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原　著

Effects of supplemental water-soluble β-carotene on IgA induction in the intestine and mammary glands of lactating mice

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Summary

The present study was conducted to clarify the effects of supplemental water-soluble β-carotene (WS-BC) in maternal mice during pregnancy and lactation on IgA induction in the intestine and mammary glands of lactating mice and IgA transfer from maternal milk to neonatal mice. From 6.5 days postcoitus to 14 days postpartum, maternal mice were fed rodent feed or 50 mg/kg WS-BC-supplemented rodent feed. Supplemental WS-BC increased the numbers of IgA antibody-secreting cells (ASC) in the ileum and mammary glands of lactating mice, but the numbers of IgA ASC in the jejunum and IgA concentrations in the serum, jejunum, ileum, mammary glands and feces were not affected by the treatment. Supplemental WS-BC increased IgA concentrations in the stomach contents of neonatal mice at 2 days of age, but supplemental WS-BC had no effects on IgA concentrations in serum, stomach contents, intestines and feces of neonatal mice at 14 days of age. Supplemental WS-BC increased the mRNA expressions of CCL25 in the ileum of lactating mice and tended to increase the mRNA expressions of IgA C-region in the ileum. These results imply that supplementation of WS-BC in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the ileum and mammary glands of lactating mice.

Keywords: water-soluble β-carotene, IgA induction, mammary gland, lactating mice

Introduction

Passive immunity is critical to the survival and health of neonates, and colostrum or milk is a source of nutrients and immune components for neonates. Immunoglobulin (Ig) antibodies are main immune components in colostrum[1)], and IgA is the most abundant Ig isotype in mucosal secretions and provides protection against microbial antigens at mucosal surfaces[2, 3)]. Passive immune protection of the newborn gastrointestinal tract is dependent on an active process of IgA antibody-secreting cells (ASC) accumulation in lactating mammary glands of the mother, because IgA antibodies produced from IgA ASC in the mammary glands are secreted into milk[4)].

The β-carotene or vitamin A metabolite, all-*trans* retinoic acid (RA), plays important roles in the differentiation of immune-competent cells and the proliferation and expansion of lymphocytes[5)]. Supplemental vitamin A and fat-soluble β-carotene (FS-BC) enhance the immune system in neonates[5, 6)], but β-carotene deficient calves were found to have a higher incidence of diarrhea in the first week of life[7)]. Additionally, supplemental FS-BC may be useful for enhancing colostral IgG_1 or IgA concentrations in β-carotene-borderline or deficient Japanese Black cows[8, 9)].

β-carotene is a typical fat-soluble carotenoid, and supplemental FS-BC at 30 and 50 mg/kg in the diet in maternal mice during pregnancy and lactation increased the numbers of IgA ASC in the mammary glands and ileum of lactating mice and enhanced IgA transfer from maternal milk to neonatal mice[10, 11)]. Water-soluble β-carotene (WS-BC) is a carotenoid form that has been developed as a food colorant[12)], but it is not clear whether supplemental WS-BC enhances IgA induction in the small intestine and mammary glands of lactating mice.

The present study was conducted to clarify the effects of supplemental WS-BC at 50 mg/kg in the diet on IgA induction in the small intestine and mammary glands of lactating mice and IgA transfer from maternal milk to

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neonatal mice.

Material and Methods

Animals and diets

Pregnant ICR mice (n = 15) at 6.5 days postcoitus were purchased from Clea Japan (Tokyo, Japan). They were housed in individual polycarbonate cages and maintained in an air-conditioned room (24±2℃) under controlled lighting conditions (light-dark cycle, 14:10 h). They received humane care as treated in accordance with 'Regulation on Animal Experimentation at Kyoto University' (Animal Research Committee, Kyoto University, revised 2007).

Pregnant mice were randomly allocated to the control or WS-BC group at 6.5 days postcoitus (dpp). Mice in the control group (n = 7) were fed rodent feed (Oriental Yeast, Tokyo, Japan) from 6.5 days postcoitus to 14 days postpartum (dpp), and those in the WS-BC group were fed 50 mg/kg WS-BC supplemented rodent feed from 6.5 days postcoitus to 14 dpp. The rodent feed contained 55.3% NFE, 23.6% CP, 5.1% crude fat, 5.8% crude ash, 1283IU/100 g vitamin A and 9.1 mg/100 g vitamin E. Products of WS-BC (Product name: ROVIMIX BETA-CAROTENE 10%) were donated by DSM Japan K.K. (Tokyo, Japan), and WS-BC was mixed with the rodent feed at 50 mg/kg in the diets in the β-carotene group. All the neonatal mice were alive by 2 dpp, and the numbers of pups for each mother were reduced to ten neonatal mice at 2 dpp. Then, neonatal mice born to each mother and the maternal mice were dissected at 14 dpp.

All mice were allowed free access to water and feed. Body weights and feed intake of mice and body weights of neonatal mice were measured at 10.00 hours every day.

Sample collection

Blood samples from maternal mice of the control and WS-BC groups were obtained by cardiac puncture under anaesthesia with Avertin (2,2,2-tribromoethanol, Sigma-Aldrich Chemical, St Louis, MO, USA) at 14 dpp, and then mammary glands, jejunum and ileum were removed after euthanasia by cervical dislocation. The samples of mammary glands, jejunum and ileum were immediately frozen in dry ice-cooled isopentane (2-methylbutane, Wako Pure Chemicals, Osaka, Japan) for immunohistochemical analysis or frozen in liquid nitrogen and stored at −80℃ for quantitative RT-PCR.

Blood samples from neonatal mice at 14 dpp were obtained by incising their hearts and collecting with hematocrit tubes under anesthesia with Avertin, and then small intestine, stomach contents and rectum feces were rapidly removed. Samples of stomach contents were obtained at 2 dpp. According to the previous studies[10, 11], IgA concentration in stomach contents was represented as milk IgA level. The samples were pooled for all neonatal mice born to each mother at 2 or 14 dpp for the analysis of IgA immunoassay. The samples of small intestine were frozen in liquid N_2 and stored at −80℃ and the samples of stomach contents and rectum feces were stored at −20℃.

Blood samples from maternal or neonatal mice were left to stand at room temperature for 1 h or 30 min and then centrifuged at 3000 rpm for 15 min or 10000 rpm for 5 min, respectively. The samples of serum were stored at −20℃.

IgA immunoassay and immunohistochemical analysis

IgA immunoassay of serum, stomach contents, small intestine and feces and immunohistochemical analysis of mammary glands, jejunum and ileum were determined as previously described[10]. IgA concentrations were measured using the Affinity purified Goat anti-Mouse IgA Coating Antibody, Mouse Reference Serum and HRP Conjugated Goat anti-Mouse IgA Detection Antibody (Bethyl Laboratories, Montgomery, AL, USA). The assay was performed according to the manufacturer's protocol of Mouse IgA ELISA Quantitation Set (Bethyl Laboratories, Montgomery, AL, USA).

The sections obtained from the immunohistochemical analysis were examined under an epifluoresence microscope (BX50, Olympus, Tokyo, Japan), and the resulting images were analyzed by Image J software (National Institute of Health, Bethesda, MD, USA). The IgA-positive cells in the mammary glands were counted in five randomised fields from each mouse and represented as IgA ASC/field of view (field = 1160 μm × 870 μm). Those in the jejunum and ileum were counted in lamina propria of villi in five randomised villi from each mouse and represented as IgA ASC/unit area of lamina propria of villi (unit = 10000 μm^2).

Quantitative RT-PCR

The mRNA expressions of IgA C-region in the mammary gland and jejunum were examined by quantitative RT-PCR. Total RNA was extracted using RNeasy mini kit (Qiagen). cDNA was synthesised using 2 μg of total RNA with a SuperScript VILO cDNA Synthesis kit (Life technologies). Twenty-fold diluted cDNA (2 μl) was used as a template in a reaction with 0.3 μM of each primer and Power Up™ SYBR® Green Master Mix (Life technologies) in a volume of 20 μL and each sample was analyzed in duplicate. Four serial dilutions were set up to determine Ct values and reaction efficiencies for all primer pairs.

Real-time PCR was performed using StepOnePlus Real-time PCR system (Life technologies) with the cycle parameters: 50℃ for 2 min and 95℃ for 2 min followed by forty cycles of PCR reaction at 95℃ for 3 s and 60℃ for 30 s. Melting curve analysis was performed after the amplification under the following condition: 95℃ for 15 s, 60℃ for 1min, heat increment up to 95℃, and 95℃ for 15 s. The primers for IgA C-region were as follows: forward 5'-TGCAC AGTTA CCCAT CCTGA-3', reverse: 5'-GCACC AGCAC TTCTT TAGGG-3'. The primers for CCL25 were as follows: forward: 5'-CCTTC AGGTA TCTGG AGAGG AGATC-3', reverse: 5'-CAAGA TTCTT ATCGC CCTCT TCA-3'. The primers for CCL28 were as follows: forward 5'-TGGCA AAAGC CACAT TCATA-3', reverse: 5'-CATGC CAGAG TCGAA CAGAA-3'. The amount of IgA C-region, CCL25 and CCL28 mRNA was normalized to GAPDH by the $\Delta\Delta C_T$ method. The primers for GAPDH were as follows: forward: 5'-TGTGT CCGTC GTGGA TCTGA-3', reverse: 5'-CCTGC TTCAC CACCT TCTTGA-3'. The data analysis was performed with the StepOne Software V 2.3.

Statistical analysis

In the present study, data of one mouse in the control group were removed from statistical analyses, because the numbers of IgA ASC in the mammary glands, jejunum and ileum in the mouse were 38.1, 11.8 and 12.2, which were 2 to 3 times higher than those in the other mice.

Data from bodyweight and feed intake of maternal mice and bodyweight of neonatal mice during prepartum or postpartum periods were analyzed by least squares ANOVA using the general linear models procedure of SAS[13]. The model was as follows:

$$Y_{ijk} = \mu + T_i + M_{(i)j} + D_k + TD_{ik} + e_{ijk}$$

where μ is the overall mean, T_i is the effect of treatment, $M_{(i)j}$ is the random variable of a mice nested in treatment, D_k is the effect of day, TD_{ik} is the interactions, and e_{ijk} is the residuals.

The general linear model procedure of SAS[13] was used to analyse the effects of treatment on some variables in maternal mice and neonatal mice. Significance was declared at $P < 0.05$.

Results

Bodyweight, feed intake and water intake of maternal mice during prepartum and postpartum periods and bodyweight of neonatal mice were not affected by the treatment (Figure 1). In maternal mice, the numbers of IgA ASC in the ileum and mammary glands of the WS-BC group were significantly higher ($P < 0.05$) than those of the control group, but the numbers of IgA ASC in the jejunum were not affected by the treatment (Table 1).

In maternal mice, IgA concentrations in the serum, jejunum, ileum, mammary glands and feces were not affected by the treatment (Table 2). In neonatal nice, IgA concentrations in the stomach contents of the WS-BC group at 2 dpp were significantly higher ($P < 0.05$) than those of the control group, but supplemental β-carotene had no effects on IgA concentrations in serum, stomach contents, intestines and feces of neonatal mice at 14 dpp.

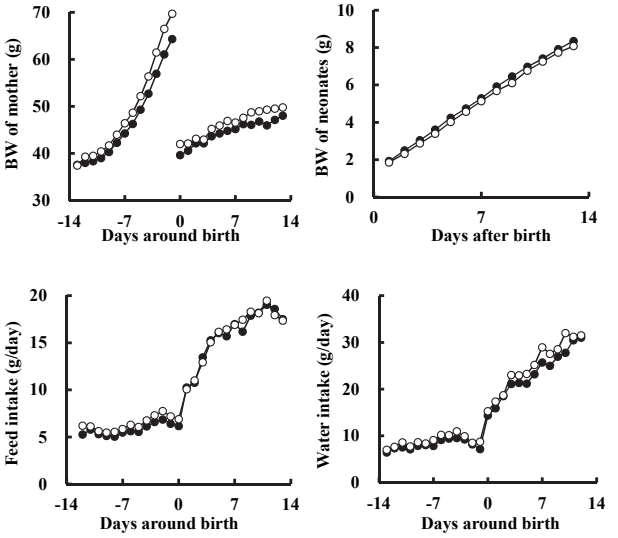

Fig. 1 Bodyweight (BW), feed intake and water intake of maternal mice and BW of neonatal mice in the control (○) and β-carotene (●) groups.

Table 1 The numbers of IgA antibody-secreting cells (ASC) in the mammary glands, jejunum and ileum of maternal mice in the control (n = 6) and water-soluble β-carotene (WS-BC, n = 8) groups at 14 days postpartum (Mean values with their standard errors).

	Control	WS-BC	*P*
Mammary gland	13.3 ± 1.0	16.3 ± 0.9	0.042
Jejunum	4.7 ± 0.6	5.0 ± 0.5	0.748
Ileum	5.2 ± 0.4	6.8 ± 0.4	0.037

The numbers of IgA ASC in the mammary gland were counted in five randomised fields from each mouse, and values in the jejunum and ileum were counted in lamina propria of villi in five randomised villi from each mouse.

Table 2 IgA concentration (μg/g) in serum, mammary glands, jejunum, ileum and feces of maternal mice at 14 days postpartum and serum, stomach contents, small intestine and feces of neonatal mice in the control (n = 6) and water-soluble β-carotene (WS-BC, n = 8) groups at 2 or14 days postpartum (Mean values with their standard errors).

	Days	Control	WS-BC	*P*
Mother				
Serum	14	348 ± 71	352 ± 62	0.963
Mammary gland	14	183 ± 48	215 ± 41	0.621
Jejunum	14	841 ± 155	817 ± 135	0.909
Ileum	14	902 ± 339	1262 ± 294	0.438
Feces	14	827 ± 359	851 ± 311	0.960
Neonate				
Serum	14	0.8 ± 0.2	0.9 ± 0.2	0.703
Stomach contents	2	12 ± 7[b]	35 ± 7[a]	0.050
	14	59 ± 15	87 ± 13	0.187
Intestine	14	91 ± 25	111 ± 22	0.547
Feces	14	782 ± 655	2385 ± 567	0.499

[a,b] Means within a row with different superscript letters differ (P < 0.05).

Table 3 The ratios of IgA C-region, CCL25 or CCL28 mRNA to GAPDH mRNA in the mammary glands, jejunum and ileum of maternal mice in the control (n = 6) and water-soluble β-carotene (WS-BC, n = 8) groups at 14 days postpartum (Mean values with their standard errors).

	Control	WS-BC	*P*
Mammary gland			
IgA	1.01 ± 0.30	1.08 ± 0.26	0.873
CCL28	1.51 ± 0.25	1.53 ± 0.21	0.949
Jejunum			
IgA	1.63 ± 0.84	1.35 ± 0.73	0.801
CCL25	1.15 ± 0.15	1.02 ± 0.13	0.525
Ileum			
IgA	0.38 ± 0.34	1.34 ± 0.29	0.054
CCL25	0.99 ± 0.07[b]	1.44 ± 0.06[a]	0.001

[a,b] Means within a row with different superscript letters differ (P < 0.001).

In maternal mice, the mRNA expressions of CCL25 in the ileum of the WS-BC group were significantly higher (P < 0.001) than those of the control group and the mRNA expressions of IgA C-region in the ileum of the WS-BC group tended to be higher (P < 0.10) than those of the control group (Table 3). The mRNA expressions of IgA C-region in the jejunum and mammary glands, CCL25 in the jejunum and CCL28 in the mammary glands of maternal mice were not affected by the treatment.

Discussion

IgA antibodies in milk are specific for antigens of the intestinal microflora and acts to limit penetration of commensal intestinal bacteria through the neonatal intestinal epithelium[14]. Peyer's patches in the gut-associated lymphoid tissue are the main site for the generation of IgA^+ B cells, and plasmablasts differentiated by IgA^+ B cells home preferentially to the gut lamina propria or mammary glands[2, 3]. RA is necessary for the imprinting of gut-homing specificity on T cells and the induction of gut-homing receptors on B cells and IgA ASC[3]. In the previous studies[10, 11], FS-BC supplementation at 30 and 50 mg/kg in the diet in maternal mice during pregnancy and lactation increased the numbers of IgA ASC in the mammary glands and ileum of lactating mice. In the present study, supplemental WS-BC at 50 mg/kg in the diet in maternal mice during pregnancy and lactation increased the numbers of IgA ASC in the mammary glands and ileum of lactating mice. These results indicates that supplemental WS-BC as well as supplemental FS-BC is effective for enhancing the numbers of IgA ASC in the mammary glands and ileum of lactating mice.

IgA plasma cells in the mammary glands in mice are derived from lymphoid cells in the gut-associated lymphoid tissue by homing to the mammary glands[15]. Very few IgA ASC were detected in the mammary glands of maternal mice during pregnancy and the numbers of IgA

ASC in the mammary glands increased at 14 dpp, but the numbers of IgA ASC in the jejunum and ileum were similar during pregnancy and lactation[10]. In the previous[10, 11] and present study, IgA concentrations in stomach contents of neonatal mice, which represented milk IgA level, increased drastically with age. Supplemental FS-BC at 50 mg/kg in the diet in maternal mice during pregnancy and lactation is useful for enhancing IgA transfer from maternal milk to neonates at 7 and 14 dpp[10, 11]. However, supplemental WS-BC at 50 mg/kg in the diet had no effects on IgA concentrations in stomach contents of neonatal mice at 14 dpp, although supplemental WS-BC increased IgA concentrations in stomach contents of neonatal mice at 2 dpp. Thus, compared with FS-BC, supplemental WS-BC may have little effects on IgA transfer from maternal milk to neonatal mice, which may be partly due to the weak effects of WS-BC on IgA induction in the mammary glands.

Peyer's patches in the guts are the main site for the generation of IgA^+ B cells, and plasmablasts differentiated by IgA^+ B cells are located preferentially in the gut lamina propria through the thoracic duct and blood by the expression of homing ligands and receptors[2]. Chemokines are transmembrane proteins that play important roles in innate and acquired immunity, and chemokine ligand CCL25 is selectively expressed in the small intestine and CCL28 is expressed in the mammary glands[17]. Supplemental FS-BC at 50 mg/kg in the diet increased the mRNA expression of IgA C-region in the ileum of lactating mice and the mRNA expression of IgA C-region and CCL25 in the jejunum of weanling mice [16], but supplemental FS-BC had no effects on the mRNA expression of IgA C-region in the mammary glands of lactating mice[10]. In the present study, supplemental WS-BC at 50 mg/kg in the diet increased the mRNA expression of CCL25 and IgA C-region in the ileum of lactating mice, but supplemental WS-BC had no effects on the mRNA expression of CCL28 and IgA C-region in the mammary glands. These results indicate that supplemental WS-BC may be useful for enhancing mucosal IgA induction in the ileum. However, because the mature isolated lymphoid follicles are inductive sites for the immune response and nodular lymphoid structures were observed in the distal small intestine of lactating mice[11], supplemental WS-BC may have no effects on the numbers of IgA ASC and mRNA expression of CCL25 and IgA C-region in the jejunum of lactating mice.

RA plays important roles in gut immunity and several effects of carotenoids are thought to be mediated by their metabolism to vitamin A and subsequent mediation of RA receptor (RAR) and retinoid X receptor (RXR) response pathways[5]. The increase of IgA ASC in the mammary glands and milk IgA in lactating mice by supplemental FS-BC may be mainly due to the RA-mediated immune response[10]. Also, supplemental FS-BC is effective to enhance mucosal IgA induction in the jejunum of weanling mice and the effects were activated by the mRNA expression of RAR and RXR in the jejunum[16]. The increased numbers of IgA ASC in the mammary glands in the present study may be due to the RA-mediated immune response, but further study is needed to clarify the mechanism of WS-BC for improving milk IgA in lactating mice.

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