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A normal level of adenosine deaminase activity in the red cell lysates of carriers and patients with severe combined immunodeficiency disease

(genetic disease/enzyme inhibitor/enzyme deficiency)

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ABSTRACT **The red cell lysates of two children with severe combined immunodeficiency disease (SCID) exhibited a virtually total absence of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) when standard volumes were assayed. Under these conditions the parents exhibited depressed specific activity except for one mother, whose lysate showed a normal value for activity. Upon storage of the lysate at 4°, a significant amount of activity appeared in one of the SCID children, and the activity of the heterozygous carriers was stimulated. With the use of a sensitive spectrophotometric assay based on conversion of inosine to uric acid, it was shown that the specific enzymatic activity in each of the SCID patients increased progressively as the volume of lysate assayed was lowered. With the smallest amount of lysate this specific activity was in the normal range. Similarly, the specific activity of each of the parents' lysates increased to the level of normal (or, in one case, about twice normal) as smaller volumes were assayed. The activity in the SCID patient was inhibitable by 2-fluoroadenosine and N^6-methyladenosine, known competitive inhibitors of human red cell adenosine deaminase. The lysate from the SCID patient was also shown to inhibit adenosine deaminase partially purified from a normal individual. The results are interpreted in terms of a genetically programmed production of an adenosine deaminase inhibitor in at least one variant of the severe combined immunodeficiency disease.**

Severe combined immunodeficiency (SCID) is a serious inherited disorder characterized by a virtually complete lack of both B and T cells. If a successful bone marrow transplant from a histocompatible donor cannot be performed, the disease is fatal at an early age (1). In 1972, three immunologically deficient children were described who also showed an apparently total absence in the red cell of the enzyme adenosine deaminase (ADase) (adenosine aminohydrolase, EC 3.5.4.4) (2, 3). This finding generated considerable interest since it was the first documented enzyme deletion associated with a severe defect of the immune response.

The subsequent description (4) of a number of SCID patients with a total lack of ADase in their red cells, and only traces of enzyme activity in other tissues, further substantiated a possible correlation between the enzyme defect and the disease, since both individually are rare occurrences. Evidence has since been presented that the loci for red cell ADase and HL-A are situated on separate chromosomes (5, 6). The possibility therefore emerged that adenosine deaminase deficiency was not simply the reflection of a common genetic deletion, but rather the cause of at least one form of the disease. This view was strengthened by the report that exogenously added adenosine could inhibit the proliferation of mammalian lymphoid lines as well as mouse fibroblasts (7, 8).

Abbreviations: ADase, adenosine deaminase (EC 3.5.4.4); SCID, severe combined immunodeficiency.

We report here the discovery of conditions which can restore the specific ADase activity from red cells of two SCID patients and their parents to a normal level. We interpret these results to indicate the possible presence of a bound inhibitor, dissociable upon dilution. These results imply new possibilities for the molecular basis of the immunodeficiency diseases.

EXPERIMENTAL

Materials

Adenosine deaminase was partially purified from human red cells by batch elution from DEAE-cellulose, followed by Sephadex G-100 chromatography. The purification was about 200-fold. Nucleoside phosphorylase (calf spleen; crystalline suspension in ammonium sulfate), xanthine oxidase (buttermilk, crystalline suspension in ammonium sulfate), and adenosine were purchased from Sigma Chemical Co., St. Louis. 2-Fluoroadenosine was obtained from Merck. [8-^{14}C]Adenosine (51.2 mCi/mmol; 0.02 mCi/ml) was obtained from New England Nuclear, Boston, Mass. 6-Methylaminopurine ribonucleoside was a gift of Dr. George B. Brown.

Methods

Enzymatic Assays. Adenosine deaminase activity in red cell lysates was measured at 37° in a Beckman Acta III recording spectrophotometer by a modification of the procedure of Hopkinson and coworkers (9). The basis of the assay is the conversion of the inosine produced to uric acid, which absorbs at 293 nm. The assay mixture contained 0.15 mM adenosine, 0.15 unit of xanthine oxidase, 100 mM potassium phosphate, pH 7.0, in a final volume of 1.0 ml. Nucleoside phosphorylase (0.2 unit) was added only in the assay of partially purified enzyme. The red cell lysate normally contains endogenous nucleoside phosphorylase 100-fold in excess over adenosine deaminase (9). A millimolar extinction of 12.1 (10) was used to convert the increase in absorbance to μmol of inosine produced. Full-scale deflection on the recorder was varied from 0–1.0 to 0–0.1, depending on the amount of activity present. A lag period, which increased in duration as the amount of inosine formed was lowered, was observed. It is most likely caused by a slow rate of conversion of low quantities of inosine to uric acid by the two coupling enzymes. The activities were calculated from the steady state achieved after the lag phase. A number of the lowest activities were determined from continuous recording up to 60 min to increase the accuracy of the determination. An alternative radioactivity assay was used to confirm various data points. It was based on the separation of the ra-

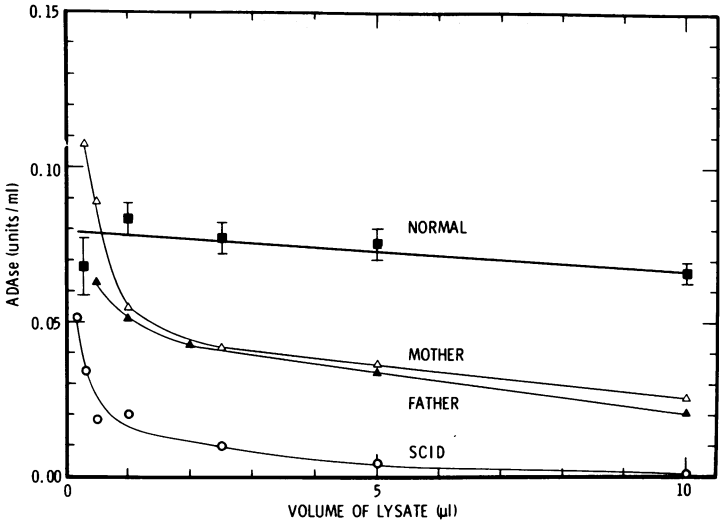

FIG. 1. Family I: specific adenosine deaminase activity as a function of the volumes of red cell lysate assayed. The data for normals were the average of determinations on five randomly selected individuals.

dioactive products on thin-layer chromatography in a butanol–0.15 M ammonium hydroxide solvent. This assay established that a small but significant quantity of adenosine deaminase contaminated various commercial preparations of xanthine oxidase. An appropriate subtraction of this activity was performed on all data points.

A unit of activity is defined as the amount of enzyme that deaminates 1 μmol of adenosine per min at 37° under steady-state conditions. Specific activity refers to the μmol of adenosine deaminated per min/ml of lysate.

Lysing of Red Cells. Red cells were separated from serum and buffy coat by centrifugation at 4° at 2000 × *g*. After washing three times with 0.154 M NaCl, a sample of cells was lysed by adding 3 volumes of 5 mM imidazole·HCl, pH 7.0. After the lysate was stirred at 4° for 45 min, it was centrifuged for 20 min at 25,000 × *g*. The supernatant was used for the various activity determinations.

CLINICAL FINDINGS

Family I. The propositus is the second child of healthy parents with no family history of immunologic disease. She had suffered from severe and recurrent infections since 10 weeks of age. In June 1971, age 10 months, she was referred to Dr. Robert A. Good at the University of Minnesota where the diagnosis of severe combined immunodeficiency (SCID) was made using the following criteria: lymphopenia, decreased immunoglobulins for age (G = 150, A = 5, M = 21 mg/100 ml) and absent or minimal response of peripheral lymphocytes to mitogens and allogeneic cells. Mixed lymphocyte cultures between the patient and her only sibling, female, showed no response although the patient had additional HL-A specificities not accounted for on a genetic basis. The patient received seven marrow transplants from her matched sibling before satisfactory immunologic competence was achieved. There were no red cell or chromosome markers to follow.

When evaluated at Memorial Sloan-Kettering Cancer Center (MSKCC), she was an active, healthy child with intact humoral and cellular immunity. Red cell adenosine deaminase (ADase) was tested for the first time and found to be missing on starch gel electrophoresis. The parents and sibling exhibited the 1-1 phenotype. On quantitative measurement in the clinical laboratory, the patient exhibited only traces of activity in the red cells; the parents had approximately half normal values and the donor sibling had normal activity. ADase was present in the patient's lymphocytes.

Family II. The propositus was referred to Dr. Robert A. Good and the Immunodeficiency Study Group at MSKCC in February, 1974 at 5 weeks of age. A male sibling had died with SCID which was diagnosed after death. Findings in the patient included an absent thymic shadow on radiologic exam of the chest, lymphopenia, markedly decreased numbers of B and T cells, abnormal immunoglobulins for age (no IgA or IgM; IgG of maternal origin = 480 mg/100 ml), and absent to diminished lymphocyte response to mitogens and allogeneic cells. His one sibling, male, was HL-A and MLC compatible with O, Rh negative red cells. The patient was A, Rh negative. Clinical laboratory determinations established that only traces of red cells and lymphocyte ADase activity were present; no activity was noted by starch gel electrophoresis. His mother and sibling had normal red cell ADase values whereas his father had a heterozygote level; ADase phenotype was 1-1.

Immunologic competence was restored in the infant after four bone marrow transplants from his sibling. He can now synthesize immunoglobulins and specific antibody; he has normal numbers of B and T cells; his lymphocytes have ADase activity and respond to mitogens. No red cell chimerism can be demonstrated at this time; he has only A negative red cells with minimal ADase by conventional clinical laboratory procedures.

RESULTS

The specific red cell ADase of a SCID child (after transplantation) and the parents, as well as that of a number of normal individuals, was studied as a function of the volume of lysate assayed (Fig. 1). At a volume of 10 μl, which is comparable to the volume used in previous studies (4, 11), the activity in the patient was essentially zero. Under these conditions, the parents' activity was depressed to a value considerably below normal, comparable to previous reports (4, 11). As the volume of lysate assayed was lowered, enzymatic activity appeared in the patient, which approached the normal range at the lowest volumes assayed. Each of the parents

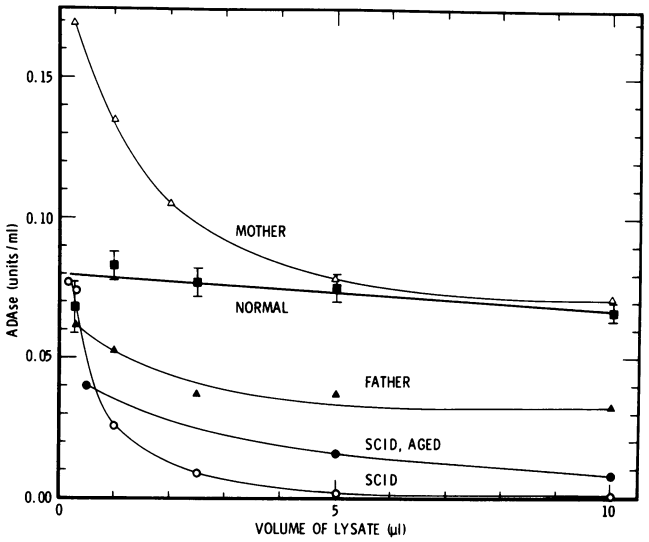

FIG. 2. Family II: specific adenosine deaminase activity as a function of the volume of red cell lysate assayed. The data for normal individuals are the same as in Fig. 1. "SCID, aged" refers to data obtained from the lysate of a SCID patient which was stored at 4° for about 1 month.

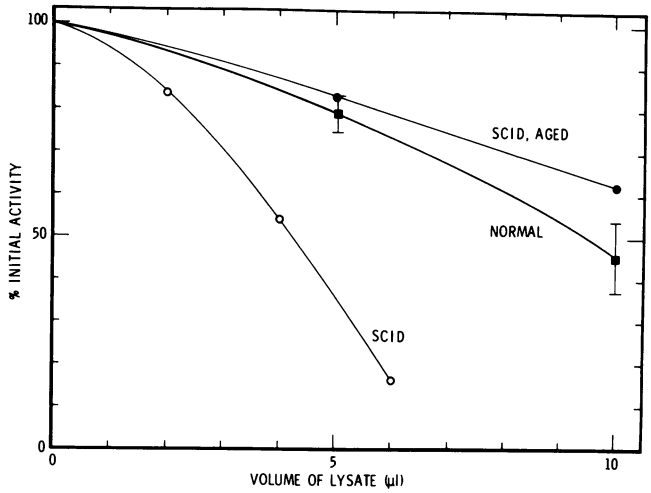

FIG. 3. Effect on the enzymatic activity of partially purified ADase of lysates from: red cells of five randomly selected normal individuals, red cells of a SCID patient, and a SCID patient's red cells that had been aged for 2 months at 4°.

showed the same increase to a normal value as the lysate volume was lowered. In contrast, the average specific activity of a series of normal individuals displayed only a small increase over the same range.

A similar study was subsequently performed on a second family containing a SCID patient who had also been successfully transplanted (Fig. 2). Again, there was observed a progressive increase in specific activity as the lysate volume was lowered for the child and the heterozygous parents. In this patient an even higher specific activity was observed. The interesting difference noted in this family was that the mother exhibited a normal specific enzymatic activity at standard lysate volumes (e.g., 10 μl). However, the drastic increase in specific activity to a value about twice normal which occurred with a lowering of the lysate volume clearly identified her as a carrier. It is of interest that one of her parents displayed an anomalously high specific ADase when 10 μl was assayed.

It was clearly of importance to provide further evidence that the activity obtained from the lysates of the SCID children was indeed due to enzymatic deamination by true red cell adenosine deaminase. We therefore performed a direct radioactive assay (see *Methods*) for the product, [^{14}C]hypoxanthine, in the absence of exogenously added xanthine oxidase. The results completely confirmed the high specific enzymatic activity observed at low lysate volumes for both patients. In addition, we tested the effect of N^6-methyladenosine and 2-fluoroadenosine on this activity. Both of these compounds at a concentration of 200 mM abolished all of the activity shown by the SCID patient in Fig. 2; the adenosine concentration for these studies was reduced to 0.075 mM. These compounds have been clearly established as potent inhibitors of the human red cell ADase (12). Thus, strong evidence exists for the enzymatic nature of the observed deamination in the patients.

When the lysate from one of the SCID children was allowed to stand at 4° for about a month, substantial activity appeared at 5- to 10-μl volumes assayed, where only traces had been observed originally (Fig. 2). Similar stimulatory effects have also been noticed with aging of the ADase lysate from all of the parents. Thus, for example, when the father of the SCID patient shown in Fig. 2 was examined after his red cells had aged at 4° for a month, there was a 70% increase in specific activity when the lysate was assayed at 10 μl. It thus became apparent that after appropriate aging the lysates of the carrier exhibited activity comparable to that observed in aged lysates from normal individuals. Similarly, after storage of both of the children's lysates at 4° for 2 months, neither could be classified as ADase deficient under conventional assay conditions.

Since the above data pointed to the possibility of a bound inhibitor in the cells of the SCID patients, it was of importance to test for the potential presence of free inhibitor. As shown in Fig. 3, the lysate from one of the SCID children clearly inhibited ADase from a normal subject to an extent greater than that observed using lysates from normal individuals. However, upon aging of the red cells, this inhibitory capacity was lost. It was only possible to test the second SCID patient after storage for 2 months at 4°, and the inhibition observed was no different from that found with normal lysates. The inhibition observed with normal individuals is most likely caused by an effect of hemoglobin on the coupled assay procedure. The addition of an amount of hemoglobin comparable to that present in the indicated lysate volumes also produced an apparent inhibition.

DISCUSSION

We have considered the possibility that the results of this study (i.e., the demonstration of full catalytic activity in the SCID children) might be a consequence of the successful bone marrow transplants. Unfortunately, pretransplant red cells were not available for testing by the methods used here. A number of lines of evidence suggest however that the transplantation itself was not the cause of the observed activity. Thus, no red cell chimerism has been demonstrated in the patient with A red cells (Family II) who received marrow from his type O sibling. In addition, both SCID children continue to have a lack of red cell enzyme when tested by conventional methods. This result is consistent with other studies which indicate that the red cells remain ADase deficient after transplant although lymphocytes and serum become positive (4). In addition, the stimulation of specific activity upon dilution is present in both parents of the two

children. Interestingly, one of the parents exhibited a red cell ADase value in the normal range, even under standard assay conditions. However, the specific activities measured at low lysate volumes indicated that this mother had the potential for abnormally high activity. Mothers of three other ADase deficient children are also known to have red cell levels well within the normal range (4).

One straightforward interpretation of the results is that in both the carriers of SCID and their homozygous children, a gene is active which programs the production of an inhibitor. This substance is apparently in equilibrium with active enzyme since normal specific activity can be restored simply by dilution. This concept gains strength from the observation that at least in one SCID patient there is sufficient free inhibitor present to inhibit partially purified enzyme. This kind of inhibition may not be generally observable in the patients, however, if the inhibitor is complexed with enzyme or other cellular components.

The data presented here represent a report of adenosine deaminase activity in the red cells of patients diagnosed as having the autosomal recessive form of SCID with ADase deficiency. However, according to previous reports a small residual amount of activity can be found in various tissues, including spleen, lymphocytes, and skin fibroblasts (4, 13, 14). These data have been previously interpreted as indicating the presence of an altered enzyme which exhibits a low specific activity. In light of the data reported here, this residual ADase might indicate that either: (*i*) the tissue-specific ADase (15) is more resistant to inhibition than the red cell form, or (*ii*) the amount of inhibitor capable of binding with ADase is reduced in tissues. In either case, it is a reasonable hypothesis that the tissues in these patients also contain a full potential for ADase activity.

In addition to containing red cell type ADase isozymes, various tissues exhibit isozymes of substantially larger molecular weight (15). According to one hypothesis, the red cell and various tissue forms of the enzyme are determined by independent genetic loci (15). The opposite conclusion of a single structural genetic locus was recently supported by the experiments of Hirschhorn and associates (16). The main evidence presented was the observation that on starch gel electrophoresis no activity could be detected in a variety of tissues from a SCID patient. If, however, as proposed here, an inhibitor is present which causes an apparent absence of ADase in all tissues of the SCID patient, the question of whether or not there exists a common genetic locus for the various forms of ADase must be considered as yet unanswered.

The effects of aging on the activity of red cell lysates are consistent with the concept of an inhibitor that is less stable than the enzyme itself. Thus, in the lysate of a SCID patient aged at 4°, activity was detectable under conditions where virtually none was observable on a fresh sample. Similarly, in all cases, the parents' activity was stimulated upon storage of lysates. It is also consistent with the presence of a labile inhibitor that an aged SCID lysate could not inhibit partially purified ADase in distinction to the inhibitory effect observed with the fresh lysate. These observations indicate that it should be possible to develop procedures to destroy the inhibitor without inactivating the enzyme itself.

Another interpretation of the data is that the SCID patient is homozygous for a somewhat altered ADase molecule in which proper conformation of the active site is maintained only in dilute solution. This would be the case if, for example, this modified enzyme polymerized into an inactive form when concentrated. At intermediate enzyme concentrations, an equilibrium would exist between an active monomer and inactive polymer. The results with the aging experiments could then be explained by a time-dependent dissociation to the active polypeptide chain. This hypothesis is actually a specialized case of the postulated inhibitor theory discussed above, since in this case the enzyme would serve as its own inhibitor.

The conclusions from these data may shed considerable light on the relationship of adenosine deaminase "deficiency" to severe combined immunodeficiency disease. A theory that was first considered (2) is that there is a large genetic deletion in the disease which affects both the enzyme and the immune response genes. This concept gained support from the initial finding that the loci for the red cell ADase and HL-A were genetically linked (17). This observation was important in the light of the fact that in mice and guinea pigs the HL-A locus is closely linked to immune response loci (18). However, it has subsequently been demonstrated in man that the HL-A locus and the locus for ADase could be assigned to separate chromosomes (5, 6).

The attractive hypothesis emerged therefore that adenosine deaminase deficiency might in fact be the direct cause of one form of immunodeficiency (12). Consistent with this theory is the fact that both defects alone are quite rare. Indeed, of the thousands of individuals who have been screened for ADase, only one case has been reported where there is an absence of the red cell form of the enzyme (19), but no evidence of a severe immunodeficiency. The possibility exists that it is adenosine itself which is the causal agent in the disease, since it has been demonstrated that relatively low concentrations of adenosine can inhibit the proliferation of certain lymphoid cell lines of mouse and human origin (7, 8). The recent report (20) of a nucleoside phosphorylase deficiency in a patient with a defect in T-cell immunity further emphasized the important role of adenosine metabolism in lymphocyte function.

The postulate of a genetically controlled production of an inhibitor is consistent with the accumulation of adenosine as one cause of SCID. However, it offers the intriguing additional possibility that it is the inhibitor itself which interferes with one or more steps in the immune response. In such a situation the ADase deficiency would be simply an indication that severe combined immunodeficiency was present, but not its cause. The more general possibility also exists that it is the postulated production of the inhibitor which is a causative agent for other forms of immunodeficiency. These cases presumably would not be characterized by an absent ADase if, for example, the particular isozymes present displayed a weak affinity for the inhibitor.

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