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Globoid Cell Leukodystrophy: Deficiency of Lactosyl Ceramide Beta-Galactosidase

(sphingolipidoses/glycolipid hydrolase/lysosomal enzymes/Krabbe's disease/galactocerebrosidase)

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ABSTRACT **Activity of lactosyl ceramide β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was found to be extremely low in enzyme preparations from liver, brain, and cultured skin fibroblasts from patients with Krabbe's disease. Leukocytes from one set of parents had enzyme levels approximately half those measured in control leukocytes. The low activity observed for this galactolipid hydrolase is the fourth enzymatic deficiency noted for this genetic disease. Beta-galactosidase activity toward galactocerebroside, psychosine, and monogalactosyl diglyceride is also low in patients with Krabbe's disease. Other lysosomal enzymes measured were found to be in the normal range. This enzymatic defect may provide a better explanation for the pathological and chemical findings previously reported for this syndrome.**

At the present time there are four distinct genetic diseases involving storage of glycolipids ending in terminal β-galactoside linkage. These include Krabbe's disease or globoid cell leukodystrophy (GLD), lactosyl ceramidosis, G_{M1} gangliosidosis (generalized gangliosidosis), and juvenile G_{M1} gangliosidosis (ref. 1, recent review). Krabbe's disease is a severe disorder of children characterized clinically by loss of skills at 6 months of age, tonic seizures, optic atrophy, convulsions, and deafness with a rapid course ending in death before 2 years of age. The most striking pathological observation is the severe lack of myelin accompanied by simultaneous damage to cortical gray matter. The globoid cells appear to be the result of storage of galactosyl ceramide from myelin and can be produced in experimental animals by injecting this lipid into the brain. A number of reports have also documented an increase in lactosyl ceramide in the brains of children with this disease (2–4). A deficiency in galactocerebroside β-galactosidase activity in this disease was first reported by Suzuki and coworkers (5, 6). In a later study they found that psychosine (galactosyl β-sphingosine) β-galactosidase was also deficient in this disease, and they presented evidence that the same enzyme was acting on both substrates (7, 8). Recently, Wenger and coworkers reported that β-galactosidase with activity toward another myelin lipid, monogalactosyl diglyceride, was deficient in Krabbe's disease (9). Further studies in this laboratory indicate that the same enzyme may be acting on the three galactolipids, all having a galactosyl residue attached in a beta linkage directly to the lipid moiety. Enzymatic studies in tissues from Krabbe's disease patients by Austin *et al.* revealed normal or above normal activities for the following enzymes: glucocerebrosidase, sphingomyelinase, ceramidase, and lactosyl ceramide β-galactosidase (10).

Lactosyl ceramidosis, a lipid storage disease involving the accumulation of lactosyl ceramide in brain and viscera has been reported in one patient (11–13). Liver tissue and fibroblast culture from this patient were found to have only 10–20% of normal lactosyl ceramide β-galactosidase activity (11, 12). Fibroblast cultures from the parents of this patient had about half normal ability to degrade this lipid. This deficiency was postulated to be the primary enzymatic defect in this disease.

In the G_{M1} ganglioside storage diseases, the activity of β-galactosidase with specificity toward synthetic substrates, glycoprotein, G_{M1} ganglioside, and asialoG_{M1} ganglioside was found to be extremely low (14). This enzyme has been partially purified and found to have activity toward the synthetic β-galactoside substrate, 4-methylumbelliferyl, and G_{M1} ganglioside (15). No activity toward galactosyl ceramide was found in this partially purified preparation (A. Norden and J. O'Brien, personal communication).

In this paper we report the inability of enzyme preparations of brain, liver, and fibroblast culture from children who died of Krabbe's disease to degrade lactosyl ceramide *via* β-galactosidase. In addition, parents of one patient were found to have half of normal activity for this enzyme in preparations of leukocytes. This is the fourth galactolipid substrate whose catabolism is extremely low in this disease.

MATERIALS AND METHODS

Enzymes. Brain and liver tissue was obtained at autopsy from controls with no history of metabolic disease, from patients who died of Krabbe's disease, and from patients with other lipid storage diseases. These tissues have been stored frozen 1–5 years at −20°. Enzyme fractions were prepared from all specimens as previously described and kept frozen after the isolation of the 11,500 × *g* and 100,000 × *g* supernatant fractions (9). Pathological control samples included brain tissue from patients who died of the following: metachromatic leukodystrophy (MLD), Tay-Sachs disease, generalized gangliosidosis, and Fabry's disease. Liver tissue was obtained at autopsy from patients with MLD, and generalized gangliosidosis, and at biopsy from a child with lipid storage disease of unknown etiology involving storage of glycolipids and phospholipids in liver and spleen. Spleen tissue from a child with Niemann–Pick disease Type C was also obtained at splenectomy and stored frozen. Fibroblast cultures from a control and a Krabbe disease patient were

Abbreviations: GLD, globoid cell leukodystrophy, Krabbe's disease; 4MU, 4-methylumbelliferyl; MLD, metachromatic leukodystrophy.

grown and harvested as previously described (9). Leukocytes were prepared from 5 or 10 ml of heparinized blood. The pellet was homogenized in a Duall microhomogenizer in about 0.5 ml of distilled water. Protein concentration was determined on all fractions before assay according to the method of Lowry *et al.* (16).

The two children with GLD were diagnosed on the basis of clinical symptoms and confirmed by enzymological and pathological findings after death. One of the children had two siblings who died at age 10 months and 19 months with similar symptoms. The second child's family was tested for galactocerebroside β-galactosidase activity in leukocytes. Both parents and one sibling were found to have half normal activity for this enzyme and one other sibling was found to have a value in the normal range.

Substrates. Lactosyl ceramide was prepared from mixed brain gangliosides (Sigma) by acid hydrolysis followed by column and thin-layer preparative chromatography. The homogeneous lipid was found to have an equal molar ratio of glucose and galactose. The structure was confirmed by mass spectrometry and was found to have approximately 60% $C_{18:0}$ and 40% $C_{20:0}$ fatty acids (17). Additional lactosyl ceramide was purchased from Miles Laboratories. This was chemically synthesized and had the structure of *N*-stearoyldihydrolactocerebroside. Both of these lactosyl ceramides were made radioactive with tritium in the terminal galactosyl residue by the method of Radin *et al.* (18). The radioactive product was repurified on 0.5-mm silica gel thin-layer plates in either chloroform–methanol–water (60–35–8, by volume) or chloroform–methanol–water (24–7–1, by volume). Acid hydrolysis of the tritiated lactosyl ceramide revealed that at least 98% of the radioactivity was present in the terminal galactosyl residue. A small amount of radioactivity was present in the lipid portion of the molecule but this did not interfere with the assay or subsequent workup. Other glycolipids were prepared from the acid hydrolysis of gangliosides or as described previously (9). Sugars were separated on silica gel thin-layer plates in *n*-butanol–acetone–water (20–25–5, by volume). The end product of the enzymatic hydrolysis was identified as galactose after isolation of the upper phase and deionization with Amberlite MB-3. The following fluorescent substrates were purchased from Koch-Light: 4-methylumbelliferyl-β-GlcNAc (4MU-β-GlcNAc), 4-methylumbelliferyl-β-Gal (4MU-β-Gal), and 4-methylumbelliferyl-α-Gal (4MU-α-Gal).

Assay Procedures. The synthetic fluorescing substrates, and the galactocerebroside and monogalactosyl diglyceride β-galactosidase were assayed as described previously (9). Optimum conditions for lactosyl ceramide β-galactosidase were determined with enzyme preparations from control tissues. Our optimum conditions for hydrolysis are significantly different from the conditions reported by Radin *et al.* (18) and Gatt and Rapport (19). The [^{3}H]lactosyl ceramide was diluted with unlabeled lactosyl ceramide to a specific activity of approximately 500 cpm/nmol before assay. About 50 nmol of this lipid was added to a test tube with 400 μg of sodium taurocholate (Calbiochem) and 20 μg of oleic acid (Schwarz-Mann) and the chloroform–methanol (2–1, by volume) mixture was evaporated to dryness with nitrogen. To this residue was added 0.1 ml of acetate buffer (1 M, pH 4.4) and enzyme plus distilled water to a final volume of 0.2 ml. After incubation for 1 or 2 hr the incubation mixture was cooled on ice, 0.1 ml of galactose solution (0.5 mg/ml) was added followed by 1.5 ml of chloroform–methanol (2–1). After mixing, the lower phase was removed and the upper phase was washed with 1 ml of Folch theoretical lower phase. Exactly 0.5 ml of the upper phase was counted in the liquid scintillation fluid described previously (9). Control tubes contained labeled substrate plus heat denatured enzyme (100°, 5 min) and active enzyme kept at 0° until workup. These control tubes gave consistently 100 cpm which is 0.4% of the counts added.

TABLE 1. *Galactosyl ceramide and lactosyl ceramide β-galactosidase activity**

Substrate tissue	Galactosyl ceramide 100,000 × *g*	Galactosyl ceramide 11,500 × *g*	Lactosyl ceramide 100,000 × *g*	Lactosyl ceramide 11,500 × *g*
Brain				
Controls(3)	8.4–17.4	3.3–10.7	24.6–30.0	6.9–16.3
Tay-Sachs	15.8	9.7	17.9	5.1
MLD	6.9	5.7	12.7	12.2
Generalized gangliosidosis	8.9	10.0	21.5	24.2
Fabry's	27.8	7.4	50.0	15.8
Krabbe's(2)	0–0.05	0–0.03	0–0	0–0
Liver				
Controls(2)	0.57–2.43	0.68–0.84	2.03–6.2	2.2–12.9
Generalized gangliosidosis	2.2	4.2	6.5	2.9
MLD	1.3	1.3	1.8	2.4
Lipidoses(?)	3.9	5.2	3.9	5.2
Krabbe's	0	0	0	0
Spleen				
Niemann–Pick Type "C"	2.4	1.5	10.1	6.7
Fibroblasts	*Total homogenate*		*Total homogenate*	
Control	1.8		6.0	
Krabbe's	0.02		0.04	

* Enzyme activities are expressed as nmol of galactose cleaved per mg of protein per hr at 37°.

RESULTS

Lactosyl ceramide β-galactosidase activity was easily measured in all tissues examined except those derived from patients who died of Krabbe's disease (Table 1). In addition, these same patients were deficient in β-galactosidase activity toward galactosyl ceramide and monogalactosyl diglyceride (9). Activity toward other lysosomal enzymes was near normal (Table 2). The 11,500 × *g* supernatant fractions were found to have considerable enzymatic activity, especially in those derived from patients with lysosomal diseases. The expected deficiency of activities of 4-methylumbelliferyl-β-galactosidase in generalized gangliosidosis and 4-methylumbelliferyl-α-galactosidase in Fabry's disease is clearly noted. Fibroblast culture from a patient with GLD also clearly demonstrated the lack of lactosyl ceramide β-galactosidase activity. Leukocyte preparations were tested to determine the carrier status of one set of obligate heterozygotes using galactocerebroside, monogalactosyl diglyceride, and lactosyl ceramide as substrates. Table 3 shows that both parents had approximately one-half normal leukocyte activity towards all three substrates indicating the carrier status for Krabbe's disease. Activities of other lysosomal enzymes tested in these

TABLE 2. *Enzymatic activities* of other lysosomal enzymes*

Substrate tissue	4MU-β-Gal		4MU-α-Gal		4MU-β-GlcNAc	
	100,000 × *g*	11,500 × *g*	100,000 × *g*	11,500 × *g*	100,000 × *g*	11,500 × *g*
Brain						
Controls(3)	64.5–229	40.7–132	16.6–40.0	8.8–16.0	557–1548	599–936
Tay-Sachs	208	139	110	50.0	397	1565
MLD	96.2	63.6	38.3	25.1	1000	1336
Generalized gangliosidosis	9.61	15.5	30.9	65.4	1826	3193
Fabry's	181	63.8	0	2.3	1068	649
Krabbe's(2)	56.3–68.0	77.9–105	30.7–31.8	23.3–50	1544–1947	2070–2790
Liver						
Controls(2)	915–2304	396–413	69.2–115	58.2–73.4	1382–1461	1513–3389
Generalized gangliosidosis	15.4	20.2	154	283	7090	13,571
MLD	966	552	332	215	2150	1336
Lipidoses(?)	615	736	158	15.1	3942	4670
Krabbe's	698	279	51.9	55.1	7481	1628
Spleen						
Niemann–Pick Type "C"	193	173	158	209	2699	2881
Fibroblasts	*Total homogenate*		*Total homogenate*		*Total homogenate*	
Control	376		75		2403	
Krabbe's	516		148		3347	

* Enzyme activities are expressed as nmol of 4-methylumbelliferone cleaved per mg of protein per hr at 37°.

TABLE 3. *Enzymatic activities* in leukocytes*

Subjects	Substrates: Galactosyl ceramide	Lactosyl ceramide	Monogalactosyl diglyceride	4MU-β-Gal
Controls(5)				
average	3.6	6.3	1.0	60.4
range	2.9–5.3	5.1–8.8	0.7–1.8	47.9–74.9
Father of patient with Krabbe's disease	2.03	3.58	0.41	71.2
Mother of patient with Krabbe's disease	1.34	2.39	0.33	51.2

* Enzyme activities are expressed as nmol of galactose or 4-methylumbelliferone cleaved per mg of protein per hr at 37°.

parents were in the normal range. The activity of lactosyl ceramide β-galactosidase in Tay-Sachs disease, generalized gangliosidosis, Fabry's disease, Niemann–Pick Type C disease, and the unknown lipidosis was normal or above normal. Therefore, the demyelination observed in Tay-Sachs disease, generalized gangliosidosis, and MLD could not account for the absence of measurable activity for this enzyme in tissues from Krabbe's disease. Mixing experiments between control and Krabbe's disease enzyme preparations produced the expected amount of hydrolysis for lactosyl ceramide, indicating the absence of soluble inhibitors in the Krabbe's disease tissues. Lactosyl ceramide β-galactosidase activity was not present in tissues from GLD patients when measured at other pH values between 3 and 6. Competitive inhibition of lactosyl ceramide β-galactosidase by endogenous galactosyl ceramide in the Krabbe's disease tissues was not expected in light of the lack of "true" storage in this syndrome. No galactosyl ceramide or lactosyl ceramide could be seen on lipid extraction of the enzyme preparations used for assay.

DISCUSSION

These results clearly demonstrate the inability of enzyme preparations from patients with GLD to degrade lactosyl ceramide via β-galactosidase (Table 1). Both parents of a child who died of this syndrome had about 50% of control activity for this enzyme in their leukocytes (Table 3). This enzyme deficiency is the fourth reported for this genetic disease (5, 7, 9). The catabolism of galactosyl ceramide, psychosine, and monogalactosyl diglyceride has also been found to be extremely low. This new defect may explain some of the pathological and chemical findings reported for this disease and not adequately explained by the previously reported enzymatic defects. Involvement of gray matter in Krabbe's disease has been reported although it is often overlooked because of the severe lack of myelin (0.4% of normal) (20). The storage of lactosyl ceramide in the brain of patients with Krabbe's disease has been reported (2–4). However, Eto and Suzuki could not find any storage of this compound (21). Svennerholm and Vanier reported a marked increase in the concentration of lactosyl ceramide and certain gangliosides in brain tissue from patients with Krabbe's disease (2). They even indicated that a deficiency of lactosyl ceramide β-galactosidase could explain their findings. However, no enzyme studies were reported and they refer to the paper by Austin and collaborators which reported that this enzyme activity is increased in Krabbe's disease (10). Our data demonstrates that patients with Krabbe's disease have little, if any, ability to degrade lactosyl ceramide, as well as galactocerebroside and monogalactosyl diglyceride. This discrepancy is probably due to the change in assay conditions and the substantially higher activity for this enzyme measured in this laboratory.

Why is there so little myelin in the brains of these children? Apparently the synthesis of myelin and its constituents is turned off early in the life of the child (fetus). Suzuki and coworkers have postulated that psychosine may be the agent that causes destruction of the myelin-forming cells (7). However, psychosine has not been reported to be present in normal tissue or in brain tissue from patients with GLD. Perhaps lactosyl ceramide (cytolipin H) is the agent causing the severe symptoms observed in this disease. Lactosyl ceramide can occur throughout the body. It is the major glycolipid in white blood cells, and is an intermediate in the biosynthesis and degradation of erythrocyte glycolipids, gangliosides, kidney trihexosyl ceramide, and other more glycosylated sphingolipids.

A defect in the catabolism of lactosyl ceramide might be expected to be manifested in a more generalized storage pattern. One case of a genetic disease involving the storage of lactosyl ceramide and a defect in its catabolism via β-galactosidase has been reported (11). However, storage of lactosyl ceramide is seen in other storage diseases in which the primary enzymatic defect is known not to be lactosyl ceramide β-galactosidase (22–24). These include Tay-Sachs disease, generalized gangliosidosis, Hurler's, Hunter's, and Sanfilipo syndromes. A decrease in β-galactosidase activity toward synthetic substrates as well as G_{M1} ganglioside has been reported in these disorders. Only in generalized gangliosidosis has this enzyme defect been demonstrated to be the primary enzymatic lesion. The activity of galactocerebroside β-galactosidase in the one case of lactosyl ceramidosis has not been reported. In light of our results with lactosyl ceramide β-galactosidase in GLD, this should be examined. Possibly a structural gene mutation involving the activity of this enzyme toward lactosyl ceramide but not galactocerebroside could explain the difference in phenotype observed. However, the almost total lack of activity for lactosyl ceramide β-galactosidase in our Krabbe's disease patients does not present a picture of visceral involvement. It is hoped that answers to these questions will be found with further study.

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