deficiency and combined immunodeficiency disease. Clinical data on one of the children with ADA deficiency (Mi. Re.) have been summarized previously [3], and clinical information on the other (Ja. Da.) will be independently reported by other authors. Two obligate heterozygote cell lines were established from the parents of another child with ADA deficiency and combined immunodeficiency disease [7]. Twenty amniotic fluid specimens were obtained from either therapeutic abortions performed for social reasons or from amniocenteses performed for chromosomal evaluations. The gestational age at the time the cells were obtained varied from 69 to 162 days.

The cells were established in Dulbecco-Vogt medium with 20% fetal calf serum containing penicillin and streptomycin. The cultured cells were harvested after 14 days with

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[1] Department of Pediatrics, University of Washington School of Medicine, Seattle, Washington 98195.

the use of 0.05% trypsin. After centrifugation, the cells were suspended in 5 vol distilled water followed by freezing and thawing three times in a dry ice–acetone bath. The homogenates were centrifuged at 10,800 *g* for 20 min to remove cell debris; the clear supernatants (about 5 mg protein/ml) were used for assays.

Adenosine deaminase activity in the cell extracts was determined by measuring the rate of absorbance change at 293 nm using a modification of the method described by Hopkinson et al. [8]. Each milliliter of the reaction mixture contained 0.06 M phosphate buffer (pH 7.5), 0.15 mM adenosine, 0.05 U nucleoside phosphorylase (Sigma Co., Saint Louis), and approximately 0.1 U xanthine oxidase (Sigma Co.). The reaction was initiated by adding 20 μl of the homogenate to 2 ml of reaction mixture at 25°C. An increase of the optical density at 293 nm was recorded against a blank for 10–20 min using a Gilford 2400 S spectrophotometer with the full range of the recorder adjusted to 0.2 absorbance units. One unit of ADA activity was defined as the deamination of 1 μmol adenosine/min. Specific activity was expressed as units per gram of protein. Protein was estimated by the method of Lowry et al. [9].

Starch gel electrophoresis was performed vertically at 5 V/cm for 18 hr at 4°C in sodium phosphate buffer, pH 7.0 (0.005 M for gels and 0.1 M for trays). The gels were stained for ADA activity according to the method described by Spencer et al. [4].

RESULTS

The isozyme pattern observed from the 26 normal fibroblast lines revealed the expected isozymes for red cell ADA and a slower migrating tissue-specific ADA. Twenty-five of the cell lines had an isozyme pattern consistent with ADA 1, and one cell line had an ADA 1-2 pattern. In each instance, the position of the tissue-specific ADA was the same.

TABLE 1

SPECIFIC ACTIVITY AND PHENOTYPE OF ADA IN CULTURED SKIN FIBROBLASTS FROM CONTROLS, PATIENTS WITH COMBINED IMMUNODEFICIENCY DISEASE AND ADA DEFICIENCY, AND FROM OBLIGATE HETEROZYGOTES FOR ADA DEFICIENCY

	Specific Activity (U/g Protein)	ADA Phenotype
Cultured skin fibroblasts:		
Controls ($N = 26$)	14.6 ± 6.8* (6.4–34.0)	ADA 1, ADA 2-1, TS
Obligate heterozygotes for ADA deficiency:		
Ch. Fi.	9.2, 7.9, 6.0	ADA 1, TS
Da. Fi.	8.6, 5.1, 7.8, 6.3	ADA 1, TS
Patients with ADA deficiency and combined immunodeficiency disease:		
Mi. Re.	0.13, 0, 0, 0	TS†
Ja. Da.	1.3, 1.9	TS‡
Amniotic fluid cells ($N = 20$)	14.3 ± 6.7* (6.5–31.4)	ADA 1, ADA 2-1, TS

NOTE.—TS = tissue-specific isozyme band.

* ± 1 SD.

† An extremely weak band observed when a threefold increase in protein was applied to the gel.

‡ Weak band that migrated slightly faster than normal.

The isozyme pattern from the 20 amniotic fluid cell cultures revealed a pattern similar to that observed in the skin and consisted of both red cell and tissue-specific bands. Nineteen had an ADA 1 isozyme pattern, and one cell line an ADA 2-1 pattern.

The specific activity of ADA within the skin fibroblast cultures was quite variable. The range varied between 6.4 and 34 U/g protein with a mean of 14.6 U/g protein ± 6.8 (SD). The ADA activity from the 20 amniotic fluid cell cultures was found to range from 6.5 to 31.4 U/g protein with a mean of 14.3 U/g protein ± 6.7 (SD). The two obligate heterozygotes had ADA 1 phenotypes and mean specific activities of 7.0 and 7.7 U/g protein. These values were in the low normal range but were greater than the lowest values obtained from cultures of normal skin fibroblasts.

Cultured skin fibroblasts from an infant (Mi. Re.) with combined immunodeficiency disease had ADA activity less than 1% of normal. At the usual protein concentration used for starch gel electrophoresis (5 mg/ml), no detectable ADA activity could be observed. However, by using a more concentrated preparation (15 mg/ml) of the extract and prolonging the incubation of the gel, a very weak ADA band was seen in the region of the tissue-specific ADA. In the cultured cells obtained from another child (Ja. Da.) with ADA deficiency, definite residual activity could be measured and was approximately 1.6 U/g protein, or 10% of the mean of normal fibroblasts. On starch gel electrophoresis, the mutant enzyme migrated slightly faster toward the anode than the tissue-specific enzyme from normal cells (fig. 1).

Preliminary studies of the unpurified variant enzyme indicate a decrease in the maximum reaction velocity, a pH optimum between 6.5 and 7.5 in 0.05 phosphate buffer, and a K_m of 1.9×10^{-5}M for adenosine. The latter two properties are similar to that found for unpurified ADA in normal skin fibroblasts (optimum pH 6.5–7.5, $K_m = 2 \times 10^{-5}$M). The greatest difference between the variant and normal enzyme was in heat denaturation: the enzyme from normal fibroblasts lost 80% of activity within 30 min of incubation at 65°C, while the variant enzyme demonstrated no inactivation under these conditions.

DISCUSSION

Adenosine deaminase deficiency was first recognized by Giblett and co-workers [2] who reported an association between combined immunodeficiency disease of childhood and the absence of ADA in red cell hemolysates from two unrelated children. Because each child presented with the clinical syndrome of combined immunodeficiency disease, it was suggested that the enzyme deficiency within the lymphocytes might be causally related to the failure of lymphocyte function. Alternatively, they suggested that a partial chromosomal deletion might have involved both the red cell ADA locus and a closely linked locus for immunoresponsiveness. Subsequent to their report, at least 14 cases have been identified with ADA deficiency [3]. Each case except one was detected because the subject was afflicted with clinical and physiological stigmata of combined immunodeficiency disease.

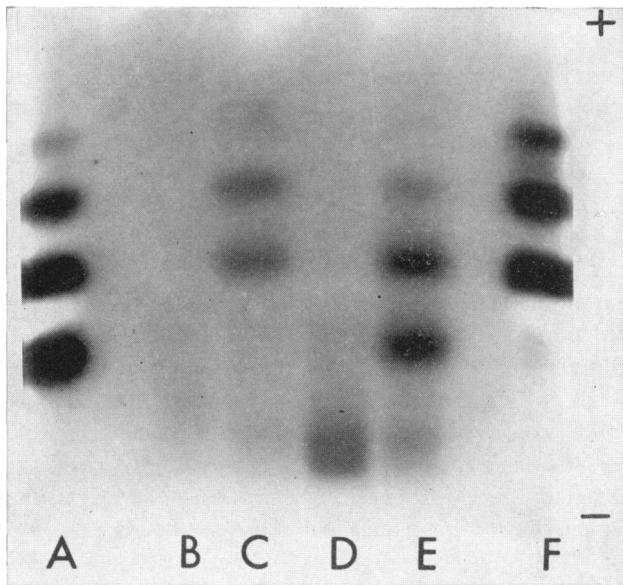

FIG. 1.—Isozyme patterns of ADA in cultured cells. *Channels A* and *F,* ADA 2-1 and ADA 1 phenotypes from erythrocytes; *channel B,* extract from the child (Ja. Da.) with 10% ADA activity showing only a faintly staining ADA band migrating slightly faster than the usual tissue-specific band; *channel C,* skin fibroblast culture showing the principal isozyme band of ADA 1 and a less intensely staining tissue-specific band; *channel D,* skin fibroblast culture showing predominantly the tissue-specific band; *channel E,* ADA 2-1 and a slower migrating tissue-specific band found in cultured amniotic fluid cells.

The expression of ADA in cultured fibroblasts is of major importance because of its potential for genetic studies and for the prenatal detection of ADA deficiency. Edwards and co-workers [10] have previously demonstrated that skin fibroblasts contain isozyme patterns similar to those found in erythrocytes and have an additional tissue-specific ADA. They suggested that within the fibroblast there are at least two separate loci controlling the expression of adenosine deaminase.

Akedo et al. [11] and Nishihara et al. [12] have purified adenosine deaminase from human lung and stomach and have determined that two major molecular species of ADA exist: one of mol wt 47,000 and one of mol wt 230,000. They believe the primary gene product is the low molecular weight species and that a "tissue conversion factor" exists which aggregates the low molecular weight protein to the large molecular weight enzyme. This "conversion factor" has been isolated and studied by Nishihara et al. [12]. The absence of the red cell ADA isozymes and a significant deficiency of the tissue-specific ADA from the fibroblast cultures of the two patients with combined immunodeficiency disease in this report, as well as the absence of both forms of the enzyme from the fibroblasts of a similarly affected patient [13], support the concept of a single genetic locus for ADA. Hirschhorn et al. [13] were able to offer evidence that ADA in most, if not all, tissues is under the control of a single gene by showing that kidney, liver, intestine, spleen, heart, and

muscle of an affected child contained neither the red cell isozymes nor the tissue-specific isozymes of ADA corresponding to each of the organ types.

Preliminary reports of ADA activity in amniotic fluid cells were offered by Chen and Scott [14] and by Hirschhorn and Beratis [15] who suggested that the specific activity of the enzyme in amniotic fluid cells may be similar to that seen in cultured skin fibroblasts. The present study shows that amniotic fluid cells have isozyme patterns and ADA specific activities closely resembling values obtained from cultured skin fibroblasts. There was no evidence that the ADA in amniotic fluid cells was different from the enzyme present in skin fibroblasts. This implies that the characterization of ADA in amniotic fluid cells may be used diagnostically for the prenatal evaluation of fetuses at risk for ADA deficiency.

We have evaluated one fetus at 16 weeks of gestation who was at risk for ADA deficiency. The cultured amniotic fluid cells revealed ADA activity of 11.4 and 11.1 U/g protein from two separate culture flasks and a phenotype of ADA 1. Red cells obtained at birth from the umbilical cord had an ADA 1 phenotype and activity of 31.6 U/g hemoglobin, well within our normal range. There is no clinical or laboratory evidence of combined immunodeficiency disease at 6 months of age.

The range of ADA values which exists in fibroblasts and amniotic fluid cells may not allow their use for accurate heterozygote detection. However, we have reported that heterozygotes within a family with ADA deficiency may be determined with an accuracy of 90% by the quantitation of ADA erythrocyte hemolysates [7].

The single clinical exception to the association of combined immunodeficiency disease with ADA deficiency is the case of a young native bushman from southwest Africa [16]. This male child is apparently healthy in spite of the absence of ADA in his erythrocytes as demonstrated by starch gel electrophoresis. He may represent the first clinical evidence for genetic heterogeneity at the ADA locus. Jenkins [16] suggested that the child may have an unstable form of the enzyme with sufficient residual activity in leukocytes and other tissues for normal function, but the enzyme may be effectively absent in the anucleate mature circulating red cell.

The activity of ADA observed in the two children with combined immunodeficiency disease was less than 1% and 10% of the mean of normal fibroblasts. This enzymatic evidence is compatible with the existence of genetic heterogeneity in children with ADA deficiency. Preliminary characterization of the residual enzyme from fibroblast cultures of the child (Ja. Da.) with 10% activity indicates differences in electrophoretic mobility, heat stability, and maximum reaction velocity from the normal enzyme. A similar type of mutation in another patient has been presented by Hirschhorn et al. [17].

The finding of residual enzyme activity in the fibroblasts of the children with ADA deficiency precludes the previous suggestion of a chromosomal deletion involving the ADA locus and a nearby immunoresponsive locus closely linked to the HL-A locus. Recently, however, Creagan et al. [5] have shown that ADA is on chromosome 20 and that the locus for HL-A is syntenic with the phosphoglucomutase$_3$ locus which is assigned to chromosome 6. Thus linkage between the loci for HL-A and ADA is incompatible.

SUMMARY

Adenosine deaminase (ADA) could be quantitated and the isozyme pattern characterized in cultured amniotic fluid cells. In 20 amniotic fluid cell cultures the mean specific activity was 14.3 U/g protein ± 6.7 (SD) and compared favorably with values of 14.6 U/g protein ± 6.8 (SD) observed in 26 cultures of skin fibroblasts. In cultures of skin fibroblasts established from two obligate heterozygotes for ADA deficiency, the specific activity of ADA was 7.0 and 7.7 U/g protein.

The ADA isozyme pattern that existed in cultures of amniotic fluid cells was the same as that observed in cultured skin fibroblasts. This identification of the same apparent enzyme may permit the prenatal diagnosis of that form of combined immunodeficiency disease caused by ADA deficiency.

Residual enzyme activity of less than 1% and 10% of the mean of normal fibroblasts could be measured in cultured fibroblasts from two unrelated children with ADA deficiency and combined immunodeficiency disease. The tissue-specific enzyme from cultured skin fibroblasts from the child with 10% residual activity had a faster electrophoretic mobility and greater heat stability than normal ADA. This enzymatic evidence indicates that at least two mutant alleles exist at the locus for ADA which predispose to combined immunodeficiency disease when present in the homozygous state.

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