Studies of Immunomodulating Actions of Carotenoids. II. Astaxanthin Enhances *In Vitro* Antibody Production to T-Dependent Antigens Without Facilitating Polyclonal B-Cell Activation

Harumi Jyonouchi, Lei Zhang, and Yoshifumi Tomita

Abstract

Previously we have shown that astaxanthin, a carotenoid without provitamin A activity, enhances in vitro *antibody (Ab) production to sheep red blood cells in normal B6 mice. In this study, we further attempted to examine the mechanisms of this enhancing action of carotenoids on specific Ab production* in vitro *in relation to different antigen (Ag) stimuli, cytokine production, and T- and B-cell interactions in both normal and autoimmune strains of mice. When the actions of carotenoids were tested in normal strains of mice, we found that astaxanthin enhanced* in vitro *Ab production to T cell-dependent Ag, but not to T-independent Ag, and did not augment total immunoglobulin production. Astaxanthin exerted maximum enhancing actions when it was present at the initial period of Ag priming. This action of astaxanthin was abolished when T cells were depleted from spleen cell suspensions and appeared to require direct interactions between T and B cells. The results also indicated that carotenoids may modulate the production of interferon-τ in this assay system. When the actions of carotenoids were tested in autoimmune-prone MRL and NZB mice, the enhancing action of astaxanthin on* in vitro *Ab production was less significant. Furthermore, carotenoids did not potentiate or augment spontaneous Ab and immunoglobulin production by spleen cells in these strains.*

Taken together, carotenoids without provitamin A activity may be able to augment in vitro *specific Ab production to T cell-dependent Ag partly through affecting the initial stage of Ag presentation without facilitating polyclonal B-cell activation or autoantibody production.*

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Introduction

Both epidemiological and animal studies indicate that carotenoids with or without provitamin A activity exert chemoprophylactic actions on certain cancers (1,2). This can be partly attributed to their oxygen-quenching capacity and their provitamin A activity (1–3). However, their actions on the immune system are not thoroughly understood and remain the focus of considerable dispute.

Previously we reported that astaxanthin, a carotenoid without provitamin A activity, can enhance *in vitro* antibody (Ab) production [to sheep red blood cells (SRBC)] in normal B6

H. Jyonouchi and L. Zhang are affiliated with the Division of Immunology, Department of Pediatrics, 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 889-21, Japan.

mice without enhancing lymphocyte proliferation or augmenting interleukin- (IL) 2/IL-4 production (4). This enhancing action of astaxanthin on *in vitro* Ab production may not be beneficial to the state of autoimmunity because it may further augment autoantibody production and polyclonal B cell activation, which are common features of autoimmunity (5). However, in past studies employing autoimmune MRL mice, we found that the dietary supplementation of astaxanthin appears to delay the onset of autoimmunity in this strain (6). In the present study, we further attempted to explore the mechanisms of this enhancing action of carotenoids on *in vitro* Ab production and examined whether carotenoids exacerbated the state of autoimmunity. Namely, we studied the effects of carotenoids on *in vitro* Ab production in relation to antigen (Ag) stimuli, total immunoglobulin (Ig) production, and cytokine production in both normal [mainly C57BL/6 (B6)] and autoimmune-prone [New Zealand Black (NZB) and MRL] mice. β-Carotene and astaxanthin were employed as representatives of carotenoids with and without provitamin A activity, respectively.

The results obtained in this study reveal that astaxanthin enhances *in vitro* Ab production to T cell-dependent Ag (TD-Ag) in normal strains of mice. This enhancing action was abolished by the depletion of T cells. Astaxanthin must be present in the initial stage of Ag priming to exert its action at optimal levels. Both astaxanthin and β-carotene appeared to have no effect on total Ig production and Ig/Ab production potentiated by nonspecific B cell activation or T cell-independent Ag (TI-Ag). Also, they did not enhance spontaneous Ig/Ab production, which is evident in autoimmune strains of mice. Although the production of IL-2/IL-4 appeared not to be significantly altered by carotenoids as reported previously (4), carotenoids may modulate the production of interferon-τ (IFN-τ) in this culture system. Thus this study revealed that carotenoids may augment specific Ab production partly through exerting actions directly or indirectly on T cells, especially T-helper cells.

Materials and Methods

Mice

C57B/6, DBA/2, BALB/c, MRL/lpr/lpr (MRL), and NZB mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in the animal facility at the University of Minnesota (Minneapolis, MN). The mice were housed in groups of five per cage and fed regular laboratory chow (Purina Lab Chows 5010, Purina Mills, Richmond, IN). The chow contained 23.5% protein, 5.8% fiber, 7.3% ash, 4.5 ppm carotene, and 4.25 kcal/g total digestible nutrients. The mice were sacrificed in a CO_2 chamber, as approved by the Laboratory Animal Medicine Ethics Committee at the University of Minnesota (Minneapolis, MN), and their spleens were removed. Unless otherwise stated, two- to three-month-old mice were used in each experiment.

Cell Suspensions

The spleen was removed aseptically, and single cell suspensions were prepared by crushing the spleen between two sterile glass slides. Spleen cells were suspended in RPMI 1640 with 5% calf serum (CS). Debris was removed by passing cell suspensions through coarse filters. T cells were depleted by rosetting cells with neuraminidase-treated SRBC and Ficoll-Hypaque density gradient, as previously reported (7). We observed <1% of E-rosetting T cells in cell suspensions after removing T cells in this way. In some experiments, cell suspensions enriched for T cells by E rosetting were also employed. Namely, T cell-enriched cell suspensions were placed in ice-cold NH_4Cl solution for five minutes to lyse SRBC and washed three times in phosphate-buffered saline (PBS), pH 7.4 (7). More than 95% of the cells in T cell-enriched cell suspensions expressed Thy-1 and CD5 (pan T cell markers).

In Vitro Ab Production Assay by Plaque Formation

Spleen cell suspensions were washed twice in PBS with 5% CS and resuspended in RPMI 1640, 10% CS, 2-mercaptoethanol (10^{-6} M), penicillin + streptomycin, and *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid. Then cells were primed with Ag in a CO_2 incubator at 37°C for five days (4). SRBC (1% SRBC, 50 μl/ml; Colorado Serum, Denver, CO) and Keyhole Limpet hemocyanin modified with trinitrophenol (TNP-KLH, 50 μg/ml) were used as representatives of TD-Ag, and lipopolysaccharide modified with trinitrophenol (TNP-LPS, 2 μg/ml) was used as a representative of TI-Ag. Then cells were harvested, washed once, and resuspended in complete Hanks' balanced salt solution (HBSS) and subjected for plaque formation cell (PFC) assay as reported previously (4). 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 agar (Sigma Chemical) in HBSS], mixed well and plated on microscope slides. The slides with solidifed agar-cell mixture were immersed in guinea pig complement solution (Pel-Freeze, Rogers, AZ) preabsorbed with SRBC. After incubation at 37°C for three to four hours, the plaques developed were counted. When cells were primed with SRBC, SRBC in HBSS (30–40%) was used for the PFC assay. When cells were primed with TNP-LPS or TNP-KLH, TNP-treated SRBC in HBSS (40%) was used. The numbers of PFC were expressed per 10^6 initially cultured cells or per 10^6 viable cells.

Enzyme-Linked Immunosorbent Assay for IgG, IgM, IFN-τ, and IL-10

Ig present in the culture supernatant were studied by a standard solid-phase enzyme-linked immunosorbent assay (ELISA) (8). A 96-well flat-bottomed microtiter plate (F96 Maxisorp, Nunc, Naperville, IL) was coated with goat anti-mouse Ig (5 μg/ml) overnight in 0.1 N $NaHCO_3$ coating buffer, pH 9.6, with 0.02% NaN_3. After the plate was washed with rinse buffer (PBS, pH 7.4, 0.05% Tween 20), samples were diluted with dilution buffer [0.05 M tris (hydroxymethyl)aminomethane, pH 8.1, 1 mM $MgCl_2$, 0.15 M NaCl, 0.05% Tween 20, 0.02% NaN_3, and 1% bovine serum albumin] and incubated at room temperature (RT) 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 RT for two hours. The color was developed by addition of substrate solution (*p*-nitrophenyl disodium phosphate, 1 mg/ml in 0.05 M $NaHO_3$, pH 9.8, 10 mM $MgCl_2$). Optical density (OD) at 410 nm (OD_{410}) was read by ELISA reader. Monoclonal mouse IgM and IgG (Sigma Chemical) were used as standards. IFN-τ concentration was also studied by ELISA assay (9). Namely, samples were incubated on the plate coated in antibody against mouse IFN-τ (3.5 μg/ml; Genzyme, Cambridge, MA) for two hours at RT, washed with rinse buffer, and then incubated with a second Ab (2 μg/ml of anti-IFN-τ, clone XMG1.2; Pharmingen, San Diego, CA) for one to two hours at RT. Then the plate was washed again and incubated with anti-rat Ig-alkaline phosphatase conjugate (Pharmingen) for one hour at 37°C. OD_{410} was read with a reference at OD_{630}. For IL-10 ELISA assay, samples were incubated at RT for two hours on the plate coated with anti-IL-10 Ab (5 μg/ml; Pharmingen) (10). The plate was washed with rinse buffer and incubated with biotinylated rat anti-mouse IL-10 Ab (5 μg/ml; Pharmingen) at RT for two hours. The plate was washed and incubated with streptoavidin conjugated with peroxidase (Jackson Immune Research Laboratories, West Grove, PA) for one hour at 37°C, and the color was developed by addition of the same substrate buffer used in the mouse Ig ELISA. OD_{410} was read with a reference at OD_{490}.

IL-2/IL-4 Assay

The IL-2/IL-4-like activities in harvested samples were assessed by studying the growth of an IL-2/IL-4-dependent CTTL2 cell line (11). Namely, CTTL2 cells were washed twice with RPMI 1640 and 5% fetal calf serum and incubated in a 96-well microtiter plate (Costar; 5 ×

10^3 cells/well) in the presence of samples and incubated for 24 hours. 3-(4,5-Dimethyltiazol-2-*yl*)-2-5-diphenyl tetrazolium bromide (20 µl of 1.5 mg/ml in PBS; Sigma Chemical) was added to each well. The plate was further incubated for four hours in a CO_2 incubator, and 100 µl of 0.04 HCl in isopropanol were added to each well. OD_{570} was read by an ELISA reader with a reference at OD_{630}.

Reagents

Crystalline forms of astaxanthin and β-carotene were kindly provided by Hoffmann-La Roche (Nutley, NJ). Stock solution (10^{-4} M) of both carotenoids in absolute ethanol were prepared on the day of each experiment and kept in the dark. Further dilutions were prepared with culture media during the experiment. The control, ethanol (0.01%) without carotenoids, did not alter the results of bioassays used in this study, as reported elsewhere (4).

Statistics

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

Results

Effects of Carotenoids on In Vitro Ab Production in Normal and Autoimmune-Prone Strains

Previously we demonstrated the enhancing actions of astaxanthin on *in vitro* Ab production in B6 mice (4). In this study, we tested this action of carotenoids in several strains of mice. As representatives of normal strains of mice, we employed B6, DBA/2, and BALB/c mice. Autoimmune-prone NZB and MRL mice were also tested as representatives of a strain with relatively T cell-independent autoimmune features and a strain with T cell-dependent autoimmune features, respectively (5,12). β-Carotene and astaxanthin were tested as representatives of carotenoids with provitamin A activity and without provitamin A activity, respectively. We employed the concentration (10^{-8} M) that was most effective in previous studies (4). Spleen cells from two- to three-month-old mice were primed *in vitro* with SRBC for five days, as detailed in **Materials and Methods**, and the numbers of cells producing Ab (to SRBC) were examined by PFC assay. Results obtained are summarized in Table 1. Astaxanthin appeared to moderately enhance PFC formation in normal strains of mice, but no significant enhancing actions were obtained when NZB mice were tested. MRL mice, which are poorly responsive to specific Ab stimuli, may have responded more in the presence of carotenoids, but the changes of PFC numbers were not statistically significant. Ethanol used to dissolve carotenoids (final concn 0.01%) did not affect PFC formation in this assay system, as reported elsewhere (4).

We then tested the effects of carotenoids on *in vitro* Ab production against other Ags. TNP-KLH, another TD-Ag, and TNP-LPS, a TI-Ag, were employed for that purpose. The results obtained from three experiments are summarized in Table 2. Both carotenoids appeared to enhance PFC formation moderately in response to TNP-KLH in B6 mice, although the results were not statistically significant. The specific Ab production to TNP-LPS was not affected by the addition of carotenoids to the culture medium in either B6 or NZB mice. Ethanol added to the culture as solvent for carotenoids (0.01%) in these experiments did not significantly change PFC formation in either strain. NZB mice, whose B cells are polyclonally activated, were shown to produce higher numbers of PFC spontaneously without Ag priming, consistent with previous reports (reviewed in References 6 and 13) (Table 2). The addition of carotenoids did not enhance spontaneous PFC formation in this strain. Carotenoids did not enhance spontaneous PFC formation in any of the strains tested in this study (data not shown).

Table 1. Effects of Carotenoids on *In Vitro* Ab Production (to SRBC) in Normal and Autoimmune-Prone Strains of Mice[a,b]

Mouse Strain	*n*	Ab Production[c,d] Control	β-Carotene[e] (10^{-8} M)	Astaxanthin[f] (10^{-8} M)	*P* Value
			Normal		
B6	5	46 ± 7	42 ± 7	87 ± 9*	<0.01
		(177 ± 18)	(168 ± 33)	(136 ± 25)*	<0.005
DBA/2	3	90 ± 9	102 ± 37	162 ± 12*	<0.01
		(232 ± 44)	(206 ± 13)	(361 ± 24)*	<0.1
BALB/c	3	84 ± 16	75 ± 20	127 ± 5	<0.1
		(142 ± 18)	(120 ± 20)	(218 ± 28)	<0.1
			Autoimmune prone		
NZB	5	54 ± 14	33 ± 7	49 ± 11	
		(200 ± 70)	(168 ± 53)	(181 ± 44)	
MRL	5	6 ± 3	23 ± 13	22 ± 11	
		(33 ± 14)	(85 ± 37)	(92 ± 37)	

a: Spleen cells were obtained from 2- to 3-mo-old mice. Spleen cells from each mouse were tested separately in 3–5 expts.

b: Nos. of plaque formation cells (PFCs) formed spontaneously in B6, DBA/2, BALB/c, NZB, and MRL mice were 4.6 ± 0.6, 0.8 ± 0.2, 1.0 ± 0.6, 7.7 ± 1.9, and 1.5 ± 1.0/10^6 initially cultured cells, respectively.

c: Values are means ± SEM expressed as PFCs per 10^6 initially cultured spleen cells; values in parentheses are PFCs per 10^6 viable cells; *n*, no. of mice.

d: Cells were primed with sheep red blood cells (SRBC) in the presence of β-carotene or astaxanthin.

e: Ethanol (0.01%) used to dissolve carotenoid did not affect PFC formation by murine spleen cells.

f: Statistical significance (by Student's *t* test) is as follows: *, different from value obtained when cells were cultured in the absence of carotenoids (Control).

In two experiments, carotenoids did not significantly enhance *in vitro* Ab production to TNP-KLH in MRL mice.

Effects of Carotenoids on Total Ig Production

Because the results stated above may suggest that carotenoids preferentially enhance Ab production to TD-Ag in normal B6 mice but not in autoimmune-prone mice, we also examined whether they exert any action on total IgG and IgM production in response to TD-Ag in both B6 and autoimmune-prone strains. Spleen cells (2×10^6/ml) were incubated for seven days in the presence of TNP-KLH or SRBC, and total IgM and IgG in the harvested supernatant were measured by ELISA assay, as described in **Materials and Methods.** The results of four experiments are summarized in Table 3. NZB mice spontaneously produced large amounts of IgM, as reported previously (6,13), but this elevated Ig production was not further augmented by the presence of astaxanthin and β-carotene. Ag stimuli provided by TNP-KLH or SRBC did not further augment IgM production in the NZB strain, irrespective of the presence of both carotenoids. Total IgM and IgG production was also elevated in MRL mice compared with that of B6 mice, but Ig production by MRL spleen cells was not affected by Ag stimuli or by the presence of carotenoids. In B6 mice, total IgM production potentiated by these TD-Ag was not further augmented in the presence of astaxanthin or β-carotene. We obtained similar results for total IgG production in both strains (data not shown). Namely, total IgG production was elevated in both NZB and MRL mice compared with B6 mice and was not further augmented by carotenoids. IgG production potentiated by TD-Ag, TI-Ag, and nonspecific TI stimuli was not augmented by the presence of carotenoids in B6 mice. We also studied the Ig levels in the supernatant of PFC assay after five days of culture (part of

Table 2. Effects of Carotenoids on *In Vitro* Ab Production in Response to TNP-KLH and TNP-LPS in B6 and NZB Mice[a,b]

Additive	Ab Production[c,d]		
	Control	TNP-KLH (50 μg/ml)	TNP-LPS (2 μg/ml)
	B6		
Control	2.2 ± 1.0 (16 ± 9)	27 ± 10 (130 ± 32)	233 ± 49 (576 ± 128)
β-Carotene (10^{-8} M)		42 ± 9 (164 ± 43)	186 ± 31 (596 ± 122)
Astaxanthin (10^{-8} M)		36 ± 5 (211 ± 54)	216 ± 41 (544 ± 137)
	NZB		
Control	16 ± 9 (70 ± 42)	20 ± 10 (68 ± 17)	166 ± 47 (477 ± 23)
β-Carotene (10^{-8} M)		12 ± 7 (150 ± 75)	157 ± 68 (485 ± 111)
Astaxanthin (10^{-8} M)		27 ± 6 (151 ± 35)	180 ± 63 (564 ± 88)

a: Spleen cells were obtained from 2–3 mice (8–12 wks old) of each strain.
b: Effect of carotenoids on spontaneous PFC formation was minimal, as reported previously (4). Ethanol (0.01%) used to dissolve carotenoids did not affect PFC formation.
c: Cells were primed with Keyhole Limpet hemocyanin modified with trinitrophenol (TNP-KHL) and lipopolysaccharide modified with trinitrophenol (TNP-LPS).
d: Values are means ± SEM of 3 expts expressed as PFCs per 10^6 initially cultured spleen cells; values in parentheses are PFCs per 10^6 viable cells.

Table 3. Effects of Carotenoid on *In Vitro* Total IgM Production in Response to TD-Ag (TNP-KLH and SRBC) in B6 and NZB Mice[a,b]

	IgM Production[c]		
	Control	β-Carotene (10^{-8} M)	Astaxanthin (10^{-8} M)
	B6		
Control	49.6 ± 26.1	59.3 ± 20.9	26.6 ± 5.6
TNP-KLH	208.4 ± 90.5	387.9 ± 168.4	147.1 ± 22.4
SRBC	91.0 ± 35.6	94.6 ± 28.1	78.4 ± 22.4
	NZB[d]		
Control	1,070 ± 296	1,117 ± 296	1,084 ± 325
TNP-KLH	1,255 ± 339	1,111 ± 273	1,101 ± 277
SRBC	1,015 ± 275	1,117 ± 317	1,094 ± 315

a: Supernatant of murine spleen cells from 2 or 3 NZB or B6 mice (8–12 wks old) was harvested after culture of cells for 7 days with TNP-KLH (50 μg/ml) or SRBC (0.05%).
b: TD-Ag, T cell-dependent antigen.
c: Values are means ± SEM of 4 expts expressed in ng/ml.
d: Production of immunoglobulin M (IgM) was significantly greater in NZB than in B6 mice ($p < 0.01$) and was not enhanced in the presence of carotenoids.

Table 4. Effects of Carotenoids on Cytokine and Ig Production in the Culture of Ag (SRBC) Priming[a–c]

	IL-2/IL-4, U/ml	IFN-τ, U/ml	IL-10, U/ml	IgM, ng/ml	IgG, ng/ml
		B6			
Control	<1	0.5 ± 0.1	<1	513 ± 74	96.7 ± 25.9
SRBC	<1	1.4 ± 0.7*	<1	649 ± 149	76.9 ± 32.5
SRBC + astaxanthin	<1	1.0 ± 0.2*	<1	620 ± 128	82.1 ± 41.1
SRBC + β-carotene	<1	1.1 ± 0.3	<1	430 ± 67	94.9 ± 35.0
		NZB			
Control	<1	0.4 ± 0.1	<1	1,287 ± 208	172 ± 9
SRBC	<1	1.1 ± 0.5	<1	1,302 ± 175	169 ± 9
SRBC + astaxanthin	<1	0.9 ± 0.2	<1	1,306 ± 158	160 ± 12
SRBC + β-carotene	<1	0.4 ± 0.1	<1	1,260 ± 149	173 ± 9
		MRL			
Control	<1	1.0 ± 0.1	<1	1,061 ± 230	175 ± 23
SRBC	<1	1.9 ± 0.3	<1	917 ± 184	165 ± 32
SRBC + astaxanthin	<1	1.1 ± 0.2	<1	1,024 ± 193	169 ± 25
SRBC + β-carotene	<1	1.1 ± 0.2	<1	988 ± 177	183 ± 19

a: Murine spleen cells were incubated for 5 days for *in vitro* Ab production, as described in **Materials and Methods.** Cells were primed with SRBC (0.05%) in the presence of carotenoids (10^{-8} M), and supernatant was harvested at the end of the culture and used for assays of interleukin- (IL) 2/IL-4 activity, interferon- (IFN) τ, IL-10, IgM, and IgG. Ethanol (0.001%) used as a solvent for carotenoids did not alter values of parameters tested. IL-2/IL-4 activity was tested by employing IL-2/IL-4-dependent CTTL2 cell line. As positive controls, murine recombinant IL-2 and IL-4 (Genzyme) were used in each experiment. Activities of IL-2 and IL-4 were completely blocked by monoclonal anti-IL-2 and anti-IL-4 antibodies in this assay system. IFN-τ, IL-10, IgM, and IgG were all measured by enzyme-linked immunosorbent assay, as detailed in **Materials and Methods.**

b: Values are means ± SEM of 4 expts; for each expt, spleen cells were obtained from 2 mice (8–12 wks old) of each strain.

c: Statistical significance is as follows: *, higher than Control ($p < 0.05$) by Student's t test.

these data were shown in Table 4) and found no significant increase of IgM or IgG production in either B6 or NZB mice in the presence of carotenoids, irrespective of the Ag stimuli provided. Furthermore, astaxanthin did not facilitate Ig production potentiated by lipopolysaccharide (a nonspecific B cell activator) or TNP-LPS (TI-Ag) in B6, NZB, or MRL mice (data not shown). Thus carotenoids do not seem to enhance spontaneous or Ag-potentiated total Ig production.

Effects of Carotenoids on Cytokine Production in the Culture of In Vitro Ab Priming

Because carotenoids appeared to augment specific Ab production in this assay system, we further studied their effects on the production of cytokines, which are involved in the process of B cell differentiation. Namely, the culture supernatant was harvested after Ag (SRBC) priming for five days, and the concentrations of IL-2/IL-4, IL-10, and IFN-τ were measured as described in **Materials and Methods.** The results obtained in four experiments employing B6, NZB, and MRL mice are summarized in Table 4. Table 4 shows that IL-2- and IL-4-like activity was almost undetectable in all strains tested, irrespective of the presence of carotenoids. IL-10 production did not appear to be elevated in any of these strains of mice. Again, we observed significantly elevated IgM and IgG levels in the culture supernatant of NZB and MRL spleen cells. However, elevated levels of IgM and IgG were not further increased in the presence of astaxanthin and β-carotene. IgM concentrations in this culture

system were higher than those in the Ig production assay, which may be associated with the higher cell concentrations employed in this culture system (5×10^6/ml vs. 2×10^6/ml in Ig production). IFN-τ levels appeared to be slightly increased after Ag (SRBC) priming in all strains (Table 4). Interestingly, astaxanthin and β-carotene appeared to slightly decrease IFN-τ concentration in the supernatant; however, the difference detected was fairly minimal and not statistically significant.

Role of T Cells on the Actions of Carotenoids

Because carotenoids appeared to enhance specific Ag stimuli to TD-Ag, we further tested whether T- and B-cell interactions were required in this process. For that purpose, we depleted T cells from spleen cell suspensions by E rosetting, as described in **Materials and Methods**, and then studied the actions of carotenoids on PFC formation. In other experiments, T-depleted spleen cells were cocultured with purified T cells in a diffusion chamber, which prevents direct cognitive interactions between T cells and other lineage cells but allows T cells to release humoral factors to provide nonspecific help for normal B cell maturation. Then Ag (SRBC) primed cells were harvested and tested for PFC formation.

The results of four experiments were summarized in Figure 1. The depletion of T cells totally abolished the enhancing actions of astaxanthin. Furthermore, coculture of T cells and T-depleted spleen cells without direct contact failed to produce Ab effectively, indicating that astaxanthin may exert actions in the initial process of Ag priming (Ag presentation). To test this possibility, we then studied the time course of the enhancing action of astaxanthin on *in vitro* Ab production. That is, spleen cells were primed with SRBC, and astaxanthin (10^{-8} M) was added at Days 0–4 of the culture of Ag priming. Then cells were harvested on Day 5, and PFC formation was tested. The enhancing action of astaxanthin on *in vitro* Ag production (Ag:SRBC) was most prominent when astaxanthin was added at Day 0 of the culture of Ag priming (Figure 2). No enhancing action was observed when astaxanthin was added to the culture at Day 2 or later. These results may support our initial assumption that astaxanthin enhances Ab production partly through T helper cells in the initial stage of Ag presentation.

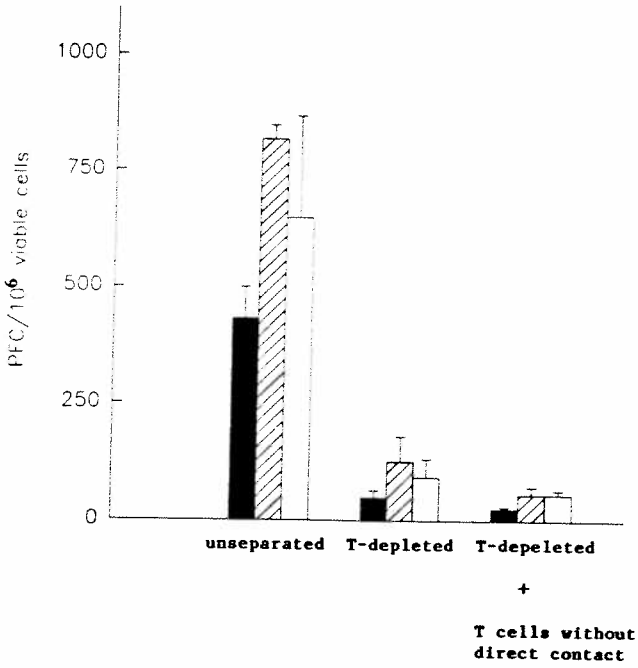

Figure 1. Number of plaque formation cells (PFCs) per 10^6 viable cells in unseparated, T-depleted, and T cells + T-depleted cells in a diffusion chamber without direct contact between T cells and T-depleted spleen cells when cells were primed with sheep red blood cells in the presence of medium only *(filled bars)*, astaxanthin (10^{-8} M) *(hatched bars)*, and β-carotene (10^{-8} M) *(open bars)*. PFC numbers were significantly reduced when T cells were depleted or when T cells were not permitted to have direct contact with other lineage cells.

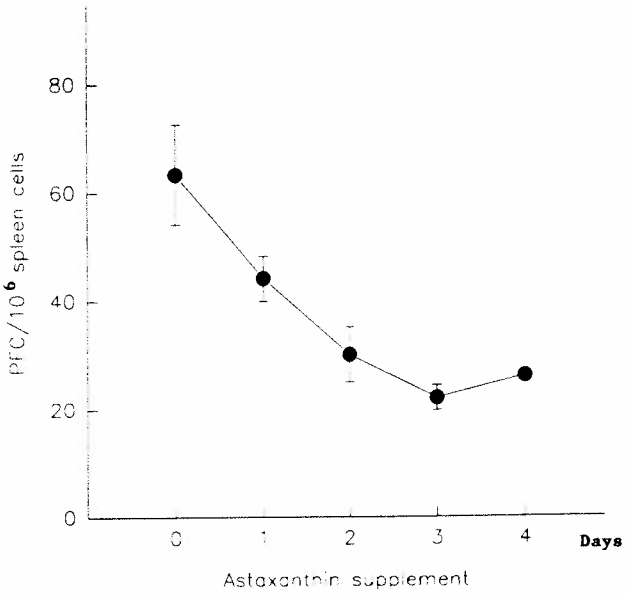

Figure 2. PFC numbers per 10^6 initially cultured cells. Each data point represents mean ± SEM of triplicate samples. Results are from 1 representative expt in a series of 3 expts. Astaxanthin supplementation (10^{-8} M) was done at Days 0–4 of culture of antigen priming. Its enhancing actions were most prominent when astaxanthin was added on Day 0.

Discussion

Our previous findings indicate that carotenoids without provitamin A activity can enhance Ab production *in vitro* in normal B6 mice. This study was undertaken to address the following questions regarding this action of carotenoids: 1) Can carotenoids enhance Ab production in other strains of mice? 2) Can carotenoids augment total Ig production or polyclonal Ab production or preferentially enhance specific Ab production? 3) What is the action of carotenoids on *in vitro* Ab production? 4) Is there a possibility that such an enhancing action of carotenoids may exacerbate the state of autoimmunity?

For these purposes, we tested the actions of carotenoids on Ab production in both normal and autoimmune strains of mice against various Ag stimuli. Total Ig and cytokine production was assessed as well. NZB and MRL strains of mice were employed as representatives of autoimmune-prone mice. NZB mice spontaneously develop autoimmune hemolytic anemia and immune complex-based glomerular nephritis by one year of age (5,13). B cells obtained from young NZB mice (<3 mos old) already appeared to be polyclonally activated and produced large amounts of IgM *in vitro*, which appeared to be relatively independent of T cell regulation (5,15). In contrast, the pathogenesis of autoimmunity found in MRL mice has been largely attributed to abnormal T cells and inability of MRL mice to induce tolerance by negatively selecting autoreactive T cell clones (12). Recent advances in molecular biology revealed that *lpr* gene carried by MRL mice is a mutant gene coding for *fas* protein, a protein necessary for apoptosis or programmed cell death (16). It appears that MRL mice fail to express FAS protein on their cell surface secondary to a point mutation of *lpr* gene, resulting in the inability to eliminate autoreactive T cell clones (16–18). Therefore we reasoned that NZB and MRL mice would be appropriate to employ in this study as animal models of B cell-dominant autoimmunity and T cell-dominant autoimmunity, respectively.

The results obtained demonstrated that astaxanthin preferentially enhances Ab production to TD-Ag in normal strains, but its action on Ab production to TI-Ag may not be significant. Actions of β-carotene on *in vitro* Ab production were less prominent than those of astaxanthin, as reported previously (4). Total IgM and IgG production potentiated by specific Ag stimuli was not altered by carotenoids. Spleen cells from autoimmune-prone NZB and MRL mice produced large amounts of Ig spontaneously in culture, as reported by others (5,13,15). NZB mice produced significant numbers of PFC spontaneously without Ag priming, perhaps

reflecting the autoantibody production against erythrocytes commonly seen in this strain (5,15). However, such features of polyclonal B cell activation were not altered in the presence of carotenoids. The enhancing action of carotenoids on Ab production (to TD-Ag) appeared to be less significant in both NZB and MRL mice. Thus it may be concluded that carotenoids preferentially augment specific Ab production to TD-Ag but do not augment polyclonal B cell activation.

Previously, we showed that carotenoids do not facilitate lymphocyte proliferation in response to mitogen (4). In this study, we have also shown that the depletion of T cells abolished the enhancing action of astaxanthin on Ab production. On the basis of these observations, we may speculate that astaxanthin exerts its enhancing action in the process of Ag presentation. Perhaps once B cells are activated, as observed in NZB mice, the action of astaxanthin on specific Ab production cannot be effectively demonstrated. In MRL mice, because of the T cell dysfunction and expansion of autoreactive T cell clones, astaxanthin may not be able to effectively augment the process of Ag presentation.

Cognate interactions of T, B, and Ag presentation cells (APC) are essential to initiate specific Ab responses, and B cells can function as APC in certain circumstances (19). After interactions of these cells, T helper cells produce various cytokines, which in turn regulate the processes of B cell maturation and isotype switching of Ig (20). Key cytokines produced by T helper cells that participate in this process involve IL-2, IL-4, IFN-τ, IL-5, IL-6, and IL-10. The presence of T helper cell subsets in relation to the profile of cytokine production is now firmly established in rodents. Immature resting T helper (Th_0) cells produce only IL-2 (21). When Th cells are activated, they may differentiate into either type 1 Th (Th_1) or type 2 Th (Th_2) cells. Th_1 cells produce IL-2, IFN-τ, and lymphotoxin and mainly participate in cellular immune responses, whereas Th_2 cells produce IL-4, IL-5, and IL-10 and mainly participate in humoral immune responses (reviewed in References 22–25). Thus we speculated that because the actions of astaxanthin seem to be limited on specific Ab responses to TD-Ag, astaxanthin may even alter the production of some of these cytokines produced by Th cells. We elected to study IL-2/IL-4 activity and levels of IFN-τ and IL-10 in the supernatant of the Ag priming culture. In previous studies, we found that carotenoids did not enhance concanavalin A- or phytohemagglutinin A-potentiated IL-2 production (4). We also did not find significant IL-2/IL-4 activity in the supernatant of the Ag priming culture, irrespective of the presence of carotenoids. Because IL-2 and IL-4 are known to be rapidly consumed by Th cells after Ag stimulation, it does not exclude the possibility of the moderate increase of IL-2/IL-4 production by carotenoids, but it is unlikely that carotenoids significantly alter the production of IL-2 and IL-4. In addition, IL-10 was undetectable in the supernatant in this culture system. However, a slight difference of IFN-τ production was noted in the presence of carotenoids. Namely, elevated IFN-τ levels with the Ag priming (SRBC) appeared to be suppressed to some extent in the presence of carotenoids, although the results were not statistically significant in this study. IFN-τ can suppress the action of IL-4 and may consequently suppress the specific Ab production to TD-Ag (23,24). Therefore a slight decrease of IFN-τ may be related to the enhanced action of carotenoids on Ab production. However, because IFN-τ can exert various actions on many lineage cells, it may be difficult to attribute this observation to the direct actions of carotenoids on Th cells. Further studies examining the effects of carotenoids on Th_1 and Th_2 clones would be interesting.

We also demonstrated that astaxanthin must be present from the beginning of the culture of Ag priming to exert its enhancing action on Ab production at optimal levels. In addition, astaxanthin did not display its enhancing actions in the absence of direct interactions between T and other lineage cells. Previously we observed a slight increase of Ia expression by T cells after culturing spleen cells in the presence of carotenoids (4). These results may further support our assumption that carotenoids exert enhancing actions on *in vitro* Ab production in the initial stage of Ag presentation and cognate interaction of T and B cells. Carotenoids are

reported to influence certain functions of macrophage-monocyte lineage cells, such as trinitrophenol production and phagocytic function (1,26). Because these cells function as strong APC, it might be interesting to study the effects of carotenoids on Ag presentation by macrophage and monocytes.

The other interesting finding in this study is that carotenoids do not potentiate or augment polyclonal B cell activation. This indicates the safety of their use by patients with autoimmune diseases. In MRL mice fed astaxanthin, we observed a significant delay of the development of autoimmunity as well as lymphadenopathy (6). In this study, we did not demonstrate significant improvement of *in vitro* Ab production by carotenoids in MRL mice. The discrepancy of these findings *in vivo* and *in vitro* in these studies employing MRL mice may be partly attributed to the rather artificial cell culture system employed. It is possible that carotenoids may modulate the immune system more effectively *in vivo*. It would be very interesting to study the specific Ab production *in vivo* in MRL and control strains of mice supplemented with astaxanthin.

In summary, this study revealed the possible actions of astaxanthin on Ag presentation and subsequent B lymphocyte differentiation. Because specific Ab production is a key function of the immune system to remove invading pathogens and mutated (malignant) cells, further studies regarding this subject may help us assess the chemoprophylactic actions of carotenoids on certain cancers.

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References

1. Bendich, A: "Carotenoids and the Immune Response." *J Nutr* **119,** 112–115, 1989.
2. Ziegler, RG: "A Review of Epidemiologic Evidence That Carotenoids Reduce the Risk of Cancer." *J Nutr* **119,** 116–122, 1989.
3. Krinsky, NI: "Carotenoids and Cancer in Animal Models." *J Nutr* **119,** 123–126, 1989.
4. 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 Marker Expression in *In Vitro* Culture System." *Nutr Cancer* **16,** 93–105, 1991.
5. Kastner, DL, and Steinberg, AD: "Determinants of B Cell Hyperactivity in Murine Lupus." *Concepts Immunopathol* **6,** 22–88, 1988.
6. Tomita, Y, Engelman RW, Jyonouchi, H, Day, NK, and Good, RA: "Influences of Carotenoids on the Development of Autoimmune Diseases in MRL/*mpj-lpr/lpr* Mice Fed High Calorie Diet" (abstr). *FASEB J* **5,** A1323, 1990.
7. Jyonouchi, H, Kimmel, MD, Lee, G, Kincade, PW, and Good, RA: "Humoral Factors in Very Young NZB Mice That Enhance the Maturation of Normal B Cell Precursors. Partial Purification and Characterization." *J Immunol* **135,** 1891–1899, 1985.
8. Boom, WH, Liano, D, and Abbas, AK: "Heterogeneity of Helper/Inducer T Lymphocytes. II. Effects of Interleukin 4 and Interleukin 2 Production T Cell Clones on Resting B Lymphocytes." *J Exp Med* **167,** 1350–1363, 1988.
9. Schreiber, RD, Hicks, JL, Celada, A, Buchmeier, NA, and Gray, PW: "Monoclonal Antibodies to Murine Gamma Interferon Which Differentially Modulate Macrophage Activation and Antiviral Activity." *J Immunol* **134,** 1609–1618, 1985.
10. Go, NF, Castle, BE, Barrett, R, Bieira P, Kastelein, R, et al.: "Interleukin (IL-10) Novel B Cell Stimulatory Factor: Unresponsiveness of 'XID' B Cells." *J Exp Med* **172,** 1624–1631, 1990.

11. Mosmann, TR: "Rapid Calorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays." *J Immunol Methods* **65**, 55–63, 1983.
12. Gause, WC, Steinberg, AD, and Mountz, JD: "Characteristics and Functions of CD4, CD8 T Cells in Normal and Autoimmune Mice." *Concepts Immunopathol* **6**, 89–118, 1988.
13. Theofilopoulos, AN, and Dixon, FJ: "Murine Models of Systemic Lupus Erythematosus." *Adv Immunol* **37**, 269–390, 1985.
14. Goldman, M, Druet, P, and Gleichmann, E: "T_H2 Cells in Systemic Autoimmunity: Insights From Allogeneic Diseases and Chemically-Induced Autoimmunity." *Immunol Today* **12**, 223–227, 1991.
15. Kasaian, MT, Ikematsu, H, and Casali, O: "$CD5^+$ B Lymphocytes." *Proc Soc Exp Biol Med* **197**, 226–241, 1991.
16. Watanabe-Fukunaga, R, Branana, CI, Copeland, NG, Jenkins, NA, and Nagata, S: "Lymphoproliferation Disorder in Mice Explained by Defects in *fas* Antigen That Mediates Apoptosis." *Nature Lond* **356**, 314–317, 1992.
17. Cohen, PL, and Eisenberg, RA: "*lpr* and *gld:* Single Gene Models of Systemic Autoimmunity and Lymphoproliferative Diseases." *Annu Rev Immunol* **9**, 243–270, 1991.
18. 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.
19. Myers, CD: "Role of B Cell Antigen Processing and Presentation in the Humoral Immune Response." *FASEB J* **5**, 2547–2553, 1991.
20. Rajewsky, K: "Early and Late B-Cell Development in the Mouse." *Curr Opin Immunol* **4**, 171–176, 1992.
21. Carding, SR, Hayday, AC, and Bottomly, K: "Cytokines in T-Cell Development." *Immunol Today* **12**, 239–245, 1991.
22. Mosmann, TR, and Coffman, RL: "TH1 and TH2 cells: "Different Patterns of Lymphokine Secretion Lead to Different Functional Properties." *Annu Rev Immunol* **7**, 145–174, 1989.
23. Finkelman, FD, Homes, J, Katona, IM, Urban, JF, Peckmann, MP, et al.: "Lymphokine Control of *In Vivo* Immunoglobulin Isotype Selection." *Annu Rev Immunol* **8**, 303–332, 1990.
24. Dijkmans, R, and Billiau, A: "Interferon τ. A Master Key in the Immune System." *Curr Opin Immunol* **1**, 269–274, 1988.
25. Forentino, DF, Zlotnik, A, Vieira, P, Mosmann, TR, Howard, M, et al.: "IL-10 Acts on the Antigen-Presenting Cell to Inhibit Cytokine Production by Th1 Cells." *J Immunol* **146**, 3444–3451, 1991.
26. Schwartz, J, Suda, D, and Light, G: "Beta Carotene Is Associated With the Regression of Hamster Buccal Pouch Carcinoma and the Induction of Tumor Necrosis Factor in Macrophages." *Biochem Biophys Res Commun* **136**, 1130–1135, 1986.
27. Canfield, LM, Forage, JW, and Valenzuela, JG: "Carotenoids as Cellular Antioxidants." *Proc Soc Exp Biol Med* **200**, 260–265, 1992.