Glycolipid and Mucopolysaccharide Abnormality in Fibroblasts of Fabry's Disease

Abstract. *Cultures of skin fibroblasts from a patient with Fabry's disease showed an accumulation of the glycolipid, galactosyl-galactosyl-glucosyl ceramide. Such cells also showed metachromasia on staining with toluidine blue and a markedly elevated acid mucopolysaccharide content.*

Fabry's disease (angiokeratoma corporis diffusum universale) is a generalized X-linked heritable disease characterized by the occurrence of birefringent lipid deposits throughout the body, with especially high concentrations in the kidneys, heart, and blood vessels (*1*). The predominant lipid deposited in the kidneys has been characterized as a trihexosyl ceramide (GL-3) with the following structure (*2*): gal(1→4)gal(1→4)glc-ceramide (*3*). Associated with this glycolipid in kidney is another glycolipid, gal(1→4)gal-ceramide, which accumulates to a lesser extent than the GL-3. The digalactosyl ceramide has not been found in any other tissues examined.

Extracts of biopsies of small intestine from two patients have been shown to be deficient in the enzyme that is apparently specific for the hydrolysis of the terminal D-galactose residue of the deposited trihexosyl ceramide (*4*). A biopsy of the intestine from the mother of one of the patients showed enzyme activity intermediate between that of the patient and that of a normal individual. The accumulation of GL-3 in Fabry's disease has been considered to result from an enzymic block in the stepwise degradation of globoside from erythrocytes and other tissues by specific glycosidases. Globoside isolated from kidney and erythrocytes (*5*) has the following structure: galNAc(β1→3) gal(β1→4)gal(β1→4)glc-ceramide.

Fibroblasts cultured from skin are useful in both biochemical and genetic studies of mucopolysaccharide and lipid storage diseases (*6, 7*). Accumulation of dermatan sulfate in fibroblasts has been demonstrated in a Hurler variant in which increased amounts of lipids were found in addition to the mucopolysaccharide (*8*). Recently, the presence of metachromatic granules in cultured fibroblasts has been shown to be a more widespread phenomenon since it also occurs in a variety of diseases not previously associated with abnormalities of mucopolysaccharide metabolism. Among these are Gaucher's disease, juvenile familial amaurotic idiocy, cystic fibrosis (*9*), late infantile amaurotic idiocy, Krabbe's disease, and other unrelated disorders (*10*).

We have studied cultured fibroblasts from the skin of a patient with Fabry's disease and now report the isolation of the characteristic trihexosyl ceramide and related glycolipids, together with the isolation and identification of the acid mucopolysaccharides accumulated in these cells. The patient was a 29-year-old male with the classical symptoms of Fabry's disease, who had elevated GL-3 in his plasma (*11*). Methods for tissue culture, staining of fibroblasts, and isolation and identification of mucopolysaccharides have been described (*7, 12*).

Total lipids were extracted with a mixture of chloroform and methanol (2:1) from approximately 150 mg of exhaustively washed cells that were scraped from the culture dishes and then lyophilized. The extract (300 ml) was washed with water by the Folch procedure. The crude lipids were fractionated on a small column of silicic acid (4 g) into neutral lipids, which were eluted with chloroform; glycolipids, which were eluted with a mixture of acetone and methanol (9:1); and polar lipids, which were eluted with methanol. The fraction of crude neutral glycolipids was further separated on preparative thin-layer plates developed in a mixture of chloroform, methanol, and water (100:42:6). The individual lipids were recovered by scraping areas from the plates after development in iodine vapor and elution of the lipid with chloroform-methanol. After methanolysis of the glycolipids with 0.75*N* HCl in dry methanol at 80°C for 24 hours and neutralization with solid silver carbonate, methyl esters of long-chain fatty acids were removed by extraction with hexane; the hexose methyl glycosides were estimated by gas chromatography of their trimethylsilyl ethers with D-mannitol as an internal standard for quantitation. This procedure is a slight modification of one described for the isolation and quantitative estimation of neutral glycolipids from human plasma and erythrocytes (*13*).

When stained with toluidine blue, fibroblast cultures of the patient showed marked metachromasia. Table 1 shows the increase of acid mucopolysaccharides in Fabry's. In contrast to abnormal distribution of acid mucopolysaccharides in Hurler's (*7*) and Marfan's syndromes (*12*), the distribution of mucopolysaccharides in Fabry's disease is similar to that of normal and cystic fibrosis cells (*14*).

There was an increase in the amounts of both polar lipids and glycolipids isolated from Fabry cells, as compared with normal cells (Table 2). The glycolipid fraction showed the expected increase in the concentration of GL-3, whereas glucosyl ceramide and the tetrahexosyl ceramide were present in normal amounts and lactosyl ceramide was slightly decreased. The specific accumulation of GL-3 in fibroblasts suggests the possibility of the same enzymatic defect in glycolipid metabolism found previously in biopsied tissues (*4*). Further studies are under way on the nature of the polar lipid fraction.

The relation of an accumulation of mucopolysaccharides to an inherited disorder of glycolipid metabolism is not clear. The same results that we

Table 1. Acid mucopolysaccharides isolated from normal and Fabry's fibroblasts. Results are based on isolation of acid mucopolysaccharides from ten tissue culture plates (150 mg; dry weight) with 12×10^6 to 14×10^6 cells for each 100-mm plate. Quantity of the polysaccharide is based on 33 percent hexosamine content. H, hyaluronic acid; D, dermatan sulfate; C, chondroitin sulfate.

Cell type	Acid mucopolysaccharide			
	Total (mg)	Fractions		
		H (%)	D (%)	C (%)
Normal	0.6	67	16	16
Fabry's	4.1	80	12	8

* Sum of chondroitin-4-sulfate and chondroitin-6-sulfate.

Table 2. Lipids in normal and Fabry's fibroblasts. All measurements were based on weights of lyophilized cells (dry weight). GL-1, glucosyl ceramide; GL-2, lactosyl ceramide; GL-3, trihexosyl ceramide; GL-4, tetrahexosyl ceramide (GalNAc : gal : glc, 1 : 2 : 1).

Cell type	Lipids (mg/g)				Glycolipid types (μmole/g)			
	Total	Neutral	Polar	Glycolipids	GL-1	GL-2	GL-3	GL-4
Normal	122.8	48.3	33.7	18.6	1.44	0.34	0.69	0.31
Fabry's	202.0	36.0	110.0	42.3	1.45	0.22	2.23	0.33

found with cultured cells may also have been observed in a patient with Fabry's disease (*15*). Here it was found that two types of inclusion materials were present in biopsy specimens, one of which appeared to be a mucopolysaccharide on morphological grounds.

It is possible that the deficiency of an enzyme which participates in the degradation of both glycolipids and mucopolysaccharides may result in accumulation of both types of substance. A gal→gal linkage is present in the linkage region of a number of sulfated mucopolysaccharides (chondroitin-4-sulfate and -6-sulfate, dermatan sulfate, heparin and heparitin sulfates) (*16*) but has not been demonstrated in hyaluronic acid. It differs from that of the trihexosyl ceramide in that it is internal rather than terminal and is $\beta 1 \rightarrow 3$ rather than $\beta 1 \rightarrow 4$ (*17*). Whether the galactosidase can also act as an endoglycosidase is not known. Available evidence (*4*) suggests that the galactosidase missing in Fabry's disease is highly specific for GL-3.

In view of the accumulation of acid mucopolysaccharides in a number of diseases, it is possible that other types of mechanisms may be responsible for this phenomenon. The biosynthesis, accumulation, and excretion of acid mucopolysaccharides occur on membrane systems (*18*). The distribution of polysaccharides seems similar to normal in Fabry's disease, in contrast to the specific accumulation of dermatan sulfate in Hurler's disease and in the Hurler variant, and of hyaluronic acid in Marfan's disease. Interference in the metabolism of membrane components may secondarily influence mucopolysaccharide metabolism, though experimental evidence is lacking. Further information regarding the degradation of both glycolipids and mucopolysaccharides as well as the role of glycolipids in cell physiology are necessary for definitive answers to these questions. The demonstration of defects of both glycolipid and mucopolysaccharide metabolism in cultured fibroblasts opens new possibilities for the study of such genetic disorders as well as of normal cell physiology.

REUBEN MATALON, ALBERT DORFMAN
Departments of Pediatrics and
Biochemistry, Joseph P. Kennedy, Jr., Mental Retardation Center, LaRabida–University of Chicago Institute, University of Chicago, Chicago, Illinois
GLYN DAWSON, CHARLES C. SWEELEY
Department of Biochemistry, Michigan State University, East Lansing

References and Notes

1. C. C. Sweeley and B. Klionsky, in *The Metabolic Basis of Inherited Disease*, J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, Eds. (McGraw-Hill, New York, 1966), p. 618.
2. Abbreviations: gal, galactose; glc, glucose; gal NAc, *N*-acetylgalactosamine.
3. C. C. Sweeley and B. Klionsky, *J. Biol. Chem.* **238**, PC 3148 (1963).
4. R. O. Brady, A. E. Gal, R. M. Bradley, E. Mårtenson, A. L. Warshaw, L. Laster, *New Engl. J. Med.* **276**, 1163 (1967).
5. T. Yamakawa, S. Nishimura, M. Kaminura, *Jap. J. Exp. Med.* **35**, 201 (1965).
6. B. S. Danes and A. G. Bearn, *J. Exp. Med.* **1**, 123 (1966).
7. R. Matalon and A. Dorfman, *Proc. Nat. Acad. Sci. U.S.* **56**, 1310 (1966).
8. R. Matalon, J. A. Cifonelli, H. Zellweger, A. Dorfman, *ibid.* **59**, 1096 (1968).
9. B. S. Danes and A. G. Bearn, *Lancet* **1968-I**, 1061 (1968); *ibid.* **1968-II**, 855 (1968); *Science* **161**, 1347 (1968).
10. R. Matalon and A. Dorfman, unpublished results.
11. D. E. Vance, W. Krivit, C. C. Sweeley, *J. Lipid Res.* **10**, 188 (1969).
12. R. Matalon and A. Dorfman, *Biochem. Biophys. Res. Commun.* **32**, 150 (1968). Cells grown on cover slips were rinsed with Earle's balanced salt solution, dried in air, fixed, and stained with toluidine blue O (Fisher) as described.
13. D. E. Vance and C. C. Sweeley, *J. Lipid Res.* **8**, 621 (1967).
14. R. Matalon and A. Dorfman, *Biochem. Biophys. Res. Commun.* **33**, 954 (1968).
15. H. Loeb, G. Jonniaux, M. Tondeur, P. Davis, P. E. Gregoire, P. Wolff, *Helv. Paediat. Acta* **23**, 269 (1968).
16. L. Rodén, in *Proceedings of the 4th International Conference on Cystic Fibrosis of the Pancreas*, E. Rossi and E. Stoll, Eds. (Karger, New York, 1968), p. 185.
17. The glycosidic bond between the two galactose units in GL-3 is in the beta configuration (P. D. Snyder and C. C. Sweeley, unpublished).
18. A. Horwitz and A. Dorfman, *J. Cell Biol.* **38**, 356 (1968).
19. Supported by PHS grants AM 05996, FR 00305, and AM 12434; the Chicago Heart Association and Research Paper No. 4663 from Michigan State Agricultural Experimental Station. We thank Dr. W. Krivit for the referral of biopsy material from a Fabry's patient; Drs. J. A. Cifonelli and Lennart Rodén for discussion; and Mrs. Bonnie B. Sipe and Miss Minerva Norella for technical assistance. R.M. is a Joseph P. Kennedy, Jr., scholar.

20 March 1969

Cytophaga That Kills or Lyses Algae

Abstract. *A myxobacterium* (Cytophaga N-5) *isolated from sewage kills or lyses an array of living green and blue-green algae. When assayed with* Nostoc muscorum *or* Plectonema boryanum, *plaques form like those caused by the blue-green algal virus LPP-1. This isolate lyses or inhibits mutually Gram-positive and Gram-negative eubacteria.*

In seeking new algal viruses from the waste stabilization ponds of Austin, Texas, we found a nonfilterable agent which killed or lysed (*1*) an array of living green and blue-green algae. This agent was distinguished from viruses by its wide host range and its ability to digest autoclaved algae.

This organism is Gram-negative, unicellular, rodlike with rounded ends (0.4 to 0.6 by 1.9 to 3.7 μ), and aflagellate (Fig. 1a). On agar it forms slimy, yellow colonies with thin spreading margins and, with age, produces a brown water-soluble pigment. The cells at the margins of colonies move in a gliding, flexuous manner. No fruiting bodies or microcysts are produced. Cellulose is digested; chitin is not. These characteristics relegate the organism to the order Myxobacterales and identify it as a new species of the genus *Cytophaga* (*2*) which is herein referred to as *Cytophaga N-5*.

A salient characteristic of *C. N-5* is its ability to kill or lyse a variety of green and blue-green algae (*3*). Sensitivity was determined by placing 0.1 to 0.2 ml of an actively growing culture of *C. N-5* on algal lawns. Killing or lysis occurred within 1 day to 2 weeks. Although most green and blue-green algae tested were killed or

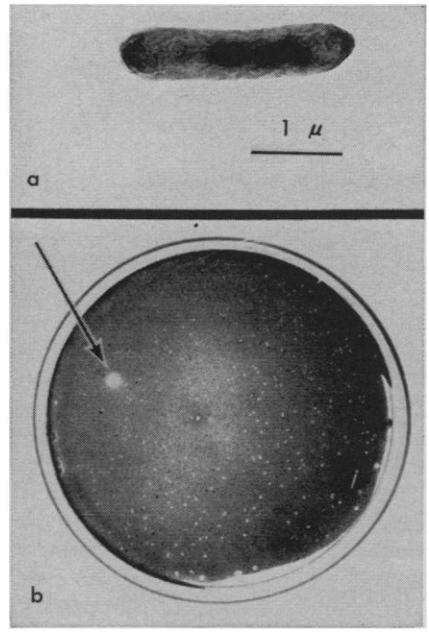

Fig. 1. (a) Electron micrograph of a cell in the log phase of growth negatively stained with 2 percent uranyl acetate. (b) Three-week-old overlay of *Cytophaga N-5* assayed with *Nostoc muscorum* in a 9-cm petri dish. Arrow indicates largest plaque, while 400 smaller plaques are visible at this dilution ($\times 10^{-7}$ cells per milliliter).