Radioimmunochemical Quantitation of Human Adenosine Deaminase

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ABSTRACT Markedly reduced or absent adenosine deaminase activity in man is associated with an autosomal recessive form of severe combined immunodeficiency disease. To further define the genetic nature of this enzyme defect, we have quantitated immunologically active adenosine deaminase (CRM) in the hemolysate of homozygous deficient patients and their heterozygous parents. A highly specific radioimmunoassay was developed capable of detecting 0.05% of normal erythrocyte adenosine deaminase. Hemolysates from nine heterozygotes (five families) showed a wide range in CRM (32–100% of normal) and variable absolute specific activities with several being at least 1 SD below the normal mean. Hemolysates from four unrelated patients showed <0.09% adenosine deaminase activity with CRM ranging from <0.06 to 5.6% of the normal mean. In conclusion, heterozygote and homozygote hemolysates from five of the eight families analyzed revealed variable levels of CRM suggesting heterogeneous genetic alteration or expression of the silent or defective allele(s) of adenosine deaminase.

INTRODUCTION

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) catalyzes the irreversible hydrolytic deamination of adenosine to produce inosine and ammonia. Markedly reduced or absent adenosine deaminase activity in man is associated with an autosomal recessive form of severe combined immunodeficiency disease and the relationship appears to be causal (1–4). Affected patients present a defect of both cellular and humoral immunity characterized clinically by severe recurrent infections with a uniformly fatal outcome if untreated. The deficiency of adenosine deaminase represents the first association of a specific enzyme defect with an inherited disorder of both T- and B-cell function. Thus, a better understanding of the nature of this association may provide substantial insight into the regulation of immune function.

More than 30 patients with adenosine deaminase deficiency and severe combined immunodeficiency disease have been described since the initial description by Giblett et al. (1) in 1972. In all cases studied, the patients exhibited trace or absent erythrocyte adenosine deaminating activity. When other tissues of some of these patients were examined, however, varying amounts of enzyme activity ranging from 1 to 30% of normal values were observed (5–7), suggesting considerable genetic heterogeneity in this disorder.

To further define the genetic nature of this enzyme defect, we have developed a sensitive and specific radioimmunoassay for the quantitation of immunoreactive adenosine deaminase protein in the hemolysate of homozygous[1] adenosine deaminase-deficient patients and their heterozygous parents.

METHODS

Materials. ^{125}I-Na carrier-free (2 mCi/nmol), was purchased from Amersham Corp. (Arlington Heights, Ill.). Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Div. of Pharmacia Inc. (Piscataway, N. J.). Todd Hewitt broth was purchased from BBL Microbiology Systems, Becton, Dickinson & Co. (Cockeysville, Md.). Cowan I strain of *Staphylococcus aureus* was a generous gift from Dr. L. A. Frohman, Department of Medicine, Michael Reese Hospital, Chicago, Ill. Normal hemolysates were obtained from 18 adults and 5 children. Hemolysates from heterozygous adenosine deaminase-deficient donors were obtained as follows: E.B. and T.T. from Dr. E. W. Gelfand, Toronto, Canada; La.L. and L.L. from Dr. F. C. Schmalstieg, Galveston, Tex.; R.W. from Dr. S. Polmar, Cleveland, Ohio; F.H. and M.H. from Dr. K. Rich, Chicago, Ill.; R.M. and D.M. from Dr. J. Cassidy, Ann Arbor, Mich. Hemolysates from homozygous adenosine deaminase-deficient donors were obtained as follows: R.M. Jr. the child of R.M.

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[1] The use of the term homozygote to describe the adenosine deaminase-deficient patients in this text and the table is meant to convey the presence of two abnormal alleles at the adenosine deaminase structural gene locus. If these abnormal alleles are different they would be more accurately described as compound heterozygotes.

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and D.M. was obtained from J. Cassidy, Ann Arbor, Mich.; B.W. from Dr. R. Buckley, Durham, N. C., and E.M. (not related to R.M. or D.M.) from Dr. D. Martin, San Francisco, Calif. Hemolysate from a healthy, homozygous adenosine deaminase-deficient donor, M.H. (not related to F.H. or M.H.) was obtained from Dr. R. Hirschhorn, New York.

Growth and preparation of S. aureus. *S. aureus* Cowan I strain containing membrane-bound Protein A was cultured in Todd Hewitt broth and prepared for use as an immunoprecipitant as previously described (8). The concentration of *S. aureus* used to precipitate a given dilution of adenosine deaminase antiserum was based on titrations performed for each new batch of bacteria.

Preparation of adenosine deaminase antiserum. Rabbit antiserum against highly purified erythrocyte adenosine deaminase (>96% pure, 545 μmol/min per mg immunologically cross-reactive material [CRM][2] was prepared and titered by a previously described technique (9). A high titer, high affinity antiserum was obtained in ≅3 mo and was used in all subsequent experiments.

Radioiodination of human erythrocyte adenosine deaminase. Human adenosine deaminase was purified to homogeneity from erythrocytes by a previously described procedure (9). The highly purified enzyme had an absolute specific activity of 545 μmol/min per mg at 37°C and exhibited all the common isozyme forms on polyacrylamide gel electrophoresis (9). This highly pure adenosine deaminase was radioiodinated essentially as described by Hunter (10). A mixture containing human erythrocyte adenosine deaminase (small form) (0.5 μg, 0.013 nmol), 1 μl ^{125}I-Na (1 mCi, 0.5 nmol), and 20 μl chloramine T trihydrate (0.7 nmol) in 100 mM sodium phosphate, pH 6.0 (buffer A) was shaken for 60 s at room temperature. The reaction was stopped by the addition of 20 μl of sodium metabisulfite (0.7 nmol). The reaction mixture was then diluted with 100 μl of buffer A containing 2 mg/ml bovine serum albumin and applied to a Sephadex G-25 (medium) column (0.7 × 28 cm). Isolated ^{125}I-adenosine deaminase was further purified on an adenosine Sepharose column (1.8 × 12 cm) (11) using 3 mM adenosine in 100 mM sodium phosphate, pH 6.0, 100 mM NaCl, 3 mM NaN_3, and 2 mg/ml bovine serum albumin as elution buffer. Each column fraction (1 ml) was analyzed for ^{125}I radioactivity by gamma counting and for immunoreactive protein by immunoprecipitation with excess adenosine deaminase antiserum and excess *S. aureus*. Peak tubes containing the highest concentration of immunoprecipitable ^{125}I-adenosine deaminase were pooled, dialyzed against 1,000 vol of 10 mM Tris/HCl, pH 7.4, and stored at 4°C. Radiochemical purity was analyzed by native and sodium dodecyl sulfate polyacrylamide gel electrophoresis by methods previously described (9).

Radioimmunoassay of adenosine deaminase. All reagents for the competitive radioimmunoassay were diluted with 10 mM Tris/HCl, pH 7.4, containing 154 mM NaCl, 3 mM NaN_3, and 2 mg/ml bovine serum albumin (buffer C). Reagents were added to disposable 6 × 50-mm glass tubes in the following order: (*a*) 50 μl of adenosine deaminase standard (concentration range 160–0.63 ng/ml), sample to be assayed or buffer C; (*b*) 50 μl of ^{125}I-adenosine deaminase (10,000 cpm); and (*c*) 50 μl of adenosine deaminase antiserum diluted to precipitate 30% of the labeled adenosine deaminase (1:20,000 vol/vol) or buffer C. The incubation tubes were blended in a Vortex mixer (Scientific Industries, Inc., Bohemia, N. Y.) and allowed to sit overnight at 4°C. Under these conditions antibody-antigen binding was found to reach equilibrium within 8 h. To each tube a suspension of *S. aureus* (50 μl) was added in 100-fold excess of that necessary to bind to all antibody (free and complexed with ^{125}I-adenosine deaminase). The tubes were mixed and immediately centrifuged for 15 min at 1,000 *g*. The resulting pellets were then washed once with buffer C (200 μl), and analyzed for ^{125}I radioactivity in a Searle model 1190 gamma counter (Searle Diagnostics Inc., subsidiary of G. D. Searle & Co., Des Plaines, Ill.). All radioimmunoassays were performed in triplicate and repeated several times over a period of several days.

Statistical methods. In the radioimmunoassay procedure, the percentage of ^{125}I-adenosine deaminase bound to antibody in the presence of unlabeled adenosine deaminase was corrected for background and nonspecific ^{125}I binding. Data were analyzed using a modified logit-log transformation (12) on a Hewlett-Packard computer model 9825A and printer model 9871A (Hewlett-Packard Co., Palo Alto, Calif.). A reiterative weighted linear regression was used to fit the best line to the standards. The values for sample displacements similarly transformed were interpolated from the standard line. The least detectable dose (that dose statistically different than zero) was taken where the lower 95% confidence limit of the counts bound in the absence of competing antigen crosses the extrapolated standard line.

Preparation of hemolysate samples. Blood samples obtained from normal and adenosine deaminase-deficient donors were washed with normal saline and frozen at −70°C for variable time periods. The blood sample from M.H. was initially centrifuged in a Ficoll-Hypaque gradient (Pharmacia Fine Chemicals) and the erythrocytes subsequently washed and frozen as described. Before assay the hemolysate samples were thawed and dialyzed overnight against buffer C (1/1,000 vol/vol). Adenosine deaminase enzyme activity and CRM in hemolysates from normal, heterozygous, and homozygous deficient subjects remained stable (within ±10%) after 2½ mo storage at both 4° and −70°C.

Enzyme and protein determinations. Adenosine deaminase was assayed using the previously described radiochemical method (13). All enzyme assays were performed using reaction mixtures containing [^{14}C]adenosine (2 mCi/mmol) at a final adenosine concentration of either 0.8 or 4 mM. Sample activities were determined in duplicate at least twice over a period of several days. Protein concentrations were determined by the method of Lowry et al. (14) using bovine serum albumin as standard.

RESULTS

Radioiodination of human erythrocyte adenosine deaminase. In developing a radioimmunoassay for adenosine deaminase, our goal was to make the assay as sensitive as possible while retaining a high degree of specificity. In part the preparation of an immunoreactive tracer (^{125}I-adenosine deaminase) with the highest possible specific radioactivity was essential for a sensitive radioimmunoassay. Adenosine deaminase was iodinated using the chloramine T method and purified by adenosine Sepharose column chromatography.

As shown in Fig. 1A, native polyacrylamide gel electrophoresis of purified ^{125}I-labeled adenosine deaminase revealed radiochemical and immunochemical homogeneity. In addition, sodium dodecyl sulfate-gel electrophoresis of this preparation also showed only one peak of radioactivity (Fig. 1B) coincident with purified unlabeled adenosine deaminase (data not shown). The

[2] *Abbreviation used in this paper*: CRM, immunologically cross-reactive material.

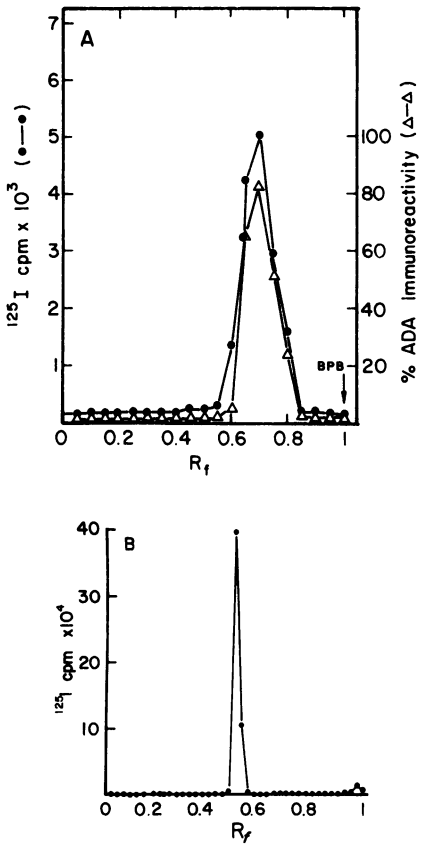

FIGURE 1 Polyacrylamide gel electrophoresis of ^{125}I-adenosine deaminase (ADA) purified by adenosine Sepharose. (A) Purified ^{125}I-adenosine deaminase was electrophoresed on a 7½% polyacrylamide gel at pH 8.9. The gel was sliced into 1.5-mm sections and analyzed by gamma counting. Subsequently, the gel slices were individually incubated in 200 μl of buffer C overnight at 4°C and the supernate analyzed for immunoprecipitable ^{125}I-radioactivity using excess adenosine deaminase antiserum and *S. aureus*. An arrow indicates the position of the bromphenol blue (BPB) tracking dye. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified ^{125}I-adenosine deaminase. The gel was sliced and analyzed by gamma counting as above.

purified ^{125}I-labeled enzyme had a minimum specific radioactivity of 15 mCi/μmol and was 82% immunoprecipitable. However the iodinated enzyme was stable for only 1–2 wk at 4°C, necessitating weekly preparation of this reagent for the radioimmunoassay.

Characteristics of the adenosine deaminase radioimmunoassay. The adenosine deaminase radioimmunoassay was found to be linear over a standard enzyme concentration range of 160–0.63 ng/ml with a slope of −1.00±0.021 (15 determinations) and a calculated least detectable dose of 0.3 ng/ml. Identical slopes are obtained using various dilutions of either purified adenosine deaminase or normal hemolysate as standards insuring that both preparations have the same affinity for the antibody.

The specificity of the adenosine deaminase radioimmunoassay was examined in the following ways. The addition of a 100-fold excess of unlabeled adenosine deaminase over that required for 50% displacement of tracer from antibody prevented >99% of tracer from binding. Further, hemolysate was treated with excess adenosine deaminase antiserum or the same concentration of control serum. After the addition of *S. aureus* to precipitate all antibodies, the supernate was analyzed by the normal radioimmunoassay technique. As expected, the hemolysate treated with control serum showed a normal level of adenosine deaminase whereas the adenosine deaminase antiserum-treated hemolysate showed <0.3 ng/ml of enzyme. These data indicated that artifactual results possibly related to nonspecific binding of tracer or antibody, could be ruled out in this assay system.

Levels of adenosine deaminase CRM were measured using serial dilutions of all hemolysate samples (triplicates) over a fourfold displacement range. Normalized values at each hemolysate concentration agreed within ±10% of each other. Hemolysate samples from heterozygous and homozygous adenosine deaminase-deficient donors were characterized further. Hemolysate samples were mixed with adenosine deaminase standard (at two different concentrations) or an equal volume of buffer C and analyzed for CRM. In all cases, the recovery of adenosine deaminase CRM was found to be quantitative.

Table I shows the characteristics of erythrocyte adenosine deaminase in hemolysates from normal, heterozygous, and homozygous deficient subjects. Adenosine deaminase activity for 18 normal adults and 5 normal children was 0.88±0.24 and 0.86±0.26 nmol/min per mg hemolysate protein, respectively. Using the radioimmunoassay, the quantity of adenosine deaminase immunoreactive protein (CRM) was determined to be 1.67±0.60 and 1.55±0.50 ng CRM/mg hemolysate protein and the mean absolute specific activity was calculated to be 563.5±46 and 553.6±14 μmol/min per mg CRM, respectively for normal adults and children. The calculated absolute specific activities compared well with the value of 515 and 538 μmol/min per mg previously reported for homogeneous preparations of erythrocyte adenosine deaminase (9, 11). The close agreement in the absolute specific activities provides further evidence for the accuracy of the adenosine deaminase radioimmunoassay used in conjunction with the enzymatic assay and confirms the absence of systematic error.

As noted in Table I, enzyme activity varied substantially from 0.53 to 1.20 nmol/min per mg hemolysate protein as did the level of CRM (0.9–2.23 ng/mg hemolysate protein) for the 18 normal adult hemolysates

TABLE I
Characterization of Erythrocyte Adenosine Deaminase

Hemolysate*	sp act	ng CRM/mg	Absolute sp act
	nmol/min/mg		*μmol/min/mg CRM*
Normal adults (18)	0.88±0.24‡ (0.53–1.20)	1.67±0.60 (0.9–2.23)	563.5±46 (531–620)
Normal children (5)	0.86±0.26 (0.62–1.2)	1.55±0.50 (1.07–2.22)	553.6±14 (540–572)
Heterozygote			
R.M.	1.35	2.4	562
D.M.	0.29	0.54	537
T.T.	0.74	1.47	500
E.B.	0.56	1.25	448
La.L.	0.68	1.16	586
L.L.	0.41	0.72	569
M.H.	0.56	0.94	595
F.H.	0.60	1.12	535
R.W.	0.33	1.02	320
Homozygote			
R.M., Jr.	$<0.8 \times 10^{-3}$	<0.001	—
B.W.	$<0.8 \times 10^{-3}$	0.007	—
E.M.	$<0.8 \times 10^{-3}$	0.052	—
M.H.	$<0.8 \times 10^{-3}$	0.087	—

* Protein concentrations for hemolysates ranged from 287 to 156 mg/ml.
‡ Mean±1 SD range given in parentheses.

analyzed. However, using linear regression analysis, the level of enzyme activity and CRM for each sample correlated well ($r = 0.98$, 18 pairs) suggesting that the catalytic activity per molecule was similar although the concentration of enzyme varied in each hemolysate sample.

As shown in Table I, hemolysate samples from unrelated parents of adenosine deaminase-deficient patients with severe combined immunodeficiency disease showed a wide range in adenosine deaminase specific activities from 0.29 to 1.35 nmol/min per mg hemolysate protein with easily detectable quantities of CRM ranging from 0.54 to 2.4 ng CRM/mg hemolysate protein. However, the calculated adenosine deaminase absolute specific activity (micromoles per minute per milligram CRM) for the majority of the heterozygous samples in this study appeared to be within the normal adult range. The obvious exceptions were hemolysates of E.B., T.T., and R.W. which were at least 1 SD below the mean. CRM in these three heterozygote hemolysates showed the same affinity toward adenosine deaminase antibody as did the normal erythrocyte enzyme. Further analysis of the adenosine deaminase from these subjects revealed a normal K_m for adenosine and complete inhibition of enzyme activity in the presence of the competitive enzyme inhibitor erythrohydroxynonyl adenine. Unfortunately, the hemolysates from the affected children of these heterozygote parents were either not available for analysis or were not suitable for analysis because of recent blood transfusions (with the exception of R.M., Jr.).

The hemolysates from homozygous adenosine deaminase-deficient subjects all showed undetectable enzyme activity of <0.09% of the normal mean activity (representing the lower limit of our enzymatic assay) (Table I). Enzyme activity resulting from mixing normal and adenosine deaminase hemolysate was additive suggesting the absence of an adenosine deaminase inhibitor in these samples. The hemolysate of R.M. Jr., the child of D.M. and R.M., failed to exhibit any detectable CRM i.e., <0.06% of normal child mean CRM. However, the other homozygous deficient patients analyzed, B.W., E.M., and M.H., did reveal detectable CRM, of apparently normal affinity, representing 0.4, 3, and 5.6% of the normal child mean CRM, respectively. Unfortunately, hemolysates from the parents of this latter group of patients were not available for analysis at the time of this study.

DISCUSSION

Patients with adenosine deaminase deficiency and severe combined immunodeficiency disease have been reported to exhibit virtually no enzyme activity in their erythrocytes, whereas other tissues of these patients demonstrate variable amounts of residual adenosine deaminating activity (5–7). However, the adenosine deaminase enzymatic assay used in all of these studies could only detect biologically active protein and so may have underestimated genetic expression of the adenosine deaminase alleles.

In a previous study of fibroblast cell strains from three adenosine deaminase-deficient subjects, an immunoassay based on enzyme activity revealed that one cell strain with trace adenosine deaminase activity had immunoreactive protein (CRM) representing 35% of normal, whereas two other adenosine deaminase-deficient cell strains demonstrated <5% of normal CRM (15). In this report we have described the development of a specific radioimmunoassay for erythrocyte adenosine deaminase which is at least 100 times more sensitive than the previously published immunoassay (15). In addition, we have used this immunoassay for the analysis of hemolysate from nine heterozygotes and four homozygotes with adenosine deaminase deficiency, representing a combined total of eight different families.

The radioimmunoassay was linear over a broad adenosine deaminase concentration range and was sensitive to an enzyme concentration of 0.3 ng/ml. This assay would be sensitive to a calculated enzyme

activity of 0.17 nmol/min per ml based on an absolute specific activity of 563 μmol/min per mg CRM (Table I). The specificity of the radioimmunoassay for the analysis of adenosine deaminase in hemolysate was verified by finding no detectable CRM in normal hemolysate pretreated with excess adenosine deaminase antiserum and by finding no detectable CRM in one of the homozygous adenosine deaminase-deficient hemolysates (R.M., Jr.).

This radioimmunoassay was used in conjunction with a sensitive enzymatic assay to characterize adenosine deaminase in the hemolysate of normal subjects and of adenosine deaminase-deficient subjects. The radioimmunoassay and the enzymatic assay for adenosine deaminase were equally sensitive capable of detecting 0.06% of normal mean CRM and 0.09% of normal mean enzyme activity, respectively. As shown in Results, the specific activities of adenosine deaminase in the hemolysates of the heterozygous parents ranged from 0.29 to 1.35 nmol/min per mg with several samples falling within the normal adult range. The quantity of adenosine deaminase CRM among these samples also showed a wide range. However, the calculated absolute specific activity for adenosine deaminase (micromoles per minute per milligram CRM) in the majority of hemolysates from heterozygotes appeared to fall within the normal range. Samples E.B., T.T., and R.W. were the exceptions with calculated absolute specific activities at least 1 SD below the normal adult mean. Storage for 2½ mo at either 4° or −70°C did not significantly influence the stability of the enzyme activity or CRM in these samples suggesting that our measurements did not reflect artifacts due to enzyme denaturation.

Variable genetic expression of the "silent" or apparently defective adenosine deaminase allele was even more apparent in the hemolysate of the homozygous deficient subjects. The hemolysate from R.M., Jr., revealed no detectable enzyme activity or CRM, consistent with the calculation of a normal absolute specific activity for adenosine deaminase in the hemolysate of both parents (R.M. and D.M.). In this family, the data suggested that only the "normal" adenosine deaminase allele was expressed in the hemolysate of the parents whereas the inherited "silent" or defective alleles did not produce a gene product immunologically detectable by this antiserum or alternatively produced a gene product which may have been rapidly degraded in vivo. The hemolysates of B.W., E.M., and M.H. however, all had easily detectable quantities of adenosine deaminase CRM ranging from 0.4 to 5.6% of the normal child mean CRM. From this type of analysis it was impossible to determine whether our CRM measurement reflected the expression of both adenosine deaminase alleles or only one producing an immunoreactive gene product. M.H. has also been shown previously to be genetically different from other patients with adenosine deaminase deficiency and severe combined immunodeficiency disease by other criteria as well (16); whereas M.H. has been shown to be deficient in erythrocyte adenosine deaminase (<1% of normal), his lymphocytes have 22% of normal enzyme activity. Further, this subject has been reported to be healthy and immunologically normal (16).

In conclusion, heterozygous and homozygous hemolysate samples from five of the eight total families analyzed revealed apparent variable genetic expression of the "silent" or defective adenosine deaminase allele(s). Further, hemolysate from some heterozygous subjects had quantities of the normal gene product which brought total enzyme quantities to a normal mean level suggesting possible autologous regulation of adenosine deaminase in the reticulocytes of these subjects.

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