Immunomodulating Actions of Carotenoids: Enhancement of *In Vivo* and *In Vitro* Antibody Production to T-Dependent Antigens

Harumi Jyonouchi, Lei Zhang, Myron Gross, and Yoshifumi Tomita

Abstract

Previously, we demonstrated an enhancement of in vitro *antibody (Ab) production in response to T-dependent antigens (TD-Ag) by astaxanthin, a carotenoid without vitamin A activity. The effects of β-carotene, a carotenoid with vitamin A activity, and lutein, another carotenoid without vitamin A activity, on* in vitro *Ab production were examined with spleen cells from young and old B6 mice. In addition, the* in vivo *effects of lutein, astaxanthin, and β-carotene on Ab production were studied in young and old B6 mice.*

Lutein, but not β-carotene, enhanced in vitro *Ab production in response to TD-Ags. The depletion of T-helper cells prevented the enhancement of Ab production by lutein and astaxanthin.* In vivo *Ab production in response to TD-Ag was significantly enhanced by lutein, astaxanthin, and β-carotene. The numbers of immunoglobulin M- and G-secreting cells also increased* in vivo *with the administration of these carotenoids when mice were primed with TD-Ags. Antibody production in response to TD-Ags* in vivo *and* in vitro *was significantly lower in old than in young B6 mice. Astaxanthin supplements partially restored decreased* in vivo *Ab production in response to TD-Ags in old B6 mice. Lutein and β-carotene also enhanced* in vivo *Ab production in response to TD-Ags in old B6 mice, although to a lesser extent than did astaxanthin. However, none of the carotenoids had an effect on* in vivo *or* in vitro *Ab production in response to T-independent antigen.*

These results indicate significant immunomodulating actions of carotenoids for humoral immune responses to TD-Ags and suggest that carotenoid supplementation may be beneficial in restoring humoral immune responses in older animals.

(Nutr Cancer **21**, *47–58, 1994)*

Introduction

Carotenoids demonstrate significant immunomodulating actions and suppress tumorigenesis (1–4). Because certain carotenoids can act as precursors of vitamin A (provitamin A carotenoids) and numerous studies have demonstrated various immunomodulating activities for retinoids, the conversion of carotenoids to retinoids may be a significant factor in this activity (5,6). However, in subjects with adequate vitamin A intake, other properties of

H. Jyonouchi and L. Zhang are affiliated with the Division of Immunology, Department of Pediatrics, and M. Gross is affiliated with the Department of Epidemiology, University of Minnesota, Minneapolis, MN 55455. Y. Tomita is affiliated with the Laboratory of Animal Nutrition and Biochemistry, Animal Science Division, Department of Agriculture, Miyazaki University, Miyazaki, Japan 889-21.

carotenoids may be important for their immunomodulating activities: antioxidant and singlet oxygen-quenching activities and possibly other unknown activities (4). In fact, significant immunomodulating activities are demonstrated by carotenoids without vitamin A activity: canthaxanthin increases mitogen responses and cytokine production *in vivo* (7,8). We showed previously that astaxanthin, a carotenoid without provitamin A activity, enhanced *in vitro* antibody (Ab) production in response to a T-dependent antigen (TD-Ag) in normal B6 mice (9). Our studies also demonstrated that astaxanthin enhances Ab responses by exerting actions in the initial stages of antigen (Ag) priming. This action appears to be exerted partly through T-helper cells and does not facilitate polyclonal B-cell activation in autoimmune-prone NZB and MRL mice (10).

This study examined the effects of carotenoids with and without provitamin A activity on humoral immune responses *in vivo* and *in vitro*. Because humoral immune responses are known to decline with age, the effects of carotenoids were tested in young and old B6 mice. The results showed that at least two carotenoids, without provitamin A activity, enhanced *in vivo* and *in vitro* Ab production. Moreover, these carotenoids partially restored decreased humoral immune responses in old B6 mice.

Materials and Methods

Mice

C57B/6J (B6) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in the animal facility at the University of Minnesota (Minneapolis, MN). They were housed in groups of five mice per cage and fed a nonpurified diet (Purina Lab Chows 5010, Purina Mills, Richmond, IN) containing 23.5% protein, 5.8% fiber, 7.3% ash, 4.5 ppm carotene, and 17.79 kJ/g total digestible nutrients. The carotene content of this diet provides approximately 4.5 times the requirement of vitamin A for mice (11). The mice were killed in a CO_2 chamber, as approved by the Laboratory Animal Medicine Ethics Committee at the University of Minnesota. Unless otherwise stated, young female mice (2–3 mos old) were used in the experiments. Old mice employed in this study were all retired female breeders (>11 mos old, Jackson Laboratory).

Cell Suspensions

Single cell suspensions were prepared by crushing the spleen between two sterile glass slides and suspending cells in RPMI 1640 with 50 ml/l calf serum (CS). Debris was removed by passing cell suspensions through coarse filters. $CD4^+$ T-helper cells were depleted by treating cells with monoclonal rat anti-mouse L3T4 Ab specific for murine CD4 molecule (12,13) and complement (C') (Pel-Freeze, Rogers, AR). Namely, spleen cells were incubated with anti-L3T4 Ab for 30 minutes on ice, washed well with phosphate-buffered saline (PBS), incubated with C' (1:10) at 37°C for 45 minutes, and then washed with PBS. Less than 1% of $CD4^+$ T cells were present after this procedure.

In Vitro Ab Production Assay by Plaque Formation

Spleen cell suspensions were washed twice in PBS with CS (50 ml/l) and resuspended in a medium [RPMI 1640, CS (100 ml/l), 2-mercaptoethanol (2-ME) (10^{-6} mol/l), 10^5 U/l penicillin G, 100 mg/l streptomycin, and 25 mmol/l *N*-2-hydroxypiperazine-*N'*-2-ethanesulfonic acid]. Cells were Ag primed in a 7% CO_2 incubator at 37°C for five days (9). Sheep red blood cells (SRBC, 0.5 ml/l; Colorado Serum, Denver, CO) and Keyhole limpet hemocyanin modified with trinitrophenol (TNP-KLH, 50 mg/l), as described previously (10), were used as

representatives of TD-Ag, and lipopolysaccharide modified with trintrophenol (TNP-LPS, 2 mg/l; Sigma Chemical, St. Louis, MO) was used as a representative of T-independent antigen (TI-Ag) (14). Cells were harvested, resuspended in complete Hanks' balanced salt solution (HBSS), and subjected for plaque formation cell (PFC) assay as described previously (9). Namely, 50 μl of SRBC and 100 μl of harvested spleen cells were added quickly to tubes containing 0.5 ml of preheated agar solution [5 g/l of agar (Sigma Chemical) in HBSS], mixed well, and plated on microscope slides. The slides with solidified agar-cell mixtures were immersed in SRBC-preabsorbed guinea pig complement solution (Pel-Freeze). After incubation at 37°C for three to four hours, the plaques that developed were counted under a dissecting microscope. When cells were primed with SRBC, SRBC in HBSS (300–400 ml of packed SRBC/l) were used for the PFC assay. When cells were primed with TNP-LPS or TNP-KLH, TNP-modified SRBC in HBSS (400 ml of packed SRBC/l) were used. TNP-modified SRBC were prepared as described previously (10). The numbers of PFC were expressed per 10^6 viable cells. Dilutions of these carotenoids were prepared with culture media during the experiment. All carotenoids and control ethanol were added at Day 0 of the culture of Ag priming, which was found to be most effective in the previous study (10). The organic solvent [ethanol (0.1 ml/l)] employed in this study did not alter the results of the *in vitro* bioassay used in this study, as reported elsewhere (9,10).

Enzyme-Linked Immunosorbent Assay for Immunoglobulins G and M

Immunoglobulin (Ig) levels in the culture supernatant were detected by a solid-phase enzyme-linked immunosorbent assay (ELISA) (15). A 96-well flat-bottomed microtiter plate (F96 Maxisorp, Nunc, Naperville, IL) was coated with goat anti-mouse Ig (5 mg/l) overnight in 0.1 mol/l of $NaHCO_3$ coating buffer, pH 9.6, with 0.2 g/l of NaN_3. After the plate was washed with rinse buffer (PBS, pH 7.4, 0.5 ml/l Tween 20), samples were diluted with buffer [0.05 M tris(hydroxymethyl)aminomethane (pH 8.1), 1 mmol/l of $MgCl_2$, 0.15 mol/l of NaCl, 0.5 ml/l of Tween 20, 0.2 g/l of NaN_3, and 10 g/l of bovine serum albumin (BSA)] and incubated at room temperature for two hours. Then the plate was washed with rinse buffer and incubated with a second Ab (goat anti-mouse IgG or IgM-alkaline phosphatase conjugate, 1:1,000 dilution, Sigma Chemical) at room temperature for two hours. The color was developed by adding substrate solution (*p*-nitrophenyl phosphate, 5 mg/tablet, 1 tablet/5 ml, Sigma Chemical). Optical density at 410 nm was read by an ELISA reader (Titertek Multiskan MCC/340, Titertek, Pittsburgh, PA).

In Vivo Ab Production Assay

Each B6 mouse was primed by an intraperitoneal injection of Ag (0.5 ml) and sacrificed five to six days after the Ag challenge. Spleen cells obtained as described above were analyzed by the PFC assay in the presence of SRBC or TNP-modified SRBC. The amount of SRBC (10 ml/l, 0.5 ml/mouse), TNP-KLH (200 μg/mouse), and TNP-LPS (10 μg/mouse) was based on previous studies (16). PFC numbers were determined by employing triplicate spleen cell samples from each mouse and expressed as number of PFC per 10^6 viable cells or per spleen (means ± SE). Just before use, carotenoids were diluted with RPMI 1640 supplemented with 100 ml/l of CS to be 10^{-6} mol/l and 0.5 ml per mouse was given (5×10^{-10} mol/mouse). An amount of ethanol similar to that used for dissolving carotenoids (0.5 ml of 10 ml/l ethanol in RPMI 1640 with 100 ml/l CS) was given intraperitoneally to control B6 mice. They were subjected for PFC assay five to six days later. These controls produced numbers of PFC comparable to those produced by B6 mice primed with Ag and receiving no other injections. When mice were not primed with Ag, plaque formation was not detectable.

Enzyme-Linked Immunospot Assay for IgM- and IgG-Secreting Cells

Spleen cells were adjusted to 10^9 cells/l in Iscove's modified Dulbecco medium supplemented with 5 ml/l of fetal CS, 10^5 U/l of penicillin G, 100 mg/l of streptomycin, and 2 mmol/l of glutamine. The cells (100 µl/well) were incubated overnight at 37°C in a 5% CO_2 incubator in a flat-bottomed 96-well microtiter plate (Costar, Cambridge, MA) coated with goat anti-mouse Ig (5 mg/l) and preincubated with a blocking buffer (PBS, pH 7.4 with 10 g/l of BSA) for 30 minutes at 37°C (100 µl/well). On the next day, cells were removed by vigorous washing, and goat anti-IgG or IgM antibody-alkaline phosphatase conjugate (1:1,000 in dilution buffer used for ELISA; 100 µl/well) was added to the well. The plate was incubated overnight at 4°C and washed to remove unbound antibody. Bound antibody was detected by adding gel substrate solution (75 mg/l of 5-bromo-4-chloro-3-indolyl phosphate and 150 mg/l of nitroblue tetrazolium chloride in 50 mmol/l of $NaHCO_3$, pH 9.8, with 5 mmol/l of $MgCl_2$ and 5 g/l of agar, 100 µl/well) and incubating the plate at 37°C for four hours (17,18). Each spot was considered to correspond to a cell secreting IgM or IgG. The numbers of IgM- and IgG-secreting cells were determined separately and expressed as means ± SE per spleen. Triplicate samples from each mouse were used.

Reagents

KLH (Boehringer Mannheim, Indianapolis, IN) was modified in our laboratory as reported previously (10). Crystalline forms of astaxanthin and β-carotene were kindly provided by Hoffmann-LaRoche (Nutley, NJ). Lutein was solubilized in our laboratory, as reported elsewhere (19), and its purity (>97%) was determined as described previously (20). Stock solutions (10^{-4} mol/l) of astaxanthin and β-carotene in absolute ethanol were prepared on the day of each experiment and kept in the dark, and the concentration was determined by spectrophotometer (20). Crystalline forms of carotenoids were removed before the concentration was determined by passing the stock solution through the filter (0.22 µm, Milipore, Bedford, MA) before the concentration was measured.

Statistics

Statistical analyses were performed with Student's t test or Welch's test based on the results of the F test; $p < 0.05$ was considered to be significant.

Results

Effects of Various Carotenoids on In Vitro Ab Production in Old and Young B6 Mice

The effects of astaxanthin, lutein, and β-carotene on *in vitro* Ab production in response to SRBC were tested with spleen cells from young and old B6 mice (Table 1). The concentration of carotenoids (10^{-8} mol/l) used in these experiments was optimal for astaxanthin in this assay (9).

Astaxanthin enhanced PFC formation in young B6 mice, as reported previously (9,10). Lutein supplements resulted in a statistically significant enhancement of *in vitro* Ab production. β-Carotene did not show significant enhancing actions. Spleen cells from old B6 mice produced significantly less PFC in response to SRBC than those from young B6 mice. The addition of lutein and astaxanthin enhanced PFC formation, although it was not as high as that observed in young B6 mice (Table 1). When TNP-KLH, another TD-Ag, was used to prime the spleen cells, similar results were obtained (data not shown). Total IgM and IgG

Table 1. Effects of Various Carotenoids on *In Vitro* Ig and Ab Production in Response to SRBC in Young and Old B6 Mice[a,b]

		In Vitro Ab Production Assay			
	n	Control	Astaxanthin	Lutein	β-Carotene
PFC/10^6 cells					
Young	6	132 ± 25	377 ± 55*	239 ± 30†	125 ± 26
Old	6	23 ± 5	89 ± 23†	64 ± 18†	34 ± 8
IgM, mg/l					
Young	5	229.8 ± 48.3	206.3 ± 409	94.7 ± 33.6	200.8 ± 29.3
IgG, mg/l					
Young	5	98.5 ± 16.0	85.0 ± 14.1	97.6 ± 29.8	124.8 ± 31.7

a: *In vitro* antibody (Ab) production in response to sheep red blood cells (SRBC) was assessed by plaque cell formation (PFC) assay in each mouse, and results are expressed as no. of PFC/10^6 viable cells (means ± SE). Immunoglobulin (Ig) levels in culture supernatant are expressed as mg/l (means ± SE). Data represent a summary of 6 experiments; *n*, no. of mice. Young B6 mice were 2–3 mos old and old B6 mice were 11–12 mos old. Final concentration of carotenoids in culture medium was 10^{-8} mol/l.

b: Statistical significance is as follows: *, $p < 0.005$ compared with control (Student's *t* test); †, $p < 0.05$ compared with control (Student's *t* test).

production were not altered by these carotenoids in young and old mice, consistent with our previous findings (10).

Dose Response of Lutein to In Vitro Ab Production

We determined PFC numbers produced by SRBC-primed spleen cells from young and old B6 mice in the presence of various doses of lutein in young and old B6 mice. The results of five experiments are summarized in Figure 1. Lutein exerted its maximal action at 10^{-8} mol/l. The dose response for lutein was similar to that previously observed for astaxanthin (9). In old B6 mice, lutein also enhanced *in vitro* Ab production by their spleen cells at 10^{-8} mol/l. β-Carotene did not appear to exert significant enhancing actions on *in vitro* Ab production at 10^{-7}–10^{-9} mol/l (data not shown).

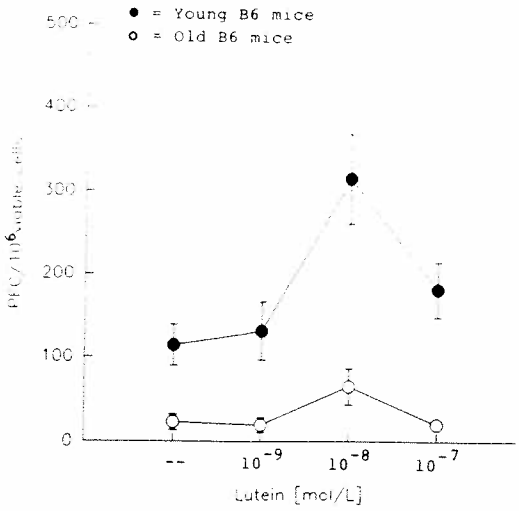

Figure 1. Changes of plaque formation cell (PFC) numbers in presence of lutein (10^{-9}–10^{-7} mol/l) in young and old B6 mice when spleen cells were primed with sheep red blood cells *in vitro*. Values are results of 5 experiments; 1 young B6 mouse and 1 old B6 mouse were used in each experiment.

Effects of T-Helper Cell Depletion From Spleen Cells

We examined the effects of astaxanthin and lutein on T-helper cell-depleted spleen cell suspensions. Spleen cells from young B6 mice were depleted of T-helper cells. Then cells were primed with SRBC in the astaxanthin- or lutein- (10^{-8} mol/l) supplemented medium. PFC numbers were compared with those of control untreated spleen cells. The results are shown in Figure 2. We obtained similar results in two additional experiments. Neither astaxanthin nor lutein exerted any enhancing action on PFC formation in the absence of T-helper cells. This is consistent with our previous observation that the removal of T cells abolished the enhancing action of astaxanthin on *in vitro* Ab production against SRBC (10).

Effects of Carotenoids on Specific Ab Production In Vivo

We initially tested the effects of astaxanthin, β-carotene, and lutein on *in vivo* Ab production in young B6 mice. The amount of carotenoids (10^{-6} mol/l, 0.5 ml/mouse ip) was chosen on the basis of the results obtained in our previous *in vitro* experiments. Young B6 mice were primed with SRBC (10 ml/l in PBS, 0.5 ml/mouse), and carotenoids were given intraperitoneally one hour before the Ag priming. Five days later, the mice were killed, and spleen cells were analyzed by PFC assay. The results of three experiments are summarized in Figure 3. Spleen weights and cell numbers were not significantly different between controls and carotenoid-injected mice. Mice given astaxanthin produced significantly higher numbers of PFC ($p < 0.001$) than controls. Lutein and β-carotene also enhanced *in vitro* Ab production (to SRBC) significantly ($p < 0.05$) compared with controls (Figure 3). These carotenoids exerted similar enhancing actions on TNP-KLH, another TD-Ag, in two experiments (data not shown). Young B6 mice were also primed with TNP-LPS, a TI-Ag (Table 2). We observed no enhancing action of the carotenoids on *in vivo* Ab production in response to TNP-LPS. These carotenoids moderately suppressed PFC formation against TNP-LPS (Table 2).

Effects of Carotenoids on Numbers of IgM- and IgG-Secreting Cells In Vivo

We also tested the effects of astaxanthin, lutein, and β-carotene on total numbers of Ig-secreting cells *in vivo*. Namely, young B6 mice were primed with SRBC for five days *in vivo;* then spleen cells were harvested, and ELISPOT assay was used to detect nonspecific

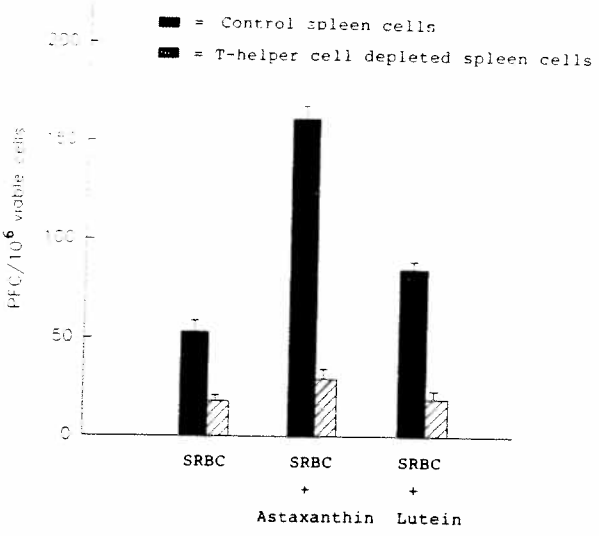

Figure 2. Murine spleen cells from 2 young B6 mice were primed with sheep red blood cells (SRBC) *in vitro* in presence of medium only, astaxanthin (10^{-8} mol/l), or lutein (10^{-8} mol/l). Spleen cells were either unseparated or depleted of T-helper cells. Results are from 1 representative experiment.

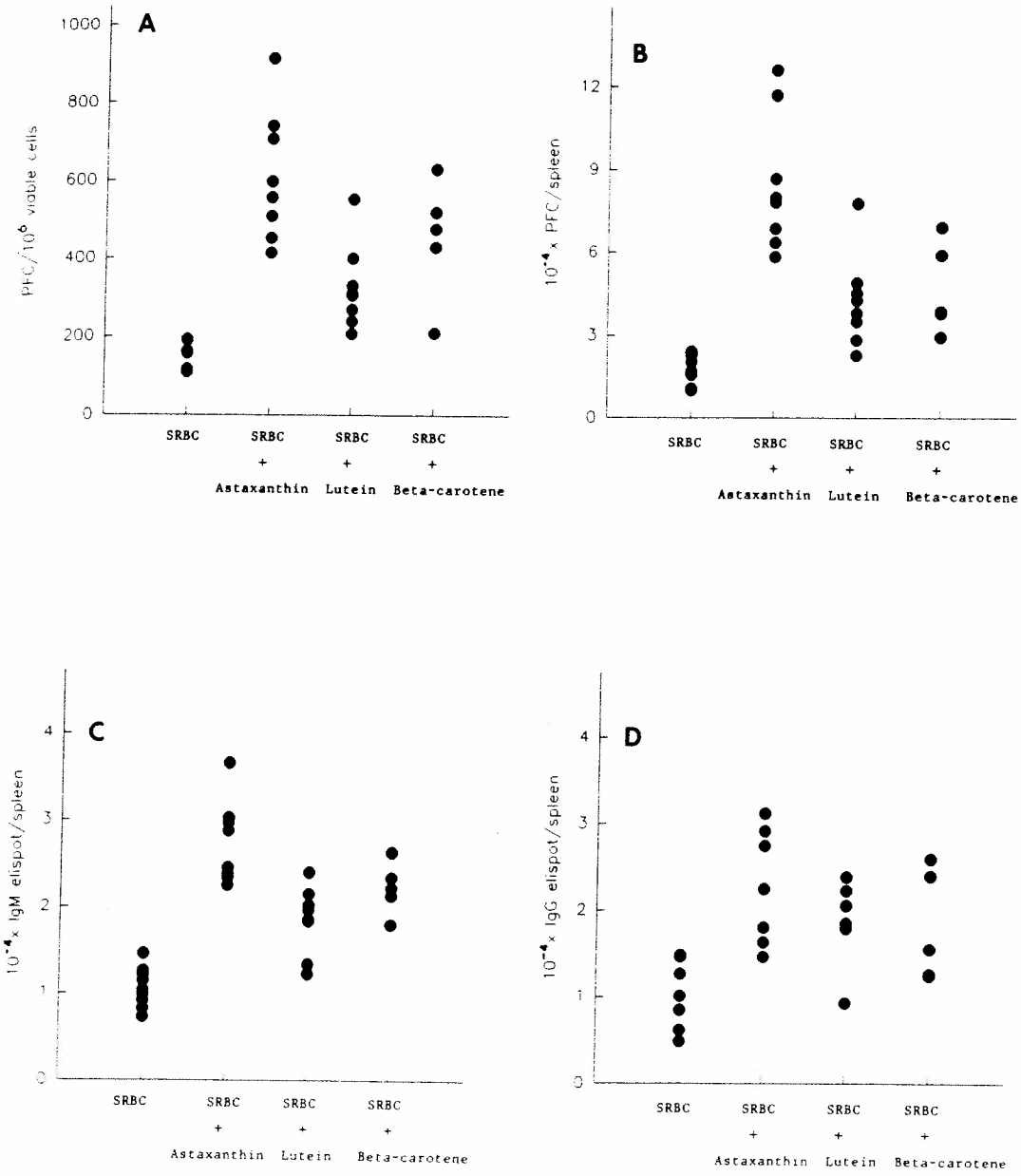

Figure 3. Effects of carotenoids on specific antibody production to SRBC *in vivo*. Each young B6 mouse (8 wk old) was given carotenoids (10^{-6} mol/l, 0.5 ml/mouse ip) on day of antigen priming, and after 5 days spleen cells were subjected to PFC assay. Control mice were given same amount of ethanol to dissolve carotenoids (10 ml/l, 0.5 ml/mouse). PFC numbers are expressed per 10^6 spleen cells (A) or per spleen (B). Numbers of IgM- (C) and IgG- (D) secreting cells in spleen are expressed per spleen. ELISPOT, enzyme-linked immunospot. Results are from 3 experiments; 8–12 young B6 mice were used in each experiment. Astaxanthin, lutein, and β-carotene significantly enhanced PFC formation compared with controls: astaxanthin, $p < 0.001$; lutein and β-carotene, $p < 0.05$. Increase of IgM-secreting cells by astaxanthin, lutein, and β-carotene compared with controls was significant ($p < 0.001$) (C), and so was increase of IgG-secreting cells by these carotenoids ($p < 0.005$) (D).

Table 2. Effects of Carotenoids on *In Vivo* Ab/Ig Production in Response to TNP-LPS and TI-Ag[a–c]

	In Vivo Ab and Ig Production Assay			
	Control	Astaxanthin	Lutein	β-Carotene
PFC	7.7 ± 1.1	4.3 ± 0.8*	5.6 ± 0.9	4.0 ± 0.8*
IgM ELISPOT	8.0 ± 0.7	9.0 ± 1.6	6.2 ± 0.7	6.4 ± 1.3
IgG ELISPOT	2.3 ± 0.4	4.7 ± 0.9*	5.1 ± 1.0*	4.2 ± 0.9

a: B6 mice were primed with trinitrophenol-lipopolysaccharide (TNP-LPS, 15 μg/mouse ip), and after 5 days spleen cells were obtained and subjected to PFC and enzyme-linked immunospot (ELISPOT) assay. The same amounts of carotenoids (10^{-6} mol/l, 0.5 ml/mouse) were given 1 hr before challenge with T-independent antigen (TI-Ag). Control mice were given same amount of ethanol to dissolve carotenoids. Results are a summary of 2 experiments; in each experiment, 12 mice (3/group) were employed.

b: Values are means ± SE × 10^4/spleen obtained in 6 mice in each group.

c: Statistical significance is as follows: *, significantly lower or higher than control ($p < 0.05$).

IgM- and IgG-secreting cells. Carotenoids were given on the day of Ag priming (carotenoids: 10^{-6} mol/l, 0.5 ml/mouse ip). Control mice were given organic solvent to dissolve carotenoids (10 ml/l of ethanol, 0.5 ml/mouse). The results of three experiments are also summarized in Figure 3. The numbers of IgM-secreting cells detected by ELISPOT assay increased significantly when mice were given astaxanthin and lutein ($p < 0.001$). However, the effect of astaxanthin appeared to be greater than that of lutein ($p < 0.01$). β-Carotene also increased the numbers of IgM-secreting cells as significantly as did astaxanthin *in vivo* ($p < 0.001$). All these carotenoids increased the numbers of IgG-secreting cells significantly compared with the controls ($p < 0.005$). The effects on the numbers of IgG-secreting cells were not statistically different among the carotenoids tested.

We also examined the changes in the numbers of IgM- and IgG-secreting cells by these carotenoids when mice were primed with TNP-LPS, because carotenoids seemed to moderately suppress *in vitro* PFC formation potentiated by TNP-LPS (Table 2). Carotenoids did not significantly alter the numbers of IgM-secreting cells *in vivo* in response to TNP-LPS but appeared to increase the numbers of IgG-secreting cells. When the same amount of LPS, a nonspecific B-cell activator, was given intraperitoneally to B6 mice, almost no PFC was detected in the spleen, but the numbers of IgM-secreting cells increased significantly (data not shown). This may reflect a nonspecific action of LPS as a polyclonal B-cell activator. This action of LPS was not affected by the supplementation of these carotenoids *in vivo*.

Effects of Carotenoids on Old B6 Mice In Vivo

Old B6 mice were primed with SRBC *in vivo*, and astaxanthin, β-carotene, and lutein (10^{-6} mol/l, 0.5 ml/mouse ip) were given one hour before the Ag priming (Table 3). Spleen cells from old mice formed less PFC than those from young B6 mice. This is consistent with the results obtained in our *in vitro* studies presented here. The administration of astaxanthin, lutein, and β-carotene at the time of Ag priming partially restored the Ab production in old B6 mice *in vivo*. The numbers of IgM- and IgG-secreting cells detected by the ELISPOT assay appeared to increase as well, in parallel to the increase of PFC numbers in response to SRBC. These carotenoids also partially restored the responses to TNP-KLH, another TD-Ag, in old B6 mice (data not shown).

Table 3. Effects of Carotenoids on *In Vivo* Ab/Ig Production in Response to SRBC in Old B6 Mice[a–c]

	In Vivo Ab/Ig Production Assay			
	Control	Astaxanthin	Lutein	β-Carotene
PFC	0.9 ± 0.1	2.6 ± 0.5*	1.9 ± 0.2†	1.7 ± 0.2‡
	(1.7 ± 0.5)	(8.5 ± 2.5)	(4.2 ± 1.7)	(4.7 ± 1.7)
IgM ELISPOT	0.8 ± 0.1	1.4 ± 0.1§	1.0 ± 0.1	0.9 ± 0.1
	(1.0 ± 0.2)	(2.8 ± 0.5)	(1.8 ± 0.4)	(2.2 ± 0.3)
IgG ELISPOT	0.20 ± 0.02	0.52 ± 0.05*	0.37 ± 0.05‡	0.29 ± 0.04
	(1.0 ± 0.2)	(2.3 ± 0.3)	(1.9 ± 0.3)	(1.1 ± 0.2)

a: Old (11- to 12-mo-old) B6 mice were primed with SRBC (10 ml/l in PBS, 0.5 ml/mouse) and given carotenoids (10^{-6} mol/l, 0.5 ml/mouse) 1 hr before Ag challenge.

b: Values are means ± SE × 10^4/spleen of 4 mice/group. Values in parentheses are aggregate data from young B6 mice (9–10/group), and some of these data are shown in Fig. 3.

c: Statistical significance is as follows: *, significantly higher than controls ($p < 0.05$); †, significantly higher than controls ($p < 0.005$); ‡, significantly higher than controls ($p < 0.02$); §, significantly higher than controls ($p < 0.01$).

Discussion

Our previous studies and those of others demonstrate that carotenoids without provitamin A activity exert significant immunomodulating actions (1,2,7–10,21). We showed that astaxanthin, a carotenoid without provitamin A activity, significantly enhances *in vitro* Ab production in response to TD-Ag, perhaps by exerting its effects during the initial stages of Ag presentation (9,10). This study was undertaken to further address the actions of carotenoids without provitamin A activity on humoral immune responses in young and old mice *in vivo* and *in vitro*. We employed carotenoids commonly found in American and Japanese diets. Shellfish are rich in astaxanthin, and green vegetables are rich in lutein (22). β-Carotene, which is widely distributed in yellow-green vegetables, was also employed as a representative of carotenoids with provitamin A activity.

The results demonstrate that astaxanthin and lutein enhance *in vitro* Ab production in response to TD-Ag, but not TI-Ag. The dose response of lutein was similar to that previously obtained for astaxanthin (9): it was most effective at approximately 10^{-8} mol/l. Considering the fact that plasma levels of carotenoids in humans are around 10^{-6} mol/l (19), this relatively low optimal concentration may be due to the availability of added carotenoids. Most carotenoids in human plasma are bound to lipoprotein; the concentration of free carotenoids that are responsible for their immunomodulating actions may be much lower (23). Similar concentrations of carotenoids are optimal in the other tissue culture system (24). Higher amounts of astaxanthin through intraperitoneal injection were also found to be ineffective in enhancing tumor immunity (Y. Tomita et al., personal communication).

We also examined the actions of lutein, astaxanthin, and β-carotene on Ab production *in vivo*. A significant increase of PFC numbers is observed with the administration of lutein and astaxanthin in mice challenged with TD-Ag (SRBC and TNP-KLH). β-Carotene, which does not demonstrate consistent enhancing action on *in vitro* Ab production, significantly enhanced Ab production in response to TD-Ag *in vivo*. The number of IgM- and IgG-secreting cells also appeared to increase in parallel to the increase in number of PFC. It may be concluded that carotenoids can significantly enhance Ab production in response to TD-Ag *in vivo* and *in vitro* in mice. This activity may be due to their antioxidant and singlet oxygen-quenching properties (4,25–29). The more prominent enhancing action of β-carotene on specific Ab production in response to TD-Ag *in vivo* may also be related to its provitamin A activity, but this requires further investigation.

In this study we have shown that 1) lutein increases Ab production in response to TD-Ag *in vivo* and *in vitro;* 2) astaxanthin and lutein require the presence of T-helper cells *in vitro* to exert their enhancing actions, which may indicate a selective action on T-helper cells; 3) these carotenoids did not augment Ab production in response to a TI-Ag but rather suppressed it; their action on humoral immune responses may be selective and dependent on the type of Ag; and 4) these carotenoids did not significantly increase total IgM or IgG concentrations in the culture supernatant. These results point to the importance of further pursuing delineation of carotenoids' actions on T-helper cells and antigen-presenting cells. We are now studying their actions on cloned Ag-specific type 1 and type 2 T-helper cells (30).

In our previous *in vitro* studies, carotenoids did not facilitate polyclonal B-cell activation in autoimmune-prone NZB and MRL mice or in response to LPS, a nonspecific B-cell activator (14). In this study, the *in vivo* Ab production in response to TNP-LPS, a TI-Ag, is suppressed by intraperitoneal administration of carotenoids. TNP-LPS is a carbohydrate antigen and may retain its ability as an endotoxin. It may induce nonspecific polyclonal B-cell activation, resulting in the production of low-affinity polyclonal IgM Ab (31). Thus, a part of the PFC formed by the challenge of TNP-LPS *in vivo* may result from polyclonal B-cell activation, but not from specific Ab responses. The carotenoids may suppress nonspecific polyclonal B-cell activation by TNP-LPS. The fact that most of the Ig-secreting cells from mice primed with TNP-LPS produced IgM may support this speculation. None of the carotenoids tested significantly decreased the numbers of IgM-secreting B cells. However, carotenoids did tend to increase the numbers of IgG-secreting cells in response to TNP-LPS. This indicates that these carotenoids may exert actions on isotype switching to facilitate the production of high-affinity specific IgG antibodies.

In aged individuals, decreases in specific Ab responses are commonly seen and have been attributed to the immunodysregulation associated with aging, most likely secondary to decreased T-helper cell functions (32,33). This may be one of the reasons for an increase in incidence of autoimmunity and malignancy in aged individuals (32,33). In this study, we found a significant reduction in the ability to produce specific Ab against TD-Ag *in vivo* and *in vitro* in old, compared with young, mice. The administration of astaxanthin, lutein, and β-carotene *in vivo* partially restores their ability to produce specific Ab against TD-Ag. It may thus be speculated that dietary carotenoids can help restore specific Ab responses in older humans, perhaps through affecting T-helper cells.

Our finding that astaxanthin consistently and significantly enhances specific Ab production *in vitro* and *in vivo* is also of interest, because we previously reported the augmentation of tumor immunity by astaxanthin (21). Furthermore we observed previously that astaxanthin significantly delays the development of autoimmunity in autoimmune-prone MRL/lpr/lpr mice (34). This autoimmunity is thought to result from abnormal T cells (35). It may be speculated that dietary carotenoids could reduce the chance of developing autoimmunity and malignancies by enhancing T-helper functions and promoting specific Ab responses. It would be interesting to examine the relationship between the incidence of autoimmunity and malignancy and the consumption of a diet rich in carotenoids in the general population.

Acknowledgments and Notes

The authors thank H. Reed for secretarial help in preparing the manuscript. This study was supported by National Institute on Aging Grant AI-25064 from the National Institutes of Health (Bethesda, MD); grants from Nikken SoHonsha (Gifu, Japan), Viking Children's Fund (Minneapolis, MN), and the Graduate School, University of Minnesota (Minneapolis, MN) (to H. Jyonouchi); Biomedical Research Support Grant, University of Minnesota (Minneapolis, MN) (to M. Gross); and a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan (to Y. Tomita). Address reprint requests to: Dr. Harumi Jyonouchi, Box 610 UMHC, University of Minnesota, Dept. of Pediatrics, 420 Delaware St. S.E., Minneapolis, MN 55455.

Submitted 27 May 1993; accepted in final form 30 September 1993.

References

1. Bendich, A: "Carotenoids and the Immune Response." *J Nutr* **119,** 112–115, 1989.
2. Krinsky, NI: "Carotenoids and Cancer in Animal Models." *J Nutr* **119,** 123–126, 1989.
3. Ziegler, RG: "A Review of Epidemiologic Evidence That Carotenoids Reduce the Risk of Cancer." *J Nutr* **119,** 116–122, 1989.
4. Canfield, LM, Forage, JW, and Valenzuela, JG: "Carotenoids as Cellular Antioxidants." *Proc Soc Exp Biol Med* **200,** 260–265, 1992.
5. West, CE, Rombout, JH, van der Zijpp, AJ, and Sijtsma, SR: "Vitamin A and Immune Function." *Proc Nutr Soc* **50,** 251–262, 1991.
6. Ross, AC: "Vitamin A Status: Relationship to Immunity and the Antibody Response." *Proc Soc Exp Biol Med* **200,** 303–320, 1992.
7. Bendich, A, and Shapiro, SS: "Effect of β-Carotene and Canthaxanthin on the Immune Responses of the Rat." *J Nutr* **116,** 2254–2262, 1986.
8. Shklar, G, and Schwartz, J: "Tumor Necrosis Factor in Experimental Cancer Regression With α-Tocopherol, β-Carotene, Canthaxanthin and Algae Extract." *Eur J Cancer Clin Oncol* **24,** 839–350, 1988.
9. Jyonouchi, H, Hill, RJ, Tomita, Y, and Good, RA: "Studies of Immunomodulating Actions of Carotenoids. I. Effects of β-Carotene and Astaxanthin on Murine Lymphocyte Functions and Cell Surface Market Expression in *In Vitro* Culture System." *Nutr Cancer* **16,** 93–105, 1991.
10. Jyonouchi, H, Zhang, L, and Tomita, Y: "Studies of Immunomodulating Actions of Carotenoids. II. Astaxanthin Enhances *In Vitro* Antibody Production to T-Dependent Antigens Without Facilitating Polyclonal B Cell Activation." *Nutr Cancer* **19,** 269–280, 1993.
11. Subcommittee on Laboratory Nutrition: *Nutrient Requirements of Laboratory Animals,* 3rd ed. Washington, DC: Natl Acad Sci, 1978.
12. Wilde, DB, Marrack, P, Kappler, J, Dialynas, DP, and Fitch, FW: "Evidence Implicating L3T4 in Class II MHC Antigen Reactivity: Monoclonal Antibody GK 1.5 (Anti-L3T4a) Blocks Class II MHC Antigen-Specific Proliferation, Release of Lymphokines, and Binding by Cloned Murine Helper T Lymphocyte Lines." *J Immunol* **131,** 2178–2183, 1983.
13. Dialynas, DP, Quan, ZS, Wall, KA, Pierres, A, Quintans, J, et al.: "Characterization of the Murine T Cell Surface Molecules, Designated L3T4, Identified by Monoclonal Antibody GK 1.5: Similarity of L3T4 to the Human Leu-3/T4 Molecules." *J Immunol* **131,** 2445–2451, 1983.
14. Jyonouchi, H, Hill, RJ, Voss, RM, and Ishii, E: "Immunomodulating Actions of RNA and Nucleotides on Murine Lymphocytes *In Vitro.* Augmentation of Antibody Production to T-Dependent Antigens and Expansion of T-Helper Cells." *J Nutr Immunol.* In press.
15. Boom, WH, Liano, D, and Abbas, AK: "Heterogeneity of Helper/Inducer T Lymphocytes. II. Effects of Interleukin 4 and Interleukin 2 Producing T Cell Clones on Resting B Lymphocytes." *J Exp Med* **16,** 1350–1363, 1988.
16. Ishii, E, Gengozian, N, and Good, RA: "Influence of Dimethyl Myelran on Tolerance Induction and Immune Function in Major Histocompatibility Complex-Haploidentical Murine Bone-Marrow Transplantation." *Proc Natl Acad Sci USA* **88,** 8435–8439, 1991.
17. Czerkinsky, C, Nilsson, LA, Nygren, H, Ouchterlony, O, and Tarkowski, A: "A Solid Phase Enzyme-Linked Immunospot (ELISPOT) Assay for Enumeration of Specific Antibody-Secreting Cells." *J Immunol Methods* **65,** 109–121, 1983.
18. Sedgwick, JD, and Holt, PG: "A Solid-Phase Immunoenzymatic Technique for the Enumeration of Specific Antibody-Secreting Cells." *J Immunol Methods* **57,** 301–309, 1983.
19. Stacewicz-Sapuntzakis, M, Bowen, PE, Kikendall, JW, and Burgess, M: "Simultaneous Determination of Serum Retinol and Various Carotenoids: Their Distribution in Middle-Aged Men and Women." *J Micronutr Anal* **3,** 27–45, 1987.
20. Bieri, JG, Brown ED, and Smith JC: "Carotenoids in Human Plasma by High Performance Liquid Chromatography." *J Liquid Chromatogr* **8,** 473–484, 1985.
21. Tomita, Y, Himeno, K, Nomoto, K, Endo, H, and Hirohata, T: "Augmentation of Tumor Immunity Against Syngenetic Tumors in Mice by β-Carotene." *JNCI* **78,** 679–681, 1987.
22. Mangels, AR, Holden, JM, Beecher, GR, Forman, M, and Lanza, E: "The Carotenoid Content of Fruits and Vegetables: An Evaluation of Analytical Data." *J Am Diet Assoc.* In press.
23. Parker, RS: "Carotenoids in Human Blood and Tissues." *J Nutr* **119,** 101–104, 1989.
24. Bertram, JS, Pung, A, Churley, M, Kappock, TJ, Wilkins, LR, et al.: "Diverse Carotenoids Protect Against Chemically Induced Neoplastic Transformation." *Carcinogenesis* **12,** 671–678, 1991.
25. Foote, CS: In *Singlet Oxygen,* H H Wassermann and R W Murray (eds). New York: Academic, 1979, pp 139–173.
26. Terao, J: "Antioxidant Activity of β-Carotene-Related Carotenoids in Solution." *Lipids* **24,** 659–661, 1989.
27. Kurashige, M, Okimasu, E, Inoue, M, and Utsumi, K: "Inhibition of Oxidative Injury of Biological Membranes by Astaxanthin." *Physiol Chem Phys Med NMR* **22,** 27–38, 1990.

28. Palozza, P, and Krinsky, NI: "β-Carotene and α-Tocopherol Are Synergistic Antioxidants." *Arch Biochem Biophys* **297**, 184–187, 1992.
29. Krinsky, NI: "Mechanisms of Action of Biological Antioxidants." *Proc Soc Exp Biol Med* **200**, 248–254, 1992.
30. Mosmann, TR, and Coffman, RL: "TH1 and TH2 Cells: Different Patterns of Lymphokine Secretion Led to Different Functional Properties." *Annu Rev Immunol* **7**, 145–175, 1989.
31. Lynn, WA, and Golenbock, DT: "Lipopolysaccharide Antagonists." *Immunol Today* **13**, 271–276, 1992.
32. Kay, MMB: "Autoimmunity and Aging." *Concepts Immunopathol* **6**, 166–192, 1988.
33. Kay, MMB: "Immunology of Aging." In *Nutrition, Immunity and Illness in the Elderly.* New York: Pergamon, 1985, pp 97–119.
34. Tomita, Y, Jyonouchi, H, Engelman, RW, Day, NK, and Good, RA: "Preventive Action of Carotenoids on the Development of Autoimmunity in MRL-lpr/lpr Mice." *J Autoimmun.* In press.
35. Mountz, JD, Zhou, T, Elidridge, J, Berry, K, and Bluthmann, H: "Transgenic Rearranged T Cell Receptor Gene Inhibits Lymphadenopathy and Accumulation of $CD4^-$ $CD8^-$ $B220^+$ Cells in lpr/lpr Mice." *J Exp Med* **172**, 1805–1817, 1990.