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Ceramide and Ganglioside Accumulation in Farber's Lipogranulomatosis.* (32554)

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(Introduced by K. J. Isselbacher)
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Farber's lipogranulomatosis is a rare disorder of infants and children with unique clinical and pathological features. Most of the available information about this disease has been reviewed recently(1). Eight cases have been reported; the fact that two of the patients were sibs suggests that the disorder is genetically determined. The pathological findings include striking granulomata of periarticular and subcutaneous tissues and a neuronal storage process. The granulomata contain a variable number of foam cells. Histochemical studies suggest that both the foam cells and the involved neurons contain glycolipids, although one group of investigators concludes that there was polysaccharide accumulation on the basis of a study of formalin-fixed paraffin-embedded sections(2). Uzman *et al* performed biochemical analyses and reported the accumulation of a lipoglycoprotein(3).

The pathogenesis of Farber's lipogranulomatosis is unresolved. Crocker *et al*(1) have emphasized that there appear to be two seemingly unrelated elements: "a proliferative infiltrative process . . . in the skin, subcutaneous tissues, tendons and viscera, and also an apparently intrinsic nervous system handicap." The first element would tend to place the disorder in the category of the histiocytoses, while the neuronal storage process suggests an inborn error of metabolism, as in the lipidoses. Resolution of this problem requires a decision as to whether lipid accumulation is the cause of the granulomata or is secondary to some other factor which causes tissue proliferation and breakdown.

We will present biochemical analyses of post-mortem tissues from a patient with Farber's lipogranulomatosis. We found a marked excess of ceramide both within granulomatous lesions and in visceral organs in which there was little or no granuloma formation. There was also a moderate accumulation of ganglioside. These findings suggest that lipid accumulation in Farber's lipogranulomatosis is a primary event and that the syndrome should be classified as one of the lipidoses.

Material and methods. A patient (MGH Case #132-24-00) with the typical clinical and pathological features of Farber's lipogranulomatosis died when she was 11 months old.† Tissues from this patient and suitable controls were taken at the time of the autopsy and stored at —50°C. The following control samples were available: liver, 7 samples from patients varying in age from 4 months to 9 years; kidney, 2 and 3 years; lymph node, 5 samples from patients varying in age from 5 days to 4 years; lung, 10 weeks, 6 years.

The tissues were extracted, partitioned, the gangliosides concentrated and the neuraminic acid (NANA) estimated by the methods described by Suzuki(5). Lipid hexose was estimated by a modification of the orcinol method of Sørensen and Haugaard(6), and chloroform:methanol extractable protein was determined by the method of Lowry *et al*(7).

To measure liver mucopolysaccharide con-

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† The ocular changes in this patient have already been published(4); a report of the clinical and pathological features is being prepared.

tent, the defatted tissue was digested with pronase and dialyzed. The acid polysaccharides were then precipitated with cetylammonium bromide(8), and the glucuronic acid content was measured(9). For tissues such as lymph node where only small quantities were available, precipitation with cetylammonium bromide was omitted.

Isolation and identification of the major tissue ganglioside. Gangliosides were separated on 0.25 mm silica gel G plates using a solvent system of n-butanol:acetic acid:water 60:20:20 (V/V/V), and rechromatographed on a similar plate developed in n-propanol:water 7:3 (V/V). The major ganglioside component was eluted from silica gel in methanol:water 4:1, and its molar composition determined. NANA was estimated on aliquots prior to hydrolysis. The sample was hydrolyzed with a mixture of methanol, hydrochloric acid and water(10) for 18 hours at 95°C. The hydrochloric acid was evaporated in the presence of toluene:absolute ethanol 50:50 (V/V) and the residue was taken up in either chloroform or methanol:water 4:1 (V/V). An enzymatic method using hexokinase and glucose-6-P dehydrogenase was found to be the most desirable method of evaluating the amount of glucose present in the hydrolyzate. The ratio of glucose to galactose and to amine sugars was determined by gas chromatographic techniques(11). Sphingosine was estimated by the method of Lauter and Trams(12).

Isolation and identification of ceramide. Ceramide was separated from other sphingolipids by chromatography on Florisil columns using 40 cc of solvent per gram of Florisil. In the elution scheme: hexane, hexane:diethyl ether 75:25 (V/V), hexane, chloroform, chloroform:methanol 19:1 (V/V), chloroform:methanol 2:1 (V/V), the compound was contained entirely in the chloroform:methanol 19:1 fraction. With the exception of the diethyl ether, all solvents were redistilled prior to use.

Three solvent systems were used for chromatography of this ceramide fraction on 0.5 mm silica gel G plates: I. chloroform:methanol:glacial acetic acid 192:5:8 (V/V/V); II. chloroform:methanol 95:5 (V/V); III. chloroform:methanol:water 96:28:3 (V/V/V). All the spots found when material from the patient's liver was chromatographed in solvent system I were individually eluted from preparative thin layer plates; authentic ceramide derived from galactocerebroside was treated in the same manner. The material was hydrolyzed, the fatty acids extracted in hexane and the sphingosine content of the residue determined. Fatty acids were chromatographed on thin layer plates using the solvent systems suggested by Eng *et al*(13). Paper chromatograms developed in ethylacetate:pyridine:water 120:50:40 (V/V/V) and stained with silver nitrate were used to determine if sugars were present in the hydrolyzate, while the presence of ninhydrin-positive bases was established by chromatographing the residue on 0.5 mm silica gel G plates developed in chloroform:methanol:2N ammonia 160:40:4 (V/V/V).

All the spots from the 19:1 chloroform:methanol fraction of the patient's liver which were shown to contain sphingosine as well as those spots derived from authentic ceramide were further purified by desalting on Sephadex (14) and then passing the eluate through silica gel G colums and eluting with chloroform followed by 19:1 chloroform:methanol. The infrared spectrum of the material obtained from each spot was then determined.

Results. Liver tissue taken from the patient who died of Farber's disease showed some increase in all forms of sphingolipid (Table I), as well as a slight elevation in mucopolysaccharides. However, only ganglioside and ceramide were found to be elevated in all the tissues studied and the increase in gangliosides paralleled the degree to which the tissue was invaded by foam cells. The most prominent ganglioside spot in liver, lymph node and nodule on thin layer chromatography plates had the R_f values described for hematoside (5). In liver preparations this spot was found to contain sphingosine-glucose-galactose and NANA in the following ratio: 1.05:1.00:1.15:0.91; no amine sugars were detected. It was concluded, therefore, that this substance was an hematoside. Hematoside was found to account for 61.5% of the ganglioside NANA in the patient's liver and $61.6 \pm 6.5\%$ of the ganglioside NANA in five normal livers. Thus, no single ganglioside appeared to be disproportionately increased.

TABLE I. Levels of Ceramide, Gangliosides and Certain Other Constituents, in the Post-Mortem Tissues of a Patient with Farber's Lipogranulomatosis.

	Liver		Nodule	Femoral lymph node		Mesenteric lymph node		Lung		Kidney	
	Farber's	Control	Farber's	Farber's	Control	Farber's	Control	Farber's	Control	Farber's	Control
Ceramide sphingosine	4130	62 ± 11	3100	2320	41 ± 13	560	49	730	23 ± 7	280	30 ± 7
Ganglioside NANA	86 ± 27	31 ± 3	410	210	47 ± 17	77	37 ± 12				
Lower phase hexose*	359 ± 10	148 ± 40		321	258 ±138	280	208 ±173	182	212		
Lipid phosphorus	800 ± 20	810 ± 50		647	632						
Alkali stable phosphorus	204 ± 7	164 ± 8									
Proteolipid protein	1475 ± 35	2113 ±496									
Total lipid	6.93 ±0.13	3.86 ±0.34	4.59	4.98	7.80	3.06	8.62	4.19	1.8 ± 0.2	3.50	3.05 ± 0.9
Polysaccharide glucuronic acid	128 ± 5	57 ± 6				1565†	1450†				
Polysaccharide hexosamine	101 ± 2	44 ± 2									
Infiltration by foam cells	1+		3+	3+		1+		2+		0	

Values for total lipids are expressed as per cent of fresh weight; all other biochemical results (except as indicated by †) are expressed as gamma per gram of fresh weight.

* Refers to glycolipids other than gangliosides which are present in the lower phase after solvent partition according to Suzuki(5).

† Expressed as gamma per gram of dried, defatted tissue residue.

The presence of an excessive amount of ceramide in the patient's tissues was suspected after chromatographing lower phase lipids in chloroform:methanol 95:5 (V/V). The identity of the ceramide was established after it was isolated from other sphingolipids on Florisil columns; the 19:1 chloroform: methanol fraction derived from this column was essentially free of hexose and phosphorus. Material taken from this fraction was shown to contain 4 major spots when chromatographed on thin layer plates in solvent system I: spot *A*, R_f 0.63; *B*, R_f 0.39; *C*, R_f 0.33; *D*, R_f 0.13. The identity of spot *A* is unknown. However, it is also present in considerable amount in control tissue. The remaining spots contained all the sphingosine found in this fraction in the following proportions: *B* + *C*, 89.9; *D*, 10.1. The R_f values of spots *B* and *D* were identical to those of authentic ceramide in the 3 solvent systems used. Upon hydrolysis, spots *B* and *C* appeared to contain normal fatty acids and spot *D* hydroxy-fatty acids; none of the 3 spots contained hexose. The infrared spectra of the material in these spots was similar to that obtained from the authentic ceramide and agreed with the spectra previously published for this compound(15).

The accumulation of ceramide was the most pronounced biochemical abnormality since this compound, which is generally found only in trace amounts in normal tissue, accounted for as much as 13% of the lipid in the patient's organs. However, the increase in ceramide did not clearly parallel the presence of foam cells since the biochemical abnormality was most severe in the liver which was only mildly involved histologically, and was significant in the kidney where no foam cells were seen.

Samples of liver from this patient and from 4 controls were submitted to Drs. Julian Kanfer and Roscoe Brady who found that ceramidase, galacto- and gluco-cerebrosidase and sphingomyelinase activity were within normal limits.

Discussion. It is likely that the glycolipid within the foam cells of the granulomatous lesions is a ganglioside. This conclusion is based upon the fact that the ganglioside concentration was increased to a greater extent

than that of other glycolipids or polysaccharides, and in the tissues studied the degree of ganglioside excess appears to correlate with the amount of glycolipid accumulation seen in the tissue sections. The most striking abnormality was found in the subcutaneous nodule in which the ganglioside concentration was equal to that of normal brain gray matter.

Unlike what has been observed in Tay-Sachs disease(16), Hurler's syndrome(17) or generalized gangliosidosis(18), the ganglioside pattern in Farber's disease was not abnormal. This observation made it unlikely that there was a primary disturbance of ganglioside metabolism, and caused us to search for other biochemical abnormalities. The striking and consistent excess of ceramide suggests that this represents, or is closely related to, the primary biochemical disturbance. The ceramide excess has escaped detection histochemically, probably because this molecule lacks groups with specific staining reactions.

The biochemical basis for the accumulation of ceramide is unknown. The marked ceramide excess, combined with a moderate accumulation of gangliosides, and a slightly elevated cerebroside concentration, suggested that this disorder might be the result of a ceramidase deficiency. However, Kanfer and Brady have assayed this enzyme in the post-mortem liver of our patient, and found its activity to be normal. This makes it unlikely that the ceramide accumulation is due to a deficiency of this degradative enzyme, although one cannot rule out the existence of other, as yet undescribed, ceramidases which may not have been included in this enzymatic assay. Other pathogenetic mechanisms, such as the occurrence of an abnormal ceramide, or the deficiency in a lipid-protein linkage analogous to the defective mucopolysaccharide-protein linkage postulated in Hurler's disease(19), are being investigated.

Summary. An analysis of the lipids of various organs of an 11-month-old female dying of Farber's Disease indicated a marked increase in ceramide varying from 8 times normal in the kidney to 66 times normal in the liver. Ceramide made up 13% of the liver lipids. There was also a slight increase in other lipids in the liver, but aside from the ceramide, only the gangliosides were increased in all organs tested. The increased ganglioside was more marked in subcutaneous nodule and varied proportionally with the number of foam cells seen in the tissue. The mechanism by which these lipids are stored in Farber's Disease remains to be determined.

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