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POSSIBLE IMMUNOMODULATING ACTIVITIES OF CAROTENOIDS IN *IN VITRO* CELL CULTURE EXPERIMENTS

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Abstract—Immunomodulating activities of β-carotene and carotene-associated carotenoids such as canthaxanthin (β,β-carotene-4,4 dione) and astaxanthin (3,3′-dihydroxyl β,β-carotene 4,4-dione) were analyzed by *in vitro* cell culture experiments. (i) β-Carotene, canthaxanthin and astaxanthin caused significant stimulatory effects on the cell proliferative response of spleen cells and thymocytes from BALB/c mice at the concentrations of 2×10^{-8} to 10^{-7} M, although they showed the activities different from each other. (ii) Astaxanthin exhibited the highest activity on the polyclonal antibody (immunoglobulin M and G) production of murine spleen cells at the concentrations of 2×10^{-8} to 10^{-7} M but β-carotene did not cause a significant effect at a low concentration (2×10^{-8} M) although stimulated at a high concentration (2×10^{-7} M). Canthaxanthin expressed moderate activities at the same concentrations. (iii) All tested carotenoids significantly enhanced the release of interleukin-1α and tumor necrosis factor-α from murine peritoneal adherent cells at the concentrations of 2×10^{-9} to 10^{-7} M and the ranks of cytokine-inducing activities were astaxanthin > canthaxanthin > β-carotene. These results indicate that carotenoids such as β-carotene, canthaxanthin and astaxanthin have possible immunomodulating activities to enhance the proliferation and functions of murine immunocompetent cells. © 1997 International Society for Immunopharmacology.

Keywords: β-carotene, canthaxanthin, asthaxanthin, immunomodulating activity

Epidemiological studies have indicated that the low incidence of some types of cancer may be associated with the high consumption of vegetables and fruits (Willet, 1994) which contain various types of anticancer substances (Bertrum *et al.*, 1987; Hayatsu *et al.*, 1988). Amongst these antitumor substances, β-carotene has been considered an important constituent for cancer prevention (Peto *et al.*, 1981; Bertrum *et al.*, 1987). However, another investigator claimed that the clinical trial of β-carotene was not effective on cancer prevention (Smigel, 1990). Recently, a large-scale prospective study in Finland has indicated that a supplement with β-carotene significantly increased the incidence of some types of tumor in male smokers (The Alpha-tocopherol, Beta-carotene Cancer Prevention Study Group, 1994). To elucidate this discrepancy, the authors intended to analyze systematically the effects of β-carotene and β-carotene-associated carotenoids on various biochemical and biological events associated with carcinogenesis (tumor initiation, promotion, immunological regulation and so on). Recently, the author and co-researchers showed that β-carotene and canthaxanthin have potential antigenotoxic activities which were much higher than those of ascorbic acid and α-tocopherol, but lower than those of retinol and retinoic acid in the *umu* C gene expression system induced by genotoxic substances (Okai *et al.*, 1996a). Furthermore, they also indicated that β-carotene has enhancing activities for tumor promotor-induced cell proliferation and ornithine decarboxylase activity of the mouse skin fibroblast (Okai *et al.*, 1996b).

In the present study, the authors analyzed the effects of β-carotene and β-carotene-associated carotenoids, canthaxanthin and astaxanthin (Fig. 1) on the proliferation and functions of murine immunocompetent cells by *in vitro* cell culture experiments.

MATERIALS AND METHODS

Chemicals

All *trans*-β-carotene and canthaxanthin were donated from Hoffman and Roth, Japan, and astaxanthin was a gift from Dr M. Tsushima (Kyoto Pharmaceutical College). These carotenoids were dissolved in absolute ethanol to make stock solutions at 2×10^{-4} M and further diluted in the appropriate cell culture medium described below.

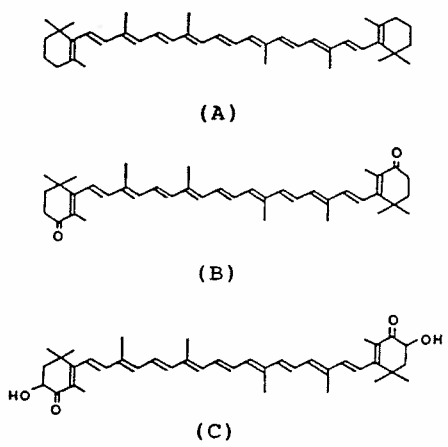

Fig. 1. Chemical structures of carotenoids: (A) β-carotene, (B) canthaxanthin and (C) astaxanthin.

Assay for the proliferative response of spleen cells and thymocytes

Proliferative response of spleen cells or thymocytes was assayed by a modification of the previous method (Okai *et al.*, 1985). Spleen cells (5×10^5) from BALB/c mice (10 weeks old, Charles-River Japan Inc., Kanagawa, Japan) were suspended in 200 μl of RPMI 1640 medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Gibco Co., New York, U.S.A.) and 25 μl of test solution was added. The cells were cultured at 37°C in 5% CO_2 and 95% humidified air for 2 days and labeled with 0.25 μCi of [^{3}H]TdR(6 Ci/mmol, Amersham, Buckinghamshire, U.K.) for last 18 h. The cells were harvested with the aid of a cell harvester and the radioactivity incorporated into the cells was counted by a Beckman scintillation counter.

Assay for antibody production by murine spleen cells

Assay for antibody production was performed by a slight modification of the previous method (Okai and Ishizaka, 1994). Spleen cells (1×10^6) from BALB/c mice (10 weeks old, Charles-River Japan Inc., Kanagawa, Japan) were suspended in 100 μl of RPMI 1640 medium–10% FBS and cultured with 25 μl of test solution in 5% CO_2 and 95% air at 37°C for 4 days in the presence or absence of carotenoids. The number of antibody-producing cells was determined by the protein A-plaque forming cell assay as follows: 25 μl 1:11 dilution of anti-immunoglobulin (anti-Ig) M or anti-Ig G serum (Cederlane, Ontario, Canada), 25 μl protein A-coupled sheep red blood cells, 25 μl guinea pig complement (10-fold dilution) and 25 μl splenocyte suspension were added to 200 μl 0.5% melted agar (Difco) with 0.05% DEAE dextran (Pharmacia-LKB, Uppsala, Sweden). The mixture was placed on Petri dishes, covered with a coverglass, incubated at 37°C for 4 h and the number of plaque-forming cells (PFC) was counted. *E. coli* lipopolysaccharide (Sigma Chemical Co., St. Louis, U.S.A.) was used for the positive control experiment.

Preparation of the peritoneal adherent cells

The peritoneal adherent cells were prepared by a slight modification of the previous method (Okai, 1984). One milliliter of 0.2% oyster glycogen (Sigma) in phosphate-buffered saline (PBS) was injected into each BALB/c mouse. After 3 days, 5 ml of PBS was injected into each mouse and peritoneal fluids of mice were harvested by suction with a pasteur pipette. The peritoneal cells were washed three times with PBS, resuspended in RPMI 1640 medium with 10% FBS and cultured in a Corster plastic culture dish at 37°C for 2 h in 5% CO_2 and 95% air. After the non-adherent cells were washed away with RPMI 1640 medium, the adherent cells were kept for 30 min at 4°C and were recovered gently with a rubber policeman. The recovered cells were resuspended with RPMI 1640 medium–5% FBS and used for further experiments.

Assay for the content of interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) released from peritoneal adherent cells of BALB/c mice

Peritoneal adherent cells (2×10^6) were suspended in 1 ml of RPMI 1640 medium supplemented with 1% FBS and appropriate doses of carotenoids were added to the cell culture medium. After incubation at 37°C for 24 h in 5% CO_2, the culture medium was recovered, frozen and kept at −80°C. The amount of IL-1α or TNF-α in the culture medium was measured by a commercial cytokine assay kit (Genzyme Co., Cambridge, MA, U.S.A.) according to the method in each kit manual. We made a serial dilution system of the test sample in a microtitration plate and compared it with the color development of standard assay data of control purified IL-1α or TNF-α using a spectrophotometer for EIA (Intermed, Tokyo, Japan).

Statistical analysis of the experimental result

The experimental result was expressed as the mean and standard deviation (S.D.) of triplicate assays. The statistical analysis of the experimental results was carried out by Students's *t*-test. In all instances, *P* values less than 0.05 were considered to be the significantly different results between the control and treated experiments.

RESU

Effects of carotenoids on t spleen cells and thymocytes

First we analyzed the e as β-carotene, cantaxanthi proliferative response of I As indicated in Table 1, responses induced by carote compared with the respon experiment by concanava nificant stimulatory effects into spleen cells in a dose- concentrations of 2×10^{-8} when we examined the effe mocyte proliferation, the si carotenoids were observed experiment (Table 2). In ad otenoids, astaxanthin exhi in both proliferation assay lowest activity and cantha erate activity.

Effects of carotenoids on an cells

Next, to analyze the effe specific function of spleer effects of carotenoids on th spleen cells by plaque-forn shown in Table 3, when th with carotenoids, enhancin antibody production were cated the increase in the P Ig G compared with the cc

Table 1. Effects of carotenoids of spleen cells fro

Test sample	[(
Control (PBS)	1
β-Carotene	
2×10^{-8} M	2
2×10^{-7} M	2
Canthaxanthin	
2×10^{-8} M	2
2×10^{-7} M	2
Astaxanthin	
2×10^{-8} M	2
2×10^{-7} M	3
Concanavalin A (2 μg/ml)	3

The values in the table are t assays.
P value was calculated by Stud

RESULTS

Effects of carotenoids on the proliferative response of spleen cells and thymocytes

First we analyzed the effects of carotenoids such as β-carotene, cantaxanthin and astaxanthin on the proliferative response of BALB/c mice spleen cells. As indicated in Table 1, although the proliferative responses induced by carotenoids were relatively weak compared with the response of the positive control experiment by concanavalin A, they showed significant stimulatory effects on [^{3}H]TdR incorporation into spleen cells in a dose-dependent manner at the concentrations of 2×10^{-5} to 10^{-7} M. Furthermore, when we examined the effects of carotenoids on thymocyte proliferation, the similar stimulatory effects of carotenoids were observed compared with the control experiment (Table 2). In addition, amongst these carotenoids, astaxanthin exhibited the highest activity in both proliferation assays. β-Carotene showed the lowest activity and canthaxanthin expressed a moderate activity.

Effects of carotenoids on antibody production of spleen cells

Next, to analyze the effects of carotenoids on the specific function of spleen cells, we examined the effects of carotenoids on the antibody production of spleen cells by plaque-forming cell (PFC) assay. As shown in Table 3, when the spleen cells were treated with carotenoids, enhancing effects on the polyclonal antibody production were observed and they indicated the increase in the PFC numbers of Ig M and Ig G compared with the control experiment, but the antibody-producing activities of carotenoids were considerably different from each other. Astaxanthin showed the highest stimulatory effects on the antibody production in a dose-dependent manner at the concentrations of 2×10^{-7} to 10^{-8} M, whereas β-carotene did not show the significant stimulatory effect at a low concentration (2×10^{-8} M), although the stimulatory effect was observed at a high concentration (2×10^{-7} M). Canthaxanthin exhibited a moderate stimulatory effect at the same concentrations (Table 3).

Effects of carotenoids on the release of tumor necrosis factor-α and interleukin-1α from murine peritoneal adherent cells

To analyze the effect of carotenoids on another function of immunocompetent cells, the effect of car-

Table 1. Effects of carotenoids on the proliferative response of spleen cells from BALB/c mice

Test sample	[^{3}H]TdR Incorporation (cpm)
Control (PBS)	1845 ± 156
β-Carotene	
2×10^{-8} M	2468 ± 237 ($P<0.05$)
2×10^{-7} M	2639 ± 214 ($P<0.05$)
Canthaxanthin	
2×10^{-8} M	2403 ± 108 ($P<0.05$)
2×10^{-7} M	2778 ± 169 ($P<0.01$)
Astaxanthin	
2×10^{-8} M	2967 ± 195 ($P<0.01$)
2×10^{-7} M	3369 ± 305 ($P<0.01$)
Concanavalin A (2 μg/ml)	34850 ± 1742

The values in the table are the mean ± S.D. of triplicate assays.
P value was calculated by Student's *t*-test.

Table 2. Effects of carotenoids on the proliferative response of thymocytes from BALB/c mice

Test sample	[^{3}H]TdR Incorporation (cpm)
Control (PBS)	527 ± 39
β-Carotene	
2×10^{-8} M	648 ± 56 ($P<0.05$)
2×10^{-7} M	762 ± 31 ($P<0.01$)
Canthaxanthin	
2×10^{-8} M	725 ± 64 ($P<0.05$)
2×10^{-7} M	823 ± 25 ($P<0.01$)
Astaxanthin	
2×10^{-8} M	807 ± 44 ($P<0.01$)
2×10^{-7} M	986 ± 62 ($P<0.001$)
Concanavalin A (μg/ml)	4825 ± 380

The data in Table are expressed as the mean ± SD of triplicate assays. *P* value is calculated by Student's *t*-test.

Table 3. Effects of carotenoids on polyclonal antibody production in mouse spleen cells

Test sample (PFC number/10^6 cells)	Antibody-producing activity	
	Ig M	Ig G
Control (PBS)	180 ± 16	215 ± 29
β-Carotene		
2×10^{-8} M	208 ± 25	226 ± 22
2×10^{-7} M	254 ± 12 ($P<0.01$)	286 ± 14 ($P<0.05$)
Canthaxanthin		
2×10^{-8} M	237 ± 15 ($P<0.05$)	307 ± 11 ($P<0.05$)
2×10^{-7} M	319 ± 28 ($P<0.01$)	356 ± 30 ($P<0.01$)
Astaxanthin		
2×10^{-8} M	336 ± 10 ($P<0.001$)	389 ± 18 ($P<0.01$)
2×10^{-7} M	428 ± 36 ($P<0.001$)	508 ± 45 ($P<0.01$)
E. coli lipopolysaccharide (25 μg/ml)	1209 ± 165	1455 ± 230

The experimental result is expressed as the mean ± S.D. of triplicate assays.
P value is calculated by a Student's *t*-test.

Table 4. Effects of carotenoids on the release of tumor necrosis factor-α from the peritoneal adherent cells

Test sample	Concentration of TNF-α (pg/ml)
Control (PBS)	18±1
β-Carotene	
2×10^{-8} M	20±2
2×10^{-7} M	32±5 ($P<0.05$)
Canthaxanthin	
2×10^{-8} M	27±4 ($P<0.05$)
2×10^{-7} M	40±6 ($P<0.01$)
Astaxanthin	
2×10^{-8} M	75±10 ($P<0.01$)
2×10^{-7} M	106±18 ($P<0.01$)

The results in the table represent the mean±S.D. of triplicate assays.
P value is calculated by Student's t-test.

Table 5. Effects of carotenoids on the release of interleukin-1α from peritoneal adherent cells

Test sample	Concentration of IL-1α (pg/ml)
Control (PBS)	8±0.7
β-Carotene	
2×10^{-8} M	10±2
2×10^{-7} M	17±3 ($P<0.05$)
Canthaxanthin	
2×10^{-8} M	20±4 ($P<0.05$)
2×10^{-7} M	32±6 ($P<0.01$)
Astaxanthin	
2×10^{-8} M	46+2 ($P<0.001$)
2×10^{-7} M	67±8 ($P<0.001$)

The values in the table are the mean±S.D. of triplicate assays.
P value is calculated by Student's t-test.

otenoids on the release of cytokines from glycogen-induced peritoneal adherent cells was studied. As indicated in Table 4, β-carotene showed a significant effect at a high concentration (2×10^{-7} M) but not at a low concentration (2×10^{-8} M). Cantaxanthin expressed moderate enhancing effects at both concentrations. Astaxanthin exhibited much higher activities than those of the other carotenoids at the same concentrations. Furthermore, the authors also studied the effect of the carotenoids on the release of IL-1α from the same cells. As shown in Table 5, the carotenoids indicated similar stimulatory effects on the release of IL-1α and the ranks of the enhancing activity were estimated to be astaxanthin > canthaxanthin > β-carotene.

DISCUSSION

The results mentioned above indicate that β-carotene and related carotenoids such as canthaxanthin and astaxanthin (Fig. 1) caused significant enhancing effects on the proliferation and other functions of mouse immunocompetent cells.

First, we found that these carotenoids showed moderate stimulatory effects on the proliferative response of spleen cells or thymocytes from BALB/c mice in the absence of mitogen (Tables 1 and 2). However, we could not detect a significant effect on mitogen-induced proliferative response of spleen cells in an *in vitro* cell culture experiment (data not shown). On the other hand, when β-carotene or canthaxanthin was orally administered to rats during a relatively long term, the spleen cells showed a much more sensitive response to mitogen than that of untreated rats (Bendich and Shapiro, 1986). Possibly, the stimulatory effects of carotenoids on the proliferative responses of spleen cells or thymocytes might be not associated with the provitamin A activity of carotenoids because β-carotene has a provitamin A activity but canthaxanthin and astaxanthin have no provitamin A activity.

Second, the present study also elucidated that β-carotene, canthaxanthin and astaxanthin caused stimulatory effects on the polyclonal antibody (Ig M and Ig G) production of mouse spleen cells (Table 3). However, the previous report showed that astaxanthin exhibited a stimulatory effect on the antibody production against SRBC but β-carotene failed to cause a stimulatory effect (Jyonouchi *et al.*, 1991). Although the exact reason for the difference of our present and previous results is not clear, a possible explanation can be considered. In the present study, β-carotene showed relatively weak activities compared with that of astaxanthin and they caused a significant effect at a high concentration (2×10^{-7} M), but not at a low concentration (2×10^{-9} M) (Table 3). In the previous report, the authors analyzed the effects of carotenoids at a specific low concentration (1×10^{-8} M). These results indicate that β-carotene has a possible enhancing activity for antibody production but the activity at a low concentration seems to be too weak to detect the enhancing effect.

As another interesting finding in the present study, carotenoids enhanced the release of IL-1α from peritoneal adherent cells as indicated in Table 5. Generally adherent cells play an important role as accessory cells in enhancing antibody production by B lymphocytes (Landahl, 1976). One of the accessory cell functions is to release or produce cytokines such as IL-1α which has been known to be one of major regulating factors for B cell differentiation (Giri *et al.*, 1984). As a possibility, the IL-1α-inducing activity of carotenoids might contribute to the enhancement of antibody production in B cells by carotenoids.

Furthermore, the present study also indicates that carotenoids caused the significant stimulatory effect on the release of TNF-α from peritoneal adherent cells which consist of macrophages as a major cell population (70–80%) judged by the analysis of cell surface marker (Mac-1). On the other hand, the previous report showed that the administration with β-carotene or canthaxanthin caused a regression of chemically induced buccal pouch tumor in the hamster which was accompanied by the induction of TNF-α of macrophages at tumor sites (Schwartz and Shklar, 1988). The same study group also demonstrated that β-carotene increased the number of TNF-α-positive cells in an *in vitro* culture experiment of murine peritoneal macrophages (Schwartz *et al.*, 1990). These observations seem to be consistent with our present results. Although whether the induction of TNF-α in macrophages by carotenoids is due to a direct effect or indirect effect by other immunocompetent cells after the carotenoid treatment has not been elucidated at present, a more detailed analysis of the mechanism of TNF-α induction by carotenoids should be required in the next study.

The results described above and the chemical structures of carotenoids (Fig. 1) seem to show a structure and functional relationship. Namely, astaxanthin exhibited the highest immunomodulating activity and β-carotene expressed the lowest activity but canthaxanthin gave moderate activity. These immunomodulating activities seem to correspond to the ranks of antioxidant and oxygen radical scavenging activities (Mascio *et al.*, 1989). Although these properties of carotenoids might cause an increase of the cell viability of immunocompetent cells, a more detailed analysis for the mechanism of carotenoid-induced immunomodulation will be required in the next study.

Generally it might be difficult to link the immunological activity of carotenoids in an *in vitro* experiment and *in vivo* tumorigenesis. However, based upon our present result, we intend to analyze further the effect of carotenoids on immunocompetent cells in *in vivo* tumorigenesis as the next study.

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