TUMOR GANGLIOSIDE – NATURAL OCCURRENCE OF GM_{1b}

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1. Introduction

In [1–3], difference of glycolipid compositions has been demonstrated between the island-forming cell type (maintaining cell adhesiveness) and the free cell type (lacking in cell adhesiveness) of rat ascites hepatoma cells. In general, the former was characterized by GM_3 as its sole ganglioside, while the latter was by the presence of asialo-GM_2, asialo-GM_1, fucolipid and gangliosides giving the same R_F value as GM_{1a} and GD_{1a} on thin-layer plate. However, the GM_1 synthesized in vitro from asialo-GM_1 and CMP-NeuAc by the sialyltransferase in AH 7974F (free cell type) was found to be GM_{1b} [4]. It is interesting to know whether GM_{1b} is present in the free cells or not. The present paper is concerned with the detailed analysis of ganglioside structures in AH 7974F cells.

2. Materials and methods

The cells, AH 7974F, were transplanted to rats of Moriyama inbred strain, (~150 g). After feeding animals for 4 days, the ascitic fluid was taken out and centrifuged at 600 rev./min for 10 min to sediment the tumor cells. The tumor cells were washed several times with 0.9% NaCl solution to remove red blood cells, and finally lyophilized [3]. Total lipids were extracted from the lyophilized cells (37 g) with 20 vol. C-M (2:1, v/v) and the residue was extracted again with the same volumes of C-M (1:1, v/v). The combined extracts were evaporated to dryness at 40°C under vacuum. Acidic lipids were separated from neutral lipids on a DEAE–Sephadex A-25 column [5]. The acidic lipid fraction was subjected to peracylation, which was followed by Florisil column chromatography as in [6]. By this procedure, 22 mg ganglioside was obtained from the starting materials.

Rechromatography on DEAE–Sephadex A-25 column (1.1 × 35 cm) of the total ganglioside fraction was carried out with linearly increasing ammonium acetate (AmAc) in methanol (from 0.03–0.3 M AmAc solution). The distribution of gangliosides in effluent fractions was detected by TLC (fig.1) [7]. All of the gangliosides eluted with 0.04–0.07 M AmAc (monosialo-ganglioside), which, however, could not be separated from each other on the column, were then subjected to Iatrobeads (6RS-8060, Iatron Co. Ltd., Tokyo) column chromatography (0.7 × 120 cm) using a linear gradient of C-M-W (from 83:16:0.5 to 20:80:5, v/v/v, 300 ml each) for the isolation of individual ganglioside [7]. As shown in fig.2, 5 gangliosides were purified by repeating the column chromatography at least 2 times. These gangliosides were designated as A, B, C, D and E from the top to the bottom of the thin-layer chromatogram, the mobilities of which corresponding to those of GM_3, GM_2, sialylparagloboside, GM_{1a} and GD_{1a}, respectively. The final yields of these purified gangliosides, A, B, C, D and E from 37 g dried cells were ~0.15 mg, 0.1 mg, 1.5 mg, 3.0 mg and 0.9 mg, respectively.

The sugar and fatty acid compositions were analyzed by GLC as in [3]. The long-chain bases were also analyzed by GLC as in [8].

Abbreviations: C-M-W, chloroform–methanol–water; NeuAc, *N*-acetylneuraminic acid; asialo-GM_2, GalNAc–Gal–Glc–ceramide; asialo-GM_1, Gal–GalNAc–Gal–Glc–ceramide; GM_{1a}, Gal–GalNAc–(NeuAc)–Gal–Glc–ceramide; paragloboside, Gal–GlcNAc–Gal–Glc–ceramide; MS, mass spectrometry; TLC, thin-layer chromatography; GLC, gas–liquid chromatography

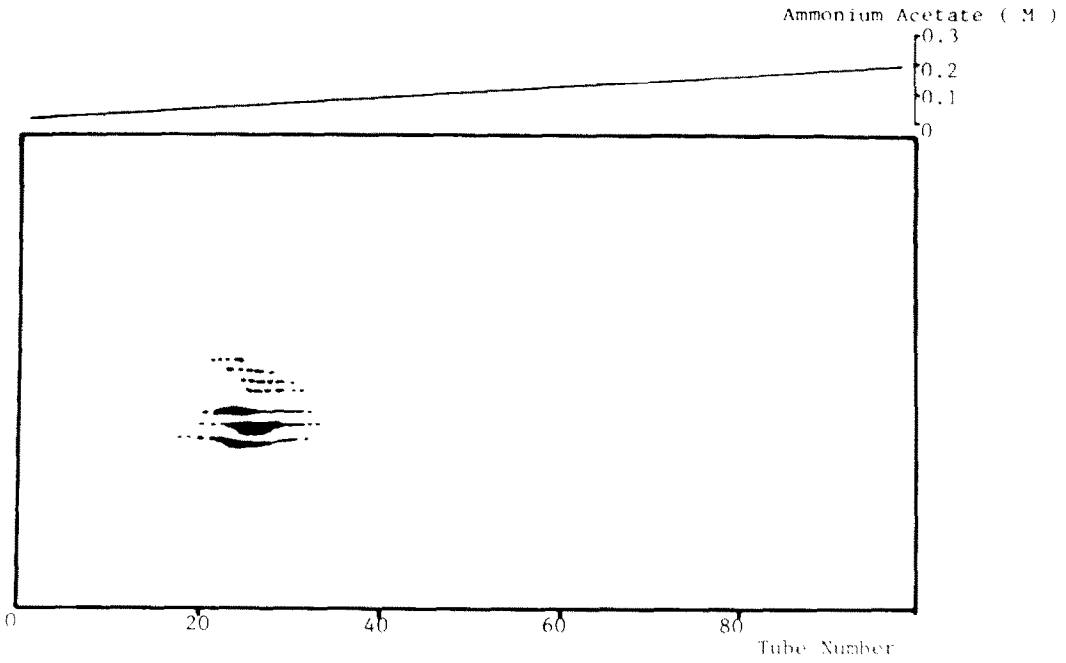

Fig.1. Elution pattern of AH 7974F gangliosides from DEAE–Sephadex A-25 column. Total ganglioside fraction (20 mg) was applied to a DEAE–Sephadex column with a linear gradient of a AmAc in methanol. Fractions (4 ml) were collected and each fraction was analyzed by TLC with a solvent system of C-M–ammonia 2.5 N (60:40:9, v/v/v). The spots were visualized by resorcinol reagent.

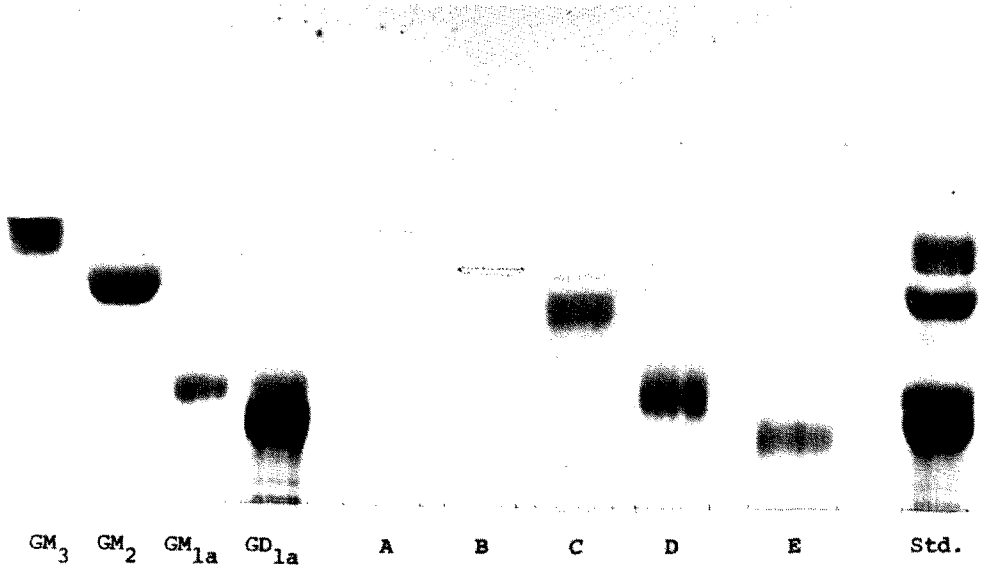

Fig.2. Thin-layer chromatogram of the ganglioside from AH 7974F. Each ganglioside was isolated by repeating (at least 2 times) Iatrobeads column chromatography as described in the text. TLC was performed with a solvent system as described in fig.1. Std., the mixture of standard gangliosides from bovine brain.

The isolated gangliosides, C and D were permethylated as in [9]. Partially methylated alditol acetates from the permethylated glycolipids were obtained as in [10]. Partially methylated neutral and *N*-acetamidoalditol acetates were analyzed on a column (1.5 m long) of 5% ECNSS-M on Chromosorb WAW 60-80 mesh, at 150°C by GLC [10–12]. The alditol acetates were also identified on a combined GC–MS system by reference to the data in [10,13–15]. GC–MS was performed as in [16].

Hydrolysis of the gangliosides C and D with neuraminidase from *Clostridium perfringens* (Sigma, type IV) was as in [17]. After incubation for 72 h at 37°C, the reaction mixture was dialyzed against distilled water (10 ml) for 3 days at 4°C. Non-dialyzable materials were analyzed by TLC. The dialyzable materials were subjected to TLC for the determination of species of sialic acid as in [18].

3. Results

3.1. *Chemical structure of ganglioside C*

Ganglioside C migrated to a position between GM_2 and GM_{1a} on a thin-layer plate (fig.2). As shown in table 1, this ganglioside was found to contain glucose, galactose, *N*-acetylglucosamine and sialic acid in the molar ratios of 1:2:1:1. Neuraminidase from *Cl. perfringens* converted it into paragloboside, which migrated between asialo-GM_2 and asialo-GM_1 (fig.3), with the liberation of *N*-acetylneuraminic acid which was identified in the dialysate of the reaction mixture by TLC. Partially methylated alditol acetates from ganglioside C were identical to those from sialylparagloboside from human erythrocytes (table 2). These studies indicated that the chemical structure of ganglioside C was as follows: NeuAc (2–3) Gal (1–4) GlcNAc (1–3) Gal (1–4) Glc–ceramide (sialylparagloboside). As shown in table 3, $C_{18:0}$ and $C_{24:1}$ are the dominant fatty acid in the ganglioside C. C-18 Dihydrosphingosine was the major (52%) long-chain base of this ganglioside detected.

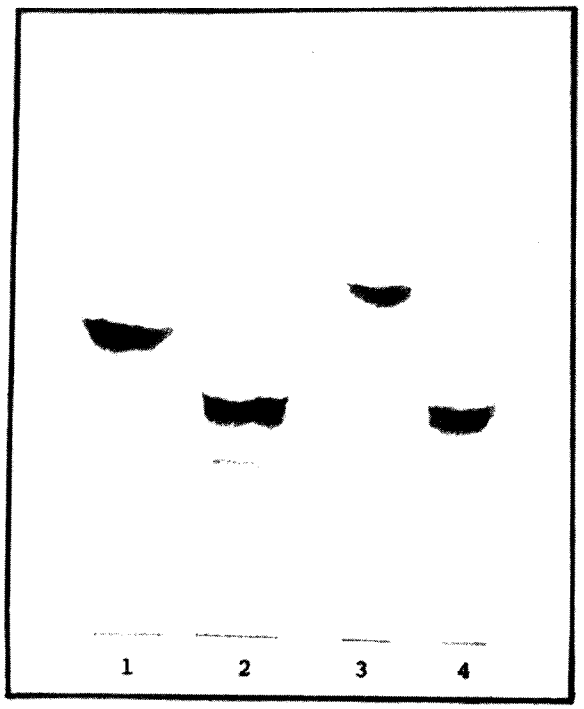

Fig.3. Thin-layer chromatogram of hydrolysis products of ganglioside C (lane 1) and D (lane 2) with neuraminidase from *Cl. perfringens*. The spots were visualized by α-naphthol/H_2SO_4 reagent. Lane 3, asialo-GM_2; Lane 4, asialo-GM_1. TLC was performed with a solvent system of C-M-W (60:35:8, v/v/v).

3.2. *Chemical structure of ganglioside D*

Ganglioside D moved identically with authentic GM_{1a} obtained from bovine brain (fig.2). GLC analysis of its sugar composition showed that ganglioside D contained glucose, galactose, *N*-acetylgalactosamine and sialic acid in the molar ratios of 1:2:1:1 (table 1). Neuraminidase from *Cl. perfringens*

Table 1
Sugar compositions of isolated gangliosides

Ganglioside	Glucose	Galactose	Galactosamine	Glucosamine	Sialic acid
C	1.0	1.7	–	0.7	0.8
D	1.0	2.2	1.0	–	1.1

Table 2
Partially methylated alditol acetates from isolated gangliosides

Alditol acetates	Glc	Gal	Gal	Gal	Gal	GalNAc	GlcNAc
Position of CH_3	2,3,6	2,3,4,6	2,3,6	2,4,6	2,6	4,6	3,6
Ganglioside							
C	+	–	–	+	–	–	+
D	+	–	+	+	–	+	–

converted the lipid into asialo-GM_1 (fig.3). This finding showed that ganglioside D was different from GM_{1a}, since GM_{1a} is resistant to the enzyme under usual incubation conditions [19]. The sialic acid released by the enzyme was found to be *N*-acetylneuraminic acid. Analysis of partially methylated alditol acetates indicated that the chemical structure of ganglioside D was as follows: NeuAc (2–3) Gal (1–3) GalNAc (1–4)Gal (1–4) Glc–ceramide (GM_{1b}). Compositions of the fatty acid and long-chain base were similar to those of ganglioside C (table 3).

4. Discussion

We have isolated 5 monosialo-ganglioside (A, B, C, D, E) from AH 7974F cell line. Two major gangliosides were characterized as sialylparagloboside and GM_{1b}. In both gangliosides, sialic acid was found to link to non-reducing terminal galactose of paragloboside and asialo-GM_1, respectively. This finding was also confirmed by resistance to galactose oxidase and NaB^3H_4 labelling technique as in [20].

Table 3
Fatty acid and long-chain base compositions of isolated gangliosides

	Ganglioside	
NAF	C	D
16:0	3.2	6.9
16:1	5.1	1.3
18:0	21.9	12.4
18:1	4.9	0.3
20:0	5.5	16.0
22:0	10.9	15.8
24:0	14.1	19.7
24:1	30.3	26.4
LCB		
d18:0	52.1	57.6
d18:1	25.3	27.6
d20:0	14.2	8.9
d20:1	8.0	6.0

NFA, normal fatty acid; LCB, long-chain base

In [3] we reported the presence of asialo-gangliosides in the free cell type of hepatomas, and GM_1 synthesized from asialo-GM_1 and CMP-NeuAc by sialyltransferase in AH 7974F cells was shown to be GM_{1b} but not GM_{1a} [4], suggesting the natural presence of GM_{1b} in the cells. In the present paper, it is described that the presence of GM_{1b} was confirmed by GLC, GC–MS and enzymatic hydrolysis studies. This is the first report on the natural occurrence of GM_{1b} in the mammalian cells.

The presence of sialyltransferase catalyzing the transfer of sialic acid from CMP-NeuAc to asialo-GM_1 to form GM_{1b} has been reported in rat brain [21]. We have also detected the enzyme activity in rat brain and liver (Y.H., T.T., unpublished results). Therefore, the enzyme is not tumor associated. From these observations, it seemed possible that GM_{1b} would be found in the cells containing asialo-GM_1. Characterization of the enzyme concerning the biosynthesis of GM_{1b} is now in progress in our laboratory.

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