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Prenatal Diagnosis of G_{M2} Gangliosidosis with High Residual Hexosaminidase A Activity (Variant B^1; Pseudo AB Variant)

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ABSTRACT. A case of infantile G_{M2} gangliosidosis with high residual β-hexosaminidase A activity toward the synthetic substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside was diagnosed prenatally. Extracts from cultured amniotic fluid cells of the fetus had a hexosaminidase A activity of 27% of total hexosaminidase but were almost completely unable to degrade [^{3}H]ganglioside G_{M2} (less than 0.5% of control values) when assayed in the presence of the natural activator protein. These results were confirmed by analyses of fetal muscle fibroblasts, liver, and brain. All tissues examined showed a profound deficiency of ganglioside G_{M2} galactosaminidase despite hexosaminidase A levels in the heterozygote range. In brain tissue, ganglioside G_{M2} content was elevated more than 4-fold. Hydrolysis of *p*-nitrophenyl glucosaminide-6-sulfate, a substrate specific for hexosaminidases A and S, by tissue extracts was also markedly reduced but the residual activities found (5% in liver, 12% in fibroblasts, and 16% in brain) were much higher than those with the physiological lipid substrate, ganglioside G_{M2}. (*Pediatr Res* 19: 1220–1224, 1985)

Abbreviations

4-MU-GlcNAc, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside
PG-6S, pNPGlcNAc-6-SO_4, *p*-nitrophenyl-2-acetamido-2-deoxy-6-sulfo-β-D-glucopyranoside

G_{M2} gangliosidoses are recessively inherited disorders of lysosomal glycolipid catabolism, characterized by massive accumulation of ganglioside G_{M2} and glycolipid G_{A2} in nervous tissue (for review see References 1–4). The numerous variant forms of this disease can be classified either biochemically or according to their clinical presentation. Biochemically, three enzymic variants can be distinguished: patients of variant B (Tay-Sachs disease) lack the lysosomal hexosaminidase isoenzyme β-hexosaminidase A (5, 6), which is responsible for the degradation of the stored glycolipids (7). In patients with variant O (Sandhoff disease) both major lysosomal hexosaminindase isoenzymes, A and B, are deficient (8, 9), due to a defect of their common subunit (10, 11). The third variant, termed AB variant (9), is not caused by the deficiency of an enzyme but by the absence of a nonenzymic activator protein required for the interaction of the water-soluble hexosaminidase A with its membrane-bound lipid substrates (12–14).

The clinical variation of G_{M2} gangliosidoses ranges from severe infantile cases with onset of neurological symptoms during the 1st yr of life and death usually occurring before the age of 4 yr to mild adult cases which may be only moderately handicapped at the age of 40 yr or more (for review see Reference 15).

Diagnosis of G_{M2} gangliosidosis is usually based on the determination of residual β-hexosaminidase A activity in serum, leukocytes, or skin fibroblasts of the patient (for review see References 3 and 16). Routine assays employ fluorogenic or chromogenic synthetic substrates, after separation of the isoenzymes or after differential heat inactivation.

Recently several late-infantile G_{M2} gangliosidosis patients were described with nearly normal β-hexosaminidase A activities when assayed with the synthetic substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (17–20). These enzymes, however, proved to be unable to cleave the physiological substrate, ganglioside G_{M2}, in the presence of the natural activator protein. They were also inactive against *p*-nitrophenyl-N-acetyl-β-D-glucosaminide-6-sulfate (19, 21), a synthetic substrate that is thought to be hydrolyzed predominantly by β-hexosaminidases A and S (22, 23). Therefore they are presumed to carry mutations in the α-subunit locus, representing allelic variants of G_{M2} gangliosidosis variant B (Tay-Sachs disease). Consequently, these variants have been referred to as variant B^1, pseudo-AB variant, or A^MB variant (for hex A mutated). It is evident that such cases can not be diagnosed reliably with the routine methods employing as substrates either 4 MU-GlcNAc or lipid substrates in the presence of detergents. Recently a sensitive assay was developed to assess the ability of extracts from cultured fibroblasts to degrade ganglioside G_{M2}, in the presence of the physiological cofactor, the G_{M2} activator protein (24, 25).

We report herein on the application of this assay system to the antenatal diagnosis of a case of G_{M2} gangliosidosis variant B with high residual activity of β-hexosaminidase A (variant B^1).

CASE REPORT

The index case, a male first child of unrelated parents, after uneventful delivery (birth weight 3,600 g, length 51 cm) showed normal development for the first 7 months. However, after a viral infection, psychomotor development deteriorated. At the age of 8 months, hyperacusis was noted. Ensuing clinical symptoms were tremor of the upper extremities (three to four times a day for several seconds), extension of the lower extremities (8–9

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Table 1. *Degradation of ganglioside G_{M2} by homogenates of skin fibroblasts from family members and of cultured amniotic fluid cells*

Subject	4-MU-GlcNAc-β-hexosaminidase		Ganglioside G_{M2} degradation (pmol/h × mg × AU*)	Degradation of PG-6S (nmol/min × mg)	Acid phosphatase (nmol/min × mg)
	Total activity (nmol/min × mg)	% Hex A			
Fibroblasts					
Proband	55.7	22.4†	2.3		16.4
Father	59.0	41.9‡	121		25.8
Mother	66.7	37.6‡	357		15.9
Infantile G_{M2} gangliosidosis	33.3	0†	6.1		24.4
Controls	44.6	76.6†‡	670		
	(33.2–57.2)	(70.3–84.1)	(540–760)		
Amniotic cells					
Fetus at risk	30.0	27.1‡	1.9	0.88	17.8
Control 1	82.0	70.5†	963	14.87	27.1
Control 2	40.1	78.7‡	526	7.22	12.5

* One activator unit (AU) is defined as the amount of activator protein required to stimulate ganglioside G_{M2} hydrolysis by 1 nmol/h × unit hexosaminidase A (7).

† Determined by isoelectric focusing.

‡ Determined by ion-exchange chromatography.

months), spastic hemiparesis, severe psychomotor retardation (13 months), quadriplegia, dementia, and deterioration of kidney function, edema, and severe cachexia (22 months). Ophthalmological examination revealed cherry red spots in the clearly pale macular region. The patient died at the age of 23 months.

Autopsy findings included cerebral atrophy particularly of the left parietotemporal region, with hydrocephalus, focal leptomeningitis, scoliosis calvae, cranial cleft, bronchopneumonia, and atrophy of all organs (spleen 12 g, liver 250 g, kidneys 45 g, heart 30 g).

Microscopic examination revealed a general storage process which was seen most distinctly in neurons. In the Purkinje cells of the cerebellar cortex, storage granules were visible in the dendrites. In thalamus and corpus mamillare, neuroaxonal dystrophy with numerous fine granular spheroids was seen, and the filament glia was multiplied. Storage was also apparent in the nerve plexes of the gastrointestinal system.

Histochemical staining revealed the presence of strongly acidic glycolipid. Lipid storage was accompanied by accumulation of autofluorescent sudanophilic material, most clearly seen in the granular cells. Electron microscopy revealed classical membraneous cytoplasmic bodies.

A partial deficiency of thermolabile hexosaminidase activity against 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide was detected in the proband's fibroblasts (Dr. A. D. Patrick, London, England). The family was referred to the laboratory in Bonn for further biochemical study.

The diagnosis of G_{M2} gangliosidosis variant B^1 was established on the basis of a profound deficiency of ganglioside G_{M2} hydrolase activity in cultured skin fibroblasts (Table 1).

Genetic counseling was provided and the couple requested prenatal diagnosis. Amniocentesis was performed at 18 wk of gestation. After 5 wk, the amniotic cells were assayed and found to be almost completely deficient in ganglioside G_{M2} hydrolase activity (Table 1). Counseling on the basis of these results led the parents to elect termination of the pregnancy.

Selected tissue specimens were processed for culture (muscle fibroblasts) or frozen at −60° C and shipped to the laboratory.

MATERIALS AND METHODS

Materials. Ganglioside G_{M2} was isolated from Tay-Sachs brain, tritiated in the N-acetyl-galactosamine moiety, repurified and its specific activity (11 Ci/mol) determined as previously described (25). PG-6S was prepared as described by Kresse *et al.* (26).

The activator protein specific for the degradation of ganglioside G_{M2} by β-hexosaminidase A (G_{M2} activator) was purified from postmortem human kidney as described (7). One activator unit was defined as the amount of activator protein that stimulates ganglioside G_{M2} hydrolysis by hexosaminidase A under the conditions used for activator quantification (7) (100 mM citrate buffer, pH 4.2, 37° C) by 1 nmol/h per enzyme U. One mg of pure activator corresponds to approximately 4,000 activator units.

Cell culture. Fibroblast cultures were established from forearm punch biopsies (skin fibroblasts) or fetal muscle tissue. The cells were grown in EPL (Institute of Sera and Vaccines, Prague, Czechoslovakia) medium consisting of medium TC 199, 0.4% lactalbumin hydrolysate, 0.4% growth promoting proteins from calf serum (27), and 5% fetal calf serum. Cultures of amniotic fluid cells were established in the same way. After the fourth passage, the cells were shipped to the laboratory in Bonn where they were cultured with Eagle's modified medium (Gibco, Grand Island, NY, cat. no. H 16) containing 10% fetal calf serum and maintained in a 5% CO_2 atmosphere. After 6–8 wk (four subcultures), confluent cells were harvested with trypsin and frozen at −20° C until assay. Amniotic fluids cells were cultured in essentially the same way but were harvested after confluency of the second subculture.

Enzyme Assays. Aqueous cell homogenates (10%, w/v) and extracts were prepared as previously described (25).

Fluorogenic substrates. β-hexosaminidase activity was monitored with 4-MU-GlcNAc (Koch-Light, Colnbrook, England) as substrate as previously described (28). For the assay of acid phosphatase and β-galactosidase, appropriately diluted samples of the homogenates were added to incubation mixtures containing 0.2 μmol of 4-MU phosphate and 10 μmol of citrate buffer, pH 5.0 (acid phosphatase) or 0.2 μmol of 4-MU-β-D-galactoside and 20 μmol acetate buffer, pH 4.3 (β-galactosidase in a total volume of 200 μl). After 30-min incubation at 37° C, the reactions were terminated by addition of 1 ml 0.2 M Na_2CO_3/0.2 M glycine solution. Fluorescence of the liberated 4-methylumbelliferone was read in a filter fluorimeter (Locarte, London, England) and compared with that of a standard solution of known concentration. Assays with PG-6S as substrate were performed as previoulsy described (19).

Table 2. *Degradation of various hexosaminidase substrates by extracts of fetal tissues*

Proband	Total β-hexosaminidase (4-MU-GlcNAc, nmol/min × mg)	Total β-hexosaminidase % Hex A*	Degradation of ganglioside G_{M2} (pmol/mg × h × AU)	Degradation of pNPGlcNAc-6-SO_4 (nmol/min × mg)	β-Galactosidase (nmol/min × mg)	Acid phosphatase (nmol/min × mg)
Brain						
At-risk fetus	3.78	46	0.34	0.122	0.63	5.16
Control 1†	5.29	78	33.1	0.944	0.54	3.97
Control 2‡	4.03	80	25.3	0.541	0.64	4.14
Liver						
At-risk fetus	10.89	26	2.54	0.205	4.06	13.17
Control 1	21.14	80	174.7	4.01	2.63	12.96
Control 2	20.77	81	145.4	3.56	4.92	9.08
Muscle fibroblasts						
At-risk fetus	42.8	31.5	3.8	1.1	6.69	18.4
Control 1	49.8	64.0	528	9.1	7.20	13.5

* Determined by isoelectric focusing.
† Pompe's disease.
‡ Down's syndrome.

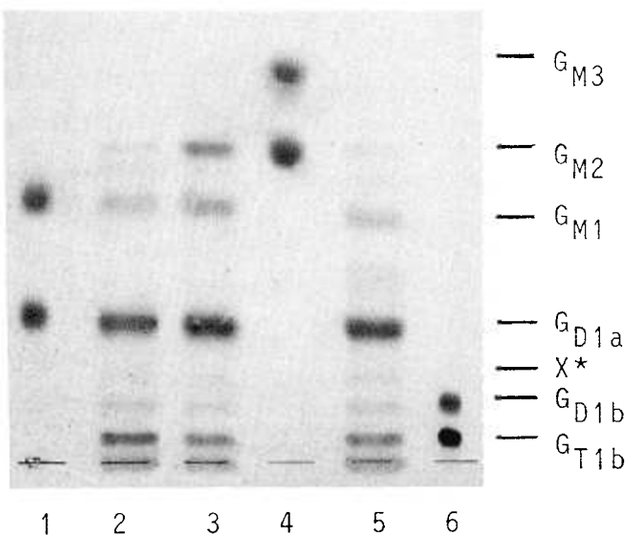

Fig. 1. Ganglioside patterns in fetal brains. Samples of fetal brains were extracted and analyzed as described in "Materials and methods." *Lanes 1, 4, and 6*, ganglioside standards; *lane 2*, fetus with Pompe's disease; *lane 3*, at -risk fetus; *lane 5*, fetus with Down's syndrome. X*, unknown compound, presumably G_{T1a}.

[³H]ganglioside G_{M2}. Degradation of ganglioside G_{M2} hydrolysis by cell extracts, in the presence of the physiological activator protein (5 activator units), was performed as previously described (25).

Isoelectric Focusing. Isoelectric focusing was performed in 15-ml columns according to Harzer (29) with carrier ampholytes, pH 3.5–10 (LKB, Bromma, Sweden) in a linear sucrose gradient from 35% to 0% (w/v). The cathode solution was 1.5% (w/v) ethylene diamine in a 50% (w/v) aqueous sucrose solution; the anode solution was 0.1% (w/v) aqueous H_2SO_4. After 18 h at 1000 V, focusing was terminated and fractions of 0.5 ml were collected. The pH value of each fraction was determined in 65 μl aliquots with a micro electrode (Radiometer, Copenhagen, Denmark) and hexosaminindase acitivty was measured with 4-MU-GlcNAc as described above.

Separation of Isoenzymes by Ion-Exchange Chromatography. Fibroblast extracts were loaded onto 0.5-ml columns of DEAE-cellulose (DE 52, Whatman, Maidstone, England) that had been equilibrated with 10 mM phosphate buffer, pH 6.0, with 50 mM NaCl. Nonadsorbed material, including hexosaminidase B, was eluted with 3 column vol of the same buffer. Hexosaminidase A was eluted with 3 vol of 0.5 M NaCl in buffer.

Table 3. *Ganglioside content of fetal brain tissues (nmol/g wet wt)**

Ganglioside	Affected fetus	Control 1 (Pompe's disease)	Control 2 (Down's syndrome)
G_{T1b}	19.3	38.5	32.0
G_{D1b}	12.3	17.9	19.8
X†	12.0	18.9	14.7
G_{D1a}	133.2	143.5	153.7
G_{M1}	96.4	90.7	84.4
G_{M2}	114.5	25.9	26.8
G_{M3}	11.7	11.1	16.4

* Extraction and analysis of glycolipids was done as described in "Materials and methods."
† Unidentified ganglioside migrating between G_{D1a} and G_{D1b}, presumably G_{T1a}

Lipid Analysis. Tissue samples were homogenized in 20 vol of chloroform/methanol (2/1; v/v) and filtered. The residues were rehomogenized with 10 vol of chloroform/methanol (1/2; v/v) and filtered again. Combined filtrates were dried under a stream of N_2. The residues were dissolved in 4 vol of chloroform/methanol (2/1; v/v) and 1 vol of 0.1 M aqueous KCl was added. After shaking, the phases were separated by centrifugation. The lower phases were reextracted twice, each time with 2 vol of theoretical upper phase (chloroform/methanol/water; 3/48/47; v/v/v). Combined upper phases were dialyzed against distilled water for 24 h and then lyophilized. The residues were taken up in chloroform/methanol (2/1; v/v; 5 ml/g tissue wet weight). Sialic acid content was determined by the method of Svennerholm (30) as modified by Miettinen and Takki-Luukkainen (31). Aliquots containing approximately 12 nmol sialic acid were applied to tlc plates (Kieselgel 60; Merck, Darmstadt, FRG). The plates were developed in chloroform/methanol/15 mM aqueous $CaCl_2$ (55/45/10; v/v/v), sialic acid-containing substances were visualized by spraying with a solution of 600 mg *p*-dimethylaminobenzaldehyde in 100 ml ethanol/conc. HCl (8/2; v/v) and subsequent heating at 150° C for 10 min. The spots were quantified with a tlc densitometer (Shimadzu CS-910, Shimadzu Europe, Düsseldorf, FRG) equipped with an automatic integrator (Shimadzu Chromatopac C-R1A) by transmission densitometry at 575 nm.

RESULTS

Cleavage of various hexosaminidase substrates by extracts of aminotic fluid cells. Cultured amniotic fluid cells were harvested and extracted with water as previously described for fibroblasts (25). Aliquots of the extracts were assayed for total β-hexosaminidase activity with 4-MU-GlcNAc, for the percentage of the hexosaminidase A isoenzyme, for their capability to hydrolyze ganglioside G_{M2} in the presence of the physiological activator protein, and for their activity against the artifical chromogenic substrate PG-6S, a substrate specifically hydrolyzed by hexosaminidases A and S (22, 23).The results are shown in Table 1.

The at-risk cells showed a clear deficiency of ganglioside G_{M2} catabolism (0.3% of the control values). In contrast, hexosaminidase A was found to make up some 27% of total hexosaminidase activity toward 4-MU-GlcNAc, a value which would usually be interpreted as indicating a carrier status rather than that of an affected patient. Degradation of pNP-GlcNAc-6-SO_4 was also strongly depressed, at approximately 10% of control values.

Fetal fibroblasts. A muscle fibroblast culture was established from fetal tissue. Biochemical examination of these cells revealed the same severe deficiency of ganglioside G_{M2} β-galactosaminidase activity (Table 2) as had been found with the amniotic fluid cells. Hexosaminidase A activity as determined with 4-MU-GlcNAc, after separation of the isoenzymes, was again in the heterozygote range and activity against pNP-GlcNAc-6-SO_4 was some 12% of that of the control (Table 2).

Fetal tissues. Diagnosis of G_{M2} gangliosidosis was confirmed in brain and liver tissue of the aborted fetus. In both tissues a severe deficiency of ganglioside G_{M2} catabolism could be demonstrated (Table 2). Hydrolysis of pNP-GlcNAc-6-SO_4 was also slower than normal but the residual activity toward this substrate was some 5% of controls in the liver and between 13 and 22% of the control values in brain tissue. Assay of β-hexosaminidase isoenzymes with 4-MU-GlcNAc, after isoelectric focusing, showed β-hexosaminidase A percentages in the low heterozygote range (Table 2), with normal isoelectric points (not shown).

Lipid analysis revealed an approximately 4-fold increase of ganglioside G_{M2} concentration in brain tissue of the affected fetus, compared to the controls (Fig. 1, Table 3), further corroborating the diagnosis of G_{M2} gangliosidosis.

DISCUSSION

Diagnosis of G_{M2} gangliosidosis is usually based on the demonstration of the deficiency of hexosaminidase A toward synthetic substrates such as 4-MU-GlcNAc. These procedures are sufficiently rapid and sensitive for routine diagnosis and for screening purposes. However, patients who synthesize a functionally altered enzyme that still cleaves these water-soluble substrates but is inactive toward glycolipids may be misdiagnosed. Several such patients have recently been described (17–20). Conversely, healthy probands with very low hexosaminidase activity have been found in the course of family screening (32–34). In one such family, the father and one daughter had low hexosaminidase activity but were clinically healthy whereas another child had died of late-infantile G_{M2} gangliosidosis (32).

The prenatal diagnosis of such cases presents a difficult biochemical problem. The most secure approach to this problem is to determine the capability of amniotic cells to degrade the physiological substrate, ganglioside G_{M2}. This can be done either by extracting the cells and measuring their ganglioside G_{M2} hydrolase activity, in the presence of the physiological activator protein, or by feeding radiolabelled ganglioside to cell cultures.

The latter method, *i.e* loading cell cultures with the substrate and studying its metabolism in intact cells, would seem preferable since any defect in the entire system, such as deficiency of enzyme or activator protein or subcellular mislocation of one of them, would be detected (35). However, the quantitative interpretation of such data becomes very difficult because the turnover rates observed do not necessarily parallel residual activities of the enzymes. Even low residual enzyme activities may be sufficient to substain a turnover rate close to that observed in controls, depending on the rate of substrate uptake by the cells. (For a theoretical discussion of this relation see Reference 36.)

In contrast, determination of the enzyme's activity *in vitro* against the ganglioside substrate in the presence of the G_{M2} activator protein gives a direct estimate of the residual activity that correlates well with the clinical status of the patients (19, 23, 25).

Applied to cultured amniotic fluid cells, this determination showed the fetus at risk to have the same genotype as his brother who died from infantile G_{M2} gangliosidosis, variant B1, at the age of 23 months (Table 1). With 4-MU-GlcNAc as substrate, hexosaminidase A activity was in the low heterozygote range whereas the G_{M2} hydrolase activity in the presence of the G_{M2} activator protein was less than 0.5% of the control values, indicating a severe infantile G_{M2} gangliosidosis. Although extensive data on amniotic fluid cells were still lacking, on the basis of this extremely low residual activity the diagnosis of G_{M2} gangliosidosis, variant B^1 was established.

This diagnosis was confirmed by the examination of fetal tissues and fibroblasts. A profound deficiency of ganglioside G_{M2} cleaving capability was demonstrated in muscle fibroblasts as well as in liver and brain tissue (Table 2). Analysis of the ganglioside pattern in brain showed a more than 4-fold increase of ganglioside G_{M2} (Table 3 and Fig. 1), the major storage compound in this disorder, even at this early stage of development. Very similar values were reported by Schneck *et al.* (37) for the brains of two fetuses with variant B of G_{M2} gangliosidosis.

Hydrolysis of pNP-GlcNAc-6-SO_4 by the tissue extracts examined was also strongly decreased to 5% (liver) and 16% (brain) of the respective control values. The use of sulfated N-acetylglucosaminide substrates for the discrimination of G_{M2} gangliosidosis variants has been described by several groups (21, 23, 38, 39). It was generally found that patients with variant B and B^1 had very low residual activities with these substrates whereas even in infantile variants O rather high activities, sometimes overlapping with the carrier or even the normal range, were found (23, 38, 39), presumably because hexosaminidase S is very active with sulfated substrates (23). Fuchs *et al.* (40) found a residual activity toward PG-6S of 6% in the sera of infantile Tay-Sachs patients, indicating that hexosaminidase B can also attack such substrates to some extent. The activity of the residual hexosaminidase A toward PG-6S will have to be determined in cases of unclear G_{M2} gangliosidosis variants before this substrate can be used safely for prenatal diagnosis.

The assay of ganglioside G_{M2} hydrolase activity in extracts of cultured skin fibroblasts, in the presence of the natural activator protein, allowed a clear differentiation between G_{M2} gangliosidosis variants of different severity (25). It remains to be shown that a similar distinction can be made from amniotic fluid cells for prenatal diagnosis. However, this method is more laborious and time consuming than the established procedures using artificial substrates and its practical use will therefore be restricted to single unclear cases.

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