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Note

Stability of Milk Gangliosides and Formation of G_{D3} Lactone under Natural Acidi Conditions

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To clarify the stability of milk gangliosides under natural acidic conditions in the stomach, the resistance of monosialoganglioside 3 (G_{M3}) and disialoganglioside 3 (G_{D3}) to gastric juice and acid solutions was investigated. The data presented here suggest that G_{M3} and G_{D3} in milk are likely to reach the intestinal tract because over 80% of the sialic acid in their structure remained intact under the same acidic conditions found in an infant's stomach.

Numerous studies have indicated that breast milk protects infants against enteric infections.[1–3] Holmgren *et al.*[3] demonstrated that the non-immunoglobulin fraction of human milk inhibits bacterial adhesion and enterotoxin binding of *Escherichia coli* and *Vibrio cholerae* to the epithelial cells of the intestinal tract. The enterotoxin inhibitor in human milk appeared to be of a ganglioside-like nature. Gangliosides have been detected in milk, particularly in the membrane fraction of the fat globule; this derived mainly from the apical plasma membrane of the secretory cells in the lactating mammary gland.[4,5] Kolstø Otnœss *et al.*[6] suggested that human milk gangliosides might be important in the protection of infants against enterotoxin-induced diarrhea. Again, human milk gangliosides showed considerably higher enterotoxin-inhibitory activity compared to those in bovine milk.[7] Although the oligosaccharide chains of gangliosides may be fundamental in protection against infection, sialic acids at the terminal of the sugar moiety are labile to low pH.[8] Thus, it would be interesting to know whether milk gangliosides reach the gastrointestinal tract with their sialic acids intact. We have reported that the predominant ganglioside in human colostrum was G_{D3} and that in mature milk was G_{M3}.[9] In this study, we have investigated the resistance of G_{M3} and G_{D3} to gastric juice and acid solutions to clarify the stability of gangliosides under natural acidic conditions in the stomach.

Gangliosides G_{M3} and G_{D3} isolated from bovine milk were purchased from Wako Pure Chemicals (Osaka, Japan). Ceramide lactoside was prepared by treating G_{M3} with neuraminidase (Sigma Chemical Co., St. Louis, MO; N-6514). Gastric juice was collected according to the method of Shay *et al.*[10] To measure the acidity of the gastric juice, an aliquot of juice was titrated with a standard solution of 0.01 N sodium hydroxide to the endpoint pH of 7.0 using a pH meter. The normality of the gastric juice was 0.11. TLC was done using silica gel 60 high performance thin layer plates (Merck, Darmstadt, Germany). Each plate was developed with chloroform–methanol–water containing 0.2% calcium chloride (55:45:10), sprayed with orcinol–sulfuric acid reagent to color glycolipids containing neutral carbohydrates, or with resorcinol–copper sulfuric acid–hydrochloric acid reagent to color glycolipids containing sialic acid; it was then incubated at 100°C for color development. The chromatogram was scanned at 505 nm for the orcinol reaction, and at 580 nm for the resorcinol reaction with a Dual-Wavelength TLC scanner CS-930 (Shimadzu, Tokyo, Japan). To evaluate their stability under acidic conditions, gangliosides (1 mg/ml) were incubated in gastric juice (pH 1.3) and acid solutions at 37°C for 2 h. The acid solutions were prepai ed by mixing adequate volumes of 0.1 M sodium acetate solutio and 0.1 M hydrochloride to obtain solutions with pH 1.3, 2.2, 3.: and 4.0. After the incubated solution was evaporated, the cor centrate was dissolved in chloroform–methanol (1:1) and spotte on a TLC plate. G_{D3} lactone was isolated from G_{D3} incubated ; pH 2.2 through an Iatrobeads 6RS 8060 (Iatron Labs, Tokyc Japan) column (15 mm × 150 mm) equilibrated with chloroform methanol (85:15). Chromatography was done with a linea gradient of chloroform–methanol (from 85:15 to 20:80) at flow rate of 0.8 ml/min, and 2-ml fractions of the eluate wei automatically collected. The elution profile was monitored by TLC and G_{D3} lactone was pooled and lyophilized after being suspende in deionized water. To investigate the reversible changes in th ganglioside structure under neutral conditions in the intestin: tract, G_{D3} lactone was incubated at 37°C for 2 h after adjustin the pH to 6.3 through 7.4 with 1 M Tris solution.

Table I represents the changes in the structure of G_{M3} and G_{D} incubated in gastric juice and acid solutions. The proportions (ganglioside were estimated by scanning the spots of incubated G_{M} and G_{D3} on the TLC plate. Little conversion of G_{M3} to ceramic lactoside occurred at pH 3.1 and 4.0, while 75% of G_{M3} wa degraded when incubated in gastric juice or in a solution with pl 1.3. On the other hand, G_{D3} lactone was formed when G_{D3} wa incubated at pHs between 1.3 and 4.0, while no formation of G_{M} was observed. The extent of G_{D3} lactone formation drastical increased with the decrease in the pH of the incubation mixtur

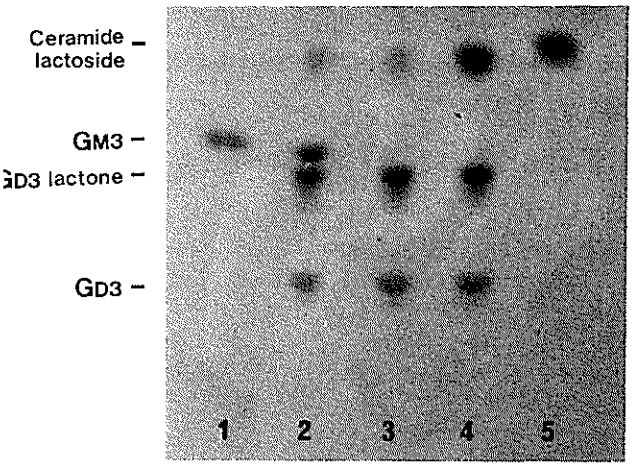
Table I. Changes in the Structure of G_{M3} and G_{D3} Incubated in Gastri Juice and Acid Solutions

Sample	Treatment[a]	Glycolipid[b]			
		Ceramide lactoside	G_{M3}	G_{D3}	G_{D3} lacton
G_{M3}	Initial	0%	100.0%	0%	0%
	pH 4.0	10.9	89.1	0	0
	pH 3.1	19.8	80.2	0	0
	pH 2.2	42.2	57.8	0	0
	pH 1.3	74.5	25.5	0	0
	Gastric juice	75.0	25.0	0	0
G_{D3}	Initial	0%	0%	100.0%	0%
	pH 4.0	7.6	0	87.3	5.1
	pH 3.1	17.3	0	52.5	30.2
	pH 2.2	20.4	0	22.5	57.1
	pH 1.3	23.8	0	7.6	68.6
	Gastric juice	23.0	0	7.5	69.5

[a] Gangliosides (1 mg/ml) were incubated in gastric juice (pH 1.3) an acid solutions (pH 1.3, 2.2, 3.1, and 4.0) at 37°C for 2 h.

[b] The proportions of glycolipid were estimated by scanning the spo of incubated G_{M3} and G_{D3} on the TLC plate.

Abbreviations: G_{M3}, monosialoganglioside 3; G_{D3}, disialoganglioside 3; TLC, thin layer chromatography.



g. TLC Profiles of Ceramide Lactoside, G_{M3}, and G_{D3} Incubated at
H 2.2.

mples were dissolved in chloroform–methanol (1 : 1) and spotted on a TLC plate. e plate was developed with chloroform–methanol–water containing 0.2% calcium ioride (55 : 45 : 10), sprayed with orcinol–sulfuric acid reagent to color glycolipids ntaining neutral carbohydrates, and incubated at 100°C for color development. ne 1, G_{M3}; 2, G_{M3} and G_{D3} incubated at pH 2.2; 3, G_{D3} incubated at pH 2.2; 4, $_{3}$ incubated at pH 2.2 and ceramide lactoside; 5, ceramide lactoside.

ble II. Changes in the Structure of G_{D3} Lactone Incubated in Neutral lutions

Sample	Treatment[a]	Ganglioside[b]	
		G_{D3}	G_{D3} lactone
G_{D3} lactone	Initial	0%	100.0%
	pH 6.3	69.5	30.5
	pH 6.8	74.2	25.8
	pH 7.1	83.2	16.8
	pH 7.4	100.0	0

[a] G_{D3} lactone (1 mg/ml) were incubated in neutral solutions (pH 6.3, 6.8, 7.1, and 7.4) at 37°C for 2 h.
[b] The proportions of ganglioside were estimated by scanning the spots of incubated G_{D3} lactone on the TLC plate.

it that of ceramide lactoside formation by release of two olecules of sialic acid remained below 24%. No decrease in the tal amount of G_{D3} and G_{D3} lactone was identified, indicating at G_{D3} lactone is stable under the acidic conditions. Thus, it is suggested that G_{D3} lactone resistant to acid would be formed the stomach to protect its structure from the attack of gastric ice. Riboni *et al.*[11] demonstrated that inner ester linkages for tonization are formed and hydrolyzed to regenerate intact ngliosides under physiological conditions in the body. Their sults support our hypothesis that G_{D3} in milk would be converted G_{D3} lactone in the stomach to protect its structure from the tack of gastric juice. Figure shows the TLC profiles of ceramide toside, G_{M3}, and G_{D3} incubated at pH 2.2. The formation of $_{3}$ lactone was demonstrated based on the different migration tterns of ceramide lactoside, G_{M3}, and G_{D3} on TLC; however, uctural analysis by nuclear magnetic resonance spectroscopy would be necessary to obtain more accurate results. Ganglioside lactones are considered to be easily formed in the presence of catalysts under anhydrous conditions.[11,12] Ando *et al.*[12] reported that G_{D3} was converted into two kinds of lactones denoted as G_{D3} lactone I and II when incubated in glacial acetic acid at 25°C for 8 h. By comparing its migration on TLC with the results obtained by Ando *et al.*,[12] we estimated that the G_{D3} lactone formed in this experiment corresponded to G_{D3} lactone I, which behaves as a monosialoganglioside. No formation of G_{M3} lactone occurred under the same experimental conditions, indicating that polysialogangliosides are more easily converted to less polar derivatives than monosialogangliosides, as suggested by Yu *et al.*[13] Table II represents the changes in the structure of G_{D3} lactone after the pH was readjusted to neutral conditions. The extent of G_{D3} reconversion from its lactone form increased with the increase in the pH of the solution; G_{D3} was completely regenerated at pH 7.4. The reformation of G_{D3} from its lactone under neutral conditions suggests that the form of G_{D3} would be important for its performance as a bioactive component in the intestinal tract. The gastric pH of infants seems to be between 3 and 5, because they are fed with breast milk at intervals of several hours.[14] Thus, our findings suggest that gangliosides G_{M3} and G_{D3} in milk are likely to reach the intestinal tract because over 80% of sialic acid in their structure remained intact under the same acidic conditions found in an infant's stomach. Although further studies are required to clarify the physiological role of milk gangliosides in infants, it may be possible that G_{M3} and G_{D3} perform as components to protect against infections in the intestinal tract of infants.

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