

Effect of Carotenoids on *In Vitro* Immunoglobulin Production by Human Peripheral Blood Mononuclear Cells: Astaxanthin, a Carotenoid Without Vitamin A Activity, Enhances *In Vitro* Immunoglobulin Production in Response to a T-Dependent Stimulant and Antigen

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Abstract

The effect of carotenoids on *in vitro* immunoglobulin (Ig) production by peripheral blood mononuclear cells (PBMNC) was examined by employing blood samples from adult volunteers and full-term newborn babies (umbilical cord blood). Under carotenoid-supplemented culture conditions, cells were stimulated by polyclonal stimulants, neoantigens, and a recall antigen (Ag), and IgM, IgA, and IgG levels in the culture supernatant were measured. β -Carotene and astaxanthin were used as representatives of carotenoids with and without vitamin A activity, respectively.

Astaxanthin enhanced IgM production in response to T-dependent Ag (TD-Ag) and a T-dependent polyclonal stimulant. Astaxanthin also augmented IgG production in response to a recall Ag. IgA production without supplemental carotenoids was negligible for all stimuli. However, in carotenoid-supplemented cultures, IgA production was significantly higher in response to a T-dependent polyclonal stimulant than in unsupplemented cultures. IgM and IgA production was augmented at 10^{-3} mol/l astaxanthin, whereas astaxanthin enhanced IgG production in response to a recall Ag at 10^{-10} – 10^{-9} mol/l. Similar enhancing actions of astaxanthin on IgM production were observed in cord blood mononuclear cells (CBMNC), although CBMNC produced less IgM than adult PBMNC. β -Carotene did not have a significant effect on human Ig production. The carotenoid actions were not demonstrated under serum-free culture conditions; serum is essential for solubilization of carotenoids. In summary, this study has shown for the first time that astaxanthin, a carotenoid without vitamin A activity, enhances human Ig production in response to T-dependent stimuli.

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Introduction

Carotenoids are widely distributed in green and yellow vegetables, fruits, and certain sea plants. Dietary intake of carotenoids and plasma carotenoid levels are positively correlated with the prevention of cancer and other degenerative diseases (1–3). The mechanisms of carotenoid biologic actions are not fully understood (1–4) and may include vitamin A activity,

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antioxidant activity, the enhancement of gap-junctional communication, and suppression of mutagenesis (1,5-8). Apart from their direct suppressive effect on tumor growth, carotenoids can modulate functions of the immune system (9,10). The enhancement of tumor immunity (9,10) as well as other carotenoid-modulating actions on the immune system have been reported, especially under conditions of immunologic stress (11-13), which may be a major factor in their chemopreventive action.

Our study of carotenoid biologic actions focuses on humoral immunity. In animal models, we have shown that carotenoids with and without vitamin A activity enhance antibody (Ab) production against T-dependent antigen (TD-Ag) *in vivo* and *in vitro* (14-16). Carotenoids seem to exert their enhancing actions partly through modulating T-helper (Th) cell functions in the initial stage of Ag priming (15). This carotenoid action occurs at carotenoid concentrations of around 10^{-8} mol/l. In this study, we examined the effects of carotenoid supplementation on human immunoglobulin (Ig) production *in vitro*. We found that astaxanthin, a carotenoid without vitamin A activity, enhances Ig production by human lymphocytes in response to T-dependent stimuli.

Materials and Methods

Study Subjects

Heparinized peripheral blood samples were obtained from healthy adult nonsmokers by venipuncture. Human cord blood samples from healthy full-term newborns were taken from the umbilicus after it was ligated, as approved by the Human Subject Committee, University of Minnesota. Blood samples were obtained on the day of the experiment.

Cell Suspensions

Peripheral blood mononuclear cells (PBMNC) and cord blood mononuclear cells (CBMNC) were separated by Ficoll-Hypaque gradients and washed three times with phosphate-buffered saline (PBS; pH 7.4) before use.

Reagents

All-*trans*- β -carotene was purchased from Sigma Chemical (St. Louis, MO). Astaxanthin was kindly provided from Hoffmann-La Roche (Nutley, NJ). Both carotenoids were dissolved in ethanol plus hexane (49:1) and filtered, and the concentrations were measured spectrophotometrically on the day of the experiment: the stock solutions typically ranged from 10^{-4} to 5×10^{-5} mol/l. Further dilution was made with the culture medium for human Ig production assay. The same amount of solvents (ethanol plus hexane) used for dissolving carotenoids did not affect Ig production by adult PBMNC when tested in five healthy adult volunteers, consistent with our previous observations in rodents. Serum-free medium was provided with serum replacement reagent (20 ml/l; TCM, Celox, Hopkins, MN).

In Vitro Ig Production

PBMNC and CBMNC were incubated at 10^9 cells/l in RPMI 1640 with fetal calf serum (FCS, 25 ml/l; Hyclone, Logan, UT), penicillin G (10^5 U/l), streptomycin (100 mg/l), 2-mercaptoethanol (10^{-6} mol/l), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (25 mmol/l), and glutamine (2 mmol/l) in 5-ml disposable plastic tubes with a cap (Costar, Cambridge, MA). Ten days after incubation in a 5% CO_2 incubator, supernatants were harvested, and IgA, IgG, and IgM levels in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (17,18). Stimuli employed to potentiate Ig production include 1) *Staphylococcus aureus* Cowan I strain (SAC, 0.05 g/l; Pansorbin, Calbiochem, San Diego, CA) as a T-independent polyclonal stimulant, 2) pokeweed mitogen (PWM, 1:1,000; GIBCO-BRL, Gaithersburg, MD)

as a T-dependent polyclonal stimulant, 3) trinitrophenol-modified keyhole limpet hemocyanin (TNP-KLH, 10 mg/l) as a TD-Ag, 4) trinitrophenol-modified lipopolysaccharide (TNP-LPS, 2 mg/l; Sigma Chemical) as a T-independent Ag (TI-Ag), and 5) tetanus toxoid (1:10,000; Wyeth-Ayerst, Philadelphia, MA) as a recall Ag. KLH (Calbiochem) was modified with TNP (Sigma Chemical) in our laboratory, as reported previously (17). The concentrations of the stimuli employed were those that produced optimal responses in healthy adults (17,18). In a previous study, no significant differences were found between males and females in Ig production (17). A 10-day incubation period was selected on the basis of the previous study to detect responses of IgM, IgA, and IgG production by human PBMNC (17).

ELISA for Human Ig

An ELISA plate (F96 Maxisorp, Nunc, Naperville, IL) was coated with goat anti-human Ig (20 mg/l; Sigma Chemical) overnight at 4°C in 0.1 ml/l NaHCO₃ (pH 9.6) with 0.2 g/l NaN₃. After the plate was washed 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(hydroxymethyl)aminomethane (pH 8.1) with 1 mmol/l MgCl₂, 0.15 mol/l NaCl, 0.05 ml/l Tween 20, 0.2 mg/l NaN₃, and 10 g/l bovine serum albumin; Sigma Chemical] and incubated at room temperature for two hours. The plate was washed extensively with rinse buffer and incubated with the second Ab (goat anti-human IgG-, IgM-, or IgA-alkaline phosphatase conjugate, 1:1,000–1:3,000 dilution; Sigma Chemical). The color was developed by addition of substrate solution (*p*-nitrophenyl phosphate, 1 g/l; Sigma Chemical), and optical density at 410 nm was read by an ELISA reader. Monoclonal human IgG, IgA, and IgM were used as standards in each plate (IgA, Sigma Chemical; IgM and IgG, ICN, Irvine, CA).

Experimental Design

Experiment 1: PBMNC were tested for *in vitro* Ig production in response to 1) polyclonal stimuli (PWM, a T-dependent polyclonal stimulant, and SAC, a T-independent polyclonal stimulant), 2) neoantigens (TNP-KLH, a TD-Ag, and TNP-LPS, a TI-Ag), and 3) a recall Ag (tetanus toxoid). Cells were cultured in a medium 1) without carotenoid supplementation (control), 2) supplemented with β -carotene (10^{-8} mol/l), and 3) supplemented with astaxanthin (10^{-8} mol/l). Ig production was tested in a total of nine healthy adult volunteers (age >19 yrs, 5 females and 4 males, nonsmokers). The optimal concentration (10^{-8} mol/l) for Ab and Ig production by rodents was effective with humans in preliminary experiments and was used in Experiment 1.

Experiment 2: CBMNC were tested for *in vitro* IgM production in response to PWM, SAC, TNP-KLH, and TNP-LPS in the presence of 1) medium alone (control), 2) β -carotene (10^{-8} mol/l), and 3) astaxanthin (10^{-8} mol/l). A total of five cord blood samples from healthy full-term newborns were tested. In each experiment, adult PBMNC from a healthy volunteer were used as controls. Tetanus toxoid was not used, because CBMNC are not primed with tetanus.

Experiment 3: The dose response of carotenoids was tested with adult PBMNC. PBMNC were stimulated by PWM and tetanus toxoid in the presence of various amounts of carotenoids. For IgM and IgA production, 10^{-10} – 10^{-7} mol/l carotenoids were supplemented to the culture. For IgG production, 10^{-14} – 10^{-7} mol/l carotenoids were added to the culture. A total of eight and five individuals were tested for Ig production in response to PWM and tetanus toxoid, respectively. Similar dose-response experiments for carotenoid action on Ig production in response to SAC were conducted in four individuals.

Experiment 4: Our previous results indicate that the solubility of carotenoids was dependent on serum concentrations of the culture medium (19). Thus the effects of carotenoids

(astaxanthin and β -carotene, 10^{-8} mol/l) on *in vitro* Ig production by PBMNC were tested in culture media supplemented with 1) no serum, 2) 25 ml/l FCS, 3) 50 ml/l FCS, and 4) 100 ml/l FCS. Serum-free medium was provided with TCM (20 ml/l) as a supplement for micro-nutrients, albumin, and transferrin; TCM does not contain lipoproteins. Ig production was potentiated by PWM and tetanus toxoid, because astaxanthin enhanced Ig production in response to these stimuli in preliminary experiments. The experiments were done in triplicates: a total of three individuals were tested.

Statistics

Student's *t* test or Welch's test was used for the comparison of data, based on the results of an *F* test; $p < 0.05$ was considered to be significant.

Results

Effects of Carotenoids on In Vitro Ig Production by Human PBMNC

Ig production by PBMNC was potentiated by polyclonal stimuli (PWM and SAC), neoantigens (TNP-KLH and TNP-LPS), and a recall Ag (tetanus toxoid) in the presence of 1) medium (control), 2) astaxanthin, and 3) β -carotene. A total of nine healthy adult volunteers were tested, and the PBMNC were obtained from each individual. IgM, IgG, and IgA production was modified by carotenoid supplementation (Figure 1) as follows.

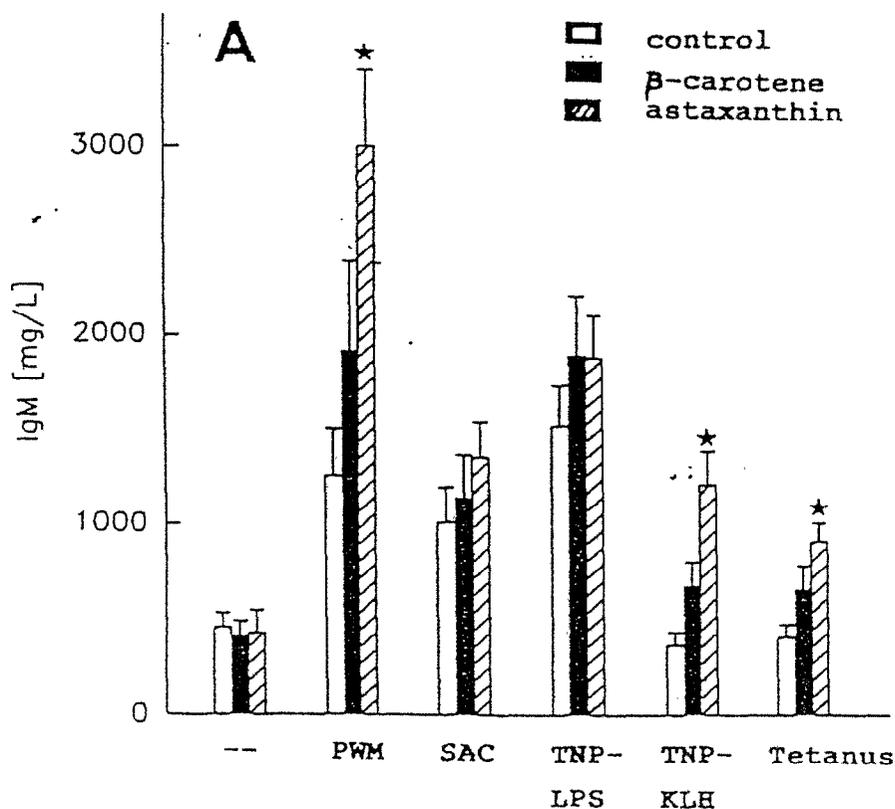


Figure 1. Immunoglobulin (Ig) production by adult peripheral blood mononuclear cells (PBMNC) in response to pokeweed mitogen (PWM, 1:1,000), *Staphylococcus aureus* Cowan I strain (SAC, 0.05 g/l), trinitrophenol-modified lipopolysaccharide (TNP-LPS, 2 mg/l), trinitrophenol-modified keyhole limpet hemocyanin (TNP-KLH, 10 mg/l), and tetanus toxoid (5 mg/l). IgM (A), IgG (B), and IgA (C) levels are expressed as means \pm SEM ($n = 9$). PBMNC were cultured with 1) medium only (control), 2) β -carotene (10^{-8} mol/l), and 3) astaxanthin (10^{-8} mol/l). *, Significantly higher than control values obtained in response to stimuli without carotenoid supplementation (P values are provided in Results); *, significantly higher than background values obtained without stimuli (P values are provided in Results).

IgG production: Astaxanthin (10^{-8} mol/l) enhanced IgG production in response to tetanus toxoid ($p < 0.01$). β -Carotene (10^{-8} mol/l) did not have a significant effect.

IgA production: IgA production in response to PWM was significantly higher in the carotenoid-supplemented cultures ($p < 0.02$ for astaxanthin and $p < 0.05$ for β -carotene vs. background values). IgA production in cultures without supplemental carotenoids was negligible for all stimuli.

Effects of Carotenoids on Ig Production by Cord Blood Lymphocytes

CBMNC from healthy full-term babies were stimulated with polyclonal stimulants (PWM and SAC) and neoantigens (TNP-LPS and TNP-KLH). Response to recall Ag was not tested: CBMNC were unprimed, and recall Ag responses were not possible (18). CBMNC produced less IgM than adult PBMNC, consistent with our previous report (18). Nevertheless, astaxanthin augmented IgM production in response to TNP-KLH ($p < 0.05$) (Figure 2). β -Carotene did not enhance IgM production in response to any of the stimuli. As reported previously (18), CBMNC produced negligible amounts of IgA and IgG in response to the stimuli. Neither carotenoid enhanced IgG and IgA production by CBMNC (data not shown).

Dose-Response Study for Carotenoids

The relationship between a carotenoid dose and its action on human Ig production was examined with PBMNC. Ig production potentiated by PWM and tetanus toxoid was examined

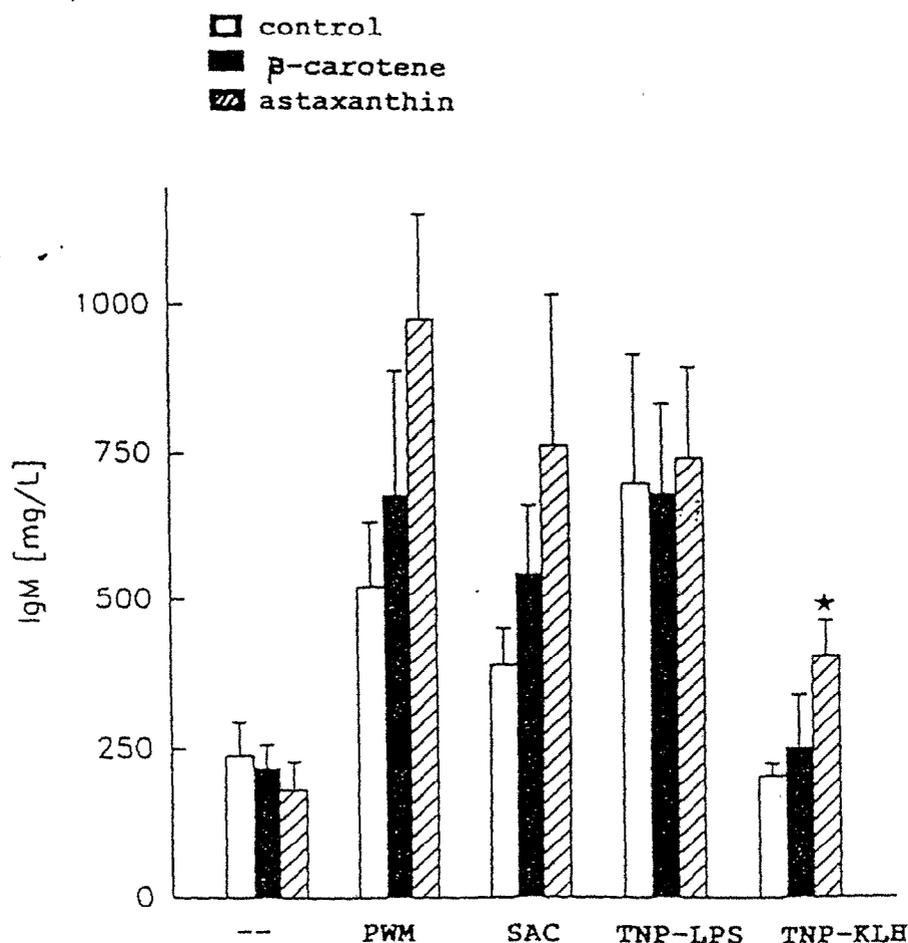


Figure 2. IgM production by cord blood mononuclear cells (CBMNC) in response to PWM (1:1,000), SAC (0.05 g/l), TNP-LPS (2 mg/l), and TNP-KLH (10 mg/l). IgM levels are expressed as means \pm SEM ($n = 5$). Concentration of carotenoids in culture was 10^{-8} mol/l. \star , Significantly higher than control values obtained in response to stimuli without carotenoid supplementation (P values are provided in Results).

for eight and five individuals, respectively. Concentrations of astaxanthin and β -carotene tested were as follows: 1) 10^{-10} – 10^{-7} mol/l for IgM and IgA production and 2) 10^{-14} – 10^{-7} mol/l for IgG production. Results are shown in Figs. 3 and 4 and summarized as follows.

IgM and IgA production: IgM and IgA production were highest at 10^{-8} mol/l astaxanthin when PBMNC were stimulated with PWM and significantly higher than controls without added carotenoids ($p < 0.005$ for IgM and $p < 0.01$ for IgA). IgM and IgA production in response to tetanus toxoid was highest with supplementation of 10^{-9} – 10^{-8} mol/l astaxanthin: the amounts of IgM and IgA produced were higher in the astaxanthin-supplemented cultures than in the unsupplemented control cultures [$p < 0.05$ for IgM (10^{-8} mol/l) and $p < 0.05$ for IgA (10^{-9} mol/l)].

IgG production: IgG production in response to PWM and tetanus was highest at 10^{-10} – 10^{-9} mol/l astaxanthin. The IgG levels were significantly higher than controls [$p < 0.05$ for PWM (10^{-10} mol/l) and $p < 0.05$ for tetanus (10^{-10} and 10^{-9} mol/l)].

Ig production in response to SAC was tested in four individuals with 10^{-10} – 10^{-7} mol/l of each carotenoid, and, consistent with previous experiments, there was no significant augmentation of Ig production (data not shown).

Effect of Serum Concentrations on Carotenoid-Enhancing Action on Human Ig Production

Ig production was measured in PBMNC cultures, in which the media contained 0, 25, 50, and 100 ml/l FCS. PWM and tetanus toxoid were used as stimuli, and the medium was supplemented with each carotenoid at 10^{-8} mol/l. Results were expressed as the percent difference in Ig production compared with control values. Control cultures contained media without carotenoid supplementation (Table 1). Astaxanthin did not enhance IgM and IgG production when cells were cultured in serum-free medium. In the presence of 25, 50, and 100 ml/l FCS in the media, astaxanthin had a similar enhancement effect on Ig production (Table 1). Astaxanthin did not enhance IgA production in response to PWM under serum-free culture conditions (Table 1).

Discussion

Our studies in rodent models demonstrate that astaxanthin, a carotenoid without vitamin A activity, enhances T-dependent humoral immunity *in vivo* and *in vitro* (14–16). In this study, we have shown an enhancing action of astaxanthin on *in vitro* Ig production by human lymphocytes. Human PBMNC produce more IgM, IgG, and IgA in carotenoid-supplemented cultures than in unsupplemented cultures. This is the first description of an enhancement of human Ig production by a carotenoid.

For the *in vitro* culture experiments with human cells, it is necessary to ensure the solubility of carotenoids. In studies using high carotenoid concentrations (10^{-6} mol/l), we confirmed that β -carotene and astaxanthin are readily solubilized in medium supplemented with 100 ml/l FCS: the carotenoids bind to lipoproteins in the media (19,20; unpublished observations). Carotenoids are stable in the culture supernatant. Moreover, astaxanthin and β -carotene are detected in washed cell pellets of carotenoid-supplemented cell cultures (19; unpublished observations). Therefore, astaxanthin is likely to be dissolved in the medium at 10^{-8} mol/l as long as small amounts of FCS are provided to the medium.

To test the serum dependence of carotenoid action in human cell cultures, we examined carotenoid action on human Ig production in the media supplemented with 0–100 ml/l FCS. The results demonstrate that the enhancement of Ig production by astaxanthin requires medium containing FCS. Solvents used for dissolving carotenoids did not affect the assay of human Ig production, consistent with our previous observations in rodents (16). Apparently, the carotenoids dissolve in serum-supplemented medium, and augmentation of human Ig production by carotenoids depends on the presence of lipoprotein-solubilized carotenoids.

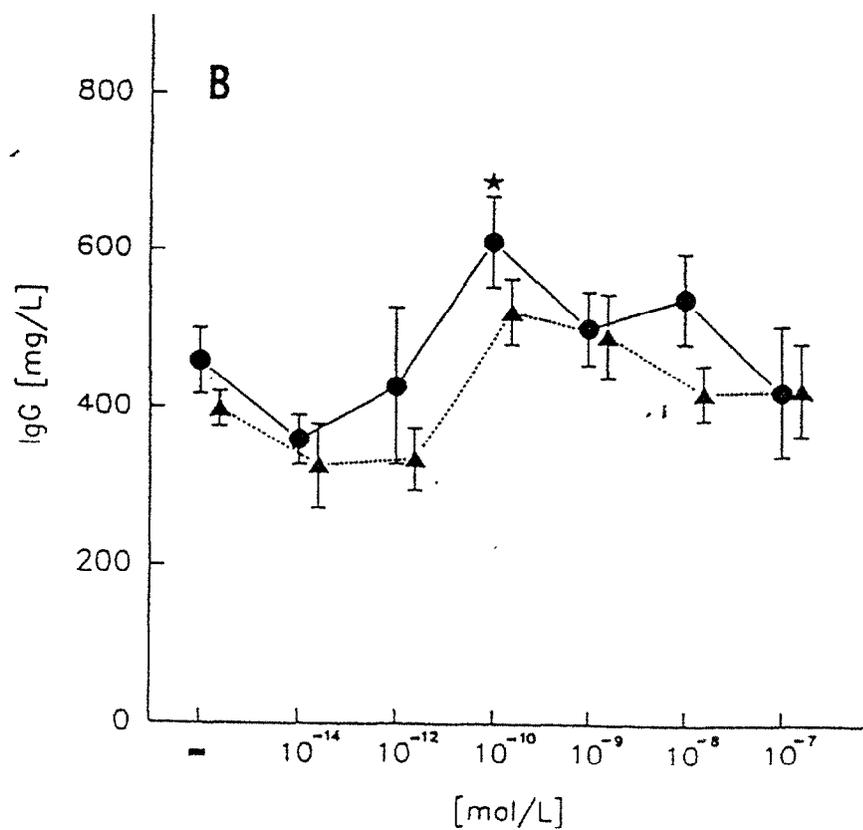
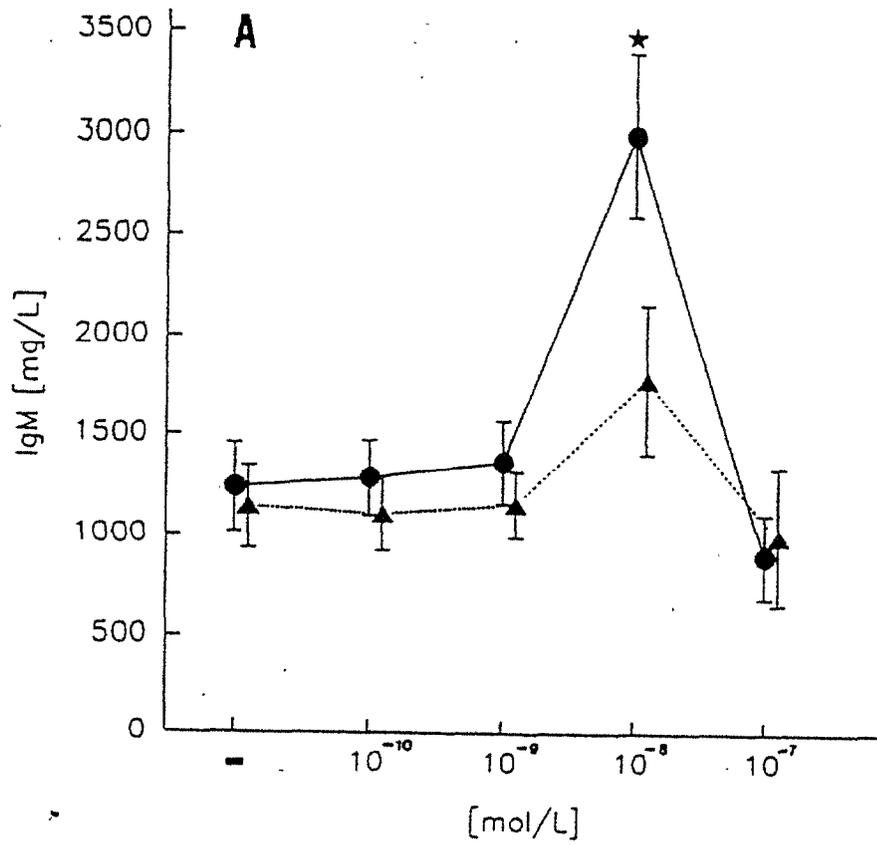
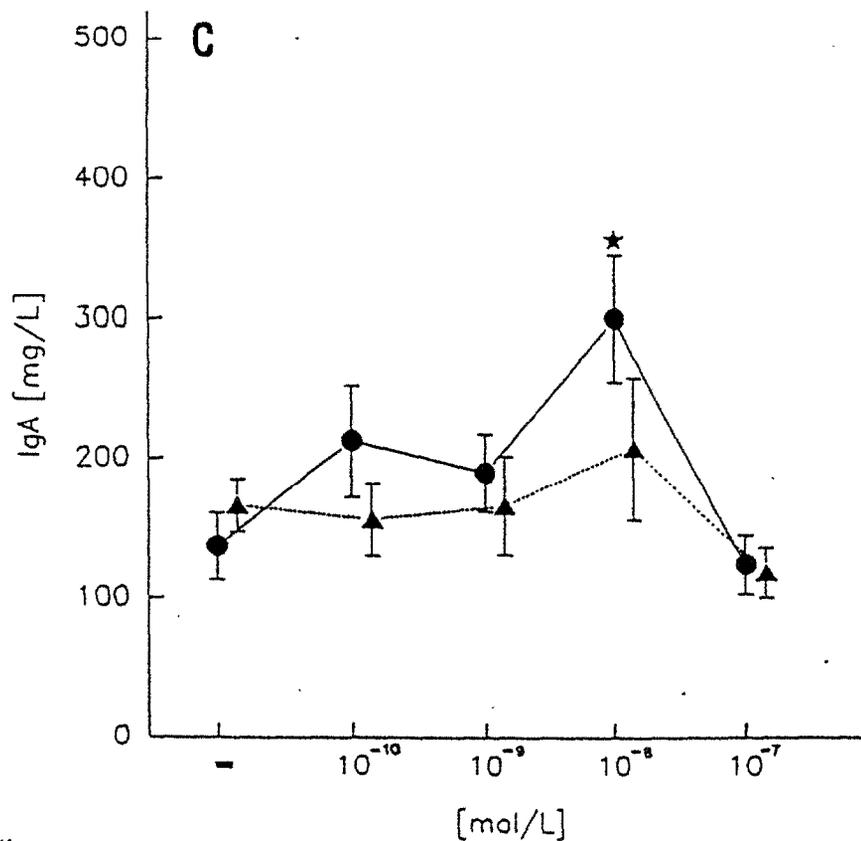


Figure 3. Effects of changes in carotenoid concentrations on IgM (A), IgG (B), and IgA (C) production in response to PWM (1:1,000). Concentrations of astaxanthin (closed circles) and β -carotene (filled triangles) tested were 10^{-10} – 10^{-7} mol/l for IgM production and 10^{-14} – 10^{-7} mol/l for IgG production. *, Significantly higher than control values obtained in response to stimuli without carotenoids (*P* values are provided in Results).



By use of the *in vitro* culture system for human Ig production, this study extends our analysis of carotenoid actions on immune responses in rodents to humans. In previous studies examining Ig production by human lymphocytes, we showed that PBMNC produce significant amounts of IgM and IgG in response to the stimuli used in this study (17). IgA production by PBMNC is negligible for any of the stimuli (17). In the astaxanthin-supplemented culture, PBMNC produce more IgM and IgG in response to a T-dependent polyclonal stimulant (PWM) and TD-Ag. Moreover, carotenoids induced a significant increase of IgA production in response to PWM, a T-dependent polyclonal stimulant. Thus astaxanthin enhances T-dependent Ig production in humans. We showed in rodent models that astaxanthin enhances Ab production through Th cells in the early stages of Ag presentation (14,15). It appears that Th cells exert this enhancing action during the process of cognitive cell-cell interactions between Th cells and Ag-presenting cells (15). These results suggest that carotenoids may also affect Th functions for Ig production in humans.

In the experimental model of tumor rejection, astaxanthin promotes tumor rejection through cytotoxic T cells (21,22). Therefore the astaxanthin effect on human Ig production may also indicate that astaxanthin, a carotenoid without vitamin A activity, preferentially affects T cell functions. This effect may be relevant for interpretations of epidemiological studies indicating carotenoid chemopreventive actions for degenerative diseases including cancer (1,2).

While studying carotenoid action in rodent models, we consistently observed that actions of astaxanthin on *in vitro* Ab production are highest at around 10⁻⁸ mol/l (14,15,23). This is also true for human lymphocytes: astaxanthin is effective at relatively low concentrations in augmenting human Ig production. Others also report that carotenoids without vitamin A activity enhance activation-marker expression by human lymphocytes at 10⁻⁹ mol/l in the culture (24). Carotenoid tumor suppression and antioxidant activity are optimal at higher concentrations (>10⁻⁶ mol/l) (1,2,5-8). Thus our findings in this study suggest that carotenoids may modulate T-dependent human Ig production by as yet undescribed mechanisms, apart from antioxidant and vitamin A activity.

Recent progress in immunology has disclosed a complex yet intricate network of immune

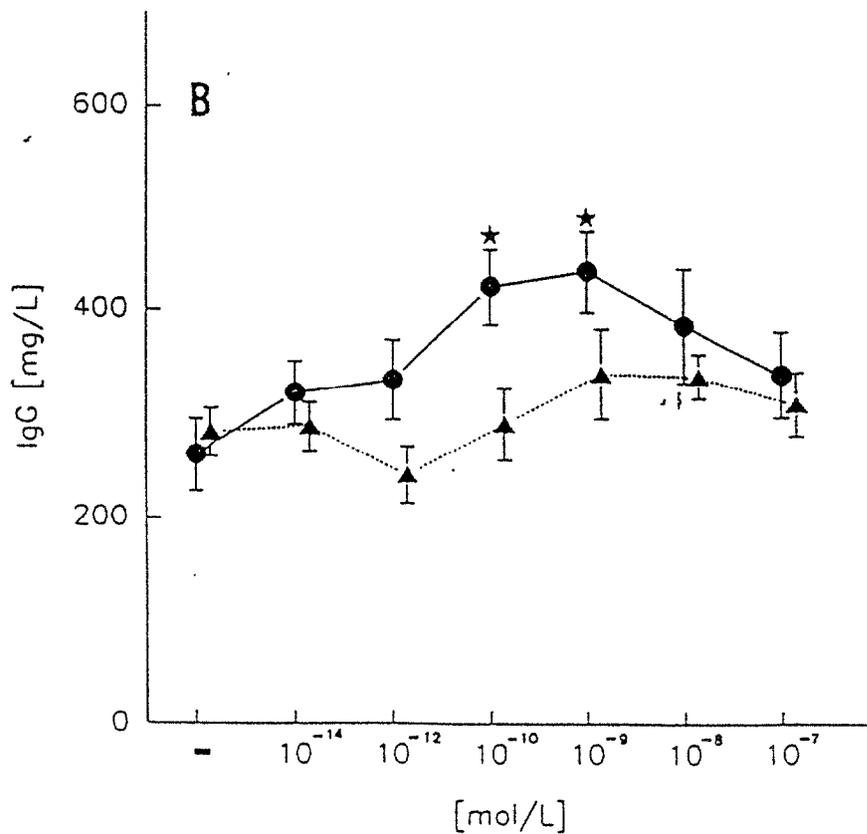
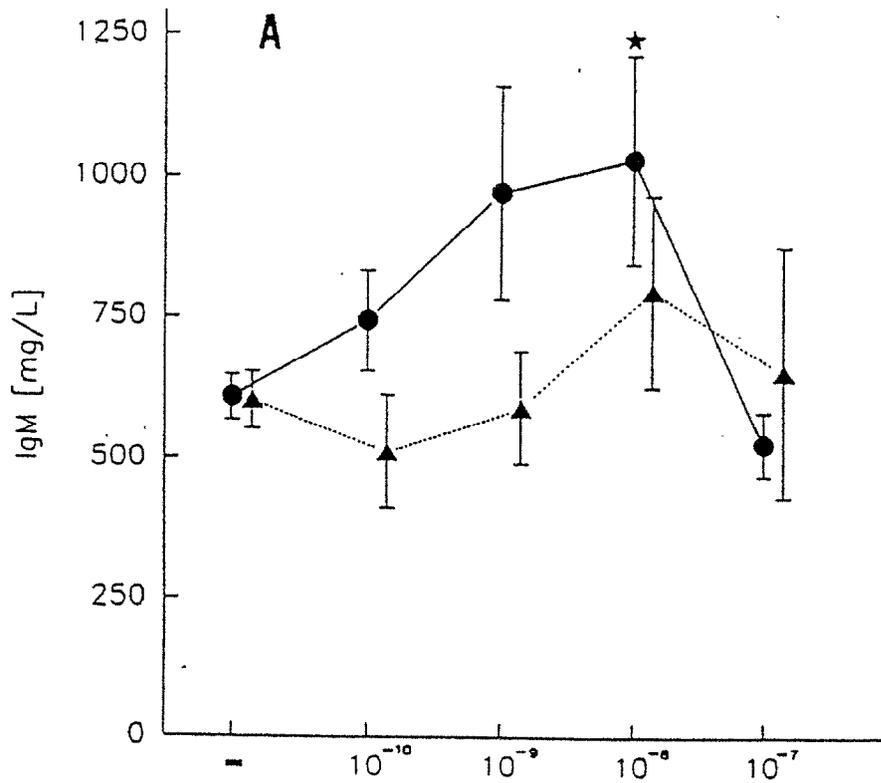


Figure 4. Effects of changes in carotenoid concentrations on IgM (A), IgG (B), and IgA (C) production in response to tetanus toxoid (5 mg/l). Concentrations of astaxanthin (closed circles) and β -carotene (filled triangles) tested were 10^{-10} - 10^{-7} mol/l for IgM production and 10^{-14} - 10^{-7} mol/l for IgG production. *, Significantly higher than control values obtained in response to stimuli without carotenoids (*P* values are provided in Results).

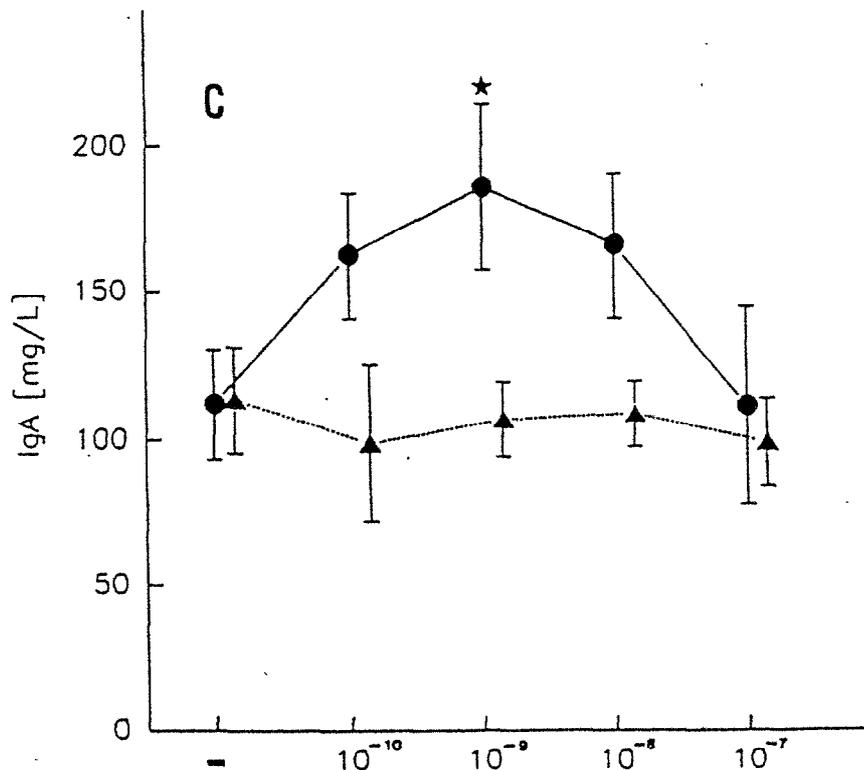


Table 1. Effect of Serum Supplementation on Ig Production by Human PBMNC—Results of Experiment 4^{a-d}

	FCS (0%)	FCS (2.5%)	FCS (5.0%)	FCS (10.0%)
<i>IgM production</i>				
Astaxanthin (10 ⁻⁸ mol/l)				
PWM (1:1,000)	45 ± 9	325 ± 86*	286 ± 41†	201 ± 17†
Tetanus (5 mg/l)	101 ± 24	280 ± 13‡	159 ± 24‡	172 ± 30‡
β-Carotene (10 ⁻⁸ mol/l)				
PWM (1:1,000)	59 ± 17	218 ± 81	135 ± 13	190 ± 70
Tetanus (5 mg/l)	87 ± 20	174 ± 27	184 ± 16	147 ± 15
<i>IgG production</i>				
Astaxanthin (10 ⁻⁸ mol/l)				
PWM (1:1,000)	83 ± 17	220 ± 23†	219 ± 18†	169 ± 5†
Tetanus (5 mg/l)	90 ± 19	182 ± 13§	169 ± 17§	144 ± 7§
β-Carotene (10 ⁻⁸ mol/l)				
PWM (1:1,000)	76 ± 16	138 ± 15	94 ± 4	112 ± 18
Tetanus (5 mg/l)	80 ± 12	133 ± 18	85 ± 11	112 ± 18
<i>IgA production</i>				
Astaxanthin (10 ⁻⁸ mol/l)				
PWM (1:1,000)	93 ± 45	158 ± 32 ³	155 ± 22‡	132 ± 3†
Tetanus (5 mg/l)	63 ± 43	110 ± 40	43 ± 13	94 ± 25
β-Carotene (10 ⁻⁸ mol/l)				
PWM (1:1,000)	75 ± 36	155 ± 58	106 ± 13	120 ± 4
Tetanus (5 mg/l)	70 ± 28	94 ± 75	57 ± 30	140 ± 56

a: Values are means ± SE of 3 replicate experiments expressed as mean percent difference in immunoglobulin (Ig) production in carotenoid-supplemented cultures compared with control values obtained in cultures without carotenoids.

b: Abbreviations are as follows: FCS, fetal calf serum; PWM, pokeweed mitogen.

c: Spontaneous Ig production without stimuli was not significantly altered by carotenoids, irrespective of serum concentrations in culture medium.

d: Statistical significance (Student's *t* test) is as follows: *, *p* < 0.05 vs. control (control values are obtained in cultures without carotenoid supplementation); †, *p* < 0.01 vs. control; ‡, *p* < 0.02 vs. control; §, *p* < 0.005 vs. control.

responses regulated by Th cells. Th cells carry out versatile regulatory actions by producing humoral factors called cytokines and cognitive cell-cell interactions with target cells through cell surface molecules (25). Th cells differentiate into type 1 and type 2 Th cells (Th1 and Th2 cells) upon activation by Ag stimuli or other stimuli (25). In studies using murine Th1 and Th2 clones, carotenoids enhance Th cell functions partly by modulating T-cell activation marker expression and cytokine production (23; unpublished observations). It is possible that carotenoids modulate the human immune system by modifying the process of Th cell activation.

Serum IgA levels are low compared with serum IgM and IgG levels. However, the largest amount of Ig produced in the body is IgA, and it is mainly present in the gastrointestinal and airway mucosa: IgA is believed to have an essential role in the first-line defense mechanisms for pathogen invasion at the mucosal surface (26). In that regard, our finding that carotenoids enhance IgA production against PWM, a T-dependent polyclonal stimulant, is interesting: dietary carotenoid could enhance IgA production of the gastrointestinal and airway mucosa and consequently reduce infection-associated inflammatory responses and lower the risk of tumor transformation.

In summary, this study demonstrates carotenoid's effect on human Ig production: this carotenoid effect may have a role in the prevention of human cancers and other degenerative disease. Further studies of biologic actions of carotenoids and their mechanisms of action in human models will aid in understanding the micronutrient role of carotenoids.

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