Effects of astaxanthin-enriched yeast on mucosal IgA induction in the jejunum and ileum of weanling mice

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ABSTRACT

The present study was conducted to clarify the effects of astaxanthin-enriched yeast on the concentration of IgA, the numbers of IgA antibody-secreting cells (ASC) and the mRNA expression of IgA C-region in the jejunum and ileum of weanling mice. Weanling mice were fed rodent feed or astaxanthin-enriched yeast-supplemented rodent feed for 7, 14 or 21 days. Supplemental astaxanthin-enriched yeast increased the numbers of IgA ASC in the jejunum and ileum after 7, 14 and 21 days of treatment. Supplemental astaxanthin-enriched yeast increased IgA concentrations in the jejunum after 21 days of treatment, but IgA concentrations in the ileum were not affected by the treatment. The mRNA expressions of IgA C-region in the jejunum after 14 and 21 days of treatment and the ileum after 14 days of treatment were enhanced by supplementation of astaxanthin-enriched yeast. These results indicate that supplementation of astaxanthin-enriched yeast is effective to enhance the numbers of IgA ASC in the jejunum and ileum and IgA concentrations in the ileum of weanling mice.

Key words: astaxanthin, IgA, ileum, jejunum, weanling mice

INTRODUCTION

Carotenoids and retinoids are present in many foods of plant and animal origin, and retinoids have the potential to mediate or induce proliferative and differentiating effects on several immune-component cells (Rühl 2007). The improvement of immune system is required to prevent diarrhea in neonates, and IgA provides protection against microbial antigens at mucosal surfaces in animals and humans (Ertesvåg *et al.* 2009; Fagarasan & Honjo 2003). Supplemental vitamin A and β-carotene enhance the immune system in neonates **(**Bendich 1989; Chew & Park 2004), but β-carotene deficient calves were found to have a higher incidence of diarrhea in the first week of life (Kume & Toharmat 2001). In the previous studies (Nishiyama *et al.* 2011a,b), supplemental β-carotene increased not only serum β-carotene concentrations in neonatal calves but also IgA transfer from maternal milk to neonatal mice.

Reactive oxygen species (ROS) are highly reactive and associated with oxidative stress in the body, and ROS can destroy cellular membranes, cellular proteins and nucleic acids (Chew & Park 2004). Carotenoids have an antioxidant activity and reduce the toxic effects of ROS in the mucosal membrane. Dietary carotenoids such as lutein, cantaxanthin, lycopene and astaxanthin do not have a pro-vitamin A activity, but these carotenoids are active to enhance the cell-mediated and humoral immune response in animals and humans (Chew & Park 2004). Astaxanthin shows notable antioxidant properties and is twice as effective as β-carotene as a scavenger of peroxyl radicals (Goto *et al*. 2001). Astaxanthin prevents the oxidative injury in human lymphocytes (Bolin *et al.* 2010; Campoio *et al.* 2011), inhibits the tumor growth (Chew & Park 2004) and ameliorates the embryonic development impaired by heat stress (Namekawa *et al.* 2010). Because β-carotene supplementation is effective to enhance mucosal IgA induction in the jejunum or ileum in weanling mice (Nishida *et al.* 2013), astaxanthin supplementation has been expected to enhance the mucosal immune induction in neonates.

The objective of the present study was conducted to clarify the effects of astaxanthin-enriched yeast on the concentrations of IgA, the numbers of IgA antibody-secreting cells (ASC) and the mRNA expression of IgA C-region in the jejunum and ileum of weanling mice.

MATERIALS AND METHODS

Animals and diets

Male weanling ICR mice (n=49) at 21 days of age 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 in accordance with “Regulation on Animal Experimentation at Kyoto University” (Animal Research Committee, Kyoto University, revised 2007).

Weanling mice were randomly allocated to the control or astaxanthin group at 21 days of age. Mice in the control group were fed rodent feed (Oriental Yeast, Tokyo, Japan) for 7 (n=8), 14 (n= 8) or 21 (n=8) days, and those in the astaxanthin group were fed astaxanthin-enriched yeast -supplemented rodent feed for 7 (n=8), 14 (n=9) or 21 (n=8) days. The rodent feed contained 55.3% NFE, 23.6% CP, 5.1% crude fat, 5.8% crude ash, 1283IU/100g vitamin A and 9.1mg/100g vitamin E. Astaxanthin was derived from dried yeast product of *phaffia rhodozyma* (Aquasta^{TM}, Naturxan Co Ltd., Colombia, USA), which contained 58.3% NFE, 20.8% CP, 9.6% crude fat, 2.6% crude ash, 1.2% astaxanthin and 1030IU/100g vitamin A. For the astaxanthin group, 1% Aquasta was mixed with the rodent feed and astaxanthin concentration in the diet was 120mg/kg, which was established about 2 times higher than the dietary β-carotene level at 50 mg/kg as previously described (Nishida *et al.* 2013). All mice were allowed free access to water and feed. Body weights and feed intake of mice were measured at 11.00 hours every day.

Sample collection

Blood samples from mice in the control and astaxanthin groups after 7, 14 and 21 days of treatment were obtained by cardiac puncture under anaesthesia with Avertin (2,2,2-tribromoethanol, Sigma-Aldrich Chemical, St Louis, MO, USA), and then jejunum, ileum and rectum feces were removed after euthanasia by cervical dislocation. The samples of 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 IgA immunoassay and semi-quantitative RT-PCR. Blood samples were left to stand at room temperature for 1 h and then centrifuged at 3000 x g for 15 min. The samples of serum and rectum feces were stored at -20℃ until IgA analysis.

IgA immunoassay and immunohistochemical analysis

IgA immunoassay of serum, jejunum, ileum and feces and immunohistochemical analysis of jejunum and ileum were carried out as previously described (Nishiyama *et al.* 2011a). IgA concentrations were 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. The sections obtained from the immunohistochemical analysis were examined under an epifluoresence microscope (BX50; Olympus, Tokyo, Japan), and the resulting images were analyzed using Image J software (National Institute of Health, Bethesda, MD, USA). IgA-positive cells in the jejunum and ileum were counted in the lamina propria of villi in eight randomised villi from each mouse and shown as IgA ASC/unit area of the lamina propria of villi (unit=10000 μm^2).

Semi-quantitative RT-PCR

The mRNA expression of IgA C-region in the jejunum and ileum was examined by semi-quantitative RT-PCR. Total RNA was extracted from homogenized jejunum and ileum samples from each mouse using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA). Complementary DNA was synthesised with oligo-(dT) primer using an SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Calisbad, CA, USA) 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-TGCACAGTTACCCATCCTGA-3, reverse: 5-GCACCAGCACTTCTTTAGGG-3. The PCR procedure was as follows: after 94°C for 5 min to denature DNA, PCR was performed for thirty-three cycles in the jejunum or thirty cycles in the ileum at 94°C for 1 min, 53°C for 1 min, 72°C for 1 min, then at 72°C for 7 min. The PCR products were electrophoresed in 2% agarose gel and stained with 1mg/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. (National Institute of Health, Bethesda, MD, USA). The relative abundance of specific mRNA was normalised by the abundance of glyceraldehydes 3-phosphate dehydrogenase (GAPDH) mRNA. The primer pairs for GAPDH were as follows: forward: 5-GGGTGGAGCCAAACGGGTC-3, reverse: 5-GGAGTTGCTGTTGAAGTCGC-3. The PCR procedure was as follows: after 94°C for 5 min to denature DNA, PCR was performed for twenty-seven cycles in the jejunum or twenty-three cycles at 94°C for 1 min, 59°C for 1 min, 72°C for 1 min, then at 72°C for 7 min.

Statistics

Data from body weight and feed intake were analysed by least squares ANOVA using the general linear model procedure of Statistical Analysis Systems (SAS 1997). 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 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 Statistical Analysis Systems (SAS 1997) was used to analyze the effects of treatment or time on some variables in mice. The differences were tested by Tukey-Kramer's multiple comparisons. Significance was declared at $P<0.05$.

RESULTS

IgA concentration in serum and tissues

Body weight gains and feed intake of mice during 21 days of treatment as well as those of mice during 7 and 14 days of treatment (data not shown) were similar between groups (Fig. 1). IgA concentrations in the jejunum of the astaxanthin group were significantly higher ($P<0.01$) than those of the control group after 21 days of treatment, but IgA concentrations in the ileum, serum and feces of mice after 7, 14 and 21 days of treatment were not affected by the treatment (Table 1). Compared with IgA concentrations of mice after 7 days of treatment, IgA concentrations in the jejunum, ileum, serum and feces increased ($P<0.001$) after 21 days of treatment.

IgA antibody-secreting cells in tissues

The numbers of IgA ASC in the jejunum of the astaxanthin group were significantly higher than those of the control group after 7 ($P<0.001$), 14 ($P<0.001$) and 21 ($P<0.01$) days of treatment (Fig. 2). The numbers of IgA ASC in the ileum of the astaxanthin group were significantly higher ($P<0.001$) than those of the control group after 7, 14 and 21 days of treatment. Compared with the numbers of IgA ASC of mice after 7 days of treatment, the numbers of IgA ASC in the jejunum and ileum of mice increased ($P<0.001$) after 21 days of treatment.

Expression of mRNA in tissues

The mRNA expression of IgA C-region in the jejunum of the astaxanthin group was significantly higher ($P<0.05$) than that of the control group after 14 and 21 days of treatment (Table 2). The mRNA expression of IgA C-region in the ileum of the astaxanthin group was significantly higher ($P<0.05$) than that of the control group after 14 days of treatment.

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 (Harris *et al.* 2006). 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 preferentially home on the gut lamina propria through the thoracic duct and blood by the expression of homing ligands and receptors (Ertesvåg *et al.* 2009; Fagarasan & Honjo 2003). The gut-associated lymphoid tissue dendritic cells rely on retinoic acid (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 (Mora & von Andrian 2009). In the previous study (Nishida *et al.* 2013), supplemental β-carotene at 50 mg/kg in the diet increased the numbers of IgA ASC in the jejunum of weanling mice after 7, 14 and 21 days of treatment and the ileum after 14 and 21 days of treatment, and the effects are mainly due to the RA-mediated immune response.

Astaxanthin is a typical fat-soluble antioxidant, which scavenges ROS and blocks lipid peroxidation (Goto *et al.* 2001). A number of endogenous antioxidants were produced in the body and scavenges harmful ROS to maintain the normal cellular function and health, but dietary sources of antioxidants are required to eliminate excessive ROS in the body under conditions of high oxidative stress (Chew & Park 2004). In the present study, the numbers of IgA ASC and IgA concentrations in the jejunum and ileum of weanling mice increased with age and supplemental

astaxanthin-enriched yeast, which contained 120 mg/kg astaxanthin in the diet, increased the numbers of IgA ASC in the jejunum and ileum of weanling mice after 7, 14 and 21 days of treatment. After weaning, the intestinal mucosa of mice is exposed to a wide variety of exogenous antigens due to the sudden change in the diet and the numbers of Ig-secreting cells are likely to increase (Nishida *et al.* 2013; Van del Heijden *et al*. 1988). Thus, our data indicate that astaxanthin-enriched yeast as well as β-carotene is effective to increase the numbers of IgA ASC in the jejunum and ileum of weanling mice.

Chemokines are transmembrane proteins that play important roles in innate and acquired immunity and chemokine ligand CCL25 is selectively expressed in the small intestine (Mora *et al.* 2006; Morteau *et al.* 2008). Supplemental β-carotene enhanced the mRNA expression of CCL25 in the jejunum of weanling mice after 14 and 21 days of treatment, which contributed to the mucosal immune induction by the increased homing of IgA ASC to the jejunum (Nishida *et al.* 2013). Because astaxanthin scavenges ROS in the mucosal membrane of intestines, the increased homing of IgA ASC to the guts in the present study may be partly due to the antioxidant-mediated immune response owing to the activated homing ligands. On the other hand, the dry yeast supplementation of *Saccharomyces cerevisiae* at 0.05% in the diet showed the increase of IgG level in colostrum and the maintenance of IgA level in milk of sows, although the dry yeast supplementation at 0.05 and 0.5% in the diet displayed immunostimulatory effects on maternal immunity (Zannello *et al.* 2013). Because of the useful effects of the dry yeast on IgA level in milk of sows, further study is needed to clarify the effects of astaxanthin or dry yeast on the homing of IgA ASC to the intestines.

Supplementation of vitamin A and carotenoids affects the immune-cell function during ontogenesis (Garcia *et al.* 2003), and vitamin A-depleted mice exhibit impaired IgA secretion in mucosal tissues of the small bowel (Mora *et al.* 2006). Supplemental β-carotene in weanling mice increased the concentrations of IgA and the mRNA expression of IgA C-region in the jejunum

after 14 and 21 days of treatment, but IgA concentrations in the ileum were not affected by β-carotene supplementation (Nishida *et al.* 2013). In the present study, supplemental astaxanthin-enriched yeast increased the concentrations of IgA and the mRNA expressions of IgA C-region in the jejunum after 21 days of treatment, but IgA concentrations in the ileum, serum and feces were not affected by astaxanthin-enriched yeast supplementation. These results suggest that a long-term supplementation of astaxanthin-enriched yeast is needed for enhancing the mucosal IgA induction in the jejunum of weanling mice owing to the increase in the concentrations of IgA, the numbers of IgA ASC and the mRNA expressions of IgA C-region.

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 pIgR (Fagarasan & Honjo 2003). Supplementation of fructo-oligosaccharides in mice after weaning increased not only the concentrations of IgA but also the expression of pIgR in the ileum and colon (Nakamura *et al.* 2004). On the other hand, 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 pIgR in mammary glands (Wang *et al.* 2013). Thus, the increased IgA concentrations in the jejunum of weanling mice after 21 days of treatment in the present study may be due to the increased expression of pIgR caused by astaxanthin-enriched yeast supplementation. However, further study is needed to clarify the exact mechanism of astaxanthin for promoting mucosal IgA induction in the intestines of weanling mice and preventing diarrhea.

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1

Table 1 IgA concentrations (μg/g) in serum, feces, jejunum and ileum of the control group after 7 (n=8), 14 (n=8) and 21 (n=8) days of treatment and the astaxanthin group after 7 (n=8), 14(n=9) and 21 (n=8) days of treatment (Mean ±SE)

	Days	Control	Astaxanthin	*P*
Serum	7	156 ± 26	123 ± 17	NS
	14	163 ± 12	164 ± 11	NS
	21	331 ± 40	362 ± 40	NS
Feces	7	233 ± 53	235 ± 49	NS
	14	391 ± 60	314 ± 53	NS
	21	546 ± 102	508 ± 77	NS
Jejunum	7	751 ± 83	736 ± 74	NS
	14	880 ± 92	951 ± 33	NS
	21	1049 ± 91	1487 ± 99	**
Ileum	7	628 ± 75	600 ± 58	NS
	14	662 ± 53	938 ± 128	NS
	21	958 ± 70	1005 ± 90	NS

***P*<0.01, NS, not significant.

2

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Table 2 The ratios of IgA C-region mRNA to GAPDH mRNA in the jejunum and ileum of the control group after 7 (n=8), 14 (n=8) and 21 (n=8) days of treatment and the astaxanthin group after 7 (n=8), 14(n=9) and 21 (n=8) days of treatment (Mean ± SE)

	Days	Control	Astaxanthin	*P*
Jejunum	7	0.66 ± 0.11	0.84 ± 0.07	NS
	14	0.85 ± 0.12	1.27 ± 0.09	*
	21	0.78 ± 0.07	1.10 ± 0.08	*
Ileum	7	0.88 ± 0.16	0.99 ± 0.11	NS
	14	1.27 ± 0.17	1.72 ± 0.10	*
	21	0.77 ± 0.08	0.89 ± 0.10	NS

*$P<0.05$, NS, not significant.
GAPDH, glyceraldehyde-3-phosphate dehydrogenase

2

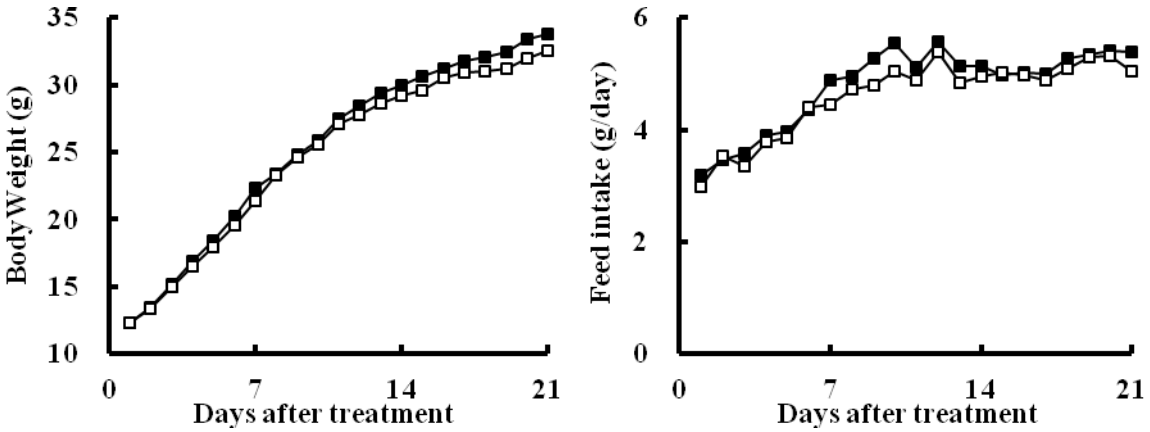

Figure 1 Body weights and feed intake of the control (■; n=8) and β-carotene (□; n=8) groups during 21days of treatment.

a) Jejunum **b) Ileum**

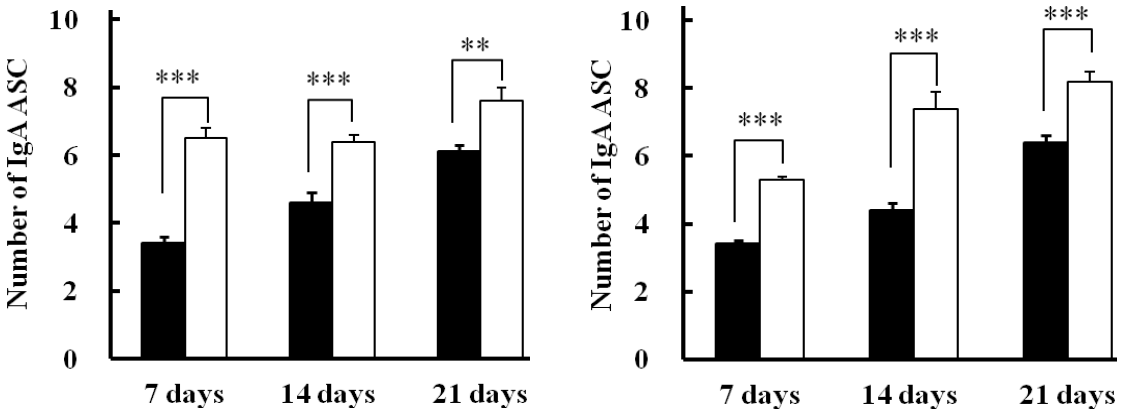

Figure 2 Numbers of IgA antibody-secreting cells (ASC) in the jejunum and ileum of the control group (■) after 7 (n=8), 14(n=8) and 21 (n=8) days of treatment and the astaxanthin group (□) after 7 (n=8), 14(n=9) and 21 (n=8) days of treatment (Mean ± SE). The numbers of IgA ASC in the jejunum and ileum were counted in the lamina propria of villi in eight randomized villi from each mouse.

***P<0.001,**P<0.01.