Effects of supplemental β-carotene on mucosal IgA induction in the jejunum and ileum of mice after weaning

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Abstract

An adequate immune system is required to prevent diarrhoea in neonates, and IgA provides protection against microbial antigens on mucosal surfaces. Although β-carotene supplementation has been expected to enhance the retinoidic acid (RA)-mediated immune response in neonates, the exact mechanism of the enhancement of mucosal IgA production in the small intestine by β-carotene is still unclear. In the present study, we investigated the effect of supplemental β-carotene on the concentrations of IgA, the numbers of IgA antibody-secreting cells (ASC) and the mRNA expressions of IgA C-region, CCL25, retinoid X receptor (RXR) a, retinoic acid receptor (RAR) a and RARy in the jejunum and ileum of weanling mice. Weanling mice were fed rodent feed or 50 mg/kg β-carotene-supplemented rodent feed for 7, 14 or 21 d. The concentrations of IgA and the numbers of IgA ASC in the jejunum and ileum of mice increased markedly with age, and supplemental β-carotene increased the concentrations of IgA, the numbers of IgA ASC and the mRNA expressions of IgA C-region, CCL25 and RARY in the jejunum after 14 and 21 d of treatment. Supplemental β-carotene increased the numbers of IgA ASC in the ileum after 14 and 21 d of treatment, but the concentrations of IgA in the ileum were not affected by β-carotene supplementation. The mRNA expressions of RXRa and RARa in the jejunum and those of RXRa and RARY in the ileum after 21 d of treatment were enhanced by β-carotene supplementation. These results indicate that β-carotene supplementation in weanling mice is effective to enhance mucosal IgA induction in the jejunum or ileum and that the effects are mainly due to the RA-mediated immune response.

Key words: Supplemental β-carotene: Jejunum: Ileum: IgA induction: Weanling mouse

Mortality and morbidity of neonates continue to be major problems in humans and animals, and their most common cause is diarrhoea. Optimal neonatal health depends on many factors related to management and nutrition, but improvement of the immune system is required to prevent diarrhoea. IgA is the most abundant Ig isotype in mucosal secretions and provides protection against microbial antigens on mucosal surfaces (1–3), and IgA antibodies produced by IgA antibody-secreting cells (ASC) in the intestines and mammary glands are mainly secreted as dimers (4, 5). Supplemental vitamin A and β-carotene enhance the immune response of neonates (6–9). The administration of vitamin A decreases the incidence of diarrhoea and mortality in malnourished children (10, 11). In previous studies (12, 13), it has been observed that β-carotene supplementation in maternal mice during pregnancy and lactation increases the numbers of IgA ASC in the mammary glands and ileum of lactating mice and enhances IgA transfer from maternal milk to neonatal mice. However, very few IgA ASC are detected in the jejunum and ileum of neonatal mice at 14 d of age and most IgA in neonatal mice may be derived from milk IgA (12). Decreased intestinal secretions of IgA in mice can be found at weaning, and the concentrations of IgA rise rapidly after weaning and reach a maximum level at 10 weeks of age (14). Thus, the enhancement of mucosal IgA induction is important for maintaining a normal immune system in neonates.

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 differentiating from IgA+ B cells preferentially home in on the gut lamina propria through the thoracic duct and blood by the expression of homing ligands and receptors (11–13). Chemokines are transmembrane proteins that play important roles in innate and acquired immunity (15–17), and chemokine ligand CCL25 is selectively expressed in the small intestine and its receptor CCR9 is expressed by almost all T cells in the small intestine and a fraction of IgA ASC (18). The interplay of CCL25 and CCR9 is likely to have a significant role in the recruitment and development of mucosal B cells (19), and the selective expression of CCL25 in the small bowel underlies the homing of CCR9+ intestinal memory T cells to the small bowel (20).

Abbreviations: ASC, antibody-secreting cells; pIgR, polymeric Ig receptor; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor.

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The vitamin A metabolite all-trans retinoic acid (RA) plays important roles in gut immunity and 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\(^{16,17}\). Several effects of carotenoids are thought to be mediated by their metabolism to vitamin A and subsequent mediation of retinoic acid receptor (RAR) and retinoid X receptor (RXR) response pathways\(^7\). RA is a highly potent activator of RAR and RXR and influences the transcription of various retinoid response genes by the activation of these receptors\(^7,21,22\). RA regulates the apoptosis of T lymphocytes through interplay between RAR and RXR, but RARα and RARγ induce opposite effects during thymic selection\(^7,25\). The expressions of RAR and RXR isotypes vary greatly in different tissues, but human lymphocytes express RXRα, RARα and RARγ\(^5,24\). Supplemental β-carotene increases the mRNA expression of IgA C-region and the numbers of IgA ASC in the ileum of lactating mice, and these effects may be mainly due to the RA-mediated immune response\(^6\). However, the exact mechanism of the enhancement of mucosal IgA induction in the intestines of neonates by β-carotene is still unclear, although β-carotene supplementation has been expected to enhance RA-mediated immune response in neonates\(^6\).

In the present study, we investigated the effect of supplemental β-carotene on the concentrations of IgA, the numbers of IgA ASC and the mRNA expressions of IgA C-region, CCL25, RXRα, RARα and RARγ in the jejunum and ileum of weaning mice. The present study demonstrates that β-carotene supplementation in weaning mice is effective to enhance mucosal IgA induction in the jejunum or ileum, because it increases the concentrations of IgA, the numbers of IgA ASC and the mRNA expression of IgA C-region in the jejunum and also the numbers of IgA ASC in the ileum. These effects may be mainly due to the RA-mediated immune response, owing to the increased mRNA expressions of CCL25, RXRα, RARα and RARγ in the jejunum and of RXRα and RARγ in the ileum.

### Materials and methods

#### Animals and diets

Male weaning ICR mice aged 21 d (\(n = 44\)) were purchased from Clea Japan (Tokyo, Japan). They were housed in individual polycarbonate cages and maintained in an air-conditioned room (24 ± 2°C) under controlled lighting conditions (14 h light–10 h dark cycle). They received humane care in accordance with the ‘Regulation on Animal Experimentation at Kyoto University’ (Animal Research Committee, Kyoto University, revised 2007).

Weaning mice were randomly allocated to the control or β-carotene group at 21 d of age. Mice in the control group were fed rodent feed (Oriental Yeast) for 7 (\(n = 8\)), 14 (\(n = 8\)) or 21 (\(n = 6\)) d, and those in the β-carotene group were fed 50 mg/kg β-carotene-supplemented rodent feed for 7 (\(n = 8\)), 14 (\(n = 8\)) or 21 (\(n = 6\)) d. The rodent feed contained a vitamin mix, and vitamin A concentration was 385 μg retinol activity equivalent/100 g, but β-carotene was not supplemented to the control group. For the β-carotene group, β-carotene was mixed with the rodent feed at 50 mg/kg. All mice were given free access to water and feed. Body weights and feed intake of mice were measured at 07.00 hours every day.

#### Sample collection

Blood samples from eight mice in the control and β-carotene groups after 7 and 14 d of treatment and six mice in the control and β-carotene groups after 21 d of treatment were obtained by cardiac puncture under anaesthesia with Avertin (2,2,2-tri-bromoethanol; Sigma-Aldrich Chemical), and then jejunum, ileum and rectal faeces were removed after euthanasia by cervical dislocation. The samples of jejunum and ileum were immediately frozen in dry ice-cooled isopentane (2-methyl-butane; Wako Pure Chemicals) for immunohistochemical analysis or frozen in liquid N\(_2\) and stored at −80°C for IgA immunoassay and semi-quantitative RT-PCR. Blood samples were left to stand at room temperature for 1 h and then centrifuged at 3000 rpm for 15 min. The samples of serum and rectal faeces were stored at −20°C until IgA analysis.

#### IgA immunoassay and immunohistochemical analysis

IgA immunoassay of serum, jejunum, ileum and faeces and immunohistochemical analysis of jejunum and ileum were carried out as described previously\(^12\). IgA concentrations were measured using the Mouse IgA ELISA Quantitation Kit (Bethyl Laboratories) and the ELISA Starter Accessory Package (Bethyl Laboratories) according to the manufacturer’s instructions. The sections obtained from the immunohistochemical analysis were examined under an epifluorescence microscope (BX50; Olympus), and the resulting images were analysed using Image J software (National Institutes of Health). IgA-positive cells in the jejunum and ileum were counted in the lamina propria of villi in eight randomised villi from each mouse and reported as IgA ASC/unit area of the lamina propria of villi (unit = 10 000 μm\(^2\)).

#### Semi-quantitative RT-PCR

The mRNA expressions of IgA C-region, CCL25, RXRα, RARα and RARγ in the jejunum and ileum were examined by semi-quantitative RT-PCR as described previously\(^12\). The relative abundance of a 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 and GAPDH were the same as those used in the previous study\(^12\). The primer pairs for CCL25 were as follows: forward: 5'–CCCTCACGTATCGAGAGGATC–3' and reverse: 5'–GAAGATTCTTATCGCCCTTTCA–3'. The PCR was carried out as follows: after 95°C for 7 min to denature DNA, PCR was conducted for thirty-three cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and then for one cycle at 72°C for 7 min. The primer pairs for RXRα and RARα were as follows: forward: 5'–GAGCAGACGTGAGGATATCAAGC–3' and reverse: 5'–GGTCAGGTCTTGGGTCTTCA–3'. The PCR was carried out as follows: after 95°C for 7 min to denature DNA, PCR was conducted for thirty-five cycles at 94°C for 1 min,
57°C for 1 min and 72°C for 1 min, and then for one cycle at 72°C for 7 min. The primer pairs for RARα were as follows: forward: 5'-AGCACCAGCTTCCAGTCTGTA-3' and reverse: 5'-AG-TGGTAGCCGGATGATTTG-3'. The PCR was carried out as follows: after 95°C for 7 min to denature DNA, PCR was conducted for thirty-seven cycles at 94°C for 1 min, 53°C for 1 min and 72°C for 1 min, and then for one cycle at 72°C for 7 min.

The primer pairs for RARγ were as follows: forward: 5'-GGGCAAGTACACCACGAACT-3' and reverse: 5'-ATCCGCAGCATTAGGTACGTC-3'. The PCR was carried out as follows: after 95°C for 7 min to denature DNA, PCR was conducted for thirty-seven cycles at 94°C for 1 min, 53°C for 1 min and 72°C for 1 min, and then for one cycle at 72°C for 7 min.

**Statistical analysis**

Data are expressed as mean values with their standard errors. Data of body weights and feed intake were analysed by least-squares ANOVA using the general linear model procedure of Statistical Analysis Systems (SAS Institute) (25). 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 (25) was used to analyse the effects of treatment or time on some variables in mice. Significance was declared at \( P<0.05 \).

**Results**

**IgA concentrations in serum and tissues**

Body-weight gains and feed intake of mice during 21 d of treatment as well as those of mice during 7 and 14 d of treatment (data not shown) were similar between the groups (Fig. 1). IgA concentrations in the jejunum of the β-carotene group were significantly higher than those of the control group after 14 (\( P<0.01 \)) and 21 (\( P<0.05 \)) d of treatment, but IgA concentrations in the serum, ileum and faeces of mice after 7, 14 and 21 d of treatment were not affected by the treatment (Fig. 2). Compared with the IgA concentrations of mice after 7 d of treatment, those in the serum (\( P<0.001 \)) and faeces (\( P<0.01 \)) increased after 14 and 21 d of treatment. IgA concentrations in the jejunum and ileum increased (\( P<0.001 \)) with age.

**Fig. 1.** Body weights (BW) and feed intake of the control (■; n 6) and β-carotene (□; n 6) groups during 21 d of treatment.

**Fig. 2.** IgA concentrations (µg/g) in the (a) serum, (b) faeces, (c) jejunum and (d) ileum of the control group (■) after 7 (n 8), 14 (n 8) and 21 (n 6) d of treatment and of the β-carotene group (□) after 7 (n 8), 14 (n 8) and 21 (n 6) d of treatment. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that of the control group: * \( P<0.05 \), ** \( P<0.01 \).
IgA antibody-secreting cells in tissues

The numbers of IgA ASC in the jejunum of the β-carotene group were significantly higher than those of the control group after 7 (P<0.05), 14 (P<0.001) and 21 (P<0.001) d of treatment (Fig. 3), and those in the ileum of the β-carotene group were significantly higher than those of the control group after 14 (P<0.001) and 21 (P<0.05) d of treatment. The numbers of IgA ASC in the jejunum and ileum of mice increased (P<0.001) with age.

Expression of mRNA in tissues

The mRNA expression of IgA C-region in the jejunum of the β-carotene group was significantly higher (P<0.01) than that of the control group after 14 and 21 d of treatment, and the mRNA expression of CCL25 in the jejunum of the β-carotene group after 14 (P<0.01) and 21 (P<0.05) d of treatment was significantly higher than that of the control group (Table 1). The mRNA expression of REXα in the jejunum and ileum of the β-carotene group was significantly higher (P<0.01) than that of the control group after 21 d of treatment. The mRNA expression of RARα in the jejunum of the β-carotene group after 21 d of treatment was significantly higher (P<0.05) than that of the control group, but that of RARα in the jejunum of the β-carotene group after 7 d of treatment was significantly lower (P<0.01). The mRNA expression of RARγ in the jejunum of the β-carotene group after 14 and 21 d of treatment was significantly higher (P<0.05) than that of the control group, and that of RARγ in the ileum of the β-carotene group after 21 d of treatment was significantly higher (P<0.05). There were no significant differences in the mRNA expressions of IgA C-region, CCL25 and RARα in the ileum between the groups.

Discussion

IgA antibodies in the intestines are specific to antigens of the intestinal microflora and act to limit the penetration of commensal intestinal bacteria through the neonatal intestinal epithelium(26). After weaning, the intestinal mucosa of mice is exposed to a wide variety of exogenous antigens due to the sudden change in diet and the numbers of Ig-secreting cells are likely to increase(14). Mean faecal IgA concentration of mice at weaning is 3-1 μg/g, but faecal IgA concentration increases rapidly until 35 d of age and thereafter remains almost constant(27). In the present study, however, the concentrations of IgA and the numbers of IgA ASC in the jejunum and ileum of weanling mice increased rapidly with age, although concentrations in the faeces and serum increased after 14 d of treatment and then remained constant.

Supplementation of vitamin A and carotenoids affects the immune cell function during ontogenesis(28), and vitamin A-depleted mice exhibit impaired IgA secretion in mucosal tissues of the small bowel(17). In the present study, supplemental β-carotene at 50 mg/kg in the diet increased the concentrations of IgA, the numbers of IgA ASC and the mRNA expression of IgA C-region in the jejunum of weanling mice after 14 and 21 d of treatment, but in the ileum, supplemental β-carotene only increased the numbers of IgA ASC. On the other hand, supplementation of β-carotene at 50 mg/kg in the diet given to maternal mice during pregnancy and lactation enhanced IgA transfer from maternal milk to neonates, owing to the increase in the numbers of IgA ASC in the mammary glands and ileum of maternal mice, but β-carotene supplementation had no effect on the numbers of IgA ASC and the mRNA expression of IgA C-region in the jejunum of weanling mice(12). In the previous(12,13) and present studies, however, β-carotene supplementation had no effect on IgA concentrations in the serum and faeces of neonatal and weanling mice. Thus, β-carotene supplementation in weanling mice is effective to enhance mucosal IgA induction in the small intestine, owing to the increase in the numbers of IgA ASC in the jejunum and ileum, but supplemental β-carotene at 50 mg/kg in the diet may have a predominant effect on IgA concentrations in the jejunum of weanling mice.

IgA antibodies in the intestines are mainly secreted as dimers after incorporation of the J chain and association with polymeric Ig receptor (pIgR), and the transport of IgA antibodies from IgA ASC into the intestines is regulated by
plgR\(^{(1,4)}\). In the murine small intestine, mature isolated lymphoid follicles are inductive sites for the immune response and nodular lymphoid structures can be observed in the distal small intestine\(^{(25)}\). On the other hand, supplementation of fructo-oligosaccharides in mice after weaning was more effective for enhancing IgA levels in the jejunum rather than in the ileum and colon, and supplemental fructo-oligosaccharides increased not only the concentrations of IgA but also the expression of plgR in the ileum and colon\(^{(1)}\). Additionally, coumestrol administration in maternal mice during pregnancy and lactation increased the number of IgA ASC in mammary glands, but had no effect on the concentrations of milk IgA and the mRNA expression of plgR in mammary glands\(^{(25)}\). These results indicate that the increased concentrations of IgA in the jejunum of weanling mice may be due to the increase in the mRNA expression of IgA C-region and the expression of plgR caused by β-carotene supplementation.

Gut-associated lymphoid tissue dendritic cells rely on RA to induce IgA class switching, and RA is essential for the imprinting of gut-homing receptors on T and B cells and contributes to IgA production\(^{(16,17)}\). CCL25 plays essential roles in intestinal homing of IgA ASC primarily by mediating their extravasation into intestinal lamina propria\(^{(28)}\). RA is important for the induction of the expression of CCR9 on activated T cells, and the blocking of RA receptors decreases the induction of gut-homing receptors\(^{(16)}\). In the present study, supplemental β-carotene increased the mRNA expressions of CCL25 and RAR\(_{γ}\) in the jejum after 14 and 21 d of treatment and those of RAR\(_{α}\) and RAR\(_{β}\) in the jejunum and of RAR\(_{α}\) and RAR\(_{γ}\) in the ileum after 21 d of treatment, although it decreased the mRNA expression of RAR\(_{α}\) in the jejunum after 7 d of treatment. These results imply that the enhanced mucosal IgA induction caused by β-carotene supplementation is mainly due to the RA-mediated immune response, owing to the increased mRNA expressions of RAR\(_{α}\), RAR\(_{α}\) and RAR\(_{γ}\) in the jejunum and of RAR\(_{α}\) and RAR\(_{γ}\) in the ileum, and β-carotene supplementation may influence the expression of chemokine receptor CCR9 on developing IgA ASC in the jejunum.

In conclusion, the present study suggests that supplementation of β-carotene in mice after weaning is useful for

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**Table 1. mRNA expressions of IgA C-region, CCL25, retinoic X receptor (RXR) α, retinoic acid receptor (RAR) α and RAR\(_{γ}\) in the jejunum and ileum of the control group after 7 (n = 8), 14 (n = 8) and 21 (n = 6) d of treatment and the β-carotene group after 7 (n = 8), 14 (n = 8) and 21 (n = 6) d of treatment\(^{*}\)**

(Mean values with their standard errors)

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GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

* mRNA expression is represented relative to IgA C-region, CCL25, RXR\(_{α}\), RAR\(_{α}\) and RAR\(_{γ}\) mRNA expression normalised by the abundance of GAPDH mRNA.
enhancing mucosal IgA induction in the intestines, owing to the increase in the concentrations of IgA, the numbers of IgA ASC and the mRNA expression of IgA C-region in the jejunum and the numbers of IgA ASC in the ileum. Foods containing animal products and pro-vitamin A carotenoids are the primary source of vitamin A, but vitamin A deficiency is associated with an increased risk of death from common childhood infections. Supplementation of β-carotene-enriched formulas may improve the immune system of neonates, because β-carotene has not been detected in four of eight brands of formula preparations and plasma β-carotene of formula-fed children is significantly lower than that of breast-fed children. However, for children aged 1–3 years in the USA, the median vitamin A intake from foods and supplements is 12.05 μg of retinol activity equivalent/d and exceeds the tolerable upper level of 600 μg/d, but does not exceed the no-observed-adverse-effect level of 6000 μg/d. Further study is needed to clarify the optimal and toxic levels of β-carotene in formulas to improve the immune system of neonates.

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The authors have no conflicts of interest to declare.

References


