ELSEVIER

Veterinary Immunology and Immunopathology
73 (2000) 331–341

Veterinary immunology and immunopathology

www.elsevier.com/locate/vetimm

Modulation of humoral and cell-mediated immune responses by dietary lutein in cats

Hong Wook Kim[a], Boon P. Chew[a,*], Teri S. Wong[a], Jean Soon Park[a], Brian B.C. Weng[a], Katherine M. Byrne[a], Michael G. Hayek[b], Gregory A. Reinhart[b]

[a]*Department of Animal Sciences, Washington State University, Pullman, WA 99164-6351, USA*
[b]*The Iams Company, Lewisburg, OH 45338, USA*

Received 2 September 1999; received in revised form 20 December 1999; accepted 28 December 1999

Abstract

The immuno-modulatory role of dietary lutein in domestic cats is unknown. Female Tabby cats (10-month old; n=56) were supplemented daily for 12 weeks with 0, 1, 5 or 10 mg lutein. Blood was collected on Weeks 0, 2, 4, 8 and 12 to assess the following: (1) mitogen-induced peripheral blood mononuclear cells (PBMCs) proliferation, (2) changes in PBMC subpopulations, (3) interleukin-2 (IL-2) production and (4) plasma immunoglobulin (Ig)G production. In addition, delayed-type hypersensitivity (DTH) response to concanavalin A (Con A) or a polyvalent vaccine was performed on Weeks 0, 6 and 12. Dietary lutein increased plasma lutein concentrations in a dose-dependent manner (p<0.001) and concentrations had not reached steady state after 12 weeks of feeding in cats given 5 or 10 mg lutein. Concentrations of plasma retinol and α-tocopherol were not influenced by diet. The DTH response to vaccine but not to Con A increased (p<0.05) in a dose-dependent manner on Week 6. Compared to control, cats fed lutein also showed enhanced Con A- and pokeweed mitogen-stimulated PBMCs proliferation. Dietary lutein also increased the percentages of CD4+ and CD21+ lymphocytes on Week 12 but had no significant effect on pan T, CD8 and MHC class II markers. Plasma IgG was higher (p<0.05) in cats fed 10 mg lutein on Weeks 8 and 12. These results support the immuno-modulatory action of lutein in domestic cats.

Keywords: Cats; Lutein; Immunity; Lymphocyte; Vaccine; DTH

* Corresponding author. Tel.: +1-509-335-1427; fax: +1-509-335-4246.
E-mail address: boonchew@mail.wsu.edu (B.P. Chew).

0165-2427/00/$ – see front matter
PII: S0165-2427(00)00152-5

1. Introduction

Carotenoids are an important group of natural pigments that possess immunomodulatory activities (Chew, 1993, 1995; Mayne, 1996). Previous studies on the biological action of carotenoids have focused on β-carotene. However, over the last decade, interest on the immune regulatory function of other carotenoids, notably lutein, lycopene, astaxanthin and canthaxanthin, has increased (Bendich and Shapiro, 1986; Jyonouchi et al., 1995; Chew et al., 1996). In vitro, astaxanthin significantly increased the proliferative response of murine thymocytes and splenocytes (Okai and Higashi-Okai, 1996) while canthaxanthin enhanced the expression of activation markers for T-helper and natural killer (NK) cells in human peripheral blood mononuclear cells (PBMCs) (Prabhala et al., 1989). In addition, Jyonouchi et al. (1994) reported that lutein enhanced antibody production in response to T-dependent antigen in mice.

Mammals lack the ability to synthesize carotenoids de novo. Earlier studies have reported that carnivores do not absorb or are poor absorbers of carotenoids (Ahmad, 1931). However, a recent survey of captive wild animals showed that exotic felids are high accumulators of carotenoids (Slifka et al., 1999). More direct uptake studies have demonstrated that domestic dogs and cats can absorb β-carotene (Chew et al., 1997; Weng et al., 1997) and lutein (Kim et al., 1998; Park et al., 1999).

In the United States alone, there are approximately 70 million cats. Little is known regarding the possible biological functions of dietary lutein. Our present objective is to study the role of dietary lutein in modulating immune response in domestic cats.

2. Materials and methods

2.1. Animals and diet

Fifty-six female Tabby cats (10-month old; Liberty Research Inc., Waverly, NY) were randomly assigned to be fed 0, 1, 5, or 10 mg lutein daily for 12 weeks. The lutein (FloraGlo^{TM} crystalline lutein, Kemin Industries Inc., Des Moines, IA) was incorporated into the basal diet (Table 1; The Iams Co., Lewisburg, OH) to a final concentration of 250 mg lutein/kg diet. Prior to being fed to the cats, the lutein-containing diet was mixed

Table 1
Nutrient composition of the basal diet

Nutrient	(g/kg)
Moisture	72
Protein	310
Ash	52
Fat	217
Crude fiber	14
Calcium	11.5
Phosphorus	8.3
Gross energy (kcal/kg)	5412

with additional basal diet to achieve the desired lutein concentration. The final diets were mixed weekly and stored at −20°C in sealed containers under nitrogen gas. Prepared food analyzed periodically showed no significant destruction of lutein. Food and water were available ad libitum. Cats were housed (7 cats/pen; 1.5 m×5.0 m) in a temperature- (20 to 22°C) and light- (14 h light) controlled facility. The research facility is AAALAC (American Association for the Accreditation of Laboratory Animal Care) approved. The research protocol was approved by the Washington State University Institutional Animal Care and Use Committee.

2.2. Extraction of plasma for high performance liquid chromatography (HPLC)

Blood was collected by jugular venipuncture into heparinized evacuated tubes on Weeks 0, 2, 4, 8 and 12 and aliquots used for HPLC analysis and for assessing immune responses. Plasma lutein, retinol and α-tocopherol were analysed by reverse phase HPLC (Alliance 2690 HPLC, Waters, Milford, MA) as previously described (Chew et al., 1996; Park et al., 1998). The identity of the eluted compounds was verified by comparing their absorption spectra with those of standard compounds using a photodiode array detector (Waters 996, Waters, Milford, MA).

2.3. Delayed-type hypersensitivity

The Delayed-type hypersensitivity (DTH) response was evaluated on Weeks 0, 6 and 12 after the initiation of lutein feeding. Cats were mildly sedated with an i.m. injection of ketamine hydrochloride (5 mg/kg; Fort Dodge, IA) and acepromazine (0.1 mg/kg; Vedco, St. Joseph, MO). The hair on the flank was clipped and the area wiped with 70% ethyl alcohol. All cats were injected i.d. with 100 μl of saline (8.5 mg/ml; control), concanavalin A (Con A; 0.5 mg/ml) to measure nonspecific immunity, and with an attenuated polyvalent vaccine (Felocell^{TM}, Pfizer, NY, NY) containing feline herpesvirus-1, feline calicivirus, feline parvovirus and *Chlamydia psittaci* to measure specific immunity. All animals were vaccinated with the same vaccine prior to the commencement of the study. This technique has previously been used to assess cell-mediated immunity in cats (Otto et al., 1993). The DTH response was assessed by measuring skin induration at 24, 48 and 72 h after injection with the aid of a digital micrometer (Mitsutoyo, Tokyo, Japan). The response was expressed as a percent increase of skin thickness compared to the same injection site at 0 h.

2.4. Peripheral blood mononuclear cells proliferation

Whole blood was used to analyze the mitogenic proliferative responsiveness of PBMCs to phytohemagglutinin (PHA), Con A and pokeweed mitogen (PWM) to mimic in vivo conditions. Heparinized whole blood was diluted 1:12 with RPMI-1640 containing 25 mM Hepes, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma, St. Louis, MO). Our preliminary studies using blood diluted 1:2, 1:4, 1:8, 1:12 and 1:16 showed the best repeatability and optimal response to mitogens with the 1:12 dilution. The mitogens were used at the following final concentrations previously determined to provide both

maximal (the higher mitogen concentration) and suboptimal (the lower mitogen concentration) proliferation: PHA (1.25 and 0.25 μg/ml), Con A (2.5 and 0.5 μg/ml) or PWM (0.125 and 0.025 μg/ml). The mixture was incubated for 72 h at 37°C in a humidified incubator under 5% CO_2 atmosphere. Four hours prior to the termination of the incubation, 20 μl of [^{3}H]-thymidine (1 μCi/well) was added. Tritiated thymidine uptake was measured by liquid scintillation and proliferative response of PBMCs was expressed as counts per minute (cpm) of stimulated cultures corrected for cpm of unstimulated cultures.

2.5. Flow cytometry

Mouse monoclonal antibodies (mAbs) used to quantitate the feline lymphocyte subsets were purchased from VMRD (Pullman, WA). Single color flow cytometry was used to quantitate the lymphocyte subset populations. Briefly, PBMCs were separated on Histopaque-1119 (Sigma, St. Louis, MO) and contaminating red blood cells were lysed in NH_4Cl (8.4 mg/ml) and cells were washed three times with phosphate buffered saline (PBS, pH 7.4). Isolated PBMCs were resuspended to 1×10^7 cells/ml in PBS supplemented with 2% gamma globulin-free horse serum, 5% goat serum and 0.2 mg/ml sodium azide. A total of 5×10^5 cells were then incubated for 30 min on ice with monoclonal antibodies which recognize feline Pan T+ cells (CF54A), CD4+ cells (CAT30A, helper T cells), CD8+ cells (25–2A, cytotoxic T cells), MHC class II+ cells (CAT82A) and CD21+ cells (F46A, mature B cells). Cells were then washed three times and secondary staining was performed by incubating cells with fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-mouse immunoglobulin (Ig) G (H+L) antibody (Caltag, Burlingame, CA) to visualize the bound mAb. Stained cells were fixed in 4% paraformaldehyde in preparation for acquisition and determined by flow cytometer (FACScan, Becton Dickinson, San Jose, CA) after gating the lymphocyte population by forward and side scatter analysis. For each sample, 5000 total events were acquired for analysis. Appropriate negative controls were included to correct for background fluorescence. Data were expressed as the percentage of positive-staining cells corrected for cells stained nonspecifically with the secondary antibody.

2.6. Interleukin-2 production

Whole blood was diluted 1:2 in RPMI-1640 and cultured for 48 h in the presence of 5 μg/ml Con A. The conditioned medium was removed and frozen at −80°C until use. The Interleukin-2 (IL-2) concentrations were determined in duplicate by ELISA using a human IL-2 kit (Intergen, Purchase, NY). The anti-human IL-2 was previously determined to cross-react with feline IL-2 and recombinant human IL-2 was used as the standard.

2.7. Measurement of plasma immunoglobulin (Ig) G

Plasma IgG concentration was determined by single radial immunodiffusion (SRID). Goat antiserum to cat IgG (Sigma Chem. Co., St. Louis, MO) was used and ring

diameters were measured using a SRID reader (Transidyne General Corp., Ann Arbor, MI). Feline IgG (VMRD, Pullman, WA) was used as the standard.

2.8. Statistical analysis

Data were analyzed by split-plot ANOVA using the General Linear Model of SAS (1991). Differences among treatment means within a sampling period were compared using Student's *t*-test. Differences with $p<0.05$ were considered as statistically significant. All values expressed are means±SEM.

3. Results

3.1. Body weight

Dietary lutein did not significantly affect changes in body weight during the experimental period. Body weight across all diets and periods averaged 1.41±0.04 kg.

3.2. Plasma lutein, Vitamin A and E

Prior to lutein supplementation, the baseline plasma lutein concentration averaged 0.036±0.005 μmol/l in all cats (Fig. 1). The presence of low concentrations of plasma lutein in unsupplemented cats is likely due to the inclusion of chicken by-products and fat in the basal diet. Lutein concentrations in unsupplemented cats remained at this concentration throughout the study period. Plasma lutein concentrations increased in a dose-dependent manner between Weeks 2 and 12 in lutein-fed cats. The increase was

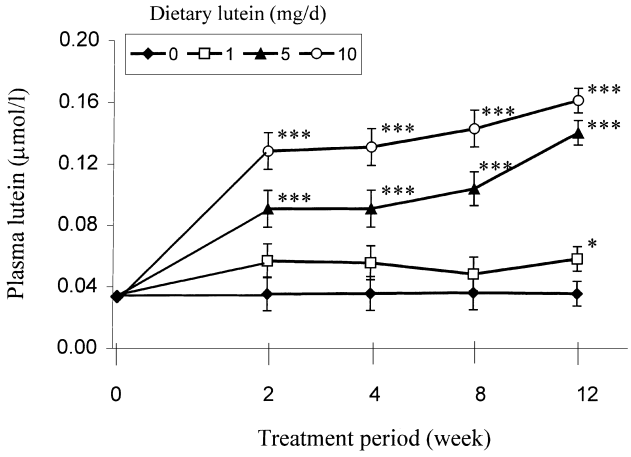

Fig. 1. Concentrations of plasma lutein from cats fed diets containing 0, 1, 5 or 10 mg lutein for 12 weeks. Values are means±SEM ($n=14$/treatment). Statistical significance is as follows: ***$p<0.001$, *$p<0.05$ compared with control group.

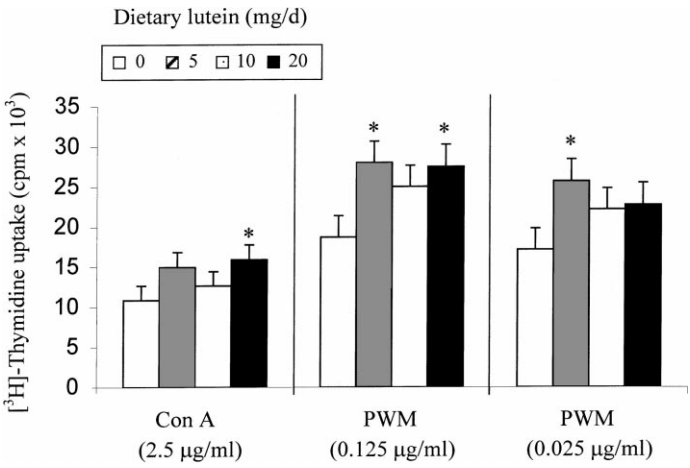

Fig. 2. Proliferative responses of PBMCs to Con A and PWM measured on Week 4. Values are means±SEM (n=14/treatment). Statistical significance is as follows: *p<0.05 compared with control group.

rapid by Week 2 and concentrations continued to increase, albeit more gradually, through Week 12 in cats fed 5 or 10 mg lutein. Lutein supplementation did not influence the concentration of plasma retinol and α-tocopherol which averaged 0.56±0.03 and 14.9±0.9 μmol/l, respectively.

3.3. Mitogen-induced peripheral blood mononuclear cells proliferation

Dietary lutein did not significantly influence spontaneous proliferation by unstimulated PBMCs and averaged 1744±148 cpm throughout the study. Generally, the proliferative response of PBMCs tended to be higher in lutein-fed cats than in unsupplemented cats. Cats fed 10 mg lutein showed significantly higher proliferative response to Con A (2.5 μg/ml) at Week 4 (Fig. 2). However, no treatment differences were observed with a suboptimal dose (0.5 μg/ml) of Con A (data not shown). Of the mitogens used, PWM induced the highest proliferative response (Fig. 2). Lutein supplementation increased PBMCs proliferation on Week 4 with 0.125 μg/ml (1 and 10 mg lutein) and 0.025 μg/ml (1 mg lutein) of PWM. There were no significant dietary effects on PHA-induced PBMCs proliferation throughout the study. Tritiated thymidine uptake with 1.25 and 0.25 μg/ml PHA averaged 6464±504 and 1934±147 cpm, respectively.

3.4. Delayed-type hypersensitivity

Prior to lutein supplementation, no differences were observed in DTH response among dietary treatment groups. The DTH response to saline as a control was low (4.3±0.6%) and not significantly different among treatments during all periods studied. There was a significant dose-related DTH response to vaccine when measured at 72 h post-injection; response was significantly higher with cats fed 5 (p<0.05) or 10 (p<0.01) mg lutein on Week 6 (Fig. 3). The similar trend in a dose-related DTH response persisted with vaccine

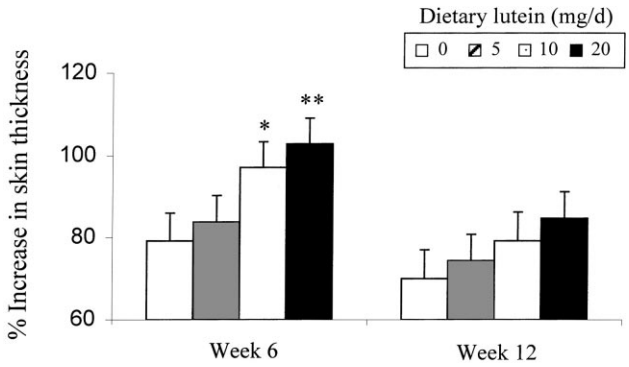

Fig. 3. Delayed-type hypersensitivity response (expressed as a percentage of skin thickness measured at 0 h) to polyvalent vaccine at Weeks 6 and 12. Skin induration was measured at 72 h post-injection. Values are means±SEM (n=14/treatment). Statistical significance is as follows: *p<0.05, **p<0.01 compared with control group.

on Week 12 at 72 h postinjection as was observed on Week 6. The DTH response to Con A was not influenced by lutein supplementation in any of the weeks.

In general, peak DTH response to Con A was observed around 24 h post-injection whereas maximal response to vaccine occurred between 48 and 72 h.

3.5. Lymphocyte subsets

The percentages of pan T+ cells, CD8+ Tc cells and MHC class II+ lymphocytes were similar among treatment groups. However, on Week 12 of supplementation, the percentages of CD4+ Th and CD21+ B cells were significantly elevated (p<0.05) in cats fed 10 mg lutein compared to unsupplemented cats (Fig. 4).

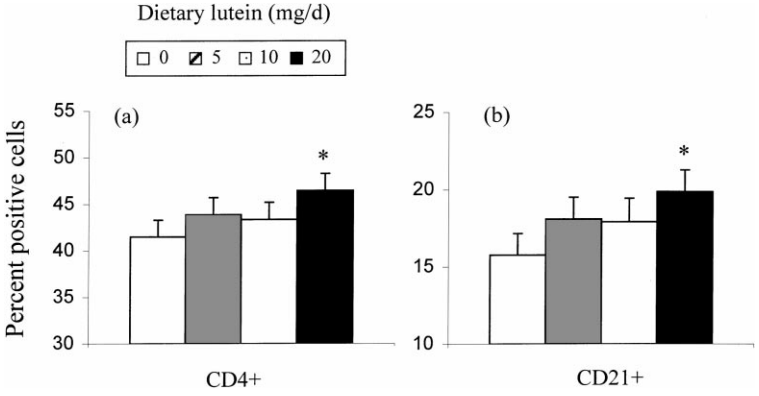

Fig. 4. Comparison of (a) CD4+ T and (b) CD21+ B lymphocyte population among treatment groups measured on Week 12. Values are means±SEM (n=14/treatment). Statistical significance is as follows: *p<0.05 compared with control group.

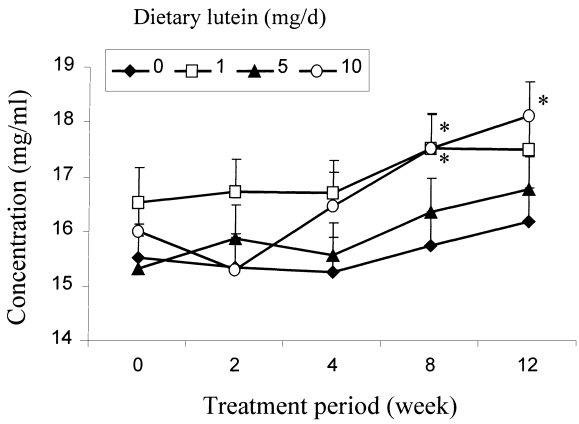

Fig. 5. Changes in plasma polyclonal IgG concentrations in cats fed 0, 1, 5, or 10 mg lutein daily for 12 weeks. Values are means±SEM (n=14/treatment). Statistical significance is as follows: *p<0.05 compared with control group.

3.6. Interleukin-2 production

There were no differences in IL-2 production throughout the experimental period. The mean concentration of IL-2 in Con A-stimulated cultures was 15.1±0.79 ng/ml across all treatment and periods.

3.7. Immunoglobulin (Ig) G production

The concentrations of plasma IgG were not different among treatment groups at the commencement of the study and averaged 15.85±0.62 mg/ml (Fig. 5). However, by Week 8 of lutein feeding, cats fed 1 or 10 mg lutein had significantly higher concentrations of plasma IgG. This trend persisted through Week 12 where plasma IgG in cats fed 10 mg lutein remained significantly higher than unsupplemented cats.

4. Discussion

The present study showed that dietary lutein modulated both cell-mediated and humoral immune responses in domestic cats. Cats have previously been considered non-absorbers or low-responders of β-carotene (Ahmad, 1931; Baker et al., 1986). However, Park et al. (1999) recently demonstrated that cats can absorb lutein into the blood and the lutein is subsequently taken up by circulating leukocytes. Results from the present study on dietary lutein uptake are in agreement with the latter report. We showed that the absorption of lutein was dose- and time-dependent.

Lutein increased the proliferative response of feline PBMCs to Con A and PWM after 4 weeks of supplementation. We previously reported a similar stimulatory effect of lutein supplementation on lymphocyte proliferative response to mitogen in mice (Chew et al.,

1996) and dogs (Kim et al., 1998). In addition, the populations of peripheral blood CD4+ T and CD21+ B lymphocytes were elevated in cats fed lutein. Kim et al. (1998) similarly reported higher percentages of CD5+, CD4+ T and MHC II+ lymphocytes in dogs fed lutein. These observations indicate that lutein affects the immune system by modulating the functional expression of cell surface markers. Watson et al. (1991) reported that β-carotene significantly increased the expression of CD4 and IL-2 receptor cell surface markers in human PBMC. Therefore, the enhanced lymphocyte proliferation by dietary lutein is likely mediated via the alteration of surface marker expression on lymphocytes.

In this study, lutein did not influence Con A-induced IL-2 production despite observed increases in Con A- and PWM-induced PBMCs proliferation. These results are consistent with Chew et al. (1996) who reported increased PHA-stimulated mitogenesis and no significant influence on IL-2 production in mice fed 0.1 or 0.4% lutein. Also, β-carotene supplementation has been shown to increase IL-2 receptors in human (Prabhala et al., 1991; Watson et al., 1991). Why dietary lutein did not stimulate IL-2 production even though mitogen-induced PBMC proliferation was stimulated and the populations of CD4+ and CD21+ cells were increased cannot be explained at this time. Perhaps, the ex vivo condition used in this study did not reflect in vivo response.

The DTH reaction is a good indicator of events occurring at the effector phase of the cell-mediated immune response in vivo. Our data further illustrate the modulatory action of lutein in DTH response. Dietary lutein increased DTH response to vaccine in a dose-related manner at Weeks 6 and 12. The increase in the population of CD4+ T lymphocytes in this study may partly explain the enhanced DTH response observed in lutein-supplemented cats because CD4+ T cells play an important role in inducing the DTH response and activated CD4+ T cells are a potent source of various cytokines. However, the mechanism by which dietary lutein modulates the DTH response cannot be explained in the present study because cytokines such as IFN-γ and TNF-α and many other cell types not measured may be involved.

Available information on the effects of lutein on antibody production is limited. In the present study, dietary lutein increased plasma IgG concentrations in cats. Jyonouchi et al. (1994) showed that lutein and astaxanthin enhanced in vitro antibody production in response to T cell-dependent antigens and increased the number of IgG-secreting cells in mice. Similarly, murine splenocytes incubated with astaxanthin, β-carotene or canthaxanthin showed higher polyclonal antibody production in vitro (Okai and Higashi-Okai, 1996). The elevated concentration of plasma IgG in the present study was observed without a specific antigenic challenge. However, cats were injected intradermally with a polyvalent vaccine and with Con A at Weeks 0, 6 and 12 for assessing DTH response. These antigenic challenges could prime the memory B cells, thereby allowing lutein to stimulate the memory B cells to secrete IgG. Therefore, dietary lutein seems to possess an anamnestic effect on the humoral immune response.

Carotenoids can enhance antigen presentation due to their unique physical structures. Jyonouchi et al. (1993, 1994) suggested that astaxanthin may modulate antigen presentation to T helper cells because of its polar end-groups. These polar end-groups may increase the rigidity and mechanical strength of the cell membrane (Britton, 1995). Consequently, physical changes to the cell membrane may influence antigen presentation that largely depends on cell-to-cell interaction (Jyonouchi et al., 1996). Like astaxanthin,

lutein also possesses a hydroxyl group on each of the β-ionone rings. Thus, lutein may exert similar actions on antigen presentation.

In summary, dietary lutein modulates both the cell-mediated and humoral immune response in domestic cats. Our present study adds to the small body of evidence presently available supporting an immuno-modulatory role of dietary lutein in humans and in other animals. The health-promoting action of lutein in domestic cats, therefore, remains to be elucidated.

Acknowledgements

This work was supported by The Iams Co., Lewisburg, OH and the Agricultural Research Station, College of Agriculture and Home Economics, Washington State University, Pullman, WA.

References

Ahmad, B., 1931. The fate of carotene after absorption in the animal organism. Biochem. J. 25, 1195–1204.

Baker, H., Schor, S.M., Murphy, B.D., DeAngelis, B., Feingold, S., Frank, O., 1986. Blood vitamin and choline concentrations in healthy domestic cats, dogs, and horses. Am. J. Vet. Res. 47, 1468–1471.

Bendich, A., Shapiro, S.S., 1986. Effect of β-carotene and canthaxanthin on the immune responses of the rat. J. Nutr. 116, 2254–2262.

Britton, G., 1995. Structure and properties of carotenoids in relation to function. FASEB J. 9, 1551–1558.

Chew, B.P., 1993. Role of carotenoids in the immune response. J. Dairy Sci. 76, 2804–2811.

Chew, B.P., 1995. Antioxidant vitamins affect food animal immunity and health. J. Nutr. 125, 1804S–1808S.

Chew, B.P., Wong, M.W., Wong, T.S., 1996. Effects of lutein from marigold extract on immunity and growth of mammary tumors in mice. Anticancer Res. 16, 3689–3694.

Chew, B.P., Weng, B.C., Park, J.S., Wong, T.S., Combs, R.L., Hayek, M.G., Reinhart, G.A., 1997. Uptake of β-carotene by plasma and lymphocytes in cats. FASEB J. 10, A447.

Jyonouchi, H., Zhang, L., Tomita, Y., 1993. Studies of immunomodulating actions of carotenoids. II. Astaxanthin enhances in vitro antibody production to T-dependent antigens without facilitating polyclonal B-cell activation. Nutr. Cancer 19, 269–280.

Jyonouchi, H., Zhang, L., Gross, M., Tomita, Y., 1994. Immunomodulating actions of carotenoids: enhancement of in vivo and in vitro antibody production to T-dependent antigens. Nutr. Cancer 21, 47–58.

Jyonouchi, H., Sun, S., Gross, M., 1995. Effects 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. Nutr. Cancer 23, 171–183.

Jyonouchi, H., Sun, S., Mizokami, M., Gross, M., 1996. Effects of various carotenoids on cloned, effector-stage T-helper cell activity. Nutr. Cancer 26, 313–324.

Kim, H.W., Chew, B.P., Wong, T.S., Park, J.S., Weng, B.C., Cerveny, C.G., Byrne, K.M., Hayek, M.G., Reinhart, G.A., 1998. Dietary lutein stimulates cell-mediated immune response in the canine. FASEB J. 12, A966.

Mayne, S.T., 1996. Beta-carotene, carotenoids and disease prevention in human. FASEB J. 10, 690–701.

Okai, Y., Higashi-Okai, K., 1996. Possible immunomodulating activities of carotenoids in in vitro cell culture experiments. Int. J. Immunopharmac. 18, 753–758.

Otto, C.M., Brown, C.A., Lindl, P.A., Dawe, D.L., 1993. Delayed hypersensitivity testing as a clinical measure of cell-mediated immunity in the cat. Vet. Immunol. Immunopathol. 38, 91–102.

Park, J.S., Chew, B.P., Wong, T.S., 1998. Dietary lutein from marigold extract inhibits mammary tumor development in BALB/c mice. J. Nutr. 128, 1650–1656.

Park, J.S., Chew, B.P., Wong, T.S., Weng, B.C., Kim, H.W., Hayek, M.G., Reinhart, G.A., 1999. Dietary lutein uptake by blood and leukocytes in domestic cats. FASEB J. 13, A441.4.

Prabhala, R.H., Maxey, V., Hicks, M.J., Watson, R.R., 1989. Enhancement of the expression of activation markers on human peripheral blood mononuclear cells by in vitro culture with retinoids and carotenoids. J. Leuk. Biol. 45, 249–254.

Prabhala, R.H., Garewal, H.S., Hicks, M.J., Sampliner, R.E., Watson, R.R., 1991. The effects of 13-*cis*-retinoic acid and beta-carotene on cellular immunity in humans. Cancer 67, 1556–1560.

SAS, 1991. SAS/STAT User's Guide. SAS Institute Inc., Cary, NC.

Slifka, K.A., Bowen, P.E., Stacewicz-Sapuntzakis, M., Crissey, S.D., 1999. A survey of serum and dietary carotenoids in captive wild animals. J. Nutr. 129, 380–390.

Watson, R.R., Prabhala, R.H., Plezia, P.M., Alberts, D.S., 1991. Effect of β-carotene on lymphocyte subpopulations in elderly humans: evidence for a dose-response relationship. Am. J. Clin. Nutr. 53, 90–94.

Weng, B.C., Chew, B.P., Park, J.S., Wong, T.S., Combs, R.L., Hayek, M.G., Reinhart, G.A., 1997. β-Carotene uptake by blood plasma and leukocytes in dogs. FASEB J. 10, A447.