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Changes of the ganglioside pattern and content in human fibroblasts by high density cell population subculture progression

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In this study we show that the ganglioside content and pattern of human skin fibroblasts change along the process of cell subculture progression by varying the cell density.

GM3, GD3 and GD1a were components of the total cell ganglioside mixtures extracted from cells, but GD1a was in all the extracts a minor component or very scant. Other gangliosides present in traces were not characterised. The fibroblast ganglioside content of 52 pools of cells obtained from 5 different cell lines cultured at variable cell density ranged from 2.0 to 13.1 nmoles per mg of cell protein. The molar ratio between GM3 and GD3 varied from 418 to 0.6 in the ganglioside mixtures, as determined by densitometric quantitative analysis after thin layer chromatographic separation.

Both the ganglioside content and the GM3/GD3 molar ratio were constant along several passages of subculture progression performed by plating cells collected at confluence. Instead, when the subculture progression was performed by plating cells collected at a few days after reaching confluence, a progressive increase of the ganglioside content was observed. GD3 increased proportionally more than GM3 so that a progressive decrease of the ratio between GM3 and GD3 was observed. In some experiments, GD3 was very scant at the beginning of the progression, while it was near 30% after 5 passages under these conditions. The progressive increase of GD3 along the high density cell population subculture progression was associated to a moderate increase of the mRNA GD3 synthase.
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Abbreviations: **Ganglioside and glycosphingolipid nomenclature is in accordance with Svennerholm [29], and the IUPAC-IUBMB recommendations [30].**

LacCer, ß-Gal-(1-4)-ß-Glc-(1-1)-Cer; GM3, II^{3}Neu5AcLacCer, α-Neu5Ac-(2-3)-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer; GD3, II^{3}Neu5Ac$_2$-LacCer, α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer; GM1, II^{3}Neu5AcGgOse$_4$Cer, ß-Gal-(1-3)-ß-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer; GD1a, IV^{3}Neu5AcII^{3}Neu5AcGgOse$_4$Cer, α-Neu5Ac-(2-3)-ß-Gal-(1-3)-ß-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer; Neu5Ac, *N*-acetylneuraminic acid; Cer, ceramide, *N*-acyl-sphingosine; Sph, sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadecene. EMEM, Eagle's minimum essential medium; FCS, fetal calf serum; PBS, phosphate-buffered saline.

Introduction

Gangliosides [1], sialic acid containing glycosphingolipids, are inserted with the lipid moiety in the outer layer of the plasma membranes of vertebrate cells. Their oligosaccharide moiety faces the external medium; this makes them free to interact with soluble extracellular molecules and with the hydrophilic portion of the same or other cell membrane components. Gangliosides in the cell membranes are organized in domains [2,3] containing proteins involved in signal transduction [4,5], where it is believed that they play an important role in modulating biological functions.

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Cell cultures are a model to study the functional properties of membrane sphingolipids. At this purpose, skin fibroblasts are widely used: they are prepared from several mammalian species (comprising humans), are used for comparison studies when prepared from healthy and ill subjects, reflect the cellular genic potential and are used as a model for studies related to neuronal diseases. GM3 is the main ganglioside of cultured fibroblasts, while GD3 and GD1a are minor components [6,7]. Nevertheless, the ganglioside pattern and content of cultured fibroblasts have been shown to be very variable [8–10].

In this study we present data suggesting that a high content of GD3 in human fibroblasts is associated to the maintenance of a high cell density and to the extension of the cell membrane contacts.

Materials and methods

Materials

The commercial chemicals were the purest available, common solvents were distilled before use and deionized water, obtained by a MilliQ system (Millipore), was distilled in a glass apparatus. High performance silica gel precoated thin-layer plates (HPTLC Kieselgel 60, 10 × 10 cm) were purchased from Merck GmbH; Mega priming kit and nylon membrane Hybond N^+ from Amersham International; Express Hyb solution from Clontech; *Vibrio cholerae* sialidase, trypsin, bovine serum albumin and oligo dT [11–17] cellulose from Sigma; PVDF membranes from Millipore; DNAse and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase from Life Technologies; random hexamers from Pharmacia; Taq DNA polymerase from Roche. R24 IgG anti-GD3 monoclonal antibody was from NIH; DH2 IgG anti-GM3 monoclonal antibody [18] was kindly provided by Professor S.-i. Hakomori (Pacific Northwest Research Institure, Seattle). Gangliosides GM1, GD1a, GD3 and GM3 were prepared as previously described [11–13].

Cell cultures

Human skin fibroblasts were obtained by the punch technique from normal children (age 6–12 month). Cells were cultured and propagated as monolayers in 75 cm^2 culture flasks at 37°C in a humidified atmosphere containing 5% CO_2, using EMEM supplemented with 10% FCS [14]. Cells were harvested at different density corresponding to 2 days after plating, at confluence (determined by phase contrast microscopy) and 5 days after reaching confluence. The study has been carried out on 5 different cell lines.

The cell protein and DNA contents were determined as previously described [15,16].

Cell lipid extraction and characterization

Cell cultures were washed twice with PBS, scraped off with a rubber policeman, centrifuged at 1000 g for 15 min and subjected to lipid extraction followed by ganglioside purification by partitioning [17].

Lipids were separated by HPTLC with the solvent system chloroform-methanol-0.2% aqueous $CaCl_2$, 55:45:10, by vol. After HPTLC separation, ganglioside spots were made visible by treatments with a *p*-dimethylaminobenzaldehyde reagent followed by heating at 120°C for 10 min [19], with anisaldehyde reagent followed by heating at 130° for 15 min [20], or by staining with anti-ganglioside antibodies [21] and *Vibrio cholerae* toxin [22]. Gangliosides GM3, GD3 and GD1a were purified by the far eastern blotting [23] and characterized. GM3, GD3 and GD1a dissolved in 50 μL of water, were treated at 37°C for 2 h with 1 mU of *Vibrio cholerae* sialidase, to yield lactosylceramide, a mixture of GM3 and lactosylceramide, and GM1, respectively.

Gangliosides separated by TLC were quantified using a Biorad GS-700 imaging densitometer; ganglioside-bound sialic acid was determined by the resorcinol-HCl method [24]. Pure GM3, GD3 and Neu5Ac were used as the reference standard in the quantitative determinations.

RNA and DNA preparation and northern analysis

Sequential isolation of DNA and total RNA was performed from fibroblast cell cultures using SV total RNA isolation system (Promega) according to the manufacturer's specifications. Poly-adenylated mRNA was obtained by column chromatography fractionation on oligo dT [11–17] cellulose [25]. Five to eight μg of poly-A mRNA were size fractionated through 1.4% formaldehyde agarose gel and transferred to a nylon membrane Hybond N^+ [26]. Radioactive probes were prepared from a PCR specific fragment amplified from the GD3 synthase encoding region, using synthetic complementary primers as described below. To have the highest probe specific activity, probe double labeling was performed with α^{32}P dCTP and dATP using the Mega priming kit (Amersham) according to the manufacturer's specifications. In order to magnify the intensity of the signals, blots were hybridized at 65°C using the Express Hyb (Clontech) solution as recommended by the manufacturer. Washing conditions were performed according to standard protocols [26]; auto-radiography was performed at −80°C with an exposure time of fourteen days, using BioMax X ray films with intensifying screens. A loading control was performed with a PCR generated probe obtained from the β actin gene (data bank a.n. X00351).

Reverse transcription—polymerase chain reaction for generation of the GD3 probe

Reverse transcription of fibroblast mRNA was performed as described [27] with a minor modification. 3 μg of each RNA sample was treated with DNAse 1 (1 U/μg of RNA) (Gibco-BRL), according to the manufacturer's specifications. RNA was converted to cDNA using the MMLV reverse transcriptase (200 U/μg of RNA), random hexamers (2.5 μM, Pharmacia) and dNTPs (1 mM each dATP, dCTP, dGTP, dTTP) in a final volume of 20 μL.

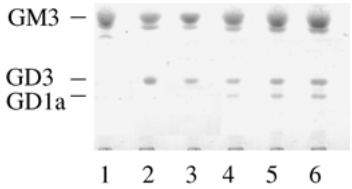

Figure 1. TLC of the ganglioside mixtures extracted and purified from two different human skin fibroblast lines (lanes 1, 2 and 3, and lanes 4, 5 and 6, respectively) cultured at different cell density. Lanes 1 and 4: low density; lanes 2 and 5: confluence; lanes 3 and 6: high density.

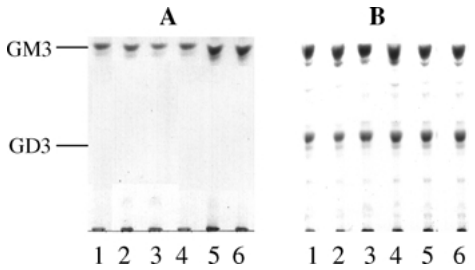

Figure 2. TLC of the ganglioside mixtures extracted and purified from human skin fibroblasts at stage 15 to 20 (lanes 1 to 6) of doubling in culture. In the progression process, cells were harvested, at each stage, at confluence. Panel A, gangliosides from a cell line having low content of GD3; panel B, gangliosides from a cell line having high content of GD3.

RT-PCR efficiency was tested using primers (243 & 244) designed from the HPRT gene [28]. A negative PCR control using no DNA template was also performed.

RT-PCR for the GD3 synthase gene was performed with the specific primers from nt 612 to nt 630 (Forward primer 5'TACATCTTCCCCGTCTACC3') and from nt 1573 to 1554 (Rev primer 5'CCTTTCTTCTTCCATTGTTC) designed in the GD3 synthase coding region (databank a.n. X77922), using, as template, cDNA obtained from pre-confluent, confluent, and post-confluent fibroblast cells. A product of correct size (962 bp) was detected after 35 cycles using the following PCR conditions: 45 sec at 94°C, 1 min at 55°C, 1 min at 72°C in a programmable Thermal Cycler using Taq DNA polymerase according to the manufacturer's instruction.

Results

Human skin fibroblasts in culture contained three main gangliosides (Figure 1). These were purified and characterized as GM3, GD3 and GD1a. Other minor components were not characterised.

GM3 was always a main cell component, being from about 50% to about 100% of the total ganglioside content; at the contrary, GD3 was from less than 1% to about 50% of the total ganglioside content. GD1a was present in very small amounts. Examples of the ganglioside patterns are shown in Figure 1.

The cell ganglioside content was very variable in 52 subcultures, deriving from 5 different cell lines, collected at a wide range of cell density. It varied from 2.0 to 13.1 nmoles of ganglioside/mg protein.

We prepared subcultures by doubling progression. The cell ganglioside content and pattern were quite constant up to the 30th passage, if cells, at each stage, were harvested at confluence (Figure 2 and Table 1). Nevertheless, in a subculture progression comprising a very high number of passages, the GD3 percentage content slowly and progressively decreased after the 30th doubling (see Table 1). Microscopy studies showed that until about 20°–25° passage in colture cells maintained a quite constant elongated morphology at confluence. Then cells progressively became larger and the cell border more irregular, thus displaying a morphology that allowed only partial contacts between plasma membranes. As we can see in Table 1, the GM3/GD3 molar ratio significantly increases between 24° and 33° passage, when the morfology of the cells progressively changes, then remains constant. As an example, Figure 3 shows for comparison the microscopy pictures of subcultures at the 42th (panel A) and 12th (panel B) passage of the doubling progression.

The GM3/GD3 molar ratios determined in cells grown at different density are reported in Figure 4. Cells at confluence (450 ± 50 μg protein/dish, 1.4 ± 0.2 μg DNA/dish) were used to prepare three subcultures that were harvested two days after plating (175 ± 20 μg protein/dish, 0.5 ± 0.1 μg DNA/dish),

Table 1. GM3/GD3 molar ratios in cultured human fibroblasts, deriving from 4 different cell lines, along four different experiments of doubling passages. Replating were performed using cells that were collected at confluence or 5 days after reaching confluence (over-confluence). The ganglioside ratios were determined by densitometry after TLC separation of the ganglioside mixtures

	GM3/GD3 molar ratio													
	Subculture number													
Doubling from cells at	*5*	*6*	*7*	*8*	*9*	*10*	*12*	*14*	*16*	*19*	*20*	*24*	*33*	*42*
Confluence, cell line 1									3.8			3.9	7.0	6.6
Confluence, cell line 2					12.9		14.2				14.3			
Confluence, cell line 3		12.7					13.3	13.8		12.8				
Over-confluence, cell line 4	87.0	12.9	14.0	4.2	3.0	3.4								

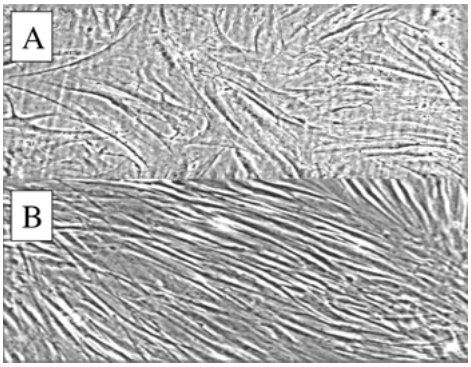

Figure 3. Light microscopy of human skin fibroblasts at confluence in culture. Panel A, cells at the 42th stage of subculture; panel B, cells at the 12th stage of subculture.

at confluence and 5 days after confluence (740 ± 80 μg protein/dish, 2.0 ± 0.2 μg DNA/dish). From each subculture three new sub-subcultures were prepared when reaching low cell density, confluence density or high density. Figure 4 shows that the GM3/GD3 molar ratio progressively decreased from cells grown at low density to cells grown at high density. All the data on the GM3/GD3 molar ratio in cells grown at different density are reported in Table 2. In a further experimental model, cell subcultures having low content of GD3, were subjected to a series of doubling passages having care to harvest cells always 5 days after confluence. Figure 5 shows that within 5 passages the GD3 content become relevant, the GM3/GD3 molar ratio decreasing from about 87 to 3.4.

Figure 6 shows the molar content of gangliosides GM3 and GD3 in two subculture progressions where cells were always

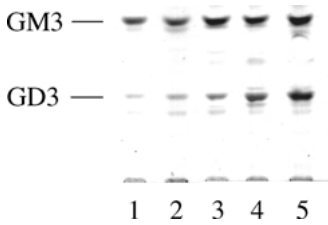

Figure 5. TLC of the ganglioside mixtures extracted from fibroblasts at stages 13 to 17 of doubling in culture (lanes 1 to 5). Cells were collected 5 days after reaching confluence.

collected 5 days after confluence. The contents of both GM3 and GD3 increased progressively along the doubling progression but the most relevant increase of GD3 yielded a continuous decrease of the GM3/GD3 molar ratio.

To verify a possible role between GD3 synthase and GD3 content we analysed the content of GD3 synthase mRNA in cells grown at different densities. Figure 7 (top panel) shows the GD3 synthase mRNA from cells harvested two days after plating, at confluence and 5 days after confluence. The GD3 synthase mRNA obtained from cells harvested two days after plating was very low (lane 1), but it moderately and progressively increased harvesting cells at confluence (lane 2) and then 5 days after confluence (lane 3).

Discussion

The relationship between cell ganglioside content and pattern in cultured fibroblasts has been studied as a function of many parameters [8–10], but the data are not easily comparable due

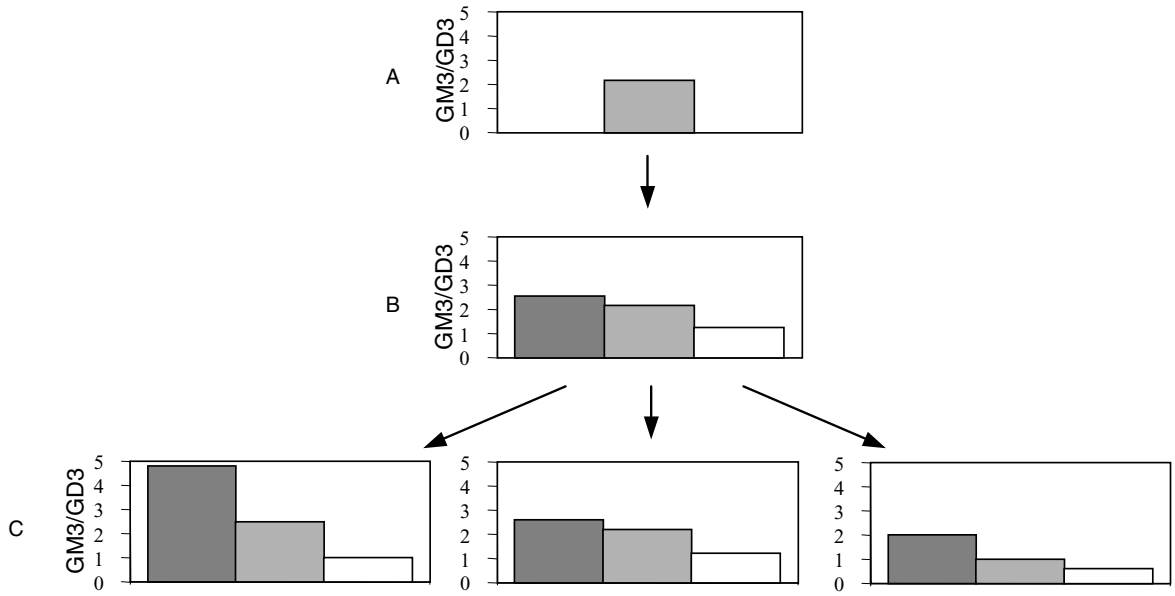

Figure 4. GM3/GD3 molar ratio in cultured human skin fibroblasts cultured at different cell densities. Ganglioside analyses were carried out on subcultures B and C, derived from the same cell population A, collected two days after plating (darkest bar at the left), at confluence (medium dark bar in the middle) and five days after confluence (open bar at right). Then, from each subculture, three sub-subcultures with different cell density were prepared.

Table 2. GM3/GD3 molar ratios in cultured human fibroblasts as a function of cell density. Each subculture series (table columns) was derived from cells with different GM3/GD3 molar ratio and comprised for cells harvested two days after plating (pre-confluence), at confluence and five days after reaching confluence (over-confluence). The eleven experiments were performed using 3 cell lines stored at different doublings.

	GM3/GD3 molar ratio										
Pre-confluence	2.5	4.8	1.8	2.0	5.1	418.0	5.4	10.0	5.6	4.2	8.4
Confluence	2.2	2.5	1.2	1.0	2.1	54.1	3.3	2.7	4.1	2.7	7.5
Over-confluence	1.2	1.0	0.7	0.6	nd	28.2	2.7	2.0	nd	1.4	1.0

Nd, not determined.

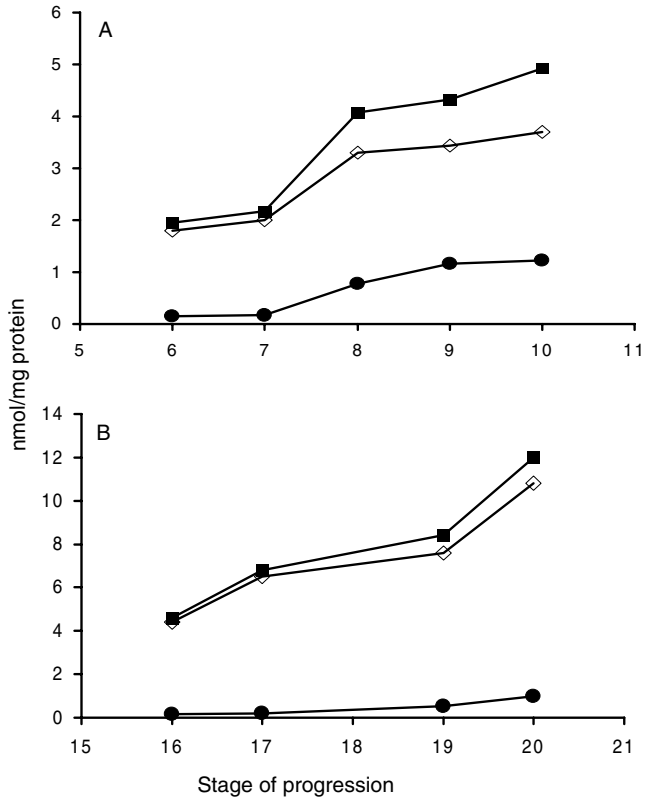

Figure 6. GM3 and GD3 molar content in two different cell lines along two different doubling progressions. Cells were always collected 5 days after reaching confluence. Panel A, stages 6 to 10; panel B, stages 16 to 20. Open diamond, GM3; closed circle, GD3; closed square, total.

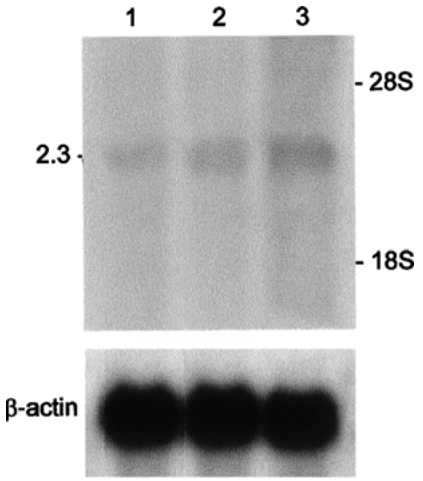

Figure 7. Northern blot analysis of GD3 synthase mRNA (upper panel) of human skin fibroblasts collected 2 days after plating, lane 1, at confluence, lane 2, and 5 days after confluence. Lower panel shows the actin loading control.

to the differences among the experimental models. Our studies suggest a relationship between the cell high content of GD3 and the high number of contacts among fibroblasts in culture. In this study we used five cell lines derived from five different subjects and obtained similar results. This suggests, also if we did not use cell clones, that our data are representative of a common cell behavior.

We prepared subculture progressions using human skin fibroblast cell lines having care to harvest cells two days after plating (low density), at confluence or five days after confluence (high density). Along the 5 days after confluence, cell number increased of about 25–30%, this corresponding to a partial cell overlaping. Under this condition we observed an increase of GD3. Moreover, a cell doubling performed with cells that at each stage of the progression were at "over-confluence" corresponded to a progressive absolute and relative increase of GD3 content (Figures 5 and 6). On the contrary, when cells were cultured at confluence, the GD3 content remained constant along the subculture progression (Figure 2). Thus, the high content of GD3 seems to be related the cell-to-cell contacts. This would be confirmed by the results on the ganglioside pattern that we found in cultures of cells that underwent to a very long subculture progression. Cells in culture, after many passages change their morphology (Figure 3) decreasing the cell-to-cell surface contacts. In the course of doubling progression, when cells reached these characteristics the content of GD3 decreased (see Table 1). The doubling of cells at cell high density led to a progressive increase of the ganglioside content (see Figure 6). Under this experimental condition, both GM3 and GD3 increased, but the increase of GD3 was in absolute more relevant of that of GM3, this leading to obtain a progressive decrease of the molar ratio between GM3 and GD3. The mechanisms that yield the cell

ganglioside pattern are very complex and comprise for both the anabolic and catabolic pathways. As first step we analysed the expression of the GD3 synthase mRNA (Figure 7). We found that the increase of GD3 was associated to a moderate increase of the GD3 synthase mRNA expression, that, by itself, seams to not support the important change of cell GD3 ganglioside content. At this moment we do not have any information on the activity of cell membrane sialidase. In the past, it has been suggested that the early stages of the cell-to-cell contacts are involved in suppressing a plasma membrane sialidase activity [8]. This, would explain the increase of the cell content of sialidase labile ganglioside GD3 due to the increase of cell-to-cell contacts. If this is true, it is necessary to consider a metabolic pathway in which the neobiosynthesized GD3 is transported to the plasma membranes, where it is partially or largely converted to GM3 to obtain the final GM3/GD3 molar ratio appropriate for the cell functionality.

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References

1 Wiegandt H, *New Compr Biochem* **10**, 199–260 (1985).
2 Schroeder L, London E, Brown D, *Proc Natl Acad Sci USA* **91**, 12130–4 (1994).
3 Hakomori S-I, Handa K, Iwabuchi K, Yamamura S, Prinetti A, *Glycobiology* **8**, xi–xix, (1998).
4 Prinetti A, Iwabuchi K, Hakomori S-I, *J Biol Chem* **274**, 20916–24 (1999).
5 Kasahara K, Watanabe Y, Yamamoto T, Sanai Y, *J Biol Chem* **272**, 29947–53 (1997).
6 Dawson G, Matalon R, Dorfman A, *J Biol Chem* **247**, 5944–50 (1972).
7 Chigorno V, Tettamanti G, Sonnino S, *J Biol Chem* **271**, 21738–44 (1996).
8 Yogeeswaran G, Hakomiri S-I, *Biochemistry* **14**, 2151–6 (1975).
9 Ohsawa T, *Exp Gerontol* **24**, 1–9 (1989).
10 Colombo I, Sottocornola E, Moretti S, Meloni MA, Pippia P, Berra B, *Biochim Biophys Acta* **1485**, 214–24 (2000).
11 Dohi T, Nores G, Hakomori S-I, *Cancer Res* **48**, 5680–5 (1988).
12 Acquotti D, Cantu' L, Ragg E, Sonnino S, *Eur J Biochem* **225**, 271–88 (1994).
13 Hauttecoeur B, Sonnino S, Ghidoni R, *Biochim Biophys Acta* **833**, 303–7 (1985).
14 Mauri L, Casellato R, Kirschner G, Sonnino S, *Glycoconjugate J* **16**, 197–203 (1999).
15 Leroy JG, Ho WM, McBrinn MC, Zielke K, Jacob J, O'Brien JS, *Pediatr Res* **6**, 752–7 (1972).
16 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, *J Biol Chem* **193**, 265–75 (1951).
17 Rago R, Mitchen S, Wilding G, *Anal Biochem* **191**, 31–4 (1990).
18 Tettamanti G, Bonali F, Marchesini S, Zambotti V, *Biochim Biophys Acta* **296**, 160170 (1973).
19 Partridge SM, *Biochem J* **42**, 238–48 (1948).
20 Stahl E, *Anisaldehyd-Schwefelsaure fur Steroide, Terpene Zucker und so w. Dunnschicht Chromatographie* (Springer Verlag, Berlin, 1962), p. 498.
21 Nobile-Orazio E, Legname G, Daverio R, Carpo M, Giuliani A, Sonnino S, Scarlato G, *Ann Neur* **28**, 190–4 (1990).
22 Wu G, Ledeen R, *Anal Biochem* **173**, 368–75 (1988).
23 Taki T, Handa S, Ishiwaka D, *Anal Biochem* **223**, 232–8 (1994).
24 Svennerholm L, *Biochim Biophys Acta* **24**, 604–11 (1957).
25 Maggi A, Susanna L, Bettini E, Mantero G, Zucchi I, *Mol Endocrinol* **3**, 1165–70 (1989).
26 Sambrook J, Russel DW, *Molecular Cloning: A Laboratory Manual*, 3rd ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001).
27 Zucchi I, Montagna C, Susani L, Vezzoni P, Dulbecco R, *Proc Natl Acad Sci USA* **95**, 1079–84 (1998).
28 Gibbs RA, Nguyen PN, McBride LJ, Koepf SM, Caskey CT, *Proc Natl Acad Sci USA* **86**, 1919–23 (1989).
29 Svennerholm L, *Adv Exp Biol Med* **125**, 11 (1980)
30 IUPAC-IUBMB Joint Commission on Biochemical Nomenclature, *Pure Appl Chem* **69**, 2475–87 (1997); *Carbohydr Res* **312**, 167–75 (1998).

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