Astaxanthin, a Carotenoid without Vitamin A Activity, Augments Antibody Responses in Cultures Including T-helper Cell Clones and Suboptimal Doses of Antigen[1,2]

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ABSTRACT Astaxanthin, a carotenoid without vitamin A activity, enhances T-dependent antigen (Ag)-specific humoral immune responses. We examined carotenoid actions on T-helper (Th) cell activity in a direct manner with reconstitution experiments; spleen Th cells were replaced with Ag-specific Type 1 and Type 2 (Th1 and Th2) Th cell clones. The Ag for the Th1 and Th2 clones were pigeon cytochrome C and rabbit γ-globulin, respectively. Astaxanthin and β-carotene augmented the number of IgM antibody (Ab)-secreting cells when unprimed B cells were incubated with Th clones and stimulated with suboptimal doses of Ag specific for each Th clone. The number of IgG Ab-secreting cells were greater with use of in vivo primed B cells than with unprimed B cells in both Th clones. Astaxanthin but not β-carotene augmented the number of IgG Ab-secreting cells when primed B cells and Th cell clones were stimulated with suboptimal doses of Ag specific for each Th clone. In the presence of optimal doses of Ag for each Th clone, neither carotenoid augmented the number of Ab-secreting cells. Astaxanthin and β-carotene may enhance the actions of both Th1 and Th2 cells for humoral immune responses with suboptimal Ag challenges; certain carotenoids may help maintain Ag-mediated immune responses at optimal levels. J. Nutr. 125: 2483–2492, 1995.

INDEXING KEY WORDS:

- *T-dependent antibody production*
- *murine T-helper cell clones*
- *astaxanthin*
- *β-carotene*
- *antibody-secreting cells*

Carotenoids, colored pigments widely distributed in vegetables, fruits and seafood, are implicated in the prevention of degenerative diseases including coronary heart disease and cancer (Gerster 1993, Morris et al. 1994, Van Poppel 1993). Numerous but not all epidemiological studies have suggested a protective effect of carotenoids against degenerative diseases (Blot et al. 1993, Li et al., 1993, Morris et al. 1994, The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group 1994, Van Poppel 1993). Several biochemical activities of carotenoids are consistent with a putative role for carotenoids in disease prevention. These include provitamin A activity, antioxidant activity, enhancement of gap-junctional communications (Hanush et al. 1995, Zhang et al. 1992), immunomodulation (Bendich 1991) and augmentation of tumor immunity (Chew et al. 1995, Tomita et al. 1987). To better understand the role of dietary carotenoids, it is important to define mechanisms of their biological and pharmacological actions, including carotenoids with and without provitamin A activity.

Astaxanthin, a carotenoid without vitamin A activity, modulates T-dependent antibody (Ab)[4] responses in rodent models (Jyonouchi et al. 1991, Jyonouchi et al. 1993, Jyonouchi et al. 1994). Our recent studies demonstrate that astaxanthin modulates T-dependent humoral immune responses in humans (Jyonouchi et al. 1995). Carotenoids may facilitate Ab responses by

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[4] Abbreviations used: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot assay; IL, interleukin; SRBC, sheep red blood cells; Th cells, T-helper cells; Th1 and Th2 cells, Type 1 and Type 2 Th cells.

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modulating T-helper (Th) cell functions (Jyonouchi et al. 1993, Jyonouchi et al. 1994). In these studies, spleen cell suspensions and relatively crude antigens (Ag) such as sheep red blood cells (SRBC) were used. To further analyze carotenoid action on T-dependent Ab responses, it is essential to examine carotenoid action on Type 1 and Type 2 (Th1 and Th2) Th cell subsets (Swain et al. 1991) and primary (dominantly IgM Ab response) and secondary (dominantly IgG Ab response) immune responses against purified protein Ag. However, such a study is extremely difficult using spleen cell suspensions because of the paucity of Ag-specific Th cell and B cells: < 1 of 10,000 Th cells respond to a primary Ag challenge (Miller 1991).

In this study we studied carotenoid action on T-dependent Ab responses in a more direct manner, using Th cell clones. Namely, Th cells in spleen cell suspensions were replaced with Th clones, and Ab responses against Ag specific for each Th clone was assessed using primed or unprimed B cells. In this way the frequency of Ag-specific Th cells is markedly increased; this permits the quantification of carotenoid action on Th cell-mediated Ab responses with each Th clone and primed and unprimed B cells in response to various doses of Ag. β-Carotene and astaxanthin were used as representatives of carotenoids with and without vitamin A activity respectively.

MATERIALS AND METHODS

Mice. B10 and DAB/2 female mice (5–6 wk old) were purchased from Jackson Laboratories, Bar Harbor, ME and maintained in the animal facility at the University of Minnesota, Minneapolis, MN. The mice were generally housed in groups of five mice per cage and fed a nonpurified diet[5] (Purina Lab Chows #5010, Purina Mills, Richmond, IN). The carotene content of this diet provides approximately 4.5 times the daily requirement of vitamin A for mice (Subcommittee on Laboratory Nutrition 1978). The mice were killed in a CO_2 chamber as approved by the Laboratory Animal Medicine Ethics Committee, University of Minnesota, Minneapolis, MN. Two to 4-mo-old mice were used in the experiments.

Cell suspensions. Spleen 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. $CD4^+$ Th cells were depleted by treating cells with monoclonal rat anti-mouse L3T4 Ab (specific for murine CD4) and guinea pig complement (Pel-Freeze, Rogers, AR). More than 98% of Th cells were removed after this procedure by flow cytometry.

Reagents. β-Carotene (Sigma, St. Louis, MO) and astaxanthin (kindly provided by Hoffman-La Roche, Basel, Switzerland) were dissolved into 5 mL of an ethanol/hexane mixture (49 parts of absolute ethanol and 1 part of hexane) and filtered with 0.45-μm filter (Millex-AP, Milipore, Bedford, MA). The concentration was measured spectrophotometrically on the day of each experiment; concentrations of the stock solution were usually ~ 10^{-4} to 10^{-5} mol/L. The prepared stock solutions were further diluted by culture media containing 100 mL/L of fetal calf serum or calf serum. The amount of hexane-ethanol mixture used for preparing carotenoid stock solution did not interfere with the bioassays employed in this study. The solubility of carotenoids in the same medium used for this study were tested using higher concentrations of carotenoids (10^{-7} to 10^{-6} mol/L) because of the limited sensitivity of HPLC methodology. With this concentration, > 80% of astaxanthin and 50% of β-carotene were solubilized and stable in the medium for several days in 5% CO_2 at 37°C. When spleen cells were incubated at higher concentrations (10^{-6} mol/L), 3–4% of astaxanthin or β-carotene was detected in cell pellets.

Maintenance of Th1 and Th2 clones. A.E7 cells (Th1 clone) were kindly provided by Marc K. Jenkins, Department of Microbiology, University of Minnesota and maintained in our laboratory. A.E7 cells were stimulated periodically (every 2–3 wk) with Ag (pigeon cytochrome C; 3 μmol/L, Sigma) and irradiated B10 spleen cells in Dulbecco's modified Eagle medium supplemented with FCS (100 mL/L), penicillin G (10^5 U/L), streptomycin (100 mg/L) and 2-mercaptoethanol (10^{-5} mol/L) (Weaver et al. 1988). CDC35 cells (Th2 clone) were kindly provided by David C. Parker, Department of Molecular Genetics and Microbiology, University of Massachusetts, Worcester, MA, and maintained in our laboratory with stimulation by Ag (rabbit γ-globulin; 25–50 mg/L, Sigma) and irradiated DBA/2 spleen cells every 2 wk in the same medium used for the A.E7 cells (Tony et al. 1985).

In vivo Ag-priming. The Ag were given two times subcutaneously 2–3 wk apart; pigeon cytochrome C (25 μmol/L in the mixture of 1 part of PBS and 1 part of Freund's complete adjuvant; 0.5 mL/dose to B10 mice) and rabbit γ-globulin (50 mg/L in the mixture of 1 part PBS and 1 part Freund's complete adjuvant 0.5 mL/dose to DBA/2 mice). Then another booster dose of Ag (the same dose) was given intraperitoneally 5–7 d before the experiment (Bradley et al. 1993).

ELISA for IgG and IgM. The Ig concentrations in the culture supernatant were detected by an enzyme linked immunosorbent assay (ELISA). A 96-well ELISA plate (F96 Maxisorp, Nunc, Naperville, IL) was coated with goat anti-mouse Ig (5 mg/L) overnight in 0.1 mol/L $NaHCO_3$, pH 9.6 with 0.1 g/L NaN_3 (coating buffer

[5] Dietary composition of a nonpurified diet (Purina Lab Chow #5008, Purina Mills) is as follows: protein 23.5 g/100g, fat 6.5 g/100g, fiber 3.8 g/100g and ash 6.8 g/100g, with gross energy of 17.44 KJ/g.

After washing the plate with rinse buffer (PBS, pH 7.4 with 0.5 mL/L Tween 20), samples were diluted with dilution buffer [0.05 mol/L Tris, pH 8.1 with $MgCl_2$ (1 mmol/L), NaCl (0.15 mol/L), NaN_3 (0.2 g/L), and bovine serum albumin (BSA; 10 g/L, Sigma)] and incubated at room temperature for 2 h. Then the plate was washed with rinse buffer and incubated with the second Ab (goat anti-mouse IgG or IgM-alkaline phosphatase conjugate, 1:1000 to 1:3000 dilution, Sigma) at room temperature for 2 h. The color developed by adding substrate solution (p-nitophenyl phosphate 1 g/L, Sigma).

ELISPOT assay for IgM and IgG antibody-secreting cells. Numbers of Ag-secreting cells were measured by enzyme-linked immunospot (ELISPOT) assay (Czerkinsky et al. 1983, Sedgwick and Holt 1983). Cloned Th1 and Th2 cells were incubated with Th cell-depleted syngeneic spleen cells; A.E7 cells (2×10^5 cells/L) and Th cell-depleted B10 spleen cells (1.25×10^9 cells/L) or CDC35 cells (2.5×10^7 cells/L) and Th cell-depleted DBA/2 spleen cells (2×10^9 cells/L). Cells were incubated for 5 d in the same medium used for maintaining the Th clones in a 24-well tissue culture plate (Costar, Cambridge, MA). Then the cells were harvested, counted and resuspended in Iscove's modified Dulbecco's medium supplemented with fetal calf serum (50 mL/L), penicillin G (10^5 U/L), streptomycin (100 mg/L) and glutamine (2 mmol/L). The harvested cells (100 μL/well) were incubated overnight at 37°C in a 5% CO_2 incubator in a 96-well microtiter plate (Costar) coated with Ag (pigeon cytochrome C; 5 mmol/L or rabbit γ-globulin; 50 mg/L) and preincubated with a blocking buffer (PBS, pH 7.4 with 10 g/L BSA) for 30 min at 37°C. The cells were removed by vigorous washing next day and goat anti-mouse IgG or IgM antibody-alkaline phosphatase conjugate (1:1000 in dilution buffer used for ELISA; 100 μL/well) was added to the well. The plate was incubated overnight at 4°C and washed again. A spot of Ab secreted by cells were detected by adding gel substrate solution [5-bromo-4-chloro-3-indolyl phosphate (75 mg/L), and nitroblue tetrazolium chloride (150 mg/L) in 50 mmol/L $NaHCO_3$, pH 9.8 with $MgCl_2$ (5 mmol/L) and agar (5 g/L), 100 μL/well] and incubating the plate at 37°C for 4–5 h. Triplicates for each sample were tested and expressed as mean number of triplicates per 10^6 viable cells based on the cell count after 5-d culture. Cell viability declines when cells are cultured > 6 d in this experimental system.

Experimental design. *Experiment 1.* Carotenoid action on Ab production was measured in experiments using unprimed B cells, various doses of Ag and cloned Th cells. Th cell-depleted spleen cells plus Th1 (A.E7) or Th2 (CDC35) clones were stimulated with Ag [pigeon cytochrome C for Th1 cell; 0.063–4 μmol/L, rabbit γ-globulin for Th2 cells; 0.625–20 mg/L] in culture supplemented with *1*) medium alone (control), *2*) astaxanthin (10^{-8} mol/L), or *3*) β-carotene (10^{-8} mol/L). Five d later, cells were harvested and numbers of Ab-secreting cells were measured by an Ag-specific ELISPOT assay. The Ig levels in the supernatant were measured by ELISA. Spleen cells were obtained from unprimed syngeneic mice; B10 mice for A.E7 cells and DBA/2 mice for CDC35 cells. A summary of the experimental design is shown in **Figure 1**. A total of five replicate experiments were done for each Th cell clone. In preliminary experiments, various concentrations of carotenoids (10^{-10} to 10^{-7} mol/L) were tested with the use of 0.5–1 mmol/L pigeon cytochrome C or 5–20 mg/L rabbit γ-globulin as Ag stimuli. The most potent enhancing action of carotenoids were observed at carotenoid concentration of 10^{-8} mol/L, consistent with our previous reports (Jyonouchi et al. 1991, Jyonouchi et al. 1993, Jyonouchi et al. 1994). Thus this concentration was used for these experiments.

Experiment 2. Carotenoids action on Ab production was tested with the use of primed B cells and the same experimental system as described in Experiment 1 (Fig. 1). Namely, Th1 (A.E7 cells) and Th2 (CDC35 cells) cell clones were stimulated with the same doses of Ag as in Experiment 1. The culture was supplemented with *1*) medium alone (control), *2*) astaxanthin (10^{-8} mol/L) or *3*) β-carotene (10^{-8} mol/L). Spleen cells were obtained from syngeneic mice (B10 for A.E7 cells and DBA/2 for CDC35 cells) that were primed in vivo with Ag specific for each Th clone. The carotenoid concentration (10^{-8} mol/L) was selected on the basis of preliminary experiments; when various concentrations of carotenoids (10^{-10} to 10^{-7} mol/L) were tested, this concentration of astaxanthin was most potent in enhancing the numbers of IgG Ab-secreting cells, consistent with our previous reports (Jyonouchi et al. 1991, Jyonouchi et al. 1993, Jyonouchi et al. 1994). A total of five replicate experiments were done for each Th clone.

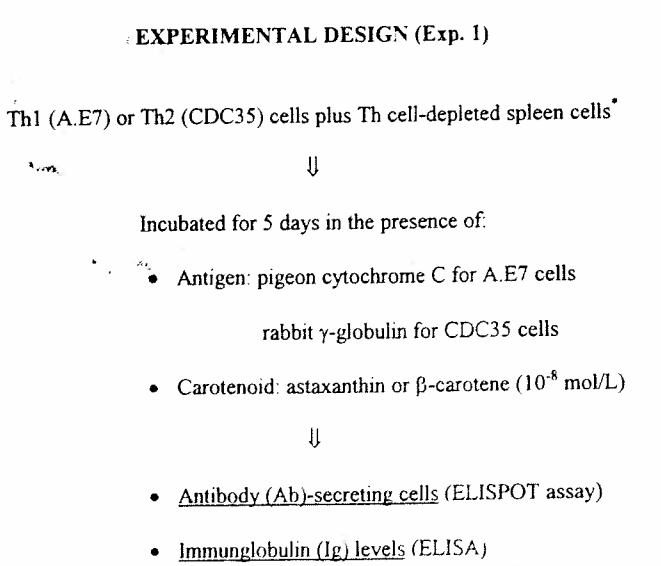


FIGURE 1 Schematic illustration of experimental design of Experiment 1.

Statistics. Numbers of Ab-secreting cells in carotenoid-supplemented cultures were compared with those in control cultures without carotenoid supplementation at each concentration of Ag in each Th clone. This analysis was done in numbers of both IgM and IgG Ab-secreting cells. Numbers of IgG Ab-secreting cells without carotenoid supplementation were compared with the use of unprimed vs. primed B cells at each concentration of Ag in both Th clones. Either Student's *t* test or Welch's test (Snedecor and Cochran 1971) were used to analyze the data on the basis of the results of *F* test (Snedecor and Cochran 1971); Student's *t* test if $F > 0.05$ and Welch's test if $F < 0.05$. Differences were considered to be significant when $P < 0.05$.

RESULTS

Carotenoid action on T-dependent Ab responses with cloned Th cells and unprimed B cells. *Experiment 1.* T-dependent Ab responses in cultures with Th cell clones were assessed by measurement of Ab-secreting cell numbers. Total Ig concentrations in the supernatant were measured to assess nonspecific Ig production secondary to polyclonal B cell activation. The results of five replicate experiments for each Th cell clone are as follows:

Numbers of IgM Ab-secreting cells. Astaxanthin augmented the number of IgM Ab-secreting cells in cultures with Th clones and suboptimal doses of Ag [cytochrome C; 0.5 μmol/L ($P < 0.05$ vs. control, **Fig. 2***A*) and rabbit γ-globulin; 5 mg/L ($P < 0.01$ vs. control, **Fig. 3***A*)]. β-Carotene also enhanced the number of IgM Ab-secreting cells in cultures with suboptimal doses of Ag [cytochrome C; 0.25 and 0.5 μmol/L, ($P < 0.05$ vs. control, Fig. 2*B*) and rabbit γ-globulin; 5 mg/L ($P < 0.05$ vs. control, Fig. 3*B*)]. Neither carotenoid increased numbers of Ab secreting cell in the presence of optimal doses of Ag (cytochrome C; 2–4 μmol/L, rabbit γ-globulin; 10–20 mg/L). In the absence of Th cell clones, numbers of both IgM and IgG Ab-secreting cells were negligible (data not shown).

Numbers of IgG Ab-secreting cells. IgG Ab-secreting cells did not increase significantly in response to Ag stimuli specific for each Th clone as compared with those generated in the absence of Ag stimuli; unprimed B cells predominantly produce IgM Ab when exposed to neoantigen.

Ig concentrations in the culture supernatant. Total IgM and IgG concentrations in culture supernatants were not significantly increased by stimulation with Ag stimuli compared with unstimulated control cultures, irrespective of carotenoid supplementation, the Th clone type or doses of Ag (data not shown).

Carotenoid action on T-dependent Ab responses with cloned Th cells and primed B

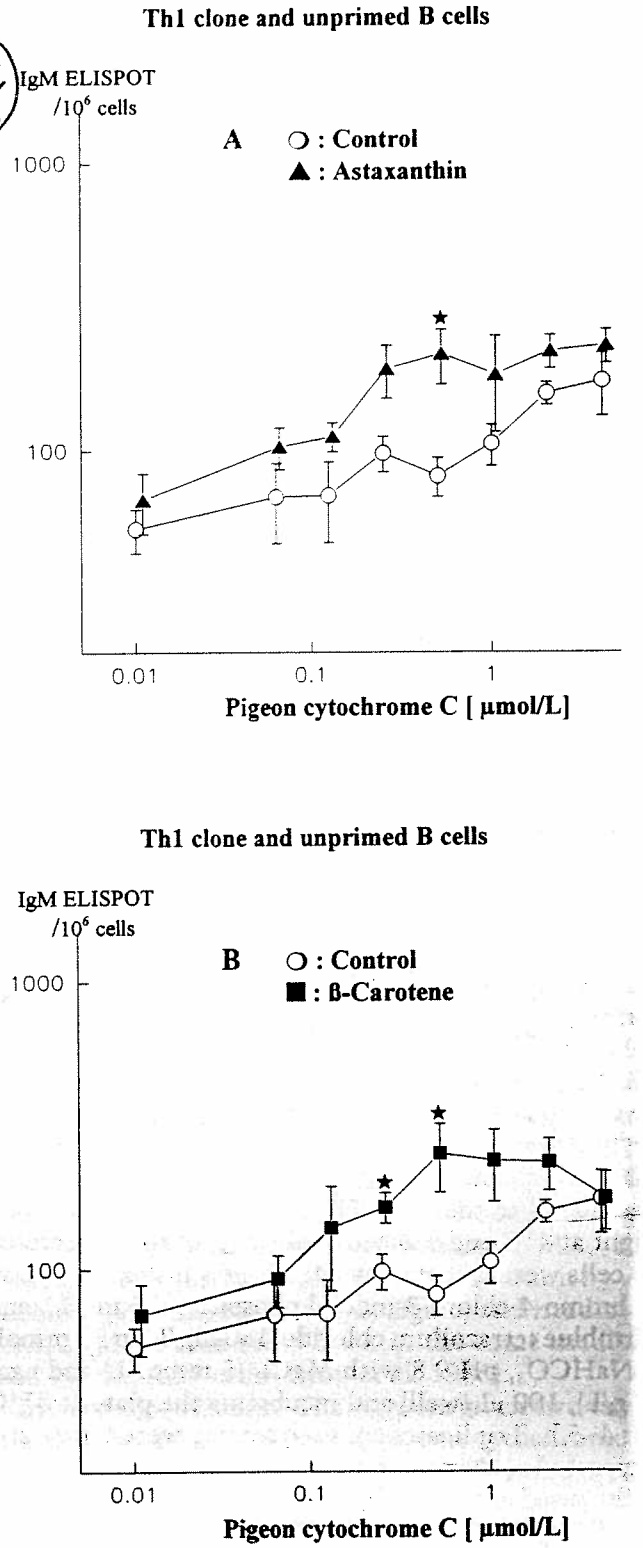


FIGURE 2 Numbers of IgM Ab-secreting cells in r sponse to various doses of pigeon cytochrome C. Th1 ce clones and Th-cell depleted B10 spleen cells were culture for 5 d, and numbers of Ab-secreting cells were detected b ELISPOT assay. Spleen cells were obtained from untreate B10 mice, depleted of Th cells and used as the source of A presenting cells and unprimed B cells. The cells were culture with astaxanthin (10^{-8} mol/L) (*A*) or β-carotene (*B*). Contro were cultured without carotenoid supplementation. Ea data point represents mean ± SEM ($n = 5$). ★ significant higher than control values, $P < 0.05$.

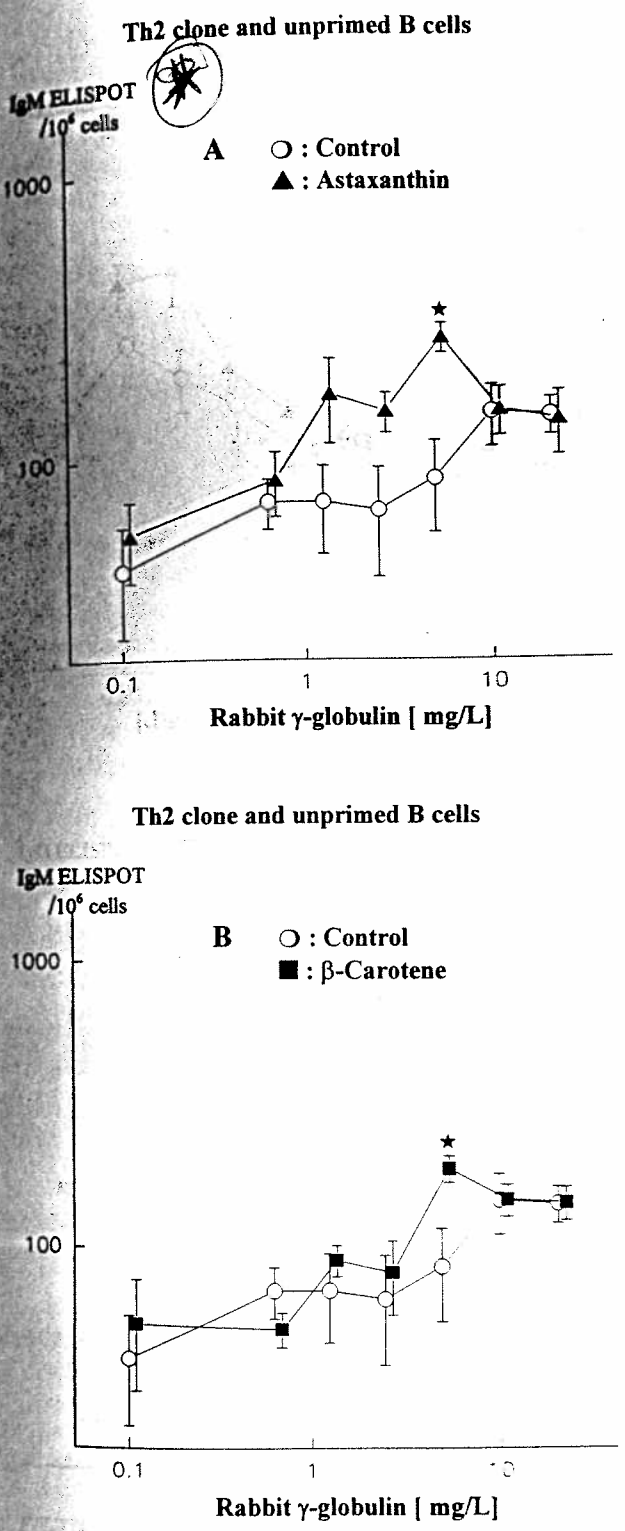


FIGURE 3 Numbers of IgM Ab-secreting cells in response to various doses of rabbit γ-globulin. Th2 cell clones and Th-cell depleted DBA/2 spleen cells were cultured for 5 d, and numbers of Ab-secreting cells were detected by ELISPOT assay. Spleen cells were obtained from untreated DBA/2 mice and used as the source of Ag-presenting cells and unprimed B cells. The cells were cultured with astaxanthin (10^{-8} mol/L) (*A*) or β-carotene (*B*). Control cells were cultured without supplementation of carotenoids. Each data point represents mean ± SEM ($n = 5$). ★ significantly higher than control values, $P < 0.01$ for astaxanthin and $P < 0.05$ for β-carotene.

cells. *Experiment 2.* Carotenoid action was assessed in the same way as described for Experiment 1 (Fig. 1), except for the use of primed spleen cells. The results of five replicate experiments are as follows:

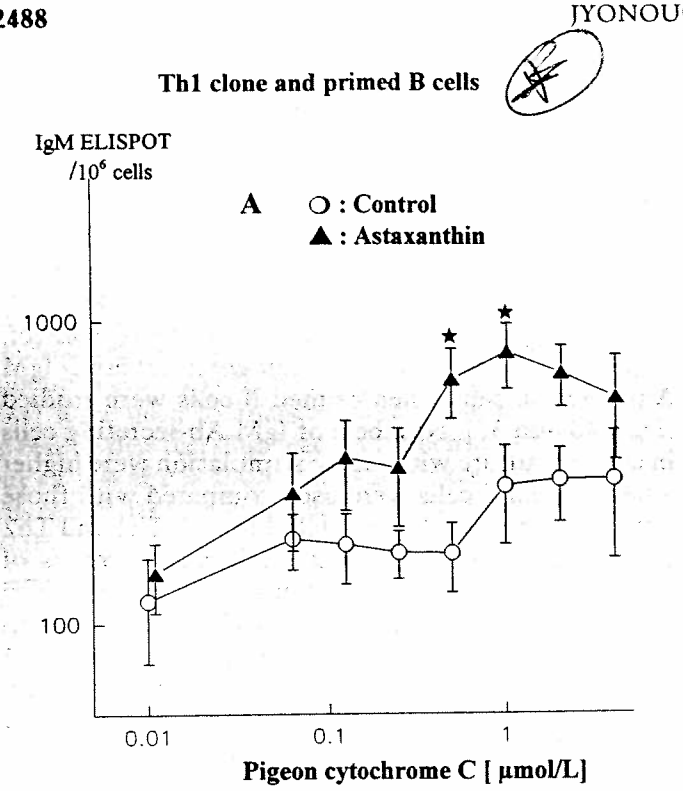
Numbers of IgM Ab-secreting cells. Astaxanthin augmented the number of IgM Ab-secreting cells, with the use of suboptimal doses of Ag [pigeon cytochrome C; 0.5 and 1 μmol/L, ($P < 0.05$ vs. control, **Fig. 4***A*), and rabbit γ-globulin; 5 mg/L, ($P < 0.05$ vs. control, **Fig. 5***A*)]. β-Carotene did not augment numbers of IgM Ab-secreting cells when primed B cells were studied (Figs. 4*B* and 5*B*). Numbers of IgM Ab-secreting cells in control culture without Ag stimulation were higher when primed B cells were used compared with those with unprimed B cells ($P < 0.05$ for both Th1 and Th2 cell clones); this may reflect the higher numbers of preexisting Ab-secreting cells in spleen in response to in vivo Ag challenge given to mice before the experiment. In the absence of Th clones, numbers of IgM and IgG Ab-secreting cells were $< 1/10^5$ cells even with the use of primed B cells (data not shown).

Numbers of IgG Ab-secreting cells. Numbers of IgG Ab-secreting cells were higher with the use of primed B cells as compared with those with unprimed B cells in Ag-stimulated cultures with both Th cell clones ($P < 0.05$ at 0.5–4 μmol of pigeon cytochrome C and at 2.5–20 mg/L rabbit γ-globulin). This is partly due to the fact that primed B cells predominantly produce IgG Ab. Astaxanthin augmented the numbers of IgG Ab-secreting cells in cultures with primed B cells, Th clones and suboptimal doses of Ag [pigeon cytochrome C; 0.5 μmol/L, $P < 0.02$ and 1 μmol/L, $P < 0.05$ vs. controls (**Fig. 6***A*) and rabbit γ-globulin; 2.5 mg/L and 5 mg/L, $P < 0.05$ vs. controls (**Fig. 7***A*)]. Neither carotenoid augmented IgG Ab production with optimal doses of Ag [pigeon cytochrome C; 2–4 μmol/L (Fig. 6), rabbit γ-globulin 10–20 mg/L (Fig. 7)]. β-Carotene did not augment the number of IgG Ab-secreting cells with the use of primed B cells, irrespective of type of the Th clone and doses of Ag (Figs. 6*B* and 7*B*).

Ig concentrations in the culture supernatants. Total IgG and IgM concentrations in the culture supernatants were not altered by carotenoid supplementation (data not shown).

DISCUSSION

This study demonstrates that astaxanthin enhances cloned Th1 and Th2 cell actions on Ab responses (measured as the number of Ab-secreting cells) in reconstitution experiments in which murine spleen cells were depleted of Th cells and replaced with cloned Th cells. This enhancing action was demonstrated with suboptimal but not optimal Ag challenges. These results are the first direct, quantitative demonstration of carotenoid effects on Ab responses of Th1 and Th2



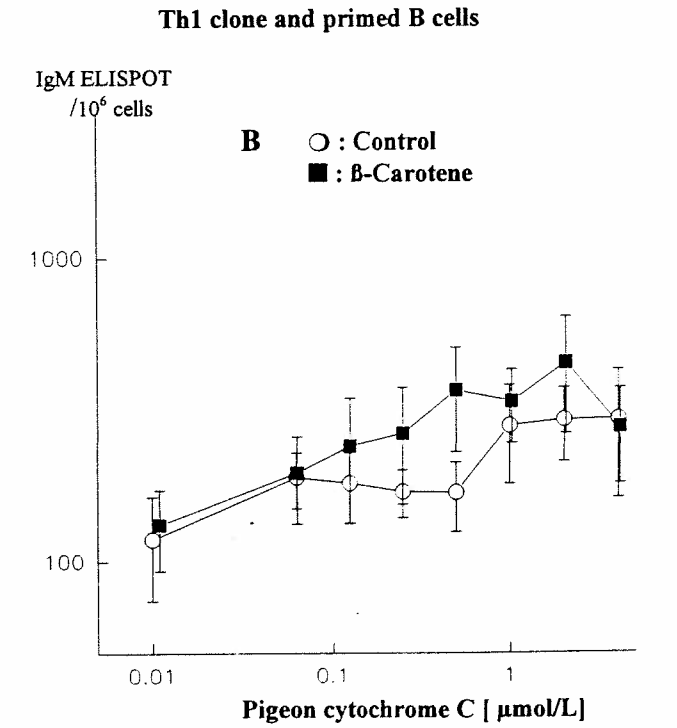
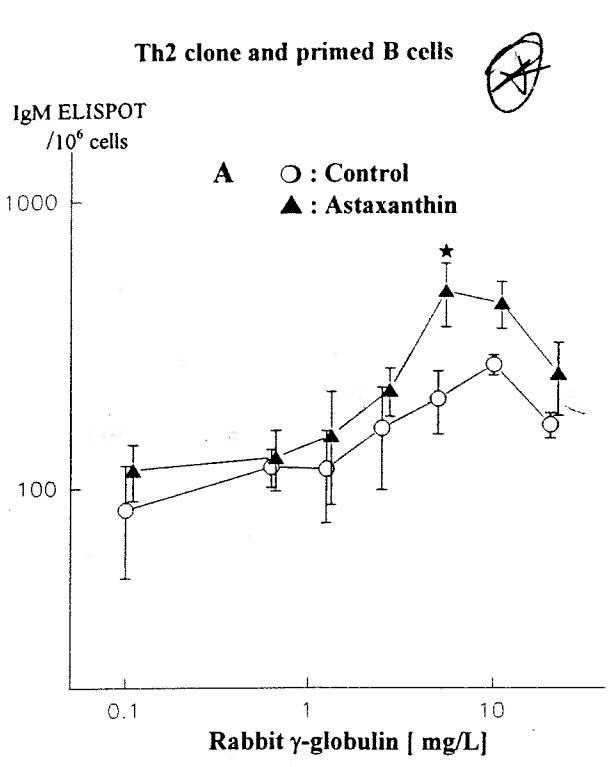


FIGURE 4 Numbers of IgM Ab-secreting cells in response to various doses of pigeon cytochrome C. Th1 cell clones and Th-cell depleted B10 spleen cells were cultured for 5 d, and numbers of Ab-secreting cells were detected by ELISPOT assay. Spleen cells were obtained from in vivo primed B10 mice and used as the source of Ag-presenting cells and unprimed B cells. The cells were cultured with astaxanthin (10^{-8} mol/L) (*A*) or β-carotene (*B*). Control cells were cultured without carotenoid supplementation. Each data point represents mean ± SEM ($n = 5$). ★ significantly higher than control values, $P < 0.05$.



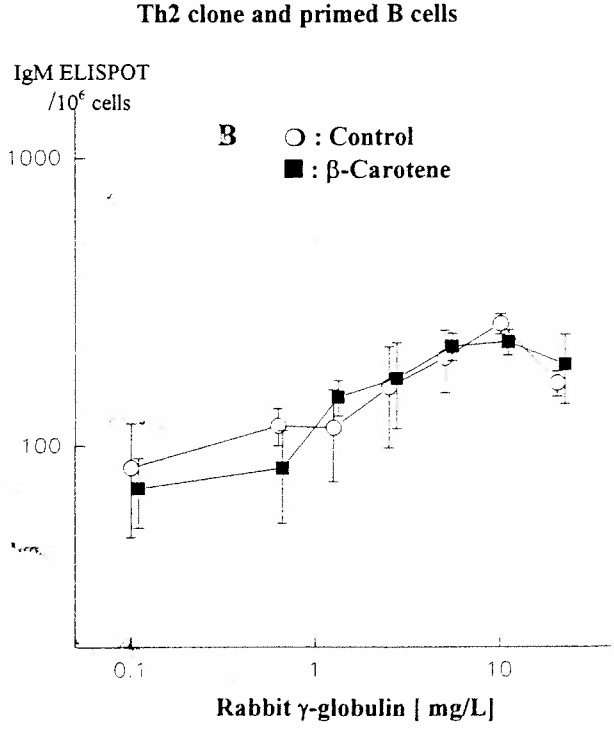


FIGURE 5 Numbers of IgM Ab-secreting cells in response to various doses of rabbit γ-globulin. Th2 cell clones and Th-cell depleted DBA/2 spleen cells were cultured for 5 d and numbers of Ab-secreting cells were detected by ELISPOT assay. Spleen cells were obtained from in vivo primed DBA/2 mice and used as the source of Ag-presenting cells and unprimed B cells. The cells were cultured with astaxanthin (10^{-8} mol/L) (*A*) or β-carotene (*B*). Control cells were cultured without carotenoid supplementation. Each data point represents mean ± SEM ($n = 5$). ★ significantly higher than control values, $P < 0.05$.

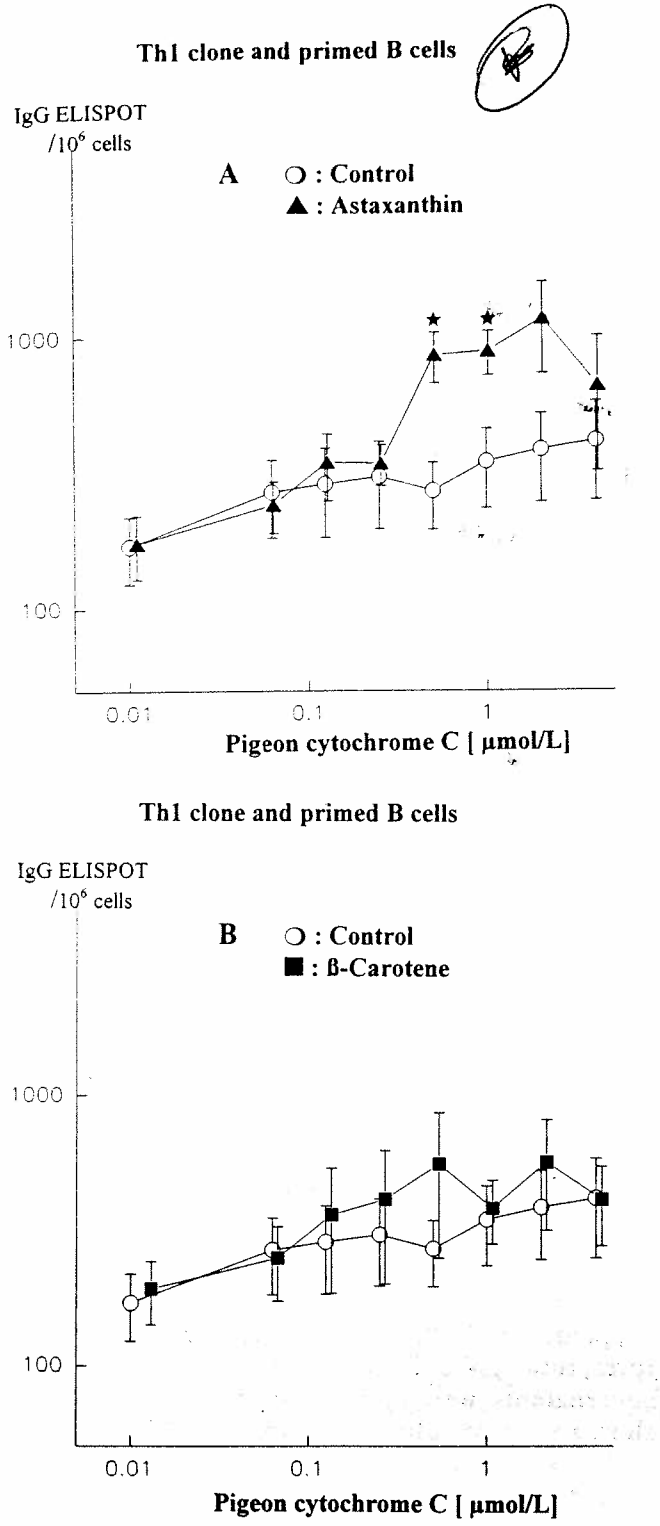


FIGURE 6 Numbers of IgG Ab-secreting cells in response to various doses of pigeon cytochrome C in the same experiment described in Figure 4. Each data point represents mean ± SEM ($n = 5$). ★ significantly higher than control values, $P < 0.02$ (pigeon cytochrome C; 0.5 μmol/L) and $P < 0.05$ (pigeon cytochrome C; 1 μmol/L). The cells were cultured with astaxanthin (*A*) and β-carotene (*B*).

cells. The experimental system used in this study may have several advantages over the previous experimental systems as follows: *1*) use of purified protein Ag with limited epitopes, *2*) much higher frequency of Ag-specific Th cells and *3*) use of primed and unprimed B cells.

Previously, we showed that astaxanthin enhances humoral immune responses by murine spleen cells (Jyonouchi et al. 1991, Jyonouchi et al. 1994). In these studies, SRBC, a crude Ag with multiple epitopes, was a source of Ag stimuli (Mond et al. 1991). Responses to SRBC were against multiple epitopes and influenced by cross-reactivity between SRBC and murine red blood cells (Mond et al. 1991). To assess carotenoid action on Th cell responses to Ag stimuli, a better model may be to use purified protein Ag with limited numbers of epitopes than to employ crude Ag.

The frequency of Th cells reactive to specific Ag is very low in unprimed animals (Miller 1991); this makes it extremely difficult to analyze carotenoid action on Ag-specific Th cells in a direct manner. To overcome this problem, our study uses Th cell clones; cloned Th cells are expanded from a single cell primed with purified protein Ag and are in the stage of effector Th cells (Croft 1994). By replacing Th cells in the spleen cell suspension with Th clones, the frequency of Ag-specific Th cells is greatly increased, and this manipulation permits the evaluation of carotenoid actions on each Th cell subset. In this study, carotenoid action was examined using both unprimed and primed B cells; although unprimed B cells predominantly produce IgM Ab, primed B cells produce more IgG Ab. In this way we are able to examine carotenoid action on both IgM and IgG Ab responses. In summary we adapted the assay system as shown in Figure 1 using Th clones. This in vivo assay permits the quantification of carotenoid action on IgM vs. IgG Ab responses against purified protein Ag specific for each Th clone.

The astaxanthin concentration used in this study was 10^{-8} mol/L. In the cultures of murine Th clones and B cells, this concentration appears to be most potent, consistent with our previous observations (Jyonouchi et al. 1991, Jyonouchi et al. 1993, Jyonouchi et al. 1994). In human cells total IgM and IgG Ab responses to T-dependent stimuli are higher at 10^{-8} to 10^{-9} mol/L astaxanthin (Jyonouchi et al. 1995). Carotenoid antioxidant activity and enhancement of gap-junctional communication have been found at higher concentrations (> 10^{-6} mol/L) (Gerster 1993, Hanusch et al. 1995, Van Poppel 1993, Zhang et al. 1992). In our preliminary experiments astaxanthin did not protect hyperoxic injury at a concentration of 10^{-8} mol/L when both an epithelial cell line and spleen cells were used as indicator cells (Jyonouchi, H., Sun, S., and Inbgar, D., unpublished observation). Because astaxanthin has negligible vitamin A activity in mammals and astaxanthin concentrations used in our study are too low for its antioxidant activity, astaxanthin's action on Th cell-mediated Ab responses may result from mechanisms not yet described. We are now carefully examining carotenoid action on each Th clone in re-

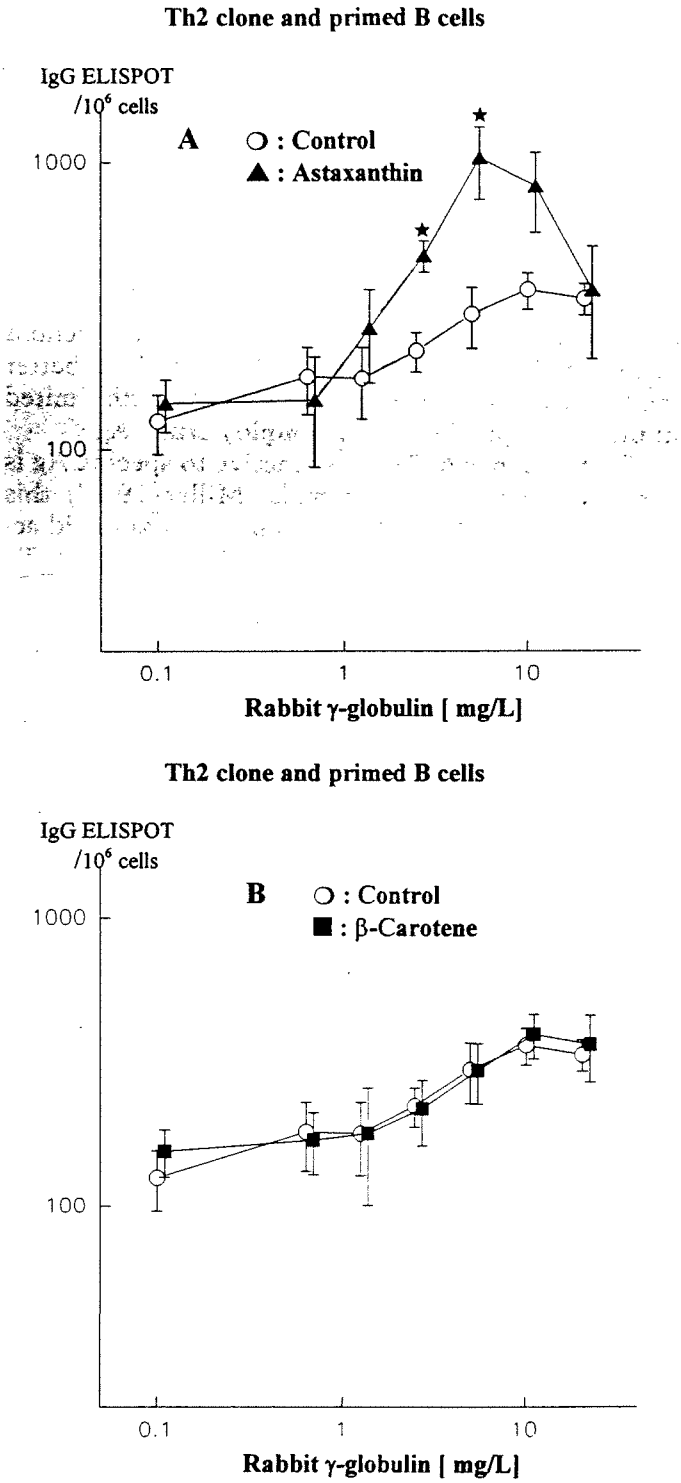


FIGURE 7 Numbers of IgG Ab-secreting cells in response to various doses of rabbit γ-globulin in the same experiment described in Figure 5. Each data point represents mean ± SEM ($n = 5$). ★ significantly higher than control values, $P < 0.005$ (rabbit γ-globulin; 2.5 mg/L) and $P < 0.05$ (rabbit γ-globulin; 5 mg/L). The cells were cultured with astaxanthin (*A*) and β-carotene (*B*).

gard to cytokine production and cell surface molecule expression.

Th cells, especially naive Th cells, are difficult to activate with suboptimal doses of Ag (Liu and Janeway 1991). At the initial stage of pathogen invasion, doses of Ag challenges may be suboptimal to elicit effective immune responses. However, nonspecific stimulants may modulate processes of Ag presentation and enhance Th cell activation. These stimulants include byproducts of inflammatory reactions such as double stranded RNA and endotoxins (Janeway 1992, Liu and Janeway 1991). This may also be true for astaxanthin's action on Th cells; astaxanthin could potentiate or facilitate Ag-specific immune responses with suboptimal Ag challenges before serious, potentially hazardous inflammatory reactions occur.

Murine Th cells consist of at least two subsets based on the patterns of cytokine secretion (Swain et al. 1991). Th1 cells produce interferon-γ and interleukin (IL)-2 and are more involved in cellular, phagocytic cell-mediated, proinflammatory immune responses. Although Th2 cells produce IL-4, IL-5, IL-10, IL-13 and are more dominant in humoral immune responses and down regulate proinflammatory responses (Fiorentiono et al. 1991, Swain et al. 1991, Zurawski and de Vries 1994). This is consistent with other's reports that Th1 cells have less helper activity for Ab production than Th2 cells (Swain et al. 1991). Most infectious pathogens induce mixed Th1 and Th2 responses. Usually, Th1 responses are dominant in the initial stage of infection. Th2 responses follow Th1 responses and are more Ag-specific and down regulate inflammatory responses (Biron 1994). The induction of Th1 and Th2 responses are largely dependent on the local microenvironment including cytokine concentrations (Paul and Seder 1994). In this study we found that astaxanthin and β-carotene enhanced Th functions exhibited by both Th1 and Th2 cell clones. Astaxanthin's actions were demonstrated for both primary (IgM) and secondary (IgG) immune responses. Therefore, our results suggest that astaxanthin, a naturally present carotenoid, could modulate Th1 and Th2 responses.

It is also noteworthy that although we detected greater numbers of Ab-secreting cells in the carotenoid supplemented cultures compared with control cultures, total IgM and IgG concentrations in the culture supernatants were not altered by carotenoids. The short period of culture (5 d) employed in our experimental system does not permit B cells in the culture to secrete large amounts of Ab in response to Ag stimuli; it usually requires > 7–10 days for Ag-specific B cells to produce enough Ab to be detected as an increase in total Ig levels in the culture supernatants. The amount of a specific Ab produced against Ag stimuli may be only a fraction of the total Ig produced in the culture and thus the total IgG and IgM concentrations are not sensitive enough to reflect changes in Ab production. The ELISPOT assay is generally more sensitive than detection of the total amounts of Ab secreted (Sedgwick and Holt 1983). We did not find significant increases in total Ig concentrations when murine spleen cells were primed with SRBC for 5 d in

the carotenoid-supplemented cultures (Jyonouchi et al. 1993). It is possible to detect increases in total IgM concentrations within 5 d in cultures including a nonspecific, polyclonal stimulant such as lipopolysaccharide. We used total Ig concentrations as markers for polyclonal B cell activation, and we did not detect any significant increase. Thus it may be concluded that carotenoids augment Th clone-mediated Ab responses but do not cause polyclonal B cell activation even in the presence of activated Th clones; this makes it unlikely that carotenoid-immunomodulating action induces autoimmune phenomena mediated by polyclonal B cell activation.

The enhancement of humoral immune responses with astaxanthin occurs with both unprimed and primed B cells. Astaxanthin may be effective in promoting secondary immune responses against pathogens and transformed cells. Secondary immune responses decline in senior citizens (> 65 y) in parallel to loss of memory T cells (Thoman and Weigle 1989). This may be associated with increased frequency of infections, cancer and autoimmunity in senior citizens. Astaxanthin's enhancing action on secondary immune responses may have a potential role in older individuals, partially restoring specific Ab responses.

In summary we have established a sensitive assay system for carotenoid action on T-dependent Ab responses and have shown astaxanthin and β-carotene's enhancing actions on Th cell clone-mediated Ab responses with suboptimal Ag challenges in a quantitative, direct manner. This experimental system may be useful for further elucidation of carotenoid biological actions and mechanisms of action.

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