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

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Abstract

The immunomodulating effects of carotenoids (β-carotene and astaxanthin) on mouse lymphocytes were studied in in vitro *culture system by use of assay for mitogen responses of spleen cells, thymocyte proliferation, interleukin 2 production, and antibody (Ab) production* in vitro *in response to sheep red blood cells. Changes of cell surface markers on spleen lymphocytes including Ia antigen (Ag), surface immunoglobulin, B220, and Thy-1 Ag were also examined.*

At a concentration of 10^{-8} M, carotenoids did not show any significant effect on mitogen responses (phytohemagglutinin P and concanavalin A) on murine spleen cells, irrespective of the concentrations of mitogens used. Interleukin 2 production by murine spleen cells was not significantly altered by carotenoids in the culture media (10^{-7} to 10^{-9} M). [^{3}H]thymidine incorporation by B6 thymocytes was somewhat enhanced in the presence of astaxanthin or β-carotene when cultured in the concentration of 10^6/ml. At higher concentrations of cells (5 × 10^6/ml), such an effect was not observed. In assays of in vitro *Ab production in response to sheep red blood cells, B6 spleen cells produced significantly more Ab-forming cells (plaque-forming cells, immunoglobulins M and G) in the presence of astaxanthin (> 10^{-8} M) but not β-carotene. Expression of Ia Ag seemed to be moderately enhanced on both Thy-1$^+$ and Thy-1$^-$ spleen cells in the presence of astaxanthin (> 10^{-9} M) but not β-carotene. The expression of Thy-1 and surface immunoglobulin seemed unchanged with the treatment of these carotenoids.*

These results indicate that immunomodulating actions of carotenoids are not necessarily related to provitamin A activity, because astaxanthin, which does not have provitamin A activity, showed more significant effects in these bioassays and also indicate that such actions of carotenoid demonstrated in this study may be difficult to explain only by its oxygen-quenching capacity.

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Introduction

Growing evidence suggests that carotenoids significantly influence immune function. For example, diets rich in carotenoids are inversely related to incidence of certain cancers (1,2). In experimental animal models, high intakes of carotenoids appear to prevent tumor development (3). It was also reported that the response to mitogens is enhanced in rats fed a carotenoid-rich diet (4). The effect of carotenoids on the immune system has been attributed to provitamin A activity, because the important role of retinoids on the immune system, especially on T cell differentiation/function, is well established (reviewed in Reference 5). However, several reports indicate that carotenoids that do not have provitamin A activity can modulate certain immune functions (6,7). It has been proposed that carotenoids function as immunomodulators through their action as oxygen scavengers, a function not performed by retinoids (1,8).

However, the mechanisms by which carotenoids influence immune functions *in vitro* and *in vivo* remain unclear. Consequently, we attempted to examine the effect of carotenoids on *in vitro* bioassays commonly used to assess immune functions. All target cell suspensions were obtained from young autoimmune-resistant C57B/6 mice (B6). β-Carotene, which has provitamin A activity, and astaxanthin, which does not have provitamin A activity, were used as representative carotenoids in this study.

The results indicate that astaxanthin, but not β-carotene, can enhance *in vitro* antibody (Ab) production by spleen cells stimulated with sheep red blood cells (SRBC). Carotenoids also seem to affect cell surface marker expression *in vitro*. These results indicate that carotenoids exert their immunomodulating actions apart from provitamin A activity as indicated by *in vivo* studies (dieting studies), perhaps in part enhancing the response to specific antigenic stimuli.

Materials and Methods

Mice

In this study, B6 mice were used as a representative of normal strains of mice. Six- to seven-week-old female B6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in the animal facility at the University of South Florida/All Children's Hospital (St. Petersburg, FL). The mice were housed in groups of five mice in each cage and fed regular lab chow (Purina Lab Chows, Purina Mills, Richmond, IN; protein 23.5%, fiber 5.8%, ash 7.3%, total digestible nutrients 76.0%, 4.25 kcal/g). At two to three months of age, they were killed by cervical dislocation instantly as approved by the animal ethics committee at the University of South Florida (Tampa, FL), and the spleen and thymus were removed.

Cell Suspensions

The spleen was removed aseptically, and single cell suspensions were obtained by crushing the spleen between two sterile glass slides and suspending tissues in phosphate-buffered saline with 5% calf serum (CS). Debris was removed after cell suspensions were placed on ice for five minutes. Thymocyte cell suspensions were obtained by gently squeezing out cells with a cell scraper (Costar, Cambridge, MA).

Mitogen Response

Spleen cells were washed twice and suspended in RPMI 1640 supplemented with 5% Nu serum (Collaborative Laboratories, Bedford, MA), pencillin and streptomycin, 1% Na pyruvate, 2% *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 1% glut-

amine, and 2-mercaptoethanol. Cell suspensions were then adjusted to 2.5 × 10^6/ml as a final concentration in a 96-well round-bottom microtiter plate (Costar) in the presence of mitogens [phytohemagglutinin P (PHA-P) and concanavalin A (Con A)] and/or carotenoids as indicated in **Results** (9). The cells were then incubated for two days in a CO_2 incubator (5%) at 37°C, pulsed with 1 μCi of [^{3}H-*methyl*]thymidine, and harvested 12–16 hours after the pulse. [^{3}H]thymidine incorporation was detected by a scintillation counter. In assays of thymidine incorporation, Stimulation Index was calculated by the average value of triplicate samples divided by average control value.

Interleukin 2 Production

Mouse spleen cells were washed twice and resuspended in the medium as described above. Cells (10^6/ml) were incubated in a 24-well flat-bottom culture plate (Costar) in the presence of medium only, PHA-P (20 μg/ml), or Con A (5 μg/ml) for 48 hours at 37°C with or without carotenoids. The supernatant was then harvested and frozen at −20°C until use. Interleukin 2 (IL-2) like activity in the harvested supernatant was detected by studying [^{3}H]thymidine incorporation by IL-2-dependent cell lines (HT-2) as described before (10). Briefly, various amounts of supernatants were added to HT-2 cells in a 96-well microtiter plate (Costar) (5 × 10^4/ml) and HT-2 cells were incubated overnight, pulsed with 1 μCi of [^{3}H]thymidine, and harvested four hours later. Rat IL-2 (Collaborative Laboratories) was used in each experiment as standard. IL-2 activity was calculated by comparing the thymidine incorporation by HT-2 cells stimulated by the standard IL-2. HT-2 cells were maintained in RPMI 1640, P + S, 2-ME, and 10% fetal calf serum in the presence of 10 U/ml rat IL-2.

Thymocyte Proliferation Assay

Thymocytes from B6 mice were washed twice and resuspended in medium as described above. Cells were then placed in a 96-well microtiter plate (Costar) (either 10^6/ml or 5 × 10^6/ml as final concentration) on the basis of bioassays for IL-1- or IL-2-like activities (11). Cells were stimulated with various amounts of carotenoids in the presence or absence of PHA-P (0.1 μg/well). Mouse recombinant IL-2 and recombinant human or mouse IL-1 (Genzyme, Boston, MA) were used as control. Cells were incubated at 37°C for three days in 5% CO_2 incubator and pulsed with 1 μCi of [^{3}H]thymidine and harvested 12–16 hours later.

In Vitro AB Production in Response to SRBC by Plaque Formation

Single-cell suspensions of mouse spleen cells were washed twice in phosphate-buffered saline supplemented with 5% CS. The cells were then resuspended in RPMI 1640, 10% CS (Hyclone, Logan, UT), 1% Na pyruvate, penicillin and streptomycin, 2% HEPES, 1% glutamine, and 2-mercaptoethanol (10^{-6} M). SRBC were washed three times, the buffy coat was removed, and the SRBC were resuspended in the same medium. Cells were then incubated in a 24-well plate (Costar) (5 × 10^6/ml, 1 ml/well) for five days in 10% CO_2 incubator at 37°C. Antigenic stimulation was provided by the addition of 50 μl of 1% SRBC to each 1-ml spleen cell suspension. The cells were then harvested, washed twice, and resuspended in 0.5 ml Hanks' balanced salt solution (HBSS), and cell viability was examined. Fresh SRBC suspension was prepared by washing SRBC twice and resuspending in incomplete HBSS (30–40% SRBC). Then 50 μl of SRBC suspension and 100 μl of harvested spleen cell suspension were added quickly to the tubes containing 0.5 ml of preheated agar solution [0.5% agar (Sigma Chemical, St. Louis, MO) in complete HBSS heated at 42°C], mixed well, and plated on microscope slides precoated with 0.025% agar. Guinea pig

complement solution (1:30 in HBSS, GIBCO-BRL, Gaithersburg, MD) was then applied to the trays of slides that were inverted to immerse the solidified agar-cell mixture with the complement solution. The slides were incubated at 37°C for two to three hours, and the plaques developed were counted under a dissecting microscope. The numbers of plaque-forming cells (PFC) were expressed per 10^6 initially cultured cells.

Cell Surface Marker Analysis

To test the effect of carotenoids on Ia expression, mouse spleen cells in the RPMI 1640 medium supplemented with 10% CS and other additives were incubated for two days at 37°C ($1–2 \times 10^6$ cells/ml) in 24-well tissue culture plates (Costar) in the presence of various amounts of carotenoids. Cells were then harvested and treated with cell lysis buffer [0.17 M $(NH_4)_2Cl_2$, pH 7.3] on ice for five minutes, washed twice, and subjected to immuno-fluorescence studies. Ia expression was detected by staining spleen cells with biotin-conjugated monoclonal anti-mouse Ia antibody (Meiji Institute of Health Science, Tokyo, Japan) and phycoerhythrin-conjugated streptavidin (Becton-Dickinson, San Jose, CA). Thy-1^+ cells were detected by staining with monoclonal anti-mouse Thy-1 antibody-fluorescein isothiocyanate (FITC) conjugate (Becton-Dickinson). Preimmunized goat serum-FITC conjugate (Tago, Burlingame, CA) was used as the control antibody. Ia^+ T lymphocytes (activated T lymphocytes) were detected by doubly staining cells with anti-Ia and anti-Thy-1 antibodies as described above. Then cells were analyzed by flow cytometer (EPICS Elite, Coulter, Miami, FL). Thy-1^-, Ia^+ lymphocytes were assumed to be B cells, but B lymphocyte population was also detected by staining cells with other pan-B cell surface markers, namely, surface immunoglobulin (sIg) and B220. B220 antigens (Ag) are originally reported to be expressed on most of mature B cells in peripheral blood in mice (12). FITC-conjugated anti-mouse immunoglobulin M (IgM) [$F(ab)_2$ fragment] was used for staining sIg, and rat monoclonal B220 Ab was used for staining B220 Ag. This monoclonal Ab (mAb) was prepared in our laboratory by precipitating IgM mAb (RA3-3AI/6.1, ATCC, Lockville, MA) in 0.1 M NaCl and conjugating with biotin. Streptoavidin-phycoerythrin conjugate (Becton-Dickinson) was used as second Ab for staining B220 Ag.

Reagents

Rat IL-2 (Collaborative Laboratory), recombinant human and mouse IL-1 (Genzyme), PHA-P (Difco, Baxter Scientific, Ocala, FL), and Con A (Pharmacia-LKB, Uppsala, Sweden) were purchased from commercial sources as indicated. Crystalline forms of astaxanthin and β-carotene were kindly provided by Hoffman-La Roche (Nutley, NJ). Stock solutions (10^{-4} M) of astaxanthin and β-carotene in absolute ethanol were made on the day of each experiment and kept in the dark. Further dilutions were prepared with absolute ethanol and then the culture media during the experiment. As control, ethanol of the same concentration used for dissolving carotenoids was employed in each experiment. Absolute ethanol was selected to be the solvent for carotenoids that is least likely to change the chemical structures of carotenoids. To prepare initial solutions, finely powdered carotenoids were diluted in ethanol by uniform suspension with a glass rod. The use of sonication was avoided because it often causes a colloidal precipitation of carotenoids, especially astaxanthin.

Statistics

The significance of results were obtained by comparing the data with control values. After F test, P values were obtained by either Student's t test or Welch's test.

Results

Influence of Carotenoids on Mitogen Responses In Vitro

Studies in which experimental diets were employed showed that the mitogen response of lymphocytes was enhanced in animals fed carotenoid-rich diets (4). Therefore, we first tested whether carotenoids at physiological concentration (10^{-8} M) in the culture medium could enhance the mitogen response by murine spleen lymphocytes. In our three initial experiments the concentrations of PHA-P and Con A were those regularly used for assessing immune functions of mice on normal or special diets in our department (1–2 μg/ml) (13). In this assay, neither β-carotene nor astaxanthin significantly changed responses of spleen cells to mitogens as shown in Table 1. Ethanol, which was used as the solvent for carotenoids, did not show any significant effect. Next, we investigated whether carotenoids could enhance the response in the presence of suboptimal concentrations of mitogens (0.1–1.0 μg/ml PHA-P or Con A), because the presence of optimal concentrations of mitogens may have prevented detection of actions of carotenoids. However, as shown in Figure 1, neither β-carotene nor astaxanthin changed mitogen responses of B6 spleen cells significantly at a concentration of 10^{-8} M. In this particular experiment, the optimal responses to PHA-P were observed at concentrations of 0.5 μg/ml, which we experience sometimes, although we most often observed optimal responses at 1 μg/ml.

Influence of Carotenoids on Thymocyte Proliferation

Because retinoids are known to influence maturation of T lineage cells (5), we sought to determine whether carotenoids enhance proliferation of thymocytes (immature T lymphocytes) obtained from B6 mice. Thymocyte cell suspensions were incubated for three days in the presence of carotenoids (10^{-7} to 10^{-8} M) with or without PHA-P (0.1 μg/ml), and the proliferative responses were determined by [^{3}H]thymidine incorporation. Cell concentrations of 10^6/ml and 5×10^6/ml were used in this assay system, which was adapted from the assay for thymocyte proliferation employing IL-1 or IL-2 (11). Thymocytes proliferated slightly better in the presence of astaxanthin or β-carotene in the absence of PHA-P when they were incubated in low cell concentration (10^6/ml) (Figure 2). At higher concentrations

Table 1. β-Carotene and Astaxanthin Do Not Enhance Proliferation Response to Mitogens by Spleen Cells

	Cells Incubated With[a]		
	Control	β-Carotene (10^{-8} M)	Astaxanthin (10^{-8} M)
Control[b]			
PHA-P[b,c]			
1 μg/ml	4.6 ± 1.2	4.3 ± 1.2	4.3 ± 1.2
2 μg/ml	5.5 ± 1.0	5.2 ± 0.7	5.2 ± 0.9
Con A[d]			
1 μg/ml	43.0 ± 4.3	43.1 ± 3.1	41.1 ± 4.4
2 μg/ml	114.4 ± 40.2	118.2 ± 27.4	111.7 ± 23.3
4 μg/ml	82.4 ± 17.2	80.2 ± 21.3	73.7 ± 21.0

a: Values are means ± SE expressed in terms of [^{3}H]thymidine incorporation (SI units).
b: Summary of 5 separate expts. In each expt spleen cells were obtained from 2 or 3 B6 mice and pooled. With ethanol used for dissolving carotenoids, there was no significant difference in mitogen responses compared with control values cultured without carotenoids (final concn of ethanol 0.01%).
c: Abbreviations are as follows: PHA-P, phytohemagglutinin P; Con A, concanavalin A.
d: Summary of 3 expts.

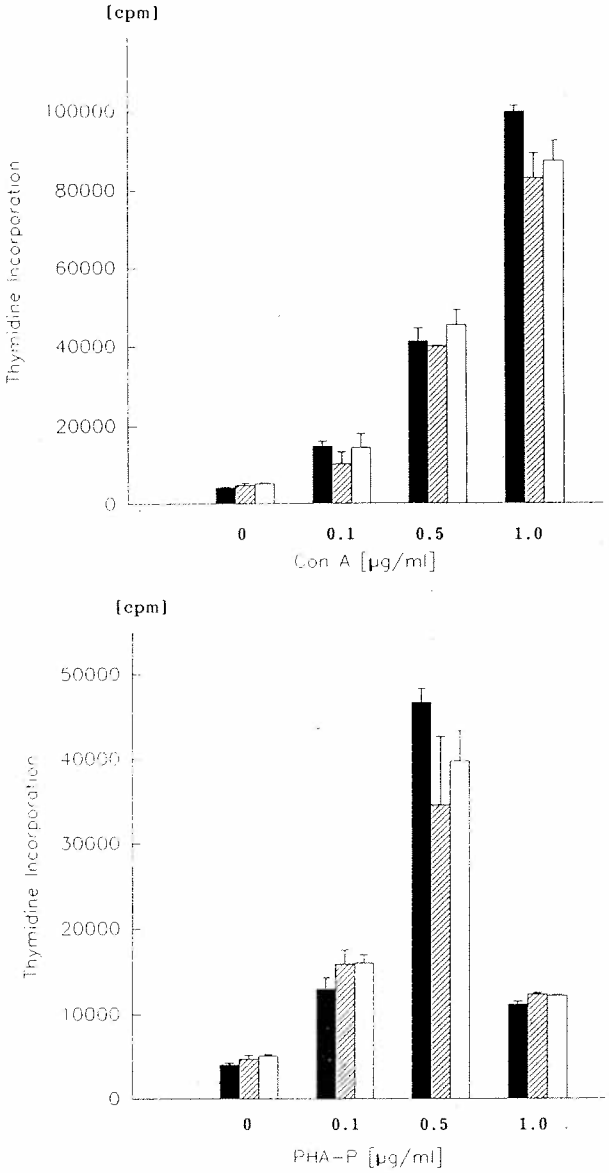

Figure 1. Proliferation responses of B6 spleen cells to concanavalin A (Con A) (A) and phytohemagglutinin P (PHA-P) (B) (0.1–1.0 μg/ml). Values represent average ± SE of triplicate samples in medium only (*solid bars*), 10^{-8} M β-carotene (*hatched bars*), and 10^{-8} M astaxanthin (*open bars*).

of cells (5×10^6/ml), enhancing action of carotenoids on thymocyte proliferation was not observed; nor were any significant enhancing effects observed in the presence of PHA-P (0.1 μg/ml) or PHA-P plus IL-2 (10 U/ml) (data not shown).

Effects of Carotenoids on IL-2 Production In Vitro

Among the various cytokines, IL-2 represents a key growth factor for T lineage cells and is known to influence the immune functions significantly. In fact, IL-2 suppresses the growth of malignant cells by potentiating cytotoxic killer cell activity, and recombinant IL-2 is now on trial for treatment of cancer patients (14,15). Given the antitumor effect of carotenoids that has been demonstrated in *in vivo* studies (3), we examined whether carotenoids could influence the production of IL-2 *in vitro*. B6 spleen cells were cultured in the presence of 10^{-7} and 10^{-9} M astaxanthin or β-carotene for two days with medium only or with PHA-P (20 μg/ml) or Con A (5 μg/ml), and the supernatant was harvested and tested for IL-2-like activity by using IL-2-dependent cell line (HT-2) (see **Methods**). As shown in Figure 3, the presence of neither β-carotene nor astaxanthin (10^{-8} M) in the culture medium enhanced IL-2 production by spleen cells, irrespective of the stimulant used (PHA-P or Con A). This is the

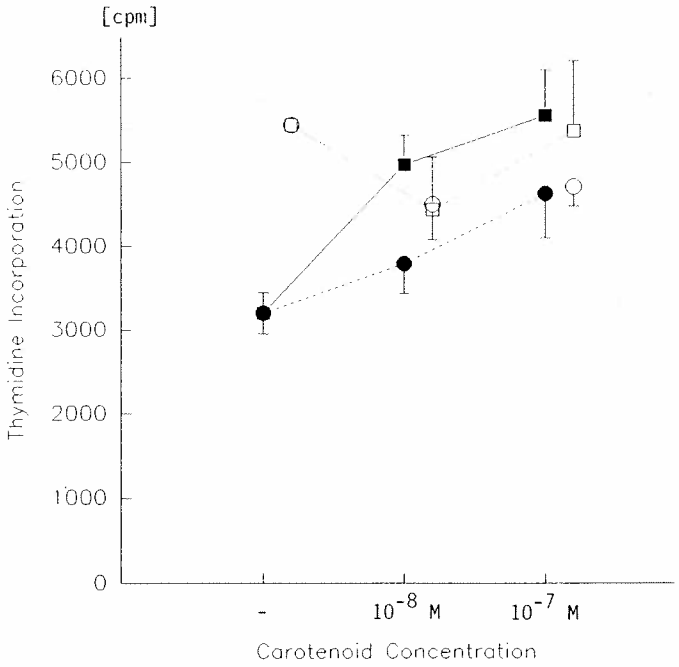

Figure 2. Proliferative responses of B6 thymocytes in presence of 10^{-8} M β-carotene (*solid circles*) and 10^{-8} M astaxanthin (*solid squares*) at cell concentration of 10^6/ml. Response was also examined at higher cell concentrations (5×10^6/ml) in presence of β-carotene (*open circles*) or astaxanthin (*open squares*). Values represent average ± SE of triplicate samples.

result of one representative experiment in a series of three experiments. Nor was a significant effect of carotenoids observed with concentrations of 10^{-7} or 10^{-9} M. In the absence of stimulants, no significant IL-2-like activity was generated, regardless of whether carotenoids were present or absent (data not shown).

In Vitro Ab Production

Carotenoids or retinoids have been reported to reduce the frequency or severity of infection in certain infectious diseases (16,17). Thus it is suspected that carotenoids may modify humoral immunity. To address this question, we studied the effects of carotenoids on Ab production in response to SRBC by spleen lymphocytes *in vitro*. Namely, B6 spleen cells were primed with SRBC *in vitro* for five days in the presence or absence of carotenoids. In initial experiments, cell viability seemed to be better in the presence of 10^{-8} M astaxanthin and the numbers of Ab-producing cells against SRBC. PFC seemed to increase in the presence of astaxanthin in culture media (Table 2). β-Carotene did not seem to increase the numbers of PFC significantly. When astaxanthin was added to the initial culture medium, plaques tended to be larger in size and plaque formation occurred after a shorter incubation period when the agar-cell mixture was incubated with complement solutions. When spleen

Table 2. Astaxanthin But Not β-Carotene Enhances *In Vitro* Ab Production by B6 Spleen Cells[a]

Cells Incubated With	No. of PFC[b,c]
Control	14 ± 8*
SRBC	112 ± 21
SRBC + β-carotene (10^{-8} M)	119 ± 15
SRBC + astaxathin (10^{-8} M)	225 ± 21*

a: Values are means ± SE expressed per/10^6 initially cultured cells.
b: Abbreviations are as follows: Ab, antibody; PFC, plaque-forming cell; SRBC, sheep red blood cell.
c: Statistical significance is as follows: *, significantly higher than values obtained from cells incubated with SRBC alone or SRBC plus β-carotene ($p < 0.01$).

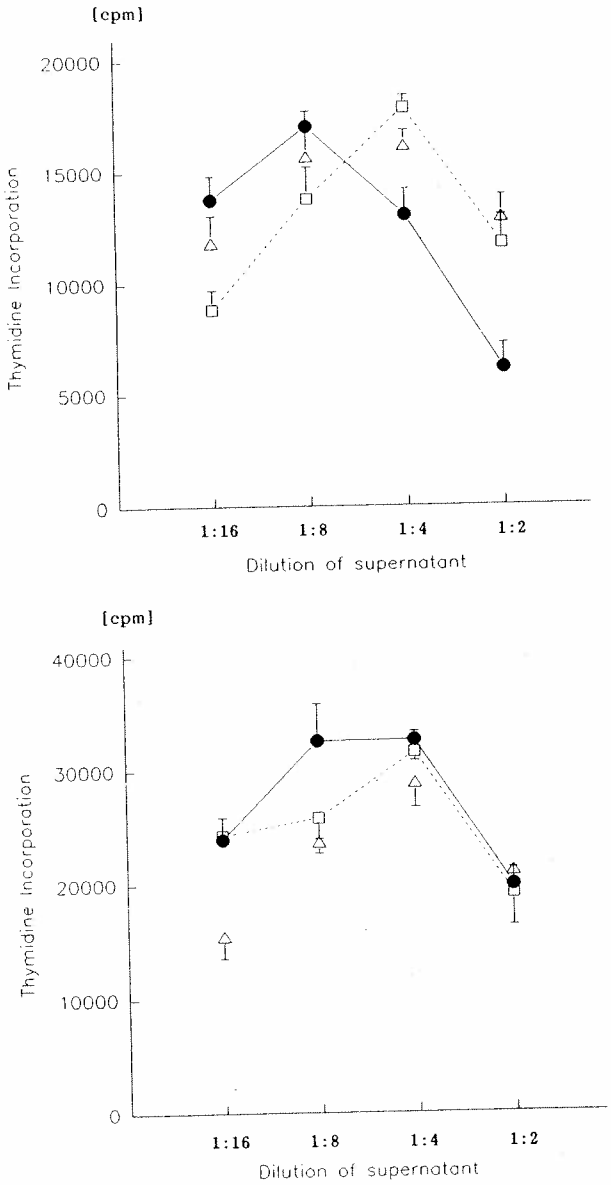

Figure 3. Interleukin 2-like activity in supernatants of spleen cell cultures in the presence of medium only (*solid circles*), β-carotene (*open circles*), and astaxanthin (*open triangles*). Cells were stimulated with PHA-P (20 μg/ml) (A) or Con A (5 μg/ml) (B). Concentration of carotenoids used was 10^{-8} M in this expt. Values represent average ± SE of triplicate samples.

cells were incubated for five days, higher numbers of PFC were produced, irrespective of the presence or absence of carotenoids (data not shown). Thus, in further experiments cells were incubated (primed) with SRBC *in vitro* for five days before PFC assay.

Next, we tested whether astaxanthin could enhance PFC formation by spleen cells in a dose-dependent manner. As shown in Figure 4, astaxanthin exerted moderate enhancing effects on PFC formation at concentrations of 10^{-7} to 10^{-9} M, but the effect was not necessarily dose dependent. Astaxanthin (10^{-10} M) did not show a significant effect on *in vitro* Ig production. We also examined whether astaxanthin could enhance production of a particular isotype of Ab. For that purpose, PFC assay was also done in the presence of goat anti-mouse IgG Ab (1:1,000) or goat anti-mouse IgM Ab (1:2,000) in the agar-cell mixture. The percent decrease of numbers in PFC in the presence of these Ab was not changed between controls and those treated with astaxanthin (Fig. 4 and data not shown). These results indicate that astaxanthin may enhance *in vitro* Ab production unrelated to isotype of Ig. In the absence of SRBC as Ag stimuli, spontaneous Ig production was negligible in the presence or absence of carotenoid (Table 2).

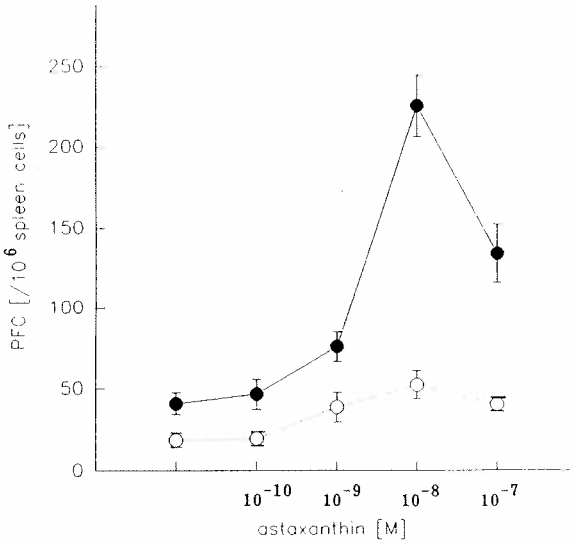

Figure 4. Number of plaque-forming cells (PFC) formed by murine spleen cells in response to various doses of astaxanthin. Ethanol (0.01% or 0.05%) did not affect numbers of PFC formed in this expt. PFC number formed in response to sheep red blood cells in absence (*solid circles*) and in presence of anti-immunoglobulin G antibody (*open circles*) are shown with various concentrations of astaxanthin controls. Values represent average ± SE of triplicate samples.

Cell Surface Marker Expression by Mouse Spleen Cells

We further examined whether carotenoids could modulate the cell surface marker expression, including Ia Ag, on mouse spleen cells in *in vitro* culture. B6 spleen cells were washed twice and incubated in medium as for mitogen assays for two days (10^6/ml, 2 ml/well in 24-well culture plate) (Costar) and subjected to analysis of expression of cell surface markers including B220, sIg, Ia, and Thy-1. Ia expression seemed to be moderately enhanced in the presence of astaxanthin ($>10^{-8}$ M) in both Thy-1^+ and Thy-1^- cell populations as shown in Figure 5 and Table 3. In the presence of carotenoids, the expression of sIg and Thy-1 seemed to be unchanged. Interestingly, B220 Ag expression seemed to be slightly decreased in the presence of astaxanthin (Figure 5). The effects of β-carotene on Ia and B220 expression by spleen lymphocytes was not as significant as those of astaxanthin. Taken together, astaxanthin seemed to modulate the expression of certain cell surface markers on spleen cells, which may be related in part to its enhancing action of *in vitro* Ab production by spleen cells *in vitro*.

Discussion

Although the immunomodulating actions of retinoids are well established, not much attention has yet been paid to the actions of carotenoids, because their effects were simply thought to be related to their provitamin A activity. However, several epidemiological studies indicate that diets rich in carotenoids are associated with a lower risk of developing certain cancers, which has not been observed in similar studies with vitamin A–rich diets

Table 3. Astaxanthin Moderately Increased Thy-1^+, Ia^+ Cells in *In Vitro* Culture System

	Thy-1^-, Ia^+ Cells	Thy-1^+, Ia^+ Cells
Control	25.0 ± 0.8[a]	4.5 ± 0.4[b]
Astaxanthin (10^{-8} M)	29.6 ± 0.9	6.3 ± 0.4
β-Carotene (10^{-8} M)	28.2 ± 2.6	4.5 ± 0.4

a: Frequency of Thy-1^-, Ia^+ or Thy-1^+, Ia^+ cells is expressed as average percent ± SE in spleen lymphocyte population in 3 separate expts. B6 spleen cells were cultured as described in **Methods** in absence or presence of carotenoids for analysis of cell surface marker expression.
b: Statistical significance is as follows: $p < 0.05$ compared with control value.

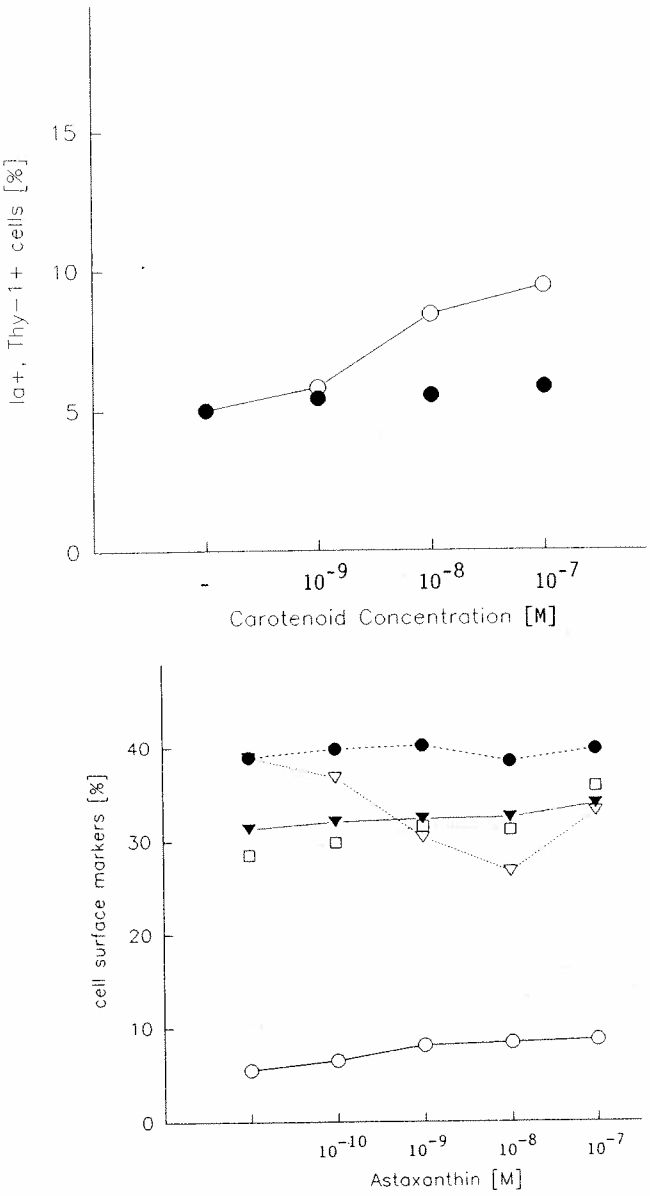

Figure 5. A: changes in Thy-1$^+$, Ia$^+$ cell population *in vitro* in presence of various amounts of β-carotene (*solid circles*) or astaxanthin (*open circles*). B: changes of Thy-1$^+$ (*solid circles*), serum immunoglobulin (sIg$^+$), (*solid inverted triangles*), B220$^+$ (*open inverted triangles*), Ia$^+$ and Thy-1$^+$ (*open circles*), and Thy-1$^+$, Ia$^+$ cell populations (*open squares*) in presence of astaxanthin (10^{-10} to 10^{-7} M). Control ethanol (0.01% or 0.05%) did not change expression of any of these cell surface markers.

(1,2). Recent studies using experimental animal models impressively demonstrated chemoprotective effects of carotenoids on tumor growth (3,18–21). Moreover, carotenoids that do not have provitamin A activity (canthaxanthin and astaxanthin) have been shown to suppress tumor growth (1,23). This tumoricidal activity of carotenoids may be attributable to enhancement of activity of natural killer cells, macrophages, or cytotoxic T cells (20,23–25). Bendich and Shapiro (4) showed that rats fed either canthaxanthin or β-carotene showed increased responses to mitogens when spleen cells were cultured *in vitro* compared with controls. Perhaps carotenoids should be considered to possess important immunomodulatory functions distinct from the properties of provitamin A. Because the immunologic effects of carotenoids have been inferred but not yet elucidated in epidemiological and dietary studies, we investigated mechanisms of actions of carotenoids by *in vitro* culture systems. We studied the effects of β-carotene [provitamin A activity (+)] and astaxanthin [provitamin A activity (-)] in bioassays routinely used for assessing murine lymphocyte functions. B6 mice were chosen for study as a representative normal strain. In prior studies using syngeneic tumor system, astaxanthin was found to be the most effective among non-provitamin A carotenoids tested for augmenting tumor immunity (Tomita and

co-workers, unpublished observations); thus astaxanthin was used as a representative of carotenoid without provitamin A activity.

We studied the effects of carotenoids on the proliferation of spleen lymphocytes (mature lymphocytes with a few cells in S phase) and thymocytes (immature lymphocytes with many cells in S phase). Our data demonstrate that neither β-carotene nor astaxanthin had a significant influence on proliferative responses of spleen lymphocytes in the presence of suboptimal or optimal concentrations of mitogens (PHA-P and Con A). Furthermore, no significant influence on thymocyte proliferation in the presence of PHA-P was observed. Astaxanthin and β-carotene slightly enhanced proliferation of thymocytes in the absence of PHA-P when cells were incubated at a concentration of 10^6/ml. At higher cell concentrations (5×10^6/ml), no significant enhancing effects were observed. Thus, we concluded that carotenoids do not stimulate or support nonspecific proliferation of mature or immature lymphocytes *in vitro*. Slight enhancement of thymocyte proliferation and increased viability of thymocytes by carotenoids may be related to an oxygen-scavenging function of carotenoids.

We also examined whether the increased tumoricidal activity observed on carotenoid-fed animals was related to the enhanced production of IL-2. IL-2 was originally identified as the key T cell growth factor needed to sustain paracrine proliferation of activated T cells (13), and its antitumor activity is thought to be secondary to the nonspecific activation of cytotoxic T cells (14). Our previous results from *in vivo* experiments also indicate that cytotoxic T cells may be important in chemoprotective actions of carotenoids (Tomita and others, unpublished observations). However, carotenoids did not enhance the IL-2 production by spleen lymphocytes at concentrations of 10^{-7} to 10^{-9} M, irrespective of the presence of mitogens or the kinds of mitogens present in the culture media. These results are consistent with our observations that carotenoids do not enhance lymphocyte proliferation in response to nonspecific stimulation (mitogens). Therefore it is difficult to explain the chemoprotective action of carotenoids on tumor growth by nonspecific activation of T lymphocytes.

The possibility that carotenoids may enhance the immune responses to specific antigenic stimuli was also examined. For that purpose we used the assay of *in vitro* Ab production by spleen B lymphocytes in response to SRBC in culture as the model system. Our results indicate that astaxanthin can enhance *in vitro* Ab production moderately at concentrations of 10^{-7} to 10^{-9} M. The effects of β-carotene were marginal. Interestingly, in the presence of carotenoids the viability of spleen cells in culture seemed to be improved compared with controls (up to 2- to 3-fold). Thus, we initially attributed the enhancing effects of astaxanthin on *in vitro* Ab production to its oxygen-scavenging functions. β-Carotene is reported to have fewer oxygen-scavenging functions than astaxanthin (26), which may explain the difference in the effects of these two carotenoids on *in vitro* Ab production.

On the other hand, the presence of T cells was essential to induce effective Ab production by B cells. It is thought that T cells are required for recognition of Ag and for activation of B cells to produce Ab through various soluble factors including IL-2, IL-4, and IL-6 (27). Prabhala and co-workers (28) reported recently that both carotenoids and retinoids can upregulate the expression of certain cell surface markers on human peripheral mononuclear cells *in vitro*. They observed increased expression of cell surface markers for T helper cells and natural killer cells and increased numbers of cells bearing lymphocyte activation markers, including HLA-DR Ag, IL-2 receptor, and transferrin receptors (28). The increased expression of such surface markers was exerted by both retinoids and carotenoids (28). Among these activation markers, major histocompatibility complex (MHC) class II molecules (HLA-DR for humans) are crucial for specific Ag response by T helper cells when antigens are presented by Ag presenting cells (29). Thus, we also explored the possibility that the moderate enhancing effects of astaxanthin on *in vitro* Ab production (in response to SRBC) may be related to the enhanced expression of Ia antigen (MHC II molecules for

mouse). As shown in **Results**, our data indicate that astaxanthin can enhance Ia expression on spleen cells, perhaps including both T and B lymphocyte populations, when cells were cultured in the presence of astaxanthin ($> 10^{-9}$ M) for two days. β-Carotene did not enhance Ia expression on spleen cells. On the basis of these results, it may be concluded that the enhancing effects of astaxanthin in this assay can be attributable in part to its upregulatory action on the Ia expression of spleen lymphocytes. The apparent lack of upregulatory action of Ia Ag by β-carotene may also be related to marginal enhancing effects on *in vitro* Ab production.

B220 (pan-B cell marker) expression is moderately suppressed by astaxanthin, but the expression of Thy-1 (pan-T cell marker) and sIg seemed to be unchanged by both carotenoids. Therefore the modulating action of astaxanthin on cell surface marker expression is difficult to explain by nonspecific effects of cell membranes or oxygen-scavenging functions. When analyzing spleen cell surface markers by flow cytometry, a window for lymphocyte populations was set up. However, contamination of monocyte populations in small percentages may be possible. Thus, apparent upregulatory action of astaxanthin on Ia expression may also be exerted on monocyte populations. Further studies, with respect to effects of carotenoids on cell surface marker expression, are required to employ purified populations of T cells, B cells, and monocytes. We are now in the process of performing these studies.

Both carotenoids used in this study belong to the "isoprenoid group"; astaxanthin differs from β-carotene only by modified ionone rings with diketo- and dihydroxylation. The presence of hydroxy/keto groups on two β-ionone rings in astaxanthin may be related to its altered immunomodulating actions compared with β-carotene, possibly changing its interactions with the cell membranes, considering their hydrophobic chain structure. However, actions of carotenoids exerted on lymphocyte or other lineage cells were virtually unknown. Further studies using different types of carotenoids are necessary to determine what chemical structure is essential in the immunomodulating actions exerted by carotenoids, and we then may be able to explore the metabolism of carotenoids in cells in cell culture system.

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