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# Selective terminal α2–3 and α2–6 sialylation of glycosphingolipids with lacto-series type 1 and 2 chains in human meconium

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Human meconium was found to contain two kinds of gangliosides with the same carbohydrate sequences belonging to the lacto-series. They were detected by TLC-immunostaining with monoclonal antibodies directed to the NeuAcα2–6Gal and $Lc_4Cer$ structures. One of these two gangliosides, a major one, which migrated on TLC to a position below that of standard $IV^3NeuAcnLc_4Cer$ from human erythrocytes, reacted with the antibody to NeuAcα2–6Gal. The other minor one, which migrated on TLC to a position corresponding to standard $IV^3NeuAcnLc_4Cer$, was detected with the antibody to $Lc_4Cer$ only when the plate, on which the individual gangliosides were separated, was subjected to prior treatment with *Vibrio cholerae* sialidase. The structures of the gangliosides, each identified by means of permethylation analysis and enzyme treatment after isolation with antibody monitoring, were shown to be $IV^6NeuAcnLc_4Cer$ for the former and $IV^3NeuAcLc_4Cer$ for the latter, indicating that the lacto-series type 2 ($nLc_4Cer$) and 1 ($Lc_4Cer$) chains are sialylated at different linkages, α2–6 and α2–3, respectively. $IV^6NeuAcLc_4Cer$ and $IV^3NeuAcnLc_4Cer$ were not detected, even in trace amounts, on TLC-immunostaining with the monoclonal antibodies. The concentrations of $IV^6NeuAcnLc_4Cer$ and $IV^3NeuAcLc_4Cer$ were 448 and 18 nmol/g dry wt of human meconium.

Lacto-series ganglioside; Monoclonal antibody

## 1. INTRODUCTION

Controlled glycosphingolipid synthesis occurring during germinal proliferation, differentiation and morphogenesis indicates the existence of a program believed to be quite closely concerned with the guidance and regulation of such processes genetically as well as epigenetically. Tissue-characteristic expression of glycosphingolipids has also been thought to be the outcome of such regulated biosynthetic processes. However, in cancer, unregulated metabolism of glycosphingolipids, such as the expression of glycosphingolipids characteristic of the fetal stage, is frequently observed. Although the molecular mechanism and biological significance of the shift in the metabolism in cancer cells to that in the fetal stage are not entirely clear, precise analysis of the mode of glycosphingolipid expression in cells in the fetal, adult and cancerous stages may provide valuable information for understanding this phenomenon. To characterize the glycosphingolipids in the human fetal stage, meconium has been shown to be a useful material. By utilizing two monoclonal antibodies, directed towards the $Lc_4Cer$ and NeuAcα2–6Gal structures, respectively, we were able to demonstrate the occurrence of distinctly different pathways in the human fetal stage for the synthesis of gangliosides with the same carbohydrate sequences.

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*Abbreviations:* $Lc_4Cer$, Galβ1–3GlcNAcβ1–3Galβ1–4Glcβ1–1ceramide; $nLc_4Cer$, Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1ceramide



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## 2. MATERIALS AND METHODS

### 2.1. *Glycosphingolipids from human meconium*

Extraction of total lipids from human meconium (A and O donors), followed by fractionation into neutral and acidic lipids by DEAE-Sephadex (A-25, acetate form) column chromatography, was carried out as described previously [1,2]. The acidic glycosphingolipids were prepared from the acidic fraction by saponification and dialysis, and then purified by ganglioside-mapping [3] and high-performance liquid chromatography (HPLC) on a column (2 cm internal diameter × 25 cm) packed with Iatrobeads (6RS8010). Monosialogangliosides were eluted from the HPLC column with a linear gradient prepared from chloroform/methanol/water (55:45:2 and 20:80:2, by vol.) and monitored by TLC-immunostaining with monoclonal antibodies.

### 2.2. *TLC-immunostaining*

A mouse monoclonal antibody, Y916 (IgM), to the NeuAc$\alpha$2–6Gal structure was obtained by immunizing mice (Balb/c) [5] with the total gangliosides from bovine milk fat globule membranes [6] and then hybridizing the sensitized lymphocytes with mouse myeloma cells (SP2), and a human monoclonal antibody, HMST-1 (IgM), to $Lc_4Cer$ by hybridizing the lymphocytes from a patient with uterine endometrial adenocarcinoma with mouse myeloma cells (P3X63Ag8U1). Gangliosides from human meconium were chromatographed on a thin-layer plate (Polygram; Macherey-Nagel, Duren, FRG) with a solvent system of chloroform/methanol/0.5% calcium chloride in water (55:45:10, by vol.). The plate was incubated with the blocking buffer (1% polyvinylpyrrolidone (PVP), 1% ovalbumin and 0.02% $NaN_3$ in phosphate-buffered saline (PBS)) at 37°C for 1 h, followed by about 120 ng protein of the monoclonal antibody to NeuAc$\alpha$2–6Gal in 3% PVP in PBS at 37°C for 2 h. For the reaction with the monoclonal antibody against $Lc_4Cer$, the plate was treated with 200 mU sialidase (*Vibrio cholerae*; Calbiochem-Behring, FRG) in 7 ml of PBS at 37°C for 2 h prior to the blocking step. After washing the plate 5 times with 0.1% Tween 20 in PBS, the antibody bound on the TLC plate was detected with peroxidase-conjugated anti-mouse IgM (Cappel Lab., Cochraville, PA, USA) and anti-human IgM (Cappel Lab.) antisera for mouse and human monoclonal antibodies, respectively, and the enzyme substrates ($H_2O_2$ and 4-chloro-1-naphthol) as described [4].

### 2.3. *Structural determination of gangliosides*

The structures of the purified gangliosides were determined by TLC-immunostaining, exoglycosidase treatments and permethylation analysis [4,6]. The exoglycosidases used were sialidase, $\beta$-galactosidases (*S. pneumoniae* and jack bean, Sigma) and *N*-acetyl-$\beta$-D-glucosaminidases (bovine kidney, Boehringer Mannheim; and jack bean, Sigma).

## 3. RESULTS

### 3.1. *Acidic glycosphingolipids of human meconium*

As shown in fig.1 and as already reported [7], sulfatide was present in the highest concentration in human meconium, and the concentration varied among individual specimens. The mean value for 20 individuals was 54.24 $\mu$mol/g dry wt; this was 13 times higher than that of GM3 (4.03 $\mu$mol/g dry wt), which comprised about 85% of the total gangliosides. Surprisingly, the concentration of sulfatide in human meconium was higher than that of phospholipids (6.39 $\mu$mol/g dry wt), the molar ratio of phospholipids, cholesterol and sulfatide being 1.0:10.4:8.5. Thus, glycosphingolipids, particularly sulfatide, are significantly accumulated in human meconium.

### 3.2. *TLC-immunostaining of acidic glycosphingolipids from human meconium*

As shown in fig.1, the total human meconium acidic glycosphingolipids, corresponding to 20 $\mu$g of GM3-sialic acid, were subjected to TLC and detected with orcinol reagent (A), the monoclonal antibody to NeuAc$\alpha$2–6Gal (B) and the monoclonal antibody to $Lc_4Cer$ after neuraminidase treatment of the plate (C), respectively. The ganglioside (ganglioside 2), which migrated to a position below that of standard $IV^3NeuAcnLc_4Cer$ from human erythrocytes on TLC and which was present in a concentration of 448 nmol/g dry wt of human meconium, was positively stained with the monoclonal antibody to NeuAc$\alpha$2–6Gal. When the plate was first treated with sialidase and then examined for reactivity with the monoclonal antibody to $Lc_4Cer$, a positive spot (ganglioside 1), which, however, was not observable with orcinol reagent, was clearly detected between GM3, the largest ganglioside, and the second largest ganglioside (ganglioside 2). It was clear that the ganglioside with NeuAc$\alpha$2–6Gal was negative with the monoclonal antibody to $Lc_4Cer$ after sialidase treatment of the plate.

### 3.3. *Structures of the gangliosides detected with monoclonal antibodies*

By monitoring the reactivities of gangliosides with the monoclonal antibodies to NeuAc$\alpha$2–6Gal and $Lc_4Cer$, two gangliosides were isolated, as shown in fig.1. Ganglioside 1, which did not react with the monoclonal antibody to NeuAc$\alpha$2–6Gal but reacted with the monoclonal antibody to $Lc_4Cer$ after sialidase treatment, migrated to a

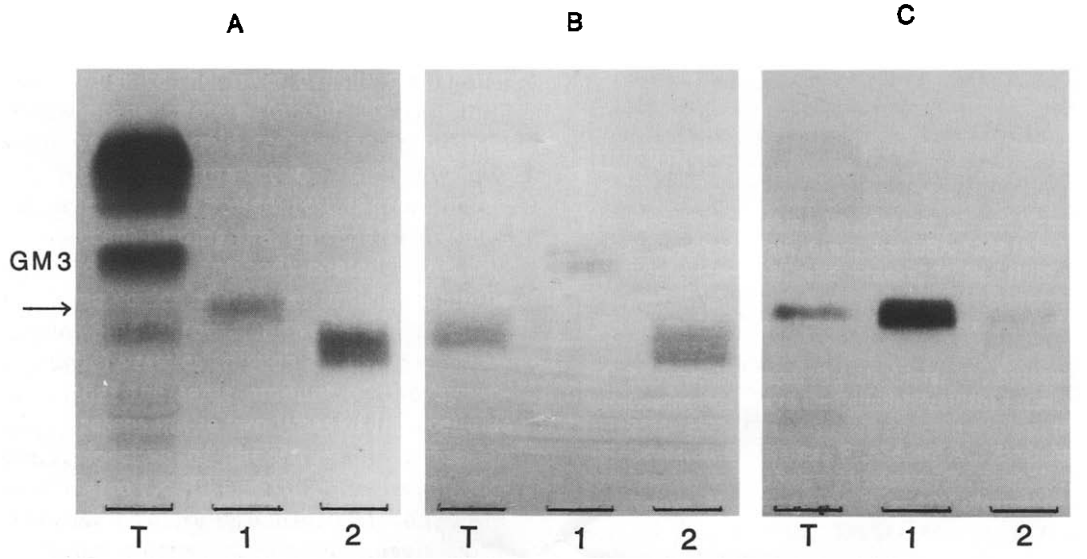


Fig.1. TLC of total acidic glycosphingolipids (T), and isolated gangliosides 1 and 2 from human meconium. Total acidic glycosphingolipids, corresponding to 20 μg of GM3-sialic acid, and gangliosides 1 and 2, corresponding to 5 μg and 10 μg of sialic acid, respectively, were applied on a TLC plate and then developed with chloroform/methanol/0.5% calcium chloride in water (55:45:10, by vol.). After cutting the plate, the spots were detected with orcinol reagent (A), and by TLC-immunostaining with the monoclonal antibody to NeuAcα2–6Gal (B) and the monoclonal antibody to $Lc_4Cer$ after treatment of the plate with *V. cholerae* sialidase (C). The major acidic glycosphingolipids migrating to above GM3 was sulfatide. The arrow indicates the position of standard $IV^3NeuAcnLc_4Cer$ from human erythrocytes.

position similar to that of standard $IV^3$ $NeuAcnLc_4Cer$ from human erythrocytes, and its concentration in human meconium was 18 nmol/g dry wt. Ganglioside 2, the second largest ganglioside in human meconium, migrated to a position lower than that of $IV^3NeuAcnLc_4Cer$ on TLC and reacted with the monoclonal antibody to NeuAcα2–6Gal but did not react with the monoclonal antibody to $Lc_4Cer$ after sialidase treatment. Both gangliosides 1 and 2 were susceptible to *V. cholerae* sialidase, which caused the release of *N*-acetylneuraminic acid as a sole sialic acid, and the products migrated on TLC to the same positions as $Lc_4Cer$ and $nLc_4Cer$. However, although the asialo derivative of ganglioside 1 was identified as $Lc_4Cer$ with the respective monoclonal antibody, ganglioside 2 was again confirmed to contain no $Lc_4Cer$ at all. Jack bean β-galactosidase completely cleaved both asialogangliosides 1 and 2 to form trihexaosyl ceramides, but *S. pneumoniae* β-galactosidase, which specifically reacts with β1–4 linkages but not with β1–3 linkages [8], only cleaved asialoganglioside 2 completely. These results indicate that the nonreducing terminals of asialogangliosides 1 and 2 are β-galactose, and that of ganglioside 2 is linked only through a β1–4 linkage, which is not present in asialoganglioside 1. The trihexaosyl ceramides obtained from asialogangliosides 1 and 2 on treatment with jack bean β-galactosidase were cleaved sequentially with *N*-acetyl-β-glucosaminidase, followed by β-galactosidase, and the final products were identified as glucosyl ceramides, indicating that gangliosides 1 and 2 have the same carbohydrate sequence, NeuAc-Gal-GlcNAc-Gal-Glc-ceramide. On permethylation analysis, as shown in fig.2, ganglioside 2 gave 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl galactitol and 1,3,5-tri-*O*-acetyl-2-deoxy-2-methylacetamido-4,6-di-*O*-methyl glucitol in the ratio of 1:2:1. Thus, the structure of ganglioside 1 was concluded to be NeuAcα2–3Galβ1–3GlcNAcβ1–3Galβ1–4Glcβ1–1ceramide, $IV^3NeuAcLc_4Cer$. On the other hand, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl galactitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl galactitol and 1,4,5-tri-*O*-acetyl-2-deoxy-2-methylacetamido-3,6-di-*O*-methyl glucitol were obtained from ganglioside 2 in the ratio of 1:1:1:1 (fig.2). This indicates that

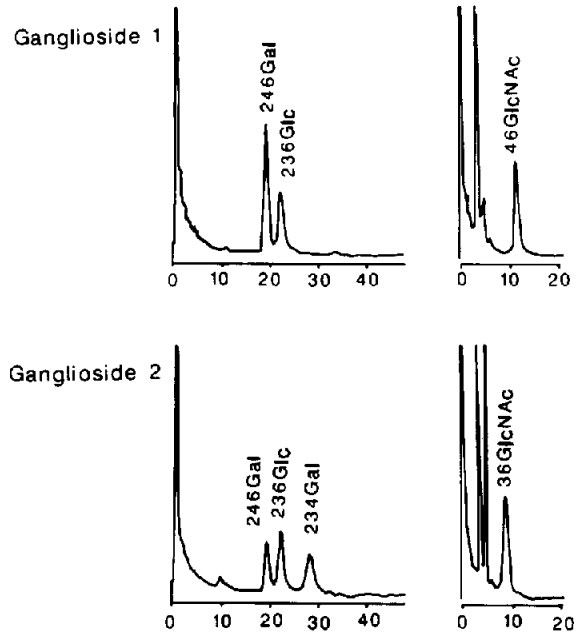


Fig.2. GLC of the partially methylated aldohexitol acetates derived from gangliosides 1 and 2. The GLC columns for the left and right chromatograms contained 3% ECNSS-M on Chromosorb W (100–200 mesh) and 2% OV-17 on Celite 545 (100–120 mesh), respectively. The scale indicates the retention time (min).

the structure of ganglioside 2 is NeuAc$\alpha$2–6-Gal$\beta$1–4GlcNAc$\beta$1–3Gal$\beta$1–4Glc$\beta$1–1ceramide, IV$^6$NeuAcnLc$_4$Cer. As a result, gangliosides with lacto-series type 1 and 2 chains in human meconium were found to be sialylated selectively at $\alpha$2–3 and $\alpha$2–6 linkages, respectively, and mixed structures such as IV$^3$NeuAcnLc$_4$Cer and IV$^6$NeuAcLc$_4$Cer could not be entirely detected by chemical and immunochemical procedures. The concentrations of IV$^6$NeuAcnLc$_4$Cer and IV$^3$NeuAcLc$_4$Cer, as determined by TLC densitometry, were 448 and 18 nmol/g dry wt of human meconium.

## 4. DISCUSSION

As clearly shown in this communication, gangliosides with lacto-series type 1 and 2 chains in human meconium are sialylated at different linkages. The monoclonal antibody to NeuAc$\alpha$2–6Gal reacted with a single molecule, which was the second largest ganglioside in human meconium and which did not contain type 1 chain, even in a trace amount. Also, the molecule showing a positive reaction with the monoclonal antibody to Lc$_4$Cer and migrating to within the region of sialyltetrahexaosyl ceramides was completely negative with the monoclonal antibody to NeuAc$\alpha$2–6Gal. Therefore, mixed forms of the molecules, with respect to the sialic acid linkages and the asialocarbohydrate structures, that is, IV$^3$NeuAcnLc$_4$Cer and IV$^6$NeuAcLc$_4$Cer, were not present in human meconium. Probably due to the difference in the specificities of the sialidases used for TLC detection of Lc$_4$Cer, we might not have been able to detect III$^6$NeuAcLc$_4$Cer, which was reported previously in human meconium [9]. Although IV$^6$NeuAcnLc$_4$Cer was detected as a minor component in normal adult human erythrocytes, liver and spleen [10,11], its synthesis is characteristically activated in human colorectal and lung carcinomas, which leads to cancer-associated antigenicity [12,13]. In addition, Lc$_4$Cer, particularly in the sialylated form with an $\alpha$2–3 linkage, was shown to be highly accumulated in several human cancers including teratocarcinomas [14] (Iwamori et al., unpublished). The two lacto-series gangliosides with the same carbohydrate sequences in human meconium are oncofetal antigens. Since IV$^3$NeuAcnLc$_4$Cer is frequently observed in adult human tissues and cells, our finding of the occurrence of IV$^6$NeuAcnLc$_4$Cer and IV$^3$NeuAcLc$_4$Cer in meconium indicates that a metabolic shift to formation of different linkages occurs developmentally, which may reflect the alteration of glycosyl transferases with different substrate specificities responsible for the synthesis of lacto-series gangliosides. Analysis of the enzyme kinetics for the lacto-series gangliosides in combination with the sensitive detection with monoclonal antibodies should shed light on the functional significance of the programmed expression of cell surface glycoconjugates and the underlying molecular mechanisms.

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