

Inhibition of Immunoglobulin E Production in Allergic Model Mice by Supplementation with Vitamin E and β -Carotene

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A diet containing different amounts of vitamin E (α -tocopherol; 0.5 mg, 5 mg, 10 mg or 50 mg per 100 g diet) was supplemented to BALB/c mice for 6 weeks. These mice were subcutaneously immunized twice with ovalbumin (OVA). A passive cutaneous anaphylaxis (PCA) analysis demonstrated that the mice fed on the diet containing 5 mg of vitamin E produced the highest level of the OVA-specific immunoglobulin E (IgE) antibody. A lower level of serum IgE was found in the mice supplemented with 0.5 mg, 10 mg and 50 mg of vitamin E. A sandwich ELISA analysis showed that the pattern of the total IgE antibody level among these four groups was the same as that of the allergen-specific IgE. In a separate experiment, 5 mg of vitamin E and/or 50 mg of β -carotene was supplemented to the basal diet containing vitamin E as α -tocopherol acetate (5 mg) in order to evaluate the effect of their combination on OVA-specific and total IgE production in the mice. The supplementation with β -carotene alone had no effect on OVA-specific or total IgE production. In contrast, supplementation with vitamin E plus β -carotene effectively suppressed both the antigen-specific and total IgE antibodies. The serum vitamin E and β -carotene levels were increased by supplementation with the respective compounds. These results strongly suggest that the combination of dietary vitamin E and β -carotene suppressed IgE production and would therefore help to prevent the type-I allergic reaction.

Key words: IgE antibody; vitamin E; β -carotene; allergy

A number of epidemiological studies have shown an inverse relationship between fruit and vegetable consumption and the incidence of allergic diseases.^{1–3)} These studies apparently indicate that a high intake of fresh fruit and vegetables decreased wheeze, allergic rhinoconjunctivitis, and allergic eczema in adults and children. A variety of food constituents in fruit and vegetables is likely to relieve these symptoms. Fruit and vegetables are rich in such antioxidants as

vitamin C, vitamin E, carotenoids and flavonoids. These antioxidants have been suggested to affect cellular immunological functions, including cytokine production.⁴⁾ For example, a higher intake of vitamin E has been found to increase the production of IL-2, a typical Th 1 cytokine.⁵⁾ Forgarty *et al.*⁶⁾ have implied that a higher intake of vitamin E suppressed the incidence of allergic disease by inhibiting allergen-specific IgE production, as the IgE antibody is well known to participate in the type-1 allergic reaction. It is therefore likely that the antioxidative action of dietary vitamin E was responsible for the anti-allergic effect of fruit and vegetables.

However, there is little evidence for the suppressive effect of dietary vitamin E and other antioxidants on allergen-specific IgE production *in vivo*. This present study was therefore conducted to determine whether or not supplementation with vitamin E alone or a combination of vitamin E and β -carotene, a typical carotenoid from plant foods, would affect the IgE production in allergic model mice. The results clearly demonstrate that the combination of these two antioxidants effectively suppressed allergen-specific IgE production.

Materials and Methods

Animals. Six-week-old female BALB/c mice and 10 to 12-week-old female Wistar rats were purchased from SLC Japan, (Hamamatsu, Japan). The animals were housed under standard laboratory conditions (25°C, 60% humidity; 12 h light and 12 h dark cycle). The mice were given the experimental diets, and the rats were maintained on a standard pelleted diet. The experiments were performed in accordance with the Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science of 1987).

Supplementation with vitamin E at different amounts to the diet. In the first experiment, a vitamin

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Table 1. Composition of the Diets in the First Experiment¹

	Group			
	vitamin E (mg/100 g of diet)			
	0.5	5	10	250
	g/100 g of diet			
Vitamin-deficient casein	20	20	20	20
D,L-Methionine	0.3	0.3	0.3	0.3
Stripped corn oil ²	5	5	5	5
Lard	5	5	5	5
α -Cornstarch	40	40	40	40
Sucrose	20	20	20	20
Cellulose	4.9995	4.995	4.99	4.95
Vitamin E-deficient vitamin mixture	1	1	1	1
Mineral mixture	3.5	3.5	3.5	3.5
Choline chloride	0.2	0.2	0.2	0.2
<i>d,l</i> - α -Tocopherol	0.0005	0.005	0.01	0.05

¹ The dietary components, except stripped corn oil, were purchased from Oriental Yeast, Japan.

² Stripped corn oil was purchased from Funabashi Nojou, Japan.

Table 2. Composition of the Diets in the Second Experiment¹

	Group			
	A	B	C	D
	g/100 g of diet			
Casein	20	20	20	20
D,L-Methionine	0.3	0.3	0.3	0.3
Corn oil	5	5	5	5
Lard	5	5	5	5
α -Cornstarch	40	40	40	40
Sucrose	20	20	20	20
Cellulose	4.75	4.745	4.7	4.695
Vitamin mixture	1	1	1	1
Mineral mixture	3.5	3.5	3.5	3.5
Choline chloride	0.2	0.2	0.2	0.2
Sodium cholate	0.25	0.25	0.25	0.25
<i>d,l</i> - α -Tocopherol	0	0.005	0	0.005
β -Carotene	0	0	0.05	0.05

¹ The dietary components were purchased from Oriental Yeast, Japan.

E-deficient diet was prepared with vitamin-deficient casein (Oriental Yeast Co., Chiba, Japan), stripped corn oil (Funabashi Noujo Co., Funabashi, Japan), lard, an α -tocopherol-deficient vitamin mixture (Oriental Yeast Co.), and other components. One hundred g of this diet was supplemented with 0.5, 5, 10, or 50 mg of vitamin E (*dl*- α -tocopherol; Sigma Co., St Louis, MO, USA) (Table 1). The BALB/c mice were divided into four groups, each group of mice (5 animals per group) being fed on the diet containing each amount of vitamin E for 6 weeks *ad libitum*.

Supplementation with vitamin E and/or β -carotene. Table 2 shows the composition of each diet given to the mice for the second experiment. The diet containing 0.25% sodium cholate, 1% vitamin mixture, 20% casein, 5% corn oil, 5% lard, and 3.5% mineral mixture was used as the basal diet. This vita-

min mixture contained 0.5% α -tocopherol acetate. The diet was supplemented with vitamin E (*dl*- α -tocopherol; 5 mg per 100 g of diet) alone, β -carotene (50 mg per 100 g of diet) or both vitamin E and β -carotene (5 mg and 50 mg per 100 g of diet, respectively). Dietary supplementation with sodium cholate has markedly enhanced the accumulation of β -carotene in mice.⁷⁾ The BALB/c mice were divided into 4 groups, each group of mice (5 animals per group) being respectively fed with the basal diet (group A), vitamin E diet (group B), β -carotene diet (group C) and vitamin E plus β -carotene diet (group D) for 6 weeks *ad libitum*.

Immunization. The mice were immunized with 1 μ g of ovalbumin (OVA) in 0.5 ml of saline without any adjuvant by a subcutaneous injection. We have already demonstrated that a high titer of IgE was obtained by injecting mice with from 1 to 10 μ g of OVA without any adjuvant.⁸⁾ The day of changing from the commercial diet to the experimental diet is designated as day 0. The first and second immunizations were carried out on days 21 and 35, respectively. The mice were bled one week after the second immunization, the sera being stored at -20°C until needed for the assay.

Evaluation of the OVA-specific IgE antibody. The serum IgE antibody level was evaluated by the passive cutaneous anaphylaxis (PCA) reaction according to the technique described previously.⁹⁾ Briefly, sera obtained from the mice of each group were mixed at the same volume. The mixed serum sample was diluted 100, 200, and 300 times with PBS, and 50 μ l of a diluted serum sample was injected intracutaneously into the shaved back of female Wistar rats. Two days after sensitization, the PCA reaction was elicited by an intravenous injection of 1 ml of PBS containing 1 mg of OVA and 5 mg of Evans blue dye. The appearing blue spots were read 30 min after the challenge. The PCA titer record represents the greatest dilution that gave a skin reaction. The animal studies for PCA experiments were conducted at least three times to check the reproducibility of the results.

Measurements of the total IgE and antigen-specific IgG₁ contents. Total IgE was measured by using a Quantitation ELISA kit (Benthy; Montgomery, TX, USA). OVA-specific IgG₁ was evaluated by sandwich ELISA, using the detecting antibody for IgG₁ (Benthy; Montgomery, TX, USA). The ELISA method has been described elsewhere.⁹⁾ In brief, 100 μ l of captured IgE or OVA (1 μ g/ml of PBS) was added to each well of a 96-well microplate (Nalge Nunc; Roskilde, Denmark) and incubated overnight at 4°C . The wells were blocked with 1% BSA in TBS at room temperature for 30 min, and then washed three times

with TBS containing 0.05% Tween 20. The wells were incubated with the antiserum at room temperature for 1 hour and then washed three times with Tween 20-containing TBS. The antibodies bound to the captured IgE or antigen in the wells were further treated with the peroxidase-conjugated adequate antibody. The second antibodies bound to IgE or IgG₁ in the wells were incubated for 3 hr at room temperature with *O*-phenylenediamine (0.4 mg/ml) and 0.012% H₂O₂ in a 50 mM citrate-phosphate buffer (pH 5.0), the peroxidase reaction being stopped by the addition of 2 M H₂SO₄. The absorbance of the reaction mixture in each well was determined at 490 nm with a microplate reader Model 450 (Bio-Rad; Hercules, CA, USA).

Quantification of vitamin E and β -carotene in the serum. The serum vitamin E content was measured by reversed phase HPLC with fluorescence detection as described previously.¹⁰ Briefly, 30 μ l of serum was mixed with 250 μ l of ethanol containing 1 mM butyl hydroxytoluene (BHT) and 250 μ l of hexane containing 1 mM BHT, after the addition of the internal standard, δ -tocopherol. The mixture was separated by centrifugation at 3,000 rpm for 5 min. The hexane layer was transferred, and the solvent was evaporated in a nitrogen stream. The resulting residue was dissolved in a mixture of methanol and ethanol (1:1, v/v), before being injected into a column of TSK-gel Octyl-80Ts (4.6 $\times$ 150 mm; Tosoh, Tokyo, Japan) and eluted with methanol and water (93:7, v/v) at a flow rate of 1 ml/min. Vitamin E (α -tocopherol) was detected at an excitation wavelength of 295 nm and emission wavelength of 325 nm.

The serum β -carotene content was also measured by the HPLC method, using 8-apocarotenal as an internal standard. Fifty μ l of serum was mixed with 250 μ l of methanol containing 1 mM BHT after the addition of 8-apocarotenal. β -Carotene was extracted with 250 μ l of hexane containing 1 mM BHT. After centrifugation at 30,000 rpm for 5 min, the hexane layer was collected, and the methanol-water layer was extracted again with 1 ml of chloroform and 2 ml of hexane. The resulting extracts were combined and brought to dryness in a nitrogen stream. The residue was dissolved in hexane and injected into a column of TSK-gel Octyl-80Ts (4.6 $\times$ 150 mm; Tosoh, Tokyo, Japan), this being eluted with a mixture of methanol, acetonitrile, dichloromethane, and water (7:7:2:0.16, v/v/v/v). The eluent was monitored by the absorbance at 450 nm.

Results

Effect of different amounts of vitamin E on OVA-specific and total IgE production

Little difference was apparent in the growth curves of BALB/c mice supplemented with different

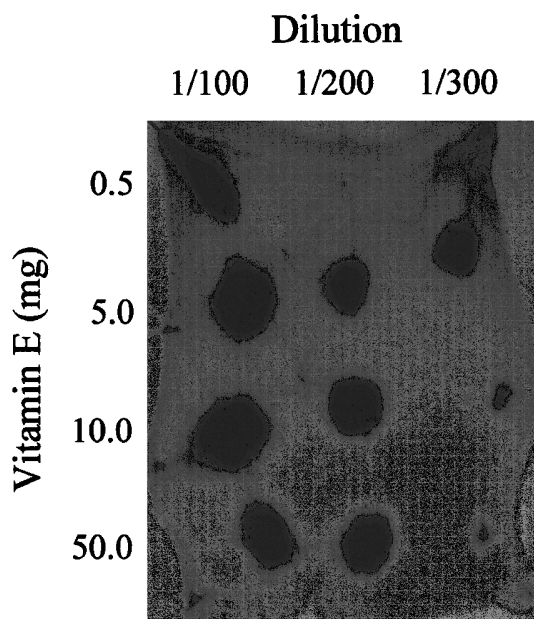


Fig. 1. PCA Reaction in the Mice Supplemented with Different Amounts of Vitamin E.

Mixed sera of mice immunized with OVA were analyzed. Mice were fed with the diet supplemented with *dl*- α -tocopherol at 0.5, 5, 10, or 50 mg/100 g of diet. The amount of *dl*- α -tocopherol is designated on the left side, and the dilution (1:X) of the sera is designated on the upper side.

amounts of vitamin E. Figure 1 shows the result of the PCA analysis for OVA-specific IgE production in BALB/c mice supplemented with various amounts of vitamin E. This figure is a typical result, and we confirmed its reproducibility by repeated experiments. The size of the blue spot expresses the serum concentration of OVA-specific IgE. In the mice fed on the diet supplemented with 0.5 mg vitamin E, the spot could only be visualized in 100-times-diluted sera. In contrast, the spot was clear even in 300-times-diluted sera of the mice supplemented with 5 mg of vitamin E. The mice in the groups with 10 mg and 50 mg of vitamin E supplementation afforded smaller spots in 300-times-diluted sera as than those that supplemented with 5 mg of vitamin E. These results demonstrate that OVA-specific IgE production was highest in the mice with 5 mg of vitamin E supplementation. Figure 2 shows the effect of supplemented vitamin E on the concentration of total IgE, as measured by the sandwich ELISA method. The highest level of total IgE was found in the mice with 5 mg of vitamin E supplementation.

Combined effect of vitamin E and β -carotene on OVA-specific IgE production

The body weight gain of the mice fed with a diet containing vitamin E and/or β -carotene was no different from those fed on the basal diet containing vitamin E as α -tocopherol acetate (5 mg). Figure 3 shows a typical example of the effect of supplement-

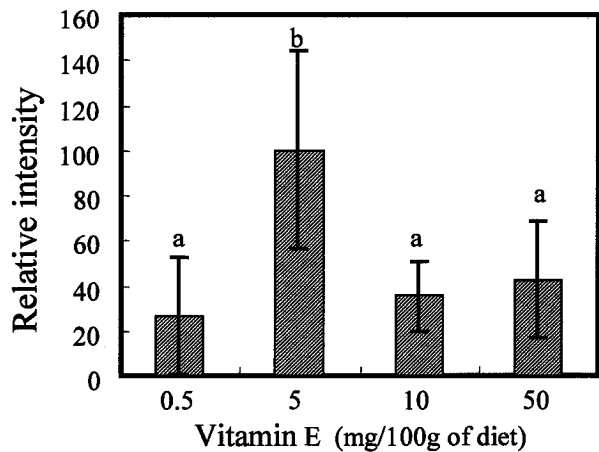


Fig. 2. Effect of Supplementation with Vitamin E on Total IgE Production.

Total IgE was estimated by using a standard curve of mouse IgE hybridoma. These values show the relative intensity to that of 5 mg of vitamin E group. Bars with different letters are significantly different ($P < 0.05$) as determined by the Bonferroni/Dunn multiple-comparison test.

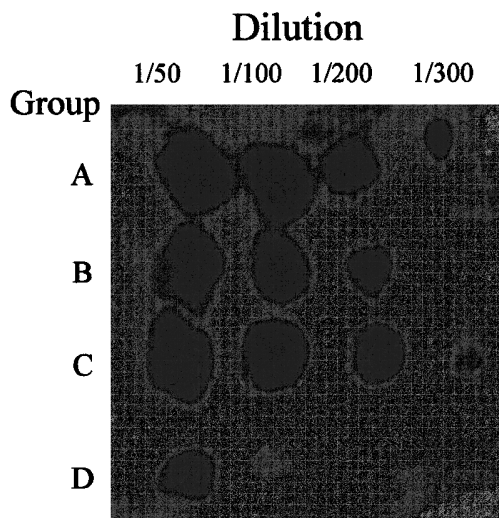


Fig. 3. PCA Reaction in the Mice Supplemented with Vitamin E and/or β -Carotene.

Mixed sera of mice immunized with OVA were analyzed. Mice were fed with the basal diet containing vitamin E as α -tocopherol acetate (5 mg/100 of g diet) (group A) or the diet supplemented with *dl*- α -tocopherol at 5 mg/100 g of diet (group B), β -carotene at 50 mg/100 g of diet (group C) or *dl*- α -tocopherol at 5 mg plus β -carotene at 50 mg/100 g of diet (group D). The dilution (1:X) of the sera is designated on the upper side.

ing vitamin E and/or β -carotene on the PCA assay for measuring serum OVA-specific IgE production. As already described, we confirmed its reproducibility by repeated experiments. Supplementation with β -carotene alone exerted no effect on the IgE production (group C), as indicated by the fact that blue spot clearly emerged in 300-times-diluted sera similarly to the control case (group A; basal diet containing

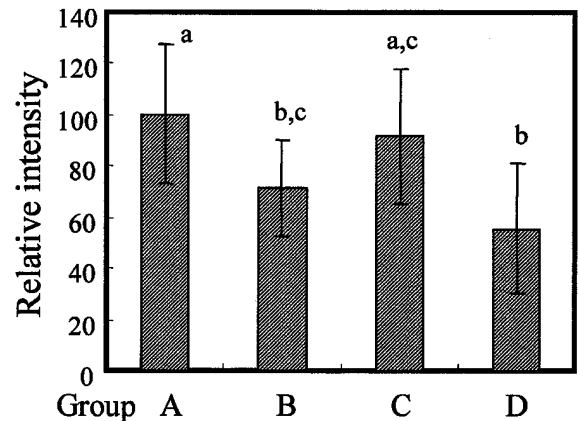


Fig. 4. Effect of Supplementation of Vitamin E and/or β -Carotene on Total IgE Production.

Total IgE was estimated by using a standard curve of mouse IgE hybridoma. These values show the relative intensity to that of group A. The total amounts of vitamin E and β -carotene per 100 g of diet were 5 mg (α -tocopherol acetate) and 0 mg (β -carotene) (group A), 10 mg (5 mg of α -tocopherol acetate plus 5 mg of α -tocopherol) and 0 mg (group B), 5 mg (α -tocopherol acetate) and 50 mg (β -carotene) (group C), 10 mg (5 mg α -tocopherol acetate plus 5 mg of α -tocopherol) and 50 mg (β -carotene) (group D). Bars with different letters are significantly different ($P < 0.05$) as determined by the Bonferroni/Dunn multiple-comparison test.

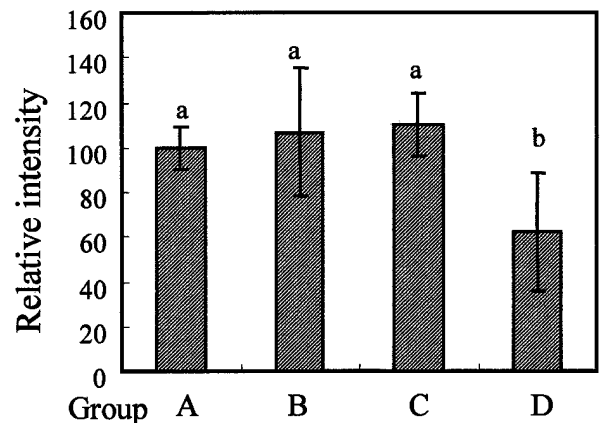


Fig. 5. Effect of Supplementation of Vitamin E and/or β -Carotene on OVA-Specific IgG₁ Production.

These values show the relative intensity to that of group A. The total amounts of vitamin E and β -carotene per 100 g of diet were 5 mg (α -tocopherol acetate) and 0 mg (β -carotene) (group A), 10 mg (5 mg of α -tocopherol acetate plus 5 mg of α -tocopherol) and 0 mg (β -carotene) (group B), 5 mg (α -tocopherol acetate) and 50 mg (β -carotene) (group C), 10 mg (5 mg of α -tocopherol acetate plus 5 mg of α -tocopherol) and 50 mg (β -carotene) (group D). Bars with different letters are significantly different ($P < 0.05$) as determined by the Bonferroni/Dunn multiple-comparison test.

vitamin E as α -tocopherol acetate). In contrast, combined supplementation with the two compounds gave no spot in the 200-times-diluted sera (group D), although a substantial spot appeared in the case of supplementation with vitamin E alone (group B).

Table 3. Concentration of α -Tocopherol and β -Carotene in the Sera

	Group			
	A*	B*	C*	D*
α -tocopherol (μ M)	13.85 $\pm$ 0.75 ^a	19.28 $\pm$ 2.13 ^b	12.88 $\pm$ 0.39 ^a	19.58 $\pm$ 3.00 ^b
β -carotene (nm)	n.d.	n.d.	13.72 $\pm$ 6.89 ^a	19.02 $\pm$ 11.48 ^a

Each value is the means $\pm$ SD, n = 5.

Data were analyzed by the Bonferroni/Dunn multiple-comparison procedure ($P < 0.001$).

A* 5 mg of α -tocopherol acetate, B* 5 mg of α -tocopherol acetate plus 5 mg of α -tocopherol, C* 5 mg of α -tocopherol acetate plus 50 mg of β -carotene, D* 5 mg of α -tocopherol acetate plus 50 mg of β -carotene.

Thus, vitamin E and β -carotene cooperatively suppressed serum OVA-specific IgE production.

Effect of vitamin E with or without β -carotene on the total IgE and OVA-specific IgG₁ production

In the experiment with vitamin E and/or β -carotene, the mice given both vitamin E and β -carotene showed a lower serum total IgE concentration than those given the basal diet alone (Fig. 4). However, supplementation with vitamin E alone or with β -carotene alone had a little effect on total IgE production. Furthermore, the group fed on the diet supplemented with both vitamin E and β -carotene showed a lower antigen specific IgG₁ level in the serum than the other groups (Fig. 5). Nevertheless, there was no difference in the antigen specific IgG₁ level among the control group, vitamin E alone group and β -carotene alone group.

Serum concentration of vitamin E and β -carotene

In the feeding experiment with vitamin E and/or β -carotene, the basal diet was supplemented with α -tocopherol acetate in the vitamin mixture at the level of 5 mg/100 g of diet. Thus, vitamin E was supplied to all the groups, of which the groups supplemented with vitamin E alone (group B) and with both vitamin E and β -carotene (group D) had the higher vitamin E concentration (Table 3). Serum β -carotene was only detected in the groups supplemented with β -carotene alone (group C) and with vitamin E plus β -carotene (group D).

Discussion

It has been well documented that IgE antibodies play an essential role in mediating the type I hypersensitivity response in an allergic reaction.¹¹⁻¹³ In this study, we examined the effect of vitamin E on the allergen-specific and total IgE production by an animal model, because vitamin E has been suggested to act as a modulator of the immune function. The conventional diet for animal studies contains vitamin E (α -tocopherol acetate or α -tocopherol nicotinate) at a level of 5 mg/100 g of diet as shown in our second experiment. Therefore, the result from our first experiment clarifies that both a low vitamin E diet and

high vitamin E diet suppressed the antigen-specific and total serum IgE production more than the conventional vitamin E diet (5 mg/100 g of diet) (Figs. 1 and 2). Lower IgE production in the case of the low vitamin E diet (0.5 mg per 100 g of diet) may be explained by impairment of the immune function due to vitamin E deficiency. On the other hand, antioxidative action of vitamin E is likely to have functioned in suppressing IgE production with the high vitamin E diet (10 and 50 mg per 100 g of diet). Inagaki *et al.*¹⁴ have reported that the administration of vitamin E (α -tocopherol acetate) lowered the serum IgE production in allergenic mice. However, they used aluminum hydroxide as an adjuvant for enhancing the allergenic reaction. It has been suggested that aluminum hydroxide served as a synergistic promoter for lipid peroxidation *in vitro*^{15,16} and that its adjuvant action may be derived from cellular redox regulation.⁸ Therefore, vitamin E may have affected the adjuvant action of aluminum hydroxide when vitamin E and aluminum hydroxide were administered simultaneously. We used no adjuvant in this work in order to identify the direct effect of vitamin E on IgE production. It is thus concluded that the administration of vitamin E could suppress IgE production without affecting the adjuvant action.

IgE production requires the induction of the NF- κ B signaling cascade in B and T lymphocytes or antigen-presenting cells,^{17,18} and ROS promotes this signaling pathway.^{19,20} N-acetylcysteine (NAC), a thiol antioxidant, has been implied to suppress the production of IgE by elevating the cellular GSH/GSSG ratio.²¹ It has been also indicated that a higher GSH level suppressed the induction of Th-2 cytokine which is responsible for the cellular production of IgE.²¹ Cellular reactive oxygen species, either invading from the extracellular fluid or generated within a cell, oxidize GSH, resulting in a decrease in the cellular GSH level.²² Thus, the antioxidative action of vitamin E is likely to have been responsible for elevation of the cellular GSH level and the resulting suppression of IgE production. Alternatively, Li-Weber *et al.*²³ have indicated that vitamin E blocked the binding of transcription factors, NF- κ B and AP-1 to the binding site of the IL-4 promoter, which is

thought to be the essential event for inducing IgE isotype switching. However, further studies are required to understand the action of vitamin E on allergenic symptoms.

In a separate experiment, we found that dietary β -carotene alone exerted no effect on serum IgE production and that supplementation with vitamin E without β -carotene suppressed the IgE production as shown in the results from the first experiment. Nevertheless, the combination of vitamin E and β -carotene substantially blocked both the antigen-specific and total IgE production (Figs. 3 and 4). Although the addition of both vitamin E and β -carotene to the diet suppressed the total IgG₁ production (Fig. 5), the concentration of IgG_{2a} in the serum was affected by neither vitamin E nor β -carotene (data not shown). A similar effect was observed by the addition of vitamin E with β -carotene on the levels of IgE and IgG₁, both Th2-type immunoglobulins. On the other hand, the amount of IgG_{2a}, a Th1-type immunoglobulin, might greatly vary among each animal. This phenomenon may reflect the synergistic antioxidative activity of vitamin E and β -carotene, as indicated by Palozza and Krinsky²⁴⁾ and Niki *et al.*²⁵⁾ One of the authors²⁶⁾ has already reported that vitamin E protected β -carotene from free radical-induced oxidative degradation when the two compounds coexisted. The provitamin A activity of β -carotene, as well as its antioxidative action, may also be included in the combined effect of vitamin E and β -carotene on IgE production. All-*trans* and 13-*cis* retinoic acids, which are β -carotene metabolites, have been reported to inhibit CD40 plus IL-4-mediated IgE production in human peripheral B lymphocytes, although all-*trans* retinoic acid did not show such inhibition in an *in vivo* model using OVA-sensitized mice.^{27,28)} Stephensen *et al.*²⁹⁾ have demonstrated that 9-*cis* retinoic acid enhanced Th2 development *via* the retinoid X receptor pathway. In an experiment using splenocytes from T cell receptor transgenic mice, all-*trans* retinoic acid was found to enhance the T helper 2 cell development.³⁰⁾ These different results do not prompt the notion that retinoic acid participates in IgE production in all cases. It is therefore unclear whether or not dietary β -carotene acts as a provitamin A for inhibiting IgE production. A high intake of an antioxidant may induce a harmful effect by acting as a prooxidant or through an unknown mechanism. However, a high dose of vitamin E and/or β -carotene seemed not to exert any undesirable effect in our experiments, because the growth curve alone was not affected by a higher administration of these antioxidants (data not shown). It is likely that the a higher level of vitamin E in the serum prevented β -carotene from oxidation. Further studies are required to know whether β -carotene acts as a provitamin A and/or an antioxidant.

In conclusion, supplementation of vitamin E with

β -carotene in the diet was capable of effectively suppressing IgE production in allergic model mice. It should be noted that the beneficial effect of dietary antioxidant obtained from animal studies does not promise a practical the application to disease prevention. Nevertheless, the result of this study seem to warrant further studies on the effect of a high intake of plant foods rich in both vitamin E and β -carotene on reducing of the risk of allergic symptoms.

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