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Infantile Sialidosis: A Phenocopy of Type 1 G_{M1} Gangliosidosis Distinguished by Genetic Complementation and Urinary Oligosaccharides

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SUMMARY

A clinical description of an apparently classical case of type 1 G_{M1} gangliosidosis is presented. The patient was the first-born child of first cousins. She was diagnosed at 6 weeks and died at 6 months. β-Galactosidase activity was deficient in cultured fibroblasts using [^{3}H]G_{M1} ganglioside and [^{3}H]ceramide-lactose as substrates. Genetic complementation studies performed after cell fusion between cultured fibroblasts from the patient and from two other type 1, one type 2, and one juvenile G_{M1} gangliosidosis strain were positive with all strains.

Subsequent studies revealed an increased excretion of a sialic acid-containing hexasaccharide in the patient's cells. Parents' fibroblasts contained normal levels of β-galactosidase. The case emphasizes the variability of the clinical expression in sialidosis and the importance of demonstrating a primary gene defect in establishing a diagnosis of an inborn error of metabolism.

INTRODUCTION

The infantile type of G_{M1} gangliosidosis has been recognized as a distinct clinical entity for 20 years [1–3]. At The Hospital For Sick Children we have seen seven such patients in the past 10 years. In each case, the diagnosis has been apparent on clinical grounds and has been supported by the typical radiographic appearance and confirmed

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by measurement of β-galactosidase activity in leukocytes, cultured fibroblasts, or tissues obtained at autopsy.

Two years ago an infant presented at our clinic who so closely resembled the other patients that the diagnosis was made after clinical examination alone. Nevertheless, a skin biopsy was obtained for fibroblast culture. The fibroblasts had less than 1% of normal β-galactosidase activity with 4-methylumbelliferyl galactoside. Studies of complementation with type 1 G_{M1} cells and examination of urinary oligosaccharides clearly showed that although phenotypically the baby had type 1 G_{M1} gangliosidosis [4], genotypically she had a different disease. The urine contained increased sialyloligosaccharides and established the diagnosis of sialidosis [5].

CASE REPORT

The baby (E. C.) was the first child of first cousins who came to Canada from a small village in the Azores. The mother, 23, and father, 26, were healthy, but two of the mother's sisters had died at approximately one year. The causes of death were unknown.

At birth, the infant had a "peculiar appearance" to her face, ascites, and periorbital edema. The edema cleared at the end of the first week, and she was discharged at eight days of age. Two weeks later she returned to her family doctor with diarrhea and was found to have hepatosplenomegaly. She was referred to The Hospital for Sick Children because of the organomegaly, her odd appearance, and continuing diarrhea.

First Admission

On examination at 6 weeks (fig. 1), she was thin, weighing 4.18 kg, (10th percentile for her age). Her length was 57 cm (75th percentile), and her head circumference was 38.5 cm (50th percentile). Her face had the dish-shaped configuration common in type 1 G_{M1} gangliosidosis, and her head was brachycephalic in shape. The bridge of her nose was flattened, and she had mild hypertelorism. The anterior fontanelle was open and full, measuring 4 × 2.5 cm. Her corneas were hazy, but a cherry-red spot could be visualized. Her liver was firm but not hard, and palpable 2 cm below the right costal margin. The spleen was 3 cm below the left costal margin. She had no hernias but had a diffuse telangiectasia over her lower abdomen and in her conjunctivae. Her limbs were of normal proportions, and she had good movement in all joints. When held in the upright position, she had a pronounced dorsal gibbus in the thoracolumbar region of her spine. Her heart sounds were normal, and there were no murmurs. She had no edema.

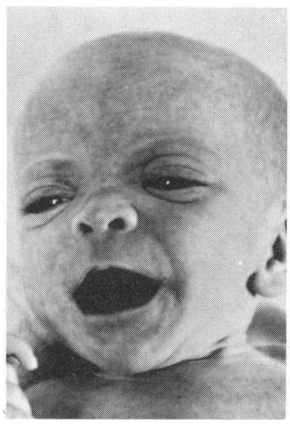

FIG. 1.—Patient shows typical facies of infant with type 1 G_{M1} gangliosidosis. Note hypertelorism, broad nasal bridge, and brachycephaly.

Radiographs showed changes typical of G_{M1} gangliosidosis (fig. 2). The overall trabecular pattern of the bones was coarse and dense. Periosteal cloaking was observed in the forearm bones and in the mandible. The ribs were widened and showed anterior flaring. There was a dorsolumbar gibbus with beaking and hooking of the body of L1 and an abnormally short antero-posterior diameter in all the vertebrae. A failure of normal metaphyseal-diaphyseal modeling gave the metaphyses in the upper and lower limbs a splayed appearance with cortical thinning. Early modeling changes in the metacarpal bones resulted in a loss of the diaphyseal waist and squaring off in contour with thinning of the cortices from within. There was a dense and amorphous texture of all the bones of the skull, particularly the temporal bones—the mastoid portions of which showed no pneumatization.

Vacuolated lymphocytes were seen on a stained blood film. The hemoglobin was 9.1 g/dl, and the platelet count 72,000/cmm, but other indices were normal. The bone marrow contained large numbers of foamy histiocytes but reduced megakaryocytes.

White blood cell β-galactosidase activity [3] was less than 0.07 nmoles/mg protein per hr (controls 162 $\pm$ 49 nmoles), while β-hexosaminidase [6] and arylsulfatase activities [7] were normal. Liver function studies showed a normal serum glutamic oxalic transaminase and bilirubin, but the total serum protein was only 4.0 g/dl with low albumin (2.7 g/dl) and gamma globulin (0.2 g/dl) fractions.

A skin biopsy was obtained for fibroblast culture (table 1). The diarrhea responded to regular therapy.

Second Admission

Two months later the patient returned to the hospital with watery diarrhea and tachypnea. She was sickly and emaciated, weighing 4.6 kg and responded only to painful stimuli. In addition to earlier findings, she now had a heart rate of 140 and respiratory rate of 60. There was indrawing of her chest on breathing. Rales were heard over the right chest. There were no heart murmurs, but she had moderate peripheral edema, and the liver was now 5 cm below the right costal margin.

She was treated with ampicillin and Lasix and placed in oxygen. Over a six-day period her chest and edema cleared.

Final Admission

At 6 months she again returned to the hospital with a two-day history of fever and cough. She had a temperature of 40.5°C, grunting respirations, and rales. Her chest X-ray showed not only bilateral infiltrations but cardiomegaly. Her serum chemistries (pH —7.21, pCO_2 —24 mm Hg, HCO^-_3 —9 meq/1, Na^+ —72 meq/1, Cl^- —121 meq/1, blood urea nitrogen —88 mg/dl) indicated metabolic acidosis and renal failure. Her hemoglobin was only 5.3 g, and the platelet count, 36,000. She did not respond to supportive therapy and died two days after admission. Permission for necropsy was denied.

MATERIALS AND METHODS

Source and Growth of Cells

Culture conditions have been described previously [9]. The fibroblast strains were from the present case and from patients with various forms of G_{M1} gangliosidosis (table 1). Strain 126 was from a child with I-cell disease. One normal strain was also included as a control.

The patient's cells, strain 652, did not grow more than 2–4 generations when thawed from ampoules frozen in liquid nitrogen. At the end of the study, all strains were examined for evidence of mycoplasma contamination by broth or agar assay and UDP-phosphorylase assay after at least two passages in antibiotic free medium (P. Quinn, personal communication, 1978). No evidence of mycoplasma was obtained by assay or by examination of cell appearance or growth rate during the study.

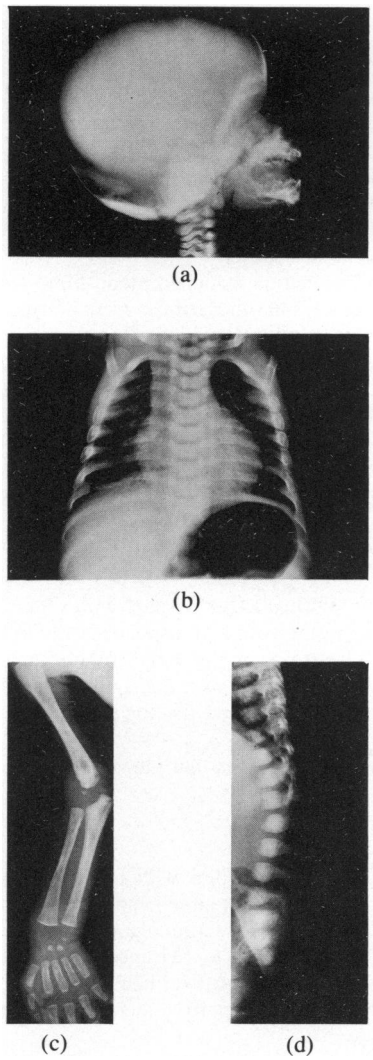

FIG. 2. —Typical features of G_{M1} gangliosidosis: *a*, Head. Note dense amorphous texture of all bones in skull and periosteal cloakings of the mandible; *b*, Chest. Ribs are Hurleroid, heart moderately enlarged, but lung fields are clear; *c*, Arm. Periosteal cloaking in all bones. Abnormal metaphyseal-diaphyseal modeling and coarse texture are also shown; *d*, Spine. Classical beaking of L1 is accompanied by kyphosis and antero-posterior shortening of vertebrae.

Enzyme Assays

The washed cells were scraped with a rubber policeman, suspended in saline, and washed twice. The pelleted cells were lyophilized, and the dry residue suspended in saline and mixed well. The extract was centrifuged at 31,000 *g* for 30 min at 2°C, and the supernatant fluid used for assay. G_{M1} gangliosidase and lactosylceramide β-galactosidase were measured as described previously [10]. The activity with 4-methylumbelliferyl-β-galactoside was measured in a final

TABLE 1

ENZYME ACTIVITY IN CULTURED FIBROBLASTS WITH VARIOUS β-GALACTOSIDASE DEFECTS

STRAIN	PATIENT	CLINICAL DIAGNOSIS	ENZYME ACTIVITY (NMOLES/MG PROT/HR)*		
			4MU-Gal	[^{3}H]G_M	[^{3}H]CDH
116†	D. C.	G_{M1} type 1	3.30	0.72	2.26
511†	C. K.	G_{M1} type 1	6.25	0.79	1.87
87†	K. L.	G_{M1} type 2	6.20	...	...
300§	H. S.	G_{M1} juvenile	11.25	2.73	4.95
652	E. C.	present case	...	7.26	3.96
126	K. F.	I-cell disease	3.85	...	...
160	M. L.	normal	333.3	72.0	25.71

* Substrate: 4MU-Gal = 4 methylumbelliferyl-β-D-galactose; [^{3}H]G_M = [^{3}H]G_M ganglioside; [^{3}H]CDH = [^{3}H]ceramide-glucosyl-galactoside.

† Not previously reported but cited by O'Brien [8].

‡ Provided by Dr. P. Hösli.

§ [24].

volume of 0.21 ml containing 1.2 mM substrate, 0.125 M acetate buffer, pH 4.0, enzyme. After 2 hrs incubation at 37°C, 2 ml of 0.1 M 2-amino-2-methyl-1, 3-propanediol buffered to pH 10.4 was added and fluorescence measured [6]. Protein was determined by the procedure of Lowry et al. [11].

Cell Fusion

Cells were fused as described previously [9] except that fusion was produced in the presence of polyethylene glycol (PEG 1000, Baker Chemical, Phillipsburg, N. J.) made up to 50% vol/vol with serum-free α-medium [12]. Fusions were done on confluent cell mixtures in 2 cm^2 Linbro wells (ICN Pharmaceutical, Cleveland, Ohio) in the presence of 0.2 ml of PEG solution for 1 min at room temperature. Following fusion, the cells were washed rapidly with six 1 ml aliquots of phosphate buffered saline (PBS) [13], followed by the addition of α-medium containing 15% fetal calf serum for incubation at 37°C for 3–4 days before assay.

β-Galactosidase Activity In Situ

β-Galactosidase activity was monitored by a modification of the procedure of Lake [14]. Cells were sparsely plated in chambered slides (Lab-Tech Products, Naperville, Ill.) or Linbro wells and incubated overnight in α-medium at 37°C. The attached cells were washed with 0.9% saline and fixed for 5 min in 10% formalin in saline. The cells were assayed for β-galactosidase activity by incubating them overnight at 37°C in the presence of 5-chloro-3-bromo-indolyl-β-D-galactoside (Vega-Fox Biochemicals, Tucson, Arizona) in the described buffer solution, except that the sodium chloride concentration was increased to 0.15 M [14].

Protocal and Scoring of Results

The design of each experiment was to test each β-galactosidase deficient strain in three different fusions: (1) self-fusion of each mutant strain (negative controls); (2) fusion of test strains with an I-cell disease strain, which all mutant G_{M1} gangliosidosis strains were expected to complement (positive controls); (3) fusion between mutant strains (test fusion). For the positive control fusions, mutant strains were fused with an I-cell disease strain, since cells from the latter lack β-galactosidase activity but are from a child with a genetically distinct disorder [15]. The positive and negative controls insured that each strain being tested was competent to complement in each experiment and provided upper and lower limits of expected results. Results were determined by comparing test fusions with positive and negative control fusions and scoring test

fusions "+" for presence or "0" for absence of complementation. Normal cells were also self-fused to compare with positive controls. All complementation experiments were repeated at least twice.

Urinary Oligosaccharide Studies

During the second hospital admission, urine was collected for 3 days for analysis of urinary oligosaccharides. The oligosaccharides were separated by thin layer chromatography on silica gel G. An aliquot (20 μl of a 24-hr urine) was applied to the chromatogram as a 1 cm wide band. The plates were developed with butanol:acetic acid:water (2:1:1 vol/vol) for 12 hr, dried, and redeveloped for a further 12 hr. The bands were located by spraying with 0.2% orcinol in 20% sulfuric acid. After heating the covered plates for 10 min at 100°C, the reddish-brown bands appeared [16].

Similar plates were sprayed with resorcinol reagent [17] to identify sialic acid-containing oligosaccharides. The sprayed plates were covered with a clean glass plate, and the color developed by heating to 100°C for 10–15 min. Purple resorcinol-positive bands appeared.

Both free and bound sialic acid were determined in urine before and after hydrolysis in 0.1 NH_2SO_4 at 80°C for 1 hr. The samples were chromatographed on Pasteur pipette columns containing 0.5 ml of Dowex 1 × 2 resin in H_2O. Sialic acid was eluted with 0.84 M formic acid and assayed by the Aminoff modification of the Warren assay [18].

Preliminary compositional studies were also done on the accumulated oligosaccharides. Details of these procedures have been described previously [19, 20]. Briefly, the proton magnetic resonance spectra of purified oligosaccharide fractions were obtained, and the oligosaccharides were digested by acid hydrolysis for identification of the resulting sugars and amino-sugars by gas-liquid chromatography and mass spectrometry.

RESULTS

β-Galactosidase Activities in the Cell Strains

The levels of enzyme activity in the mutant cell strains assayed with both natural and synthetic substrates are compatible with those described in patients with G_{M1} gangliosidosis (table 1). Strain 126 from a patient with I-cell disease also showed a deficiency of β-galactosidase activity. In the present case (strain 652), I-cell disease was ruled out by demonstrating normal levels of hexosaminidase and arylsulfatase in the cultured cells and their absence in the culture medium.

Analysis of β-galactosidase levels in white blood cells from the parents revealed normal activity: mother, 176 nmoles/mg protein per hr; father, 247 nmoles; controls, 162 ± 49 nmoles.

Complementation Studies

Cells from each strain were examined for the hydrolysis of the indolyl-β-galactoside substrate in situ. Normal cells hydrolyzed this compound to produce indigo, which was deposited as insoluble blue-green granules in the cytoplasm around the clear nucleus. After approximately 2 hrs incubation, the cells began to show staining and continued to deposit granules for at least 16 hrs. Mutant cells lacking β-galactosidase were unable to hydrolyze the substrate, and the cells remained clear throughout the incubation. However, areas of densely packed, mutant cells showed light staining so that it was important to assay only sparsely plated cells.

In the complementation experiments, self-fusion of individual mutant or normal fibroblast strains always gave staining results similar to those of the unfused parent

strains. The indigo staining pattern in normal self-fused cells (fig. 3*a*) shows a heavy deposition of indigo in the cytoplasm, leaving clearly visible nuclei. A typical fused cell is seen in the center, while heavily stained mononucleate parental cells are visible at the periphery. Self-fusions of strains from the present case (652/652, fig. 3*b*) and a classical type I G_{M1} gangliosidosis strain (116/116, fig. 3*c*) show both mononucleate and multinucleate cells devoid of indigo particles. In the test fusion (652/116, fig. 3*d*), however, multinucleate cells are shown with clear deposition of indigo around the nuclei. Mononucleate cells in the test fusion failed to show indigo deposits. Indigo staining was also observed in the positive control fusions when either strain 652 or 116 was fused with strain 126 (I-cell disease). We conclude that strains 652 and 116 complemented in heterokaryons. In addition, all pairwise combinations of fusions were made involving strains 87, 116, 300, and 652. Fusions between strain 652 and any of the G_{M1} mutants always produced indigo staining of multinucleate cells. However, no

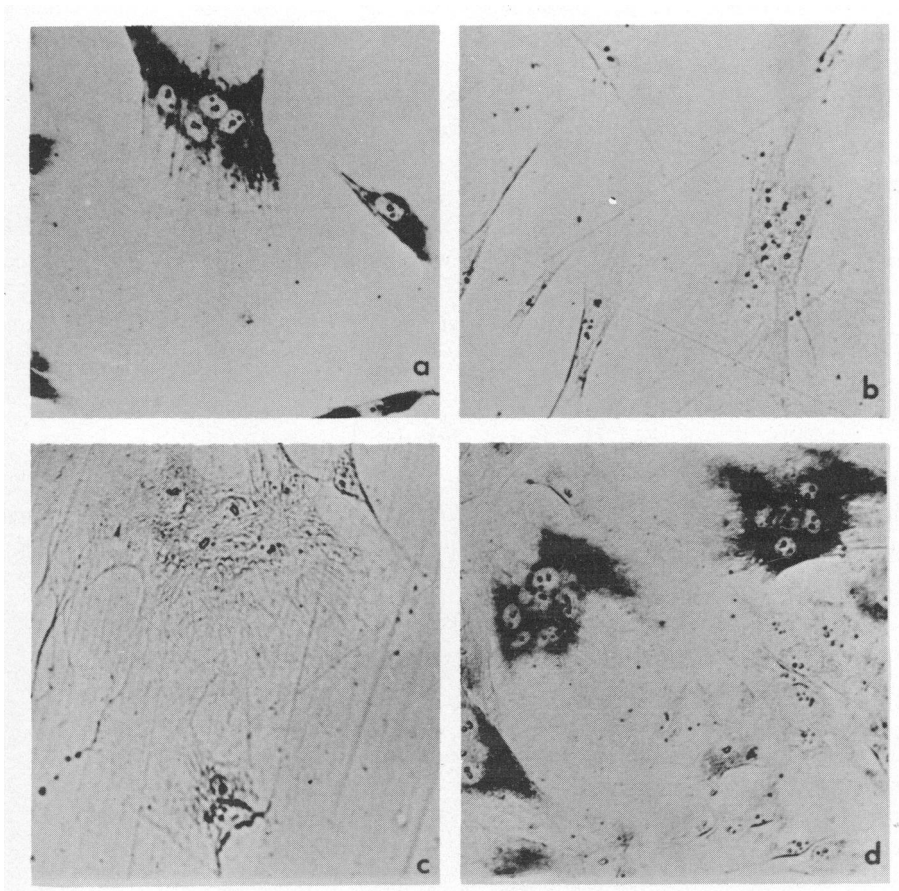

FIG. 3.—Indolyl-β-galactosidase staining patterns obtained after cell fusion. *a*, fusion of normal cells; *b* and *c*, self-fusion controls for complementation test in *d*. Fibroblast strains used: *a*, 160/160; *b*, 652/652; *c*, 116/116; and *d*, 652/116.

staining of cells was observed when the remaining mutants were fused in any pairwise combination. Each of these mutants always gave positive results when fusions with strain 126 were done in parallel (table 2).

Mixing mutant strains in the absence of PEG did produce occasional cells which became stained with the indigo particles. Most of these cells were found to be binucleate or, more rarely, trinucleate and appeared to represent heterokaryons produced by spontaneous fusion.

Urinary Oligosaccharides

In classical type 1 G_{M1} gangliosidosis, several oligosaccharides are excreted in urine (fig. 4). The most nonpolar of these is a pentasaccharide [20]: β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3)-β-Man-(1→4)-GlcNAc. The major nonpolar oligosaccharide in the urine of our patient migrated more slowly than the G_{M1} pentasaccharide (fig. 4) and stained positively with resorcinol, indicating it contained sialic acid. Analysis by proton magnetic resonance and gas liquid chromatography demonstrated it also contains galactose, mannose, and *N*-acetyl-glucosamine in a molar ratio 1:2:2. The thin layer chromatograms compare with those of O'Brien [16] and suggest the structure is identical to Strecker's compound II [21] α-NeuNAc-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3)-β-Man-(1→4)-GlcNAc.

Urinary Sialic Acid

The patient's urine contained .98 μmoles free sialic acid/ml (controls .06–.08), and after mild acid hydrolysis, 7.01 μmoles/ml were released (controls .2–.3). The values are clearly elevated. It is difficult to compare the values to those reported by others [22, 23], because they have assayed quantity against different parameters (e.g., per mg protein, or per 24 hrs). Other reports, however, do not show the 10-fold increase in free sialic acid which we noted.

DISCUSSION

Our patient, whose clinical appearance was that of the infantile, type 1 G_{M1} gangliosidosis, was found to complement four other cases of this disorder in cultured fibroblasts. The complementation technique used was adapted from an enzyme

TABLE 2

COMPLEMENTATION BETWEEN FOUR G_{M1} GANGLIOSIDOSIS STRAINS, AN I-CELL DISEASE STRAIN AND THE PRESENT CASE OF SIALIDOSIS

Strain	116	511	87	300	652	126
116	0*	0	...	...	...	...
511	0	0	...	...	...	...
87	0	0	0	...	...	...
300	0	0	0	0	...	...
652	+	+	+	+	0	...
126	+	+	+	+	+	0

NOTE. —See table 1 for explanation of strains.

* 0 = Absence of complementation; + = presence of complementation.

histochemical procedure described by Lake [14]. He showed that indolyl-hexosides could be used on tissue slices or cells to detect enzyme activity visually. We overcame the absence of quantitation by incorporating both positive and negative control fusions with each complementation test. The positive control fusions with strain 126, derived from a patient with I-cell disease, were run in parallel with the test fusions to provide a measure of the upper limit of activity staining that could be expected for complementing G_{M1} gangliosidosis mutants. Thus, the decision about whether a test fusion showed complementation depended on comparing its stain intensity with the range obtained between the self and positive control fusions.

This study shows a lack of a correlation between clinical and genetic heterogeneity. The cells of the index case, a patient who clinically resembled patients with type 1 G_{M1} gangliosidosis, complemented two other type 1 strains. Thus, these strains belong to separate complementation groups despite the clinical identity of the patients. In addition to the two type 1 strains, the second complementation group also includes a type II strain and strain 300, derived from a child with a milder, juvenile form of the disorder.

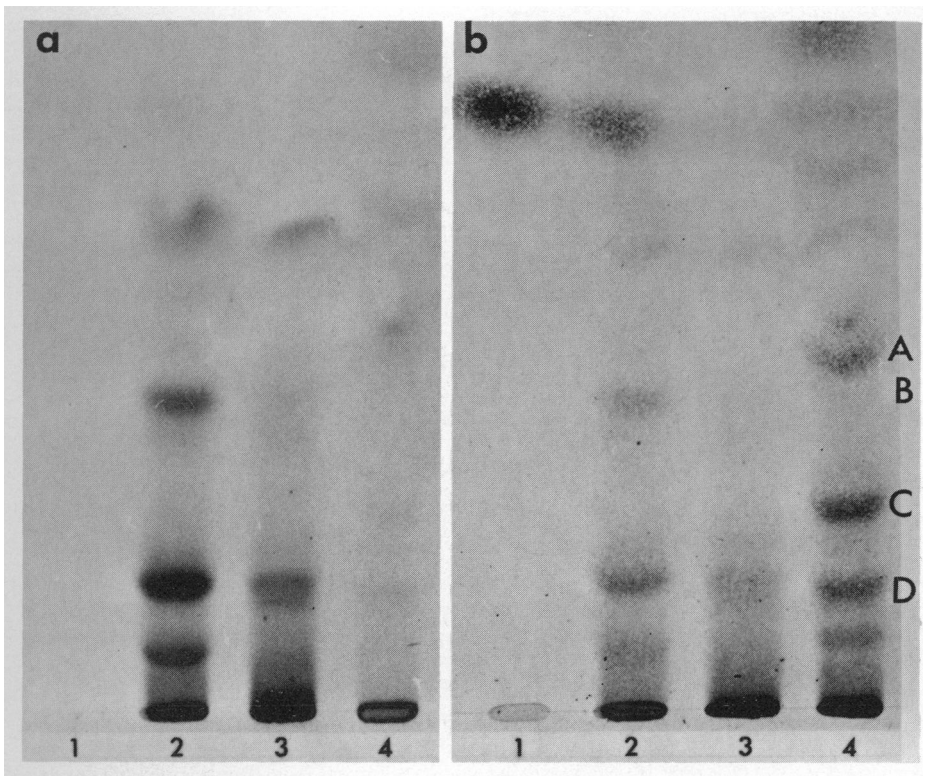

FIG. 4.—Thin layer chromatograms of urinary oligosaccharides developed in butanol: acetic acid:water (2:1:1) twice, 12 hrs each. (*a*) spots located with orcinol spray; (*b*) spots located with resorcinol spray (see text). *Lane 1*, lactose; *lane 2*, patient's urine; *lane 3*, I-cell disease urine; *lane 4*, G_{M1} gangliosidosis urine. *Band A*, pentasaccharide (see text); *band B*, hexasaccharide with terminal NeuNAc (Strecker's compound II [20]).

In addition to the classical infantile (type 1) and juvenile (type 2) patients with G_{M1} gangliosidosis, many variants have been described [24–27]. Most of these patients are adults and have a slowly progressive disease that began at 10–15 years of age. Some patients, previously thought to have G_{M1} gangliosidosis on the basis of a β-galactosidase deficiency in leukocytes or cultured fibroblasts, have now been shown to have sialidosis [26–29]. None of these cases, however, demonstrated the phenotypic expression of classic type 1 G_{M1} gangliosidosis. Our patient had those physical features and is presented here to emphasize the importance of appropriate laboratory diagnosis in association with clinical observation.

Phenotypic variability is now recognized in the sialidoses [5]. Increasing use of thin layer chromatography of urinary oligosaccharides as a diagnostic assay in patients with undiagnosed inborn errors of metabolism will, no doubt, expand our knowledge of the variability. The present case clearly stresses the value of using more than one type of assay as a confirmatory study in any investigation of a patient with a lysosomal storage disorder. This infant had the typical low levels of β-galactosidase seen in type 1 G_{M1} gangliosidosis, yet that enzymopathy proved to be secondary when an appropriate gene dose response was not found in the parents.

The nature of the low levels of β-galactosidase activity in these patients is not clear. Of the recognized infantile-onset cases of sialidosis, defects in β-galactosidase have only been described in this infant [5]. Several of the juvenile onset-type patients described in the same report did have low levels of β-galactosidase activity, but these were usually variable, being present in some tissues while absent in others. Interestingly, the juvenile onset patients who have a secondary β-galactosidase defect do not store G_{M1} ganglioside in the central nervous system [25]. Although there are surely many variants of G_{M1} gangliosidosis, this case indicates that all deserve adequate investigation to establish a primary enzyme defect.

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