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Changes of gangliosides and sialic acid in human milk during lactation

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Abbreviations: HPTLC, high performance thin layer chromatography

Abstract

To elucidate the possible biological function of gangliosides in human milk, the content and composition of gangliosides in human milk during lactation were examined. Gangliosides were rich in human milk at the earlier stages of lactation. The major ganglioside was GD3. The content of GD3 decreased, while that of GM3 increased in the later stages of lactation. GD1a, and polysialogangliosides were rich in the milk at 6 days of postpartum. Levels of sialic acid and sialidase, a ganglioside-hydrolyzing enzyme, were also studied. The content of sialic acid and sialidase activity were decreased in the later stages of lactation. As the results, GD3, GD1a, polysialogangliosides, sialic acids (lipid-bound and protein-bound), and sialidase were rich in human colostrum and these components may play a role in immune system.

Keywords: gangliosides, human milk, sialic acid

Introduction

The fat in human milk is the major energy source for newborn infants (Jensen, 1989). Specific components of milk fat are essential for brain development, particularly in myelination (Clandinin *et al.*, 1980; Crawford *et al.*, 1981), retinal function (Neuriger *et al.*, 1984), and protection from infection (Kabara, 1984; Isaacs *et al.*, 1990). Human milk fat contains 98% triglycerides. Most of the polar lipids such as phospholipids and cerebrosides are found in the milk fat globule membranes (Laegreid and Otnaess, 1986). Laegreid and Otnatess (1987) and Takamizawa (1988) have found that gangliosides from human and bovine milk inhibited the enterotoxins from *Vibrio cholerae* and *Escherichia coli*.

Gangliosides are sialic acid containing glycosphingolipids mainly located on the outer surface of mammalian cell plasma membranes (Stults *et al.*, 1989). Gangliosides are particularly abundant in neural tissues, comprising 10% of the total lipids. Gangliosides are well known to participate in various membrane related biological functions that are associated with cell growth, development, differentiation, and transformation (Hakomori, 1981; Hannun and Bell, 1989). Gangliosides are involved in selective binding of ions, bacterial toxins, viruses and peptide hormones and possibly in the transduction of external signals to the interior of the cell (Nojiri *et al.*, 1986; Daniotti *et al.*, 1992).

In order to elucidate the role of gangliosides in human milk, we analyzed the gangliosides composition, sialic acid contents, and the activities of sialidase (EC 3.2.1.18, neuraminidase) of human milk from different individuals at different stages of lactation

Materials and Methods

Materials

Human milk samples were obtained from healthy mothers who aged 25-30 and admitted to Han Gang Sung Sim hospital and stored immediately at -70°C until analysis. Precoated high performance thin layer plates (HPTLC, silica gel 60) were purchased from Merck (Darmstadt, Germany). *Clostridium perfringens* neuraminidase, purified GM3, GD3, GD1a, GT1b gangliosides, and bovine brain gangliosides were purchased from Sigma (St. Louis, U.S.A.). Resorcinol were purchased from Wako (Osaka, Japan). Other chemicals were used as the best grade from local source available.

Preparation of gangliosides from human milk

Human milk, either fresh or from storage, were centrifuged at 35,000 *g* for 60 min (Bartholomew *et al.*, 1973). The upper lipid layer was used to isolate gangliosides. Gangliosides were isolated by extraction and solvent partition, essentially as described by Folch *et al.*, (1957) and Suzuki (1965). Briefly, milk fat was homogenized with 10 vol. of chloroform/methanol/water (5.3:10.6:3, v/v/v) and extracted. The extract was subjected to Folch's partition to obtain a crude

ganglioside fraction. The combined upper aqueous phase were partially evaporated and dialyzed against distilled water for 18 h. After dialysis, the material was lyophilized and dissolved in a small amount of methanol. Crude ganglioside fractions were analysed by HPTLC.

High performance thin layer chromatography (HPTLC)

Ganglioside fractions were analysed by HPTLC using silica gel 60 HPTLC precoated glass plates developed in chloroform/methanol/0.2% $CaCl_2 \cdot 2H_2O$ in water (55:45:10, v/v) or n-butanol/0.2% $CaCl_2 \cdot 2H_2O$ in water (80:20, v/v). For visualization of gangliosides, the plates were sprayed with resorcinol (Svennerholm, 1956) and placed face down on a clean glass plate. Then the plates were heated at 120°C for 15 min until gangliosides were revealed on the plate as purple bands. Bovine brain gangliosides were used as standards.

Determination of sialic acids

The total sialic acid content of human milk was estimated after hydrolysis in 50 mM H_2SO_4 at 80°C for 1 h or after treatment of *Clostridium perfringens* neuraminidase. The hydrolysate was cooled to room temperature and salic acid was quantified by the 2-thiobarbituric acid method (Warren, 1959; Aminoff, 1961) and periodate-resorcinol method (Jourdian *et al.*, 1971). For neuraminidase digestion, gangliosides were incubated at 37°C for 20-22 h with *Clostridium perfringens* neuraminidase in 10 mM sodium acetate buffer, pH 5.4.

Preparation of enzyme fraction containing human milk sialidase

After centrifugation, middle supernatant fluid of human milk was diluted with 2 vol. of 10% ethanol in distilled water at 0°C. Solid ammonium sulfate was slowly added with stirring to 40% of saturation. The precipitate was removed by centrifugation and discarded. The supernatant solution was brought to 70 % of saturation with solid ammonium sulfate and was allowed to stand until the precipitate became flocculent. The precipitate was then collected by centrifugation at 35,000 *g* for 10 min, dissolved in a minimal volume of 5 mM potassium phosphate buffer, pH 6.8 and dialyzed against the same buffer overnight. The retentate was used as enzyme fraction.

Assay of sialidase activity

The assay mixture contained 40 μg of bovine brain gangliosides (7.5 nmol as bound sialic acid) as substrate, 0.1 M sodium acetate buffer (pH 4.5) and enzyme fraction (about 0.3 mg as protein). After bovine brain gangliosides in methanol were dried under the nitrogen gas, 0.1% sodium cholate solution was added to the reaction mixture which was incubated for 16 h at room temperature in order to form the stable micelle and facilitate reaction of enzyme. After incubation of assay mixture at 37°C for 90 min, the reaction was terminated by immediate freezing and the released sialic acid was determined by measuring absorbance at 532 nm and 549 nm as described previously (Warren, 1959).

Results and Discussion

Changes of the gangliosides from human milk at various stages of lactation

Changes of the gangliosides composition in human milk during lactation were seen by HPTLC. Human milk contained two major gangliosides, GM3 and GD3 (Figure 1A). GD3 was the major component in human milk at 6 days of postpartum, and GM3 became the major component in the milk after 60 days of postpartum. These results were well correlated with the report of Takamizawa *et al.* (1986). However, we also detected the presence of GD1a and unidentified polysialogangliosides in the milk at the earlier stages of lactation using HPTLC plate supplementary with concentration zone (Figure 1B). These GD1a and several polysialogangliosides may affect immune system as well as GD3. Total gangliosides contents decreased with continuing stages of lactaion as shown in Figure 1B. To confirm this result, we quantified ganglioside-bound sialic acid with periodate-resorcinol reagent (Jourdian *et al.*, 1971).

The concentration of gangliosides in human milk during lactation are summarized in Table 1. The concentration of ganglioside-bound sialic acid in human milk was maxial at 6 days of postpartum, and then decreased with following stages of lactation. In mature milk, gangliosides content was almost constant regardless of lactation periods. The concentration of ganglioside-bound sialic acid at 182 days of postpartum decreased to 20% of the coroctrum. Therefore,

Table 1. Concentrantion of ganglioside-bound sialic acid in human milk gangliosides fraction during lactation.

Days of lactation	Sialic acid (μmol/100 ml milk)
6	2.50 ± 0.01
60	0.61 ± 0.01
115	0.66 ± 0.16
132	0.54 ± 0.02
158	0.70 ± 0.09
171	0.90 ± 0.21
182	0.64 ± 0.02

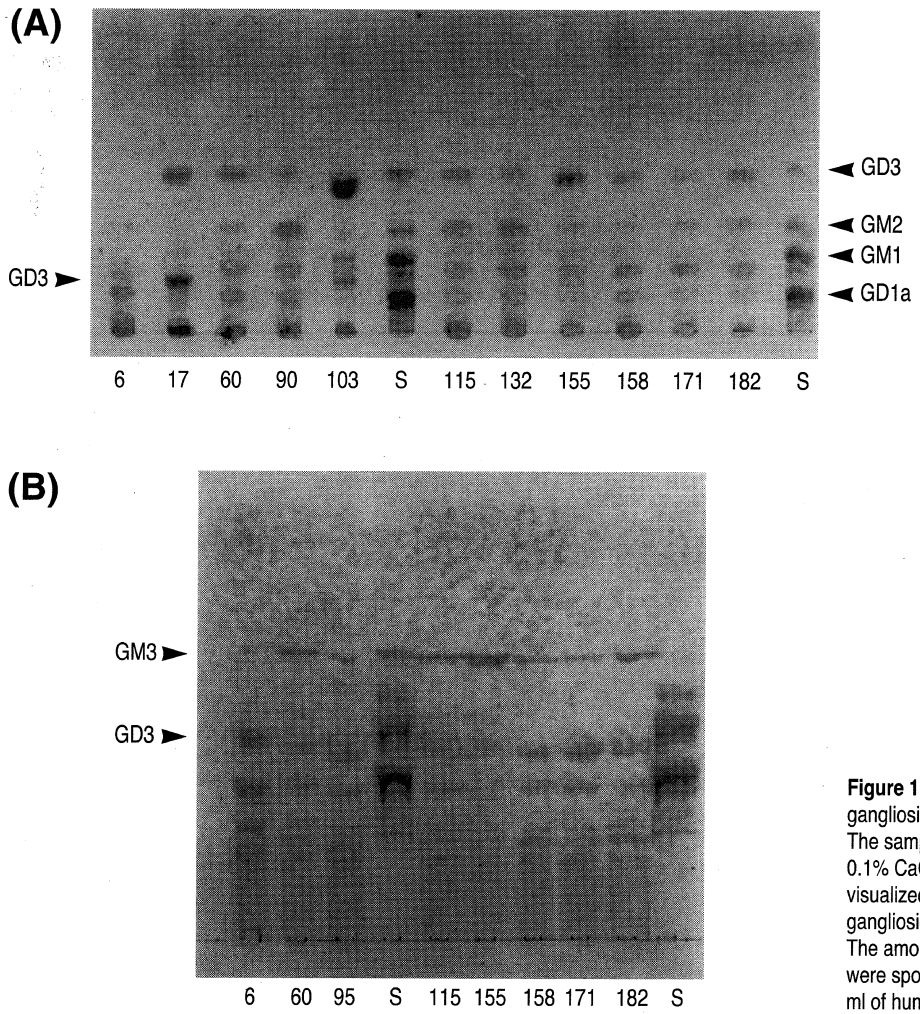

Figure 1. HPTLC chromatograms of the isolated gangliosides from human milk during lactation. The samples were developed in n-propanol: 0.1% $CaCl_2$ (80:20, v/v). Gangliosides were visualized with resorcinol. Bovine brain gangliosides was used as the standard (s). **(A)** The amounts of 4.5 nmol as bound sialic acid were spotted. **(B)** A corresponding amounts of 1 ml of human milk were spotted. The HPTLC plate with concentration zone was used.

Table 2. Sialic acid contents of human milk during lactation.

Days of lactation	Sialic acid μ mol / 100 ml Milk		
	lipid fraction	protein fraction	total milk
6	11.4 ± 0.1	299 ± 29.5	334 ± 30
60	5.1 ± 0.3	93 ± 4.5	105 ± 3
115	7.6 ± 2.1	96 ± 3.5	110 ± 4
132	4.1 ± 0.6	79 ± 0.8	92 ± 8
158	5.3 ± 0.5	81 ± 0.4	91 ± 4
171	5.4 ± 1.0	83 ± 2.1	91 ± 3
182	5.0 ± 0.8	82 ± 3.2	87 ± 4

Table 3. Sialidase activity in human milk during lactation. The reaction mixture, in a volume of 0.3 ml, containing 0.1 M sodium acetate buffer (pH 4.99) and enzyme solution (0.3 mg protein), and 40 mg bovine brain gangliosides (7.5 nmol as bound sialic acid), were incubated at 37°C for 90 min. The enzyme solution at ammonium sulfate fractionation step were employed.

Days of lactation	Specific activity (nmol/h/mg)
6	3.19 ± 0.14
10	2.30 ± 0.21
17	1.78 ± 0.06
60	1.06 ± 0.12
132	0.32 ± 0.09
182	0.72 ± 0.15

gangliosides (GD3, GD1a, and polysialogangliosides), and sialic acids were rich in human colostrum.

The changes of sialic acid concentrations and sialidase activities, from human milk during lactaion

We have already observed the changes of ganglioside concentration as ganglioside-bound sialic acid from ganglioside fraction of human milk. Thus, we attempted to find out the trends of sialic acid concentration from glycolipid and glycoprotein fraction of human milk. Table 2 shows that most of sialic acids in human milk were bound to protein fraction and the total sialic acid contents in the milk at 6 days of postpartum were higher than the following stages of lactation. The pattern of change of total sialic acid from human milk was equivalent to that of the ganglioside from human milk. To understand the cause of the high concentration of gangliosides, compositions and glycoconjugate bound sialic acids concentration in colostrum, we examined the activities of sialidase from different stages of human milk. Since the highest contents of ganglioside and sialic acid were found in early stages of lactation, the activities of sialidase might be relevant. As shown in Table 3, activities of sialidase were higher at the early stages of lactation.

We found that human milk of the earlier postpartum contained polysialogangliosides, sialic acids (ganglioside-bound, glycolipid-bound, and glycoprotein-bound), as well as high activities of sialidase. It has been reported that GD3 was expressed generally during earlier stages of cell proliferation and decreased with further stages of development (Daniott *et al.*, 1992). Since bovine milk does not have GM3 during whole lactation period (Puente *et al.*, 1992), but human milk has GM3 in the later stages of pospartum, an additional supply of gangliosides and/or sialic acids to bovine milk powder for neonatal feeding could be postulated.

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