

Effects of dietary n-6 and n-3 fatty acids and vitamin E on the immune response of healthy geriatric dogs

Jean A. Hall, DVM, PhD; Katie A. Tooley, MS; Joseph L. Gradin, PhD; Dennis E. Jewell, PhD; Rosemary C. Wander, PhD

Objective—To determine the effect of dietary n-6 to n-3 fatty acid ratios and α -tocopheryl acetate concentration on immune functions and T cell subpopulations in healthy dogs.

Animals—Thirty-two 7- to 10-year old female Beagles.

Procedure—For 17 weeks, dogs were fed food that contained low (1.4:1) or high (40:1) ratios of n-6 to n-3 fatty acids in combination with 3 concentrations of all *rac*- α -tocopheryl acetate (low, 17 mg/kg of food; medium, 101 mg/kg; high, 447 mg/kg). Dogs were inoculated twice with a keyhole limpet hemocyanin suspension at 13 and 15 weeks.

Results—After 12 weeks, dogs consuming low concentrations of α -tocopheryl acetate had lower percentages of CD8+ T cells, compared with dogs consuming medium or high α -tocopheryl acetate concentrations. Also, dogs consuming low α -tocopheryl acetate concentrations had higher CD4+ to CD8+ T cell ratios. On day 4 of week 15, the percentage of CD8+ T cells was highest in dogs fed medium concentrations of α -tocopheryl acetate, compared with other dogs; however, the CD4+ to CD8+ T cell ratio was higher only in dogs fed low concentrations of α -tocopheryl acetate with high concentrations of n-3 fatty acids. Dogs consuming low concentrations of n-3 fatty acids with medium concentrations of α -tocopheryl acetate had the largest delayed-type hypersensitivity (DTH) skin test response.

Conclusions and Clinical Relevance—An optimum amount of dietary α -tocopheryl acetate concentration, regardless of the dietary n-6 to n-3 fatty acid ratio, stimulates the CD8+ T cell population. Effects of an optimum amount of dietary α -tocopheryl acetate concentration on the DTH response are blunted by dietary n-3 fatty acids. (*Am J Vet Res* 2003;64:762–772)

The immunomodulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids (PUFAs) have been reviewed.¹⁻⁴ It is thought that n-3 PUFAs

exert their effects by modulating signal transduction, gene expression, or both within inflammatory and immune cells.⁵ Fish oils are rich in the n-3 PUFAs, eicosapentaenoic acid (20:5[n-3]), and docosahexaenoic acid (22:6[n-3]). Consumption of fish oils by humans or animals in feeding studies decreases lymphocyte proliferation, T cell-mediated cytotoxicity, natural killer cell activity, macrophage-mediated cytotoxicity, monocyte and neutrophil chemotaxis, major histocompatibility class II expression and antigen presentation, production of proinflammatory cytokines (interleukin [IL]-1, IL-6, and tumor necrosis factor), and adhesion molecule expression, as reviewed by Calder.⁵

Feeding fish oil also reduces cell-mediated immune responses.⁵ We have previously shown that consumption of a food rich in n-3 fatty acids reduces the **delayed-type hypersensitivity (DTH)** skin response in aged Beagles.⁶ We have also shown that feeding aged Beagles a fish oil-enriched food for 8 weeks with an n-6 to n-3 fatty acid ratio of 1.4:1, followed by inoculation with a unique antigen, **keyhole limpet hemocyanin (KLH)** suspension, results in significant changes in total lymphocyte count and T cell subpopulations.⁷ After inoculation, total lymphocyte count increased, and the percentage of CD4+ T cells and the CD4+ to CD8+ T cell ratio decreased in dogs consuming food rich in n-3 fatty acids.

Currently, canine food that is marketed contains greater amounts of n-3 PUFAs than traditional canine food, but the optimum amount of α -tocopheryl acetate that these foods should contain is unclear. There are concerns that feeding an increased amount of PUFAs should be accompanied by increased intake of α -tocopheryl acetate to prevent increased peroxidation of lipids. For dogs, it is recommended that an α -tocopheryl acetate-to-PUFAs ratio of 0.5:1 (milligrams: grams) should be maintained.⁸ We have shown in Beagles, similar to what has been reported for humans and rats, that plasma α -tocopheryl concentration is not dependent on the dietary ratio of n-6 to n-3 fatty acids as long as α -tocopherol concentration is expressed relative to the total lipid content of plasma.⁹ Nonetheless, in our earlier study on Beagles, it was unclear whether the change in DTH response was explained solely by the difference in the n-3 fatty acid ratio in food or whether it was related to changes in the vitamin E intake of the dogs. Further studies were indicated to define the appropriate concentration of α -tocopheryl acetate to add to the food when n-3 fatty acid supplementation occurred.

Immunologic function is compromised in aged animals. Cellular immunity declines with age, leading to a

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From the Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331-4802 (Hall, Tooley, Gradin); the Science and Technology Center, Hill's Pet Nutrition Inc, 1035 NE 43rd St, Topeka, KS 66617-1587 (Jewell); and the Department of Nutrition and Foodservice Systems, School of Human Environmental Sciences, University of North Carolina, Greensboro, NC 27402-6170 (Wander).

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Address correspondence to Dr. Hall.

tendency of failure of the immune system to eradicate early clones of malignant cells and to an increased incidence of infections in the elderly. In humans, the DTH skin response decreases with age.^{10,11} The number of circulating T lymphocytes also may be slightly affected by aging.¹² Populations of CD4+ and CD8+ T cells are affected by aging.¹² In dogs (German Shepherd Dogs and Beagles), age-associated changes in peripheral blood cell values have been described.^{13,14} The number of WBCs decreases with age; this decrease is caused by decreases in immature and mature neutrophils as well as in lymphocytes. In addition, lymphocyte proliferation decreases with age in dogs.^{13,14} Fluorescence-activated cell-sorting techniques have also been used to characterize changes in specific populations of peripheral blood leukocytes in relation to age in Labrador Retrievers.¹⁵ Those dogs had, with increasing age, a significant increase in the CD3+ and CD8+ T cell populations, a significant decrease in the CD4+ T cell population, and a corresponding decrease in the CD4+ to CD8+ T cell ratio.

Results of nutritional supplementation studies^{12,16,17} in elderly humans indicate that modest increases in micronutrient intake improve immunity and reduce the risk of infection in relation to placebo controls. This suggests that reduced immune function in relation to age in dogs may also, in part, be alleviated through micronutrient supplementation.¹⁵ Because there is little information about the impact of increased concentration of dietary n-3 fatty acids in aged dogs, healthy aged Beagles were chosen for the study reported here. Because vitamin E supplementation has been shown to enhance the DTH skin response in healthy elderly persons,¹⁸⁻²⁰ our goal was to determine whether the effect of fish oil on the DTH skin response in Beagles was related to the concentration of α -tocopheryl acetate included in the food.

Thus, the purpose of the study reported here was to evaluate immune system function and T cell subpopulations in geriatric Beagles fed food containing 2 concentrations of n-3 fatty acids, while varying the concentration of α -tocopheryl acetate. The α -tocopheryl acetate was administered at the recommended concentration (17 mg/kg of food),⁸ at the concentration sometimes used in commercially available dog food (101 mg/kg of food), and at a pharmacologic concentration, which is approximately 45 times the National Research Council requirements for dogs (447 mg/kg of food). Data from human studies suggest that there is likely a therapeutic range for many micronutrients, including vitamin E, and that over supplementation may be harmful.²¹ In particular, we wanted to know whether an interaction occurred between dietary fatty acids and α -tocopheryl acetate that would stimulate the immune system of aged Beagles. The hypothesis tested was that dietary α -tocopheryl acetate would modulate immune response regardless of dietary n-6 to n-3 fatty acid ratio fed to healthy geriatric Beagles.

Materials and Methods

Dogs—Thirty-two healthy female geriatric (7 to 10 years old) Beagles that weighed between 8.1 and 15.9 kg were obtained for use in the study from a colony maintained

at Hill's Pet Nutrition Incorporated (Topeka, Kan) for use in research. All dogs had been immunized against canine distemper, parvovirus, and rabies, and none had chronic systemic disease, as determined on the basis of results of physical examination, CBC determination, serum biochemical analysis, urinalysis, and fecal examination for parasites. For 13 weeks before the study, all dogs consumed a commercial food^a with a low concentration of n-3 fatty acids, compared with the concentration of n-6 fatty acids. The ratio of n-6 to n-3 fatty acids was 18:1. The source of n-3 fatty acids in this food was 90% plant-derived from α -linolenic acid (soybean oil). The concentration of α -tocopheryl acetate was 32 mg/kg of food (as fed basis). During the study, dogs were housed in indoor runs and fed once daily in the morning. Room temperature was maintained at 20°C, and lighting was controlled for 12 hours light and 12 hours dark each day. Dogs were ranked on the basis of body weight and assigned to 6 groups (5 or 6 dogs/group) such that body weights were evenly distributed among all groups. The experimental protocol was reviewed and approved by the Oregon State University Animal Care and Use Committee in accordance with principles outlined by the National Institutes of Health.²²

Food—Six experimental foods were prepared by a commercial company.^b Food varied in the amount of n-3 fatty acids and α -tocopheryl acetate they contained. Three of the foods contained a minimal amount of n-3 fatty acids (0.5 g/kg of food, wet-weight basis) and, thus, had a high ratio of n-6 to n-3 fatty acids (40:1, low n-3 food). Three foods contained a larger amount of n-3 fatty acids from Menhaden fish oil (mean of 6.2 g/kg of food, wet-weight basis) and had a low ratio of n-6 to n-3 fatty acids (1.4:1, high n-3 food). The amount of 18:3(n-3) fatty acid (α -linolenic acid) was low in both foods and roughly equivalent. Three amounts of all *rac*- α -tocopheryl acetate were added to the low and high n-3 food. The 3 amounts added were the required amount (17 mg/kg of food [low]), the amount sometimes used in commercially available food (101 mg/kg of food [medium]), and a pharmacologic dose (447 mg/kg of food).²¹ The group of dogs receiving a high ratio of n-6 to n-3 fatty acids (38.4:1) and a low concentration of α -tocopheryl acetate (17 mg/kg of food as fed), which emulates a standard dog food, served as a control group. The n-3 fatty acid content of food, expressed as the ratio of n-6 to n-3 fatty acids, and α -tocopheryl acetate concentration of food were determined (Appendix).

Ingredients of the basal food (by weight) included water (54.8%), turkey (20.3%), corn (15.0%), pork liver (4.5%), soy meal (2.0%), beet pulp (1.0%), and vitamins and minerals (0.4%). Rice hulls were used as the carrier for the vitamin premix. Each kilogram of the vitamin premix contained 25,000 μ g of cholecalciferol, 7,500 mg of nicotinic acid, 5,000 mg of calcium D-pantothenate, 21,800 mg of thiamine mononitrate, 1,250 mg of riboflavin, 2,430 mg of pyridoxine hydrochloride, 250 mg of folic acid, 50 mg of biotin, and 50 mg of vitamin B-12. Calcium carbonate was used as the carrier for the mineral mix. Each kilogram of the mineral mix contained 80 g of zinc as zinc oxide, 6.0 g of manganese as manganese oxide, 280 g of iodine as calcium iodate, 1.0 g of cobalt as cobalt carbonate, 180 mg of selenium as selenium selenite, and 2.5 g of copper as copper chloride. The remaining 2% of the food was provided as added oil. Food enriched with n-3 fatty acids contained Menhaden fish oil, whereas food enriched with n-6 fatty acids contained corn oil. Foods were analyzed at a laboratory at the Woodson-Tenent Laboratories (Des Moines, Iowa), and results revealed that they were within expected analytical variance of the target nutrient composition. Mean nutrient composition (by weight) was 77.4% moisture, 5.8% protein, 4.5% fat, 1.3% ash, 0.7% crude fiber, and 10.3% carbohydrate. Fatty acid composition of the 6 foods was determined (Appendix).

Experimental protocol—Dogs were fed their respective food for 17 weeks. Body weight was measured once each week. Blood samples were collected before the study was initiated and again after dogs consumed the food for 12 weeks. Blood samples were obtained from all dogs on the morning of the same day. Food was withheld for 24 hours before collection of blood samples. Samples were collected into evacuated tubes containing EDTA (final concentration 1.5 g/L), and plasma was harvested. Blood samples were used for CBC determination and characterization of T cell subpopulations by flow cytometry.

The feeding trial was continued for an additional 5 weeks (for a total of 17 weeks) during which time *in vivo* immune assays were performed. At 13 weeks, dogs were inoculated of a KLH suspension.⁶ Blood was collected on days 1 and 4 after the initial inoculation of KLH. A second injection of KLH was given 2 weeks after the first inoculation (at week 15) as a booster. Blood was collected again on days 1 and 4 after the second inoculation with KLH. The DTH skin test was also performed 1 day after the second inoculation. At 17 weeks, the serum antibody titer against KLH was determined.

DTH skin test—The DTH skin test is an *in vivo* indicator of specific cell-mediated immune responsiveness by T cells and is measured as swelling and induration following an intradermal challenge. Dogs were sensitized with KLH suspension administered IM (500 µg of KLH emulsified in 1.0 mg of T1501 adjuvant⁴ for a total volume of 0.5 mL) at week 13 after the initiation of the food trial. The KLH and adjuvant were combined in an oil-water emulsion as described by Woodard,²³ except that the ingredients were sonicated rather than ground. Briefly, KLH (1.0 g/L), hexadecane (50 mL/L), Tween 80 (35 mL/L), Span 80 (15 mL/L), and T1501 (2 mL/L) were emulsified and added to physiologically normal saline (0.9% NaCl) solution. Fourteen days later (week 15), a second IM injection (0.5 mL) was given. One day after the second inoculation, the intradermal tests were performed. To accomplish this, a large rectangular patch was gently clipped on the lateral side of the chest of each dog. Individual disposable tuberculin syringes were filled with heat-aggregated KHL, saline solution, the negative control, or a histamine base⁶ (0.1g/L). The histamine base served as a positive control for the immediate skin test reaction. A 25-gauge needle was used to inject 0.05 mL of each of these intradermally. The 0.05-mL dose of heat-aggregated KLH consisted of approximately 3 mg of KLH. The KLH was heat aggregated according to the method of Exon et al.²⁴ Briefly, 120 mg of soluble KLH¹ dissolved in 6 mL of physiologically normal saline solution was heat aggregated in an 80°C water bath for 1 hour. The resultant gel was centrifuged twice at 400 × g for 10 minutes, removing the saline solution layer each time. The gel was then dispersed by passing it through a 23-gauge needle once and through a 25-gauge needle twice, carefully avoiding air bubbles. The sites of injection were marked with a felt marker. No chemical restraint was needed for dogs. Examinations were made at 15 and 30 minutes and at 24, 48, 72, and 96 hours after intradermal injections. Reactions were recorded according to the diameter of induration and degree of erythema. According to the manufacturer's instructions, a reaction larger than the negative control was considered to be a positive reaction. If a positive reaction to the saline solution control was observed, the diameter of its induration was subtracted from the other positive reactions. However, by 24 hours, no reactions to the saline solution control were observed. Histamine produced an induration typically 20 mm larger than the saline solution control at 15 minutes, after which the reaction subsided. These controls ruled out trauma or the volume of substance injected as the cause of the DTH response. The test was administered to all dogs by the same person, who was blind to the treatments.

Serum antibody titer against KLH—Humoral immune response was measured as antibody response to KLH. Dogs received an IM injection with a KLH vaccine (0.5 mL), as already described, at weeks 13 and 15. Serum was collected for determination of serum antibody titer against KLH at 17 weeks, which was 2 weeks after the KLH was administered as a booster. The humoral immune response for KLH was measured by a modification of an indirect ELISA procedure previously described by Woodard.²³ Briefly, enzyme immunoassay microtitration plates were coated with 0.1 mL of Dulbecco's PBS solution (DPBSS; pH, 7.4; 0.01 mol/L) containing 5 mg/L of KLH (5 mg/L), covered with parafilm to prevent evaporation, and refrigerated overnight or until needed. Before use, plates were inverted to remove excess coating buffer and washed 3 times with DPBSS containing 0.05% Tween 20. Serum samples (in quadruplicate) were then placed into wells and serial 1:4 dilutions made. Dilutions ranged from 1:100 to 1:409,600. The final volume in each well was 0.05 mL. Positive and negative control wells were included on each plate. Coated plates containing serum samples were incubated for 1 hour at 37°C while gently rotating on a platform. Plates were washed 3 times with DPBSS-Tween 20 to remove unbound antibody. Antibody against dog immunoglobulin G (IgG) was conjugated with alkaline phosphatase and diluted 1:5,000 with DPBSS-Tween 20. Subsequently, 0.1 mL was added to each well for a 1-hour incubation at 37°C. Plates were washed 3 times with DPBSS-Tween 20. Phosphate substrate (*p*-nitrophenyl phosphate disodium × 6H₂O; 1 g/L) was then added to each well (0.15 mL/well). After addition of substrate, plates were allowed to incubate at room temperature (approx 20°C) until the mean absorbance of the positive control equaled 1.0. The positive control was serum from a dog inoculated in a previous experiment. Because not all plates developed color at the same rate, a positive control serum sample was used rather than a specifically timed incubation. Otherwise, data from plate-to-plate and day-to-day could not be compared statistically. After allowing the positive control to reach a mean absorbance of 1.0, the entire plate was read at dual wavelengths, 405 and 492 nm, on a spectrophotometer. The results were expressed as the log of the titer.

Determination of CBC and WBC count and differential—Determination of CBC was performed by use of a hematology analyzer, and determination of WBC count and differential was performed by microscopic examination of blood smears after Wright-Giemsa staining. Determination of CBC was performed at 0 and 12 weeks. Determination of WBC count and differential was performed each time lymphocytes were examined by flow cytometry.

Isolation of blood mononuclear cells for analysis of surface-marker expression by flow cytometry—Peripheral blood mononuclear cells were isolated according to the methods of Coligan et al,²⁵ which were modified as follows. Cells were separated from a 1:1 dilution of blood and DPBSS by layering the blood-DPBSS mixture over a density gradient⁸ and centrifuging for 30 minutes at 900 × g at 4°C. We mixed 20 mL of blood with 20 mL of DPBSS and layered 9 mL of the blood-DPBSS mixture over 3 mL of the density gradient in each of four 15-mL centrifuge tubes. Cells were washed by adding excess DPBSS (3 times the volume of the cells) and centrifuged for 10 minutes at 400 × g. Cells were resuspended in approximately 0.5 mL of DPBSS with 0.1% sodium azide and 1.0% bovine serum albumin (PAB). The cell concentration was determined by use of an automated cell counter. An aliquot of the suspension was used to assess purity of peripheral blood mononuclear cells by light microscopy, and cell viability was assessed by trypan blue exclusion. Cells (10⁶) were added to each well of a 96-well,

round-bottom microtitration plate. The PAB was used to adjust the final volume in each well to maintain a constant ratio of number of cells to reagent volume.

The following reagents were then added to the wells: fluorescein-labeled monoclonal antibodies against canine CD4^h and CD8,ⁱ biotin-labeled monoclonal antibody against CD69,^j phycoerythrin-conjugated human recombinant IL-2,^k and immunoglobulins (mouse IgG1^l and rat IgG2a^m) as isotypic-matched controls for nonspecific fluorescence. Streptavidin-spectral redⁿ was added as the second-step reagent for biotin-labeled monoclonal antibodies. The canine CD4 and CD8 monoclonal antibodies were isolated, as reported by Gebhard et al.²⁶ Canine IL-2 receptor (IL-2R) was detected by use of human IL-2, as reported by Somberg et al.²⁷ The amount used in each test was determined from recommendations of manufacturers and preliminary titration experiments. The plate was vortexed, and the cells were incubated in the dark for 30 minutes on ice. After incubation, 125 μ L of PAB was added to each well. The plate was gently vortexed and then centrifuged at 210 \times g for 3 minutes. The cells were washed once more with 125 μ L of PAB and then resuspended in 175 μ L of PAB. Cells were then filtered into a flow vial and rinsed with an additional 300 μ L of PAB. Cell viability was confirmed by propidium iodide staining. Cell suspensions were evaluated immediately by flow cytometric analysis.

Analysis of surface-marker expression by flow cytometry—Multicolor flow cytometric analysis was performed. All data were collected by list-mode acquisition and analyzed by use of special software.^o

The percentage of fluorescein isothiocyanate (FITC)-labeled T cells in the mononuclear cell population was determined by comparison with FITC-only isotypic controls. The percentages of FITC-positive T cells were obtained as the mean of 2 determinations. The percentages of the FITC-positive T cells that were phycoerythrin IL-2 positive or biotin-CD69 positive were determined by setting the region on the isotypic control for nonspecific staining at approximately 5%.

Kinetics of canine IL-2R and CD69 expression—The kinetics of canine IL-2R expression after KLH stimulation were previously determined.¹ Because the percentage of cells expressing IL-2R was highest on day 4 after inoculation with KLH, IL-2R expression was assessed on day 4 in this study.

CD69 is rapidly inducible on various hematopoietic cells upon stimulation. Thus, it serves as an early activation antigen on activated T and B cells, activated macrophages, and natural killer (NK) cells. Also known as very early activation antigen MLR-3 and Leu-23, CD69 is a member of the NK cell gene complex family of signal transducing receptors.²⁸ CD69 reportedly diminishes or disappears after 1 or 2 days of activation.^{29,30} CD69 expression was assessed in 2 dogs not used in this study on days 0 through 4 after inoculation of KLH. The kinetics for CD69 expression was less definitive than the kinetics for IL-2R expression. The percentage of CD4+ T cells expressing CD69 was marginally higher on day 1 after inoculation with KLH, maintained a high degree of expression through day 3, and returned to baseline on day 4. Therefore, CD69 expression on CD4+ and CD8+ T cells was assessed on 1 day after inoculation with KLH in this study.

Fluorescent bead phagocytosis—Peripheral blood mononuclear cells were isolated according to the methods of Coligan et al.²⁵ and Krakowka et al.³¹ In short, cells were separated from a 1:1 dilution of blood and DPBSS by layering the blood-DPBSS mixture over a density gradient⁸ and centrifuging for 30 minutes at 900 \times g. Cells were washed twice in Hank's balanced salt solution and resuspended in RPMI 1640 supplemented with penicillin (100,000 U/L), streptomycin (100 mg/L), L-glutamine (2 mmol/L), and 10% fetal calf serum. An aliquot of the cell suspension was used to count

cells, and cell viability was assessed by trypan blue exclusion. Cells were placed into 75 cm² tissue culture flasks and incubated for 4 hours at 37°C in 5% CO₂. After incubation, the adherent cells (macrophages) were rinsed with DPBSS and then harvested.

Aliquots of 1 \times 10⁶ macrophages in Eagle's minimum essential medium plus 10% fetal calf serum were placed into 1.5-mL microcentrifuge tubes. A latex bead suspension^p was added to the cells to achieve a final bead-to-macrophage ratio of 25:1. The final volume in the tubes was adjusted to 1 mL with Eagle's minimum essential medium plus 10% fetal calf serum. The cells were incubated on a shaker for 2 hours at 37°C. Macrophage engulfment of fluorescent beads was assessed by use of a flow cytometer.

Plasma fatty acid profile and α -tocopherol concentration—The fatty acid profile of plasma samples was determined by use of gas chromatography, as described elsewhere,³² with heptadecanoic acid as an internal standard. The concentration of various fatty acids was expressed as g/100 g of fatty acids. The α -tocopherol concentration in plasma was measured by use of high-performance liquid chromatography and a fluorometric detector, as described elsewhere,³³ and expressed as μ mol/mmol of total lipid.

Statistical analysis—All data were normally distributed about the mean, and variances were equal. Data are reported as mean (\pm SEM) values. A 2-factor ANOVA, with the 2 different fatty acid-containing foods and the 3 amounts of dietary α -tocopheryl acetate as the 2 factors, was used to determine significant differences among the 6 foods for values at the start of the study, at 12 weeks, on days 1 and 4 following the first inoculation at week 13, and on days 1 and 4 following the second inoculation on week 15.³⁴ To determine the effect of food on the dependent variables, the differences between week 12 and time 0 were calculated. To determine whether the response to inoculation differed among dogs fed the various foods, the differences between week 12 and days 1 and 4 after the first inoculation and the differences between week 12 and days 1 and 4 after the second inoculation were calculated. Data were analyzed for CD69 and IL-2R expression on days 1 and 4, respectively, after each inoculation. If a significant interaction was detected, mean values for each food were compared by use of a least-significant difference Bonferroni-adjusted post-hoc test. When we did not detect a significant interaction, mean values for main effects were compared. If the main effect for the concentration of α -tocopheryl acetate in food was significant, then mean values of the cells at each concentration of α -tocopheryl acetate were compared by use of a 1-way ANOVA and a least-significant difference Bonferroni-adjusted post-hoc test.³⁴ If the main effect for the concentration of n-3 fatty acid in food was significant, then each amount of n-3 fatty acid intake was compared by use of a *t*-test for the 2 groups. To determine the effect of inoculation at each of the time points after inoculation, the same statistical analyses were followed by use of differences between week 12 and the inoculation date. All other statistical tests (not involving flow cytometric analysis) were done by use of a 2-way ANOVA, taking the initial data and comparing them to the final data (week 12). A value of $P \leq 0.05$ was considered significant. Data were evaluated by use of a statistical computer program.^q

Results

Plasma fatty acids—Fatty acid composition of plasma at the beginning of the study was not significantly different among groups of dogs (Table 1). After

feeding each of the 6 foods for 12 weeks, plasma fatty acid composition changed substantially; no significant interactions were found between dietary n-3 fatty acid content (high or low) when combined with the different α -tocopheryl acetate concentrations, nor was there a direct effect from feeding α -tocopheryl acetate. Consequently, the effect of dietary n-3 fatty acid content on plasma fatty acid composition was determined (Table 2). As anticipated, the sum of n-6 fatty acids, sum of n-3 fatty acids, and ratio of n-6 to n-3 fatty acids were dependent upon the amount of n-3 fatty acids in the food. After consumption of the high n-3 food, compared with the low n-3 food, the plasma sum of n-6 fatty acids was significantly ($P < 0.001$) lower (36.4 ± 0.9 vs 54.3 ± 1.0 g/100 g of fatty acids), and the plasma ratio of n-6 to n-3 fatty acids was significantly ($P < 0.001$) lower (2.1 ± 1.8 vs 27.4 ± 1.9), whereas the plasma sum of n-3 fatty acids was significantly ($P < 0.001$) higher (17.0 ± 0.6 vs 2.0 ± 0.6 g/100 g of fatty acids). These changes were primarily reflected in the significantly ($P < 0.001$) lower plasma concentrations of 18:2(n-6) fatty acids (21.4 ± 1.1 vs 29.1 ± 1.1 g/

100 g of fatty acids) and 20:4(n-6) fatty acids (14.3 ± 0.8 vs 23.6 ± 0.8 g/100 g of fatty acids) and in the significantly ($P < 0.001$) higher plasma concentrations of eicosapentaenoic acid (8.0 ± 0.4 vs 0.3 ± 0.8 g/100 g of fatty acids) and docosahexaenoic acid (6.4 ± 0.3 vs 0.7 ± 0.3 g/100 g of fatty acids) of dogs fed food high and low in n-3 fatty acids, respectively. Plasma concentrations of saturated fatty acids did not differ significantly ($P = 0.8$) between dogs fed the high and low n-3 food, whereas the plasma sum of monounsaturated fatty acids was significantly ($P = 0.007$) higher and the plasma sum of the PUFAs was significantly ($P = 0.003$) lower after consumption of high n-3 food.

DTH skin test and serum antibody titer against KLH—Results of the DTH skin test, which measures the cell-mediated immune response, indicated that no significant interaction existed between the dietary ratio of n-6 to n-3 fatty acids and amount of α -tocopheryl acetate in the food at 15 and 30 minutes and 24 and 48 hours after intradermal injection. At 72 and 96 hours after intradermal injection, however, significant ($P <$

Table 1—Mean (\pm SEM) plasma concentration of fatty acids in geriatric Beagles prior to dietary intervention with food that differed in the ratio of n-6 and n-3 fatty acids and amount of α TA* (low, 17 mg/kg of food; medium, 95 to 106 mg/kg; high, 446 to 448 mg/kg) for 12 weeks

Variables	Low n-3 fatty acid content			High n-3 fatty acid content			P values		
	Low α TA	Med α TA	High α TA	Low α TA	Med α TA	High α TA	n-3†	α TA‡	Interactions§
Sum of SFAs	32.9 \pm 0.3	32.5 \pm 0.3	33.0 \pm 0.3	32.7 \pm 0.3	33.0 \pm 0.4	33.6 \pm 0.3	0.24	0.17	0.42
18:1(n-9)	10.9 \pm 0.4	10.6 \pm 0.4	10.5 \pm 0.4	10.9 \pm 0.4	10.7 \pm 0.4	9.8 \pm 0.4	0.61	0.16	0.54
Sum of MUFAs	16.0 \pm 0.5	15.8 \pm 0.6	15.4 \pm 0.6	16.7 \pm 0.5	15.8 \pm 0.6	14.7 \pm 0.6	0.94	0.09	0.55
18:2(n-6)	23.6 \pm 0.5	22.9 \pm 0.5	23.3 \pm 0.5	22.7 \pm 0.5	22.8 \pm 0.5	23.4 \pm 0.5	0.45	0.61	0.57
20:4(n-6)	24.0 \pm 0.5	24.3 \pm 0.5	24.6 \pm 0.5	24.1 \pm 0.5	23.3 \pm 0.6	24.6 \pm 0.5	0.44	0.33	0.51
20:5(n-3)	0.2 \pm 0.1	0.3 \pm 0.1	0.3 \pm 0.1	0.3 \pm 0.1	0.4 \pm 0.1	0.2 \pm 0.1	0.90	0.46	0.65
22:6(n-3)	0.6 \pm 0.1	0.7 \pm 0.1	0.6 \pm 0.1	0.6 \pm 0.1	0.6 \pm 0.1	0.6 \pm 0.1	0.67	0.67	0.55
Sum of PUFAs	50.7 \pm 0.6	51.2 \pm 0.7	51.1 \pm 0.7	50.0 \pm 0.6	50.3 \pm 0.7	51.2 \pm 0.7	0.35	0.47	0.71
Sum of (n-6)	48.0 \pm 0.5	48.3 \pm 0.6	48.5 \pm 0.6	47.4 \pm 0.5	47.7 \pm 0.6	48.5 \pm 0.6	0.34	0.33	0.80
Sum of (n-3)	2.6 \pm 0.1	2.9 \pm 0.1	2.8 \pm 0.2	2.7 \pm 0.1	2.6 \pm 0.1	2.7 \pm 0.1	0.27	0.78	0.65
n-6 to n-3	18.4 \pm 0.9	17.1 \pm 1.0	17.3 \pm 1.2	17.9 \pm 0.9	18.9 \pm 1.0	18.4 \pm 1.0	0.37	0.95	0.50

*Fed as all-*rac*- α -tocopheryl acetate. †P value for the n-6 to n-3 fatty acid ratio factor. ‡P value for the dl- α -tocopheryl acetate factor. §P value for the interaction between the n-6 to n-3 fatty acid ratio and dl- α -tocopheryl acetate.
 SFAs = Saturated fatty acids. MUFAs = Monounsaturated fatty acids. PUFAs = Polyunsaturated fatty acids.
 See Appendix for composition of fatty acids (g/kg of food) in diets.

Table 2—Mean (\pm SEM) plasma concentration of fatty acids in geriatric Beagles after dietary intervention for 12 weeks with food that differed in the ratio of n-6 and n-3 fatty acids and amount of α TA* (low, 17 mg/kg of food; medium, 95 to 106 mg/kg; high, 446 to 448 mg/kg)

Variables	Low n-3 fatty acid content			High n-3 fatty acid content			P values		
	Low α TA	Med α TA	High α TA	Low α TA	Med α TA	High α TA	n-3†	α TA‡	Interactions§
Sum of SFAs	30.6 \pm 0.5	30.9 \pm 0.5	31.9 \pm 0.5	31.1 \pm 0.4	30.9 \pm 0.5	31.7 \pm 0.5	0.77	0.10	0.71
18:1(n-9)	7.1 \pm 0.6	6.7 \pm 0.7	6.9 \pm 0.6	7.7 \pm 0.6	8.0 \pm 0.6	8.2 \pm 0.6	0.05	0.92	0.79
Sum of MUFAs	12.4 \pm 1.1	11.7 \pm 1.3	11.6 \pm 1.1	13.9 \pm 1.1	15.5 \pm 1.1	14.7 \pm 1.1	0.007	0.90	0.59
18:2(n-6)	30.1 \pm 1.1	27.6 \pm 1.1	29.5 \pm 1.1	20.8 \pm 1.0	21.0 \pm 1.1	22.3 \pm 1.1	0.0001	0.29	0.38
20:4(n-6)	23.3 \pm 0.8	24.3 \pm 0.9	23.3 \pm 0.8	15.7 \pm 0.7	13.8 \pm 0.8	13.3 \pm 0.8	0.0001	0.34	0.16
20:5(n-3)	0.2 \pm 0.7	0.4 \pm 0.6	0.2 \pm 1.0	8.1 \pm 0.4	8.5 \pm 0.4	7.4 \pm 0.4	0.0001	0.56	0.76
22:6(n-3)	0.7 \pm 0.3	0.7 \pm 0.3	0.6 \pm 0.3	6.2 \pm 0.2	6.5 \pm 0.3	6.5 \pm 0.3	0.0001	0.90	0.71
Sum of PUFAs	56.3 \pm 1.1	56.7 \pm 1.2	55.7 \pm 1.1	54.3 \pm 1.0	52.9 \pm 1.1	53.0 \pm 1.1	0.003	0.64	0.69
Sum of (n-6)	54.4 \pm 0.9	54.7 \pm 1.1	53.8 \pm 0.9	37.4 \pm 0.9	35.4 \pm 0.9	36.4 \pm 0.9	0.0001	0.61	0.46
Sum of (n-3)	1.9 \pm 0.6	2.2 \pm 0.6	1.9 \pm 0.6	16.9 \pm 0.5	17.5 \pm 0.6	16.6 \pm 0.6	0.0001	0.61	0.86
n-6 to n-3	29.2 \pm 1.9	25.0 \pm 1.9	28.1 \pm 1.9	2.2 \pm 1.7	2.0 \pm 1.9	2.2 \pm 1.9	0.0001	0.46	0.53

See Table 1 for key.

0.001 and $P = 0.003$, respectively) interactions were found between the dietary ratio of n-6 to n-3 fatty acids and the amount of α -tocopheryl acetate in the food. Dogs that consumed food with the high n-3 fatty acid content had similar DTH skin test reactions for all concentrations of α -tocopheryl acetate fed. However, in

dogs that consumed food with the low n-3 fatty acid content, the diameter of induration for DTH skin test reaction was markedly larger in dogs fed the medium amount of α -tocopheryl acetate (Fig 1).

The humoral immune response to KLH was assessed 2 weeks after the second injection of KLH by measuring the production of antibody against KLH. No significant interactions or significant main effects caused by amount of n-3 fatty acid or α -tocopheryl acetate in the food were found. Log of the titers ranged from 9.14 ± 0.19 to 9.84 ± 0.19 .

Lymphocyte count—In terms of the lymphocyte count, no significant interactions were found between the ratio of n-6 to n-3 fatty acids and the amount of α -tocopheryl acetate in the diet of dogs fed the 6 foods at any of the time points assessed. Significant differences in lymphocyte counts were observed as a result of the ratio of n-6 to n-3 fatty acids and the amount of α -tocopheryl acetate in the food. When the difference in lymphocyte count between weeks 12 and 13 (on day 1 after inoculation of KLH) was calculated, a significant ($P = 0.05$) difference was observed as a result of the amount of α -tocopheryl acetate in the food (Fig 2). Dogs receiving the high amount of α -tocopheryl acetate had significantly higher lymphocyte counts, compared with dogs receiving the low amount of α -tocopheryl acetate (1960 ± 300 vs 1170 ± 270 cells/ μ L, respectively). Also, a significant ($P = 0.01$) difference was observed in the lymphocyte count as a result of the amount of α -tocopheryl acetate in the food when the difference between weeks 12 and 15 (on day 4 after the second inoculation of KLH) was calculated. Again, dogs receiving the high amount of α -tocopheryl acetate had a significantly higher lymphocyte count, compared with dogs receiving the low and medium α -tocopheryl acetate food (2320 ± 280 , 1450 ± 250 , and 1940 ± 280 cells/ μ L, respectively). A significant ($P = 0.05$) difference in lymphocyte count was observed as a result of the ratio of n-6 to n-3 fatty acids when the difference between weeks 12 and 13 (day 4 after inoculation of KLH) was calculated. In dogs receiving the high n-3 fatty acid food, the lymphocyte count was significantly higher, compared with dogs receiving the low n-3 fatty acid food (2240 ± 220 and 1690 ± 220 cells/ μ L, respectively).

T cell subpopulations—At the beginning of the study, no significant differences were found among the 6 groups of dogs in the percentage of CD4+ T cells, the CD4+ to CD8+ T cell ratio, the percentage of CD4+ T cells that were IL-2R+, or the percentage of CD8+ T cells that were IL-2R+. The same was true for the percentage of CD4+ and CD8+ T cells that expressed CD69. A significant ($P = 0.002$) difference in the percentage of CD8+ T cells was found at time 0 among the 6 groups

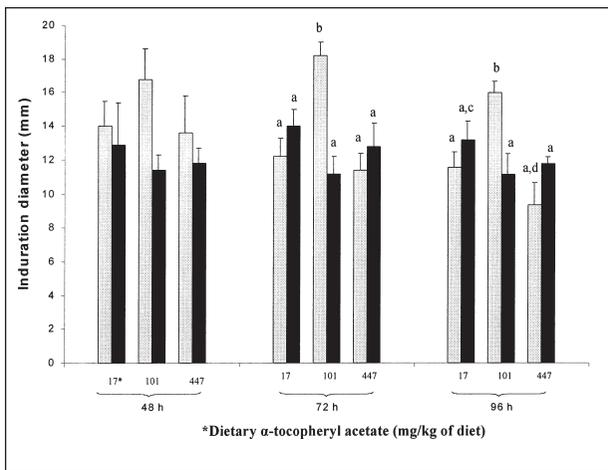


Figure 1—Effect of feeding dogs 6 foods containing high (black bars) or low (gray bars) amounts of n-3 fatty acids and 3 differing concentrations of α -tocopheryl acetate (low = 17 mg of α -tocopheryl acetate/kg of food; medium = 101 mg/kg; high = 447 mg/kg) on the delayed-type hypersensitivity skin test at 48, 72, and 96 hours. Dogs were challenged with an intradermal injection of keyhole limpet hemocyanin (KLH) to which they had been previously sensitized. Each bar represents the mean (\pm SEM) induration diameter after dogs had consumed their respective food for 15 weeks. A significant ($P < 0.05$) interaction was found between concentration of dietary α -tocopheryl acetate and n-3 fatty acids at 72 and 96 hours; therefore, effects of α -tocopheryl acetate at each amount of n-3 fatty acids were compared separately. ^{a,b,c,d}Different letters above bars at each time indicate values that differed significantly ($P \leq 0.05$).

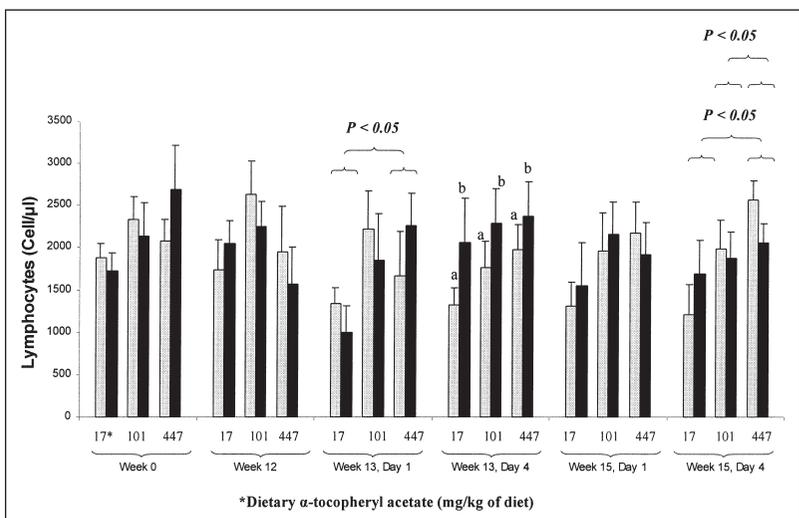


Figure 2—Effect of feeding dogs 6 foods containing high (black bars) or low (gray bars) amounts of n-3 fatty acids and 3 differing concentrations of α -tocopheryl acetate on the number of peripheral blood lymphocytes at week 0, week 12, and designated time points after inoculation with KLH (week 13, day 1; week 13, day 4; week 15, day 1; week 15, day 4). Each bar represents the mean (\pm SEM) number of peripheral blood lymphocytes. A main effect of dietary α -tocopheryl acetate concentration on lymphocyte count was found on at week 13, day 1 and at week 15, day 4. Groups whose values differed significantly ($P \leq 0.05$) are indicated. A main effect of dietary n-3 fatty acid concentration was found at week 13, day 4. ^{a,b}Different lower case letters above bars indicate values that differed significantly ($P \leq 0.05$).

of dogs on the basis of group assignments for the amount of α -tocopheryl acetate in the food. Dogs assigned to receive food with the medium amount of α -tocopheryl acetate had a higher percentage of CD8+ T cells, compared with dogs assigned to receive food with low and high amounts of α -tocopheryl acetate (25.5 ± 1.8 , 18.1 ± 1.7 , and $19.4 \pm 1.8\%$, respectively).

After 12 weeks, a significant ($P = 0.001$) difference in the percentage of CD8+ cells was found among groups of dogs consuming the 6 foods (Fig 3). This difference was attributed to the amount of α -tocopheryl acetate in the food. The percentages of CD8+ T cells of dogs fed food with low, medium, and high amounts of α -tocopheryl acetate were 13.7 ± 1.6 , 23.4 ± 1.7 , and $18.5 \pm 1.7\%$, respectively. The percentage of CD8+ T cells of dogs consuming the low and medium α -tocopheryl acetate food was significantly ($P = 0.0003$) different from one another as were the percentage of CD8+ T cells of dogs consuming the low and high α -tocopheryl acetate food ($P = 0.05$). The CD4+ to CD8+ T cell ratio was also significantly ($P = 0.02$) different among dogs consuming the 6 foods at 12 weeks. This difference was also attributed to the amount of α -tocopheryl acetate in the food. The CD4+ to CD8+ T cell ratio of dogs consuming the low α -tocopheryl acetate food (2.3 ± 0.3) was significantly ($P = 0.01$) different from dogs consuming the medium α -tocopheryl acetate food (1.5 ± 0.3), as well as from dogs consuming the high α -tocopheryl acetate food (1.5 ± 0.3 ; $P = 0.02$). The CD4+ to CD8+ T cell ratio of dogs consuming the medium and high α -tocopheryl acetate food was not significantly different from one another.

When the difference between values at week 12 and time 0 was calculated, the CD4+ to CD8+ T cell ratio was significantly ($P = 0.05$) different among dogs consuming the 6 foods based on the amount of α -tocopheryl acetate in the food. The CD4+ to CD8+ T cell ratio was significantly ($P = 0.02$) different among dogs consuming the low and high α -tocopheryl acetate food (0.5 ± 0.2 and $-0.06 \pm 0.2\%$, respectively).

After the first inoculation of KLH, no significant interaction or significant main effect caused by amount of n-3 fatty acid or α -tocopheryl acetate in the food was found for percentage of CD4+ or CD8+ T cells, nor for the CD4+ to CD8+ T cell ratio. There also were no significant differences among dogs consuming the 6 foods for CD69 and IL-2R expression, regardless of whether the impact was measured on day 1 after the first inoculation (week 13), as was done for the CD4+ and CD8+ T cells expressing CD69, or on day 4 after the first inoculation (week 13), as was done for CD4+ and CD8+ T cells expressing IL-2R.

After the second inoculation of KLH, the only significant ($P = 0.05$) interaction between the ratio of n-6 to n-3 fatty acids and the amount of α -tocopheryl acetate in the food was for the CD4+ to CD8+ T cell ratio on day 4 of week 15 (Fig 3). The CD4+ to CD8+ T cell ratio was significantly higher in dogs consuming the high n-3 fatty acid-low α -tocopheryl acetate food, compared with all other dog groups. When the differences in the CD4+ to CD8+ T cell ratio between week 12 and day 4 of week 15 were compared, significant ($P = 0.01$) differences were observed between the low n-3

fatty acid-low α -tocopheryl acetate food and the high n-3 fatty acid-low α -tocopheryl acetate food (-0.3 ± 0.23

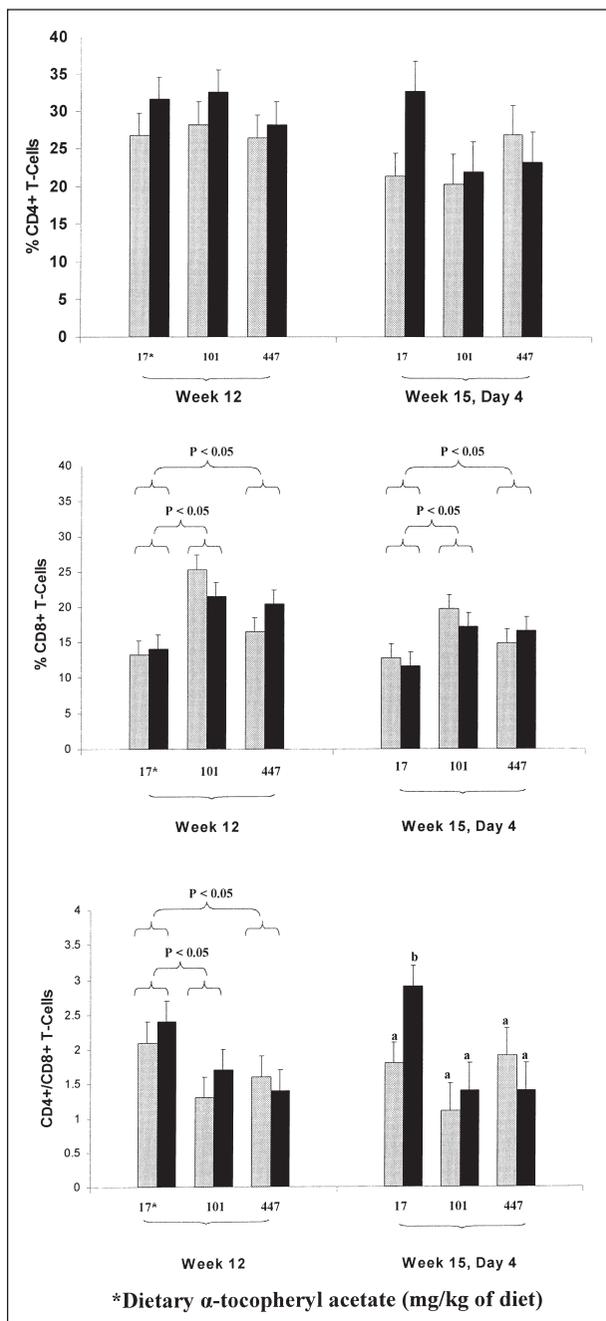


Figure 3—Effect of feeding dogs 6 foods containing high (black bars) or low (gray bars) amounts of n-3 fatty acids and 3 differing concentrations of α -tocopheryl acetate on the mean percentage of CD4+ and CD8+ T cells, and the CD4+ to CD8+ T cell ratio, after dogs had consumed their respective food for 12 weeks or 15 weeks and 4 days. Dogs were inoculated with KLH twice at 13 and 15 weeks. Each bar represents the mean (\pm SEM) percentage of T cells. A significant interaction was found between concentration of dietary α -tocopheryl acetate and n-3 fatty acids at 15 weeks day 4 for the CD4+ to CD8+ T cell ratio; therefore, effects of α -tocopheryl acetate at each amount of n-3 fatty acids were compared separately. ^{a,b}Different lower case letters above bars indicate values that differed significantly ($P \leq 0.05$). A significant main effect of α -tocopheryl acetate concentration was found at week 12 and for CD8+ T cells and for the CD4+ to CD8+ T cell ratio, and at day 4 of week 15 for CD8+ T cells. Groups whose values differed significantly ($P \leq 0.05$) are indicated.

and 0.5 ± 0.23 , respectively). In addition, the CD4+ to CD8+ T cell ratio of dogs consuming the high n-3 fatty acid-low α -tocopheryl acetate food and the high n-3 fatty acid-medium α -tocopheryl acetate food were significantly ($P = 0.02$) different (0.5 ± 0.23 and -0.3 ± 0.25 , respectively). The amount of dietary α -tocopheryl acetate in the 6 foods fed to dogs significantly ($P = 0.03$) influenced the percentage of CD8+ T cells by week 15, day 4. The percentage of CD8+ T cells was highest in dogs fed the medium amount of α -tocopheryl acetate food ($18.5 \pm 1.6\%$), compared with dogs fed the low and high amounts of α -tocopheryl acetate (12.2 ± 1.6 and $15.8 \pm 1.7\%$, respectively).

Fluorescent bead phagocytosis by macrophages—No significant interaction or significant main effect caused by amount of n-3 fatty acid or α -tocopheryl acetate in the food was found for the percentage of macrophages engulfing fluorescent beads. The percentage of cells engulfing latex beads ranged from 23.1 ± 2.1 to $26.8 \pm 1.9\%$ at the beginning of the study to 21.9 ± 2.9 to $30.9 \pm 2.6\%$ after 12 weeks of dietary intervention.

CBC and WBC count and differential—In terms of the CBC and WBC count and differentials, some significant interactions were found between the ratio of n-6 to n-3 fatty acids and the amount of α -tocopheryl acetate among dogs fed the various foods at the beginning of the study. Significant differences in measurements among dog groups included hemoglobin ($P = 0.03$), hematocrit ($P = 0.04$), and RBC count ($P = 0.05$). These differences were the result of variability in values among dogs at the beginning of the study. No significant differences among dogs consuming the 6 foods at the end of the feeding trial were found for the leukogram and hemogram variables measured. Also, no significant differences in WBC count were observed as a result of either the n-6 to n-3 fatty acids ratio or the concentration of α -tocopheryl acetate in the food.

Discussion

The changes in plasma fatty acid profiles after feeding the 6 experimental foods were as expected. Plasma concentrations of arachidonic acid were significantly lower in dogs fed the high n-3 fatty acid food. In addition, plasma concentrations of linoleic acid in dogs fed the high n-3 fatty acid food were significantly decreased. The reason for this is that the precursor of the n-6 fatty acid family is linoleic acid (18:2[n-6]), and in animal tissues, linoleic acid is converted into arachidonic acid. Thus, with less linoleic acid substrate, less arachidonic acid was produced. Furthermore, the amounts of eicosapentaenoic acid and docosahexaenoic acid in plasma were significantly increased in dogs fed the high n-3 fatty acid food. These findings are reflected in the plasma sums of the n-6 and n-3 fatty acids, as well as in the plasma ratio of n-6 to n-3 fatty acids.

Plasma α -tocopherol concentrations increased significantly in dogs fed the high amounts of α -tocopheryl acetate, compared with dogs fed the low amounts of α -tocopheryl acetate at both concentrations of n-3 fatty acid foods. This increase was signifi-

cant even when plasma α -tocopherol concentrations were expressed relative to total plasma lipid concentrations (cholesterol plus triglycerides). The latter is the more accurate way of assessing plasma α -tocopherol concentrations.⁹

After inoculation of KLH, lymphocyte count was influenced by the amount of α -tocopheryl acetate in the food on day 1 of week 13 and on day 4 of week 15. Lymphocyte count increased after inoculation of KLH in dogs receiving the high amounts of α -tocopheryl acetate in their food. In a previous study,⁷ we showed that total lymphocyte count increased in dogs consuming the high n-3 fatty acid food after inoculation of KLH. It appears that the concentration of vitamin E in the food outweighs the effect of n-3 fatty acid content on peripheral blood lymphocyte count.

The DTH skin reaction provides an *in vivo* assay to assess cell-mediated immune function. Delayed-type hypersensitivity is antigen specific, T cell dependent, and a recall response that manifests itself as an inflammatory reaction, which does not become apparent until roughly 24 hours after exposure to the antigen and may not reach peak intensity for several days. At 72 and 96 hours, results of the DTH skin test revealed a significant interaction between the ratio of n-6 to n-3 fatty acids and the amount of α -tocopheryl acetate in the food. At these time points when high concentrations of n-3 fatty acids were fed to dogs, the DTH skin test results were not significantly different for dogs consuming each of the 3 concentrations of α -tocopheryl acetate. However, when the low n-3 fatty acid foods were fed, dogs consuming either low or high concentrations of α -tocopheryl acetate in the food had a similar DTH skin test response, and both reactions were significantly smaller than those in dogs fed food containing medium amounts of α -tocopheryl acetate. Thus, a high n-3 fatty acid food suppressed the DTH response, whereas a low n-3 fatty acid food with an optimal concentration of vitamin E produced a large DTH reaction.

Study results have indicated that vitamin E supplementation can significantly enhance the DTH response in elderly human subjects. Supplementation with vitamin E (800 mg dl- α -tocopheryl acetate) for 1 month resulted in a significantly increased DTH response in elderly human subjects.³⁵ In another study, these same researchers reported increases in the DTH response of elderly human subjects when provided a dietary supplement of 60, 200, or 800 mg of vitamin E/d for 4 months.²⁰ The greatest increase in DTH response was observed in the group of people receiving 200 mg/d, and those researchers concluded that 200 mg/d represented the optimum amount of vitamin E supplement needed to maximize the DTH response in elderly human subjects. This observation suggests that there may be a threshold dose for the immunostimulatory effect of vitamin E. In our study, dogs receiving food with low concentrations of n-3 fatty acids and medium amounts of α -tocopheryl acetate had the maximum DTH response, which appears to mimic the findings in human subjects. In dogs, there appears to be an upper limit to the beneficial effects of vitamin E.

None of the foods in our study were deficient in

α -tocopheryl acetate, and the food containing the most α -tocopheryl acetate had approximately 45 times the amount required for dogs.⁹ All of the dogs had plasma concentrations of α -tocopherol that were within the reference range for Beagles before and after the feeding trial.⁹

The mechanisms by which vitamin E enhances the immune response have yet to be fully explained; however, evidence suggests that vitamin E works by reducing prostaglandin synthesis, decreasing the formation of free radicals, or both.^{35,36} Reactive oxygen species, especially H_2O_2 that is produced by activated macrophages, depress lymphocyte proliferation.³⁷ Vitamin E has been shown to decrease H_2O_2 formation by polymorphonuclear cells.³⁸ Furthermore, the immunosuppressive effect of vitamin E deficiency appears to be linked to increased free radical reactions, which lead to greater production of prostaglandin E_2 . Dietary supplementation with vitamin E has been shown to improve T cell responsiveness by reducing macrophage prostaglandin E_2 production.¹⁹

The humoral immune response was assessed by measuring the production of antibodies against a foreign protein, KLH. This protein was chosen because it is not a protein the Beagle's immune system would likely have come into contact with before. The log of the antibody titer against KLH was unaffected in these dogs by feeding either the low or high n-3 fatty acid food, regardless of the amount of vitamin E fed. These results are consistent with those of our previous study⁶ in which antibody production against KLH was not affected by changing the ratio of n-6 to n-3 fatty acids in the food. In healthy elderly human subjects, antibody titers against tetanus antigens were not significantly altered with dietary vitamin E supplementation.²⁰ In another study, dietary supplementation with arachidonic acid (either 200 mg/d or 1.5 g/d for the first 15 days of the study) to young healthy men had no effect on serum antibody titers against influenza vaccine.³⁹ Thus, other study results indicate that dietary supplementation with vitamin E or fatty acids do not affect antibody titers.

An ex vivo assay used to evaluate the immune system was the measurement of macrophage engulfment of fluorescent beads. The data were not significantly different at any time point during our study (baseline, 12 weeks, or the difference between baseline and 12 weeks). We had expected to see a decrease in percentage of macrophages engulfing beads in dogs fed high n-3 fatty acid food. Food enriched in fish oil (along with olive and evening primrose oil) has been shown to suppress expression of IL-2R after mitogenic stimulation of spleen lymphocytes.^{40,41} Because fish oil suppresses expression of IL-2R and IL-2 activates macrophages,⁴² we expected to see a smaller percentage of macrophages engulfing beads in dogs fed high n-3 fatty acid food. In our study, however, expression of IL-2R on CD4+ and CD8+ T cells was not significantly different among dogs in the 6 food groups; thus, it is not surprising that the percentage of macrophage engulfment of fluorescent beads was similar for all groups.

The percentage of CD4+ T cells was not significantly altered by any of the 6 experimental foods.

Compared with dogs of other groups, the percentage of CD8+ T cells at the beginning of our study was significantly ($P = 0.002$) different in dogs assigned to the group to be fed the medium amount of α -tocopheryl acetate, regardless of the concentration of n-3 fatty acids. The percentage of CD8+ T cells of those same dogs continued to be significantly ($P = 0.001$) different at 12 weeks into the feeding trial. The CD4+ to CD8+ T cell ratio was also significantly ($P = 0.02$) altered by the amount of α -tocopheryl acetate in the food at 12 weeks. When the difference between 12 weeks and the beginning of our study was calculated, the ratio was still significantly altered ($P = 0.02$), indicating that the 6 experimental foods had an effect on the CD4+ to CD8+ T cell ratio.

For the most part, inoculation of KLH had little effect on cell surface markers. After the second inoculation (15 weeks and 4 days), CD8+ T cells significantly decreased ($P = 0.03$). The CD4+ T cells also decreased, but not significantly ($P = 0.09$). In both cases, this was a direct result of the amount of α -tocopheryl acetate in the food. The CD4+ to CD8+ T cell ratio was also significantly decreased after the second inoculation, only this time a significant ($P = 0.05$) interaction between the ratio of n-6 and n-3 fatty acids and the amount of α -tocopheryl acetate in the food was observed. This interaction was caused primarily by the α -tocopheryl acetate factor. Even though changes in the percentage of CD4+ and CD8+ T cells were not large, when the 2 terms were combined into a ratio term, the changes became a more powerful representation of the 2 individual markers. Inoculation of KLH had no effect on the IL-2R or CD69 cell surface markers on either CD4+ or CD8+ T cells.

It is interesting to speculate that vitamin E enhanced the DTH skin test response by enhancing CD8+ T cell numbers, suppressing the CD4+ to CD8+ T cell ratio, or both. Dietary n-3 fatty acids then blunted the effects of the optimum dose of vitamin E. Formerly, it was thought that only CD4+ T cells mediated the DTH response, but it has been shown in several systems that CD8+ and CD4+ T cells can mediate the DTH response.⁴³ External antigens, including many contact sensitizers, chemical and protein respiratory allergens, viral antigens, metabolic products of drugs, and autoantigens, can enter the endogenous pathway to be presented to CD8+ T cells.⁴³ Doyle et al⁴⁴ have shown that inoculation of KLH into mice stimulates accumulation in the draining lymph nodes of CD8+ T cells, which preferentially contain interferon- γ mRNA *ex vivo* and secrete interferon- γ protein *in vitro*. However, results of their study indicated that depletion of CD8+ T cells and, thus, CD8+ T cell-derived interferon- γ resulted in no obvious perturbation of the CD4+ T cell anti-KLH response. Thus, it is unclear what role the CD8+ T cells play in shaping the anti-KLH response.⁴⁴

The results from our study are similar to those of some previously reported studies. Kelley et al⁴⁵ found that supplementation of healthy young men with docosahexaenoic acid resulted in no effects on proliferation of peripheral blood mononuclear cells, DTH skin response, number of T cells producing IL-2, the

helper-to-suppressor T cells ratio in circulation, or serum concentrations of IgG, C3, or IL-2R. Another study by Kelley et al³⁹ had similar results when young men received arachidonic acid supplementation. Supplementing with arachidonic acid did not alter the absolute number or percentage (for B, T, helper, suppressor, and NK cells) of lymphocytes in the circulation, nor did it alter helper-to-suppressor T lymphocytes ratio. A vaccine was given in the second study by Kelley et al. Results of the 2 studies by Kelley et al^{39,45} indicated that neither inoculation nor feeding either high n-3 or high n-6 fatty acid food had any significant effect on composition of lymphocyte subsets. The data from 2 other studies^{46,47} do differ from these results. However, there are differences in experimental designs, species of animals, and methods between these 2 studies and ours, which could account for variation in effects.

^aScience Diet Canine Maintenance, Hill's Pet Nutrition Inc, Topeka, Kan.

^bHill's Pet Nutrition Inc, Topeka, Kan.

^cSigma Chemical Co, St Louis, Mo.

^dTetronic 1501, BASF Corp, Parsippany, NJ.

^eHistatrol, Center Laboratories, Port Washington, NY.

^fCalbiochem-Novabiochem, La Jolla, Calif.

^gHistopaque 1077, Sigma Chemical Co, St Louis, Mo.

^hAnti-canine CD4-FITC (CM 12.125), Custom Monoclonals International, West Sacramento, Calif.

ⁱAnti-canine CD8-FITC (CM 1.140), Custom Monoclonals International, West Sacramento, Calif.

^jAnti-cat/dog B/T activation marker (CM 2.58), Custom Monoclonals International, West Sacramento, Calif.

^kR&D Systems, Minneapolis, Minn.

^lFITC-labeled Mouse IgG1, (kappa) isotype control and biotin-labeled mouse IgG1, (kappa) isotype control, PharMingen, San Diego, Calif.

^mPhycocerythrin-labeled rat IgG2a, (kappa) isotype control, PharMingen, San Diego, Calif.

ⁿSouthern Biotechnology Associates Inc, Birmingham, Ala.

^oWinList, Verity Software House Inc, Topsham, Me.

^pL75 micron, Polysciences Inc, Warrington, Pa.

^qSAS, version 6.12, SAS Institute Inc, Cary, NC.

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Appendix

Composition of α -tocopheryl acetate (α TA) and selected fatty acids in 6 experimental foods fed to geriatric Beagles for 17 weeks

Dietary components	Experimental diets					
	Low n-3 fatty acid content			High n-3 fatty acid content		
Ratio of n-6 to n-3 fatty acids	38.4:1	40.6:1	41.0:1	1.29:1	1.31:1	1.48:1
Dietary α TA content*	Low α TA	Med α TA	High α TA	Low α TA	Med α TA	High α TA
α TA, as-fed basis (mg/kg)	17	106	446	17	95	448
α TA, dry-matter basis (mg/kg)	76	465	2,010	76	436	1,870
	Fatty acid (g/kg of food)†					
14:0	0.2	0.2	0.2	1.4	1.4	1.4
16:0	7.3	7.5	7.4	7.6	7.1	8.5
18:0	2.4	2.5	2.5	2.5	2.3	2.7
Sum of SFAs (g/kg of food)‡	9.9	10.2	10.1	11.6	10.9	12.7
16:1(n-7)	1.0	1.1	1.1	2.4	2.5	2.7
18:1(n-9)c	13.9	14.5	14.5	10.2	9.0	11.4
Sum of MUFAs (g/kg of food)§	15.1	15.8	15.8	13	11.8	14.5
18:2(n-6)	18.8	19.9	20.1	7.6	7.2	8.8
18:3(n-3)	0.5	0.5	0.5	0.6	0.5	0.6
18:4(n-3)	< 0.1	< 0.1	< 0.1	0.6	0.6	0.6
20:4(n-6)	0.4	0.4	0.4	0.5	0.5	0.5
20:4(n-3)	< 0.1	< 0.1	< 0.1	0.3	0.3	0.3
20:5(n-3)	< 0.1	< 0.1	< 0.1	1.9	1.9	1.9
22:5(n-3)	< 0.1	< 0.1	< 0.1	0.4	0.4	0.4
22:6(n-3)	< 0.1	< 0.1	< 0.1	2.5	2.2	2.5
Sum of PUFAs (g/kg of food)	19.7	20.8	21.0	14.1	13.3	15.3
Sum of n-6 fatty acids	19.2	20.3	20.5	8.1	7.7	9.3
Sum of n-3 fatty acids	0.5	0.5	0.5	6.3	5.9	6.3

*Fed as all-*rac*- α -tocopheryl acetate. †Analysis performed by Woodson-Tenent Laboratories (Des Moines, Iowa).

‡Sum of the saturated fatty acids (SFAs) was determined as follows: 8:0 + 10:0 + 11:0 + 12:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0 + 22:0 + 24:0. §Sum of the monounsaturated fatty acids (MUFAs) was determined as follows: 14:1 + 15:1 + 16:1(n-7) + 17:1 + 18:1(n-9)c + 18:1(n-7) + 18:1(n-9)t + 20:1(n-9) + 22:1(n-9) + 24:1. ||Sum of the polyunsaturated fatty acids (PUFAs) was determined as follows: 18:2(n-6) + 18:3(n-6) + 18:3(n-3) + 18:4(n-3) + 20:2(n-6) + 20:3(n-6) + 20:3(n-3) + 20:4(n-6) + 20:4(n-3) + 20:5(n-3) + 21:5(n-3) + 22:2(n-6) + 22:4(n-6) + 22:5(n-6) + 22:5(n-3) + 22:6(n-3).