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Globoid Cell Leucodystrophy (Krabbe's Disease): Deficiency of Galactocerebroside β-Galactosidase*

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Abstract. Profound deficiency of a specific enzyme, galactocerebroside β-galactosidase, has been demonstrated in the brains, liver, and spleen of three patients with Krabbe's globoid cell leucodystrophy. The activity of this enzyme was normal in a variety of other cerebral diseases, including those with similarly devasted white matter. The lack of enzyme activity was not due to an inhibitor in the tissue, nor is it due to a shift in the pH optimum. The deficiency of galactocerebroside β-galactosidase as the primary enzymatic defect can account for the morphological and biochemical characteristics of this disease better than the previously reported deficiency of cerebroside-sulfatide sulfotransferase.

Globoid cell leucodystrophy (Krabbe's disease) is a hereditary metabolic disorder of children, characterized morphologically by almost total absence of myelin, severe gliosis, and the presence of characteristic multinucleated globoid cells in the white matter. A disproportionate lack of sulfatide, compared to cerebroside, in white matter has been considered the chemical hallmark of this disease,[1] and the recent report of deficient cerebroside-sulfatide sulfotransferase activity[2] appeared to have provided the enzymatic basis for the loss of sulfatide.

However, during recent analytical studies of Krabbe's disease, including isolation and chemical analysis of myelin,[3] we observed that the concentration of sulfatide in white matter was actually in the same range as in other white matter disorders in which almost total loss of myelin also occurs, such as Schilder's disease[4] or spongy degeneration of the brain[5] (sulfatide in percentage of dry weight: 0.22 in Krabbe's disease, 0.20 in spongy degeneration, and 0.05 and 0.31 in Schilder's disease). In these other diseases, however, cerebroside was also decreased drastically, roughly proportional to the loss of sulfatide. The concentration of cerebroside in white matter of globoid cell leucodystrophy was, on the other hand, relatively well preserved. The myelin isolated from our case had normal concentrations of cerebroside and sulfatide,[3] a finding that seemed inconsistent with the theory of chemically abnormal myelin, lacking in sulfatide, owing to deficient cerebroside-sulfatide sulfotransferase. We also found it difficult to reconcile other characteristics of this disease with the postulated lack of sulfotransferase activity. For instance, the characteristic globoid cells contain high concentrations of galactocerebroside[6] and can be elicited experimentally by galactocerebroside introduced into the brain.[7] Furthermore, significant activity

of cerebroside-sulfatide sulfotransferase has been found recently in two cases of globoid cell leucodystrophy.[8]

These considerations led us to believe that, in the past, we had placed undue emphasis on the lack of sulfatide. We therefore considered the possibility that relative preservation of cerebroside might be more important than the loss of sulfatide, and we examined the activity of the hydrolytic enzyme, galactocerebroside β-galactosidase. This enzyme has been extensively studied in rat brain by Radin and co-workers,[9-12] and our task was to apply their techniques to post-mortem human tissues. Data presented in this paper show that there is profound deficiency of galactocerebroside β-galactosidase activity in tissues of patients with globoid cell leucodystrophy.

Materials and Methods. Tissue specimens used for this study, including controls, were all obtained post mortem and kept frozen at −20°C or lower for periods of 5 months to 5 yr.[13] All specimens had been thawed at least once during the storage period.

Preparation of substrate: Galactocerebroside, specifically labeled by tritium at carbon 6 of the galactose moiety, was prepared from commercial bovine spinal cord cerebroside (Applied Science Labs.) by oxidation with galactose oxidase (Worthington Biochemical Corp.) and reduction by tritium-labeled sodium borohydride (New England Nuclear Corp.).[14] After purification by solvent partitioning and column chromatography, the final preparation had a specific activity of 50,000 cpm/mμmole. The cerebroside bands on thin-layer chromatogram (solvent: chloroform-methanol-water = 70:30:4) contained 97% of the total activity, and the only radioactive product after acid hydrolysis was identified as galactose by paper chromatography and counting. This labeled cerebroside was diluted with unlabeled cerebroside to give a specific activity of 4800 cpm/mμmole, and used as the substrate for subsequent enzyme assays. Substrates used for *p*-nitrophenyl glycosidases were all commercial products purchased from Pierce Biochemicals or Mann Research Labs.

Assay of galactocerebroside β-galactosidase: Most of the enzyme assays were performed on homogenates of gray and white matter, liver, and spleen. Weighed tissue was homogenized at a concentration of 5 mg tissue/0.1 ml with a buffer solution (50 mM Tris-HCl, 10 mM $MgCl_2$, 8 mM mercaptoethanol) containing 2% sodium taurocholate.[12] This homogenate was sonicated in an ice-bath for 1 min,[10] and then frozen and thawed four times to obtain activation of lysosomal enzymes which include galactocerebroside β-galactosidase. The incubation system and the procedures after incubation to assay the radioactivity of liberated galactose were those of Bowen and Radin,[12] except that their enzyme preparation was replaced by whole homogenate and that 200 μg of labeled cerebroside was added instead of 100 μg to minimize isotopic dilution by endogenous substrate. Blank tubes containing boiled homogenate were included, and the measured activity was corrected for the blank values. There was no spontaneous, nonenzymatic cleavage of galactocerebroside since blank values remained constant during a 3-hr incubation. In experiments to determine the pH curve of the enzyme in human brain, the pH 4.5 citrate buffer in the standard system was replaced by citrate-phosphate buffer of appropriate pH. A few experiments were also performed with crude enzyme preparations which were designed to eliminate endogenous substrate.[12] In these instances, the procedures described by Bowen and Radin[12] were followed exactly from enzyme preparation to the final assay of the liberated galactose. With either homogenate or prepared enzyme, reaction was linear for at least 3 hr, which was the usual incubation period.

Assay of *p*-nitrophenyl glycosidases: For the purpose of comparison, activity of lysosomal glycosidic enzymes, β-glucosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, and *N*-acetyl-β-galactosaminidase were also assayed with appropriate *p*-nitrophenyl glycosides used as substrates.[15]

TABLE 1. *Galactocerebroside β-galactosidase in gray matter.*

Specimen		Age (yr)	Galactocerebroside β-galactosidase*	p-Nitrophenyl β-Glycosidases† β-Galactosidase	β-Glucosidase	N-Acetyl-β-glucosaminidase	N-Acetyl-β-galactosaminidase
Krabbe's disease	1	1.5	12.1	2.6	1.8	64.5	8.2
	2	1	10.8	2.8	1.0	61.9	8.0
	3	1.5	5.7	3.0	4.0	113	13.4
Schilder's disease		10	104	2.3	2.7	86.7	11.5
G_{M1}-gangliosidosis							
Early onset		2	353	0.5	9.1	229	39.5
Late onset		3	189	0.4	8.2	126	15.9
Tay-Sachs disease		2	113	4.6	7.2	57.6‡	8.8‡
G_{M2}-gangliosidosis with total hexosaminidase deficiency		2.5	109	11.1	26.5	4.0	0.7
Hurler syndrome		11	136	1.9	2.6	69.8	8.5
Gaucher's disease (infantile)		?	85	2.5	0.7	41.9	5.3
Niemann-Pick disease		1	107	4.7	4.7	119	14.5
Metachromatic leucodystrophy		12	141	4.7	5.4	109	15.2
			Mean ± standard deviation = 123 ± 32§				
Normal		0.3	99	3.0	0.6	29.0	4.0
Normal		21	127	3.2	1.2	54.6	6.7
Normal		57	138	...	...	...	...
Normal		72	127	3.7	3.6	64.2	9.5
			Mean ± standard deviation = 123 ± 17				

* mμmoles/hr/gm. † μmoles/hr/gm. ‡ Hexosaminidase component A was deficient as demonstrated previously.[21] § Calculated by eliminating the value for early onset G_{M1}-gangliosidosis which appears pathologically high.

Results. Galactocerebroside β-galactosidase in the brain (Tables 1 and 2): In both gray and white matter of three brains from patients with globoid cell leucodystrophy, galactocerebroside β-galactosidase activity was 5–10% of the mean activity of the normal or pathological controls brains. There was no difference between normal and pathological control brains. Pathological controls were chosen to represent genetically determined metabolic disorders and also those diseases in which histological devastation of white matter occurs. In globoid cell leucodystrophy, β-galactosidase was not deficient when assayed with the synthetic substrate despite the deficiency of galactocerebroside β-galactosidase, indicating that only this specific β-galactosidase was deficient. This is in contrast to G_{M1}-gangliosidosis in which we found that galactocerebroside β-galactosidase was normal or even higher than normal whereas β-galactosidases assayed with *p*-nitrophenyl β-galactoside were extremely deficient, as previously demonstrated by Okada and O'Brien.[16] In globoid cell leucodystrophy, the other lysosomal *p*-nitrophenyl β-glycosidases were all normal in gray matter and higher than normal in white matter. This hyperactivation of uninvolved lysosomal enzymes in diseased tissue is a common finding among pathological tissues, as shown in many pathological controls.

Enzyme activities in liver and spleen (Table 3): The activity of galactocerebroside β-galactosidase was 5% normal in liver and 12% normal in spleen,

TABLE 2. *Galactocerebroside β-galactosidase in white matter.*

Specimen		Age (yr)	Galactocerebroside β-galactosidase*	p-Nitrophenyl β-Glycosidases† β-Galactosidase	β-Glucosidase	N-Acetyl-β-glucosaminidase	N-Acetyl-β-galactosaminidase
Krabbe's disease	1	1.5	17.7	6.0	1.4	134	15.5
	2	1	21.8	5.6	1.8	209	25.0
	3	1.5	7.5	7.7	3.3	215	25.6
Schilder's disease		10	211	3.8	1.6	240	27.6
G_{M1}-gangliosidosis							
Early onset		2	335	...	...	...	...
Late onset		3	193	0.4	7.6	102	12.4
Tay-Sachs disease		2	158	3.5	11.0	114‡	12.5‡
G_{M2}-gangliosidosis with total hexosaminidase deficiency		2.5	210	9.7	21.3	4.3	0.6
Hurler syndrome		11	130	0.9	0.7	49.5	6.0
Gaucher's disease (infantile)		?	191	1.5	0.4	25.0	3.0
Niemann-Pick disease		1	147	2.7	2.8	69.7	9.5
Metachromatic leucodystrophy		12	197	4.2	3.8	127	17.1
			Mean ± standard deviation = 197 ±59				
Normal		0.3	236	2.7	1.8	19.4	3.2
Normal		21	254	2.2	1.1	24.1	3.3
Normal		57	161	...	...	...	...
Normal		72	143	1.8	0.8	24.1	3.3
			Mean ± standard deviation = 199 ± 55				

* mμmoles/hr/gm. † μmoles/hr/gm. ‡ Hexosaminidase component A was deficient as demonstrated previously.[21]

while *p*-nitrophenyl β-galactosidase was twice normal in the liver and much higher than normal in the spleen. Therefore, in these visceral organs, galactocerebroside β-galactosidase was also severely deficient in globoid cell leucodystrophy, without deficiency of *p*-nitrophenyl β-galactosidase. Again some degree of activation of other lysosomal hydrolytic enzymes was noted, particularly in the spleen.

Discussion. Validity of the assay system: For most of our assays of galactocerebroside β-galactosidase, we used whole homogenate. Brain homogenates are known to cause errors due to different degrees of isotopic dilution by endog-

TABLE 3. *Galactocerebroside β-galactosidase in visceral organs.*

Specimen	Age (yr)	Galactocerebroside β-galactosidase*	p-Nitrophenyl β-Glycosidases† β-Galactosidase	β-Glucosidase	N-Acetyl-β-glucosaminidase	N-Acetyl-β-galactosaminidase
Liver						
Krabbe's disease 3	1.5	6.4	35.4	5.4	315	42.4
Normal	Newborn	125	43.1	4.0	119	42.4
Normal	30	113	34.0	2.8	178	27.7
Spleen						
Krabbe's disease 3	1.5	20.4	54.6	7.0	431	62.6
Normal	Newborn	157	20.0	2.1	81	30.3
Normal	30	186	12.4	0.7	172	20.2

* mμmoles/hr/gm. † μmoles/hr/gm.

enous substrate.[9, 10] This effect was greatest in normal white matter which had the highest concentration of endogenous galactocerebroside. All pathological white matter contained less than the normal amount of cerebroside. Therefore, isotopic dilution affected normal control white matter most, giving values somewhat lower than the true activity. For the amount of labeled cerebroside added in our assays, the estimated isotopic dilution by normal white matter was 1:1. In white matter of globoid cell leucodystrophy, the dilution was negligible, because only 10% of the normal amount of cerebroside was present in the tissue. If corrected for differences of isotopic dilution, the discrepancy between normal and Krabbe's disease white matter would be even greater. For practical reasons, therefore, we used the whole homogenate system instead of enzyme preparations. To ascertain the validity of this assumption, however, experiments were also performed with enzyme preparations designed to eliminate endogenous substrate by solubilization of enzyme by sonication and sodium taurocholate and its reprecipitation at pH 3;[12] the deficiency of galactocerebroside β-galactosidase in Krabbe's disease was even more striking than with the whole homogenate system (2–3% normal). Substrate dilution was not a problem in gray matter where the maximum dilution would not exceed 10%, or in visceral organs where endogenous galactocerebroside was not present.

Although we did not investigate the optimal conditions for cleavage of galactocerebroside in our homogenate system, the activities of control human white matter, when corrected for dilution of radioactive substrate by endogenous cerebroside, were comparable to values reported for rat brain.[12]

Validity of deficient galactocerebroside β-galactosidase: Several possibilities had to be excluded before we could conclude that tissues of patients with globoid cell leucodystrophy are deficient in galactocerebroside β-galactosidase. Postmortem treatment and storage conditions of the tissue were not responsible for the deficiency. The brain of patient 1 was removed within a few hours after death and kept in deep freeze for 7 months. Details about patient 2 were not available, but the organs of patient 3 were removed within 2 hr of death and stored in deep freeze for 5 months. All normal controls were treated the same way except that the time intervals between death and autopsy were generally longer. Most of the pathological controls had been stored much longer, some up to 5 yr, and thawed several times during this period. The high activities of the four *p*-nitrophenyl glycosidases in globoid cell leucodystrophy indicate satisfactory preservation of tissue. The deficiency was not merely due to the extreme devastation of the tissue, because gray matter, much less involved histologically, and the histologically normal liver and spleen also showed the same deficiency. Even for white matter, galactocerebroside β-galactosidase activity was normal in Schilder's disease in which there is also almost complete loss of myelin and oligodendroglia. White matter in the case of total hexosaminidase deficiency also exhibited an extremely severe loss of myelin but no loss of galactocerebroside β-galactosidase. The presence of an inhibitor in the tissue of globoid cell leucodystrophy was ruled out by mixing experiments: there was no decrease of cerebroside cleavage by normal brain homogenates when homogenate of Krabbe's disease brain was added to the incubation. Although all

standard assays were performed at pH 4.5, experiments were also done to ascertain that this deficiency was not due to a shift of the pH optimum of galactocerebroside β-galactosidase in Krabbe's disease. The activity was uniformly deficient throughout the pH range of 4.1 to 8.1, whereas there was an optimum around pH 4.5 for normal brain, as reported previously for rat brain.[9] The deficiencies of galactocerebroside β-galactosidase in gray and white matter were statistically highly significant, because of the number and the relatively narrow ranges of normal and pathological controls. The data for visceral organs were not sufficient for statistical treatment. The two normal controls consisted of one younger and one older individual compared to the patient with Krabbe's disease. Since these two controls were very close in the activities of galactocerebroside β-galactosidase, it is highly likely that the enzyme is also deficient in these visceral organs of the patient.

Relation to cerebroside-sulfatide sulfotransferase deficiency: Our data unequivocally demonstrated a profound deficiency of galactocerebroside β-galactosidase in globoid cell leucodystrophy. In this disease this is the second enzyme to be reported deficient; the other is cerebroside-sulfatide sulfotransferase.[2] Although the possibility of simultaneous deficiency of two enzymes or existence of two different diseases cannot, at this moment, be completely excluded, we must examine available data carefully before we draw conclusions. Both the previous report of deficient sulfotransferase and our data of deficient galactocerebroside β-galactosidase need to be confirmed. Sulfotransferase is, like other synthetic enzymes, relatively unstable and we were unable to assay it in our specimens because the conditions of our specimens were unsatisfactory. In one recent study, significant activity of sulfotransferase was found in one biopsy and one post-mortem specimen from patients with Krabbe's disease.[8] The post-mortem case studied was our patient 3. Sulfotransferase was measured on the unfrozen specimen within 2 hr of death. The original report by Bachhawat *et al.*[2] on deficient sulfotransferase included four control specimens (five, including one pathological control). Differences among the control values were sometimes almost 20-fold, despite the careful selection in regard to age and post-mortem treatment. It is unlikely that an enzymatic activity in carefully matched normal individuals differs by 20-fold. These data suggest a somewhat unstable nature of the assay procedure for cerebroside-sulfatide sulfotransferase. Therefore, we feel that further studies on additional cases and a larger number of control specimens are necessary to establish conclusively the deficiency of cerebroside-sulfatide sulfotransferase in globoid cell leucodystrophy.

Galactocerebroside β-galactosidase deficiency as the primary defect of Krabbe's disease: If galactocerebroside β-galactosidase deficiency is indeed the primary enzymatic defect of globoid cell leucodystrophy, we should be able to explain the morphological and biochemical characteristics of the disease on the basis of this deficiency. We must explain (*a*) almost total loss of myelin and oligodendroglia, (*b*) normal chemical composition of remaining myelin,[3] (*c*) morphological evidence of decrease in the amount of myelin during the illness,[17] (*d*) massive infiltration of globoid cells, and (*e*) absence of overt accumulation of cerebroside despite a block in the degradative pathway. These questions can

be resolved by two rather unique features of brain galactocerebroside. First, cerebroside is virtually nonexistent in the brain before myelination, and it is almost exclusively a constituent of myelin and oligodendroglia, as shown by almost complete loss of cerebroside in white matter of severely demyelinated brains.[4, 5] Second, galactocerebroside appears to be unique among sphingoglycolipids in its ability to elicit globoid cell infiltration when injected into normal rat brain.[7]

The following chronological steps could occur in the brain of a patient with galactocerebroside β-galactosidase deficiency. Before myelination, there is practically no cerebroside in the brain. Therefore, lack of cerebroside β-galactosidase is of little consequence, although the enzyme is normally present in low concentrations even at this premyelination stage.[12] As soon as myelination begins, which is just before birth in humans, newly formed myelin begins to undergo normal turnover.[18] This period coincides with a rapid rise of cerebroside β-galactosidase activity in normal brain.[12] In patients with Krabbe's disease, cerebroside from the catabolized myelin cannot be disposed of owing to the enzyme deficiency. This cerebroside elicits globoid cell infiltration. While the globoid cell reaction in normal brain subsides when digestion of excess cerebroside is complete,[19] globoid cells in Krabbe's disease remain permanently because cerebroside cannot be degraded. As myelination proceeds, more myelin turns over and more globoid cell infiltration results. However, before myelination can proceed much further, ever-increasing globoid cells overwhelm the oligodendroglial cells that can no longer survive this self-inflicting insult, and soon die. When the stage of massive death of oligodendroglial cells is reached, rapid myelin breakdown occurs, because myelin is an extension of oligodendroglial cell membrane, contributing more cerebroside to elicit further rapid increase of globoid cells. However, when all oligodendroglial cells die and all myelin is broken down, resulting in the maximum globoid cell infiltration, there will be no further production of myelin and, cerebroside being almost exclusively localized in myelin and oligodendroglia, no further production of cerebroside. Therefore, the total amount of cerebroside that can accumulate in the brain during the short life span of the patient is limited by the small amount of myelin produced before death of all oligodendroglial cells at what would have been normally a very early stage of myelination. This hypothesis explains the normal chemical composition of myelin in this disorder. The disease consists, therefore, both of early arrest of myelination and destruction of existing myelin. Both are the result of death of oligodendroglial cells but not the result of chemically abnormal myelin as previously postulated.

The above hypothesis explains the known disease process of globoid cell leucodystrophy satisfactorily. We suggest, therefore, that globoid cell leucodystrophy is characterized by genetically determined deficiency of galactocerebroside β-galactosidase. Despite lack of overt accumulation of galactocerebroside, it then belongs to the group of inherited disorders of sphingolipid metabolism involving lysosomal hydrolytic enzymes, that includes many lipid storage diseases such as Niemann-Pick disease, Gaucher's disease, various ganglioside storage disorders, metachromatic leucodystrophy, Fabry's disease,

and Wolman's disease. We must remember, however, that the possibilities of simultaneous deficiency of two enzymes and existence of two genetically different diseases have not been completely excluded.

Work is currently under way to determine whether or not galactocerebroside β-galactosidase can be assayed by our system conveniently on readily available material such as peripheral leucocytes, cultured fibroblasts, or amniotic fluid cells, and then to determine the enzyme activity in these materials from patients with Krabbe's disease and heterozygous gene carriers.[20] If heterozygous carriers show the enzyme activity intermediate between normal and homozygous patients, it will support our assumption that deficiency of galactocerebroside β-galactosidase is the primary genetically determined defect of globoid cell leucodystrophy. Then assay of galactocerebroside β-galactosidase could be useful for clinical and intrauterine diagnosis, and detection of heterozygous carriers of globoid cell leucodystrophy.

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