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Sialyl Oligosaccharides in the Milk of Japanese Women: Changes in Concentration during the Course of Lactation

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Abstract: The concentrations of the six major sialyl oligosaccharides found in the milk of 24 Japanese women were determined using reverse phase high performance liquid chromatography of the 3-methyl-1-phenyl-5-pyrazolone derivatives of the oligosaccharides. The milk was collected at 4, 10, 30 and 100 days postpartum. The concentrations of all six oligosaccharides decreased during the course of lactation. The concentrations of 6′-sialyllactose, sialyllacto-*N*-tetraose b and disialyllacto-*N*-tetraose decreased only after 10 days postpartum, whereas those of sialyllacto-*N*-tetraose a and sialyllacto-*N*-neotetraose c as well as 3′-sialyllactose decreased from 4 days postpartum. Large differences were observed between present data and those previously reported for Italian women, both in terms of the content and in the variation pattern of each oligosaccharide during the course of lactation. These differences may be caused by the different ethnicity of the donors as well as the assay methods used in the studies.

Key words: human milk, sialyl oligosaccharide, lactation period, PMP, reverse phase HPLC

The oligosaccharide fraction is the third largest solid component in human milk, after lactose and lipid.[1] Human colostrum contains 20 to 24 g/L of oligosaccharides, whereas human mature milk contains 12 to 14 g/L. Human milk oligosaccharides usually have a reducing lactose unit to which Gal, GlcNAc, Fuc and/or NeuAc has been attached through the action of various glycosyltransferases.[2–4] To date, the presence of over one hundred oligosaccharides has been elucidated in human milk by various methods including mass spectrometry.[5,6] Human milk oligosaccharides are classified into neutral and acidic, based on the presence or absence of a negative charge. Most of the acidic oligosaccharides are sialyl oligosaccharides, containing NeuAc attached to a penultimate Gal residue or GlcNAc residue, *via* an α2-3 or α2-6 linkage formed through the actions of various sialyltransferases.[4]

The sialyl oligosaccharides are generally believed to be of biological significance for human infants.[7] Breast-fed infants appear to have fewer or less severe gastrointestinal and respiratory infections during the first year of life than formula-fed infants.[8] It is thought that the sialyl saccharides act as growth factors for beneficial intestinal bacteria such as *Bifidobacterium*.[9] The reduction in pH of the infant colon resulting from lactose fermentation by *Bifidobacterium* causes inhibition of the growth of *Escherichia coli* and other pathogenic organisms.

The sialyl milk oligosaccharides also inhibit the attachment of pathogenic organisms[8,10–12] such as *Helicobacter pylori* and of cholera toxin, to intestinal epithelial cells and the binding of rotavirus to the colon.

In addition, sialic acid in milk oligosaccharides has been thought to be used as a material for the synthesis of brain gangliosides and sialyl glycoproteins.[7] Breast milk may be an important source of sialic acid for newborn infants, whose livers may not be able to synthesize sufficient sialic acid.[13]

The sialic acid content of bovine milk is much lower than that of human milk.[14] This implies that sialyl oligosaccharides should be added to infant formulas based on bovine milk constituents. Although the total sialic acid content of human milk at several lactation periods has

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Abbreviations: HPLC, high-performance liquid chromatography; HPAEC, high-pH anion-exchange chromatography; PMP, 3-methyl-1-phenyl-5-pyrazolone; Glc, D-glucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Fuc, L-fucose; NeuAc, *N*-acetylneuraminic acid; Xyl, D-xylose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose; LSTa, sialyllacto-*N*-tetraose a; LSTb, sialyllacto-*N*-tetraose b; LSTc, sialyllacto-*N*-neotetraose c; DSLNT, disialyllacto-*N*-tetraose; 3′S3FL, 3′-sialyl-3-fucosyllactose; LNT, lacto-*N*-tetraose; LNnT, lacto-*N*-neotetraose.

been reported,[15-18] there are few data on the concentrations of individual sialyl oligosaccharides in human milk during the course of lactation.[19,20] Accordingly, we have studied the concentrations, during the course of lactation, of six major sialyl oligosaccharides contained in the milk of Japanese women milk, using high-performance liquid chromatography (HPLC) of their 3-methyl-1-phenyl-5-pyrazolon (PMP) derivatives.[21] We have compared our values with those reported previously for the milk of Japanese and Italian women.[19,20]

MATERIALS AND METHODS

Materials. Milk samples were collected at 4, 10, 30 and 100 days postpartum from 24 Japanese women who had given birth at Obihiro Kosei General Hospital. All samples (about 3 to 5 mL) at each lactation period were collected by hand and transferred into sterilized tubes, which were then stored at −40°C until used.

3′-Sialyllactose (3′-SL) and 6′-sialyllactose (6′-SL) were purchased from Dextra Laboratories Ltd., UK. Sialyllacto-*N*-tetraose a (LSTa), sialyllacto-*N*-tetraose b (LSTb) and disialyllacto-*N*-tetraose (DSLNT) were from Seikagaku Co., Japan. *N*-Acetylneuraminyl-lacto-*N*-neotetraose c (LSTc) was obtained from Sigma Chemical Co., USA. 3-methyl-1-phenyl-5-pyrazolone (PMP) was purchased from Kishida Chemical Co., Ltd., Japan. All other chemicals were analytical grade except for acetonitrile which was of HPLC grade.

Isolation of the acidic oligosaccharides fraction from human milk. The milk samples were thawed and 1 mL of each was extracted with 4 volumes of chloroform/methanol (2:1, v/v). The emulsion was centrifuged at 3500 rpm for 30 min at 4°C and the lower chloroform layer and denatured protein were discarded. The upper layer was collected, evaporated, and freeze-dried. The resulting powder was called the 'carbohydrate fraction.' The recovery of the carbohydrate was measured using 7.0% (w/v) lactose solution, *i.e.*, a concentration similar to that in human milk. Following extraction with 4 volumes of chloroform/methanol (2:1, v/v), the recovery of lactose in the upper layer was 90.8±3.3%.

The carbohydrate fraction was dissolved in 1 mL of water, and the solution was passed through a Bio Gel P-2 (Bio Rad Laboratories, USA, Extra fine, <45 μm) column (φ2.6×100 cm, 40°C) which had been calibrated with 2 mg of each of galactose (monosaccharide), lactose (disaccharide) and raffinose (trisaccharide). Elution was done with water at a flow rate of 15 mL/h and 95 fractions of 5 mL were collected. Aliquots (0.25 mL) of each fraction were analyzed for hexose using the phenol-H_2SO_4 method[22] and for sialic acid with the periodate-resorcinol method.[23] Peak fractions were pooled and freeze-dried.

Of these, fractions which contained both neutral and acidic oligosaccharides (Peaks 2 and 3 in Fig. 1) were dissolved in 1 mL of 50 mM Tris-HCl buffer (pH 8.7) and subjected to anion exchange chromatography on a DEAE Sephadex A-50 (Amersham Pharmacia Biotech UK Ltd., UK) column (φ1.0×40 cm) equilibrated with the same buffer. The unadsorbed components were eluted with 100 mL of the same buffer, while the adsorbed components were eluted with 300 mL of a linear gradient from 0 to 1.0 M NaCl in 50 mM Tris-HCl buffer (pH 8.7). Elution was done at a flow rate of 15 mL/h and fractions of 5 mL were collected. Aliquots (0.25 mL) of each fraction were analyzed for hexose. Peak fractions were pooled and freeze-dried. The acidic oligosaccharides were contained in the fractions whose elution volume (65 mL) was retarded in relation to the void volume (20 mL), which contained the unadsorbed components, and also in the fractions which eluted with about 0.133 M NaCl in the buffer. These fractions were combined, dissolved in 1 mL of water, and passed through a Bio Gel P-2 column (φ2.6×100 cm) under the same conditions as above. Peak frac-

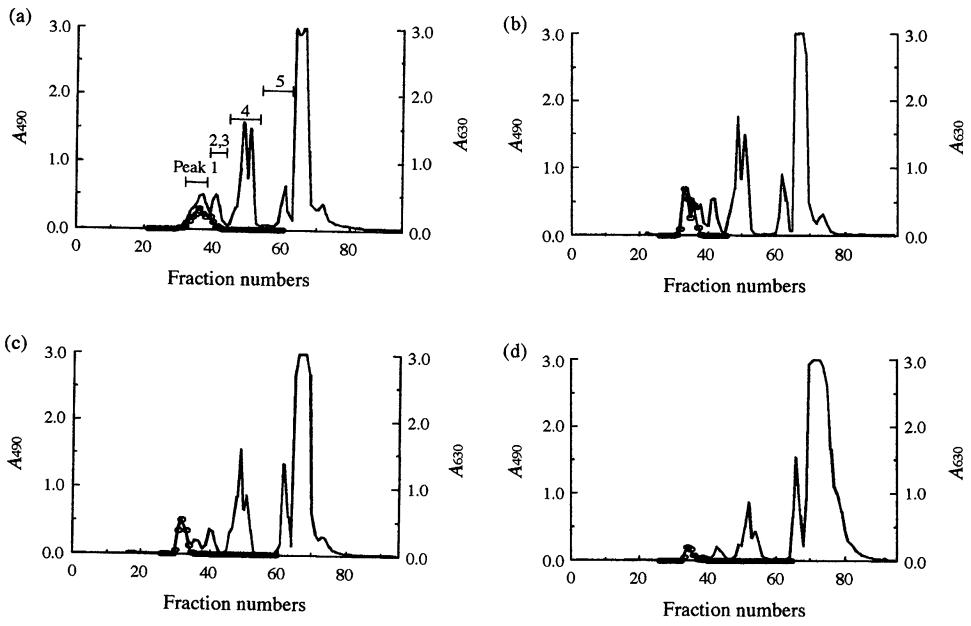

Fig. 1. Bio Gel P-2 (φ2.6×100 cm) chromatograms of the carbohydrate fractions of the milk of Japanese women.

Each fraction (5 mL) was analyzed for hexose using the phenol-H_2SO_4 method at 490 nm (—) and for sialic acid using the periodate-resorcinol method at 630 nm (○). A_{490}, absorbance at 490 nm; A_{630}, absorbance at 630 nm. The carbohydrate fractions were separated from the milk at (a) 4, (b) 10, (c) 30 and (d) 100 days postpartum.

tions were pooled and freeze-dried. This material was mixed with Peak 1 of the first gel chromatography with a Bio Gel P-2 (see Fig. 1), and designated as the acidic oligosaccharide fraction.

PMP derivatisation of the acidic oligosaccharides fraction. PMP derivatisation of the acidic oligosaccharides was done according to the method of Honda *et al.*[21] The dried sample was dissolved in 120 μL of water containing 15 μL of Xyl solution (0.1 mg/mL) as an internal standard. Then 150 μL of 0.5 M PMP in methanol and 30 μL of 1.5 M NaOH were added to the mixture. The reaction mixture was mixed and incubated in a heatblock at 70°C for 2 h. After cooling the mixture to room temperature, 100 μL of 0.5 M HCl and 1 mL of chloroform were added to the solution and mixed for 10 s using a Vortex mixer. The bottom chloroform layer was carefully removed using a Pasteur pipet and discarded. This step was repeated twice. The top aqueous layer was evaporated and freeze-dried for HPLC analysis. Derivatisation of a mixture of standard saccharides (each 100 μg) was done by the same procedure.

Quantification of PMP-labeled sialyl oligosaccharides. The PMP-labeled acidic oligosaccharides fraction was dissolved in 100 μL of water and the solution was filtered through a syringe-driven filter unit Millex-LG (Millipore Co., USA, 0.20 μm, hydrophilized PTFE membrance). Of the filtered solution, 10 μL were injected into the HPLC column and subjected to reverse-phase HPLC using an Inertsil ODS-3V column (ϕ 4.6×250 mm, packed with 5 μm particles having 10 nm pore size, GL Sciences Inc., Japan) at 40°C in a column oven CTO-10ACvp (Shimadzu Co., Japan). The elution was controlled using a pump LC-10ATvp (Shimadzu) equipped with a system controller SCL-10Avp (Shimadzu). Elution was carried out with 100 mM potassium phosphate buffer (pH 6.5) containing 15% acetonitrile at a flow rate of 1 mL/min. Detection was done with a UV-detector CM-8020 (Tosoh, Japan) by monitoring the absorption of the eluates at 245 nm. Peak areas were calculated using a chromatography data system CLASS-VP.

Each sialyl oligosaccharide was identified by comparison with the relative retention time of each peak with the peak of internal Xyl. The ratio of peak area of each PMP-labeled standard (1 μg/mL) to that of internal Xyl was determined, prior to the determination of each oligosaccharide content in the sample. The quantitative value for each PMP-labeled sialyl oligosaccharide was obtained by calculation of each peak area in relation to that of internal Xyl. Quantitative values were shown by using the mean concentration±standard deviations. The reproducibilities of peak area of each PMP-labeled standard were shown by using relative standard deviations, when the determination of each oligosaccharide content was performed three times.

RESULTS

Representative Bio Gel P-2 chromatograms of carbohydrate fractions extracted from the milk at each lactation stage are shown in Fig. 1. Each of the fractions resolved into five peaks. The acidic oligosaccharides fraction, composed of column fractions which had reacted positively in the periodate-resorcinol reaction, was prepared from the combined peaks 1 to 3 as described in MATERIALS AND METHODS. Peaks 2 and 3 has been separated into neutral and acidic oligosaccharide fractions by the anion exchange chromatography on a DEAE Sephadex A-50 (see MATERIALS AND METHODS). The peak area of the acidic oligosaccharides fraction decreased relatively to the other peaks from days 4 to 100 postpartum.

The six sialyl oligosaccharides quantified in this study are listed in Table 1. Figure 2 shows a representative

Table 1. The six sialyl oligosaccharides quantified in human milk collected at 4, 10, 30 and 100 days postpartum.

3′-SL	NeuAc α 2-3 Gal β 1-4 Glc
6′-SL	NeuAc α 2-6 Gal β 1-4 Glc
LSTa	NeuAc α 2-3 Gal β 1-3 GlcNAc β 1-3 Gal β 1-4 Glc
LSTb	Gal β 1-3 [NeuAc α 2-6] GlcNAc β 1-3 Gal β 1-4 Glc
LSTc	NeuAc α 2-6 Gal β 1-4 GlcNAc β 1-3 Gal β 1-4 Glc
DSLNT	NeuAc α 2-3 Gal β 1-3 [NeuAc α 2-6] GlcNAc β 1-3 Gal β 1-4 Glc

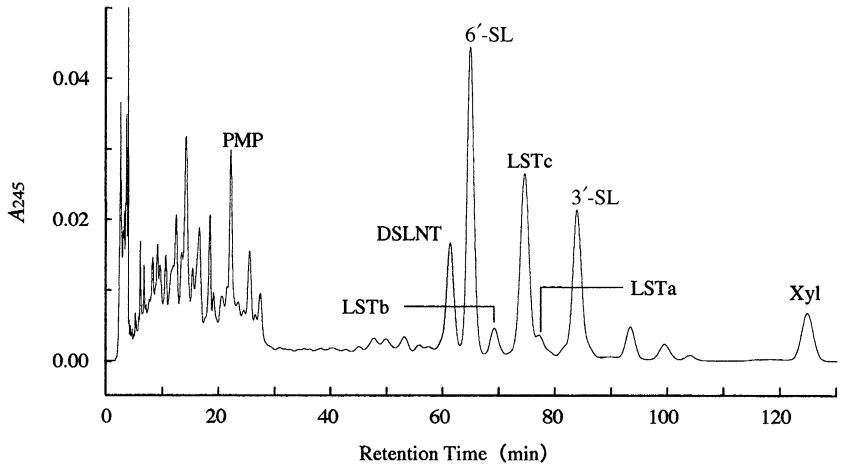

Fig. 2. High-performance liquid chromatogram of the PMP derivatives of the acidic oligosaccharides fraction separated from human milk at 4 days postpartum.

A_{245}, absorbance at 245 nm. The fraction had been prepared by gel chromatography on a Bio Gel P-2 followed by anion exchange chromatography on a DEAE Sephadex A-50. For other details, see Methods.

Table 2. The concentration of each oligosaccharide in human milk collected at 4, 10, 30 and 100 days postpartum.

	Days of lactation			
	4 (n=24)	10 (n=24)	30 (n=24)	100 (n=23)
3′-SL	69.2±56.7*	53.8±45.7	43.1±31.4	39.3±44.2
	0.0–208.7	3.1–179.0	0.0–100.3	0.0–160.0
6′-SL	409.8±274.1	412.2±398.8	275.5±233.1	95.7±158.4
	0.0–1199.4	2.1–1706.0	1.5–779.1	0.7–746.4
LSTa	103.7±58.8	82.7±39.4	52.1±25.0	37.0±16.0
	20.3–264.2	28.3–156.4	18.2–120.2	0.0–75.8
LSTb	56.2±39.0	53.8±30.6	43.6±27.0	28.8±24.7
	3.2–180.2	13.5–127.9	3.8–102.8	0.0–85.0
LSTc	293.6±210.9	145.0±155.7	73.9±60.4	38.9±66.7
	0.0–678.2	9.3–516.6	4.3–236.5	0.0–294.7
DSLNT	199.4±140.7	176.4±133.5	110.7±74.5	56.1±61.6
	0.0–559.8	30.5–555.0	0.0–268.3	0.0–236.1
Total**	1131.7	923.9	598.8	295.8

*Upper values are mean±SD and lower are minimum and maximum values (mg/L). **The sum of the above oligosaccharides quantified in human milk in this study.

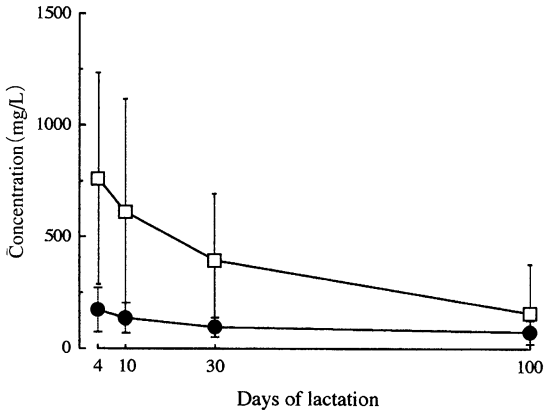

Fig. 3. Changes in the concentration of α2-3-linked (●) and α2-6-linked (□) sialyl oligosaccharides of human milk during the course of lactation.

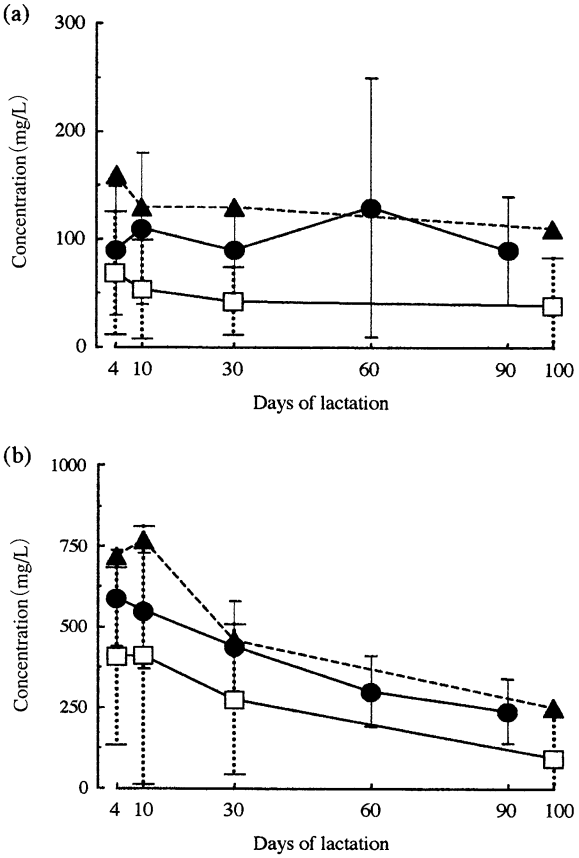

Fig. 4. Changes in the concentration of (a) 3′-SL and (b) 6′-SL of human milk during the course of lactation.

(□) The values obtained in this study. (●) The values reported by Coppa *et al.* (1999)[20]. (▲) The values reported by Idota *et al.* (1994)[19].

chromatogram of the HPLC of the PMP derivatized acidic oligosaccharides fraction.

Since lactose, lacto-*N*-tetraose (LNT) and lacto-*N*-neotetraose (LNnT) were not detected in the HPLC of the standard acidic oligosaccharides, there could have been little decomposition of sialyl oligosaccharides during the PMP derivatisation.

Reproducibilities were expressed as relative standard deviations. Reproducibilities of peak areas for 3′-SL-PMP. 6′-SL-PMP, LSTa-PMP, LSTb-PMP, LSTc-PMP and DSLNT-PMP were 2.5, 2.7, 3.0, 0.9, 6.6 and 3.1%, respectively.

Table 2 shows the mean concentration and its standard deviation of each sialyl oligosaccharide at each lactation period. Significant reductions in the concentrations of the oligosaccharides were observed during the course of lactation. From 4 to 10 days postpartum, the concentrations of 6′-SL, LSTb and DSLNT remained more or less constant but after that time they decreased. By contrast, the concentrations of 3′-SL, LSTa and LSTc decreased from 4 days postpartum onwards. The total sialyl oligosaccharide concentration, which was calculated by summing all the saccharides determined in this study, decreased from 1132 mg/L at 4 days postpartum to 296 mg/L at 100 days. However, the concentration of individual oligosaccharides greatly varied among the donors.

The total content of α2,6-linked sialyl oligosaccharides (6′-SL, LSTb and LSTc) was four times higher than that of α2,3-linked sialyl oligosaccharides (3′-SL and LSTa) in the colostrum collected at 4 days postpartum (Fig. 3).

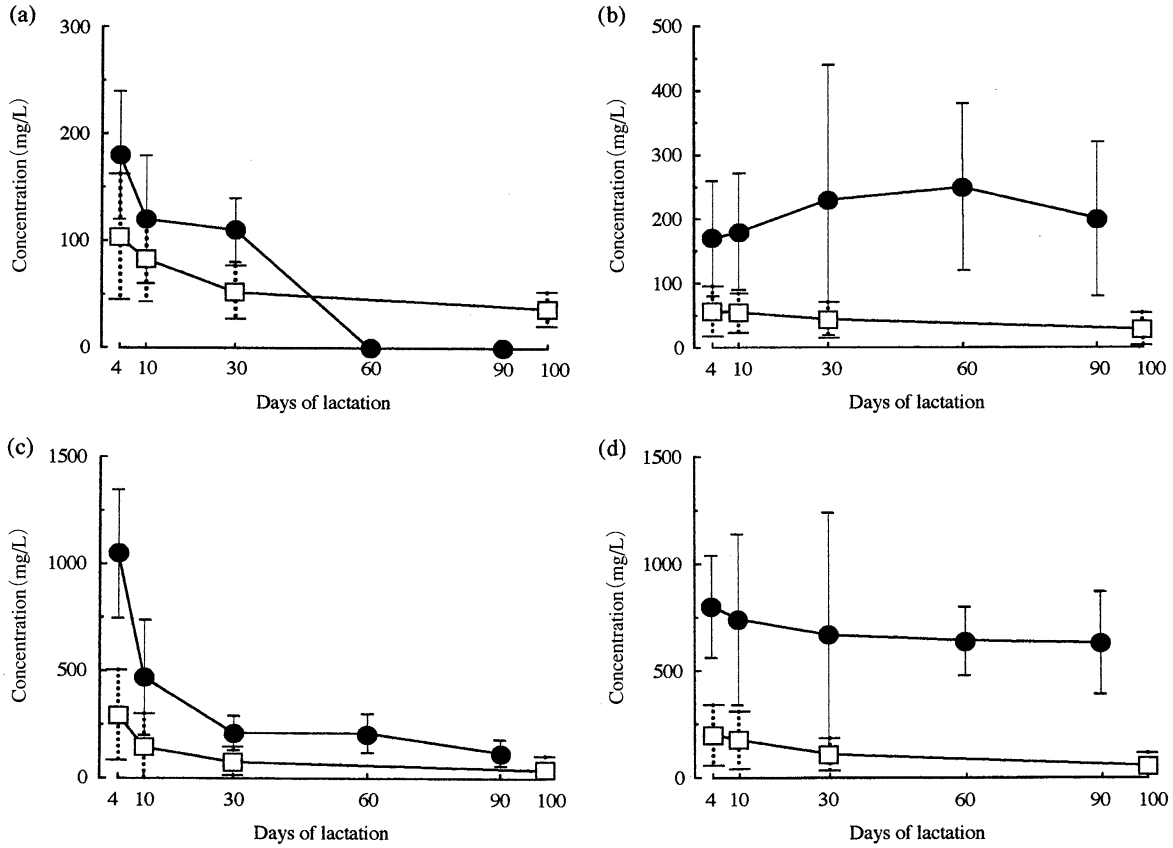

Fig. 5. Changes in the concentration of (a) LSTa, (b) LSTb, (c) LSTc and (d) DSLNT of human milk during the course of lactation. (□) The values obtained in this study. (●) The values reported by Coppa *et al*. (1999)[20].

The concentration of both kinds of oligosaccharides decreased to about 20 and 40%, respectively, of those of the colostrum, by 100 days postpartum, at which time the content of α2,3-linked sialyl oligosaccharides was similar to that of α2,6-linked sialyl oligosaccharides.

DISCUSSION

There are only a few previous studies in which individual human milk oligosaccharides have been quantified.[20,24,25] The present study is the first to use PMP derivatisation for the determination of sialyl oligosaccharides in human milk. In this method, the oligosaccharides were derivatized with PMP through the aldehyde group at the reducing end, forming a semiacetal, which then reacted with another molecule of PMP to form an acetal.[21]

Recently, Shen *et al*. determined the level of 7 sialyl oligosaccharides such as 3′-SL, 6′-SL, 3′-S3FL (3′-sialyl-3-fucosyllactose), LSTa, LSTb, LSTc and DSLNT in the milk using high-performance capillary electrophoresis.[26] However, the samples which they used in the study were pooled ones, and they did not determine concentration of each sialyl oligosaccharide in the milk of individual donors during the course of lactation.

Large differences were observed in the concentration of each oligosaccharide between our data and the values reported by Coppa *et al*.[20] for Italian women (Figs. 4, 5). Their values were almost all higher than ours, except that LSTa was not detected at 60 and 90 days postpartum. One reason for this difference between the studies may be the relatively small sample number of donors used (24 in our study, 18 in that of Coppa *et al*.). Another reason may be the difference in ethnicity. Our previous study on the neutral oligosaccharides in the milk of Japanese women showed that the concentrations of LNT and LNnT, the core units for LSTa, LSTb and LSTc, were lower than those of the milk of Italian women.[25] It was shown that the increasing and/or decreasing patterns of the content of 6′-SL, LSTc and DSLNT during the course of lactation were similar in both studies, while those of 3′-SL, LSTa and LSTb were different. This suggests that the variations, during the course of lactation, in mammary gland α2-3 sialyltransferase activity may be different in Japanese compared with Italian women.

In addition, there were differences in methodology used for separating and determining these sialyl oligosaccharides. In our HPLC of the PMP derivatives of the acidic oligosaccharides, which had been separated by gel filtration and anion exchange chromatography, we used reverse phase chromatography. By contrast, Coppa *et al*. used high pH anion exchange chromatography (HPAEC) of fractions separated by centrifugation and membrane filtration of the milk[20] Our method removed most of the oligosaccharides other than the six sialyl oligosaccharides determined in this study, which simplified the quantification. In Coppa *et al*.'s procedure, on the other hand, some peaks of the oligosaccharides being determined may have overlapped with those of others, of which there are more than 100 in human milk.[5,6]

Cataldi *et al*. commented on the poor reproducibility of retention times in the analysis of carbohydrates by HPAEC.[27] This would be another disadvantage of the

method used by Coppa *et al*. Highly reproducible separations of carbohydrates cannot be obtained by HPAEC because the adsorption of carbon dioxide and consequent production of carbonate ions progressively reduces the number of anion exchange sites available during the elution procedure.

We believe, in addition, that our method, *i.e.* reverse phase HPLC of PMP derivatives of the milk oligosaccharides, circumvents several problems. The method produces highly reproducible results and is very sensitive, requiring as little as 0.1 μg/mL of a sialyl oligosaccharide for quantitation.[28] It is thus an available method for the determination of the sialyl oligosaccharides of milk and other biological fluids.

Potentially, one problem could be the release of free sialic acid during the derivatisation procedure. Indeed, Kakehi *et al*. reported the release of sialic acid during the pyridylamination of sialyl oligosaccharides.[29] The data of Fu *et al*., however, suggest that this is not a problem in the case of PMP derivatisation, as illustrated by their excellent recovery of the PMP derivative of 3′-SL.[28] Fu *et al*. determined the 3′- and 6′-SL content of human blood plasma and urine by reverse phase HPLC of their PMP derivatives. They obtained recoveries of 98.9% (1 μg/mL) and 102.3% (4 μg/mL) of plasma 3′-SL and 116% (10 μg/mL) and 108% (50 μg/mL) of urine 3′-SL. In addition, they obtained a standard linear curve when the observed values for 3′- and 6′-SL were plotted against their concentrations.

In our experiments, we used 1.5 μg of xylose as an internal standard during the PMP derivatisation of the acidic oligosaccharide fractions. Thus the content of the sialyl oligosaccharides was determined by comparison of the area under each HPLC peak with that of the xylose; we decided that this method would provide more accurate values than those based on determinations of percentage recovery.

Idota *et al*. reported on the concentrations of 3′-SL and 6′-SL in the milk of 2720 Japanese women at several stages of lactation.[19] Their values were at least twice as high as those of this study. This difference may be caused by the methodological differences between the two studies in the separation and determination of SL contents. Idota *et al*. used normal phase chromatography with Unisil Q NH2 in the HPLC of fractions separated by centrifugation, extraction with ethanol and membrane filtration of the milk. Nevertheless, the patterns of variation in the concentrations of both SLs during the course of lactation were similar in the two studies.

Our study showed that in early lactation the concentration of α2-6-linked sialyl oligosaccharides was significantly greater than that of α2-3-linked sialyl oligosaccharides, but by 100 days postpartum their concentrations became more similar (Fig. 3). This suggests that the α2-6 sialyltransferase activity of the mammary gland is higher than the α2-3 sialyltransferase activity in early lactation, and that the activity of the former decreases more strongly during the course of lactation. It has been shown that LSTb is used as substrate in preference to LSTa in the biosynthesis of DSLNT.[30] This is consistent with the fact that the concentration of LSTb was lower than that of other α2-6-linked sialyl oligosaccharides.

It is well recognized that human mature milk contains more sialyl oligosaccharides than does bovine mature milk.[15,17,31] Cow colostrum collected immediately after parturition contains over 1.5 g of sialyl oligosaccharides per liter, but their concentration rapidly declines after 24 h postpartum.[32] These facts suggest that sialyl oligosaccharides are more significant for human infants than for calves. In addition, there is a difference between humans and cows in the kinds of sialyl oligosaccharides in their milk or colostrum.[33] 6′-SL is the dominant sialyl saccharide in human milk in early lactation, while 3′-SL is dominant in bovine colostrum. Human milk or colostrum contain LSTa, LSTb and LSTc (whose core units are LNT or LNnT), whereas bovine colostrum contains no more than trace amounts of these oligosaccharides.[32]

Milk sialyl oligosaccharides are believed to have biological significance as anti-infection factors against some pathogenic microorganisms and/or as nutrients for growth of the infant brain (see INTRODUCTION). Our data suggest that, for optimum nutrition, infant formulas derived from bovine milk should be supplemented with sialyl oligosaccharides.

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日本人女性の乳中に含まれるシアリルオリゴ糖の泌乳期における経時的変動

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日本人女性の乳中に含まれる6種のシアリルオリゴ糖濃度の泌乳期に伴う変動を，PMP誘導体化を用いた逆相HPLCにより定量した．DSLNT，6′-SLおよびLSTb濃度は出産後10日目までの変化は少なく，それ以降減少したが，LSTc，LSTaおよび3′-SL濃度は出産後4日目から100日目まで減少し続けた．本研究と以前に報告されたイタリア人女性の乳でのデータでは，泌乳期の経過に伴う各オリゴ糖の濃度およびその変動パターンに大きな差が見られた．これらの差はドナーの人種差によるばかりではなく，両者の定量方法の違いによっても生じたものと考えられる．また，本研究と以前に報告された日本人女性の乳中における3′-SLおよび6′-SLの定量データとは，泌乳期による変動パターンが類似していた．