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Vitamin B_6–Responsive and –Unresponsive Cystathioninuria: Two Variant Molecular Forms

Abstract. *Cystathionase activity in a lymphoid cell line extract from a vitamin B_6–responsive patient with cystathioninuria was increased strikingly by pyridoxal phosphate. Immunodiffusion with antiserum to human hepatic cystathionase showed identity between this cystathionase protein and cystathionase from an extract of normal lymphoid cells. Neither an increase in cystathionase activity nor immunochemical identity was found using extract of cells from a B_6-unresponsive patient.*

Primary cystathioninuria (*1*) is the result of an inherited deficiency of cystathionase [L-cystathionine cysteine-lyase (deaminating), E.C. 4.4.1.1] (*2*), which cleaves cystathionine to cysteine and α-ketobutyrate, the last step on the pathway of conversion of methionine to cysteine. Of the approximately 25 cases of cystathioninuria that have been reported, all but two (*3, 4*) have demonstrated a diminution of the excretion of cystathionine after administration of massive doses of vitamin B_6. This report presents definitive in vitro evidence that in B_6-responsive cystathioninuria there is an increase in the requirement for vitamin B_6 and that the coenzyme acts directly by stimulation of the cystathionase protein. Evidence also is presented that B_6-responsive and B_6-unresponsive cystathionase deficiencies arise from different mutations.

The defect was investigated in long-term lymphoid cell lines because cystathionase activity in cultured skin fibroblasts is, as we and others (*5*) have found, either absent or very low and liver is less readily available. Lymphoid cell lines from previously reported B_6-responsive (*6*) and B_6-unresponsive (*3*) patients and from one B_6-responsive obligate heterozygote were established by utilizing phytohemagglutinin stimulation of leukocytes and incubation with Epstein-Barr virus (*7*). Cystathionase was assayed in cell lysates prepared by freezing and thawing, under conditions established as optimal for normal lymphoid lines. The cysteine formed was measured directly, either colorimetrically with acid ninhydrin (*8*) or radioactively with L-[^{35}S]-cystathionine as substrate (*9*). The product formed by extracts both of normal cells and of cells from a B_6-responsive patient was confirmed to be cysteine by oxidation with performic acid and separation by high-voltage electrophoresis of the radioactive cysteic acid formed (*10*). The production of cysteine was linear with protein concentration up to 1.0 mg per tube and with time up to 5 hours.

In the absence of added pyridoxal phosphate (PALP) an extract of normal cells showed considerable cystathionase activity (Fig. 1). In contrast, extracts of cells from patients with either B_6-responsive or B_6-unresponsive cystathioninuria exhibited no measurable cystathionase activity when assayed without added coenzyme. The addition of PALP at a concentration of 0.25 m*M* increased the cystathionase activity of the extract of normal cells slightly. A total of 15 normal cell lines was examined in the presence of 0.25 m*M* PALP and found to have a mean specific activity of 29.5 ± 1.95 (standard error) nmole of cysteine per milligram of protein per hour.

In the presence of 0.25 m*M* PALP: Extract of cells from the patient with B_6-responsive cystathioninuria had a specific activity of 4.6 nmole mg^{-1} hour^{-1} (approximately 15 percent of the mean control value). Extract of cells from this patient's father had a specific activity of 17.7 nmole mg^{-1} hour^{-1}, which is intermediate between the mean control value and that of the affected line and gives evidence for an autosomal recessive manner of inheritance. Extract of cells from the patient with B_6-unresponsive cystathioninuria had no discernible activity.

In the presence of increasing concentrations of PALP: The extract from normal cells showed little change in cystathionase activity to 0.75 m*M* and a slight decrease at 1.0 m*M* (Fig. 1). Extract of cells from the B_6-responsive patient had a steady increase in cystathionase activity; at 1.0 m*M* the specific activity was 9.3 nmole mg^{-1} hour^{-1} (31 percent of the mean normal value). Extract of cells from the B_6-unresponsive patient had no discernible cystathionase activity, even at 1.0 m*M* coenzyme.

The activities of *S*-adenosylmethionine

Table 1. Blocking of antibody-binding sites for cystathionase by cell extract from a patient with B_6-responsive cystathioninuria. See text for experimental details. The data below represent one of two experiments. The values are averages of duplicate determinations.

γ-Globulin	Extract		Inhibition of cystathionase activity in normal cell extract (%)
	First preincubation	Second preincubation	
None (buffered saline)		Normal cells	0
Preimmune globulin		Normal cells	3.0
Anticystathionase globulin		Normal cells	54.2
Anticystathionase globulin	B_6-responsive cells	Normal cells	39.8
Anticystathionase globulin	B_6-unresponsive cells	Normal cells	54.8

decarboxylase (E.C. 4.1.1.50) (*11*), a B_6-dependent enzyme, and of N^5-methyltetrahydrofolate-homocysteine methyltransferase (E.C. 2.1.1.13) (*12*) in extracts of both mutant cell types were within the range of normal activity. Addition of extract of each of the mutant cells to an extract of normal cells did not inhibit cystathionase activity.

Since cystathionase has been shown to be virtually absent from second trimester human fetal liver (*13, 14*), it was of interest to determine whether the lack of enzymatic activity in either the B_6-responsive or the B_6-unresponsive forms of cystathioninuria might represent failure of synthesis of the apoenzyme. In addition to being of theoretical genetic interest, such information is of potential practical value if enzyme replacement is considered: a genetic mutant incapable of producing the enzyme protein may treat normal enzyme used for replacement therapy as a foreign antigen and develop an immune response. Normal human liver cystathionase was purified and antiserums were obtained from two male New Zealand albino rabbits (*15*). Antiserum obtained from one rabbit was monospecific for cystathionase after absorption with human plasma proteins and was used for immunodiffusion studies. Antiserum from the second rabbit contained as well faint traces of two to three antibodies to contaminating liver proteins; it was used in immune-inhibition experiments after γ-globulin fractionation (*16*) and concentration to contain 28 mg of total protein per milliliter. Double immunodiffusion was performed on micro agarose plates (Hyland Laboratories). A single immunoprecipitin band of identity was present between the extract of human liver and that of the normal lymphoid cell line (Fig. 2A); this band joined a precipitin band from the extract of cells from the B_6-responsive patient. However, no precipitin band formed with the extract of cells from the B_6-unresponsive patient; this lack of cross-reactivity persisted even at a protein concentration for this extract of 110 mg/ml.

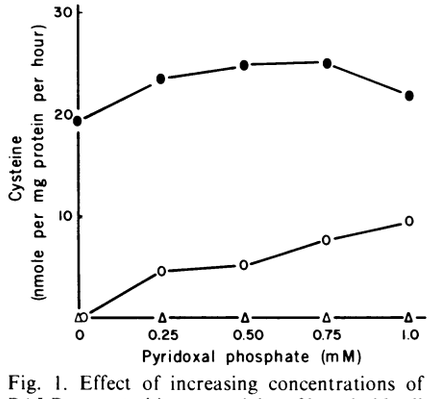

Fig. 1. Effect of increasing concentrations of PALP on cystathionase activity of lymphoid cell lines from a normal donor (●), a patient with B_6-responsive cystathioninuria (○), and a patient with B_6-unresponsive cystathioninuria (△). Similar curves were obtained in two separate experiments. The points are averages of duplicate assays. The incubation mixture contained 5.0 m*M* cystathionine and increasing concentrations of PALP (0 to 1 m*M*) in a total volume of 0.5 ml. All incubation mixtures were brought to *p*H 8.7. Incubation time was 3.5 hours at 37°C. Boiled enzyme blanks with each concentration of PALP were used to rule out the possibility of artifacts due to nonenzymatic reaction of PALP.

After the gel was washed to remove unreactive components, the antigen-antibody complexes were tested for cystathionase activity (*14*) by use of a stain employing cystathionine as substrate and reduction of nitro blue tetrazolium (Fig. 2B). This is feasible because the enzyme-antienzyme complex in immunodiffusion analysis usually retains some enzymatic activity, for the antibody seldom combines with the enzyme at the catalytic site (*17*). Enzymatic activity in the immune complexes formed with the extracts of normal liver and of normal lymphoid cells was demonstrated, but no cystathionase activity was demonstrable with the extracts of either the B_6-responsive or the B_6-unresponsive cells. When 1.0 m*M* PALP was added to the cell extract from the B_6-responsive patient before immunodiffusion, cystathionase activity was demonstrated in the antigen-antibody complex (Fig. 2C). Addition of PALP before immunodiffusion had no effect on the extract of cells from the B_6-unresponsive patient. These immunochemical results confirm the finding that when large amounts of PALP are added to an extract of cells from the B_6-responsive patient, cystathionase activity is expressed.

Finkelstein *et al.* (*18*), who found that cystathionase activity was greatly reduced in an extract of liver from a patient with B_6-responsive cystathioninuria compared to extracts of normal liver, reported that in vitro addition of 0.05 m*M* PALP did not increase the activity of cystathionase in the extract from the patient's liver more than it increased cystathionase activity in extracts from normal liver. The effect of larger amounts of PALP was not tested. In contrast, Frimpter (*2*) found a striking increase in cystathionase activity when PALP was added to extracts of liver from two patients with B_6-responsive cystathioninuria, compared to that found when PALP was added to extracts of normal liver. However, Frimpter did not show that the increase in cysteine formation was caused neither by stimulation of an alternative pathway of cysteine formation by PALP nor by nonenzymatic effects of the PALP. We performed an immune-inhibition experiment to rule out these possibilities.

Extracts of the B_6-responsive cells were preincubated at 37°C for 40 minutes with increasing amounts of γ-globulin fraction of antiserum; subsequently, cystathionase activity was measured in the presence of the precipitate formed by the enzyme-antibody complex. In this system, in contrast to immunodiffusion analysis, complete inhibition of catalytic activity can take place, because in the presence of antibody excess steric hindrance by the antibody is maximized (*17*). Inhibition of cystathionase activity (measured in the presence of 1.0 m*M* PALP) was approximately linear with in-

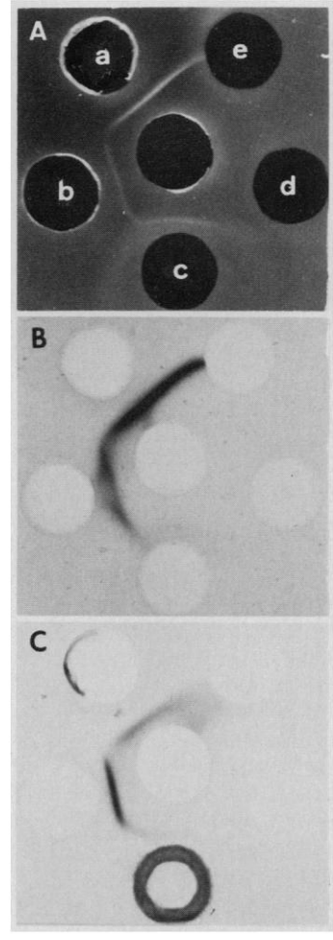

Fig. 2. Agarose double-immunodiffusion plate after 24 hours at 23°C. Monospecific rabbit antiserum to human liver cystathionase (10 μl) was placed in the center well. Outer wells contained extracts from human liver (a) and the following lymphoid cell extracts: normal (b), B_6-responsive cystathioninuric (c), and B_6-unresponsive cystathioninuric (d). Well e contained saline. All extracts from lymphoid cell lines had total protein contents of 65 to 70 mg/ml. (A) Unstained immune precipitate. (B) Same as (A) and stained for cystathionase activity. (C) Result obtained when 1.0 m*M* PALP was added to all extracts before immunodiffusion and then plate was stained as in (B).

creasing antibody concentration up to 40 percent inhibition with 0.5 μl of γ-globulin preparation; complete inhibition was produced with 5 μl. Preimmune γ-globulin did not inhibit enzymatic activity. This experiment gives a quantitative confirmation of the specific cross-reactivity of the cystathionase protein in B_6-responsive lymphoid cell lines with the antibody developed to the normal liver enzyme. Taken together with the immunodiffusion results, which directly show cystathionase activity staining of the abnormal enzyme when PALP is added (Fig. 2C), this experiment demonstrates that the effect of PALP is on the abnormal protein and not on a possible alternative pathway of cysteine formation. In addition, chemical catalysis of the cleavage of cystathionine is ruled out because product formation is proportional to residual enzyme protein and not to the concentration of cofactor, which was the same in all assay tubes.

The presence of cross-reacting, inactive apoenzyme in the cells from the B_6-responsive patient and its absence from the cells from the B_6-unresponsive patient were further confirmed in the following manner (Table 1). Crude extracts of the two cystathionase-deficient cell lines, containing 0.5 to 0.6 mg of protein, were preincubated separately at 37°C for 40 minutes with the amount of the γ-globulin fraction of antiserum previously shown to produce approximately 50 percent inhibition of the cystathionase activity of an extract of normal cells. The normal cell extract (0.43 mg of protein per milliliter) was then added, and the mixture was incubated again for 40 minutes. After the second incubation, substrate was added and cystathionase was assayed without addition of PALP in order to measure only the normal enzyme. It was found that the extract from the B_6-responsive cells had partially neutralized the inhibitory action of the antibody on the normal enzyme, resulting in a decrease in inhibition from 54.2 to 39.8 percent. The extract from the B_6-unresponsive cells, on the other hand, did not alter the degree of inhibition of the normal enzyme by the antibody. When the experiment was repeated with twice the concentration of extract from the B_6-responsive cells, inhibition of the normal cell extract decreased further to 29.5 percent; however, doubling the concentration of the extract from the B_6-unresponsive cells had no effect. These experiments demonstrate that the protein from the B_6-responsive cells is capable of blocking the antibody-combining sites for the normal enzyme; protein from the B_6-unresponsive cells cannot do so.

Identification of a catalytically inactive, cross-reacting cystathionase protein in lymphoid cell lines from a patient with B_6-responsive cystathioninuria and the lack of cross-reacting material in the cells from a B_6-unresponsive patient indicate that the two forms of primary cystathioninuria arise from different mutations, which cause different molecular defects. The B_6-responsive form results from the synthesis of an aberrant enzyme protein exhibiting altered interaction with the coenzyme, thereby resulting in an inherited increase in the requirement for vitamin B_6. The B_6-unresponsive form of cystathioninuria results from the complete absence of cystathionase or its reduction to a level undetectable by the enzymatic or the immunological methods employed. It is also possible that there may be present an enzyme protein so greatly altered that it has lost both cystathionase activity (even in the presence of excess cofactor) and the determinants necessary for recognition by specific antibody.

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Differentiation of T Cells in Nude Mice

Abstract. *Cells bearing the T-cell differentiation alloantigens TL and Thy-1 were enumerated in preparations of spleen and lymph node cells of* nu/nu *mice. Healthy* nu/nu *mice had few or no demonstrable* TL^+ *or* $Thy\text{-}1^+$ *cells whereas mice with viral hepatitis, including some born of* nu/nu × nu/nu *matings, had many. Healthy* nu/nu *mice were treated daily with the thymic hormone thymopoietin or with ubiquitin, polypeptides that induce the differentiation of* $TL^+Thy\text{-}1^+$ *cells from* $TL^-Thy\text{-}1^-$ *precursors in vitro. After 14 days, 20 to 40 percent of their spleen cells were of the* $TL^+Thy\text{-}1^+$ *phenotype typical of thymocytes and 10 to 25 percent of their lymph node cells were* $TL^-Thy\text{-}1^+$*, typical of later T-cell differentiation. Similar frequencies of such cells were found in untreated* nu/nu *mice suffering from severe viral hepatitis. These data conform to the current view that prothymocytes are preprogrammed cells whose maturation to thymocytes, normally induced in the thymus by thymopoietin, can be triggered by other agents under abnormal circumstances. Tests of T-cell function were not included in this study.*

The pleiotropic recessive mutant *nu* in mice (*1*) causes deficient growth of hair and failure to develop a normal thymus (*2, 3*). While there are reports of a thymic anlage in *nu/nu* embryos (*3*), this remains to be substantiated, and in postnatal life the thymus of *nu/nu* mice is absent or represented by a small epithelial rudiment lacking lymphocytes (*4*). The deficiency of T-cell function in *nu/nu* mice is attested by their inability to reject foreign skin grafts (*5*), with even avian skin being accepted for long periods (*6*).

The impaired development of T cells in *nu/nu* mice can be ascribed to lack of the maturation stimulus or stimuli normally provided by the epithelial thymus, because *nu/nu* mice have normal numbers of precursor cells capable of being induced to express the several cytotypic markers that distinguish members of the T-cell lineage. This is shown most incisively in the Komuro-Boyse (KB) assay in which cells from the spleen or bone marrow of *nu/nu* mice are induced in vitro to express these cell surface components, which include TL,