The Ratio of Dietary (n-6) to (n-3) Fatty Acids Influences Immune System Function, Eicosanoid Metabolism, Lipid Peroxidation and Vitamin E Status in Aged Dogs[1–4]

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ABSTRACT We studied the effects of feeding experimental diets containing (n-6) to (n-3) fatty acid ratios of 31:1, 5.4:1, and 1.4:1 to 20 healthy female geriatric Beagles (9.5–11.5 y) for 8–12 wk on various indices of the immune response. Compared with the 31:1 diet, consumption of the 5.4:1 and 1.4:1 diets significantly increased (n-3) fatty acids in plasma (2.17 ± 0.64, 9.05 ± 0.64, 17.46 ± 0.64 g/100 g fatty acids, respectively, $P < 0.0001$). Although supplementation with (n-3) fatty acids did not significantly alter the humoral immune response to keyhole limpet hemocyanin (KLH), it significantly suppressed the cell-mediated immune response based on results of a delayed-type hypersensitivity (DTH) skin test. The DTH response after intradermal injection of KLH at 24 h was significantly lower in the group consuming the 1.4:1 diet compared with the group consuming the 5.4:1 ($P = 0.02$) or the 31:1 diets ($P = 0.04$), and remained significantly suppressed at 48 h in the group fed 1.4:1 relative to the group fed 31:1. After consumption of the 1.4:1 diet, stimulated mononuclear cells produced 52% less prostaglandin E_2 (PGE_2) than those from dogs fed the 31:1 diet (224 ± 74 and 451 ± 71 pmol/L, respectively, $P = 0.04$). Plasma concentration of α-tocopherol was 20% lower in dogs fed the 1.4:1 diet compared with those fed the 31:1 diet ($P = 0.04$), and lipid peroxidation was greater in both plasma ($P = 0.03$) and urine ($P = 0.002$). These data suggest that although a ratio of dietary (n-6) to (n-3) fatty acids of 1.4:1 depresses the cell-mediated immune response and PGE_2 production, it increases lipid peroxidation and lowers vitamin E concentration. J. Nutr. 127: 1198–1205, 1997.

KEY WORDS: • *(n-3) fatty acids* • *immune system* • *prostaglandin E_2* • *delayed-type hypersensitivity* • *dogs*

The potential therapeutic benefits of dietary supplementation with (n-3) fatty acids, found in large concentrations in fish oil, have aroused great interest. However, experimental data are few concerning the correct ratio of (n-6) to (n-3) fatty acids in the diet that is necessary to maximize benefits (Neuringer et al. 1988).

The beneficial effects of (n-3) fatty acids are derived in part from their effect on the immune system. Metabolism of arachidonic acid (AA),[6] derived from the (n-6) fatty acid, linoleic acid, and eicosapentaenoic acid (EPA), derived from the (n-3) fatty acid, α-linolenic acid, leads to the generation of eicosanoids such as prostaglandins and leukotrienes. The eicosanoids derived from AA and EPA have very similar molecular structures but markedly different biologic effects. For example, the EPA-derived eicosanoids are in general much less potent inducers of inflammation than the AA-derived eicosanoids. Consequently, a predominance of (n-6) fatty acids will result in a proinflammatory status with production of prostaglandins of the 2 series and leukotrienes of the 4 series. As the relative amount of (n-3) fatty acids increases, more prostaglandins of the 3 series and leukotrienes of the 5 series are produced. These eicosanoids are considered to be less inflammatory (Shapiro et al. 1993). A reduction in the amount of the more inflammatory products from AA, prostaglandin E_2 (PGE_2) and leukotriene B_4, has been implicated as an underlying mechanism for the anti-inflammatory effects of fish oil (Meydani and Dinarello 1993). The immune response also may be altered by changes in the production of immunologic

[1] Presented in part at Experimental Biology 96, April 1996, Washington, DC [Wander, R. C., Hall, J. A., Gradin, J. L., Du, S.-H., & Jewell, D. E. (1996) Effect of dietary (n-6) to (n-3) fatty acid ratios on eicosanoid metabolism and T-cell subpopulations in aged dogs. FASEB J. 10: A2876 (abs.)].

[2] Funded in part by Hill's Pet Nutrition, Inc., P.O. Box 1658, Topeka, KS 66601–1658.

[3] Oregon State University Agricultural Experiment Station technical paper 11066.

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[6] Abbreviations used: AA, arachidonic acid; DBI, double-bond index; DHA, docosahexaenoic acid; DPBS, Dulbecco's phosphate buffered saline; DTH, delayed-type hypersensitivity; EPA, eicosapentaenoic acid; HBSS, Hanks' balanced salt solution; IL, interleukin; KLH, keyhole limpet hemocyanin; PGE_2, prostaglandin E_2; PMN, polymorphonuclear leukocytes; PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric reactive substances.

Manuscript received 19 July 1996. Initial review completed 20 August 1996. Revision accepted 20 February 1997.

TABLE 1

Physical characteristics of the beagles[1]

Dietary (n-6):(n-3)	Low (n-3) 31:1	Medium (n-3) 5.4:1	High (n-3) 1.4:1
n	6	7	7
Age, *y*	10.2 ± 0.11	10.5 ± 0.27	10.1 ± 0.37
Weight, *kg*	12.5 ± 1.1	11.5 ± 0.9	11.5 ± 0.9

[1] Values are means ± SEM.

mediators such as cytokines. Ultimately, these effects are manifest by changes in cell-mediated immunity as demonstrated by the delayed-type hypersensitivity (DTH) skin test.

The effect of these fatty acids on the immune response may be different in older animals. Meydani, S. et al. (1991) studied the effect of dietary (n-3) fatty acids on cytokine production and lymphocyte proliferation in young and older women and found the changes to be more dramatic in older women.

A possible adverse effect of high levels of dietary (n-3) fatty acids is that their accumulation in tissues may make those tissues more vulnerable to lipid peroxidation, especially if peroxidation overwhelms the normal antioxidant mechanisms. Increased intake of (n-3) fatty acids without adequate antioxidant protection could result in increased free radicals and lipid-oxidative by-products.

Consequently, the purpose of this study was to determine the effect of feeding healthy female geriatric Beagle dogs three different diets with varying (n-6) to (n-3) ratios over an 8- to 12-wk period. We report here the effect of these three diets on in vitro and in vivo indices of the immune response, namely, γ-immunoglobin (IgG) antibody production and the DTH skin test to a specific T-dependent antigen, keyhole limpet hemocyanin (KLH), and eicosanoid production of PGE_2. We also report the effect of these three diets on lipid peroxidation, obtained by measuring plasma and urine lipid peroxide levels, and on vitamin E status, obtained by measuring vitamin E concentrations in plasma.

MATERIALS AND METHODS

Animals. Twenty healthy female geriatric Beagles (9.5–11.5 y) were chosen for the study. All dogs had been vaccinated (canine distemper, parvovirus and rabies) and were determined to be free of chronic systemic disease on the basis of physical examination, complete blood count, serum biochemical evaluations, urinalysis and fecal examination for parasites. Before the study, all dogs were consuming diets with a low concentration of (n-3) fatty acids compared with the concentration of (n-6) fatty acids (Science Diet Canine Maintenance, Hill's Pet Nutrition, Topeka, KS). During the study, dogs were housed in indoor runs and fed once daily. They were ranked according to body weight and assigned to three groups, such that body weights were evenly distributed across all groups. Physical characteristics of the dogs in each of these groups are given in **Table 1**.

The experimental protocol was reviewed and approved by the Oregon State University Animal Care and Use Committee according to the principles outlined by the National Institutes of Health (NRC 1985).

Diets. The three experimental diets were prepared by Hill's Pet Nutrition, Topeka, KS. Six dogs were fed a diet with a low concentration of (n-3) fatty acids; the (n-6) to (n-3) fatty acid ratio was 31:1. Seven dogs were fed a diet with a medium concentration of (n-3) fatty acids; the (n-6) to (n-3) fatty acid ratio was 5.4:1. Seven dogs were fed a diet with a high concentration of (n-3) fatty acids; the (n-6) to (n-3) fatty acid ratio was 1.4:1. The basal diet ingredients 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 a carrier for the vitamin premix, which contained 25,000 μg/kg cholecalciferol, 175,000 mg/kg *dl*-α-tocopherol acetate, 7500 mg/kg nicotinic acid, 5000 mg/kg calcium D-pantothenate, 21,770 mg/kg thiamine mononitrate, 1250 mg/kg riboflavin, 2431 mg/kg pyridoxine hydrochloride, 250 mg/kg folic acid, 50 mg/kg biotin and 50 mg/kg vitamin B-12. Calcium carbonate was used as the carrier for the mineral mix, which contained 80 g/kg zinc as zinc oxide, 6.0 g/kg manganese as manganese oxide, 280 mg/kg iodine as calcium iodate, 1.0 g/kg cobalt as cobalt carbonate, 180 mg/kg selenium as selenium selenite and 2.5 g/kg copper as copper chloride. The remaining 2% was provided by added oil. The source of oil for the (n-3) enriched diet was fish oil (Zapata Protein, Reedville, VA). The source of oil for the (n-6) enriched diet was corn oil (Mazola, Englewood Cliffs, NJ). The expected nutrient composition by weight was 71.8% moisture, 7.4% protein, 6.0% fat, 2.0% ash, 0.6% crude fiber and the remainder carbohydrate. The diets were analyzed by Woodson-Tenent Laboratories (Des Moines, IA) and shown to be within expected analytical variance of these targets. The fatty acid composition of the three diets is given in **Table 2**. Total vitamin E levels of the two fish oil diets were adjusted at 137 ± 8 mg/kg, the concentration in the low (n-3) diet, with an (n-6) to (n-3) fatty acid ratio of 31:1, by adding the appropriate amount of α-tocopherol.

Study design. Dogs were fed their respective diets for 12 wk. Body weight was measured once weekly. Energy levels were adjusted so that the dogs neither gained nor lost weight. Changes in body weight throughout the study were <10%, with the exception of one dog in the high (n-3) diet group, with an (n-6) to (n-3) fatty acid ratio of 1.4:1. This dog was hyperactive and frequently refused to eat

TABLE 2

Composition of selected fatty acids of the experimental diets[1]

Dietary (n-6):(n-3)	31:1	5.4:1	1.4:1
Fatty acid		*g/kg diet*	
12:0	<0.1	<0.1	<0.1
14:0	0.4	0.85	1.9
16:0	10.6	9.15	10.35
18:0	3.4	2.9	3.2
Σ SFA[2]	14.6	13.2	15.85
16:1(n-7)	1.8	2.25	3.7
18:1(n-9)*cis*	19.4	15.1	14.05
Σ MUFA[3]	21.4	17.6	18.25
18:2(n-6)*cis*	24.1	17.1	10.35
18:3(n-3)	0.7	0.7	0.85
20:4(n-6)	0.5	0.5	0.65
20:5(n-3)	<0.1	1.05	3.0
22:5(n-3)	<0.1	0.2	0.5
22:6(n-3)	<0.1	0.95	2.65
Σ PUFA[4]	25.5	21.1	19.4
Σ (n-6)[5]	24.7	17.8	11.2
Σ (n-3)[6]	0.8	3.3	8.2
(n-6):(n-3)	31:1	5.4:1	1.4:1
DBI[7]	52.3	50.3	59.3

[1] Analysis performed by Woodson-Tenent Laboratories (Des Moines, IA).

[2] Sum of the saturated fatty acids: 8:0 + 10:0 + 11:0 + 12:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0 + 22:0 + 24:0.

[3] Sum of the monounsaturated fatty acids: 14:1 + 15:1 + 16:1(n-7) + 17:1 + 18:1(n-9)*cis* + 18:1(n-7) + 18:1(n-9)*trans* + 20:1(n-9) + 22:1(n-9) + 24:1.

[4] Sum of the polyunsaturated fatty acids: 18:2(n-6)*trans* + 18:2(n-6)*cis* + 18:3(n-6) + 18:3(n-3)*cis* + 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).

[5] Sum of the (n-6) fatty acids.

[6] Sum of the (n-3) fatty acids.

[7] DBI (double-bond index) = 2[18:2 (n-6) + 20:2 (n-6)] + 3[18:3 (n-3) + 20:3 (n-6)] + 4[20:4 (n-6)] + 5[20:5 (n-3) + 22:5 (n-3)] + 6[22:6 (n-3)], where the concentration of each fatty acid is expressed as g/100 g fatty acids.

all her food in the allotted 30-min feeding period. Her weight loss was 13.7%. The values for this dog were included in the analyses because they were within the ranges observed for the other dogs.

Blood samples were collected into evacuated tubes containing EDTA (final concentration 1 g/L) after food was deprived for 24 h for in vitro immunologic and biochemical measurements before beginning the study and at 8 wk. The feeding trial was continued for an additional 4 wk during which time in vivo immune response studies were performed.

Quantitation of PGE2 from stimulated mononuclear cells. Peripheral blood mononuclear cells were isolated according to the methods of Coligan et al. (1992) and Krakowka et al. (1987). Briefly, cells were separated from a 1:1 dilution of blood and Dulbecco's phosphate-buffered saline (DPBS; Sigma Chemical, St. Louis, MO) by layering the blood-DPBS mixture over Histopaque 1077 (Sigma) and centrifuging 30 min at 900 × *g*. Cells were washed twice in Hanks' balanced salt solution (HBSS, Sigma) and resuspended in RPMI 1640 supplemented with 100,000 U/L penicillin, 100 mg/L streptomycin, 2 mmol/L L-glutamine and 10% fetal calf serum (Sigma) to the original blood volume. An aliquot of the cell suspension was used to determine cell numbers with a hemocytometer, and cell viability was assessed by trypan blue exclusion. The remaining mononuclear cells were centrifuged for 10 min at 400 × *g* and resuspended in RPMI 1640 containing 5% fetal calf serum for a final concentration of 2×10^9 cells/L.

Mononuclear cells (5 mL of 2×10^9 cells/L cell suspension) were transferred to 25-mL tissue culture flasks (Corning, Corning, NY) and incubated for 4 h at 37°C in an atmosphere of 5% CO_2. Nonadherent cells were decanted and the adherent cells were washed twice with 5 mL RPMI 1640 (no fetal calf serum) to remove any residual nonadherent cells. Five milliliters of RPMI 1640 (5% fetal calf serum) containing 30 mg/L lipopolysaccharide (*E. coli* 055:B5; Sigma) was added to the adherent cells, which were then incubated for 40 h at 37°C and 5% CO_2. Previous timed-incubation studies showed maximal production of PGE_2 at 40 h. The supernatant was clarified by centrifugation for 10 min at 1000 × g and filtered (0.45-μm filter). Cell-free supernatant was stored at −70°C for subsequent PGE_2 analysis. A flask containing medium only was processed identically and supernatant was harvested for use as a control. PGE_2 concentration was determined by Ken Allen, Department of Food Science and Human Nutrition, Colorado State University, using RIA (Bottje et al. 1993).

Delayed-type hypersensitivity skin test. A DTH skin test was performed at 10.5 wk as described below. 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 intramuscularly (500 μg of KLH emulsified in 1.0 mg of T1501 adjuvant for a total volume of 0.5 mL) on d 60 after the initiation of the feeding. The KLH and adjuvant were combined in an oil-water emulsion as described by Woodard (1989), except that the ingredients were sonicated rather than ground. Briefly, 1.0 g/L KLH, 50 mL/L hexadecane, 35 mL/L Tween 80, 15 mL/L Span 80 and 2 mL/L T1501 were emulsified and added to normal saline solution. Fourteen days later (d 74), a second 0.5-mL intramuscular injection was given. One day later (d 75), the intradermal skin 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 KLH; saline (0.9%), the negative control; or histamine base (0.1 g/L), a positive control for the immediate skin test reaction of a sensitive dog when challenged with an offending antigen (Histatrol, Center Laboratories, Port Washington, NY). 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 ~3 mg of KLH. The KLH was heat aggregated according to the method of Exon et al. (1990). Briefly, 120 mg soluble KLH (Calbiochem-Novabiochem, La Jolla, CA) dissolved in 6 mL normal saline solution was heat aggregated in an 80°C water bath for 1 h. The resultant gel was centrifuged twice at 400 × *g* for 10 min, removing the saline 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 the dogs. Examinations were made at 15 and 30 min, at 24, 48, 72 and 96 h, and at 7, 10 and 14 d after intradermal injections. Reactions were recorded according to the diameter of induration and 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 control was observed, the diameter of its induration was subtracted from the other positive reactions. However, by 24 h, no reactions to the saline control were noted. Histamine produced an induration typically 20 mm larger than the saline control at 15 min, 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 by the same person to all dogs.

Keyhole limpet hemocyanin antibody titer. Humoral immune response was measured as antibody response to KLH. Dogs were injected intramuscularly with 0.5 mL KLH vaccine, as described above, on d 60 and 74. Serum was collected for KLH antibody titer on d 89. Humoral immune response for KLH was measured by a modification of an indirect ELISA procedure previously described by Woodard (1989). Briefly, enzyme immunoassay microtitration plates (ICN Biomedicals, Horsham, PA) were coated with 0.1 mL of DPBS (pH 7.4; 0.01 mol/L) containing 5 mg/L of KLH, covered with parafilm to prevent evaporation, and refrigerated overnight or until needed. Before use, plates were inverted to remove excess coating buffer and washed three times with DPBS containing 0.05% Tween 20. Serum samples (in quadruplicate) were then placed into wells and serial 1:2 dilutions made. 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 h at 37°C while rotating on a platform at 120 rpm. Plates were washed three times with DPBS-Tween 20 to remove unbound antibody. Antibody against dog IgG was conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) and diluted 1:5000 with DPBS-Tween 20. Subsequently, 0.1 mL was added to each well for a 1-h incubation at 37°C. Plates were washed three times with DPBS-Tween 20. Phosphate substrate (*p*-nitrophenyl phosphate disodium · $6H_2O$; 1 g/L; Sigma) was then added to each well (0.15 mL/well). After addition of substrate, plates were allowed to incubate at room temperature until the mean absorbance of the positive control equaled 1.0. The positive control was serum from a dog vaccinated in a previous experiment. Because not all plates developed color at the same rate, a positive control serum 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 an MR 700 spectrophotometer (Dynatech Laboratories, Alexandria, VA). The results are expressed as mean absorbance at 1:8000 and 1:16,000 dilutions of the serum.

Others. Plasma and urine thiobarbituric reactive substances (TBARS) were measured as previously discussed (Wander et al. 1996b). The fatty acid profile of the plasma was measured by gas chromatography as previously described (Song and Wander 1991) using heptadecanoic acid as an internal standard. The concentration of the fatty acids was expressed as g/100 g fatty acids (Meydani et al. 1993). Double-bond index (DBI) was calculated in the following way: DBI = 2[18:2(n-6) + 20:2(n-6)] + 3[18:3(n-3) + 20:3(n-6)] + 4[20:4(n-6)] + 5[20:5(n-3) + 22:5(n-3)] + 6[22:6(n-3)].

Plasma cholesterol, triglyceride and high density lipoprotein cholesterol concentrations were determined by methods previously discussed (Wander et al. 1996a). The total lipid content of plasma was described as the sum of the cholesterol and triglyceride concentrations of plasma (Thurnham et al. 1986). The α-tocopherol concentration in plasma was measured simultaneously by HPLC using a fluorometric detector (Wander et al. 1996a) and expressed as μmol/L, μmol/mmol lipid, and μmol/(L · DBI). Urine creatinine was measured by the Jaffe reaction (Wander 1996b). Urine creatinine was used as an internal reference to normalize the concentration of urine TBARS.

Statistical analysis. Data are reported as means ± SEM. A one-factor ANOVA was used to determine significant differences (Cochran and Cox 1957). For one variable (PGE_2), the data were blocked on weight and a 2-factor ANOVA was used. If a significant difference was found, an LSD post-hoc test (Cochran and Cox 1957) was used to determine the cause of the significant difference. Values were

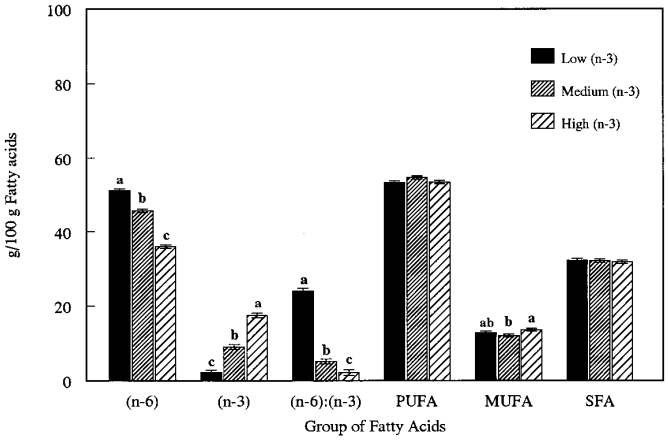

FIGURE 1 Effect of diets of different (n-3) fatty acid concentrations on the concentration of fatty acids in plasma of geriatric Beagle dogs. Each bar represents the fatty acid concentration after the dogs had consumed the respective diets for 8 wk. Values are means ± SEM, $n = 6$ or 7. Significant differences were established using a one-factor ANOVA followed by a LSD post-hoc test. Within a group of fatty acids, bars with different letters above them are significantly different ($P \leq 0.05$). Abbreviations used: (n-6), sum of (n-6) fatty acids; (n-3), sum of (n-3) fatty acids; PUFA, sum of polyunsaturated fatty acids; MUFA, sum of monounsaturated fatty acids; SFA, sum of saturated fatty acids.

considered significantly different at $P \leq 0.10$. The data were evaluated using the SAS (Version 6.1, SAS Institute, Cary, NC) General Linear Models procedures. All data were normally distributed about the mean and variances were equal. For all measurements, the following number of dogs comprised each group: 6, 7 and 7 for high, medium and low, respectively, (n-6) to (n-3) fatty acid ratios unless otherwise indicated.

RESULTS

The fatty acid composition of the plasma of the dogs before the study began was the same (data not shown) except for 22:5(n-3) (docosapentaenoic acid). There was enough variation in initial levels of this one fatty acid that the value measured in the group that was to be supplemented at the medium level of (n-3) fatty acids (1.00 ± 0.04 g/100 g fatty acids) was significantly lower than the values measured in the groups to be supplemented at the low (1.23 ± 0.05 g/100 g fatty acids) or high (1.13 ± 0.04 g/100 g fatty acids) levels ($P = 0.008$).

Supplementation of the diet with fish oil produced dramatic changes in the fatty acid profile (**Fig. 1**). The sum of the (n-3) fatty acids increased in a dose-response fashion among the three supplemented groups ($P = 0.0001$). These differences were accompanied by significant differences in the sum of the (n-6) fatty acids among the three groups ($P = 0.0001$), which occurred primarily because of lower levels for linoleic acid and AA in the dogs given fish oil. The linoleic acid level was similar in the low and medium groups (28.67 ± 0.70 and 28.22 ± 0.65 g/100 g fatty acids, respectively), but was 21.60 ± 0.65 g/100 g fatty acids in the high group. AA concentration was 21.32 ± 0.63, 16.55 ± 0.59 and 13.76 ± 0.59 g/100 g fatty acids in the low, medium and high groups, respectively; all means differed significantly.

The concentration of saturated and polyunsaturated fatty acids (PUFA) did not differ significantly among the three groups (Fig. 1). There was a minor but significant difference among the three groups in concentration of monounsaturated fatty acids ($P = 0.04$); the concentration measured in the high group was significantly greater than that measured in the medium group ($P = 0.01$), but not significantly different than the value measured in the group given no fish oil.

Initially, the concentration of cholesterol, triglycerides and HDL cholesterol did not differ among the three groups (data not shown). After two of the groups consumed the fish oil–containing diets, the concentration of plasma cholesterol tended to differ among the three groups ($P = 0.06$) (**Fig. 2**). The level measured in the high group was significantly lower than the level measured in the low group ($P = 0.02$) but did not differ significantly from the level measured in the medium group. The concentration of HDL cholesterol was significantly different among the three groups ($P = 0.04$) and decreased as the intake of (n-3) fatty acids increased. Despite the fact that triglyceride concentration in plasma has been shown on numerous occasions to be lowered by the consumption of fish oil in humans (Harris 1996), the concentration in the Beagles was not affected (Fig. 2).

There was no significant difference in the plasma concentration of α-tocopherol among all dogs before supplementation occurred. After consumption of the diets, the presence of significant differences depended upon the units used to express tocopherol concentration. Plasma concentration of α-tocopherol was lower in dogs fed the high (n-3) diet compared with those fed the low or medium (n-3) diet ($P = 0.04$) (**Fig. 3**). Plasma concentration of α-tocopherol was also expressed as a function of plasma total cholesterol and triglyceride, and as a function of DBI. No significant difference was noted in α-tocopherol concentration for dogs fed the low, medium and high (n-3) diets (16.0 ± 1.5, 16.9 ± 1.4, and 18.1 ± 1.4 μmol/mmol lipid, respectively). When plasma concentration of α-tocopherol was expressed relative to the DBI, significant differences were noted among the three diet groups ($P = 0.002$). The value measured when the high (n-3) diet was consumed (0.346 ± 0.030 μmol/L · DBI) was significantly lower than that measured when the low (0.525 ± 0.032 μmol/L · DBI; $P = 0.0007$) or medium (n-3) diet was consumed (0.460 ± 0.030 μmol/L · DBI; $P = 0.01$). The values measured when the low and medium (n-3) diets were consumed did not differ.

Initially, the level of lipid peroxidation in plasma and urine did not differ among the groups and averaged 1.50 ± 0.12 μmol TBARS/L plasma and 1.12 ± 0.07 nmol TBARS/μmol creatinine in urine. Consumption of the fish oil diets increased

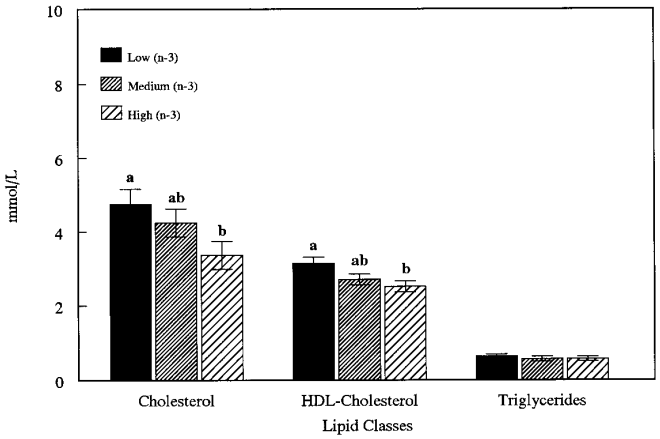

FIGURE 2 Effect of diets of different (n-3) fatty acid concentrations on lipid classes in plasma of geriatric Beagle dogs. Each bar represents the concentration after the dogs had consumed the respective diets for 8 wk. Values are means ± SEM, $n = 6$ or 7. Significant differences were established using a one-factor ANOVA followed by a LSD post-hoc test. Within a lipid class, bars with different letters above them are significantly different ($P \leq 0.05$).

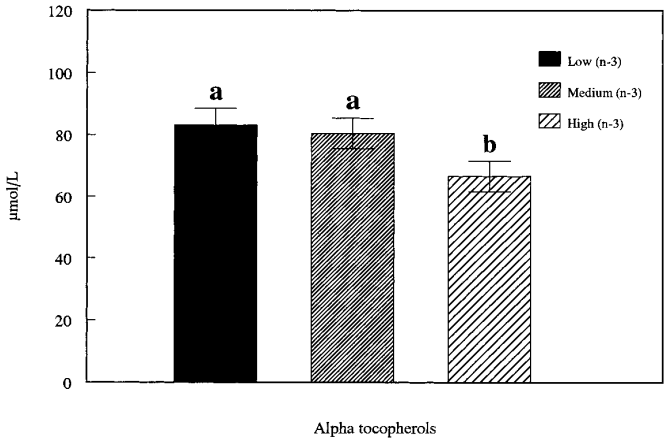

FIGURE 3 Effect of diets of different (n-3) fatty acid concentrations on plasma α-tocopherol concentration of plasma in geriatric Beagle dogs. Each bar represents the concentration after the dogs had consumed the respective diets for 8 wk. Values are means ± SEM, n = 6 or 7. Significant differences were established using a one-factor ANOVA followed by a LSD post-hoc test. Bars with different letters above them are significantly different ($P \leq 0.05$).

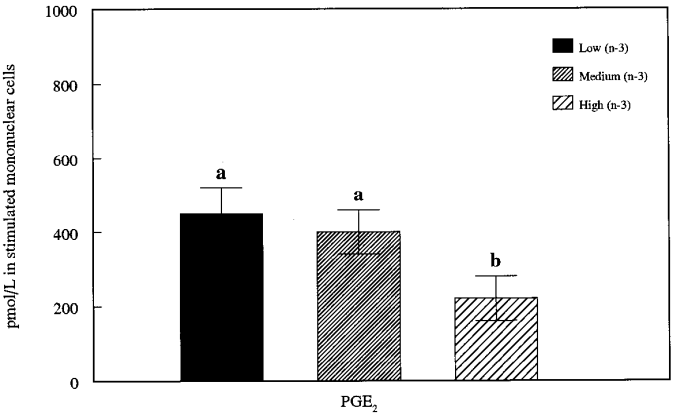

FIGURE 5 Effect of diets of different (n-3) fatty acid concentrations on the concentration of prostaglandin E_2 (PGE_2) in stimulated mononuclear cells of geriatric Beagle dogs. Each bar represents the concentration after the dogs had consumed the respective diets for 8 wk. Values are means ± SEM, n = 6 or 7. Significant differences were established using a two-factor ANOVA followed by a LSD post-hoc test. Bars with different letters above them are significantly different ($P \leq 0.07$).

lipid peroxidation in both plasma ($P = 0.03$) and urine ($P = 0.002$) (**Fig. 4**). In plasma, only the diet containing the greatest amount of (n-3) fatty acids increased lipid peroxidation. In urine, on the other hand, both medium and high dietary levels of (n-3) fatty acids increased TBARS. Urinary TBARS were about 60% greater in the groups consuming the high and medium (n-3) diets compared with the group consuming the low (n-3) diet ($P = 0.002$).

The concentration of PGE_2 in stimulated mononuclear cells was influenced by the level of (n-3) fatty acids in the diet. Initially, the concentration of PGE_2 did not differ among the dogs (data not shown). However, after consumption of the fish oil diets, the concentration differed (**Fig. 5**) ($P = 0.07$). Because cell numbers were not determined after cell purification by adhesion, it remains uncertain whether the difference in PGE_2 was due to a true difference in PGE_2 production as a result of (n-3) fatty acid feeding, or simply a difference in adhering properties resulting from (n-3) fatty acid feeding. The concentration of PGE_2 tended to decrease as the fish oil concentration of the diet increased. The difference was significant between the low and high (n-3) diet groups ($P = 0.04$). The concentration measured in the high (n-3) group was different than the value measured in the medium (n-3) group ($P = 0.07$).

Feeding an enriched (n-3) diet significantly suppressed the cell-mediated immune response based on DTH test results. The diameter of induration at 24 h was significantly smaller in the group consuming the high (n-3) diet compared with the groups consuming the medium ($P = 0.02$) or low ($P = 0.04$) (n-3) diets (**Fig. 6**). The diameter of induration at 48 h was also significantly smaller in the group consuming the

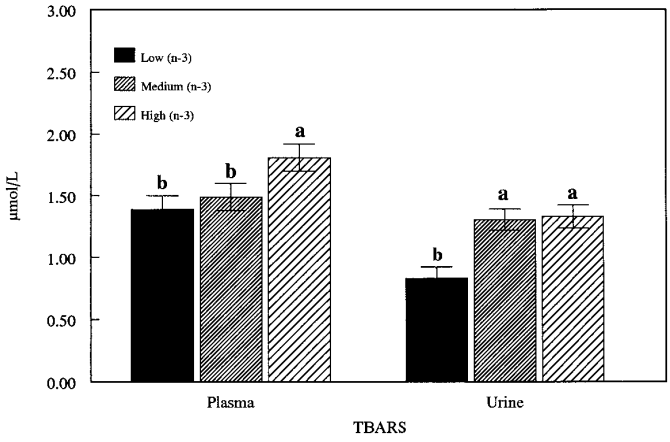

FIGURE 4 Effect of diets of different (n-3) fatty acid concentrations on lipid peroxidation in plasma and urine measured by the concentration of thiobarbituric acid reactive substances (TBARS) in geriatric Beagle dogs. Each bar represents the concentrations in plasma or urine after the dogs had consumed the respective diets for 8 wk. Values are means ± SEM, n = 6 or 7. Significant differences were established using a one-factor ANOVA followed by a LSD post-hoc test. Within a group of TBARS, bars with different letters above them are significantly different ($P \leq 0.05$).

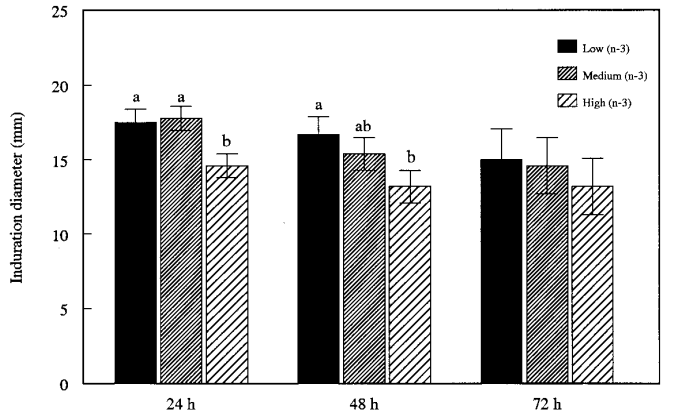

FIGURE 6 Effect of diets of different (n-3) fatty acid concentration on the delayed-type hypersensitivity (DTH) skin test in geriatric Beagle dogs challenged with an intradermal injection of keyhole limpet hemocyanin (KLH) to which they had been previously sensitized. Each bar represents the induration diameter after the dogs had consumed their respective diets for 75 d. Values are means ± SEM, n = 6 or 7. Significant differences were established using a one-factor ANOVA followed by a LSD post-hoc test. Bars with different letters above them are significantly different ($P \leq 0.05$).

high (n-3) diet compared with the group consuming the low (n-3) diet ($P = 0.05$). After 72 h, no significant differences were noted.

The humoral immune response to KLH was not significantly altered by supplementation with (n-3) fatty acids. Antibody production to KLH, determined at serum dilutions of 1:8000 (mean absorbance at dual wavelength of 405 and 492 nm ranged from 0.97 ± 0.16 in the medium group to 1.15 ± 0.16 in the high group) and 1:16,000 (mean absorbance at dual wavelength of 405 and 492 nm ranged from 0.52 ± 0.10 in the medium group to 0.72 ± 0.11 in the low group) did not differ among the three groups.

DISCUSSION

Animal and human studies suggest that supplementation with fish oil, an excellent source of (n-3) fatty acids, has beneficial effects on atherosclerotic and atherothrombotic disorders as well as on autoimmune and inflammatory diseases such as arthritis and colitis (Kremer et al. 1987, Kromhout et al. 1985, Meydani and Dinarello 1993, Stenson et al. 1992). Although these effects are positive, fish oil supplementation may also have negative effects such as increased lipid peroxidation (Meydani, M. et al. 1991, Wander et al. 1996b). The amount of (n-3) fatty acids or the best ratio of (n-6) to (n-3) fatty acids in the diet that would be necessary to maximize benefits and minimize negative effects has not been established.

The purpose of this study was to examine both sides of this issue by feeding diets supplemented with different ratios of (n-6) to (n-3) fatty acids to an older population of animals. Aged dogs were studied because immune system responsiveness gradually declines with age (Kay 1978) and lipid peroxidation may be more pronounced in this population (Harman 1982). In addition, there is a higher incorporation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into plasma lipids of older women (Meydani, S. et al. 1991) and older male rats (Suzuki et al. 1985) after fish oil consumption. Our results demonstrate that feeding a diet with a high content of (n-3) fatty acids, such that the (n-6) to (n-3) fatty acid ratio is 1.4:1, has significant effects on several of these variables in healthy female geriatric Beagle dogs.

Plasma cholesterol and HDL cholesterol were lower in dogs fed the diet high in (n-3) fatty acids compared with those receiving the diet low in (n-3) fatty acids. The lack of a hypotriglyceridemic effect in the dogs in our study is consistent with previous studies in dogs (Harris 1996).

The T-cell mediated immune response, as indicated by the DTH skin response, was suppressed, whereas the humoral immune response was unaffected by high dietary (n-3) fatty acids. The reduced DTH skin response in dogs with a high intake of (n-3) fatty acids is consistent with previously reported data. Meydani et al. (1993) observed a decrease in the induration index in normolipidemic humans consuming a low fat, high fish diet for 24 wk compared with when they consumed a low fat, low fish diet. Yoshino and Ellis (1987) showed that rats fed fish oil concentrate had lower DTH responses than those fed water, oleic acid or safflower oil. The mechanism underlying the change in the DTH response is not clear. Meydani et al. (1993) showed that production of cytokines interleukin (IL)-1β, tumor necrosis factor and IL-6 by mononuclear cells was significantly lower in humans after consumption of a low fat, high fish diet compared with a low fat, low fish diet. The decrease in cytokine production may contribute to decreased antigen-presenting cell activity and thus, a decrease in DTH response. An effective immune response may also be blunted following (n-3) fatty acid consumption because dietary fats influence major histocompatibility class II (Ia) antigen expression on cellular membranes, and thus T-lymphocyte proliferation (Huang et al. 1992).

Accompanying the decrease in DTH response was a decrease in PGE_2 production by stimulated mononuclear cells from dogs with a high intake of (n-3) fatty acids. Decreased PGE_2 production has previously been demonstrated in response to increased (n-3) fatty acid intake. Wu et al. (1996) showed that diets enriched in (n-3) PUFA reduced the production of PGE_2 by peripheral blood mononuclear cells from nonhuman primates in response to concanavalin A or phytohemagglutinin stimulation by >90%.

PGE_2 production is also affected by aging, perhaps because of increased activity of cyclooxygenase (Hayek et al. 1994). Peripheral blood mononuclear cells from healthy elderly subjects synthesized significantly more PGE_2 than did those from young subjects (Meydani et al. 1990). Increased PGE_2 production has also been demonstrated in aged mice (Meydani et al. 1986). The combination of increased (n-3) fatty acid intake and aging has pronounced effects on PGE_2 production. Meydani, S. et al. (1991) showed that the decrease in PGE_2 production after (n-3) fatty acid supplementation was more dramatic with increasing age.

It is tempting to speculate that the effects of fish oil observed in the dogs fed diets with high levels of (n-3) fatty acids resulted from a decrease in the production of PGE_2, as has been suggested previously (Meydani and Dinarello 1993). Eicosanoids regulate the production of several cytokines. Therefore, if an increase in (n-3) PUFA intake altered eicosanoid production, then it would also be expected to affect cytokine production and hence biologic function (Meydani and Dinarello 1993). Because cells of the immune system are the main source and major target for cytokines, changing cytokine production could have profound consequences on the immune response. However, it is generally agreed that an increase, not a decrease, in the concentration of PGE_2 suppresses T cell-mediated function (Meydani 1995). In this study, the decrease in both PGE_2 concentration and the DTH response suggests that the suppressive effect of diets enriched in (n-3) PUFA was independent of the decrease in PGE_2 production.

Even though the dogs receiving the high (n-3) fatty acid diets had α-tocopherol concentrations within the published normal range for Beagle dogs (58.05 ± 13.93 μmol/L; range 42.72–75.00 μmol/L) (Pillai et al. 1993), the depressed DTH response may be related to plasma vitamin E concentrations in our dogs. A deficiency in vitamin E intake has been shown to suppress the immune response in species ranging from rodents to humans (Meydani 1995). Some studies (Alexander et al. 1995) but not necessarily all (Wander et al. 1996a) have shown that high intakes of (n-3) fatty acids lower plasma concentrations of α-tocopherol. Consequently, an increased concentration of α-tocopherol may be required when diets high in (n-3) fatty acids are consumed (Muggli 1989). In the present study, α-tocopherol concentration in each diet was similar and exceeded the calculated requirements by six to eight times, according to the formula described by Muggli (1989). Despite this, plasma α-tocopherol concentration was significantly lower in dogs fed the high (n-3) fatty acid diet when the data are expressed on a molar basis. Further, this effect prevailed if the α-tocopherol concentrations are expressed relative to the DBI, which is perhaps a better indication of vitamin E status when long-chain PUFA are consumed. Therefore, the suppressed DTH response in this study may be related to changes in the vitamin E status of the dogs.

An alternative explanation for the decrease in the immune reactivity lies in the increased level of lipid peroxidation.

Plasma and urine lipid peroxide concentrations increased in this study in dogs with a high intake of (n-3) fatty acids. Meydani, M. et al. (1991) showed that supplementation of young and older women with (n-3) fatty acids increased plasma malondialdehyde levels, and that this increase was greater in the older women than in the younger women. Wander et al. (1996b) measured an increase in urine TBARS and an increase in the plasma and urine concentrations of the specific thiobarbituric acid–malondialdehyde adduct in postmenopausal women given a fish oil supplement. Although the impact of lipid hydroperoxides may stem from their effect on the activity of cyclooxygenase and thus the production of PGE_2 (Warso and Lands 1983), their effect may also be independent of this modulatory activity. Zoschke and Messner (1984) have shown that human lymphocyte mitogenesis was suppressed by lipid peroxidation products. A rise in lipid peroxide level induced by (n-3) fatty acids could also have contributed to the decrease in DTH skin test responses noted by Meydani et al. (1993). Therefore, the decrease in cell-medicated immunity observed in this study may be the result of an increase in the formation of lipid peroxidation products following (n-3) fatty acid supplementation. Although increased production of lipid peroxidation products in the group consuming high (n-3) might be related to decreasing α-tocopherol concentration in the plasma, α-tocopherol may not be the only factor protecting against oxidative stress.

The humoral immune response, demonstrated by the production of antibodies to a foreign protein, KLH, was not significantly affected by the ratio of (n-3) to (n-6) fatty acids in the diet. This observation does not agree with previous work. Virella et al. (1989), studying human peripheral blood mononuclear cell cultures, found that B cell immunoglobulin production in response to pokeweed mitogen in vitro was depressed by the addition of EPA. In the same study, B cell function assessed by measuring circulating immunoglobulin levels in response to tetanus toxoid was depressed during ingestion of fish oil. The latter study was performed on only one healthy human volunteer whose age was not disclosed. We do not know what effect, if any, age had on the B cell response in this study. Primary antibody response decreases in many species, including dogs, with age (Jaroslow et al. 1974).

In summary, we have shown that consumption of a diet enriched in (n-3) fatty acids reduced DTH skin response, changed the plasma lipid profile, and decreased the production of PGE_2 and plasma concentration of α-tocopherol while increasing lipid peroxidation in geriatric Beagle dogs. Further studies are required to define the appropriate level of α-tocopherol to be consumed when (n-3) fatty acid supplementation occurs and the possible effect of lipid peroxidation products on immune function.

ACKNOWLEDGMENT

The authors express their appreciation for the technical assistance with the PGE_2 assay provided by Ken G. Allen, Department of Food Science and Human Nutrition, Colorado State University.

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