Supplemental β-carotene increases IgA-secreting cells in mammary gland and IgA transfer from milk to neonatal mice

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Abstract
Mortality of neonates continues to be a major problem in humans and animals. IgA provides protection against microbial antigens at mucosal surfaces. Although β-carotene supplementation has been expected to enhance retinoic acid-mediated immune response in neonates, the exact mechanism by which β-carotene enhances IgA production is still unclear. We investigated the effect of supplemental β-carotene for maternal mice during pregnancy and lactation on IgA antibody-secreting cells (ASC) in mammary gland and guts and on IgA transfer from milk to neonatal mice. Pregnant mice were fed untreated or 50 mg/kg β-carotene-supplemented diets from 6.5 d postcoitus (dpc) to 14 d postpartum (dpp). Supplemental β-carotene increased the numbers of IgA ASC in mammary gland (P<0.05) and ileum (P<0.001), and also mRNA expression of IgA C-region in ileum (P<0.05) of maternal mice at 14 dpp, but few IgA ASC were detected in mammary gland at 17.5 dpc. IgA concentration in stomach contents, which represents milk IgA level, was significantly higher (P<0.01) in neonatal mice born to β-carotene-supplemented mothers at 7 and 14 dpp, and IgA concentration in serum, stomach contents and faeces increased (P<0.001) drastically with age. These results suggest that β-carotene supplementation for maternal mice during pregnancy and lactation is useful for enhancing IgA transfer from maternal milk to neonates owing to the increase in IgA ASC in mammary gland and ileum during lactation.

Key words: Supplemental β-carotene; Mammary gland; IgA transfer; Neonatal mice

Mortality and morbidity of neonates continue to be major problems in humans and animals, and their most common disease is diarrhoea. Supplemental vitamin A and β-carotene enhance the immune system in neonates, and β-carotene has pro-vitamin A activity(1–5). Vitamin A deficiency is associated with an increased risk of death from common childhood infections, and supplementation of vitamin A decreases diarrhoea and mortality in malnourished children(6,7). Additionally, β-carotene-deficient calves were found to have a higher incidence of diarrhoea and mortality in the first week of life(8,9). Foods containing pro-vitamin A carotenoids are the primary source of vitamin A, and β-carotene is abundant in some vegetables and fruits(10,11). Because animals are not able to synthesise retinoids de novo(11), supplementation of β-carotene-enriched foods may be effective to enhance the immune system in humans and animals.

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(6,12–14). IgA antibodies produced from IgA antibody-secreting cells (ASC) in mammary glands are secreted mainly as dimers after incorporation of the J chain and association with a transmembrane epithelial glycoprotein known as polymeric-Ig receptor(12,15). 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(16,17). Therefore, passive immune protection of the newborn gastrointestinal tract is dependent on an active process of IgA ASC accumulation in the lactating mammary gland of the mother(18).

The gut-associated lymphoid tissue is the largest immunological tissue in the body. Peyer’s patches 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 through the thoracic duct and blood(6,12–14). Recent studies(19,20) showed that vitamin A metabolite, all-trans retinoic acid (RA), plays important roles in gut immunity and that 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. Several effects of carotenoids are thought to be mediated by their

Abbreviations: ASC, antibody-secreting cells; dpc, days postcoitus; dpp, days postpartum; RA, retinoic acid.

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metabolism to vitamin A and subsequent mediation of RA receptor and retinoid X receptor response pathways.(21) Mice and rats efficiently convert β-carotene to vitamin A but absorb carotenoids intact only when they are provided in the diet at supraphysiological levels(10). However, the exact mechanism of β-carotene for enhancing IgA transfer from mothers to neonates is still unclear, although β-carotene supplementation has been expected to enhance RA-mediated immune response in neonatal mice.

We investigated the effect of supplemental β-carotene for maternal mice during pregnancy and lactation on the number of IgA ASC and mRNA expression of IgA C-region in mammary gland and guts and on IgA transfer from maternal milk to neonatal mice. The present study demonstrated that β-carotene supplementation for maternal mice during pregnancy and lactation is effective to increase the number of IgA ASC in mammary gland and ileum and of milk IgA during lactation, and that the effects may be mainly due to the RA-mediated immune response. Additionally, most IgA in neonatal mice may be derived from milk IgA and β-carotene supplementation enhances IgA transfer from maternal milk to neonatal mice.

Materials and methods

Animals and diets

Pregnant ICR mice (n 28) were purchased from Clea Japan (Tokyo, Japan). They were housed in polycarbonate cages and maintained in an air-conditioned room (24 ± 2°C) under controlled lighting conditions (light–dark cycle, 14:10 h). All mice were allowed free access to water and treated in accordance with ‘Regulation on Animal Experimentation at Kyoto University’ (Animal Research Committee, Kyoto University, revised 2007).

Maternal mice were fed untreated (control group) or 50 mg/kg β-carotene-supplemented (β-carotene group) diets from 6·5 postcoitus (dpc) to 14 d postpartum (dpp). The diets in the control group contained a vitamin mix, but β-carotene was not detected in the vitamin mix. Seven maternal mice from each group were dissected at 17·5 dpc (maternal mice during pregnancy) and 14 dpp (maternal mice during lactation). At birth, the average litter sizes of mice born from control and β-carotene groups were 13·9 and 15·0, respectively, and all neonatal mice were used in the experiment. All the neonatal mice were alive by 7 dpp, and subsets of neonatal mice in each group, except five female and five male neonatal mice born to each mother, were dissected at 7 dpp. Then, five female and five male neonatal mice born to each mother were dissected at 14 dpp.

Sample collection

Body weights of maternal mice before birth and food intake of maternal mice throughout the experiment were measured at 10.00 hours every day. Body weights of neonatal mice were measured at 10.00 hours every day. Blood samples from maternal mice at 17·5 dpc and 14 dpp were obtained by cardiac puncture under anaesthesia with Avertin (2,2,2-tribromoethanol; Sigma-Aldrich Chemical, St Louis, MO, USA), and then mammary gland, jejenum and ileum were removed, immediately frozen in dry ice-cooled isopentane (2-methylbutane; Wako Pure Chemicals, Osaka, Japan) for immunohistochemical analysis or frozen in liquid N2 and stored at −80°C for semi-quantitative RT-PCR. Blood samples from neonatal mice at 7 and 14 dpp were obtained by incising their hearts and collecting with haematocrit tubes under anaesthesia with Avertin, and then small intestine, stomach contents and rectum faeces were rapidly removed. At 7 dpp, samples of blood, small intestine, stomach contents and rectum faeces of neonatal mice born to each mother were pooled, and the samples were separately pooled for female or male neonatal mice born to each mother at 14 dpp. The samples of small intestine were frozen in dry ice-cooled isopentane for immunohistochemical analysis or frozen in liquid N2 and stored at −80°C for IgA analysis and semi-quantitative RT-PCR. The slices of stomach contents and rectum faeces were stored at −20°C until IgA analysis.

IgA immunoassay

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 10 000 rpm for 5 min, respectively. Serum was fractionated for IgA analysis. Stomach contents and rectum faeces were thawed, strongly vortexed in cold PBS containing protease inhibitor (Complete Mini; Roche, Basel, Switzerland) and centrifuged at 15 000 rpm for 20 min at 4°C. Small intestine was homogenised using Sample Grinding Kit (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer’s instructions and centrifuged at 15 000 rpm for 10 min at 4°C. Each supernatant was fractionated for IgA analysis.

IgA concentration was measured using the Mouse IgA ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX, USA) and ELISA Starter Accessory Package (Bethyl Laboratories) according to the manufacturer’s instructions. Plates obtained from the procedures were read at 450 and 620 nm with a Microplate Reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA).

Immunohistochemical analysis

Fresh frozen sections (6 μm thick) mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Aldrich Chemical, Milwaukee, WI, USA) were fixed in 10% neutral-buffered formalin (Wako Pure Chemicals) for 10 min at room temperature. After washing in PBS (pH 7·4), the sections were incubated with 1% bovine serum albumin and 5% normal donkey serum in PBS containing 0·02%
Tween-20 (blocking solution) for 20 min, successively with rabbit anti-mouse IgA (1:400 in the blocking solution; Open Biosystems, Huntsville, AL, USA) for 2 h at room temperature. After washing in PBS containing 0·02% Tween-20, the sections were incubated with Alexa fluor 555 donkey anti-rabbit IgG (1:500 in the blocking solution; Invitrogen, Carlsbad, CA, USA) for 90 min at room temperature. The sections were washed in PBS containing 0·02% Tween-20, mounted with glycerol (Wako Pure Chemicals) and then examined under a confocal laser scanning microscope (FV300; Olympus, Tokyo, Japan). The resulting images were analysed by ImageJ software (National Institute of Health, Bethesda, MD, USA).

The IgA-positive cells in mammary gland were counted in eight randomised fields from each mouse and represented as IgA ASC/field of view. Those in 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 = 10 000 μm²).

**Semi-quantitative RT-PCR**

The mRNA expression of IgA C-region in the tissue was examined by semi-quantitative RT-PCR. Total RNA was extracted using an RNeasy mini kit (Qiagen, Maryland, CA, USA). Complementary DNA was synthesised with oligo (dT) primer using an SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen) from 4 μg RNA of each sample. The PCR was performed using Pt PCR Super-Mix kit (Invitrogen). The primer pairs for IgA C-region were as follows: forward: 5’-TGACAGTTACCACAC-CTGA-3’, reverse: 5’-GCACCAAGACTCTTTAGGG-3’. The PCR procedure was as follows: after 95°C for 7 min to denature DNA, PCR was performed for thirty-five cycles at 95°C for 1 min, 53°C for 1 min, 72°C for 1 min, then for thirty cycles for most tissues of maternal mice or thirty-five cycles for mammary gland of pregnant mice and intestine of neonatal mice at 72°C for 5 min. 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 ImageJ software. The relative abundance of specific mRNA was normalised by the abundance of glyceraldehyde-3-phosphate dehydrogenase mRNA. The primer pairs for glyceraldehyde-3-phosphate dehydrogenase were as follows: forward: 5’-GGGTGGAGCCAAACGGG-TC-3’, reverse: 5’-GGAATTCGTGTTGAAGTGC-3’. The PCR procedure was as follows: after 95°C for 7 min to denature DNA, PCR was performed for twenty-five cycles at 95°C for 1 min, 59°C for 1 min and 72°C for 1 min for tissues of maternal mice or thirty cycles for intestine of neonatal mice. The final extension step was performed at 72°C for 5 min.

**Statistics**

Data are expressed as mean values with their standard errors. Data from body weight and food intake were analysed by least squares ANOVA using the general linear model procedure of Statistical Analysis Systems (SAS Institute, Cary, NC, USA). The model was as follows:

\[ Y_{ijk} = \mu + T_i + M_{ij} + D_k + TD_{ik} + e_{ijk}, \]

where \( \mu \) is the overall mean, \( T_i \) is the effect of treatment, \( M_{ij} \) is the random variable of mice nested in treatment, \( D_k \) is the effect of sampling day, \( TD_{ik} \) is the interactions and \( e_{ijk} \) is the residuals. The general linear model procedure of Statistical Analysis Systems was used to analyse the effects of treatment on some variables in maternal mice or neonatal mice at 7 dpp, the effects of treatment, sex and their interaction on some variables in neonatal mice at 14 dpp and the effect of age on some variables in neonatal mice. Significance was declared at \( P<0.05 \).

![Fig. 1. Least squares means of (a) body weights (BW) and food intake of maternal mice in control (●) and β-carotene groups (○), and (b) BW of their neonatal mice in control (●) and β-carotene groups (○).](https://doi.org/10.1017/S0007114510003089)
Results

Body weight and food intake

Body weight gains and food intake of maternal mice dissected at 14 dpp as well as those of maternal mice dissected at 17.5 dpc (data not shown) were similar between the groups (Fig. 1). In maternal mice, body weights increased \( (P < 0.001) \) during pregnancy and food intake increased \( (P < 0.001) \) rapidly after birth. Body weight gains of neonatal mice were similar between the groups, but body weights at 7 and 14 dpp were significantly lower \( (P < 0.01) \) in neonatal mice born to \( b \)-carotene-supplemented mothers.

IgA concentration in serum and tissues

Serum IgA concentrations of maternal mice at 17.5 dpc and 14 dpp were not affected by treatment (Table 1). Compared with IgA concentration in serum of neonatal mice at 7 dpp, serum IgA concentration of maternal mice was about 1000 times higher. IgA concentrations in stomach contents of neonatal mice born to \( b \)-carotene-supplemented mothers were significantly higher \( (P < 0.01) \) than those of control mice at 7 and 14 dpp. Although no significant differences were obtained in serum, small intestine and faeces of neonatal mice, their IgA concentrations were slightly higher in mice born to \( b \)-carotene-supplemented mothers at day 14 (Table 1). Compared with IgA concentrations of neonatal mice at 7 dpp, IgA concentrations in serum \( (P < 0.001) \), stomach contents \( (P < 0.001) \), small intestine \( (P < 0.01) \) and faeces \( (P < 0.001) \) increased drastically at 14 dpp, but their IgA concentrations were not affected by sex.

IgA antibody-secreting cells in tissues

The numbers of IgA ASC in jejunum and ileum of maternal mice at 17.5 dpc were not affected by treatment (Table 2). The numbers of IgA ASC in mammary gland \( (P < 0.05) \) and ileum \( (P < 0.001) \) of maternal mice fed \( b \)-carotene were significantly higher than those of control mice at 14 dpp, but there was no significant difference in jejunum. Few \( (> 2) \) IgA ASC were detected in mammary gland of maternal mice at 17.5 dpc and jejunum and ileum of neonatal mice at 14 dpp in each group (data not shown).

Expression of mRNA in tissues

The mRNA expression of IgA C-region in ileum was significantly higher \( (P < 0.05) \) in maternal mice fed \( b \)-carotene at 14 dpp, but there were no differences in the mRNA expression of IgA C-region in mammary gland and jejunum (Table 2). At 17.5 dpc, there were no significant differences in mRNA expression of IgA C-region in mammary gland, jejunum and ileum of maternal mice between the groups. The mRNA expression of IgA C-region in jejunum significantly decreased \( (P < 0.01) \) in neonatal mice born to \( b \)-carotene-supplemented mothers at 14 dpp, but this was not affected by sex.

Discussion

The importance of adequate consumption of high-quality colostrum or milk for acquisition of optimal nutrition and passive immunity is widely recognised in neonates\(^1,2\). In the present study, the nutritional status in maternal and neonatal mice was thought to be normal because the patterns of food intake and growth rate of maternal or neonatal mice agree with those in previous reports\(^16,24\) and low body weights in neonatal mice born to \( b \)-carotene-supplemented mothers may be due to more competition for available milk. Supplemental \( b \)-carotene for maternal mice during pregnancy and lactation increased the number of maternal IgA ASC in mammary gland during lactation, although very few IgA ASC were detected during

### Table 1. IgA concentration (\( \mu \text{g/g} \)) in serum of maternal mice at 17.5 dpc and 14 dpp, and IgA concentration (\( \mu \text{g/g} \)) in serum, stomach contents, intestine and faeces of neonatal mice at 7 and 14 dpp in control and \( b \)-carotene groups

(Mean values with their standard errors)

<table>
<thead>
<tr>
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<td>Mean</td>
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<td></td>
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<td>79</td>
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<td>26.6</td>
<td>3.5</td>
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<tr>
<td></td>
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<td>4.5</td>
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<td>9.7</td>
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<tr>
<td></td>
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<td>84.1</td>
<td>18.8</td>
<td>129.6</td>
<td>22.4</td>
</tr>
<tr>
<td>Faeces</td>
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<td>25.8</td>
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<td>19.1</td>
</tr>
<tr>
<td></td>
<td>14 dpp</td>
<td>356</td>
<td>69</td>
<td>567</td>
<td>110</td>
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pregnancy. Additionally, higher IgA concentration in stomach contents, which represented milk IgA level\(^1\text{a}\), was observed in neonatal mice born to β-carotene-supplemented mothers.

The majority of Ig in murine milk is IgA, and milk IgA provides protection against microbial antigens in neonates\(^\text{15}\). Gut-associated lymphoid tissue dendritic cells become most important during late lactation\(^\text{27}\). The mammary gland develops new vasculature during pregnancy, and it is colonised primarily by IgA-containing B cells during lactation\(^\text{26}\). As a result, milk IgA transfers the ability to exclude luminal bacteria from mothers to neonatal mice, and this process is not critically dependent on antibody specificity\(^\text{16}\). Compared with IgA concentration at day 7 in neonatal mice, the rapid increase in IgA concentration was observed not only in stomach contents but also in serum, small intestine and faeces at day 14 in the present study. On the other hand, the decreased mRNA expression of IgA C-region in jejunum in neonatal mice born to β-carotene-supplemented mothers at day 14 may be partly due to the delay in neonatal mucosal immune induction. However, intestinal secretions of IgA in mice at weaning could hardly be found, and then the amounts of IgA rose drastically and reached a maximum concentration at 10 weeks of age\(^\text{29}\). Because very few IgA ASC were detected in jejunum and ileum of neonatal mice born to β-carotene-supplemented mothers at day 14 may be partly due to the delay in neonatal mucosal immune induction. However, intestinal secretions of IgA in mice at weaning could hardly be found, and then the amounts of IgA rose drastically and reached a maximum concentration at 10 weeks of age\(^\text{29}\). Because very few IgA ASC were detected in jejurnum and ileum of neonatal mice at day 14, most IgA in neonatal mice may be derived from milk IgA, and β-carotene supplementation enhances IgA transfer from maternal milk to neonatal mice.

IgA plasma cells in the mammary gland in mice are derived from the lymphoid cells in gut-associated lymphoid tissue by homing to the mammary gland\(^\text{17}\). Homing of ASC to the intestinal mucosa requires the

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**Table 2. Numbers of IgA antibody-secreting cells (ASC) and mRNA expression of IgA C-region in mammary gland, jejunum and ileum of maternal mice at 17.5 d postcoitus (dpc) and 14 d postpartum (dpp), and the mRNA expression of IgA C-region in jejunum and ileum of neonatal mice at 14 dpp in control and β-carotene groups**

<table>
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<th>β-Carotene Mean</th>
<th>SE</th>
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</tr>
<tr>
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<td>0.6</td>
<td>8.8</td>
<td>1.0</td>
</tr>
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<td>0.4</td>
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<tr>
<td>Ileum</td>
<td>17.5 dpc</td>
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<td>Mother</td>
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<tr>
<td>Mammary gland</td>
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<td>Jejunum</td>
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<td>Ileum</td>
<td>14 dpp</td>
<td>1.53</td>
<td>0.21</td>
<td>1.73</td>
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GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

* The numbers of IgA ASC in mammary gland were counted in eight randomised fields from each mouse, and values in jejunum and ileum were counted in lamina propria of villi in five to eight randomised villi from each mouse. The mRNA expression is represented relative to the IgA C-region mRNA expression normalised by abundance of GAPDH mRNA.
expression of integrin α4β7(6,14). RA is important to induce α4β7 and CCR9 on activated T cells, and blocking RA receptors decreases the induction of gut-homing receptors(19). We demonstrated that β-carotene supplementation increased mRNA expression of IgA C-region and the number of IgA ASC in the ileum during lactation, but in the mammary gland, β-carotene supplementation only increased the number of IgA ASC. In murine small intestine, mature isolated lymphoid follicles are inductive sites for the immune response, and nodular lymphoid structures are observed in the distal small intestine(30). The numbers of IgA ASC have been shown to be greatly reduced in the small bowel of vitamin A-deficient mice, but retinoids are not absolutely required for IgA production in tissues other than the small intestine(6,20). Supplemental β-carotene probably increases expression of the chemokine receptor CCR9, and this homing receptor has been shown to be vital in the accumulation of IgA ASC in the intestine. Additionally, maternal IgA ASC primed in the gut homes to the mammary gland during late pregnancy and lactation(17,29), and homing to the lactating mammary gland has been shown to be highly dependent on the expression of CCR10 and α4β1(6,31). The data presented in the present paper suggest that β-carotene supplementation may influence the expression of the chemokine receptor CCR10 and α4β1 integrin on developing IgA ASC. However, further study is needed to clarify the exact mechanism by which β-carotene promotes homing from the ileum to the mammary gland.

In conclusion, the present study suggested that supplementation of β-carotene for maternal milk to neonates owing to the increase in IgA ASC in mammary gland and ileum during lactation.

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References


