GANGLIOSIDES OF HUMAN MECONIUM – DETECTION OF A POSSIBLE FETAL ANTIGEN

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1. Introduction

When it was shown that ganglioside GM1 specifically bound and inactivated cholera toxin [1] it was of interest to study the occurrence of this ganglioside in intestinal mucosa, the target organ for the toxin. By comparative studies of the small intestinal mucosa in man, pig and beef we showed that there was a direct relation between the binding ability for cholera toxin and the ganglioside GM1 content [2], which suggested that ganglioside GM1 is the natural specific receptor for cholera toxin. Intestinal mucosa of adult man had a remarkably low concentration of ganglioside GM1, and it was of importance both from a theoretical point of view, but also for the profylaxis and treatment of cholera and allied intestinal infectious diseases [3] to know the actual ganglioside GM1 concentration in intestinal mucosa during development. For the determination of the ganglioside concentration during the fetal stage we used meconium, which is the first faeces of the newborn and consists mainly of extruded mucosal cells from the intestine. The formation of meconium starts early during the fetal period, and it gives a readily available material for the examination of the glycolipid pattern in intestinal mucosa during fetal life. The chemical structure of the neutral glycolipids has been the subject for a few studies [4,5] and two reports have given thin-layer chromatographic evidences for the existence of gangliosides [5,6]. To our knowledge this is the first quantitative determination and characterization of the gangliosides in human meconium.

Abbreviations: TLC, thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; TMS, trimethylsilyl-; GLC–MS, gas–liquid chromatography–mass spectrometry; NeuAc, *N*-acetylneuraminic acid; gal, galactose; glcNAc, *N*-acetylglucosamine; glc, glucose

Nomenclature: The gangliosides have been designated according to [24]. GM3, II^3NeuAc-LacCer; GD3, $II^3(NeuAc)_2$-LacCer; GM1, II^3NeuAc-$GgOse_4$Cer; GD1a, IV^3NeuAcII^3-NeuAc-$GgOse_4$Cer; GT1b, IV^3NeuAc$II^3(NeuAc)_2$-$GgOse_4$Cer; 3'-LM1 and 6'-LM1, shorthand designation of IV^3-$nLcOse_4$-Cer and IV^6-$nLcOse_4$Cer, respectively

2. Experimental

2.1. *Material*

Samples of meconium were collected at the delivery ward, Department of Obstetrics and Gynecology, Östra sjukhuset, University of Göteborg, and at the Obstetric Service, County hospital, Mölndal. Silica gel 60, 230–400 mesh, TLC and HPTLC plates, silica gel 60, were obtained from Merck AG, Darmstadt. Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Uppsala. The anion exchange resin Spherosil-DEAE-dextran was a gift from Institute Mérieux, Lyon [7]. *Vibrio cholerae* sialidase (EC 3.2.1.18) was obtained from Behringwerke AG, Marburgh-Lahn. β-Galactosidase (EC 3.2.1.32) and β-*N*-acetylhexosaminidase isolated from jack beans were gifts from Drs S. C. and Y.-T. Li, Tulane University LA.

2.2. *Extraction and isolation of the glycosphingolipids*

Meconium was pooled from 42 full-term children. Only the first portion passed within 24 h from birth, was collected and stored at −20°C until extraction. The meconium (380 g) was extracted with 20 vol. and re-extracted with 10 vol. (w/v) of chloroform/methanol/water (4:8:3, by vol.) [8]. The total lipid extract was evaporated to dryness, and dissolved in chloroform/methanol/water (60:30:4.5, by vol.). Low-molecular contaminants were removed by chro-

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matography on Sephadex G-25 [9]. The lipid extract was applied to a Spherosil–DEAE-dextran anion-exchange column for the separation of the lipids into neutral and acidic lipids. The neutral lipids were eluted with chloroform/methanol/water (60:30:4.5, by vol.) and the gangliosides with a discontinuous gradient of potassium acetate in methanol according to their number of sialic acids [7]. The sulphoglycolipids were eluted together with the monosialogangliosides.

The individual gangliosides and sulphoglycolipids were isolated by silica column chromatography and preparative thin-layer chromatography [8]. The neutral glycosphingolipids were isolated from an aliquot of the neutral lipid fraction by peracetylation and chromatography on Florisil [10].

2.3. *Analytical methods*

Ganglioside sialic acid was assayed with the resorcinol method [11]. The distribution of individual gangliosides was determined by densitometry as in [8]. The total amount of neutral glycosphingolipids was determined as sphingosine base with the methyl orange method [12]. Monohexosylceramide and sulphatide were assayed with the orcinol procedure [13]. The carbohydrate moieties of the glycolipids were analysed by GLC as their alditol acetates [14]. The fatty acid and sphingosine base composition were analysed as in [15]. The 2-hydroxy fatty acids and the normal fatty acids were separated as their methyl esters by TLC developed in methylene chloride. The hydroxy fatty acids were then analysed by GLC as their TMS-derivatives.

2.4. *Partial acid and enzymic hydrolyses*

Partial acid hydrolysis of gangliosides was performed in 1 M formic acid for 30 min in a boiling water bath. Hydrolysis with *Vibrio cholerae* sialidase was performed in 0.01 M Tris–maleate buffer (pH 6.5) containing 4 mM $CaCl_2$, with and without 0.05% (w/v) Triton X-100. The incubations were done at 37°C for 16 h. The glycolipid products of the hydrolyses were analysed by HPTLC. Degradation with exoglycosidases was done as in [16].

2.5. *Permethylation studies*

The gangliosides were permethylated, hydrolysed, reduced, acetylated and analysed by GC-MS as in [17].

Table 1
Concentration of glycosphingolipids (μmol/kg meconium) in pooled meconium

Neutral glycolipids	Acidic glycolipids	
	Sulphoglycolipids	Gangliosides
2900	1400	480

3. Results

The concentrations of the different glycosphingolipid classes in meconium are given in table 1. The dominating neutral glycosphingolipid fraction was monohexosylceramides, which represented 65 M% of them, galactosylceramide constituted 45% and glucosylceramide 20%. The meconium was also rich in complex neutral glycosphingolipids with 4 or more sugars (~30% of the total), while di- and triglycosylceramides were minor components.

Sulphoglycolipids dominated among the acidic glycolipids, and occurred at 1400 μmol/kg meconium, galactosylceramide-3-*O*-sulphate constituted >95% of them. The concentration of gangliosides, expressed as μmol NeuAc/kg meconium, was 480 μmol/kg. The mono-, di- and polysialogangliosides represented 450, 20 and 8 μmol/kg, respectively. The ganglioside pattern expressed as percentage sialic acid is given in table 2. GM3 was the major ganglioside, and made up >80% of the total ganglioside sialic acid. The second most abundant ganglioside, 9% of the total, was a glucosamine-containing ganglioside, which had a dif-

Table 2
Ganglioside pattern of meconium

Ganglioside	%NeuAc
Monosialogangliosides	94.0
GM3	81.3
3′-LM1	0.8
6′-LM1	9.0
GM1	<0.5
Disialogangliosides	4.3
GD3	1.2
GD1a	1.9
Polysialogangliosides	1.7
GT1b	1.5

Table 3
The relative migration rate (R_{GM1}) by thin-layer chromatography of 3′-LM1 and 6′-LM1 in different solvent systems compared with that of GM1

Ganglioside	Propanol/ 0.25% KCl (3:1) (by vol.)	Propanol/ water/ ammonia (75:25:5) (by vol.)	Chloroform/ methanol/ 2.5 M ammonia (60:40:9) (by vol.)	Chloroform/ methanol/ 0.25% KCl (60:35:8) (by vol.)
3′-LM1	1.09	1.33	1.47	1.03
6′-LM1	0.95	0.88	0.87	0.82

ferent thin-layer chromatographic mobility than the major glucosamine-containing ganglioside, 3′-LM1, of human tissues (table 3). Several minor monosialogangliosides were also detected by TLC, but owing to the low amount of them, only 3′-LM1 (0.8%) and GM1 (<0.5%) were identified. This latter result clearly demonstrates that the concentration of ganglioside GM1 is very low also during the fetal development.

Component analyses of the major glucosamine containing ganglioside showed it to contain sphingosine, *N*-acetylneuraminic acid, glucose, galactose and *N*-acetylglucosamine in the molar ratio 1:1:1:2:1. Mild acid hydrolysis, or sialidase treatment with detergent added to the incubation buffer, totally removed the sialic acid, and gave a neutral tetraglycosylceramide. Further degradation with β-galactosidase, β-*N*-acetylhexosaminidase, and the combined action of both, showed the sequence of the sugars to be gal–glcNAc–gal–glc–cer. The results of the permethylation analyses are given in table 4. The structural analyses suggest the following structure of the major glucosamine-containing ganglioside of meconium: NeuAcα2→6galβ1→4glcNAcβ1→3galβ1→4glc–cer.

There was a large difference in the ceramide composition of the two gangliosides, GD1a and GT1b, belonging to the gangliotetraose series, compared to the other glycolipids isolated from meconium. Ganglioside GD1a and GT1b had a ceramide composition similar to that found in the corresponding gangliosides isolated from brain tissue, with high proportion of stearic acid, and sphingenine and eicosasphingenine as the dominating sphingosine bases. The glucosamine-containing ganglioside contained almost only 2-hydroxy fatty acids, with 16:0–22:0 as the dominating ones, and had a high proportion of phytosphingosine. A similar ceramide composition was also seen in galactosylceramide and in sulphatide. Ganglioside GM3 contained about equal amounts of normal and 2-hydroxy fatty acids, and had also a high proportion of phytosphingosines.

4. Discussion

In meconium, two gangliosides made up ~90% of the total ganglioside NeuAc. The major one was GM3, which is the common ganglioside in all extraneural human tissues, and the other one contained glucosamine. Structural analyses showed this latter ganglioside to be NeuAcα2→6-neolactotetraosylceramide (6′-LM1). This ganglioside was different from the major glucosamine containing ganglioside in other

Table 4
Composition of permethylated carbohydrates in the major glucosamine-containing ganglioside isolated from meconium, and in the corresponding desialylated substance

	2,3,4,6-Me_4 Gal	2,3,4-Me_3 Gal	2,4,6-Me_3 Gal	2,3,6-Me_3 Glc	3,6-Me_2 GlcNAcNMe
Ganglioside	0	1	1	1	1
Desialylated substance	1	0	1	1	1

human tissues [18] by the linkage of the sialic acid to the terminal galactose residue, NeuAcα2→6gal instead of NeuAcα2→3gal. This has been shown to be a minor component in human erythrocyte membranes [19]. Chromatographical evidence exists for its presence in human liver and spleen [20], but it has not been found in adult human intestinal mucosa [2,21].

The NeuAcα2→6gal linkage is hydrolysed much slower than the NeuAcα2→3gal linkage by *Vibrio cholerae* sialidase [22], and for the complete degradation of 6′-LM1 to the corresponding desialylated substance, detergent had to be added to the incubation mixture. The resistance to the action of sialidase could explain the high proportion of this ganglioside in meconium, and why the normally dominating glucosamine containing ganglioside in human tissues (3′-LM1) was only a minor component. Most of the gangliosides isolated from meconium (~90%) were sialidase labile, which is contradictory to the suspicion that the high proportion of NeuAcα2→6-neolactotetraosylceramide was caused by degradation of sialidase labile gangliosides. Our interpretation of the high proportion of NeuAcα2→6-neolactotetraosylceramide in meconium is that the activity of the sialyltransferases are different in the fetuses than in adults. This interpretation is supported by the observation that acidic oligosacharides isolated from meconium contained only the NeuAcα2→6gal linkage [23]. The ganglioside NeuAcα2→6-neolactotetraosylceramide might then be the first fetal antigen of glycolipid nature isolated and characterized.

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References

[1] Holmgren, J., Lönnroth, I. and Svennerholm, L. (1973) Infect. Immun. 8, 208–214.
[2] Holmgren, J., Lönnroth, I., Månsson, J.-E. and Svennerholm, L. (1975) Proc. Natl. Acad. Sci. USA 72, 2520–2524.
[3] Holmgren, J. (1981) Nature 292, 413–417.
[4] Karlsson, K.-A. and Larson, G. (1978) FEBS Lett. 87, 283–288.
[5] Karlsson, K.-A. and Larson, G. (1981) J. Biol. Chem. 256, 3512–3524.
[6] Magnani, J. L., Brockhaus, M., Smith, D. F., Ginsburg, V., Blaszczyk, M., Mitchell, K. F., Steplewski, Z. and Koprowski, H. (1981) Science 212, 55–56.
[7] Fredman, P., Nilsson, O., Tayot, J.-L. and Svennerholm, L. (1980) Biochim. Biophys. Acta 618, 42–52.
[8] Svennerholm, L. and Fredman, P. (1980) Biochim. Biophys. Acta 617, 97–109.
[9] Wells, M. A. and Dittmer, J. C. (1963) Biochemistry 2, 1259–1263.
[10] Saito, T. and Hakomori, S.-I. (1971) J. Lipid Res. 7, 449–452.
[11] Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604–611.
[12] Lauter, C. J. and Trams, E. G. (1962) J. Lipid Res. 3, 136–138.
[13] Svennerholm, L. (1956) J. Neurochem. 1, 42–53.
[14] Holm, M., Månsson, J.-E., Vanier, M.-T. and Svennerholm, L. (1972) Biophys. Acta 280, 356–364.
[15] Månsson, J.-E., Vanier, M.-T. and Svennerholm, L. (1978) J. Neurochem. 30, 273–275.
[16] Svennerholm, L., Månsson, J.-E. and Li, Y.-T. (1973) J. Biol. Chem. 248, 740–742.
[17] Svennerholm, L., Vanier, M.-T. and Månsson, J.-E. (1980) J. Lipid Res. 21, 53–64.
[18] Li, Y.-T., Månsson, J.-E., Vanier, M.-T. and Svennerholm, L. (1973) J. Biol. Chem. 248, 2634–2636.
[19] Watanabe, K., Powell, M. E. and Hakomori, S.-I. (1979) J. Biol. Chem. 254, 8223–8229.
[20] Wiegandt, H. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 1049–1056.
[21] Keränen, A. (1975) Biochim. Biophys. Acta 409, 320–328.
[22] Drezniek, R. (1967) Biochem. Biophys. Res. Commun. 26, 631–638.
[23] Herlant-Peers, M.-C., Montreuil, J., Strecker, G., Dorland, L., Van Halbeek, H., Veldink, G. A. and Vliegenthart, J. F. G. (1981) Eur. J. Biochem. 117, 291–300.
[24] Svennerholm, L. (1977) Eur. J. Biochem. 79, 11–21.