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Immunology

Characterization of the residual adenosine deaminating activity in the spleen of a patient with combined immunodeficiency disease and adenosine deaminase deficiency

(enzyme deficiency)

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ABSTRACT **A number of infants with an autosomal recessive form of combined immunodeficiency disease also lack adenosine deaminase (adenosine aminohydrolase; EC 3.5.4.4) activity in their erythrocytes. Other tissues from these infants contain only a few percent of the adenosine-deaminating activity present in corresponding normal tissue. The residual adenosine-deaminating activity in extracts from the spleen of a combined immunodeficient, adenosine deaminase-deficient patient was compared with adenosine deaminase from normal spleen. Affinity and immunoadsorbant column chromatography revealed distinct differences between the adenosine-deaminating activity in the patient's spleen and adenosine deaminase from normal spleen. The point of maximum activity and general configuration of the pH optimum curves were also different. *erythro*-9-(2-Hydroxyl-3-nonyl)adenine, a potent inhibitor of adenosine deaminase from normal spleen, had relatively little effect on the activity from the patient's spleen. In contrast, adenine was a better inhibitor of the activity in the patient's spleen than it was of the enzyme from normal tissue. An adenosine-deaminating activity with the same characteristics and specific activity as that in the patient's spleen was also isolated from normal spleen. These results suggest that the adenosine-deaminating activity in the spleen of this patient is not due to a mutant form of adenosine deaminase.**

Adenosine deaminase (adenosine aminohydrolase; EC 3.5.4.4) from human tissue is heterogeneous with respect to molecular weight (1–4). The enzyme in erythrocytes is a single polypeptide chain of molecular weight 36,000–38,000 (5, 6). In other tissue, adenosine deaminase is present either as a complex of molecular weight 230,000–440,000, depending on the source, or as a mixture of this larger form and a low molecular weight form of approximately the same size as the enzyme from erythrocytes. Current evidence suggests that erythrocyte adenosine deaminase and the catalytically active subunit of the higher molecular weight forms are products of the same gene (7, 8).

A number of infants with an autosomal recessive form of combined immunodeficiency disease also lack adenosine deaminase activity in their erythrocytes (9–11). Other tissues from these infants have some adenosine-deaminating activity, but only a fraction of the level in corresponding normal tissue (12–14). In view of the suggested genetic relationship between the different molecular forms of adenosine deaminase (7, 8), it is difficult to reconcile the presence of adenosine-deaminating activity in tissues such as spleen with the complete lack of activity in erythocytes. Van der Weyden *et al.* (14) have characterized the adenosine-deaminating activity in extracts from the spleen of a combined immunodeficient patient and suggest that it is due to a mutant form of adenosine deaminase.

We have compared the residual adenosine-deaminating activity in the spleen of a patient with combined immunodeficiency disease with adenosine deaminase from normal spleen. Significant differences were found to exist between the enzyme from normal tissue and the activity in patient spleen. In addition, an activity similar to that in patient spleen was isolated from normal spleen. These results suggest that the activity in the spleen of this combined immunodeficient patient is not a mutant form of adenosine deaminase.

EXPERIMENTAL

Materials. *erythro*-9-(2-Hydroxyl-3-nonyl)adenine (EHNA) was a gift from G. B. Elion of Burroughs Wellcome Co., Research Triangle Park, NC. Tetrahydrouridine was a gift from F. Maley, Division of Laboratories and Research, New York State Department of Health, Albany, NY. Normal spleen tissue was provided by the Institute of Forensic Medicine of New York University and the City of New York, 520 First Avenue, New York, NY. Normal tissue was stored at −20° for 12 months.

Combined Immunodeficient, Adenosine Deaminase-Deficient Patient. This child was admitted to the hospital at 4 months of age because of recurrent infections. She had progressive deterioration of the immune functions ascribed to bone marrow- and thymus-derived lymphocytes during the first few months after admission, and a diagnosis of combined immunodeficiency was made. There was no detectable adenosine deaminase activity in her erythrocytes or plasma (9). Her parents had less than normal levels of adenosine deaminase in their erythrocytes. Transplantation of maternal bone marrow reconstituted immune responses ascribed to bone marrow-derived lymphocytes, but those ascribed to thymus-derived lymphocytes remained profoundly abnormal (H.J. Meuwissen, unpublished data). She contracted a fatal cytomegalovirus infection at the age of 2.5 years.

Purification of Normal Spleen Adenosine Deaminase. Adenosine deaminase from normal human spleen was purified by a combination of previously described methods for isolation of human erythrocyte (5) and kidney (8) adenosine deaminase. Purification of the low molecular weight form of adenosine deaminase resolved by gel filtration was completed by affinity column chromatography through a gel bed of Sepharose 6B to which adenosine had been attached as the ligand (5). The enzyme migrated as a single band with an apparent molecular weight of 36,000 when electrophoresed on 9% polyacrylamide in the presence of sodium dodecyl sulfate (15). Purification of

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Abbreviation: EHNA, *erythro*-9-(2-hydroxyl-3-nonyl)adenine.

the high molecular weight form of adenosine deaminase, after gel filtration, was completed by immunoadsorbant column chromatography through a gel bed of Sepharose 4B to which IgG antibody to erythrocyte adenosine deaminase had been attached (8). The yield of high molecular weight enzyme was 4%, with a specific activity of 8 units/mg of protein.

Crude Extracts. Spleen tissue, usually 0.5–1.0 g, was homogenized in 0.1 M potassium phosphate (pH 7.4)/0.02% NaN_3(1/2, wt/vol). The homogenate was centrifuged for 10 min at 27,000 × *g*. The supernatant was recovered and dialyzed against 1 liter of 0.1 M potassium phosphate (pH 7.4)/0.02% NaN_3 overnight at 3°. The specific adenosine-deaminating activity in extracts from the patient's spleen and from two normal spleens was 0.7×10^{-3}, 50×10^{-3}, and 77×10^{-3} units/mg of protein, respectively.

Adenosine-Deaminating Activity. Adenosine-deaminating activity was measured by the method of O'Donovan (16) as previously described (5). Adenosine-deaminating activities of purified spleen adenosine deaminase, crude extracts from normal and patient's spleen, and the nonimmunoreactive aminohydrolase isolated from normal spleen were also determined by the method of Hopkinson *et al.* (17). One unit of adenosine-deaminating activity is defined as the amount of enzyme required to hydrolyze 1 μmol of adenosine per min by the method of O'Donovan.

Protein. Protein concentration was estimated by the method of Lowry *et al.* (18), with bovine serum albumin as the comparative standard.

Estimation of Molecular Weight. Molecular weight was estimated by gel filtration through a calibrated column (1.5 × 80 cm) of Sephadex G-200 according to the method of Andrews (19).

pH Optima. The mixed buffer used to study activity as a function of pH contained 0.125 M sodium citrate, 0.125 M sodium phosphate, and 0.018 M sodium borate. Adjustments in pH were made with 10 M NaOH.

Tissue Staining. Thin sections of the patient's spleen were stained for bacteria by the method of Brown and Brenn (20).

RESULTS

Immunoadsorbant and Affinity Column Chromatography. Crossreaction of adenosine deaminase from normal spleen with antibody to adenosine deaminase isolated from erythrocytes was demonstrated by immunoadsorbant column chromatography. A crude extract from normal spleen was passed through a column of gel to which IgG antibody to erythrocyte adenosine deaminase had been attached (Fig. 1*A*). Of the 0.6 unit of adenosine-deaminating activity applied, 95% was retained by the column. Most of the nonadsorbed activity was associated with particulate matter, which was separated from soluble proteins and other small molecules by the molecular sieving effect of the gel. Specificity of binding was established by passing the same amount of extract from normal spleen through a control column of bed material prepared with IgG antibody to superoxide dismutase (5). Both the high and low molecular weight forms of adenosine deaminase present in the soluble protein fraction from normal spleen passed through the control column (Fig. 1*B*). Recovery of activity was 75%. A similar extract from the patient's spleen was also passed through these columns. Recovery of activity was 45% from the anti-adenosine deaminase column and 63% from the anti-superoxide dismutase column.

Extracts from normal and patient's spleen were also compared by affinity column chromatography. Passage of an extract from normal spleen through a column of epoxy-activated

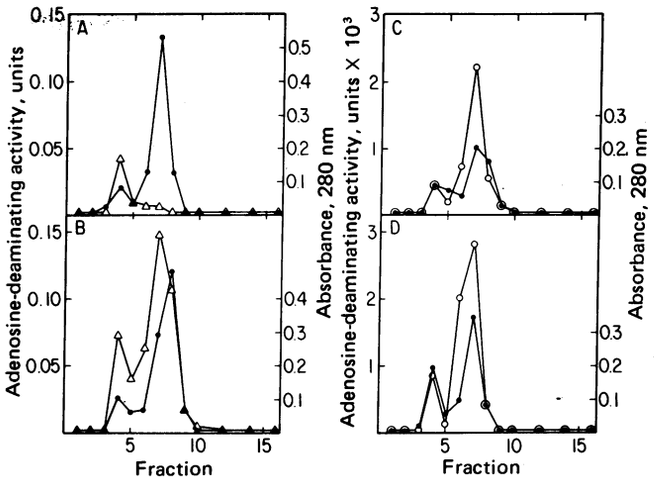

FIG. 1. Column chromatography through bed material prepared with rabbit IgG antibody to adenosine deaminase or superoxide dismutase, both isolated from human erythrocytes. Crude extracts (0.5 ml) from normal and patient's spleen were passed through immunoadsorbant columns (0.9 × 11 cm) at a rate of 1.5 ml/hr. Fractions of 1 ml were collected. Adenosine-deaminating activity (△, ○) is expressed in units/fraction. Protein (●) was measured at 280 nm after 25-μl aliquots of the fractions were diluted with 1 ml of water. (*A*) Normal spleen (△), anti-adenosine deaminase column; (*B*) normal spleen (△), anti-superoxide dismutase column; (*C*) patient's spleen (○), anti-adenosine deaminase column; (*D*) patient's spleen (○), anti-superoxide dismutase column.

Sepharose 6B to which adenosine had been attached as the ligand resolved the adenosine-deaminating activity into two components. Using partially purified preparations from normal spleen we found that low molecular weight spleen adenosine deaminase was retarded by the column, but the high molecular weight form was not. The adenosine-deaminating activity in patient spleen extract was not retarded by the column.

Isolation of a Nonimmunoreactive Adenosine-Deaminating Activity from Normal Spleen. As shown above, passage through an anti-adenosine deaminase immunoadsorbant column removed all but 1% of the adenosine-deaminating activity in the soluble protein fraction of an extract from normal spleen. To determine if the column had been overloaded, we prepared an additional extract from normal spleen and passed it through a fresh column of anti-adenosine deaminase bed material. An aliquot of the soluble protein fraction from this elution was applied to a second column of fresh anti-adenosine deaminase bed material. The recovery of activity from this column was 93%. Based on the amount of adenosine-deaminating activity retained by the first column, the second column should have had a binding capacity for adenosine deaminase at least 100 times beyond what was applied. Similar results were obtained with extracts from a second normal spleen. The nonimmunoreactive aminohydrolase represents about 1–2% of the total adenosine-deaminating activity of normal spleen. Based on these results, the aminohydrolase from normal spleen has about the same specific activity as the aminohydrolase in the patient's spleen.

Effect of EHNA. The effect of EHNA, a potent inhibitor of adenosine deaminase (21), on the adenosine-deaminating activity in patient spleen and on purified adenosine deaminase from normal spleen was examined. The purified adenosine deaminase referred to in this and subsequent studies was the high molecular weight form of the enzyme isolated from normal spleen. This was used because the patient's spleen apparently does not contain the lower molecular weight form of the

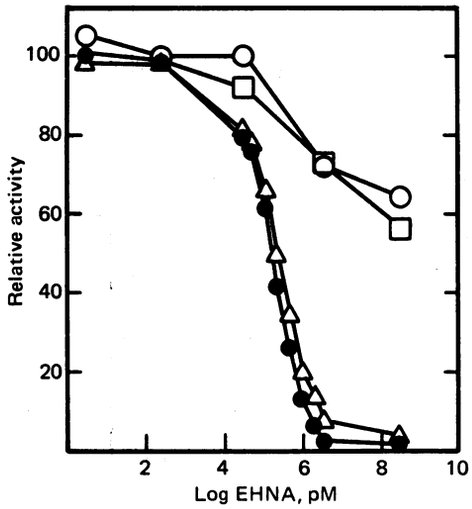

FIG. 2. Inhibition of adenosine deaminase by EHNA. Appropriate amounts of normal spleen extract (△), patient's spleen (○), purified spleen adenosine deaminase (●), and nonimmunoreactive aminohydrolase from normal spleen (□) were combined with increasing amounts of EHNA in 75 μl of assay buffer. The solutions were thoroughly mixed and then incubated for 30 min at 37°. After they were cooled in ice, 25 μl of 4 mM adenosine was added to each reaction mixture followed by incubation for 90 min at 37°. The mixtures were cooled in ice and the reagents for determination of ammonia added. The concentration of EHNA shown in the figure is that present in the mixture after addition of adenosine. Adenosine-deaminating activity is expressed as a percentage of the activity present when EHNA was not added.

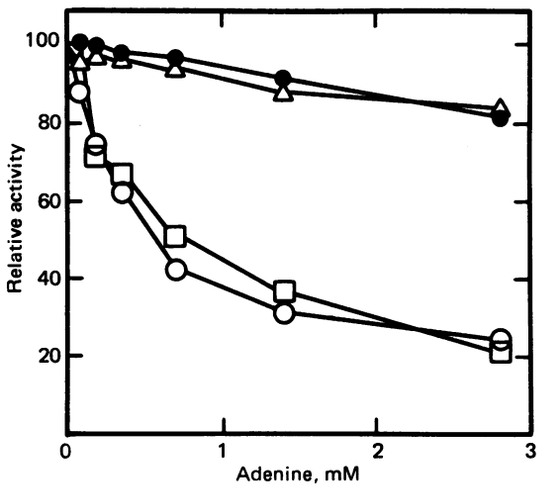

FIG. 3. Inhibition of adenosine-deaminating activity by adenine. Appropriate amounts of normal spleen extract (△), patient's spleen (○), purified spleen adenosine deaminase (●), and nonimmunoreactive aminohydrolase isolated from normal spleen (□) were combined with increasing amounts of adenine in 75 μl of assay buffer. After the mixture was cooled in ice, 25 μl of 4 mM adenosine was added to each reaction mixture followed by incubation for 60 min at 37°. The mixtures were cooled in ice and the reagents for determination of ammonia added. Adenine is shown as a final concentration after the addition of adenosine. Adenosine-deaminating activity is expressed as a percentage of the activity present when adenine was not added.

enzyme. At 3.5 μM EHNA, 98% of the activity of purified adenosine deaminase was inhibited while the activity from patient spleen was reduced by 34% (Fig. 2). Raising the concentration of EHNA to 350 μM increased the inhibition of the patient's spleen adenosine-deaminating activity by an additional 8%. EHNA was also a relatively poor inhibitor of the nonimmunoreactive aminohydrolase isolated from normal spleen (Fig. 2). The failure of 350 μM EHNA to completely inhibit deamination of adenosine by the crude extract from normal spleen was probably due to the presence of the nonimmunoreactive aminohydrolase.

Effect of Adenine. A number of purine and pyrimidine bases and nucleosides were tested to see if a naturally occurring substrate other than adenosine could be found for the aminohydrolase in the patient's spleen. Adenine, guanine, guanosine, cytosine, and cytidine did not act as substrates. Although not a substrate, adenine inhibited the deaminating activity in extracts of the patient's spleen and the nonimmunoreactive aminohydrolase from normal spleen (Fig. 3). In contrast, adenine had little effect on crude or purified preparations of spleen adenosine deaminase (Fig. 3).

Effect of pH. The effect of pH on deamination of adenosine by adenosine deaminase and on the activity in the patient's spleen extracts were compared. Typical of adenosine deaminase from other human tissue, the enzyme from normal spleen hydrolyzed adenosine over a broad range of pH with an optimum near pH 7.0. Relative to adenosine deaminase the activity from the patient's spleen increased more gradually from pH 4.5, reaching a maximum at pH 6.5, and then fell off more sharply above pH 7. The activity profile of the nonimmunoreactive aminohydrolase isolated from normal spleen was much the same as that from the patient's spleen.

Additional Studies on Nonimmunoreactive Aminohydrolase from Normal Spleen. The nonimmunoreactive aminohydrolase eluted between the high and low molecular weight forms of adenosine deaminase from normal spleen when subjected to gel filtration through Sephadex G-200 (Fig. 4). The peak of aminohydrolase activity was detected by inhibiting adenosine deaminase activity with EHNA. The aminohydrolase freed of contamination with adenosine deaminase by immunoadsorbant column chromatography had a similar elution

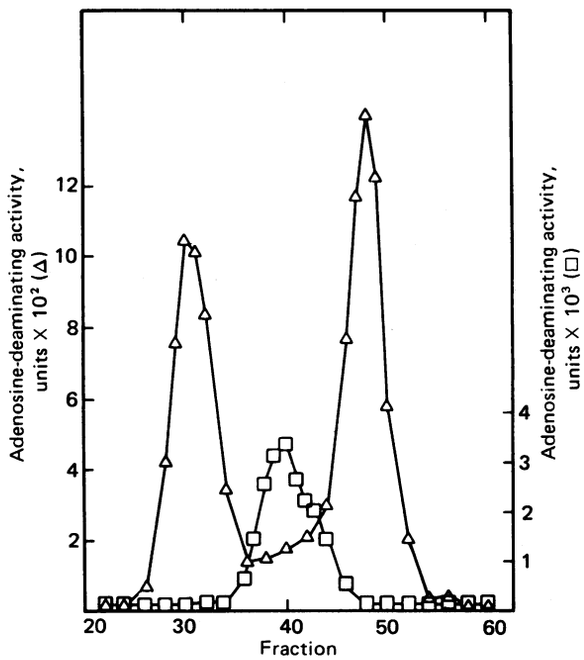

FIG. 4. Gel filtration of a crude extract from normal spleen. One milliliter of crude extract was applied to a column (1.5 × 80 cm) of Sephadex G-200. Adenosine-deaminating activities, expressed as units/fraction, were measured with (□) and without (△) EHNA (0.35 mM) in the standard reaction mixture.

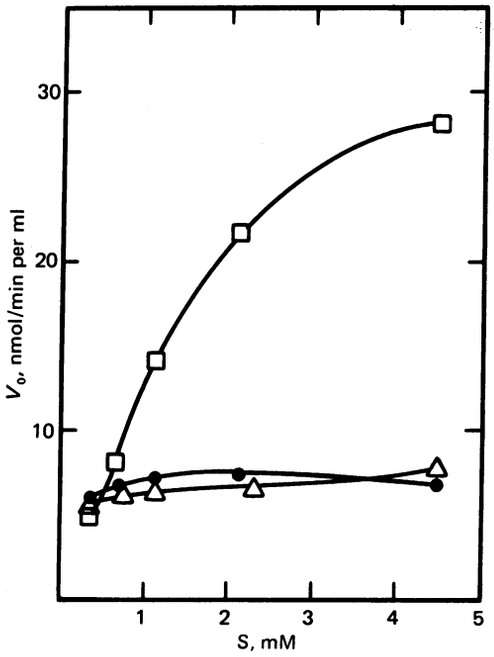

FIG. 5. Substrate (S) dependence of adenosine-deaminating activity. Appropriate amounts of normal spleen extract (△), purified spleen adenosine deaminase (●), and nonimmunoreactive aminohydrolase isolated from normal spleen (□) were assayed in the presence of increasing concentrations of adenosine. Activities are expressed as the initial velocity of hydrolysis at the indicated concentration of adenosine.

volume. Based on this elution volume, the aminohydrolase corresponds to a protein of molecular weight 92,000. Cytidine-deaminating activity present in these extracts had an elution volume similar to that of the nonimmunoreactive aminohydrolase. The two activities were differentiated with tetrahydrouridine, a potent inhibitor of cytidine deaminase (22). The adenosine-deaminating activity was not affected by 0.2 μM tetrahydrouridine, while cytidine deamination was reduced to an undectable level.

The effect of increasing adenosine concentration on the rate of hydrolysis by the nonimmunoreactive aminohydrolase and adenosine deaminase from normal spleen was also measured. The rate of hydrolysis by the aminohydrolase was concentration dependent from 0.3 to 4.5 mM adenosine (Fig. 5), with a Michaelis constant of 2 ± 1 mM. Over this same range of concentration, hydrolysis of adenosine by crude or purified preparations of adenosine deaminase from normal spleen proceeded at a nearly constant rate. This was expected since the Michaelis constant of spleen adenosine deaminase has been estimated to be about 90 μM (4).

DISCUSSION

Residual adenosine-deaminating activity has been detected in the tissues of infants with combined immunodeficiency disease and adenosine deaminase deficiency (12–14). The presence of adenosine deaminase in these tissues even at low concentration or in a less active form would demonstrate that the gene that codes for the enzyme is essentially intact and that the lack of enzyme is not the result of a gene deletion.

It has previously been shown that high molecular weight adenosine deaminase from kidney will bind specifically to a column to which antibody to low molecular weight erythrocyte adenosine deaminase has been attached (8). As shown here, both the high and low molecular weight forms of adenosine deaminase from normal human spleen also bind specifically to a similar immunoadsorbant column. In contrast, the majority of the adenosine-deaminating activity in the patient's spleen did not bind specifically to the anti-adenosine deaminase column. The significance of the small amount of activity that did appear to bind specifically is not clear.

The failure of the adenosine-deaminating activity in the extract from the patient's spleen to bind to the anti-adenosine deaminase column could be explained in several ways. Since the patient's immune system was not functioning normally, it is possible that the splenic activity was due to an infectious agent. Staining of tissue sections did not, however, reveal bacteria. Another possibility is that the activity was due to the action of a different enzyme that went unnoticed in normal spleen because it represented such a small fraction of the total adenosine-deaminating activity. In support of this hypothesis, an activity was isolated from normal spleen that did not bind to the anti-adenosine deaminase immunoadsorbant column and that accounted for only 1–2% of the extract's total adenosine-deaminating activity. Other similarities were observed between the adenosine-deaminating activity in the patient's spleen and the nonimmunoreactive aminohydrolase from normal spleen. EHNA, a potent inhibitor of adenosine deaminase, had relatively little effect on either activity. In addition, the pH optimum curves of the activities were similar and clearly distinguishable from that of spleen adenosine deaminase.

Hirschhorn *et al.* (23) have characterized what appears to be a "mutant" form of adenosine deaminase in fibroblasts derived from patients with combined immunodeficiency disease and adenosine deaminase deficiency which is clearly different than the aminohydrolase described here. The activity resembles tissue adenosine deaminase from normal fibroblasts in molecular weight and K_m but differs in electrophoretic mobility and heat stability. A similar activity was detected in fibroblasts from obligate heterozygotes. Further investigation will be required to determine if a relationship exists between the adenosine-deaminating activity in spleen and the "mutant" enzyme in fibroblasts.

The nonimmunoreactive aminohydrolase isolated from normal spleen had a molecular weight intermediate between the high and low molecular weight forms of spleen adenosine deaminase. Van der Weyden and Kelly have reported the presence of an adenosine-deaminating activity of intermediate weight in extracts from normal spleen (4). The activity described by these authors may not be the same one we have characterized since it has a Michaelis constant of 92 μM for the hydrolysis of adenosine. Van der Weyden and Kelly have also partially characterized an adenosine-deaminating activity in extracts from the spleen of a combined immunodeficient and adenosine deaminase deficient patient (14). The activity in this patient's spleen was attributed to a mutant form of adenosine deaminase intermediate in molecular weight between the high and low molecular weight forms of the normal enzyme.

Although our results are compatible with those of Van der Weyden and Kelly (4, 14), we suggest that the adenosine-deaminating activity in the spleen of the patient described here is not a mutant form of adenosine deaminase. The presence of a similar activity in extracts from the spleens of two normal individuals supports this conclusion. Further investigation will be required to determine whether the aminohydrolase in the patient's and normal spleen extracts is a modified product of the adenosine deaminase locus or of an entirely different locus. Until this issue is clarified, ascription of the residual adenosine-deaminating activity in tissue of combined immunodeficient and adenosine deficient patients to the adenosine deaminase locus is probably not warranted.

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