DEFICIENCY OF MONOGALACTOSYL DIGLYCERIDE β-GALACTOSIDASE ACTIVITY IN KRABBE'S DISEASE.

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Summary: Monogalactosyl diglyceride has previously been demonstrated to be intimately associated with brain white matter, especially myelin. Enzymes responsible for its biosynthesis and degradation have been reported to be present in rat and mouse brain. In the present study, the β-galactosidase responsible for the degradation of this brain specific compound was demonstrated to be extremely deficient in brain, liver and skin fibroblasts from patients who died of Krabbe's disease. This deficiency is the third enzymatic block demonstrated in this disorder. The β-galactosidase activity toward galactocerebroside and psychosine is also extremely deficient. This finding provides new information about the substrate recognition pattern of this enzyme and about the possible etiology of globoid cell leukodystrophy.

Krabbe's disease (globoid cell leukodystrophy) is an inherited fatal disorder of the nervous system of infants. There is a pronounced decrease in the amount of myelin although that which remains appears to be qualitatively normal(1). The white matter also shows a severe decrease in the amount of glycolipids. The first enzymatic defect described for this disorder was a decrease in the activity of β-galactosidase with activity toward galactocerebroside, a prominent myelin lipid(2,3). Recently Miyatake and Suzuki reported that Krabbe disease tissue had a pronounced deficiency in their ability to degrade another galactolipid, psychosine(4). Although this compound has not been isolated, its biosynthesis in brain tissue was published by Cleland and Kennedy(5).

Monogalactosyl diglyceride has only been isolated from nervous tissue (including human) where it has been found to be closely associated with myelin(6,7). In addition, the enzymes responsible for its biosynthesis are most active during myelination in the brains of mice and rats, and in the jimpy mutant, which fails to myelinate, there is little if any biosynthesis. Steim found the fatty acid composition to be primarily palmitic (53%) and oleic acid (22%)(8). Wenger and coworkers reported that the biosynthesis

was best stimulated by dipalmitin in the presence of UDP-galactose(9). Enzymes were also present in brain which degraded monogalactosyl diglyceride to free galactose and diglyceride(10). In the previous work cited, the β-galactosidase was not further purified, nor was it ever studied in tissues from humans. In the present study, this enzyme was assayed in enzyme preparations derived from frozen human tissues, including brain and liver as well as cultured skin fibroblasts. In addition to control human tissues, enzyme activity was looked for in two frozen brain samples, one frozen liver sample and fibroblast culture from children who died from globoid cell leukodystrophy. For comparison, a frozen liver sample was assayed from a child who died of generalized gangliosidosis, another lipid storage disease involving storage of G_{M1} ganglioside and pronounced deficiency of β-galactosidase activity with specificity for synthetic substrates and G_{M1} ganglioside(11). The results indicate an almost complete absence of β-galactosidase activity toward monogalactosyl diglyceride only in children with Krabbe's disease. This is the first example of a defect in the catabolism of a diglyceride glycolipid as compared to the usual sphingosine-base glycolipid seen in most lipid storage diseases.

Materials and Methods: Liver and brain samples were taken at autopsy from children who died of non-neurological causes and frozen at -20°C until thawed for enzyme preparation. Samples from the two Krabbe disease patients and the patient who died of generalized gangliosidosis were obtained within hours of death and immediately frozen. All samples have been frozen in plastic bags for one to five years. Previous studies have demonstrated that most lysosomal enzymes will remain active for many years at -20°C. Fibroblast cultures were grown from a forearm skin biopsy from a control and from a child with clinical Krabbe's disease. The same child died at age 19 months and is one of the two samples of Krabbe brain. The child had typical globoid cells on sectioning of the brain. The fibroblasts were grown in MEM plus 15% fetal

calf serum and were subcultured for eight doublings before assay. Enzyme fractions for assay were prepared from brain samples according to the method of Miyatake and Suzuki(12). However, the 11,500g supernate fraction was found to have substantial glycosidase activity and was therefore saved for assay. Enzyme fractions were prepared from liver samples in the same manner using 0.25M sucrose. The confluent fibroblast cultures were harvested with a rubber policeman in buffered saline and centrifuged to obtain a pellet. This pellet was suspended in distilled water (0.1 ml per confluent T-250 flask), homogenized in a Duall micro-homogenizer and used without further treatment. All the above procedures were carried out at 4°C. The protein concentration was determined according to the method of Lowry et al.(13).

Tritium-labelled galactocerebroside and psychosine were prepared according to the method of Radin and coworkers(14), and Taketomi and Yamakawa(15). Monogalactosyl dipalmitin (MGD) was prepared chemically by a new method to be described elsewhere(16). The dipalmitin derivative was synthesized because this would more resemble the natural monogalactosyl diglyceride(8). Mild alkaline hydrolysis of this MGD, followed by acetylation, gave on gas-liquid chromatography-mass spectrometry a compound identical to the galactosyl-β-3-glycerol, a compound isolated from mild alkaline hydrolysis of spinach monogalactosyl diglyceride. The synthesized ^{14}C-labelled monogalactosyl dipalmitin was diluted to approximately 600 CPM per nmole before use. The assay conditions were identical for all enzyme samples. To 0.2 mg of sodium taurocholate (Calbiochem) and 0.02 mg oleic acid (Applied Sci.) was added 50 nmoles of either ^{3}H-galactocerebroside or ^{14}C-monogalactosyl dipalmitin and the mixture was evaporated to dryness with N_2. To this residue was added 0.1 ml acetate buffer (1M, pH 4.4), enzyme and water to a total incubation volume of 0.2 ml. After incubation at 37°C for 2 or 3 hours the tubes were chilled and 0.1 ml of galactose solution (0.5 mg per ml) was added followed by 1.5 ml chloroform-methanol (2:1). After mixing, the layers were separated by a slow spin and a 0.5 ml aliquot of the upper phase was taken for counting in 10 ml

scintillation fluid (0.208 Gm NPO; 20.8 Gm PPO; 322.8 Gm naphthalene; 1000 ml ethanol; 1600 ml dioxane; 1600 ml toluene). Using this method, boiled enzyme controls had less than 0.2% of the added counts in the upper phase. The sucrose in the 11,500 g supernate fraction did not interfere with the reaction or the subsequent workup. Psychosine β-galactosidase was assayed according to the procedure of Miyatake and Suzuki(12). Synthetic substrates were assayed with the same enzyme preparations also in a total volume of 0.2 ml as follows. The 4-methylumbelliferyl (4MU) substrates were obtained from Koch-Light and 0.1 ml of each (0.5 mM 4MU-β-gal; 10 mM 4MU-α-gal; 1 mM 4MU-β-NAcglu) was used per tube plus enzyme and water to 0.1 ml. The reaction was stopped with 3.8 ml glycine-carbonate buffer (0.085 M). Readings were made on a Perkin-Elmer Fluorescence Spectrophotometer with an exciter wavelength of 365 and an analyzer wavelength of 448.

Results: The results shown in Table 1 clearly demonstrate the inability of enzyme preparations from Krabbe's disease tissues to degrade the monogalactosyl dipalmitin as well as the previously reported galactocerebroside. The psychosine β-galactosidase activity (not shown) was also greatly deficient as reported by Miyatake and Suzuki(4). Activities of other lysosomal enzymes tested were near normal or increased. In the liver from the generalized gangliosidosis patient, the activity of 4MU-β-galactosidase activity was greatly reduced (1-5% of controls), however, the galactocerebroside and monogalactosyl diglyceride β-galactosidase activity was normal. The inability of the Krabbe's disease tissues to degrade this white matter specific glycolipid, monogalactosyl dipalmitin, was just as pronounced as the defect in galactocerebroside degradation. Initial experiments carried out with labelled monogalactosyl diglyceride prepared biosynthetically from spinach chloroplasts(17) gave identical results. The assay procedure used in these studies is different from that used by Suzuki and coworkers, but in our hands it provides a simple and reliable method for measuring glycolipid β-galactosidase in all tis-

TABLE 1. Glycosidase activity*in samples of liver, brain and fibroblast cultures.

Tissue / Substrate	4MU-β-gal	4MU-β-NAcglu	4MU-α-gal	Gal-cer	MGD
BRAIN					
Controls(3)					
100,000g	64,78,229	557,971,1548	16.6,29.7,40.0	8.4,8.8,17.4	1.8,3.26,3.29
11,500g	41,49,132	599,676,936	8.8,13.5,16.0	3.3,3.64,10.7	0.73,1.4,1.55
Krabbe(2)					
100,000g	58.5,68.0	873,1544	27.1,31.8	<0.02,<0.02	<0.02,<0.02
11,500g	78.0,107	2140,2790	23.3,93.4	<0.02,<0.02	<0.02.<0.02
LIVER					
Controls(2)					
100,000g	915,2304	1382,1461	69.2,115.2	0.516,2.78	0.543,2.77
11,500g	395,413	1513,3389	58.2,73.4	0.74,0.78	0.506,0.573
Krabbe(1)					
100,000g	698	7481	51.9	<0.02	<0.02
11,500g	279	1628	55.1	<0.02	<0.02
Generalized Gangliosidosis					
100,000g	15.4	7090	153.8	2.53	1.98
11,500g	20.2	13,571	283.3	4.79	3.46
FIBROBLASTS					
Control(1)	376	2403	75	1.82	0.52
Krabbe(1)	516	3347	148	<0.02	<0.02

*Activity expressed as nmoles cleaved per mg protein per hour

sues tried, including leukocytes. No galactocerebroside was detected in lipid extracts of lyophilized samples of each of the enzyme fractions.

Discussion: The above table demonstrates the pronounced deficiency of enzyme activity from Krabbe disease tissue to degrade monogalactosyl dipalmitin, a galactolipid reported to be found in white matter of nervous system. This is the third substrate for which a deficiency in the catabolic enzyme, β-galactosidase, has been reported for this genetic disease. The first reported deficiency was in galactocerebrosidase(2) and the second was in psychosine β-galactosidase(4). Both of these galactolipids have in common the sphingosine backbone as the lipid portion with psychosine lacking the amide fatty acid. Monogalactosyl diglyceride has the galactosyl moiety attached directly to a di-acyl glycerol backbone. The possibility of the same enzyme acting on these

three substrates in which a galactosyl residue is attached directly to a lipid moiety is proposed. Further studies on this enzyme in control brain tissue have demonstrated that MGD is a potent competitive inhibitor of galactocerebrosidase and that monogalactosyl diglyceride β-galactosidase is competitively inhibited by both galactocerebroside and psychosine. This inhibition was not seen with lactosyl ceramide, G_{M1} ganglioside, glucosyl ceramide or 4MU-β-galactoside. Further studies of this enzyme are in progress. Through these studies a better understanding of the substrate recognition pattern of this β-galactosidase will arise as well as a more complete picture of the etiology of globoid cell leukodystrophy (Krabbe's disease).

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