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A New Type of Mucolipidosis with β-Galactosidase Deficiency and Glycopeptiduria

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Orii, T., Minami, R., Sukegawa, K., Sato, S., Tsugawa, S., Horino, K., Miura, R. and Nakao, T. *A New Type of Mucolipidosis with β-Galactosidase Deficiency and Glycopeptiduria.* Tohoku J. exp. Med., 1972, **107** (4), 303–315 —— Clinical, biochemical and electron microscopic studies on a patient of a new type of mucolipidosis are described. The patient is a 14-year-old Japanese boy who has coarse facies, dysostosis multiplex, neurologic deterioration, corneal clouding, macular cherry red spot, β-galactosidase deficiency, glycopeptiduria, and vacuolated cells in hepatic parenchyma, renal glomeruli, renal bone marrow and peripheral lymphocytes. ———— mucolipidosis; β-halactosidase; glycopeptiduria; cherry red spot; foam cell

During recent years, a group of storage diseases which exhibit signs and symptoms of both sphingolipidoses and mucopolysaccharidoses but cannot be classified into the actually known types of them, have been reported by several workers (Landing *et al.* 1964, Derry *et al.* 1968, Durand *et al.* 1968, Öckerman 1967, Austin *et al.* 1965, Spranger *et al.* 1968, Leroy *et al.* 1969, Maroteaux and Lamy 1966). These several disorders have recently been classified as the mucolipidoses by Spranger and Wiedemann (1970). The patient with these disorders is characterized by gargoylism-like dysmorphism, dysostosis multiplex, normal mucopolysaccharidiuria with the exception of the Austin type of sulfatidosis, vacuolated lymphocytes, foam cells in bone marrow and other organs and macular cherry red spots.

The purpose of this paper is to describe a case with characteristics of the mucolipidoses, which is probably a new type.

Case Report

U.K., a 14-year-old boy, was born on June 15, 1956, after uneventful pregnancy and labor; his birth weight was 3.0 kg. He is the sixth child of healthy parents and his paternal and maternal grandfathers were cousins (Fig. 1). Growth and development were thought to be normal during the first several years of life; however, at 3 years of age, his

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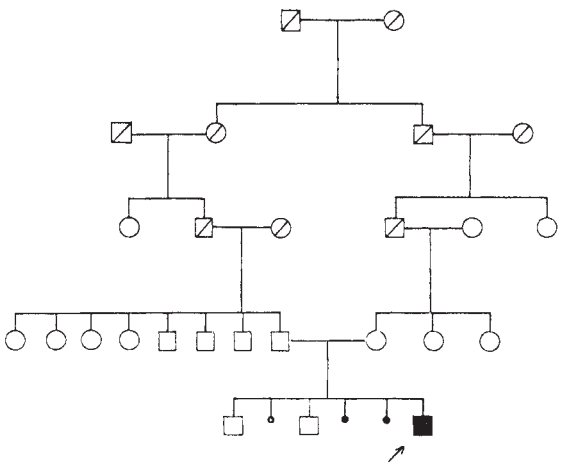


Fig. 1. Family pedigree of the patient.

grandmother noticed that his gait suddenly became unsteady. He was admitted to a hospital for eight weeks because of suspected poliomyelitis, and then for some time he complained of pain of the right lower limb. Thereafter, until ten years of age, he was considered by his parents to be similar to two healthy siblings with the exception of slow learning and slow motion.

At eleven years of age, the child was brought to us because of suspected hypothyroidism. The weight was 35.8 kg and height was 139.4 cm. Ophthalmologic examination revealed a cherry red spot in both eyes. The heart sounds were regular, and a grade II systolic ejection murmur was heard best parasternally at the second intercostal space on the left. A slight flexion contracture was seen at the distal phalanges of fingers. The examination of the deep tendon reflexes revealed a slightly generalized hyperreflexia with clonus of the quadriceps and ankles. Radiologic examination of the skull, pelvis and hands showed normal configuration. However, roentgenograms of the dorsolumbar spine showed

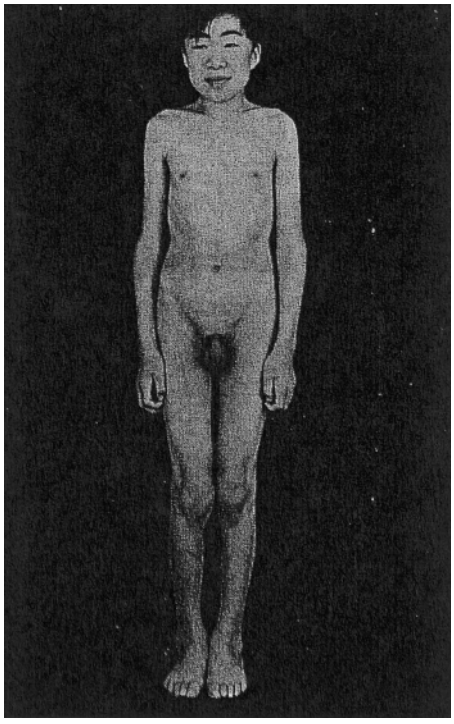


Fig. 2. Patient at 14-years old.

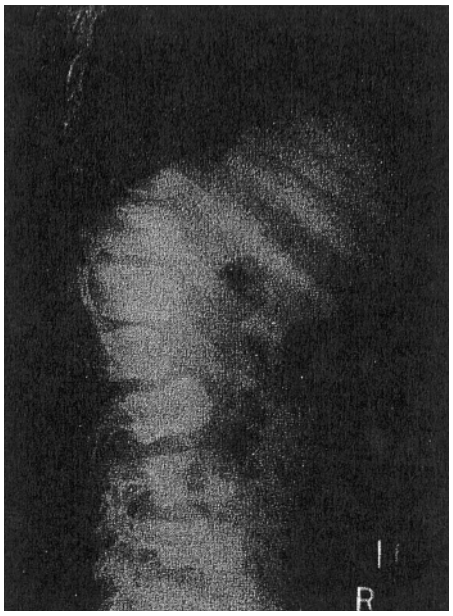
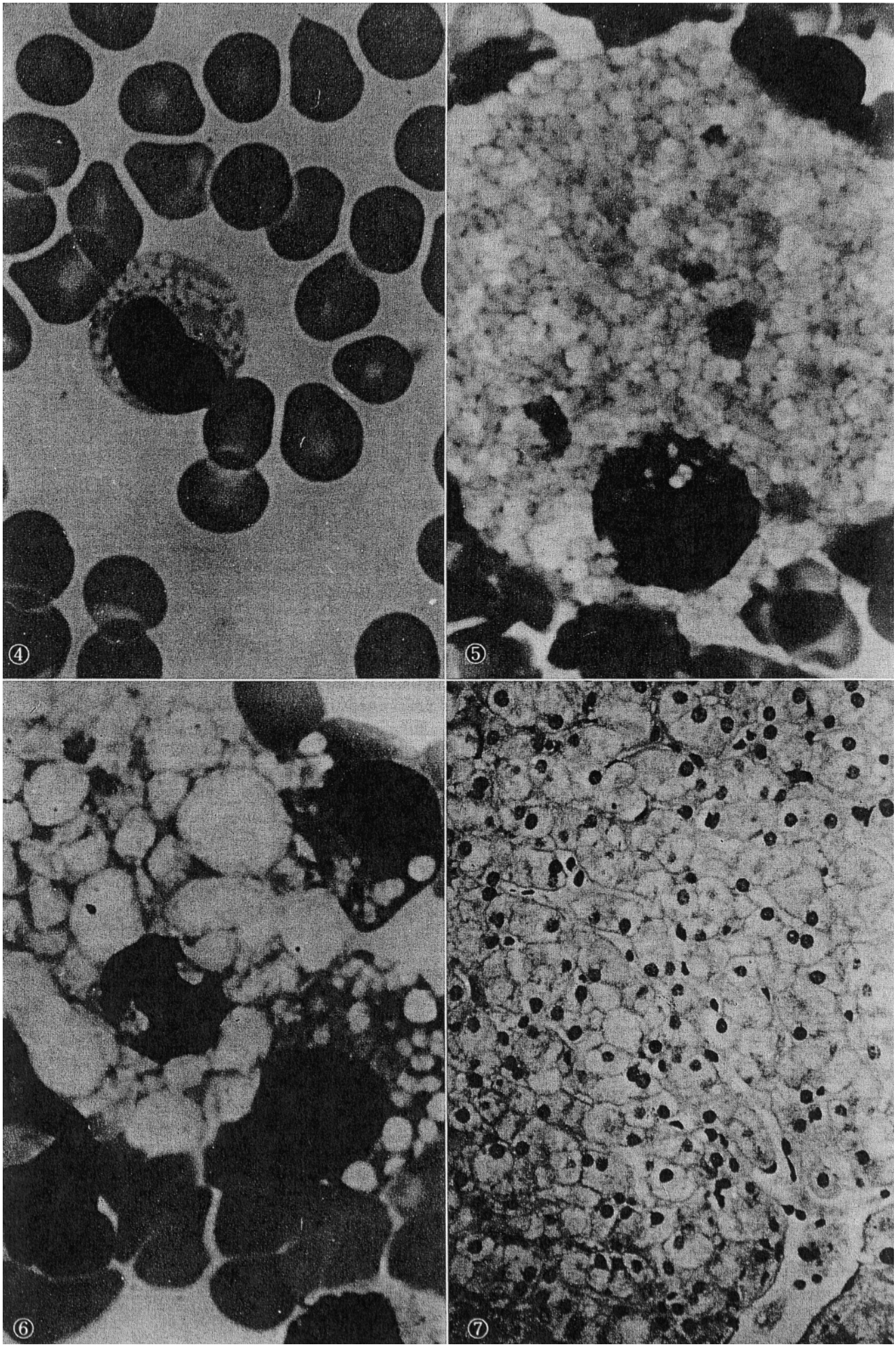


Fig. 3. Roentgenogram of dorsolumbar spine. The vertebral body of the 2nd lumbar is hypoplastic.

universal vertebra plana with anterior beaking of the vertebral bodies of the 6th~9th thoracic and a hypoplastic vertebral body of the 2nd lumbar. Urinalysis for acid mucopolysaccharides was normal.

We observed the child again when he was fourteen years old (Fig. 2). His chief complaints were poor vision, slow motion and unsteadiness of gait, which were gradually progressive. On physical examination, his height was 155.4 cm; weight, 47.0 kg; head circumference, 55 cm; these were within the normal limits. His facial features were coarse, the lips and tongue were thick, the tip of the nose was enlarged, the teeth were all carious and the gums were slightly hypertrophied. He had a pigeon chest with a flaring lower rib cage, dorsal kyphosis and pes valgus. Extension was slightly limited at the elbows and knees. Shoulder motion was slightly limited. The finger contractures, heart murmurs, cherry red spots in macular regions and bone changes (Fig. 3) were essentially unchanged. Corneal opacities were detected by slit-lamp examination. An illiterate test of vision revealed acuities of 0.2 oculus bilaterally. Hearing on gross testing was normal. An audiogram showed loss of pure tone between 3,000 and 8,000 decibels in the left ear. The liver and spleen were not enlarged. A Wechsler intelligence scale for children revealed an I.Q. of 96. The neurological examination revealed the followings: All peripheral reflexes were active and knee and ankle reflexes were hyperactive. The ankle clonus and the intension tremors of hands were noted. The finger-to-nose, heel-to-knee and arm-stopping tests were slightly hypermetric and were slow in action. The Romberg's sign was positive. Babinski's reflex was negative. An electroencephalogram showed normal pattern.

Laboratory findings: Vacuolated or abnormally granulated lymphocytes (Fig. 4) were seen in the peripheral blood, and peculiar foam cells and Buhot cells (Figs. 5 and 6) were found in the bone marrow. A complete blood count, blood urea nitrogen, fasting blood sugar, total serum proteins, albumin-globulin ratio, uric acid, cholesterol, bilirubin, serum calcium, phosphorus, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, lactic dehydrogenase, acid phosphatase, thymol turbidity, zinc turbidity were all found to be normal. Urine concentration test and phenolsulfon-phthalein excretion test were within normal limits. Alkaline phosphatase was elevated moderately.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Histologic and electron microscopic methods: Liver and kidney tissues were obtained by Menghini needle biopsy. Rectal mucosal and submucosal tissues were obtained by Crosby and Kugler intestinal biopsy capsule. Each sample was divided into two portions. The first portion was fixed in buffered formaldehyde, dehydrated in alcohols, and embedded in paraffin. Six-micron thick sections were cut and stained with hematoxylin and eosin. The second portion was used for electron microscopy. The sample was fixed immediately in 1% osmium tetroxide buffered to pH 7.4 with veronal-acetate. After fixation for 30 min at 4°C, further sectioning into smaller blocks was fixed in the original fixative. The resulting 1 to 2 mm blocks were rapidly dehydrated with increasing concentrations of alcohol at 4°C. The final steps of dehydration with absolute alcohol and two changes of propylene oxide were carried out at room temperature. The blocks were then embedded in Epon 812. Both semi-thin and thin sections were cut with a Porter-Blum ultramicrotome using glass knives. Semi-thin sections were placed on glass slides and stained with toluidine blue for light microscopy. The thin sections were mounted on copper grids, stained with uranyl acetate or lead hydroxide, and examined with Hitachi HU-11 E electron microscope.

Sampling and preparation for enzyme assay:

Liver and rectum. All biopsy samples in both controls and patient were immediately frozen in solid CO_2 and stored in solid CO_2. The tissue was homogenized in an all-glass Potter-Elvehjem homogenizer in saline and treated with a Kubota insonator (model 200 M) for 3 min at an output of 60 watts. The sonically disrupted suspension was centrifuged at $10{,}000 \times g$ for 10 min and the precipitate was discarded. The clear supernatant fluid was used for analysis of the enzyme activities and protein.

Plasma. Venous blood was taken into heparinized tubes in the morning from the controls and the patient in the fasting state, and cooled to 4°C. Plasma was separated at 4°C within 1 hr and stored frozen.

Assay of enzymes: β-Galactosidase in the liver. The enzyme activities were assayed by the modification of the Thomas procedure (1969). 0.4 ml of p-nitrophenyl-β-D-galactopyranoside (Koch-Light), 0.005 M in water; 1.4 ml of hydrochloric acid-sodium acetate buffer, 0.05 M, pH 5.0 and 0.2 ml of liver sample were incubated at 37°C for 60 min. The reaction was stopped by the addition of 0.8 ml of glycine buffer, 0.25 M, pH 10.0 (containing glycine and Na_2CO_3). The free p-nitrophenol was measured within 30 min in a spectrophotometer at 400 mμ, and p-nitrophenol was used as a standard.

β-Galactosidase in the rectum and plasma. 0.4 ml of 4-methylumbelliferyl-β-D-galactopyranoside, 0.001 M in water; 0.1 ml of acetate buffer, 1 M, pH 5.0 (rectum) or 3.75 (plasma) and 0.1 ml of samples were incubated for 60 min at 37°C. The reaction was stopped with 3 ml of glycine buffer, 0.2 M, pH 10.7. The fluorescence was read immediately in a Hitachi spectrophotofluorometer (model 203) with exciting wave-length 365 mμ and emitting wave-length 445 mμ. 4-Methylumbelliferone in glycine buffer was used as a standard.

N-Acetyl-β-Glucosaminidase in the liver. The N-acetyl-β-D-glucosaminidase activity was measured as described for β-galactosidase activity in the liver with the exception that 0.4 ml of 0.00375 M p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Koch-Light) was used as the substrate for each liver sample.

β-Glucosidase in the rectum and plasma. The β-glucosidase activities were measured as described for β-galactosidase activities in the rectum and plasma with the exception

Fig. 4. Lymphocyte with cytoplasmic vacuolation. Peripheral blood smear (May-Grünewald-Giemsa).

Fig. 5. Foam cell. Bone marrow smear (May-Grünewald-Giemsa).

Fig. 6. Peculiar bone marrow cell and Buhot-cells. Bone marrow smear (May-Grünewald-Giemsa).

that 0.4 ml of 0.001 M 4-methylumbelliferyl-β-D-glucopyranoside (Koch-Light) was used as the substrate for each sample of rectum and plasma.

Protein was assayed as described by Lowry *et al.* (1951).

Isolation and analysis of urinary acid mucopolysaccharides: The urine was centrifuged and precipitated with cetyl-pyridinium chloride (CPC); the precipitate was washed with sodium acetate-saturated 95% ethanol, dissolved in 10% sodium acetate and precipitated with 80% ethanol. Enzymatic assay of chondroitin sulfates A, B and C was carried out by method 1 of Saito *et al.* (1968), using chondroitinase ABC (Seikagaku Kogyo Co., Tokyo) on isolated acid mucopolysaccharide preparations.

Isolation, analysis and chromatography of urinary glycopeptides in non-CPC-precipitable fraction: After removal of the CPC precipitate, the supernatant was concentrated and dialyzed with Diaflo membrane UM-2 (Amicon, model 202), precipitated with sodium acetate saturated 99% ethanol; the precipitate was washed with sodium acetate saturated 95% ethanol, dissolved in 10% sodium acetate and precipitated with 80% ethanol. Subsequently, this crude non-CPC-precipitable fraction was filtered through a column (1.8×72.5 cm) of Sephadex G-25 fine with 180 ml of 0.05 M NaCl. 6 ml fraction were collected. Fractions 10~22 filtered upto the void volume from normal children and patient were combined, concentrated separately and applied to a column (1.8×80 cm) of ECTEOLA-cellulose which was eluted with 600 ml each of water, 0.02 M HCl, 1.3 M NaCl, and finally 4.0 M NaCl (Anseth *et al.* 1970). Each fraction was desalted on Sephadex G-25 column and lyophylized. Colorimetric determinations of hexuronic acid (Dische 1947), hexosamine (Svennerholm 1956), neutral sugar (Roe 1955), sulfate (Dodgson and Price 1962), sialic acid (Aminoff 1961) and quantitative estimation of glucosamine/galactosamine ratio (Gardell 1958) were made on each sample.

Results

Histological finding of liver. Hepatocytes and Kupffer cells (Fig. 7) displayed a ballooned cytoplasm and a small nucleus.

Ultrastructure. Liver. Very numerous intracytoplasmic vacuoles of different size from 0.3 to 1.0 μ were found in Kupffer cells. They were bound by a unit-membrane. In a few vacuoles, opaque globules and ring-like elements were noted (Fig. 8). In the epithelial cells a few intracytoplasmic vacuoles limited by a single membrane were noticed; some of them had an electron-dense round body (Fig. 9).

Kidney. Numerous vacuoles containing a protein-like reticulum were present in epithelial cells of glomeruli. They were bound by a unit-membrane. Some of them had ring-like and granular elements. The basement membrane was slightly hypertrophied (Fig. 10).

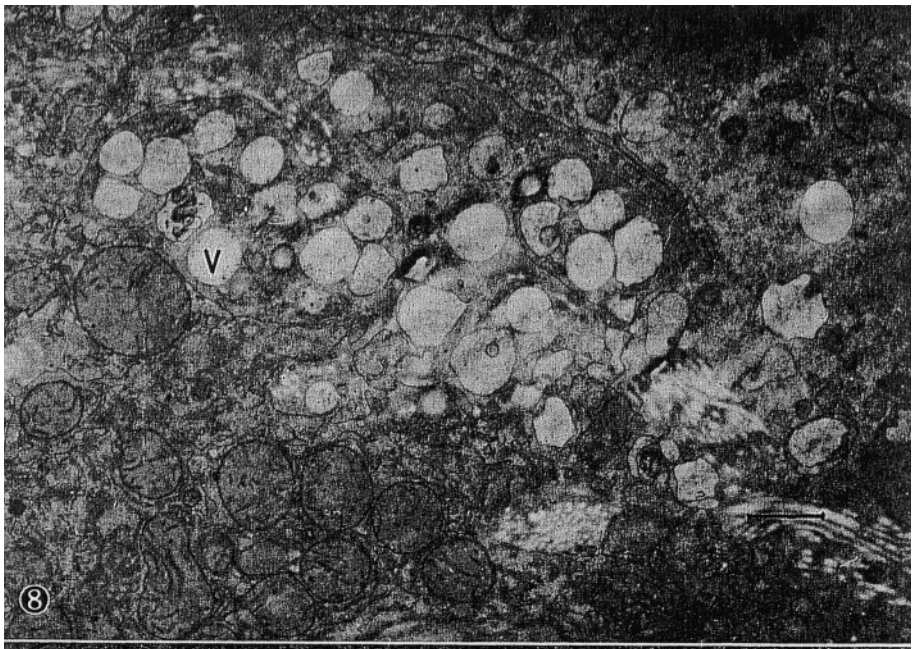
Enzymatic findings. It can be seen in Table 1 and Fig. 11 that very low levels of 4-methylumbelliferyl-β-galactosidase activity were found in rectum and plasma samples obtained from the patient. A moderate deficiency of p-nitrophenyl-β-

Fig. 7. Hepatocytes and Kupffer cells from biopsy specimen showing clear cytoplasm and small nuclei (hematoxylin and eosin, ×1,000).

Fig. 8. Liver. Kupffer cell containing numerous intracytoplasmic vacuoles. ×11,000.

Fig. 9. Liver. Hepatocyte containing a few intracytoplasmic vacuoles; some of them have electron-dense round bodies. ×11,000.

Fig. 10. Kidney. Numerous vacuoles containing a protein-like reticulum are present in epithelial cell of glomerules. ×11,000.



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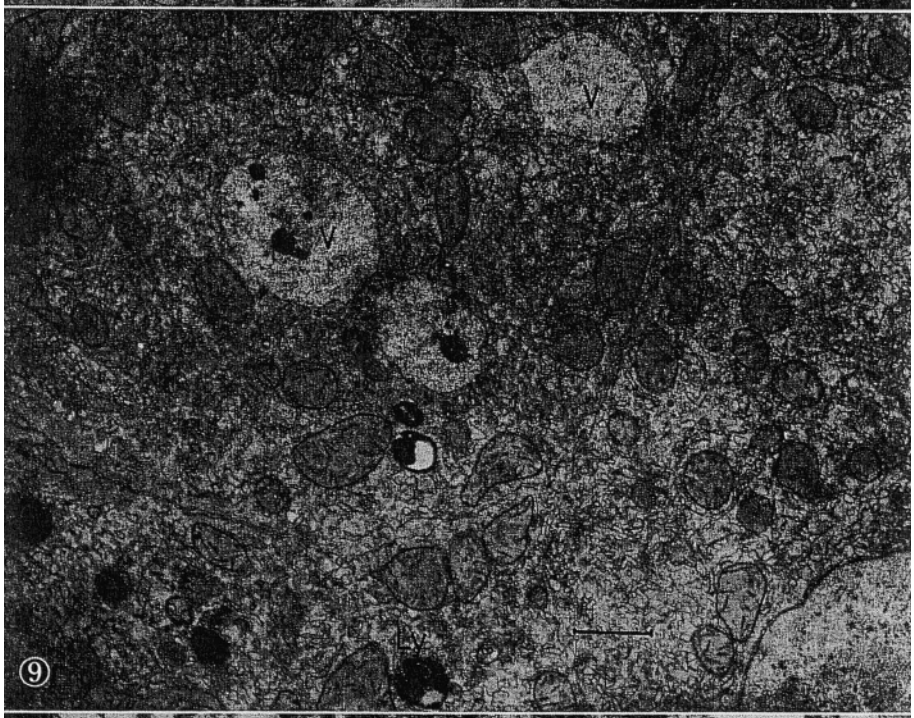

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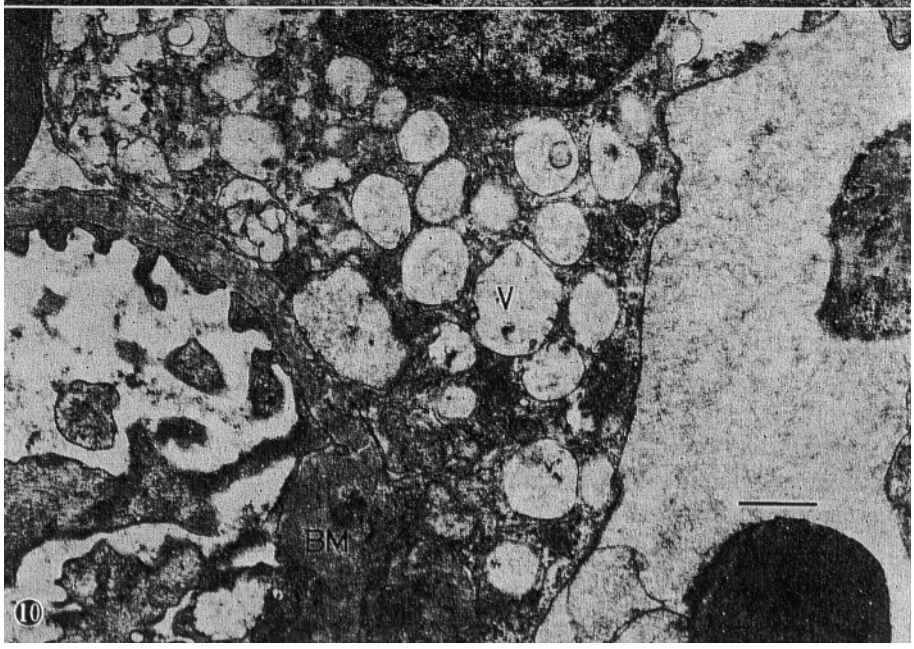

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TABLE 1. *β-Galactosidase and β-N-acetylglucosaminidase activities*

		β-Galactosidase (μmole/mg protein/hr)	β-N-acetylglucosaminidase (μmole/mg protein/hr)
Liver	Control biopsy	1) 255.0×10^{-3} 2) 247.8×10^{-3}	1) 85.2×10^{-2} 2) 36.9×10^{-2}
	Control autopsy	1) 330.0×10^{-3} 2) 255.0×10^{-3}	1) 46.2×10^{-2} 2) 24.5×10^{-2}
	Present case	1) 112.2×10^{-3} 2) 91.2×10^{-3}	1) 70.8×10^{-2} 2) 31.3×10^{-2}
Rectum	Control biopsy	1) 190.0×10^{-3} 2) 39.6×10^{-3}*	Not studied
	Present case	1) 7.0×10^{-3} 2) 2.5×10^{-3}*	Not studied
Plasma	Control Present case	650.0×10^{-6}* 36.0×10^{-6}*	Not studied

* 4-Methylumbelliferyl-β-D-galactopyranoside was used as the substrate.

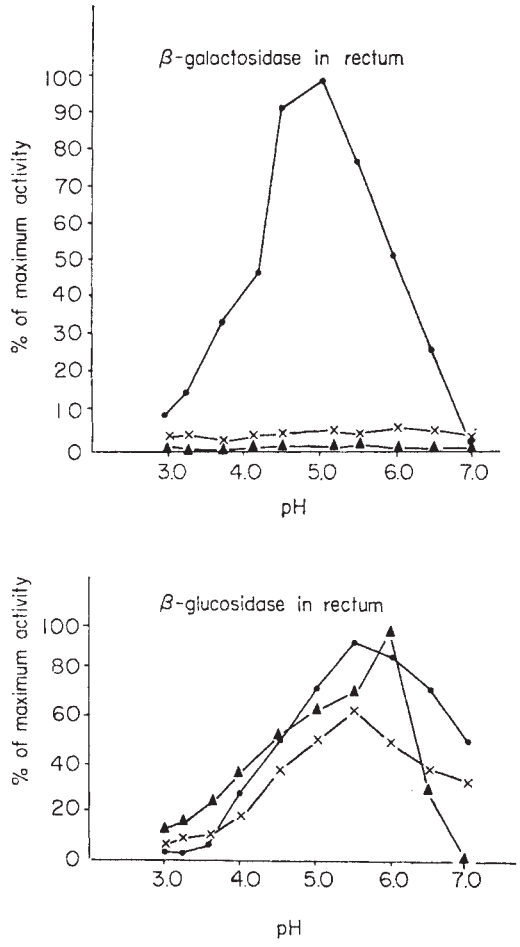

Fig. 11. pH-activity relations of β-galactosidase and β-glucosidase in rectum biopsy specimens. Profound deficiency of β-galactosidase and no deficiency of β-glucosidase are found in rectum samples of the present case.

TABLE 2. *Composition of crude acid mucopolysaccharide and glycopeptide fractions from the urine**

Constituent	CPC-precipitable fraction (mg/g creatinine)		Non-CPC-precipitable fraction (mg/g creatinine)	
	Control	Present case	Control	Present case
Uronic acid	9.5	4.3	6.9	39.8
Hexosamine	6.4	4 3	16.3	463.6
Hexose	5.8	8.8	51.3	422 5
Sulfate	3.4	6.3	85.9	130 2
Sialic acid	3.5	6.8	19.8	215.7
Protein	3 3	6.6	84.8	289 5
Total	31.9	37 1	265 0	1561.3
Dry weight (mg/g creatinine)	50.4	78.9	490.3	1817.5

* 6 liters (control) and 3 liters (present case) of urines were collected and analyzed.

TABLE 3. *Analysis of CPC-precipitable acid mucopolysaccharide fraction*

	Before chondroitinase ABC digestion (mole ratio)*	After chondroitinase ABC digestion		
		Uronic acid (% recovery)	Hexosamine (% recovery)	(mole ratio)*
Control	1.37	8.0	14.1	0.39
Present case	0.92	6.0	10.9	0.51

* Mole ratios based on hexosamine=1.0.

TABLE 4. *Proportions of urinary CPC-precipitable acid mucopolysaccharides*

	Total hexosamine (mg/g creatinine)	Chondroitin sulfate		Chondroitin	Hyaluronic acid	Heparitin sulfate+ keratosulfate
		A+B	C			
		%		%	%	%
Control	6.4	45.0	31.4	6 4	3.1	14.1
Present case	4.3	46.1	30.3	6.0	6.7	10.9

galactosidase was also found in liver samples; the range of the samples from the patient was 27.6~45.3% of the enzyme activity of controls. However, β-N-acetylglucosaminidase activities of liver samples were within normal limits. Furthermore, a deficiency of β-glucosidase in rectum samples from the patient was not found (Fig. 11).

Urinary acid mucopolysaccharides and glycopeptides. – Table 2 indicates that the amount of the total CPC-precipitable acid mucopolysaccharide fraction from the urine of patient was within normal limits. However, considerable amounts of non-CPC-precipitable crude glycopeptides were obtained in the urine of normal children and patient; furthermore, non-CPC-precipitable glycopeptide fractions from the patient were much higher than those from normal children. Table 3 indicates that after hydrolysis of the CPC-precipitable acid mucopolysaccharide fraction with

TABLE 5. *Analytical data on ECTEOLA-cellulose column fraction*

Fraction	Case	mg/g creatinine						
		Uronic acid	Hexosamine	Hexose	Sulfate	Sialic acid	Protein	Total
H_2O	Control	0.3 (0.10*)	2.9	7.4 (2.54*)	0.2 (0.13*)	1.0 (0.28*)	6 8	18.6
	Present case	0 2 (-*)	39.4	109.3 (2.76*)	0.0 (-*)	12.6 (0.26*)	40.5	202.0
0.02 M HCl	Control	0.8 (0.10*)	7.2	9.1 (1.26*)	0.0 (-*)	1.0 (0.11*)	20.4	38.5
	Present case	0.2 (0.01*)	17.8	46.0 (2.57*)	1.9 (0.20*)	12.6 (0.58*)	66.0	144.5
1.3 M NaCl	Control	0.4 (1.23*)	0.3	1.5 (4.99*)	3.0 (18.70*)	0.2 (0.54*)	2.1	7.5
	Present case	0.5 (0.06*)	7.8	13.5 (1.72*)	8.8 (2.10*)	1.6 (0.17*)	10.7	42.9
4.0 M NaCl	Control	0.3	0.0	0.0	0.3	0.0	1.2	1.8
	Present case	0.2	0.0	1.9	0.3	0.1	0.0	2.5

* Mole ratios based on hexosamine=1.0 were given in parentheses.

chondroitinase ABC, the percentage recovery of hexosamine from patient did not differ significantly from that of normal children, that is, no increase above the normal range in the excretion of keratosulfate was found. Table 4 shows that the excretion pattern of acid mucopolysaccharide fraction from patient is also within normal limits. Each fraction filtered upto the void volume on Sephadex G–25 columns contained 74.4% of hexuronic acid in normal children and 80.2% of those in the patient respectively; the hexosamine in this fraction from the former was only glucosamine, while 96.7% of the latter hexosamine comprised glucosamine. On ECTEOLA-cellulose column chromatographies, 97.1% of total hexosamine from normal children and 88.0% of those from the patient were eluted up to the concentration of 0.02 M HCl (Table 5). As shown in Table 5, glycopeptide fractions from the patient after ECTEOLA-cellulose column chromatography were also much higher than those from normal children, while composition of the glycopeptide fraction did not differ significantly between them, although high molar ratios of hexose to hexosamine were shown in 0.02 M HCl fraction of the former as compared with the latter (Table 5). The data presented indicates that a large amount of glycopeptide is excreted in the urine of the patient.

DISCUSSION

The clinical, biochemical and cytologic features of our patient are different from those encountered in any of the classical forms of genetic mucopolysaccharidoses and sphingolipidoses. Spranger and Wiedemann (1970) have tentatively classified

TABLE 6. *Comparison of data in mucolipidosis type 1, new storage disease (Goldberg et al. 1971) and our case*

	Mucolipidosis type 1	New storage disease (Goldberg *et al.* 1971)	Our case
Coarse facies	+	+	+
Mental retardation	+	+	−
Neurologic deterioration	+	+	+
Dwarfism	±	+	−
J-shaped sella	+	+	−
Lumbar vertebral beaking	+	+	+
Cloudy cornea	±	+	+
Cherry red spot	±	+	+
Hearing loss	+	+	±
Vacuolation of			
lymphocyte	+	−	+
hepatic parenchymal cell	+	−	+
Foam cell in bone marrow	+	−	+
Mucopolysaccharidiuria	−	−	−
Glycopeptiduria	+	?	+
Deficiency of β-galactosidase activity	−	+	+
β-galactosidase activity in liver	Elevated	Normal	Low
β-galactosidase activity in plasma	?	Normal	Low

mucolipidoses into eight types based on clinical, radiologic and cytologic features. Of these, our case most resembles certain cases thought by Spranger and Wiedemann (1968) to be examples of mucolipidosis type 1, as shown in Table 6. However, the fact that an enzyme deficiency was found in the liver biopsy specimen of our case is in contrast with increased levels of β-galactosidase in liver of the mucolipidosis type 1. Goldberg *et al.* (1971) have recently reported siblings who are uniquely characterized by dwarfism, gargoyle facies, mental retardation, seizures, corneal clouding, macular cherry red spot, β-galactosidase deficiency, dysostosis multiplex, hearing deficit, normal mucopolysaccharidiuria and the absence of vacuolated blood cells. A male case reported by them resembles our case because of the coarse facies, the dysostosis multiplex, the corneal clouding, the macular cherry red spot, the longevity, the β-galactosidase deficiency and the absence of abnormal mucopolysaccharidiuria. However, their case differs from our case because of the absence of vacuolated cells in hepatic parenchyma, bone marrow and peripheral blood, and the presence of normal levels of β-galactosidase in the liver biopsy specimen and in the plasma (Table 6).

With the exception of the Austin type of sulfatidosis, it has been reported by several workers that the patients with the mucolipidoses have generally shown normal urinary excretion of uronic acid containing mucopolysaccharides. Urinalysis for the uronic acid containing mucopolysaccharides in our case was also qualitatively and quantitatively normal. However, in two patients with mucolipidosis, type 1, Spranger *et al.* (1968) have detected increased amounts of

hexosamine containing uromucoid; and in one patient with G_{M1}-gangliosidosis, type 2, Wolfe *et al.* (1970) have found greatly elevated amount of undersulfated keratan sulfate-like glycosaminoglycans. The data presented indicate that our case excretes a large amount of non-CPC-precipitable glycopeptidesas compared with normal children. Furthermore, unpublished study of the authors indicates that the patients with G_{M1}-gangliosidosis, type 1 and I-Cell disease excrete also considerable amounts of glycopeptides.

The relation between the elevated level of the glycopeptide and the specific β-galactosidase deficiency in our case is not clear and requires further study.

Acknowledgment

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