Trace Nutrients Research 31 : 21－26 (2014)

Original Article

Effects of Supplemental β-Cryptoxanthin on IgA-secreting Cells 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 β-cryptoxanthin in maternal mice during pregnancy and lactation on IgA antibody-secreting cells (ASC) in the intestine and mammary glands of lactating mice. From 6.5 days postcoitus to 7 or 14 days postpartum (dpp), maternal mice were fed rodent feed or 50 mg/kg β-cryptoxanthin-supplemented rodent feed. Supplemental β-cryptoxanthin increased the numbers of IgA ASC and the mRNA expressions of IgA C-region, CCL25 and CCL28 in the jejunum at 14 dpp. Supplemental β-cryptoxanthin had no effects on the numbers of IgA ASC in the ileum and mammary glands, although supplemental β-cryptoxanthin increased the mRNA expression of IgA C-region in the ileum and mammary glands at 14 dpp. Supplemental β-cryptoxanthin had no effects on IgA concentrations in serum, stomach contents, intestines and feces of neonatal mice. These results imply that supplemental β-cryptoxanthin in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the jejunum during late lactation.

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. IgA is the most abundant Ig isotype in mucosal secretions and provides protection against microbial antigens at mucosal surfaces[1, 2)]. 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[3)].

Supplemental vitamin A and β-carotene enhance the immune system in neonates[4, 5)]. β-cryptoxanthin is rich in mandarin oranges in Japan, and β-cryptoxanthin as well as β-carotene is a typical fat-soluble carotenoid and has a pro-vitamin A activity[6)]. Vitamin A metabolite, all-*trans* retinoic acid (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[6)]. In the previous studies[7, 8)], β-carotene 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 and enhanced IgA transfer from maternal milk to neonatal mice. β-cryptoxanthin and β-carotene are inversely associated with the change of radial bone mineral density in post-menopausal female subjects[9)] and β-cryptoxanthin suppresses the adipogenesis of 3T3-L1 cells via RAR activation[10)]. Thus, β-cryptoxanthin supplementation has been expected to enhance the mucosal immune induction in lactating animals and IgA transfer from maternal milk to neonates.

Peyer's patches are the main site for the generation of IgA^+ B cells, and plasmablasts differentiated by IgA^+ B cells are preferentially homing on the gut lamina propria through the thoracic duct and blood by the expression of homing ligands and receptors[1, 11)]. The efficient homing and accumulation of lymphocytes is highly dependent on cellular adhesion molecules expressed by the vascular endothelium and their integrin ligands[12)]. Coumestrol administration in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the mammary glands during lactation owing to the activated mRNA expressions of IgA C-region and vascular cell adhesion molecule-1 (VCAM-1) in the mammary glands[13)].

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On the other hand, chemokine ligand CCL25 is selectively expressed in the small intestine and CCL28 is widely expressed in the intestinal and nonintestinal mucosal tissues[14]. Nishida et al[15]. reported that β-carotene supplementation is effective to enhance the mucosal IgA induction in the jejunum of weanling mice owing to the increased mRNA expression of IgA C-region and CCL25. However, the mechanism of β-cryptoxanthin for enhancing mucosal immune induction in lactating animals is still unclear.

The present study was conducted to clarify the effects of β-cryptoxanthin supplementation at 50 mg/kg in the diet on the numbers of IgA ASC in the small intestine and mammary glands of lactating mice and IgA transfer from maternal milk to neonatal mice.

Materials and Methods

Animals and diets

Pregnant ICR mice (n = 30) 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 β-cryptoxanthin group at 6.5 days postcoitus. Mice in the control group were fed rodent feed (Oriental Yeast, Tokyo, Japan) from 6.5 days postcoitus to 7 (n = 8) or 14 (n = 8) days postpartum (dpp), and those in the β-cryptoxanthin group were fed 50 mg/kg β-cryptoxanthin-supplemented rodent feed from 6.5 days postcoitus to 7 (n = 7) or 14 (n = 7) dpp. The rodent feed contained 55.3% NFE, 23.6% CP, 5.1% crude fat, 5.8% crude ash, 1283 IU/100 g vitamin A and 9.1 mg/100 g vitamin E. In the β-cryptoxanthin group, β-cryptoxanthin (Unitika Ltd., Uji, Japan) was mixed with the rodent feed at 50 mg/kg, which was similar to the dietary β-carotene level as previously described[7]. All the neonatal mice were alive by 2 dpp, and the numbers of pups for each mother were reduced to five female and five male neonatal mice at 2 dpp. Then, five female and five male neonatal mice born to each mother and the maternal mice were dissected at 7 or 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 12.00 hours every day.

Sample collection

Blood samples from maternal mice of the control and β-cryptoxanthin groups were obtained by cardiac puncture under anaesthesia with Avertin (2,2,2-tribromoethanol, Sigma-Aldrich Chemical, St Louis, MO, USA) at 7 and 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 semi-quantitative RT-PCR.

Blood samples from neonatal mice at 7 or 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. According to the previous studies[7, 8], IgA concentration in stomach contents was represented as milk IgA level. The samples were pooled for all neonatal mice born to each mother at 7 or 14 dpp. 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[7]. IgA concentrations were measured using the Mouse IgA ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, AL, USA) and ELISA Starter Accessory Package (Bethyl Laboratories) according to the manufacturer's instruction.

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 to eight 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 to eight randomised villi from each mouse and represented as IgA ASC/unit area of lamina propria of villi (unit = 10000 μm^2).

Semi-quantitative RT-PCR

The mRNA expression of IgA C-region, CCL25 and CCL28 in the jejunum and ileum and the mRNA expression of IgA C-region, CCL28 and VCAM-1 in the mammary glands were examined by semi-quantitative RT-PCR. Total RNA was extracted using RNeasy mini kit (Qiagen, Maryland, CA, USA). Complementary DNA was synthesised with oligo (dT) primer using SuperScript Ⅲ First-Strand Synthesis System for RT-PCR (Invitrogen) from 4 μg of RNA of each sample. The PCR was performed using Pt PCR Super Mix kit (Invitrogen). The PCR products were electrophoresed in 2% agarose gel and stained with 1 μg/ml ethidium bromide solution. After electrophoresis, the gels were recorded with a digital recorder and then mRNA expression levels were semi-quantified using Image J software. The relative abundance of specific mRNA was normalised by the abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

The primer pairs and PCR conditions used for IgA C-region, VCAM-1 and GAPDH are same as those in the previous study[7, 13]. The primer pairs for CCL25 were as follows: forward: 5'-CCTTCAGGTATCTGGAGAGGAGATC-3', reverse: 5'-CAAGATTCTTATCGCCCTCTTCA-3'. The PCR procedure was as follows: after 95℃ for 5 min to denature DNA, PCR was performed for thirty cycles in the jejunum and ileum or thirty-six cycles in the mammary glands at 95℃ for 1 min, 55℃ for 1 min, 72℃ for 1 min, then at 72℃ for 7 min. The primer pairs for CCL28 were as follows: forward 5'-TGGCAAAAGCCACATTCATA-3', reverse: 5'- CATGCCAGAGTCGAACAGAA -3'. The PCR procedure was as follows: after 95℃ for 5 min to denature DNA, PCR performed for forty-five cycles in the jejunum and ileum or thirty-seven cycles in the mammary glands at 95℃ for 1 min, 53℃ for 1 min, 72℃ for 1 min, and then at 72℃ for 7 min.

Statistical analysis

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[16]. 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[16] was used to analyse the effects of treatment or time on some variables in maternal mice and neonatal mice. Significance was declared at $P < 0.05$.

Results

IgA concentrations in serum and tissues

Bodyweight gains and feed intake of maternal mice during prepartum and 7 or 14 days postpartum periods were not affected by the treatment (Fig. 1). Bodyweight gains of neonatal mice were not affected by the treatment (data not shown).

In neonatal nice, IgA concentrations in serum, stomach contents, small intestines and feces at 7 and 14 dpp were not affected by the treatment (Fig. 2). Compared with IgA concentrations of neonatal mice at 7 dpp, IgA concentration in serum ($P < 0.001$), stomach contents ($P < 0.001$) and feces ($P < 0.05$) increased at 14 dpp. In maternal mice, serum IgA concentrations at 7 and 14 dpp were not affected by the treatment.

IgA antibody-secreting cells in tissues

In maternal mice, the numbers of IgA ASC in the jejunum of the β -cryptoxanthin group at 14 dpp were significantly higher ($P < 0.05$) than those of the control group, but the numbers of IgA ASC in the ileum and mammary glands were not affected by the treatment (Table 1). Compared with the numbers of IgA ASC of maternal mice at 7 dpp, the numbers of IgA ASC in the mammary glands ($P < 0.001$) and ileum ($P < 0.01$) increased at 14 dpp.

Expression of mRNA in tissues

In maternal mice, the mRNA expressions of IgA C-region in the jejunum ($P < 0.001$), ileum ($P < 0.05$) and the

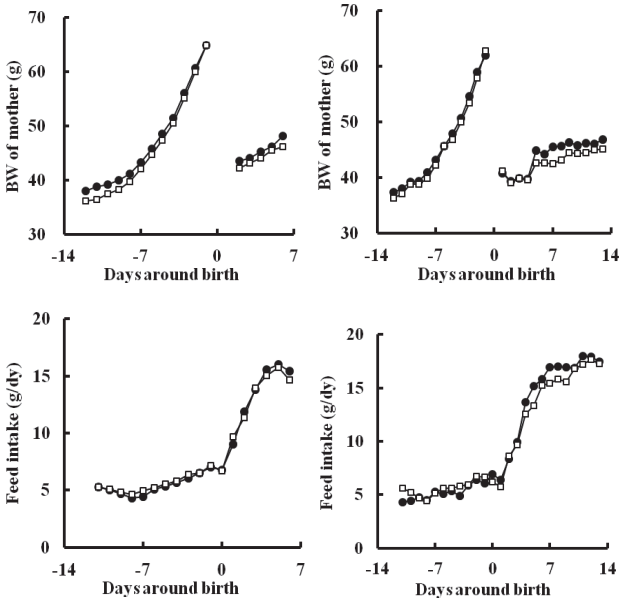

Fig. 1 Bodyweight (BW) and feed intake of maternal mice around parturition of the control (□) and β -cryptoxanthin (●) groups during prepartum and 7 or 14 days postpartum.

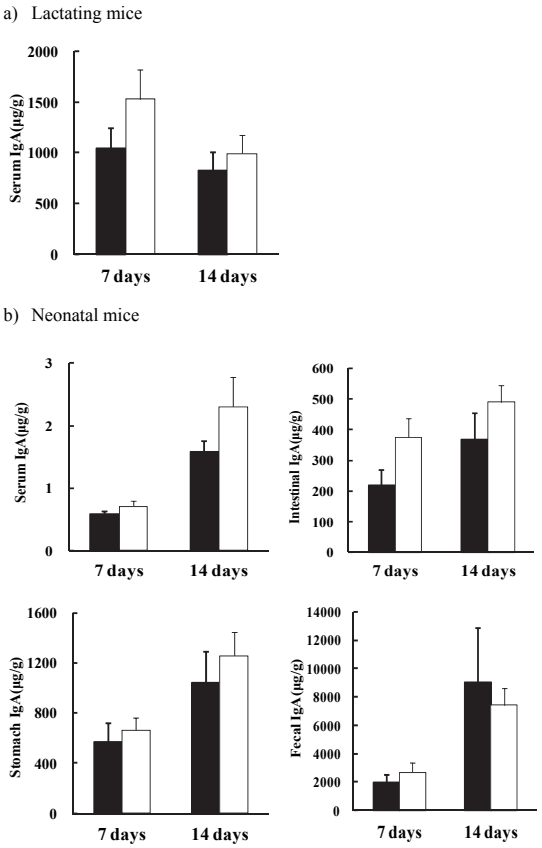

Fig. 2 IgA concentration in serum of lactating mice and serum, stomach contents, small intestine and feces of neonatal mice of the control (□) and β-cryptoxanthin (■) groups after 7 and 14 days postpartum (Mean ±SE).

Table 1 Nnumbers of IgA antibody-secreting cells (ASC) in the mammary gland, jejunum and ileum of the control and β-cryptoxanthin groups after 7 and 14 days postpartum (Mean ± SE).

	Days	Control	β-cryptoxanthin	*P*
Mammary gland	7	3.9 ± 1.0	4.1 ± 0.9	NS
	14	6.8 ± 0.4	8.9 ± 0.9	NS
Jejunum	7	10.9 ± 0.3	12.5 ± 1.0	NS
	14	10.7 ± 0.7	13.1 ± 0.6	*
Ileum	7	10.5 ± 0.9	11.6 ± 0.8	NS
	14	12.8 ± 0.6	14.0 ± 0.7	NS

$^{*}P < 0.05$

Numbers of IgA ASC in mammary gland were counted in five to eight randomised fields from each mouse, and those in jejunum and ileum were counted in lamina propria of villi in five to eight randomised villi from each mouse.

mammary glands ($P < 0.001$) of the β-cryptoxanthin group at 14 dpp were significantly higher than those of the control group (Table 2). The mRNA expressions of CCL25 ($P < 0.05$) and CCL28 ($P < 0.01$) in the jejunum of the β-cryptoxanthin group were significantly higher than those of the control group at 14 dpp, but the mRNA expressions of CCL25 and CCL28 in the ileum and VCAM-1 in the mammary glands were not affected by the treatment.

Table 2 The ratios of IgA C-region, CCL25, CCL28 and VCAM-1 mRNA to GAPDH mRNA in the mammary gland, jejunum and ileum of the control and β-cryptoxanthin groups after 14 days postpartum (Mean ± SE).

	Control	β-cryptoxanthin	*P*
Mammary gland			
IgA	0.55 ± 0.08	1.11 ± 0.07	***
CCL28	0.99 ± 0.13	1.18 ± 0.20	NS
VCAM-1	0.93 ± 0.15	0.93 ± 0.12	NS
Jejunum			
IgA	0.78 ± 0.09	1.43 ± 0.13	**
CCL25	0.50 ± 0.03	0.88 ± 0.15	*
CCL28	0.73 ± 0.04	1.42 ± 0.21	**
Ileum			
IgA	0.53 ± 0.04	0.67 ± 0.05	*
CCL25	1.29 ± 0.08	1.13 ± 0.07	NS
CCL28	1.01 ± 0.10	1.09 ± 0.11	NS

$^{***}P < 0.001$ $^{**}P < 0.01$, $^{*}P < 0.05$, NS, not significant.
CCL25, chemokine ligand 25; CCL28, chemokine ligand 28; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VCAM-1, vascular cell adhesion molecule-1.

Discussion

The mammary glands of mice develop new vasculature during pregnancy, and it is colonized primarily by IgA-containing B cells during lactation[17]. 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[7]. In the present study, the numbers of IgA ASC in the mammary glands of maternal mice at 14 dpp were about 2 times higher than those at 7 dpp, but the numbers of IgA ASC in the jejunum were similar at 7 and 14 dpp. Relative IgA mRNA levels increased dramatically beginning at birth and continued to increase through the lactation period[12], and IgA concentrations in stomach contents of neonatal mice, which represented milk IgA level, increased drastically with age in the previous[7, 8] and present study. These results indicated that the numbers of IgA ASC in the mammary glands of mice and IgA transfer from maternal mice to neonatal mice increased drastically with age during lactation, but the numbers of IgA ASC in the jejunum and ileum may be almost constant during pregnancy and lactation.

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[18]. In the previous study[7], supplemental β-carotene at 50 mg/kg in the diet in maternal mice during pregnancy and lactation increased the numbers of IgA ASC and mRNA expression of IgA C-region in the ileum during lactation, but in the mammary glands, β-carotene supplementation only increased the numbers of IgA ASC. On the other hand,

supplemental β-cryptoxanthin at 50 mg/kg in the diet in maternal mice during pregnancy and lactation increased the numbers of IgA ASC and the mRNA expression of IgA C-region in the jejunum at 14 dpp in the present study. However, β-cryptoxanthin supplementation had no effect on the numbers of IgA ASC in the ileum and mammary glands, although supplemental β-cryptoxanthin increased the mRNA expression of IgA C-region in the ileum and mammary glands at 14 dpp. These results imply that supplementation of β-cryptoxanthin in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the jejunum during late lactation.

CCL25 plays essential roles in intestinal homing of IgA ASC primarily by mediating their extravasation into intestinal lamina propria, and.CCL28 is expressed in the mucosal tissues of intestines and mammary glands[14]. Supplemental β-carotene increased the mRNA expressions of CCL25 and IgA ASC in the jejunum of weanling mice after 14 and 21 days of treatment[15]. Because supplemental β-cryptoxanthin increased the mRNA expressions of CCL25 and CCL28 in the jejunum of maternal mice at 14 dpp in the present study, the increased IgA ASC in the jejunum of lactating mice caused by β-cryptoxanthin supplementation may be due to the increased mRNA expressions of CCL25 and CCL28 in the jejunum.

IgA antibodies in milk are specific for antigens of the intestinal microflora and act to limit penetration of commensal intestinal bacteria through the neonatal intestinal epithelium[18,19]. Supplemental β-carotene at 30 and 50 mg/kg in the diet in maternal mice during pregnancy and lactation is useful for enhancing IgA transfer from maternal milk to neonates during lactation[7, 8]. However, supplemental β-cryptoxanthin had no effect on IgA concentrations in stomach contents of neonatal mice as well as the numbers of IgA ASC in the mammary glands in the present study. Thus, compared with β-carotene, supplemental β-cryptoxanthin may have little effects on IgA transfer from maternal milk to neonatal mice. However, the present study demonstrates that β-cryptoxanthin supplementation in maternal mice during pregnancy and lactation is effective to enhance mucosal IgA induction in the jejunum, because supplemental β-cryptoxanthin increased the numbers of IgA ASC and mRNA expressions of IgA C-region, CCL25 and CCL28 in the jejunum during late lactation.

Acknowledgements

This work was supported in part by Ito Kinenzaidan. Experimental β- cryptoxanthin was kindly provided by Unitika Ltd. (Uji, Japan).

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