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Profile of Nucleotides and Nucleosides of Human Milk

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Summary The content of nucleotides and nucleosides of human milk was analyzed using a newly developed method for high performance liquid chromatography. By this method it is possible to analyze nucleotides and nucleosides simultaneously. Human milk was pooled according to season, lactation period, and geographical area. Three kinds of nucleosides—cytidine, uridine, and adenosine—and 6 kinds of nucleotides —5′-CMP, 5′-UMP, 5′-AMP, 5′-GMP, 5′-IMP, and 5′-CDP—were detected. Cytidine, 5′-CMP, and 5′-CDP predominated throughout lactation. Also there seemed to be geographical differences in nucleoside composition. The overall amounts of nucleotides and nucleosides were higher in winter than in summer. No nucleosides were detected in bovine milk, nor in bovine milk-based infant formula, and bovine milk contained much less nucleotides than human milk. These results suggest that nucleosides and nucleotides found in human milk may play some important roles in the development of infants.

Key Words nucleotide, nucleoside, human milk, nonprotein nitrogen, lactation, geographical area, season, HPLC

Mother's milk is the best food for infants. Thus, knowing the composition of human milk would contribute to the understanding of infant nutrition.

It is well known that there are many differences regarding their composition between human milk and that of other species. One of them is the content of nonprotein nitrogen. In human milk, nonprotein nitrogen accounts for about 20% of the nitrogen fraction, and in bovine milk for about 5% only (*1*). Nonprotein nitrogen contains many kinds of small peptides, amino acids, amino sugars, sialic acid, urea, polyamines, nucleic acids, nucleotides and nucleosides.

Nucleotides must be essential for all kinds of living creatures, because they are the components of nucleic acids. All tissues in the body can synthesize nucleotides *de novo*, and dietary nucleotides have not been recognized to play some physiological roles for a long time. Recently, the potential importance of dietary nucleotides, especially in infants has been suggested, such as in polyunsaturated

fatty acid metabolism (*2–4*), immunological systems (*5–11*) and differentiation c intestinal cells (*12, 13*). However, there are very few reports about the content an the composition of nucleotides in human milk (*14–16*).

In general, it is said that part of orally administered nucleotides is broken dow into nucleosides and absorbed from the intestines. Also it has been reported tha nucleosides may participate in the differentiation of intestinal cells (*17*). Thes findings suggest that dietary nucleosides, along with nucleotides, may play som kind of role in infants.

The present study was undertaken to identify and characterize the content c nucleotides and nucleosides in human milk. For this purpose, we developed a ne analysis method using high performance liquid chromatography together with a ion-paired system. With this method, nucleotides and nucleosides can be analyze simultaneously.

MATERIALS AND METHODS

Human milk samples. Human milk specimens ($n=2,727$) were collecte from 2,434 mothers aged 17 to 41 living in various areas of Japan. The mil specimens were collected in 1989 from January to March (winter milk) and fro July to September (summer milk). The specimens were frozen immediately aft sampling, and transported to our laboratory. We have kept these specimens i storage at -40 or $-80°C$.

The content of nucleotides and nucleosides were analyzed in winter an summer milk pooled according to lactation period and geographical area. Th lactation periods were: 3–5, 6–10, 11–15, 16–30, 31–60, 61–120, 121–240, and 241 482 days postpartum. The numbers of specimens which constructed each lactatio ally pooled sample were 59, 103, 67, 159, 243, 194, 189, and 84, respectively. Th geographical areas were established on the basis of the classification used in th National Nutrition Survey, conducted by the Ministry of Health and Welfare c Japan (1980). The pool of milk for each geographical area was prepared by mixin equal amounts of individual specimen obtained 16 to 90 days postpartum, an stored at -40 or $-80°C$ until use. The numbers of specimens which comprise each geographically pooled sample were 42 for Hokkaido, 47 for Tohoku, 52 fc Kanto/Koshin'etsu, 50 for Chubu/Tokai, 30 for Kinki, 62 for Chugoku/Shikok and 74 for Kyushu/Okinawa.

The content of nucleotides and nucleosides of bovine colostrum, bovin matured milk, marketed milk and bovine milk-based infant formula available i Japan was also analyzed. The infant formula powder was dissolved in warm wat at a concentration of 13% (w/v).

Sample preparation for HPLC analysis. The frozen samples were thawed a room temperature and 5 ml of each sample was mixed with 10 ml of 10% (w/v trichloroacetic acid (TCA) solution. After allowing the mixtures to stand on ic for 30 min, they were centrifuged at $30,000 \times g$ for 15 min. The aqueous layers wer

recovered and the residual cream and precipitates were washed twice with 5 ml of 7.5% (w/v) TCA solution. The aqueous layers were combined, and TCA was removed by adding 20 ml of diethyl ether. The solutions were then lyophilized and stored at −20°C until the analysis. Just before the analysis, the lyophilized samples were dissolved in water and then water was added to a volume of 1 ml. After filtering through a 0.45 μm of Chromatodisk (Kurabo, Osaka, Japan), the nucleotides and nucleosides were analyzed by HPLC.

HPLC analysis. Nucleotides and nucleosides were analyzed using a Hewlett Packard HP1050 system equipped with a Capcellpak C18, type AG (4.6×500 mm, Shiseido, Tokyo, Japan) at room temperature. Solvent A was 25 mM tetrabutylammonium hydrogen sulphate −50 mM potassium phosphate (pH 3.5) and solvent B was methanol.

The pump program was as follows: 0–40 min 100% solvent A, 40–70 min 0–50% solvent B, 70–90 min 50% solvent B. After each analysis, the eluent was returned to the initial condition and equilibrated for 20 min. The flow rate was 0.5 ml/min. Nucleotides and nucleosides were detected by the absorbance at 254 nm.

Appropriate standards were used to establish the retention time of each nucleotide and nucleoside. Quantification was achieved for peak area vs. amounts of nucleotide and nucleoside standard injected. The amounts of standards were corrected for their water content.

Chemicals. Nucleotides and nucleosides for the standards were purchased from Yamasa Co. (Chiba, Japan). All other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Kanto Chemical Co. Inc. (Tokyo, Japan). Solvents were of HPLC grade and all other chemicals were of analytical grade.

RESULTS

The elution profile of the mixture of standard nucleotides is shown in Fig. 1. Nucleoside monophosphates are eluted earlier than the corresponding nucleoside di-, and triphosphates. As for pyrimidine and purine nucleotides, pyrimidine nucleotides are eluted earlier than purine nucleotides in the following order: cytidine nucleotide, uridine nucleotide, adenosine nucleotide, guanosine nucleotide and inosine nucleotide. Although not shown in the chromatogram, 5 kinds of nucleosides, cytidine, uridine, adenosine, guanosine, and inosine were also identified. The peak areas of these nucleotides and nucleosides are in linear relation with amount injected in a range between 0.01 and 15 nmol.

In the elution profile of human milk (Fig. 2), cytidine, uridine, adenosine, 5′-CMP, 5′-UMP, 5′-AMP, 5′-GMP, 5′-IMP and 5′-CDP were identified. Other nucleotides and nucleosides were presented in trace amounts or were not detected.

The contents of nucleotides and nucleosides in human milk from different lactation periods are given in Table 1. The predominant components throughout the lactation period were cytidine, 5′-CMP, and 5′-CDP. The changing patterns of

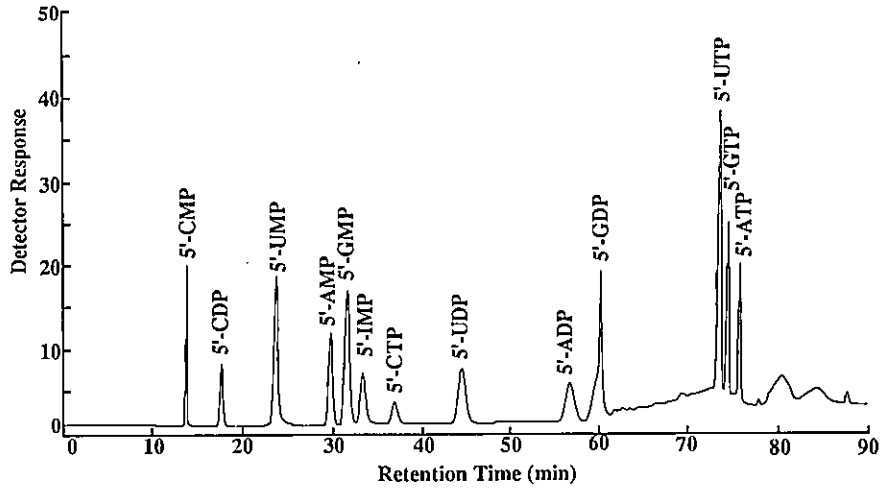

Fig. 1. Elution profile of the standard mixture of nucleotides. The standard mixture contained 10 mM for each nucleotide and was injected into the Capcell-pak C18-ion pair system (25 μl). Nucleotides were eluted with a gradient formed with 25 mM tetrabutylammonium hydrogen sulphate-20 mM potassium phosphate (pH 3.5) and methanol (see METHODS).

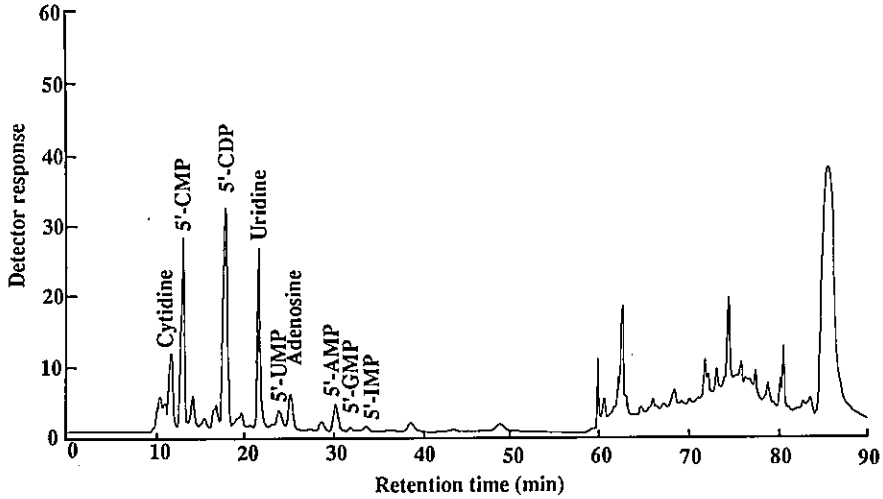

Fig. 2. Elution profile of the acid-soluble fraction from human milk. The acid-soluble fraction was obtained from 5 ml of human milk after treatment with TCA. After solubilizing lyophilized material in water (final volume = 1 ml), 25 μl of this solution was injected into the HPLC system. Conditions for the analysis were the same as indicated in the legend for Fig. 1. Components were identified by their retention time.

Table 1. Content of nucleotides and nucleosides in human milk during lactation.

(a) Winter milk (μmol/100 ml)

Component	Lactation (Postpartum days)							
	3–5	6–10	11–15	16–30	31–60	61–120	121–240	241–482
Cytidine	3.43	2.16	1.37	2.46	2.02	1.81	1.42	1.39
Uridine	1.84	0.90	2.75	3.12	3.26	2.39	1.87	1.83
Adenosine	n.d.*	n.d.	1.19	1.02	1.11	1.22	1.14	0.67
Total amount of nucleoside	5.27	3.06	5.37	6.60	6.39	5.42	4.43	3.89
5′-CMP	4.06	3.26	1.65	2.38	3.87	4.29	3.87	2.63
5′-UMP	0.11	0.27	n.d.	0.27	0.30	0.23	0.19	0.07
5′-AMP	0.35	0.36	0.28	0.35	0.26	0.23	0.21	0.20
5′-GMP	n.d.	0.09	0.09	0.08	n.d.	n.d.	n.d.	n.d.
5′-IMP	n.d.	n.d.	0.10	0.12	0.14	n.d.	n.d.	n.d.
5′-CDP	6.98	6.00	0.50	0.63	0.52	0.43	0.53	0.60
Total amount of nucleotide	11.5	9.98	2.62	3.83	5.09	5.18	4.80	3.50

*n.d. = not detected.

(b) Summer milk (μmol/100 ml)

Component	Lactation (Postpartum days)							
	3–5	6–10	11–15	16–30	31–60	61–120	121–240	241–482
Cytidine	n.d.	1.43	1.26	1.56	1.32	1.50	1.24	1.18
Uridine	3.33	1.56	1.75	1.24	1.62	1.26	0.37	1.39
Adenosine	0.67	0.71	0.46	0.32	1.62	0.65	0.49	0.78
Total amount of nucleoside	4.00	3.70	3.47	3.12	4.56	3.41	2.10	3.35
5′-CMP	2.20	0.65	0.93	0.94	1.26	0.38	0.25	0.25
5′-UMP	0.06	n.d.	0.05	0.08	0.05	n.d.	n.d.	n.d.
5′-AMP	0.22	0.18	0.16	0.15	n.d.	0.16	0.25	0.21
5′-GMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5′-IMP	n.d.	n.d.	n.d.	n.d.	0.07	n.d.	n.d.	0.09
5′-CDP	0.58	0.83	0.51	0.49	0.74	0.52	0.34	0.59
Total amount of nucleotide	3.06	1.66	1.65	1.66	2.12	1.06	0.84	1.14

*n.d. = not detected.

the amount of nucleotides and nucleosides differed between winter and summer milk. In winter, the total amount of nucleotides and nucleosides was high in milk obtained 3–5 and 31–60 days postpartum. However, in the summer, the content of these components was nearly stable during lactation, except for colostrum. Individual components showed no specific pattern of variation, such as constantly increasing or decreasing either in winter or in summer. The total amount of nucleotides and nucleosides was higher in winter than in summer.

Table 2. Content of nucleotides and nucleosides in human milk from mothers living in various areas of Japan.

(a) Winter milk (μmol/100 ml)

Component	Hokkaido	Tohoku	Kanto/ Koshin'etsu	Chubu/ Tokai	Kinki	Chugoku/ Shikoku	Kyusyu/ Okinawa
Cytidine	1.30	1.27	1.39	1.23	n.d.	1.22	2.09
Uridine	0.12	1.76	1.86	1.56	0.39	5.30	4.78
Adenosine	n.d.*	n.d.	2.76	0.38	0.71	0.27	0.24
Total amount of nucleosides	1.42	3.03	6.01	3.17	1.10	6.79	7.11
5′-CMP	3.71	2.98	2.80	2.96	1.81	0.81	0.77
5′-UMP	0.33	0.68	0.22	0.20	n.d.	n.d.	n.d.
5′-AMP	0.23	0.63	0.65	0.16	n.d.	n.d.	n.d.
5′-GMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5′-IMP	0.12	0.10	n.d.	0.09	0.21	1.11	0.34
5′-CDP	0.43	0.94	1.00	0.80	0.57	0.67	1.94
Total amount of nucleotides	4.82	5.33	4.67	4.21	2.59	2.59	3.05

*n.d. = not detected.

(b) Summer milk (μmol/100 ml)

Component	Hokkaido	Tohoku	Kanto/ Koshin'etsu	Chubu/ Tokai	Kinki	Chugoku/ Shikoku	Kyusyu/ Okinawa
Cytidine	1.12	n.d.	1.43	1.16	1.17	1.13	1.12
Uridine	0.64	0.71	0.38	0.59	0.71	4.22	0.70
Adenosine	n.d.*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total amount of nucleosides	1.76	0.71	1.81	1.75	1.88	5.35	1.82
5′-CMP	1.42	1.15	1.27	0.51	1.17	1.20	0.59
5′-UMP	0.05	0.68	0.59	0.69	0.57	n.d.	0.56
5′-AMP	0.15	0.15	n.d.	0.20	0.19	n.d.	0.12
5′-GMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5′-IMP	1.05	0.25	0.38	n.d.	n.d.	n.d.	n.d.
5′-CDP	1.31	1.19	0.79	0.77	0.77	0.13	1.08
Total amount of nucleotides	3.98	3.42	3.03	2.17	2.70	1.33	2.35

*n.d. = not detected.

Table 2(a) shows that in winter, milk from mothers living in Kanto/ Koshin'etsu, Chugoku/Shikoku, and Kyusyu/Okinawa areas contained a higher amount of nucleosides than those from mothers living in other areas. On the other hand, milk from mothers living in Kinki and Chugoku/Shikoku areas contained less amount of nucleotides. As shown in Table 2(b), in summer, milk from mothers living in Chugoku/Shikoku contained a higher amount of nucleosides, the same as in winter. However, milk from the Kanto/Koshin'etsu and Kyusyu/Okinawa areas

Table 3. Content of nucleotides in bovine milk and bovine milk-based infant formula. (μmol/100 ml)

Component	Bovine milk			
	Colostrum	Matured milk	Marketed milk	Infant formula
5′-CMP	0.19	0.99	0.56	0.70
5′-UMP	0.15	0.06	n.d.	0.08
5′-AMP	n.d.*	0.14	0.03	n.d.
5′-GMP	n.d.	n.d.	n.d.	0.08
5′-IMP	0.01	0.01	0.06	0.04
Total amount of nucleotides	0.35	1.20	0.65	0.90

*n.d. = not detected.

contained almost the same amount of nucleosides as those from other areas. Winter milk from all areas except Kinki, contained a higher amount of nucleotides and nucleosides than summer milk.

In bovine milk and bovine milk-based infant formula, neither nucleosides nor nucleoside di-, and triphosphates were detected (HPLC pattern not shown).

As shown in Table 3, the content of nucleotides was lower in bovine colostrum than in matured milk and marketed milk. However, the same as in case of human milk, 5′-CMP was the predominant component in bovine milk. The content of each nucleotide was much lower in bovine milk and bovine milk-based infant formula than in human milk. A huge peak of orotic acid was detected in the HPLC patterns of bovine milk and formula, although it was not quantified in this study.

DISCUSSION

During the last decade, it has been indicated that dietary nucleotides may play some physiologically important role especially in infants (*2–15*). In addition to nucleotides, it has been recently suggested that dietary nucleosides may also participate in the differentiation of intestinal cells (*17*). However, there have been very few reports about the content of nucleotides in human milk. Kobata et al. were the first to determine the content of nucleotides in human and bovine milk (*14*). They used ion-exchange chromatography and paper electrophoresis to isolate each nucleotide. Because of the low sensitivity of the method, they needed two liters of milk, and could not analyze the changes in composition during lactation.

Gil and Sánchez-Medina determined the changes of nucleotide content during lactation using an enzymatic method (*15*). Although this method is highly sensitive for each nucleotide, to obtain a complete profile of nucleotides a large amount of milk is needed, and the procedure is rather cumbersome. They were able to determine the content of only nucleoside monophosphates and UDP-derivatives.

Janas and Picciano analyzed nucleotides with HPLC (*16*). Although they determined the content of 9 nucleotides, they did not mention about nucleosides.

In general, it is said that part of dietary nucleotide and/or nucleic ac broken down to nucleosides and absorbed from the intestinal tract. This mean nucleosides, along with nucleotides, may also play some important roles fo physiology of infants. However, there is no report about nucleosides in hu milk.

Only our newly developed method allowed us to analyze 13 kinds of nu tides and 5 kinds of nucleosides simultaneously. Moreover, for this method we only 5 ml of milk, and it is easy to prepare the sample for the analyses.

Although all the tissues in the body can synthesize nucleotides *de no* requires many steps and much energy. It should be a great load for infants. some physiological roles of dietary nucleotides have been reported (*2–10*). So quite reasonable to provide infant nucleotide and/or nucleoside from milk.

Results presented in Table 1 show that the composition of nucleotides nucleosides in human milk changes during lactation. This suggested that the r nucleotide and nucleoside in human milk may change with lactation period. it is suggested that cytidine and cytidine nucleotides may be the key componer nucleotide and nucleoside in human milk because cytidine and cytidine nuclec were predominant all through lactation. However, in many studies about di nucleotides, the nucleotide composition has not been considered strictly, an importance of the composition of nucleotides and nucleosides has not been re ed.

It is known that food and dietary habits affect some components of hu milk, such as fatty acid composition (*18*). The results presented in Table 2 su that there may be geographical differences in nucleoside and cytidine nucle content of human milk. This suggests that food and/or dietary habits affec nucleotides and nucleosides in human milk. To clarify the geographical differ and the effect of food on nucleotides and nucleosides in human milk, we need information about the differences of dietary habits in various areas in Japan. the fate of dietary nucleotide absorbed from intestine needs to be clarified.

The origin of nucleotides and nucleosides in human milk, whether transp from serum or synthesized in the mammary glands, is unclear. The re presented here suggest that the mammary glands secrete nucleotides and nu sides actively, because the composition of nucleotides and nucleosides of hu milk is different from that of bovine milk. Moreover, it seems that nucleotide nucleosides are more important in winter, namely infants need more nucleotic winter than in summer, although the reason for this is unclear.

Bovine milk and bovine milk-based infant formula contained much less n otides and nucleosides than human milk. However, it contained a large amou orotic acid which is a precursor of cytidine monophosphate. These results su that nucleotides and nucleosides in human milk may play some important ro the development of infants. Of course, the physiological role of nucleotides a nucleosides in human milk should be examined further.

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