The Feeding of β-Carotene Down-Regulates Serum IgE Levels and Inhibits the Type I Allergic Response in Mice

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Feed containing β-carotene was administered orally to BALB/c mice immunized intraperitoneally with ovalbumin (OVA) for approximately 1 month. The titers of OVA-specific IgE, OVA-specific IgG1 and OVA-specific IgG2a in the mouse sera were determined. The OVA-specific IgE titer and OVA-specific IgG1 titer by mice fed β-carotene were significantly inhibited. On the other hand, the OVA-specific IgG2a titer in mice fed β-carotene was significantly greater than those of control mice. The OVA-specific IgE suppression of β-carotene feeding was dose-dependent. We also examined the effect of fed β-carotene on active systemic anaphylaxis. Feeding β-carotene to mice immunized with OVA inhibited the immediate reduction of the body temperature induced by antigen stimulation. Furthermore, the increase in serum histamine in the mice fed β-carotene under active systemic anaphylaxis was lower than in controls. We then examined the pattern of cytokine production by spleen cells from mice followed by restimulation with OVA in vitro. The spleen cells from the mice fed β-carotene produced more IFN-γ, IL-12 and IL-2 than those from the control group. In contrast, the spleen cells from the mice fed β-carotene produced less IL-4, IL-5, IL-6, IL-10 than those from the control group. Furthermore, analysis of IFN-γ mRNA levels of the splenocytes using the real-time quantitative RT-PCR technique revealed higher levels in the splenocytes from the mice fed β-carotene. These findings suggest that feeding β-carotene improves the helper T cell (Th1–Th2) balance, inhibiting specific IgE and IgG1 production and antigen-induced anaphylactic response.

Key words β-carotene; IgE; cytokine; Th1–Th2 balance; active systemic anaphylaxis

The incidence of type I allergic disorders is increasing; particularly, hypersensitivity to food and airborne allergens. The mechanism of Type I allergy includes a series of events, namely, production of antigen-specific IgE, binding of IgE to the FceRI receptor on mast cells or basophiles, cross-linking of IgE with newly absorbed allergens, and release of chemical mediators such as histamine and leukotrienes from cells. Inhibition of any of the steps in this sequence leads to the attenuation of allergic symptoms. Several drugs, such as corticosteroids, epinephrine, histamine antagonists, and leukotriene synthesis inhibitors, interfere with the above reactions. However, the effect of these drugs is short-lived, and thus, a more fundamental means of preventing allergies would be desirable.

It has been reported in numerous observational epidemiological studies that the intake of vegetables, including carrots, is associated with the prevention of several degenerative diseases. These reports suggest that vegetables have the potential to modulate or regulate the host immune response, resulting in an overall beneficial effect on the immune response. We have already shown evidence that the feeding of carrots to BALB/c mice sensitized with ovalbumin (OVA) inhibited IgE and IgG1 production. However, it is still unclear what kind of components in carrots could exhibit this activity.

β-Carotene is one of the major nutritional components and is present at high levels especially in carrots. On the other hand, much attention has also been paid to the role of β-carotene intake in the prevention of cancer and life-style-related diseases, such as cardiovascular disease.

In the present study, we examined the feeding of β-carotene on the production of specific IgE antibody, specific IgG1 antibody and specific IgG2a antibody. We also examined the effect of feeding β-carotene on antigen-induced active systemic anaphylactic shock (ASA), such as the decrease in body temperature and the rise of serum histamine levels. Furthermore, to clarify the active mechanism by β-carotene intake in the inhibition of specific IgE production, we examined the pattern of cytokine production by spleen cells from mice fed carrots.

MATERIALS AND METHODS

Mice and Drinking Protocols Female BALB/c mice, 3–4 weeks of age, were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Mice were maintained in a temperature (23–25°C), humidity (40–60%) and light-controlled environment. Animals were allowed free access to a powdered MF diet (Japan SLC) and water throughout the experiment. The MF diet containing β-carotene was prepared by mixing the MF diet with levels of β-carotene of 20 mg/kg. The control group (n = 5) and the β-carotene group (n = 5) had free access to the MF diet and the MF diet containing β-carotene, respectively.

As far as dose-dependent study was concerned, spiked levels in the MF diet were 0 mg/kg (n = 5), 2 mg/kg (n = 5), 10 mg/kg (n = 5) and 20 mg/kg (n = 5). The care and use of the experimental animals in this study were followed by “The Ethical Guidelines of Animal Care, Handling and Termina tion” prepared by National Institute of Health Sciences.

Immunization Protocols Two weeks after starting the administration of MF containing β-carotene, all the mice were immunized i.p. with OVA (20 µg/mouse) with an alum (Al(OH)3) adjuvant. One week later, blood was collected and
serum obtained. Ten days after the first immunization, the mice were given boosters using the same doses of the antigen. A week after the second immunization, all the mice were killed by cervical dislocation, their spleens aseptically removed, and cell suspensions made by passing them through a sterile cell strainer (Becton Dickinson, CA, U.S.A.). Blood was withdrawn and serum obtained. The serum samples were stored at −20 °C prior to analysis.14,18) For the study for the induction of ASA, the experiment procedure after the second immunization was described below.

**ELISA for Mouse Serum Anti-OVA IgE, IgG1 and IgG2a Titer** The mouse serum titer of anti-OVA IgE, IgG1 and IgG2a were determined in triplicate in 96-well microtiter plate by the method of Teshima et al. with some modifications.14,19) Fifty microliters of OVA (cosmobio Co., Ltd., Tokyo, Japan, 20 μg/ml) in 50 mM sodium carbonate buffer, pH 9.5, was added to each well of a 96-well microtiter plate, and incubated overnight at 4 °C. And each well was washed 4 times with 200 μl PBS containing 0.05% Tween 20 (PBS/Tween). Two hundreds microliters of 0.1% casein in PBS was added, and the plates were incubated for 1 h at room temperature and then each well was washed as described above. Fifty microliters of antiserum was added to each well, and the plates were incubated for 1 d at 4 °C and then each well was washed as before. Fifty microliters of rabbit anti-mouse IgE/IgG1/IgG2a (10−3 dilution in PBS containing 0.1% casein, Yamasa Shoyu Co., Ltd., Chiba, Japan) was added to each well, and the plates were incubated for 1 h at room temperature and then each well was washed as before. Fifty microliters of sheep anti-rabbit-IgG-β-galactosidase conjugate (10−3 dilution in PBS containing 0.1% casein, Amersham, U.K.) was added to each well, and the plates were incubated for 1 h at room temperature and then each well was washed as before. The wells were incubated for 1 h at 37 °C with 100 μl PBS containing 0.1 mM 4-methylumbelliferone-β-galactoside (Sigma, St. Louis, MO, U.S.A.). Finally, 25 μl of 1 M anhydrous sodium carbonate was added to each well. The fluorescence intensity of the liberated 4-methylumbelliferone was determined by a Titertek Fluoroskan reader (Flow Laboratories, Inc., Costa Mesa, CA, U.S.A.).

**Detection of IFN-γ mRNA Levels** The primers and TaqMan probes were designed as previously described.24) Primers and probes were synthesized by PE Oligofactory (Applied Biosystems, Foster city, CA, U.S.A.). Total RNA was extracted from the splenocytes using TRIzol® reagent (Life Technologies, Basel, Switzerland) according to the manufacturer’s protocol. RT was performed in a 10-μl final volume containing 1.25 U/μl MultiScribe reverse transcriptase, 5.5 μM MgCl2, 2.5 μM oligo d(T)16, 0.4 U/μl RNAse inhibitor (GeneAmp® RNA PCR kit, Applied Biosystems), and 500 μM deoxyNTPs mixture (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). The mixture was subjected to 42 °C for 60 min and inactivated at 95 °C for 5 min. The final volume was adjusted to 100 μl with RNAse-free water. The cDNA was analyzed immediately or stored at −20 °C before use.

Real-time TaqMan PCR systems for mouse GAPDH and IFN-γ were run in triplicate wells. The PCR reactions contained 400 nM of each primer, 80 nM of the TaqMan probe and commercially available PCR Mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM MgCl2, 2.5 mM deoxyribonucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 10 μl of the diluted cDNA sample in a final volume of 25 μl. The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI Prism 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Final determination was done using the comparative C_l method and is reported as relative transcription or the n-fold difference relative to a calibrator cDNA.

**Cytokine Assays of Splenocytes** Splenocytes were collected from the removed spleens of all the OVA-immunized mice (each spleen per mouse), and the cells (5×106 cells/ml) were cultured in triplicate in a 24-well culture plate and re-stimulated with OVA at a final concentration of 100 μg/ml at 37 °C for 3 d.22) The levels of IL-4, IL-5, IL-10, IL-12 (p70), and IFN-γ in the culture medium (RPMI 1640) after 3 d of co-culture with OVA were measured with an OptEIA mouse cytokine ELISA set (PharMingen). Absorbance was measured at 450 nm with a microplate reader (E-max, Molecular Devices, Sunnyvale, CA, U.S.A.).
formed by HPLC as previously described. The liver tissues or the feed materials were homogenized and saponified by addition of 60% KOH and 3% butylated hydroxytoluene in ethanol, followed by heating at 50°C for 30 min. After extraction with hexane and dichloromethane (4:1, v/v, 5.0 ml) twice, the samples were dried and then reconstituted in a sufficient quantity of the mobile phase. The samples were analyzed by HPLC, using a Shimadzu SPD-M10A VP diode array detector (Shimadzu, Kyoto, Japan) and a LiChrospher RP18-5 column (E. Merck, Darmstadt, Germany) (4.6×250 mm). The mobile phase was acetonitrile and methanol (1:1, v/v), and the flow rate was 2.0 ml/min.

**Statistical Analysis** Statistical analysis was performed using the Student's t-test. In all cases, probability (p) values below 0.05 were considered significant.

**RESULTS**

**Body Weight and the Amount of Feed Intake** We examined whether the β-carotene intake affects the body weight and feed intake. There was no significant difference in body weight during the study. Since β-carotene was thought to be unstable, we determined the β-carotene concentrations three times in the feed using the HPLC method during this study to investigate whether β-carotene could be degraded. There was no significant difference among β-carotene concentrations in the feed at the first day (20.6 μg/g), the middle day (21.0 μg/g) and that at the last day (19.8 μg/g) in this study. To examine the effect of β-carotene on the amount of feed intake, we examined the amount of feed intake for two groups. There was no significant difference between the amount of feed intake between the control group and the β-carotene group during the study. Furthermore, to examine the adsorption of β-carotene through feed intake, we determined the level of β-carotene in the liver. We could detect β-carotene in the liver of the control group because the MF feed also contains a small amount of β-carotene. As shown in Fig. 1, the levels of β-carotene in the liver of the β-carotene group were significantly higher than those of the control group.

**OVA-Specific IgE, IgG1 and IgG2a Productions in Vivo** On immunization of mice with OVA, the serum titers of specific IgE antibodies and specific IgG1 in the β-carotene group were lower than those in the control group (Figs. 2a, b), although the serum titer of specific IgG2a antibodies of the β-carotene group was significantly greater than that of the control group (Fig. 2c).

**Histamine Levels in Plasma of Mice and Body Temperature Under ASA** It has been thought that reduced specific IgE antibody production inhibits antigen-induced ASA. The body temperature of sensitized mice was measured every a minute for 8 min after intraperitoneal challenge with 1 mg of OVA (Fig. 3a). A remarkable decrease in body temperature was observed in the control group after challenge. However, a slight decrease in body temperature was observed in the β-carotene group. Blood was collected 8 min after the intraperitoneal challenge with OVA, and the serum histamine concentration was measured. However the systemic response in the β-carotene group should be weaker than that in the control group (data not shown). A marked increase in serum histamine occurred in all the mice undergoing antigen challenge (Fig. 3b). Although there was no significant difference between the serum histamine level in the fed group and that in the control group after antigen stimulation, the serum histamine level in the β-carotene group appeared to be lower than that in the control group (Fig. 3b).

**Immunophenotypes of Splenocytes** To investigate the effect of β-carotene intake on the differentiation of splenocytes immunized with OVA, we examined the phenotypic analysis of the splenocytes using FCM.

The percentage of T cells in the splenocytes isolated from mice fed β-carotene (46.2%) appears to be higher than that in the control (43.9%). The percentage of B cells in the splenocytes isolated from mice fed β-carotene (44.4%) was significantly lower than that in the control (48%). Furthermore, to assess whether β-carotene intake affects the differentiation of the T cell subsets, we analyzed the expression of CD4+ cells and CD8+ cells. Both percentages of CD4+ T cells (helper T cells) and CD8+ cells (cytotoxic T cells) in

![Fig. 1. β-Carotene Levels in the Liver of BALB/c Mice of the Control Group and β-Carotene Group](image1)

Bars represent mean values (±S.E.) of 5 mice. The asterisk indicates significant difference from the control group value (*p<0.01).

![Fig. 2. OVA-Specific-IgE (a), IgG1 (b) and IgG2a (c) Production in Cultured Splenocytes from Mice in the Control Group and the β-Carotene Group](image2)

Bars represent mean values (±S.E.) of 4 mice. The asterisk indicates significant difference from control group value (**p<0.01).
Cytokine Productions of Splenocytes in Vitro
To clarify the mechanisms involved in the inhibition of OVA-specific IgE production in mice fed β-carotene, we investigated the cytokine production of splenocytes from mice re-stimulated with OVA in vitro (Fig. 4). The splenocytes from OVA-sensitized mice re-stimulated with OVA released significant levels of IFN-γ, IL-2, IL-4, IL-5, IL-6 IL-10 and IL-12. For the non-sensitized mice, no levels of cytokine were detected in these measurements using ELISA. As shown in Fig. 4, the Th1 type cytokine levels such as IFN-γ and IL-2 produced by the splenocytes of the β-carotene group were higher than those in the control group. In addition, IL-12 productions by the splenocytes in the β-carotene group were also significantly higher than that in the control group.

Quantitation of the mRNA Level of IFN-γ Using RTPCR
The mRNA level of IFN-γ in splenocytes was analyzed using a real-time quantitative RT-PCR technique. As an
internal control, we used GAPDH mRNA expression.\textsuperscript{26} As shown in Fig. 5, the mRNA levels of IFN-\(\gamma\)/GAPDH from the splenocytes in the \(\beta\)-carotene group were significantly higher than those in the control group.

**Dose Response of \(\beta\)-Carotene Feeding on in Vitro Cytokine Productions by Splenocytes** To investigate the dose dependency of \(\beta\)-carotene feeding on the specific IgE titer reduction and the Th1 promoting effects, we examined the dose response study of \(\beta\)-carotene feeding (spiked level: 0, 2, 10, 20 mg/kg) on those effects. As shown in Fig. 6, the effects of \(\beta\)-carotene feeding on the reduction of specific IgE titer in serum appear to be dose-dependent. In addition, as shown in Fig. 6, the Th1 type cytokine levels such as IFN-\(\gamma\) and IL-12 produced by the splenocytes of the \(\beta\)-carotene group were dose-dependently increased. These results showed that the Th1 promoted activity of \(\beta\)-carotene feeding should be dose-dependent.

**DISCUSSION**

We first demonstrated that feeding \(\beta\)-carotene to mice inhibited specific IgE and IgG1 production in response to OVA in vivo, but specific IgG2a production was promoted significantly and the OVA-specific IgE suppression of \(\beta\)-carotene feeding was dose-dependent. Also, feeding \(\beta\)-carotene to mice immunized with OVA inhibited the immediate reduction of the body temperature induced by antigen stimulation. We also showed that the rise in serum histamine of the \(\beta\)-carotene group was lower compared with that of the control group and that the dietary \(\beta\)-carotene enhanced T cell development from naive T cells by OVA stimulation using FCM. These findings suggest that dietary \(\beta\)-carotene could be helpful in preventing of allergies, especially during early infancy, through suppression of the antigen-specific IgE antibody response.

In a previous study, we showed that the feeding of carrots inhibited specific IgE production and up-regulated the Th1 immune response. We presumed that the primary active substance in carrots could be \(\beta\)-carotene. According to our consideration and these results, we suggested that \(\beta\)-carotene could be the most important substance in the presentation of immediate type hypersensitivity.

It is believed that CD4\textsuperscript{+} helper T cells are subpopulations of 2 types (Th1, Th2) based on their different patterns of cytokine secretion.\textsuperscript{27–33} The balance of the 2 types of cells is considered to be important in maintaining homeostasis in the host. Once this balance becomes disturbed, various immunological diseases, such as allergies, can occur due to circumvention of the host defense mechanisms. Th1 cells secrete IFN-\(\gamma\), whereas Th2 cells produce IL-4. IFN-\(\gamma\) and IL-12 induce differentiation to Th1 from Th0 cells, whereas IL-4 induces differentiation to Th2. Therefore, it is believed that an increase in IFN-\(\gamma\) and IL-12 shifts the Th1-Th2 cell balance to predominantly Th1, and that an increase in IL-4 shifts the balance to predominantly Th2.\textsuperscript{34–38} In the present study, we showed that the amount of Th1-type cytokines such as IFN-\(\gamma\), IL-2 and IL-12 produced by spleen cells from the \(\beta\)-carotene group was higher than that from the control group, while the amount of Th2 type cytokines such as IL-4, IL-5 and IL-10 produced by spleen cells from the \(\beta\)-carotene group appears to be lower than that from the control group. IL-12 is produced by antigen-presenting cells such as macrophages, monocytes and dendritic cells.\textsuperscript{39–41} IL-12 influences the development of Th0 by facilitating the Th1 cellular immune response and by inhibiting the differentiation of lymphocytes into Th2 cells. IL-12 exists at a critical junction in the cytokine network controlling T cell and macrophage responses, through its capacity to induce IFN-\(\gamma\) production.\textsuperscript{42,43} Accordingly, these results suggested that the intake of \(\beta\)-carotene could first stimulate IL-12 production from antigen-presenting cells such as macrophages, monocytes and dendritic cells and then shifts toward the Th1 immune response. In this research, we also quantitatively showed that the feeding of \(\beta\)-carotene would promote IFN-\(\gamma\) mRNA expression in splenocytes using a real-time quantitative RT-PCR technique. This finding suggests that the \(\beta\)-carotene intake induces the enhancement of IFN-\(\gamma\) production via the promotion of IFN-\(\gamma\) mRNA expression in the splenocytes. However, the phenotype analysis of splenocytes

![Fig. 5. Determination of IFN-\(\gamma\) mRNA Level of Splenocytes](image)

The asterisk indicates significant difference from the control group value (\(* p<0.05\)). Bars represent mean values (\(\pm\)S.E.) for 5 mice.

![Fig. 6. Effects of Dietary \(\beta\)-Carotene Dose on Serum OVA-Specific IgE Titters and Cytokine Production of Mice](image)

The MF diet containing \(\beta\)-carotene was prepared by mixing the MF diet with various levels of \(\beta\)-carotene (spiked level: 0, 2, 10, 20 mg/kg). Bars represent mean values (\(\pm\)S.E.) for 5 mice. The asterisk indicates significant difference from the control group value (\(* * p<0.01\)).
showed that feeding of β-carotene appears to induce the helper T cell differentiation (Table 1). These results suggested that dietary β-carotene promotes the differentiation of T_{H1} cells, not T_{H2} cells.

Naga-fuchi et al. showed that dietary nucleotides up-regulate the antigen-specific T_{H1} immune response through the enhancement of IL-12 production and suppress the antigen-specific IgE response. 44 Matsuizaki et al. reported that feeding the Lactobacillus casei strain Shirota to mice effectively inhibited IgE production in response to OVA in vitro. 22 They also reported that the levels of cytokines produced by T_{H1} cells increased and that those of cytokines produced by T_{H2} cells decreased due to feeding the Lactobacillus casei strain Shirota to mice. 45 Shibata et al. reported that the feeding of chitin, a polymer of N-acetyl-D-glucosamine, down-regulates serum IgE levels in mice through the induction of a T_{H1} response. 46 Oka et al. showed that feeding of royal jelly suppressed the antigen-specific IgE production histamine release from mast cells in association with the restoration of macrophage function and improvement in T_{H1}–T_{H2} responses in mice immunized with DNP-KLH. 47 Several studies have shown that the induction of hypervitaminosis A in mice by supplementation of the feed induced useful suggestions in feeding β-carotene to mice. This study was partly supported by a grant from the Japan Health Sciences Foundation and by Cooperative System for Supporting Priority Research of Japan Science and Technology Corporation.

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REFERENCES

4) Matsuo N., Yamada K., Shoji K., Mori M., Sugano M., Allergy, 52, 58—64 (1997).
31) Finkelman F. D., Katona I. M., Urban J. F., Jr., Holmes J., Ohara J., J.