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Immunological Studies of β Galactosidase in Normal Human Liver and in G_{M1} Gangliosidosis

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INTRODUCTION

When assayed with synthetic artificial substrates, normal human liver contains at least two β galactosidase components which can be separated by electrophoresis [1] and by chromatography [2]. The two major components have optimal activity at pH 4 and pH 6.5 and are referred to as "acid" and "neutral" β galactosidase, respectively. Deficient activity of the acid β galactosidase has been reported in patients with G_{M1} gangliosidosis, an autosomal recessive glycolipid storage disease characterized by accumulation of G_{M1} ganglioside containing a terminal galactose residue [3–5]. We have used an antiserum against acid β galactosidase to study the immunological relationship between the two major enzyme components of normal liver and to investigate the nature of the genetic defect in G_{M1} gangliosidosis.

MATERIALS AND METHODS

Purified Acid β Galactosidase

Acid β galactosidase from human liver has been purified approximately 200-fold [6] by a modification of a previously published procedure [7]. The purified enzyme is optimally active at pH 4.0; it is stabilized by 0.1 M NaCl and is heat labile, in agreement with observations by other workers on crude liver preparations [5, 8]. The activity of our purified β galactosidase toward glycolipid substrates was kindly determined by Dr. K. Suzuki. The enzymatic activity toward G_{M1} ganglioside, asialo–G_{M1} ganglioside, and lactosylceramide was each found to be approximately 10% of the activity toward the synthetic substrate *p*-nitrophenyl-β-D-galactoside; no activity toward galactosylceramide was detected (K. Suzuki, personal communication).* Electrophoresis of the purified enzyme preparation on cellulose acetate gel at pH 5.5 [9] revealed a single band of β

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* We attribute our previous inability to detect hydrolysis of G_{M1} ganglioside [7] to the relative insensitivity of the assay employed.

galactosidase activity which corresponded in mobility with one of the bands in crude liver extracts (see below).

Preparation of Antiserum

After preparative polyacrylamide gel electrophoresis of purified β galactosidase [6], the position of the enzyme in the gels was determined by histochemical staining [10]. Gel segments containing approximately 0.3 mg of enzyme protein were then pulverized and injected subcutaneously into rabbits at 2-week intervals [11]. Adequate antibody titers were obtained after two such injections. Immunodiffusion and immunoelectrophoresis of liver extracts against this antiserum revealed two protein precipitin lines distinct from the enzymatically active β galactosidase immune complex. Since these co-purified proteins did not cross-react with β galactosidase, the precipitation of these unrelated proteins did not interfere with the assay of nonprecipitated enzymatic activity used in our experiments.

Preparation of Tissues

Liver samples were homogenized with a Tekmar Tissuemizer in 0.25 M sucrose with 0.5 M Tris buffer (pH 7.5; 20% wt/vol) and centrifuged at 105,000 *g* for 1 hr. Ammonium sulfate (350 g/liter) was added to the supernatant solution; after stirring for 20 min at 4°C, the precipitated protein was collected by centrifugation, dissolved in 0.05 M sodium phosphate buffer (pH 6.5) containing 0.14 M NaCl (phosphate-buffered saline), and dialyzed overnight against the same buffer. Extracts prepared by this procedure contain 90% of the original β galactosidase activity and are suitable for immunoprecipitation experiments.

Assay

The β galactosidase was routinely assayed with the synthetic substrate *p*-nitrophenyl-β-D-galactoside in 0.1 M acetate buffer (pH 5.0) with 0.1 M NaCl, as described previously [7]. Acid β galactosidase was assayed at pH 5.0 because the enzyme is unstable during short incubation times at lower pH [6]. Assay of neutral β galactosidase was carried out in phosphate-buffered saline. One unit of β galactosidase hydrolyzes 1 μmol of *p*-nitrophenyl-β-D-galactoside per hour.

CASE REPORT

Patient TN was a white male born after an uncomplicated pregnancy. Poor psychomotor development was noted during the first year. At 11 1/2 months of age, hypotonic quadriplegia and a significant degree of mental retardation were evident. Seizure episodes were observed at 15 months, and a cherry red macular spot was detected at that time. At 20 months of age, cells containing PAS and Oil Red O-positive material were observed in liver and intestinal mucosa biopsies. Moderate hepatosplenomegaly was present only in the last few months before death, which occurred at 2 1/2 years from aspiration pneumonia. No dysplastic changes in long bones or vertebrae were evident at the time of death. The clinical course and the autopsy findings suggested generalized gangliosidosis. This diagnosis was confirmed by demonstration of greatly increased levels of G_{M1} ganglioside in brain extracts [12] examined by thin-layer chromatography on silica gel.

Activity of β galactosidase assayed at pH 4.35 with 4-methylumbelliferyl-β-D-galactopyranoside [8] was less than 5% of normal in liver and cultured fibroblast extracts. The activities of other lysosomal enzymes were within the normal range (β glucosidase, arylsulfatase A and B) or elevated (β-D-*N*-acetylhexosaminidase A and B). Electrophoresis of liver extracts on cellulose acetate gel in 0.025 M citrate buffer, pH 5.5 [9] and pH 5.8, revealed only one band of β galactosidase activity, coincident with one of the bands in normal liver extract; the mobility of this band was different from that of

the purified acid β galactosidase. In these systems we observe three to five bands of activity in normal liver extracts; the correlation of these bands with bands A, B, and C observed by starch gel electrophoresis [13] is currently under investigation.

The patient's liver was stored frozen at −60°C for 20 months prior to the experiments reported here. Samples of liver tissue used as controls were obtained at autopsy from patients with cardiovascular disease and stored frozen.

RESULTS

Purified Acid β Galactosidase

Purified acid β galactosidase from normal human liver is optimally active at pH 4 (fig. 1*A*). This enzyme preparation was utilized to immunize rabbits. As expected, the resultant antiserum completely precipitates the purified enzyme (fig. 2, *A*).

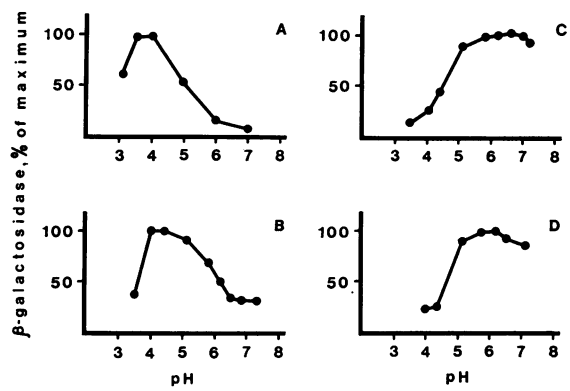

FIG. 1.—pH dependence of β galactosidase activity. Enzyme preparations containing approximately 0.2 U of activity at pH 5 were assayed for 15 min at each pH. Buffers were prepared by mixing 0.1 M citric acid and 0.2 M K_2HPO_4 in varying proportions. *A*, Partially purified acid β galactosidase; *B*, normal liver extract; *C*, nonimmunoprecipitable β galactosidase from extracts of normal liver; *D*, liver extract from patient with G_{M1} gangliosidosis.

Activity of β Galactosidase

In normal liver. The β galactosidase activity in extracts of normal human liver exhibits a broad pH optimum (fig. 1*B*). Antiserum to acid β galactosidase precipitates 75% of the total activity measured at pH 5.0 in normal liver extracts (fig. 2, *B*). The nonprecipitated enzyme is optimally active at neutral pH values between 6 and 7 (fig. 1*C*).

Thus, the broad pH dependence of total liver β galactosidase reflects the presence of two immunologically separable components, one optimally active at pH 4 and the other at pH 6.

In patient's liver. The specific activity of β galactosidase at pH 5.0 in extracts of liver from the patient with G_{M1} gangliosidosis was 17% of that of normal controls (table 1). None of this β galactosidase activity in the patient's liver was

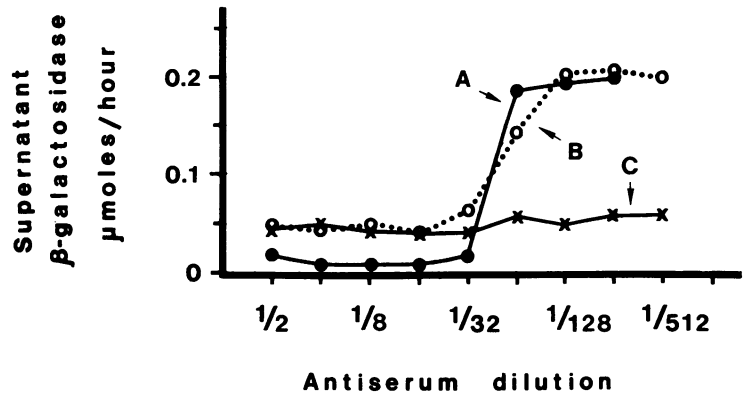

FIG. 2.—Immunotitration of β galactosidase. Enzyme preparations plus 50 μl of diluted serum were incubated in phosphate-buffered saline in a total volume of 0.15 ml for 18 hr at room temperature. Immunoprecipitates were removed by centrifugation for 5 min at 2,000 rpm. A 0.1-ml aliquot of the supernatant was assayed for 2 hr at pH 5 to determine the amount of nonprecipitated β galactosidase. *A*, purified acid β galactosidase, 0.2 U; *B*, extract of normal human liver, 0.2 U; *C*, extract of liver from patient with G_{M1} gangliosidosis, 0.05 U.

TABLE 1
β GALACTOSIDASE ACTIVITY IN EXTRACTS OF LIVER FROM NORMAL INDIVIDUALS AND PATIENT WITH G_{M1} GANGLIOSIDOSIS

	β GALACTOSIDASE ACTIVITY (U/mg PROTEIN)			
	pH 5	pH 6.5	RATIO pH 5/pH 6.5	% IMMUNO-PRECIPITABLE*
Purified acid β galactosidase	76.0	18.0	4.2	100
Normal liver extract	0.37†	0.12	3.2	75
Patient's liver extract	0.06	0.08	0.75	0

* % of total activity at pH 5 which is precipitated by antiserum to acid β galactosidase.
† Range 0.33–0.44; $N = 5$.

precipitated by the antiserum to acid β galactosidase (fig. 2, *C*). The residual β galactosidase in the deficient liver is optimally active at pH 6 (fig. 1*D*); its pH-dependent activity curve is essentially the same as that of the neutral, non-precipitable β galactosidase of normal liver (fig. 1*C*). At pH 6.5, the patient's liver had a level of galactosidase activity comparable to that of normal liver corrected for the contribution of acid β galactosidase at this pH (table 1). Thus, the genetic defect in this patient specifically inactivates acid β galactosidase without apparently affecting the activity of neutral β galactosidase.

Cross-Reacting Material in Patient's Liver

Evidence for the presence in the patient's liver of enzymatically inactive material which cross-reacts with the antiserum against acid β galactosidase was obtained by two methods.

In the first experiments, 0.1 U of purified β galactosidase was incubated with sufficient antiserum to precipitate most of the enzyme, leaving little activity in the supernate (fig. 3, *arrow*). The same volume of antiserum was then preincubated with increasing aliquots of extract from the patient's liver; after subsequent addition of the purified enzyme, increasing amounts of enzyme activity remained soluble in the supernatant (fig. 3, *A*). Thus the patient's liver contains material which binds to the same antibody sites as acid β galactosidase. (As discussed above, the residual β galactosidase in the patient's liver extract was not precipitated by the antiserum [fig. 3, *B*].)

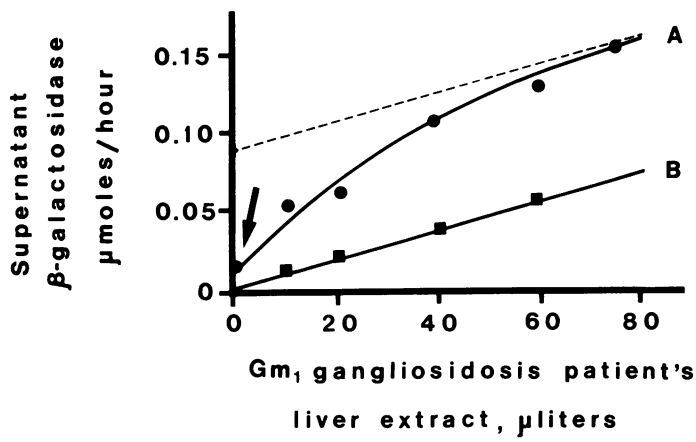

FIG. 3.—Competition between purified acid β galactosidase and liver extract from patient with G_{M1} gangliosidosis. Mixtures containing 1 μl of antiserum plus the indicated volumes of patient's liver extract were preincubated for 20 min at room temperature prior to the addition of 0.095 U of purified β galactosidase (*curve A*) or buffer (*curve B*) to a final volume of 0.15 ml. After overnight incubation and removal of immunoprecipitates, soluble β galactosidase was assayed. In the absence of patient's liver extract there was nearly complete precipitation of the purified enzyme (*arrow*). Preincubation of antiserum with liver extract displaced β galactosidase into the supernate (*curve A*). After preincubation with 75 μl of patient's liver extract, the amount of soluble activity approached the amount expected in the absence of antiserum (*dashed line*). As discussed in text, residual β galactosidase in the patient's liver is not precipitated (*curve B*).

In separate experiments, the antiserum was absorbed with lyophilized extract of the patient's liver and examined by double immunodiffusion on cellulose acetate gel [14] (fig. 4). Unabsorbed antiserum reacted with normal liver extract producing an arc of immunoprecipitate which retained enzymatic activity. However, the absorbed antiserum apparently lost the ability to precipitate acid β galactosidase from normal liver (fig. 4). As expected, the residual β galactosidase activity from the patient's liver was not precipitated by either antisera.

To determine whether other unrelated liver proteins might compete for anti-β galactosidase antibody, we compared the immunological equivalence of purified enzyme to that of β galactosidase in crude liver extracts (fig. 5). The serum equivalence of β galactosidase is identical for crude and purified enzyme. After

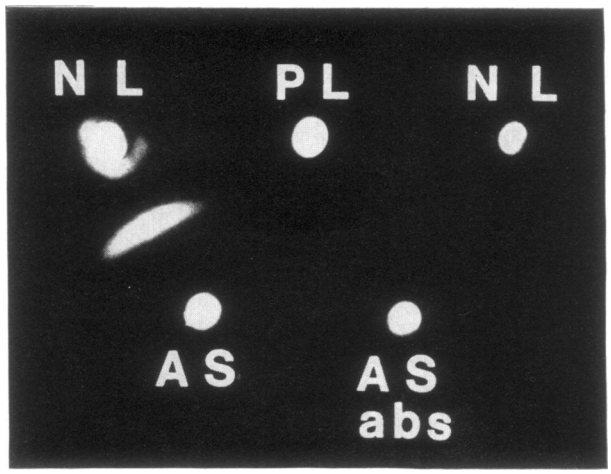

FIG. 4.—Double immunodiffusion of extracts of normal liver (NL) and liver from patient with G_{M1} gangliosidosis (PL) against antiserum to human acid β galactosidase (AS) and against the same antiserum absorbed with extracts of patient's liver (AS, abs).

Protein from the patient's liver homogenate was concentrated by precipitation with ammonium sulfate (350 g/liter). Antiserum (100 μl) was diluted and incubated with 50 mg of the liver protein for 1 hr at room temperature followed by 18 hr at 4°C. After centrifugation (40,000 *g*) for 1 hr at 4°C, the immunoprecipitate was removed and the absorption procedure repeated once. Immunodiffusion on cellulose acetate gel, which had been equilibrated in phosphate-buffered saline, for 60 hr was followed by washing in phosphate-buffered saline for 8 hr. The enzymatically active arc of immunoprecipitation between AS and NL was revealed by ultraviolet fluorescence of liberated 4-methylumbelliferone after incubation with 4-methylumbelliferyl galactopyranoside at pH 4.7.

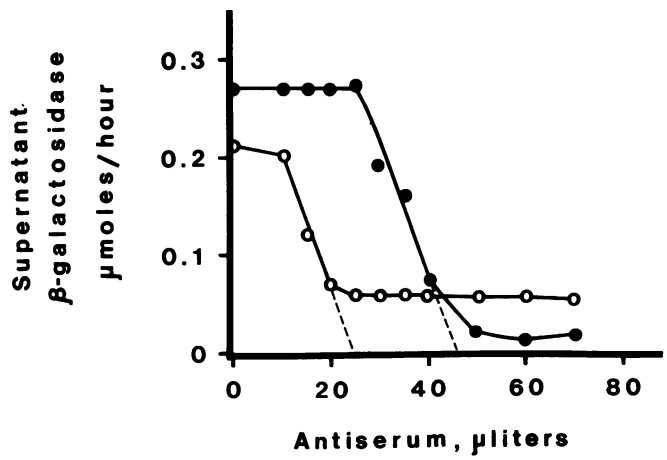

FIG. 5.—Equivalence point of purified acid β galactosidase and β galactosidase in liver extracts. Increasing aliquots of anti-β galactosidase serum (1/20 dilution) were added to tubes containing a constant amount of β galactosidase activity, either 0.27 U of purified enzyme (*closed circles*) or 0.21 U of normal liver extract (*open circles*). After overnight incubation, immunoprecipitated protein was removed and the soluble β galactosidase assayed (conditions as in fig. 2). In both cases, 1 μl of undiluted serum is equivalent to 0.12 U of precipitable β galactosidase.

subtraction of the nonimmunoprecipitable β galactosidase activity and correction for serum dilution, 1 μl of undiluted antiserum is equivalent to 0.12 U of enzyme activity in crude liver extracts and in the purified enzyme preparation. This result demonstrates that there are no other proteins in normal liver which react with the anti-β galactosidase antibody. Therefore, our observation of cross-reacting material in extracts of liver from the patient with G_{M1} gangliosidosis cannot be due to unrelated proteins.

DISCUSSION

Our results demonstrate that acid and neutral β galactosidase of normal human liver can be distinguished immunologically. Previously observed differences between the two enzymes included electrophoretic mobility, salt sensitivity, heat lability, and chromatographic behavior [1, 2, 8]. Taken together, the evidence suggests that acid and neutral β galactosidases are not closely related proteins. This is consistent with our observation that in the patient with G_{M1} gangliosidosis, a mutation which inactivates acid β galactosidase has no apparent effect on neutral β galactosidase activity.

In the liver of our patient with G_{M1} gangliosidosis, we have detected enzymatically inactive material which cross-reacts with anti-acid β galactosidase serum. The most likely interpretation is that a mutation within the structural gene for acid β galactosidase is responsible for the synthesis of inactive enzyme molecules which retain the immunological determinants of the normal enzyme. A structural gene mutation would be consistent with the observed codominant expression of alleles in heterozygotes, resulting in intermediate levels of acid β galactosidase activity in these individuals [4, 5]. The amount of cross-reacting material in the liver of our patient with gangliosidosis can be estimated from the data in figures 3 and 4 to be roughly 15%–30% of the amount of β galactosidase protein in normal liver. Further characterization of this cross-reacting material is desirable to prove that it is, in fact, inactive acid β galactosidase.

G_{M1} gangliosidosis has been differentiated into type 1 and type 2 according to differences in clinicopathological features such as age of onset, visceral involvement, bone dysplasia, and presence of cherry red macula [4, 5, 13]. The patient studied in the present work shows features of both types and cannot easily be classified according to these criteria. As more cases of G_{M1} gangliosidosis are described, the distinction between the two types becomes increasingly blurred [15]. It seems likely that most of these cases are the result of independent mutations with different phenotypic expression. The biochemical basis for the differences in clinical manifestations is still obscure, since tissues from a number of patients classified as type 1 or type 2 show the same profound deficiency of G_{M1} ganglioside β galactosidase activity when the natural substrate G_{M1} ganglioside is used [15, 16]. It had been suggested that patients with type 1 lack both acid and neutral β galactosidase activity, while patients with type 2 lack only the acid β galactosidase activity [17]. However, low levels of neutral β galactosidase activity in type 1 patients have recently been reported [5]. It seems unlikely that a single gene

mutation in type 1 G_{M1} gangliosidosis would affect two enzymes which, according to our results, appear to be immunologically unrelated. We suggest, therefore, that the reduced neutral β galactosidase activity in the liver of these patients may be secondary to in vivo inhibition by mucopolysaccharides. These accumulate in the viscera of patients with G_{M1} gangliosidosis [4] and are known to inhibit β galactosidase activity [18].

The immunological study of other cases of G_{M1} gangliosidosis may provide further evidence of genetic heterogeneity in this disease, analogous to that found in G_{M2} gangliosidosis. In Tay-Sachs disease (deficient activity of β-D-*N*-acetylhexosaminidase A), no enzymatically inactive material crossreacting with β hexosaminidase A has been found [14, 19, 20]. On the other hand, in Sandhoff's disease (deficiency of both β hexosaminidase A and B), the presence of enzymatically inactive cross-reacting material has been reported [20]. Furthermore, the cross-reactive material appears to be different in the two cases of Sandhoff's disease which were studied [21].

If enzymatically active immunoprecipitable acid β galactosidase is found to be absent in all patients with clinical variants of G_{M1} gangliosidosis, the specificity and sensitivity of the immunological identification of acid β galactosidase may also be applicable to the prenatal diagnosis of this disease. By double diffusion and histochemical staining with 4-methylumbelliferyl-β-D-galactoside, we can detect the enzyme in fewer than 10^6 cultured human cells. The presence of immunoprecipitable acid β galactosidase activity in amniotic fluid cells from normal individuals and heterozygotes would unambiguously differentiate these cells from those derived from individuals with G_{M1} gangliosidosis.

SUMMARY

An antiserum against purified "acid" β galactosidase from normal human liver did not cross-react with "neutral" β galactosidase, indicating that these two enzymes are not closely related. In the liver of a patient with G_{M1} gangliosidosis, the enzymatic activity of neutral β galactosidase was within the normal range, while acid β galactosidase activity was absent. Immunological experiments demonstrated the presence of enzymatically inactive material which cross-reacts with the antiserum to acid β galactosidase. This observation suggests that the enzyme deficiency in G_{M1} gangliosidosis results from a mutation within the structural gene for acid β galactosidase.

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NOTE ADDED IN PROOF.—Subsequent to the completion of this work, we have found that the "acid" and "neutral" β galactosidases of normal liver also differ in the pH dependence of their interaction with the inhibitor *N*-bromoacetyl-β-D-galactosylamine (M. Meisler, *Fed Proc* 33:1381, 1974).

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