Conversion of Human Erythrocyte-Adenosine Deaminase Activity to Different Tissue-Specific Isozymes

EVIDENCE FOR A COMMON CATALYTIC UNIT

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ABSTRACT Adenosine deaminase activity resides in various characteristic isozymes in red blood cells (RBC-ADA) and other tissues. Absence of RBC-ADA has been reported in a proportion of patients with autosomally inherited severe combined immunodeficiency (SCID). We have previously reported that the tissue isozymes of ADA are also deficient in children with SCID and RBC-ADA deficiency, although these isozymes differ from RBC-ADA in molecular weight, accessible SH groups, and electrophoretic mobility. The deficiency of all types of ADA in SCID implies that a catalytic unit of ADA in each isozyme is coded by the same structural gene. The relationship of RBC-ADA and the different tissue ADA isozymes is the subject of this paper.

Incubation of RBC-ADA with ADA-deficient liver, kidney, and fibroblast extracts resulted in the appearance of new isozymes of ADA. These newly generated isozymes had the physicochemical and electrophoretic characteristics of the tissue-specific isozymes obtained from normal tissues. The electrophoretic mobility of the isozyme generated appeared to depend upon the tissue utilized and corresponded to the electrophoretic mobilities of the ADA isozymes found naturally in each of the different tissues. Additionally, the genetically determined polymorphism exhibited by RBC-ADA could be detected in the isozyme generated. Incubation with normal kidney also caused conversion of the RBC isozyme to the kidney form. These findings further support the concept that the catalytic activity of each of the several forms of the ADA enzyme resides in a single molecule coded at the same genetic locus as is defective in one form of SCID. The tissue-specific isozymes, which differ in electrophoretic mobility and molecular weight, are generated by interaction of the RBC catalytic unit with tissue-specific factors present in the different tissues of normal humans and patients.

INTRODUCTION

The syndrome of severe combined immunodeficiency (SCID)[1] encompasses a group of hereditary and sporadic conditions characterized by deficits of both cellular and humoral immunity. The genetic forms are inherited as X-linked recessive or autosomally recessive traits (1). A deficiency of red cell adenosine deaminase (RBC-ADA) has recently been found in a proportion of children with autosomally inherited SCID (2–6[2]). Since the parents of these children had half the normal levels of the enzyme (6, 7),[2] a causal relationship linking the two inherited conditions was initially suspected, but two lines of evidence appeared to contradict this possibility. First, the RBC-ADA locus was thought to be genetically linked to the histocompatibility locus (8, 9) and therefore to an immune response (IR) locus (10), suggesting that a partial chromosome deletion or a frame shift mutation could have involved the ADA locus only incidentally. Second, the electrophoretic patterns of RBC-ADA in most human populations differ according to the inheritance of two autosomal allelic genes, ADA^1 and ADA^2,

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[1] *Abbreviations used in this paper:* ADA, adenosine deaminase; IR, immune response; PBS, phosphate-buffered saline: RBC, red blood cell; SCID, severe combined immunodeficiency.

[2] Parkman, R., F. S. Rosen, E. Gelfand, A. Sanderson, and R. Hirschhorn. 1975. Severe combined immunodeficiency and adenosine deaminase deficiency. *N. Engl. J. Med.* In press.

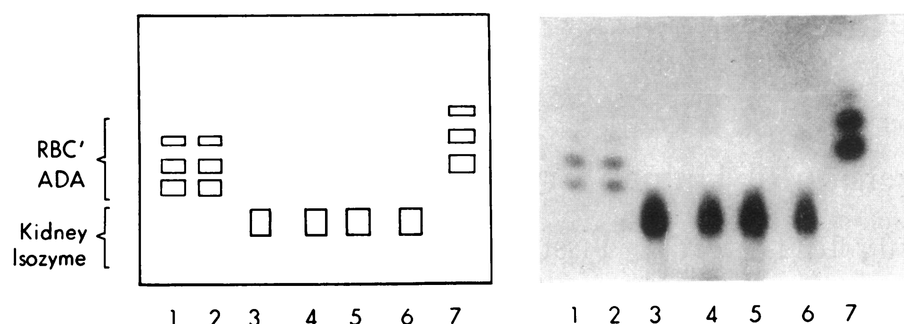

FIGURE 1 Conversion of RBC-ADA by incubation with normal kidney. Lysates of RBC's were incubated alone and with homogenates of normal kidney, in the presence and absence of SH reagents. The mixtures were subjected to starch gel electrophoresis and the areas of ADA enzyme activity visualized as described in the text. Channel 1 contains RBC lysate and demonstrates the typical triple-banded pattern of RBC-ADA (17). Channel 3 contains extracts of normal kidney and demonstrates the slower anodal mobility of normal kidney ADA isozymes (isozyme designations are defined in Fig. 3) (d, e). Channel 4 contains a mixture of RBC lysate and normal kidney extract and demonstrates the loss of all enzyme activity with the electrophoretic mobility of RBC-ADA. Total enzyme activity in this mixture was not diminished but was the sum of the enzyme activities of the individual isozymes (see text). Channel 2 contains RBC lysates in the presence of 2-mercaptoethanol, which binds to free SH groups but does not alter the electrophoretic mobility of the RBC-ADA isozyme. Channel 7 contains RBC lysate in the presence of oxidized glutathione, which binds to the SH groups, adding a negative charge and increasing the anodal electrophoretic mobility of the RBC-ADA. Channels 5 and 6 contain mixtures of RBC-ADA with normal kidney extracts in the presence of 2-mercaptoethanol and oxidized glutathione, respectively. Neither the disappearance of enzyme activity with RBC-ADA mobility nor the mobility of the kidney isozyme is altered by the presence of these SH reagents.

whereas the patterns of ADA in other tissues consist of both the red cell isozymes and additional isozymes with ADA catalytic activity, but not apparently affected by genes determining RBC-ADA. These tissue forms of ADA also differ from the red cell enzyme in mol wt (> 260,000 vs. 33,000) and the absence of reactive sulfhydryl groups. Different tissues show different characteristic patterns of these isozymes. These tissue isozymes have previously been labeled a through e on the basis of electrophoretic mobility (11–13).

We recently reported that the tissues obtained at autopsy from a child with SCID lacked not only RBC-ADA but also the tissue-specific isozymes, suggesting that all of the ADA normally produced by the various tissues is controlled by a single genetic locus (14). Akedo, Nishahara, Shinkai, Komatsu, and Ishikawa had earlier demonstrated a heat-labile factor in normal tissues that converted a small mol wt ADA to a larger molecule (15). To clarify these genetic and molecular interrelationships, we have examined the convertibility of the polymorphic low mol wt RBC-ADA enzymes of differing genotypes to the various tissue-specific high mol wt ADA isozymes, utilizing both normal tissues and extracts of tissues from children with SCID associated with RBC-ADA deficiency.

METHODS

Normal tissues and those from patients with SCID and absence of RBC-ADA (kindly supplied by Drs. H. J. Meuwissen, B. Pollara, and F. S. Rosen) were obtained at autopsy and stored frozen at $-20°C$. Tissues were thawed, homogenized in a glass-glass homogenizer in a half volume of 0.05 M Na phosphate buffer (PO_4, pH 6.5), and centrifuged in a Beckman Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) at 4°C for 2 min. Fibroblasts were trypsinized (0.25% trypsin solution A, Grand Island Biological Co., Grand Island, N. Y.), washed three times in phosphate-buffered saline, Ca^{++}, Mg^{++} free (PBS), and sonicated in half-strength PBS or 0.05 M PO_4 at pH 6.5 for 30 s at 50 W with a microtip of a Heat Systems model W 185 Sonifier (Heat Systems Ultrasonics, Inc., Plainview, N. Y.). Lymphoid line cells (kindly supplied by Dr. N. Beratis) were treated in the same manner but without trypsinisation. Normal human RBC's (4.5 ml anticoagulated with 0.5 ml 3.8% citrate), were washed three times in PBS at 1,000 g for 10 min at 4°C, the buffy coat was removed after each wash, and RBC's were diluted in 0.05 M PO_4, pH 6.5, and sonicated as above.

Gel electrophoresis and enzyme assay. Electrophoresis and detection of enzyme activity were performed as described by Spencer, Hopkinson, and Harris (12) in 11% starch gel in PO_4, pH 6.5, 0.01 M for gel buffer, and 0.10 M for bridge buffer, overnight, between cooling plates at 2.5 V/cm. The sliced starch gels were incubated at 37°C in the dark for up to 5 h with an overlay of 1% agar containing the reagents necessary for the detection of the enzyme (nucleoside phosphorylase, xanthine oxidase, Boeh-

ringer Mannheim Corp., New York, MTT tetrazolium, and phenazine methosulfate, Sigma Chemical Co., St. Louis, Mo.). Activity in extracts was quantitated by adding nucleoside phosphorylase (0.25 U/ml assay) and xanthine oxidase (0.2 U/ml assay) and measuring uric acid produced at A_{293} but as previously modified to allow for detection of smaller amounts of activity (16). The assay was linear with time of incubation and protein concentrations used.

Incubation for conversions. Various dilutions of either packed RBC's (usually 1:1) or of lymphoid line extracts (containing only RBC-ADA isozyme) were mixed with varying amounts of the different tissue extracts and incubated at room temperature for up to 5 h before electrophoresis or assay. Oxidized glutathione, 2-mercaptoethanol, or iodoacetamide (Sigma Chemical Co.) was added at a final concentration of 10 mM.

RESULTS

Extracts of normal kidney, containing only the very slowly migrating isozymes (d-e) typical of that tissue, were incubated for 5 h at 25°C with red cell hemolysates or lymphoid line extracts, containing only the RBC-ADA isozymes. The mixtures were subjected to electrophoresis in starch gel, and enzyme activity was visualized (Fig. 1). Incubation of either the hemolysate or the kidney extract alone did not cause enzyme activity to disappear, nor were alterations produced in the electrophoretic mobility of either group of isozymes (Fig. 1, channels 1 and 3). After incubation of the RBC-ADA with normal kidney extract the RBC-ADA isozyme was no longer detectable, and only enzyme activity with the electrophoretic mobility of the kidney isozyme could be seen (Fig. 1, channel 4). Total enzyme activity was unchanged; thus RBC's alone contained 0.327 U of activity, kidney alone 0.369 U of activity, and the mixture subjected to electrophoresis, 0.688 U of activity. The mobility or activity of RBC-ADA was not affected by incubation with albumin (3.2 mg/ml). RBC-ADA was incubated with oxidized glutathione, which presumably binds to the SH group of the enzyme protein, thereby adding a negative charge and increasing the anodal electrophoretic mobility (Fig. 1, channel 7) (12). This incubation did not prevent apparent conversion of RBC-ADA isozyme to kidney isozyme, nor did it alter the mobility of the isozyme generated (Fig. 1, channel 6), nor did addition of 2-mercaptoethanol prevent this conversion (Fig. 1, channel 5).

Extracts of tissues from children with SCID and RBC-ADA deficiency, which contained no detectable ADA activity on starch gel, were incubated with extracts of normal lymphoid line cells or RBC hemolysates (Fig. 2, channels 3 and 7). Such mixtures, with ADA-deficient liver, resulted in the appearance of one of the normal isozymes seen in liver (*d*) (Fig. 2, channels 1 and 2). Some of the activity of the RBC-ADA isozyme remained unchanged in electrophoretic mobility. Incubation of the same RBC-ADA with extracts of ADA-deficient kidney resulted in the appearance of enzyme

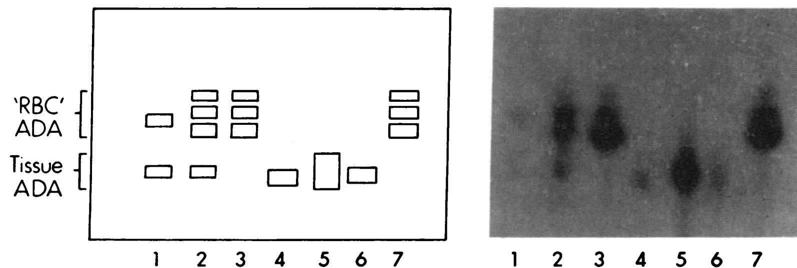

FIGURE 2 Conversion of RBC-ADA to tissue-specific isozymes by incubation with tissues deficient in ADA. Lymphoid line extracts, which contained only RBC-ADA, were incubated with extracts of kidney or liver from ADA-deficient patients. These kidney and liver extracts by themselves did not contain ADA activity detectable on starch gel electrophoresis. The mixtures of extracts of ADA-deficient tissues and lymphoid line extracts were subjected to starch gel electrophoresis and stained for ADA activity in parallel with extracts of normal liver, kidney, and lymphoid line extracts, to serve as markers of electrophoretic mobility. Channel 1 contains extracts of normal liver and demonstrates the two major isozymes of that tissue, a and d. (Isozyme designations are defined in Fig. 3). Channels 3 and 7 contain extracts of normal lymphoid line and demonstrate the typical triple-banded pattern of RBC-ADA. Channel 2 contains this same lymphoid line extract after incubation with ADA-deficient liver extract. ADA activity with the mobility of the slow-moving isozyme of normal liver (Channel 1) is now visible, in addition to the RBC-ADA. Total enzyme activity in the mixture was the sum of that present in the individual extracts (see text). Channels 4 and 6 contain extracts of normal kidney and demonstrate the slowly migrating d-e isozyme typical of that tissue. Channel 5 contains the lymphoid line extract seen in channels 3 and 7 after incubation with extracts of ADA-deficient kidney. Virtually all of the enzyme activity is now detectable as a slow-moving isozyme like that seen in normal kidney (channels 4 and 6).

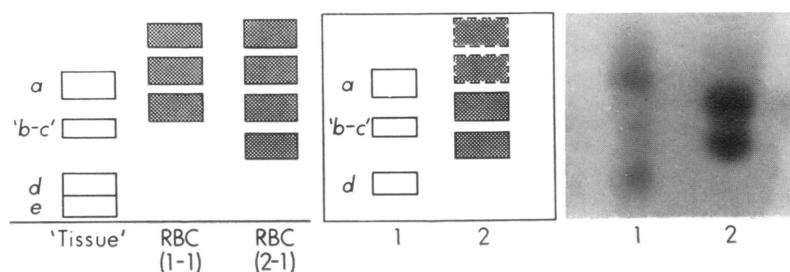

FIGURE 3 Conversion of RBC-ADA by liver to tissue isozymes. RBC-ADA was incubated with extracts of liver from an ADA-deficient child, subjected to starch gel electrophoresis, and stained for ADA activity. Various isozymes found in tissues are represented diagrammatically, with a previously suggested nomenclature (10). The isozymes specific for RBC's are indicated in the diagram as shaded areas. The RBC isozyme of both a 1-1 and a 2-1 phenotype are diagrammed to aid in identifying the isozymes generated from a 2-1 lymphoid line after incubation with liver extract. Channel 2 contains normal lymphoid line extract, demonstrating the typical pattern of a 2-1 ADA phenotype. Channel 1 contains the same lymphoid extract after incubation with an extract of ADA-deficient liver. No activity is visible as RBC-ADA, but ADA is now seen as two major areas of activity which correspond in mobility to that of the a isozyme (between the second and third bands of ADA 2-1), and the d isozyme seen in normal liver. (See Fig. 2). In addition a minor component is visible which corresponds in mobility to the b-c isozyme.

activity with the slow mobility characteristic of the tissue isozyme of normal kidney. No residual activity with the mobility of RBC-ADA was visible (Fig. 2, channel 5). On one occasion, it was possible to obtain conversion of RBC isozyme to the labile a isozyme, usually found in liver and lung extracts, which has a mobility intermediate between the first and second bands of RBC-ADA 1-1 and the second and third bands of heterozygous ADA 2-1. An extract of ADA-deficient liver was incubated with a lymphoid line of 2-1 phenotype (Fig. 3, channel 2). After incubation, no enzyme activity was seen with the mobility of the RBC isozyme, but instead all enzyme activity moved with the electrophoretic mobilities of the a, b-c, or d isozymes (Fig. 3, channel 1). Fibroblast extracts from another child with SCID converted the RBC isozyme to an isozyme with the mobility of a tissue isozyme seen in normal fibroblasts (not shown). Thus, each tissue appears to contain a factor that can convert RBC isozymes to each of the previously described, electrophoretically different species of ADA characteristic of that tissue.

There was no loss of enzyme activity during these conversions. When RBC extracts were incubated with ADA-deficient liver extracts, 10 μl of the RBC hemolysate alone contained 0.575 U of ADA while 10 μl of RBC hemolysate mixed with 10 μl of this liver extract, which converted all of the enzyme to the slower tissue form, contained 0.510 U of ADA. The liver extract alone contained too little activity to be detected under these conditions of assay. This suggests that the RBC-ADA activity was not destroyed but was converted without significant loss of activity.

The influence of the free SH groups of the RBC isozymes on the conversion was further investigated by incubating RBC's with iodoacetamide. The efficacy of this reagent in irreversibly binding the free SH groups was confirmed by demonstrating that oxidized glutathione could no longer increase the anodal electrophoretic mobility of the iodoacetamide-treated RBC's. Conversion of RBC-ADA from these RBC's to d isozyme, by incubation with ADA-deficient liver, proceeded normally, and the mobility of the tissue isozyme generated was again not altered by the presence of glutathione.

The isozyme generated in vitro by incubation of RBC's with ADA-deficient liver was further characterized by estimation of molecular weight. A mixture in which not all of the RBC enzyme was converted to isozyme of tissue mobility, as determined by gel electrophoresis, was applied to a calibrated Sephadex G-150 column and the activity of the different fractions determined. The residual RBC activity eluted at the same volume as did RBC-ADA alone. The major portion of the ADA activity was now eluted as a higher mol wt species, with the same elution fraction as the tissue isozyme of normal circulating human lymphocytes (16) (Fig. 4), with an estimated mol wt of 260,000, similar to that reported for normal liver isozyme (11[3]).

To further compare the properties of the tissue isozymes generated in vitro with those found naturally, RBC's of individuals who exhibit different polymorphic forms of RBC-ADA were used to generate tissue isozymes. It has been reported that tissue isozymes do not

[3] Hirschhorn, R. Unpublished observations.

express the polymorphism detectable in the RBC-ADA isozymes. RBC lysates from individuals homozygous for two different genetically determined, polymorphic forms of RBC-ADA (1-1 and 2-2) were incubated with extracts of ADA-deficient liver. The isozyme generated from the slower moving 2-2 RBC's could reproduceably be shown to migrate slightly less rapidly anodally than that generated from the faster moving 1-1 RBC's, although the difference observed was less than that between the two types of RBC enzymes. Thus when the distance between the slowest bands of RBC-ADA 2-2 and RBC-ADA 1-1 measured 10 mm, the distance between the tissue isozymes generated from the RBC-ADA 2-2 and RBC-ADA 1-1 measured 2.5 mm. This very small difference could easily have been undetected during electrophoresis of the tissues from rare 2-2 individuals (11).

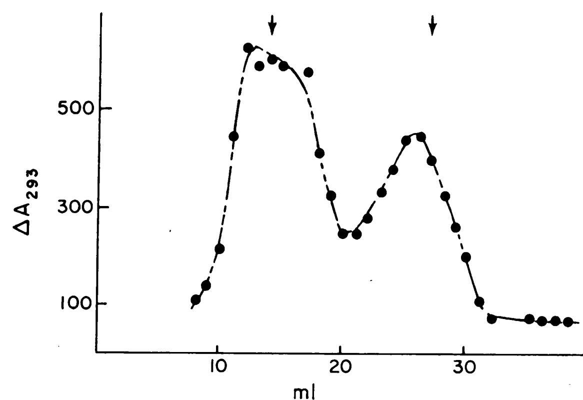

FIGURE 4 Conversion of RBC-ADA of low molecular weight to a high molecular weight ADA by incubation with ADA-deficient liver. A Sephadex G-150 column (25 × 1.5 cm) was calibrated with blue dextran, ovalbumin, endogenous nucleoside phosphorylase, cytochrome *C*, RBC-ADA, and tissue ADA from peripheral blood lymphocytes. The arrow at an elution volume of 28 ml and an estimated mol wt of 33,000 indicates where RBC-ADA was found. The arrow at elution volume of 14 ml and > 150,000 mol wt indicates where the tissue isozyme of peripheral lymphocytes was found (16). RBC-ADA was incubated with liver from an ADA-deficient child, which by itself contained no detectable ADA under these conditions. Gel electrophoresis of an aliquot of the mixture demonstrated partial conversion of the activity to a tissue isozyme (d). The mixture was applied to the Sephadex G-150 column, and 1-ml fractions were assayed for ADA activity by measuring the amount of product at A_{293} (16). ADA activity is now seen not only as the original 33,000-mol-wt form of RBC-ADA, but also as a high molecular weight form characteristic of tissue isozyme.

DISCUSSION

These experiments demonstrate that the catalytic protein of the RBC-ADA isozymes can be converted to each of four (a, b, d, and e) different tissue-specific isozymes upon incubation with the appropriate tissues. The isozymes of ADA generated in vitro from RBC-ADA are like the isozymes present in tissues other than red cells in their electrophoretic mobility, their higher molecular weight and the absence of accessible SH groups. Additionally, the generated isozymes demonstrate the genetically determined difference in electrophoretic mobility or polymorphism seen in the RBC-ADA isozymes. These findings support our previous suggestion (14) that a single genetic locus controls the enzyme activity of the several ADA isozymes and clarifies the mechanism whereby the genetic mutation responsible for RBC-ADA deficiency also results in a deficiency of the several forms of tissue ADA.

Several alternative, if less likely, interpretations of the data are possible. The loss of enzyme activity with RBC-ADA electrophoretic mobility after incubation of RBC-ADA with normal kidney, could represent inactivation rather than conversion of the RBC enzyme. However, this would require simultaneous activation of the kidney enzyme, since the total enzyme activity of the mixtures was the sum of the enzyme activities of the individual isozymes. Nor is it likely that the increased protein concentration of the mixtures exerted a nonspecific effect upon the electrophoretic mobility of the enzyme, since addition of albumin did not alter the electrophoretic mobility or enzyme activity of RBC-ADA. Finally, the appearance of enzyme activities with electrophoretic mobilities like those of tissue isozymes and the disappearance of RBC-ADA after incubation of RBC-ADA with ADA-deficient tissues might represent both an inactivation of the RBC enzyme with the simultaneous activation of previously masked tissue isozymes in the ADA-deficient tissues. Although this possibility can not be definitively eliminated without the use of purified enzymes and conversion factor, the most plausible explanation of the data is that conversion of RBC-ADA to tissue-specific isozymes took place after interaction with the previously described tissue protein (15, 17).

The fact that a single gene codes for a catalytic protein common to all of the isozymes of ADA and assignment of this gene to chromosome 20 (18, 19) and of HL-A to chromosome 6 (8, 9) argue against any chromosomal linkage between HL-A, IR genes, and RBC-ADA. We have additionally found independent assortment of HL-A haplotypes and diminished levels of RBC-ADA in families of children with SCID and ADA deficiency.[3] Therefore, a chromosomal deletion or frame shift mutation of the IR–HL-A region fortuitously involving RBC-ADA appears unlikely. The most likely explanation of the coinheritance of ADA deficiency and SCID is that the enzyme defect causes the disease. There are two possible objections to this interpretation.

First is the limitation of pathology to a single organ system in the face of a deficiency of an enzyme with widespread tissue distribution. Such restriction resulting from a genetically determined absence of a ubiquitous enzyme is not uncommon and occurs in many genetic disorders, such as the lysosomal storage diseases (20), by diverse and often poorly understood mechanisms. The enzymes of the purine salvage pathway, including ADA, may be more important for differentiation and proliferation of lymphoid cells than of other cells. Alternatively, lymphocytes may lack efficient mechanisms for dealing with increased concentrations of adenosine, AMP, adenine or cyclic AMP, all of which metabolites are known to inhibit RNA and DNA synthesis of phytohemagglutinin-stimulated lymphocytes (21). Adenosine or AMP also inhibits *de novo* pyrimidine biosynthesis in lymphoid line cells at the level of orotate (22). Secondly, a child has been reported (23) who survived the rigors of the Kalahari desert but whose RBC's lacked ADA. A different mutation in the ADA enzyme, resulting in an enzyme sufficiently stable as tissue isozyme to allow for normal functioning of the lymphoid system, could account for the lack of immunodeficiency. Absence of enzyme activity in the RBC also does not necessarily preclude the presence of residual or even normal enzyme activity in tissues, since relatively unstable enzyme may be absent from older, nonprotein-synthesizing RBC's but present in cells continually synthesizing new enzyme protein, as is seen in glucose-6-phosphate dehydrogenase variants (24). In addition we have found low levels of an electrophoretically altered ADA in fibroblasts and lymphocytes, as well as in other tissues derived from some children suffering from SCID with absence of RBC-ADA (25).[2]

It would thus appear that a single genetic defect can result in absence or diminished activity of multiple ADA isozymes. This genetically determined deficiency of the various isozymes of ADA, inherited as an autosomal recessive trait, would appear to be the primary genetic defect in one form of SCID.

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