Effect of Astaxanthin from *Phaffia Rhodozyma* on T cell Population and Responsiveness to Mitogen in Splenic Mononuclear Cells of Male Broiler Chicks

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Effect of feeding astaxanthin (Ax) provided as *Phaffia rhodozyma* on T cell population and responsiveness to mitogen, concanavalin A (Con A) or phytohemagglutinin (PHA), of splenic mononuclear cells (MNC) was determined in male broiler chicks. Chicks at 1 week of age, were fed *ad libitum* on diets containing 0, 20 or 100 ppm Ax for 7 days. Feeding the diet containing Ax decreased percentage of CD3-positive cells, an indicator of the T cell population in splenic lymphocytes. Feeding the diet containing 20 ppm Ax decreased percentage of CD4-positive cells estimated as the indicator of helper T cells, but no modulation was observed by feeding the diet containing 100 ppm Ax. Percentage of CD8-positive cells, an indicator of cytotoxic T cell, was not affected by feeding diets containing either 20 or 100 ppm Ax. T cell proliferation stimulated with Con A was enhanced by feeding diet containing 20 ppm Ax, but not affected by 100 ppm Ax. Feeding diets containing 20 and 100 ppm Ax enhanced the proliferation of splenocytes against PHA. Feeding diets containing Ax showed no effect on interleukin(IL)-2 mRNA expression in splenocytes stimulated by Con A estimated as the T cell proliferation promoting factor. IL-2 like activity was slightly, but significantly, decreased by feeding diet containing 20 ppm Ax. IFN- γ mRNA expression, estimated as a T cell activator under condition of Con A stimulation, was higher in chicks fed the diet containing 20 ppm Ax. These results suggest that proliferation and cytokine expressions of splenic T cells in response to feeding *Phaffia rhodozyma* containing Ax were independent of the dietary Ax concentration or total Ax intake. The enhancement of T cell proliferation by feeding 20 ppm Ax might be caused by changes in IFN- γ rather than IL-2 production in the chick splenocytes.

Key words : astaxanthin, *Phaffia* yeast, chicken splenocytes, T cell responsiveness, T cell population

Introduction

In chickens, a red-pigmented yeast *Phaffia rhodozyma* containing high concentration of astaxanthin (Ax) has been used as a dietary source to improve the pigmentation of broiler meats (Akiba *et al.*, 2001) and egg yolk (Akiba *et al.*, 2000). When Ax was ingested in broiler chicks, it was transported via plasma lipoprotein (mainly high density lipoprotein) and incorporated into tissues and organs (Takahashi *et al.*, 2004). However, there is a little information available on physiological pro-

perties of either Ax itself or Ax derived from the yeast in avian species although it has been reported that feeding of Ax prevented toxicity from aflatoxin in mule duckling (Cheng *et al.*, 2001).

Certain nutrients have functionality to modulate innate and acquired immune responses in chickens as well as in mammals. In mammals, it has been reported that Ax increased an antibody productions to T cell-dependent antigens (Jyonouchi *et al.*, 1995), resulted in changes in expression of antigen on membrane surface (Jyonouchi *et al.*, 1991) and enhanced cytotoxic T cell activity resulting in mod-

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ulated production of cytokine (Jyonouchi *et al.*, 2000). Furthermore, Chew *et al.* (1999) reported that splenocyte proliferative responses to phytohemagglutinin (PHA) were stimulated by Ax in mice although the increased T cell proliferation was not correlated with IL-2 production. Thus immunomodulatory effect of Ax has been partly demonstrated. However, if and how Ax affects splenic T cell proliferation in chickens has not been reported to date.

Responsiveness to mitogen and population of T cells are critical markers to represent most immune responses in animals. T cells express the cluster of differentiation (CD) molecules on their cell membrane and the detection of these molecules is useful to estimate function of T cells. CD3- (Chen *et al.*, 1986), CD4- and CD8- (Chan *et al.*, 1988) positive cells have been recognized as mature, helper and cytotoxic T cell, respectively. Interleukin (IL)-2 is an essential cytokine for fundamental immune processes in avian and mammalian species. IL-2 is a potent growth factor for a variety of cell types and plays key roles in the differentiation and the function of T cells, the development of B cells and the activation of natural killer (NK) cell (Lillehoj *et al.*, 1992). Members of the interferon (IFN) family in cytokines are produced in response to various stimuli including foreign cells, bacteria, and viral antigens and IFN- γ among the family serve as important regulators in the activation of lymphocytes and monocytes (Lillehoj *et al.*, 1992). Thus, it has been believed that IL-2 and IFN- γ are critical modulators in cellular immune responses.

In the present study, in order to elucidate mechanism of T cell responsiveness to Ax, effects of dietary supplementation of *Phaffia rhodozyma* containing high concentration of Ax on T cell population and responsiveness to mitogen were determined in splenic mononuclear cells (MNC) of male broiler chicks.

Materials and Methods

Animals and Diets

Male broiler chickens (Ross) were housed in a battery brooder with electric heater and fed on a commercial broiler starter diet until 7 days of age. Then chicks were selected and assigned to 3 dietary groups of 8 chicks with 4 replicates by keeping 2 chicks per cage. Birds were kept in each

experimental cage in temperature-controlled room (25°C). They were fed on a basal diet (CP 22.0%, ME 3.15 kcal/g, Table 1), the basal diet containing 20 or 100 ppm Ax for 7 days *ad libitum*. The red yeast, *Phaffia rhodozyma*, was kindly provided by Mercian Corporation, Tokyo, Japan. The Ax content of the freeze-dried *Phaffia* yeast was 5.5 mg/g of yeast. In our previous experiment, chicks fed diets containing 50 or 100 ppm Ax for 14 days deposited almost similar amounts of Ax in spleen (Takahashi *et al.*, 2004). From this result, dietary concentration of Ax was set up at 20 and 100 ppm. At 14 days of age, after body weight and feed intake were recorded, 4 chicks fed on each diet were slaughtered by blood letting for sampling spleens.

Preparation of Mononuclear Cell Suspension

Mononuclear cells (MNC) were isolated from spleen by density-gradient centrifugation. Collected spleen was meshed and suspended in RPMI-1640 medium (Invitrogen, Corp., Carlsbad, CA, USA) supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Corp., Carlsbad, CA, USA). The suspension was gently added on Histopaque-1077 (Sigma, St. Lois, MO, USA). Centrifugation was performed at 400 $\times$ g for 50 min at 10°C. The boundary layers between the medium with meshed spleen cells and Histopaque-1077 were collected as MNC and the resulted MNC were washed with RPMI-1640 medium 3 times.

Table 1. Basal diet composition

Ingredients	(%)
Corn meal	53.42
Soybean meal	37.35
Soybean oil	5.0
CaCO ₃	1.03
CaHPO ₄ · 2H ₂ O	1.75
NaCl	0.33
DL-methionine	0.25
Lysine-chloride	0.04
Vitamin-mix ¹⁾	0.4
Mineral-mix ¹⁾	0.43
Crude protein	22%
Metabolizable energy	3.15 kcal/g

¹⁾ See Akiba and Matsumoto (1986).

Flow Cytometry

The expression of cell surface markers on the splenic MNC was investigated by immunofluorescence. The 5×10^6 cells in $100 \mu\text{l}$ in each analysis were stained with $0.25 \mu\text{g}$ fluorescein-conjugated mouse anti-chicken monoclonal antibodies to CD3 (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) or double stained with $0.025 \mu\text{g}$ R- phcoerythrin-conjugated mouse anti-chicken monoclonal antibodies to CD4 (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) and $0.25 \mu\text{g}$ fluorescein-conjugated mouse anti-chicken monoclonal antibodies to CD8 (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) for 30 min at 4°C in a volume of $10 \mu\text{l}$ antibody solution. After washed by phosphate buffered saline, samples were analyzed within 6 hours of preparation on a FACSCalibur[®] flow cytometer (Becton Dickinson, San Jose, CA, USA) using the software CellQuest[™] Pro by first scatter-gating 50000 events on the lymphocyte population. The percentages of CD3-positive, $\text{CD4}^+ \text{CD8}^-$ and $\text{CD4}^- \text{CD8}^+$ cells in the lymphocyte population were then recorded.

Lymphocyte Proliferation

Culture of freshly isolated splenic MNC was performed in the presence of 10% of fetal bovine serum (FBS). Cells were plated at 2×10^6 cells/ml in flat-bottom 96-well culture plates in a volume of $100 \mu\text{l}$ per well and incubated at 39°C in a humidified 5% CO_2 atmosphere. T cell proliferation was induced by Con A (20 or $40 \mu\text{g}/\text{ml}$, Wako Pure Chemical Industries, Osaka, Japan) or PHA (10 or $20 \mu\text{g}/\text{ml}$, Sigma, St. Lois, MO, USA) stimulation for 48 hours. Cell proliferation was measured using Cell Counting Kit-8 (Dojindo, Osaka, Japan) according to the manufacturer's guidelines. Result was expressed as the arbitrary unit on the basis of absorbance values (absorbance_{A450nm-A655nm} of cells activity cultured with Con A or PHA/absorbance_{A450nm-A655nm} of cells activity cultured without Con A or PHA) of MNC from chicks fed the diet without Ax.

Measurement of IL-2- like Activity

The IL-2-like activity assay was carried out by a method based on T cell proliferation as described by Myers *et al.* (1992) with slight modification. Splenic MNC prepared from chicks fed on each diet were cultured at a concentration of 2×10^6

cells/ml in RPMI-1640 medium, in the absence or presence of Con A ($20 \mu\text{g}/\text{ml}$). After 24 hours incubation at 39°C in a humidified 5% CO_2 atmosphere, the supernatants (CM) were collected and the cells were removed. The CM was stored at -80°C until assayed for IL-2-like activity. The responder cells were prepared as follows ; after 2×10^6 cells/ml were incubated in the presence of ConA ($20 \mu\text{g}/\text{ml}$) for 24 hours at 39°C in a humidified 5% CO_2 , the supernatants were discarded and the cells were resuspended in RPMI-1640 medium with 5% FBS containing 0.05 M alpha methyl mannoside (Sigma, St. Lois, MO, USA) for 30 min at 39°C under 5% CO_2 . Fifty μl containing 2×10^5 of the responder cells and $50 \mu\text{l}$ of CM previously prepared were added to each well of 96-well flat bottom culture plates and incubated for 24 hours at 39°C in a humidified 5% CO_2 atmosphere. Cell proliferation was measured by an enzyme reduction assay using Cell Counting Kit-8 (Dojindo, Osaka, Japan) according to the manufacturer's guidelines. Result was expressed as the basis of arbitrary unit on the absorbance values (absorbance_{A450nm-A655nm} of cells activity cultured with CM presence of Con A/absorbance_{A450nm-A655nm} of cells activity cultured with CM absent from Con A).

Total RNA Isolation and RT-PCR

Total RNA was extracted from splenic MNC (2 ml containing 1×10^7 cells/ml) cultured in the absence or presence of Con A ($20 \mu\text{g}/\text{ml}$) for 6 hours. Cells were suspended $750 \mu\text{l}$ of TRIzol[®]-reagents (Invitrogen, Corp., Carlsbad, CA, USA) and RNA extracted according to manufacturer's instructions. RNA pellets were washed once with 75% ethanol, air dried and dissolved in $20 \mu\text{l}$ of RNase-free H_2O . RNA concentrations were determined spectrophotometrically at absorbance $A_{260\text{nm}}$, and integrity was checked by electrophoresis (100 V, 20 min) in 0.8% agarose gels containing ethidium bromide. $5 \mu\text{g}$ of total RNA was reverse-transcribed by M-MLV (Invitrogen, Corp., Carlsbad, CA, USA) in a $20 \mu\text{l}$ reaction volume using oligo (deoxythymidine)¹⁵ primer according to the manufacturer instructions.

Real Time PCR

Real-time PCR was performed using a fluorescence temperature cycler (Bio-Rad Laboratories, Hercules, CA, USA) and SYBR Green I as a double-stranded DNA-specific binding dye, according to the manufacturer's instructions (Bio-Rad

Laboratories, Hercules, CA, USA). Amplifications were carried out using 1.25 U TaKaRa TaqTM or 1.25 U TaKaRa Ex TaqTM (Takara Shuzo, Kyoto, Japan), 0.5 μ M of IL-2 and GAPDH primer, 10 $\times$ TaKaRa TaqTM buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, 1.5 mM MgCl₂] or 10 $\times$ TaKaRa Ex TaqTM buffer [2.0 mM MgCl₂], 0.05 μ l 1 : 100 diluted SYBR Green I nucleic acid gel stain (Bio-Whittaker, Inc. Molecular Applications, Rockland, ME, USA), and 1 μ l cDNA was used in a total vol of 50 μ l. The real-time PCR conditions were : preheat denature at 94°C for 3 min, annealing at 58 (IL-2), 63 (IFN- γ) and 65°C (glyceraldehyde-3-phosphate dehydrogenase used as internal standard., GAPDH) for 1 min, and extension at 72°C for 1 min. SYBR Green I fluorescence was detected at 72°C at the end of each cycle to monitor the amount of PCR product formed during that cycle. A melting curve analysis of the amplification products was performed at the end of the PCR run ; followed by gradual increase in temperature to 95°C at a rate of 0.05°C/sec with continuous measurement of fluorescence to confirm amplification of specific transcripts. The melting temperature profile for all samples of IL-2, IFN- γ and GAPDH, demonstrated single peaks at 85, 87.5 and 90.5°C, respectively. At the end of each run, melting curve profiles were recorded. Oligonucleotide sequences of sense and antisense primers were as follows : IL-2, estimated product size 428 bp (nucleotides from 39 to 466, GenBank Accession NO. AF017645) ; IFN- γ , estimated product size 288 bp (GenBank Accession NO. X99774) ; GAPDH, estimated product size 543 bp (GenBank Accession NO. AF 047874). The specificity of the amplification product was further verified by electrophoresis on a 0.8% agarose-gel following a check of the DNA sequence. Results are presented as the ratio of IL-2 and IFN- γ to GAPDH to correct for differences

in the amounts of template DNA used.

Statistical Analysis

Data were subjected to one-way analysis of variance (ANOVA) of SAS (SAS Institute, 1982. Cary, NC, USA). Mean values within the treatments were compared using Duncan's multiple range test when main effect was statistically significant ($P < 0.05$). The analysis for body weight gain, feed intake, and feed efficiency and Ax intake was done based on cage replications. Results were expressed as the mean $\pm$ standard deviation.

Results

Table 2 shows body weight gain, feed intake, feed efficiency and Ax intake in chicks fed diets containing 0, 20 or 100 ppm Ax for 7 days. Dietary supplementation for 7 days of *Phaffia* yeast to provide 20 or 100 ppm Ax had no significant effects on body weight gain, feed intake and feed efficiency when compared with the control group. Total Ax intake for 7 days was estimated 6.75 and 32.35 mg in chicks fed on diets containing 20 and 100 ppm Ax, respectively.

Fig. 1 shows percentages of CD3-, CD4- and CD 8-positive cells of T cells in splenic lymphocytes prepared from chicks fed on diets containing 0, 20 or 100 ppm Ax for 7 days. Percentages of CD3-positive cells was about 90% of total T cells in chicks fed the control diet, while those in chicks fed diets containing Ax was about 75%. Lowering effect of dietary Ax on percentages of CD3-positive cells was statistically significant in the 100 ppm Ax groups, but not in 20 ppm Ax groups. Percentages of CD8-positive cells in lymphocyte population were about 50% in all dietary groups and not affected by feeding Ax. Percentages of CD4-positive cells was significantly lower in chicks fed diet containing 20 ppm Ax than those in chicks fed the control diet, but not in chicks fed diet containing

Table 2. Effect of feeding diets with dietary supplementation of astaxanthin (Ax 0, 20 or 100 ppm) for 7 days on growth performance in male broiler chicks

Added Ax to diet (ppm)	Body weight gain (g/7 days)	Feed intake (g/7 days)	Gain to Feed	Ax intake (mg/7 days)
0	269 $\pm$ 11	338 $\pm$ 4	0.796 $\pm$ 0.026	0
20	270 $\pm$ 9	337 $\pm$ 3	0.801 $\pm$ 0.028	6.75 $\pm$ 0.06
100	254 $\pm$ 8	324 $\pm$ 2	0.785 $\pm$ 0.025	32.35 $\pm$ 0.20

Values are means ($\pm$ SD), n=4.

100 ppm Ax.

Fig. 2 shows T cell proliferation stimulated by (A) Con A or (B) PHA in MNC of chicks fed diets containing Ax for 7 days. T cell proliferation in chick fed diet containing 20 ppm Ax for 7 days was about 30% greater than that in chicks fed on diets containing 0 or 100 ppm Ax when Con A was used as a mitogen. Feeding the diet containing 100 ppm Ax showed no changes in the proliferation by challenge of Con A at concentration of 20 and 40 $\mu\text{g/ml}$ as compared to feeding the control diet. Feeding diets supplemented with Ax for 7 days significantly

enhanced the proliferative responses of T cells to PHA, regardless of PHA concentration in the incubation medium and Ax concentration of diet.

Fig. 3 shows (A) IL-2-like activity, (B) IL-2 and (C) IFN- γ mRNA expressions in MNC stimulated by Con A in chicks fed diets containing Ax for 7 days. IL-2-like activity was about 20% lower in chicks fed diets containing Ax than that in chick fed the control diet, the lowering effect in chicks fed diet containing 20 ppm Ax was statistically significant as compared with the control group. Expression of IL-2 mRNA was lower in splenocytes of

Fig. 1. Percentages of CD3-, CD 4- or CD8-positive cells in splenocytes of chicks fed diets containing 0, 20 or 100 ppm astaxanthin (Ax) for 7 days. Each bar represents the mean and standard deviation from 4 individual chickens. Means with different scripts are significantly difference ($P < 0.05$).

Fig. 2. T cell proliferation stimulated by (A) concanavalin A (Con A) or (B) phytohemagglutinin (PHA) in splenocytes of chicks fed diets containing 0, 20 or 100 ppm astaxanthin (Ax) for 7 days. AU was expressed as the basis of absorbance values (absorbance_{A450 nm-A655 nm}) of cells activity cultured with Con A or PHA/absorbance_{A450 nm-A655 nm} of cells activity cultured without Con A or PHA) of MNC from chicks fed diet without Ax. Each bar represents the mean and standard deviation from 4 individual chickens. Means with different scripts are significantly difference ($P < 0.05$).

Fig. 3. Interleukin (IL)-2-like activity (A), expression of IL-2 mRNA (B) and interferon- γ (IFN- γ) mRNA (C) in splenocytes of chicks fed diets containing 0, 20 or 100 ppm astaxanthin (Ax) for 7 days. For measurement of IL-2-like activity, splenocytes were stimulated by Con A for 24 hours. For expression of IL-2 mRNA and IFN- γ mRNA, splenocytes were stimulated by Con A for 6 hours. AU for IL-2 like activity was expressed as the ratio of absorbance_{A450 nm-A655 nm} of cells activity cultured with CM in presence of Con A/absorbance_{A450 nm-A655 nm} of cells activity cultured with CM in absence from Con A. AU for expression of IL-2 and IFN- γ mRNA was expressed as IL-2 mRNA expression/GAPDH mRNA expression and IFN- γ mRNA expression/GAPDH mRNA expression, respectively. Each bar represents the mean and standard deviation from 4 individual chickens. Means with different scripts are significantly difference ($P < 0.05$).

chicks fed diets containing 20 and 100 ppm Ax than those in chicks fed the control diet, but the effect was not statistically significant. Expression of IFN- γ mRNA was 2.5 to 3 folds greater in chicks fed diet containing 20 ppm Ax than those in chicks fed diets containing 0 or 100 ppm Ax. Pattern of changes in the expression of IFN- γ mRNA by feeding Ax was similar to that in the proliferative response of MNC to Con A.

Discussion

The present study showed that feeding 20 ppm Ax, but not 100 ppm Ax, altered T cell subpopulation in spleen lymphocyte, suggesting that the modulation of lymphocytes by Ax varies with the dietary concentration and feeding 20 ppm Ax probably modulates T cells differentiation. Percentages of CD4-positive cells were decreased and that of CD8-positive cells was not affected by feeding the diet

containing Ax 20 ppm. Thus Ax may be a novel nutrient to modify T cell differentiation in chicks, although it is not clear why the effect of Ax on T cell differentiation is not related with total Ax intake or dietary Ax concentration.

The present study also showed that T cell proliferation stimulated by Con A was enhanced by feeding the diet containing 20 ppm Ax for 7 days and that by PHA was enhanced by feeding the diet containing 20 or 100 ppm Ax. It has been reported that feeding Ax in mice enhanced lymphocyte proliferation against PHA, but not influence that against Con A and LPS (Chew *et al.*, 1999). It has been reported that Con A mainly stimulates CD8-positive T cells which specifically express at membrane of cytotoxic T cells, while PHA stimulates CD4-positive T cells which specifically express at membrane of helper T cells in chickens (Chan *et al.*, 1988). Therefore, it is presumed that Ax pro-

vided from *Phaffia* yeast stimulated proliferation of both cytotoxic and helper T cells in chick spleen.

IL-2 is an essential cytokine for fundamental immune processes and a potent growth factor for a variety of cell types, and plays key roles in T cell differentiation and function in avian species (Lillehoj *et al.*, 1992). IFN- γ serves as an important regulator in the activation of lymphocytes and monocytes (Lillehoj *et al.*, 1992). Thus, it has been believed that IL-2 and IFN- γ are critical modulators in most immune responses including T cell proliferation. An enhancement of T cell proliferation, independent of an increase in IL-2 production, by Ax has been noted in mammals (Jyonouchi *et al.*, 1991 ; Chew *et al.*, 1999). Kim *et al.* (2000 a and b) reported that lutein, a family of xanthophyll same as Ax, increased T cell proliferation but this effect was independent of IL-2 production in dogs and cats. The present study indicated that an enhancement of T cell proliferation by Ax appeared to be independent of IL-2 production in chicks when the cells were stimulated by Con A. The present experiment suggested that T cell proliferation by Ax was related to IFN- γ mRNA expression rather than IL-2 production when chicks (7 days of age) were fed the diet containing 20 ppm Ax for 7 days. However, it is unclear why the effect of Ax on T cell proliferation as well as T cell population is not dependent of dietary Ax concentration as mentioned above. Woodall *et al.* (1996) showed that dietary supplementation with 100 ppm beta-carotene lowered plasma alpha-tocopherol level in male Leghorn chicks, suggesting that an excess intake of some carotenoids in chicks would inhibit absorption of alpha-tocopherol or normal metabolism of alpha-tocopherol. Since alpha-tocopherol is an important nutrient to maintain immune responses, the reduction of alpha-tocopherol intake and its deposition in organs should be associated with impaired immune response including the proliferative response of T cells (Erf *et al.*, 1998 ; Leshchinsky and Klasing 2001). This will be one of the reasons that feeding the diet containing 100 ppm Ax showed no effect on some of immune responses observed in this study. However, it is not always sufficient to explain the effect of Ax on T cell response against mitogen from this reason since feeding diets supplemented with 100 ppm Ax for 7 days significantly enhanced the proliferative responses of T cells to

PHA. Thus further studies are needed for elucidation of immunomodulative effects of Ax on T cell population and responsiveness to antigen or mitogen.

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