Dietary (n-3) Polyunsaturated Fatty Acids Suppress Murine Lymphoproliferation, Interleukin-2 Secretion, and the Formation of Diacylglycerol and Ceramide[1,2,3]

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ABSTRACT Elucidation of the mechanism(s) by which dietary fish oil, enriched in eicosapentaenoic acid [EPA, 20:5(n-3)] and docosahexaenoic acid [DHA, 22:6(n-3)], suppresses the inflammatory process is essential in maximizing this potentially therapeutic effect. Murine T-lymphocyte function and signal transduction were examined in response to a low fat, short term diet enriched in highly purified EPA or DHA ethyl esters. For 10 d, mice were fed comparable diets containing either 3% safflower oil ethyl esters (SAF), 2% SAF + 1% arachidonic acid triglyceride (AA), 2% SAF + 1% EPA, or 2% SAF + 1% DHA. Concanavalin A-induced T-lymphocyte proliferation in splenocyte cultures was significantly suppressed by dietary EPA and DHA while AA had no effect relative to the SAF control. The suppressed proliferative response in EPA- and DHA-fed mice was preceded temporally by a significant reduction in IL-2 secretion. Kinetics of mitogen-induced diacyl-*sn*-glycerol (DAG) and ceramide production did not differ significantly between SAF and AA diet groups. In contrast, DAG production was significantly suppressed in EP- and DHA-fed mice relative to the SAF and AA groups. The reduced DAG mass was paralleled by reduced ceramide mass following EPA and DHA feeding compared to the SAF and AA groups. Thus, low dose, short term dietary exposure to highly purified EPA or DHA appears to suppress mitogen-induced T-lymphocyte proliferation by inhibiting IL-2 secretion, and these events are accompanied by reductions in the production of essential lipid second messengers, DAG and ceramide. J. Nutr. 127: 37–43, 1997.

KEY WORDS: *ceramide* • *diacylglycerol* • *(n-3) polyunsaturated fatty acids* • *T-cell* • *mice*

Decreased incidence of inflammatory diseases in Greenland Eskimos and Japanese people (Simopoulos 1991) has been attributed to their larger consumption of cold-water marine fish, enriched in eicosapentaenoic acid [EPA[5] 20:5(n-3)] and docosahexaenoic acid [DHA, 22:6(n-3)], relative to the Western diet. Clinical trials involving rheumatoid arthritis (RA) patients have shown that dietary fish oil supplementation has a therapeutic, anti-inflammatory effect (Endres et al. 1995, Kremer 1991). Experiments in rodent models of RA have also demonstrated the beneficial effects of fish oil supplementation (Morrow et al. 1993).

The T-lymphocyte is responsible for propagating both humoral and cell-mediated immunity via proliferation and subsequent secretion of a plethora of lymphokines, including interleukin-2 (IL-2), IL-4, IL-5, IL-6, etc. Dietary fish oil supplementation suppresses the T-lymphocyte proliferative response in vitro of cells from healthy humans, associated with decreased production of IL-2, a potent autocrine and paracrine T-lymphocyte growth factor (Endres et al. 1993). Most of the supplementation studies have employed large amounts of fish oil taken daily in fish oil capsules. However, another human study has shown that the incorporation of fish in the diet suppresses the delayed-type hypersensitivity (DTH) response, a T-lymphocyte dependent phenomenon, and T-lymphocyte proliferative response in vitro (Meydani et al. 1993). That study demonstrated that the anti-inflammatory properties of fish oil can be realized directly by increased fish consumption. Suppressed mitogen-induced T-lymphocyte proliferation was also observed in fish oil-fed rats (Vallette et al. 1991) and mice (Shapiro et al. 1994).

It is essential that the mechanism(s) by which dietary fish oil suppresses the immune response be elucidated in order to maximize the potential therapeutic anti-inflammatory effects without compromising important antimicrobial or antitumor immune system functions. One possible mechanism is that dietary fish oil suppresses prostaglandin (PG) formation. Dietary EPA and DHA suppress arachidonic acid- [AA, 20:4(n-6)] derived PGE_2 production (Surette et al. 1995). However, PGE_2 is itself an anti-proliferative agent for T-lymphocytes (Hwang

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[5] Abbreviations used: AA, arachidonic acid; Con A, concanavalin A; DAG, diacyl-*sn*-glycerol; DHA, docosahexaenoic acid; DTH, delayed-type hypersensitivity; EPA, eicosapentaenoic acid; IL, interleukin; PC, phosphatidylcholine; PG, prostaglandin; PI, phosphatidylinositol; PIP_2, phosphatidylinositol-4,5-bisphosphate; RA, rheumatoid arthritis; SAF, safflower oil ethyl esters; SM, sphingomyelin.

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1989); therefore, this putative mechanism is not consistent with the suppressed T-lymphocyte proliferation observed with increased dietary EPA and DHA. Another possible explanation is that EPA and DHA influence the generation of intracellular lipid second messengers. Diacylglycerol (DAG), the physiological activator of protein kinase C, and ceramide, a novel intracellular second messenger, appear to promote T-lymphocyte proliferation (Mathias et al. 1993, Szamel and Resch 1995). DAG is produced by the receptor-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) and phosphatidylcholine (PC) while ceramide is derived from sphingomyelin (SM). We have previously demonstrated that DAG is produced in a multiphasic fashion, and that ceramide exhibits a single, transient, prolonged peak in murine T-lymphocytes stimulated with concanavalin A (Con A) (Jolly et al. 1996), indicating that both DAG and ceramide play important roles in T-lymphocyte proliferation in our model system. Dietary cod liver oil, enriched in EPA and DHA, modifies the fatty acid composition of phosphatidylinositol (PI), PC and SM (Ahmed and Holub 1984). This could affect DAG and ceramide function by altering the hydrolysis of PI, PC and/or SM, yielding lower intracellular DAG and/or ceramide mass and/or influencing effector functions. Alternatively, dietary polyunsaturated fatty acids could modify lymphocyte membrane fluidity resulting in altered receptor function that could influence the generation of intracellular second messengers (Hagve 1988). Furthermore, dietary fish oil could modify the relative proportion of CD4+ or CD8+ T-cells. While fish oil has been shown to alter total spleen cell yield in mice (Fritsche and Johnston 1990), feeding purified EPA (DHA was not tested) to humans resulted in no difference in helper or suppressor T-cells (Payan et al. 1986). The later observation is in agreement with the study showing no change in blood leucocytes in rats fed fish oil (Yaqoob et al. 1995).

We have already shown that both dietary EPA and DHA enhance the (n-3) fatty acid composition of cellular DAG in murine splenocytes (Hosack-Fowler et al. 1993a). Our data clearly demonstrated that both EPA and DHA suppress the DTH response (Hosack-Fowler et al. 1993b) paralleling observations in human studies (Meydani et al. 1993). Furthermore, both EPA and DHA were incorporated into purified splenic T-lymphocyte cellular lipids (Hosack-Fowler et al. 1993b) suggesting that these fatty acids can modulate T-lymphocyte function in our murine model. In this study we extend these observations by demonstrating that dietary EPA and DHA significantly reduce Con A-induced T-lymphocyte proliferation, in part by affecting IL-2 production, and that T-cell suppression is accompanied by significant reductions in T-cell DAG and ceramide mass.

MATERIALS AND METHODS

Diets and animals. All experimental procedures using laboratory animals were approved by the Institutional Animal Care Committee of Texas A&M University. Pathogen-free, female, 6-wk-old C57Bl/6 mice (Charles River Facility, Raleigh, NC) were assigned randomly to one of four diets following an acclimation period of one week. Mice were housed five per cage in polycarbonate microisolator cages, with free access to autoclaved water. The mice were maintained at a room temperature of approximately 25°C with a 12-h light:dark cycle. Mice were given free access to semi-purified diets in powdered form for 10 d. The purified diets met the National Research Council nutrition requirements and varied only in lipid composition. The basic composition of each diet, expressed in g/kg, was 200 *g* casein, 3 *g* DL-methionine, 440 *g* sucrose, 219.8 *g* corn starch, 60 *g* cellulose, 35 g AIN-76 mineral mix, 10 *g* AIN-76 vitamin mix, 2 *g* choline bitartrate, 0.2 g tertiary butyl hydroquinone antioxidant, and 30 *g* lipid. After each diet was mixed, it was analyzed by gas chromatography and stored at −80°C. The four diet groups varied by lipid source: 30 g/kg SAF (control), 20 g/kg SAF + 10 g/kg AA, 20 g/kg SAF + 10 g/kg EPA, and 20 g/kg SAF + 10 g/kg DHA. The AA diet served as an additional control to determine whether (n-3) fatty acid effects were due to EPA and/or DHA substitution or to reduced dietary linoleic acid [18:2(n-6)] content. The SAF, EPA and DHA were obtained in ethyl ester form, and the AA was provided in triglyceride form. All ethyl esters were obtained from the National Institutes of Health Test Materials Program (Charleston, SC), and the AA was from Martek (Columbia, MD). The EPA was 99% 20:5(n-3), the DHA was 97% 22:6(n-3), the SAF was 84% 18:2(n-6), and the AA was 51.4% 20:4(n-6).

Isolation and preparation of splenic lymphocytes. Mice were killed by CO_2 asphyxiation, and each spleen was placed in 3 mL of complete medium [RPMI 1640 with 25 mmol/L HEPES (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (FBS) (Irvine Scientific), 1×10^5 U/L penicillin and 100 mg/L streptomycin (Irvine Scientific), 2 mmol/L L-glutamine, and 10 mmol/L 2-mercaptoethanol]. Spleens were dispersed using glass homogenizers, passed through a wire mesh filter to obtain a single cell suspension that was washed twice with complete medium. The number of viable cells was determined by trypan blue exclusion in a hemocytometer (Hosack-Fowler et al. 1993b).

T-lymphocyte proliferation assay. Splenic lymphocytes were cultured in the presence of 5 mg/L of the T cell mitogenic lectin Con A (Sigma, St. Louis, MO) at 2×10^5 cells per well in 96-well flat bottomed microtiter plates (Falcon; Becton Dickenson, Lincoln Park, NJ), with triplicate wells for each diet treatment. Cells were incubated for 96 h at 37°C in a 5% CO_2 in air atmosphere. For the final 6 h, cells were incubated in the presence of 37 kBq [^{3}H]-thymidine/well (New England Nuclear, North Bellerica, MA). Cells were harvested onto glass fiber filter paper discs (Whatman, Maidstone, England) using a multiple automated sample harvester unit (MASH II; MA Bioproducts, Walkersville, MD). Cellular uptake of thymidine was quantified in a liquid scintillation counter (LS 8000, Beckman Instruments, Irvine, CA). Results are expressed as the mean net Bq (stimulated minus control) of triplicate cultures (Hosack-Fowler et al. 1993b).

Interleukin-2 quantitation. Splenic lymphocytes were isolated, cultured and stimulated as described above. At 48 h post Con A-addition, 100-μL aliquots of culture supernatant were harvested and stored at −80°C. In preliminary experiments, a 48 h incubation yielded maximal IL-2 secretion relative to the 96 h culture time used for proliferation measurements (data not shown). Culture supernatant was thawed, gently vortexed, and centrifuged for 1 min at $100 \times g$. An aliquot (50 μL) of the culture supernatant was analyzed quantitatively for IL-2 using a commercially available ELISA kit (Genzyme, Cambridge, MA). The results are expressed as the mean ± SEM ($n = 5$) in pg/200,000 cells from two independent diet studies.

T-lymphocyte CD4+ and CD8+ analysis. Splenic lymphocytes were isolated as described above. The cells were resuspended in 2 mL of ACK lysis buffer (0.15 mol/L NH_4Cl, 1 mmol/L $KHCO_3$, 0.1 mmol/L Na_2EDTA in ddH_2O, pH 7.4) and incubated on ice for 5 min to remove red blood cells. The cells were washed once, and cell viability was determined by trypan blue exclusion. Cells (1×10^6) were incubated for 10 min on ice with 1 mL blocking solution (Hank's PBS with 10% heat-inactivated horse serum). A 2 mL aliquot of staining buffer (Hank's PBS with 10% FBS and 0.1 g/L NaN_3) was used to wash the cells three times. Pelleted cells were resuspended in 300 μL of staining buffer to which 50 μL (1 μg) of either mAb CD4 Quantum Red conjugated or mAb CD8 Quantum Red conjugated (Sigma Immuno Chemicals) was added. Antibody concentrations were titrated in preliminary experiments to obtain maximal fluorescence in our cell system (data not shown). The antibody/cell mixtures were wrapped in aluminum foil on a table top shaker and incubated for 1 h on ice. The T cells were washed three times with 2 mL of staining buffer, resuspended in 1 mL staining buffer, and analyzed by FACScan (Coulter Corp., Hialeah, FL). Results are expressed as the mean ± SEM ($n = 4$) percentage of total splenocytes.

Diacylglycerol and ceramide quantitation. Splenic lymphocytes were resuspended at 5×10^9 cells/L in prewarmed (37°C) complete medium to which Con A was added at a final concentration of 10 mg/L. The cells were vortexed gently and incubated in a 37°C water bath. At 2, 5, 20, 45, 60, 120 and 180 min, the cell suspension was

gently vortexed, and 1-mL aliquots of cells were transferred to a 2.0-mL microcentrifuge tube. The cell aliquot for the time 0 value was obtained immediately prior to Con A addition. Lymphocyte cellular lipids were extracted, and DAG and ceramide were quantitated using the DAG kinase assay as previously described (Jolly et al. 1996).

Statistical analysis. The data were analyzed by one-way analysis of variance at each time point using SAS (SAS 1995). Also, within each diet group, the effect of time was evaluated using one-way analysis of variance. Differences between means were tested using Duncan's multiple range test. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of dietary lipid on mitogen-induced T-lymphocyte function. Mice were fed diets enriched in either SAF, AA, EPA or DHA for 10 d. There was no significant dietary effect on body weight at the end of the study (data not shown). **Figure 1** illustrates the effects of these diets on the murine splenic T-lymphocyte proliferative response to Con A. Mice fed AA did not show a significant difference ($P > 0.05$) in proliferation relative to the SAF (control) diet. In contrast, both EPA- and DHA-fed mice showed a significant ($P < 0.05$) reduction in the T-lymphocyte proliferative response relative to AA and SAF feeding.

IL-2 is a potent autocrine and paracrine polyclonal T-lymphocyte growth factor. To determine whether alterations in mitogen-induced T-lymphocyte IL-2 production might be responsible for the suppressive effects of dietary EPA and DHA on T-lymphocyte proliferation, IL-2 secretion in the culture supernatants was measured (**Fig. 2**). The effects of dietary lipid on IL-2 secretion paralleled those seen on the proliferative response. AA feeding had no effect ($P > 0.05$) on IL-2 secretion relative to SAF-fed mice. However, both EPA and DHA feeding significantly ($P < 0.05$) suppressed IL-2 secretion compared to both AA- and SAF-fed mice.

Diet effects on splenic T-lymphocyte subpopulations. Dietary EPA and DHA could affect Con A-induced T-lymphocyte IL-2 secretion and subsequent proliferation by altering the balance of T-lymphocyte subpopulations in the spleen. **Table 1** illustrates the effects of dietary lipid on the proportions of CD4+ and CD8+ T-lymphocytes in the murine spleen. There was no significant ($P > 0.05$) difference between the proportions of CD4+ T-lymphocytes in either the SAF, AA, EPA or DHA-fed mice as determined by flow cytometry. Furthermore, there were no significant ($P > 0.05$) differences in the percentage of CD8+ T-lymphocytes in response to SAF, AA or EPA feeding. However, DHA feeding significantly ($P < 0.05$) reduced the percentage of CD8+ T-lymphocytes compared to SAF- and EPA-fed mice.

Effect of diet on splenocyte DAG kinetics in response to Con A. DAG is an important intracellular lipid second messenger in T-lymphocyte proliferation (Asaoka et al. 1991) and is produced in a multiphasic fashion in Con A-activated murine splenic lymphocytes (Jolly et al. 1996). **Figure 3***A* shows the effects of dietary lipid on Con A-induced DAG kinetics. There was no significant difference in basal DAG mass ($t = 0$) between the four diet groups. Con A induced a significant ($P < 0.05$) increase in DAG mass at 2, 60, 120 and 180 min (33, 34.6, 29.4 and 29.2 pmol/10^6 cells, respectively) compared to basal levels (22.4 pmol/10^6 cells at $t = 0$) in SAF-fed mice. A slightly different kinetic pattern was observed in AA-fed mice in that Con A stimulation induced a significant ($P < 0.05$) increase in DAG mass at 2, 5, 20, 60, 120 and 180 min (31.5, 27.8, 32.5, 28.8, 34.5 and 35.5 pmol/10^6 cells, respectively) compared to basal levels (17.7 pmol/10^6 cells). There was no significant ($P > 0.05$) difference in mitogen-induced DAG mass between AA and SAF feeding except at 5 min, at which point DAG mass was significantly ($P < 0.05$) lower relative to SAF-fed mice. In sharp contrast, Con A-induced DAG mass was significantly ($P < 0.05$) suppressed at all the time points in DHA-fed mice compared to AA-fed mice. EPA feeding also resulted in a significant ($P < 0.05$) reduction in DAG mass at 2, 5, 20, 45, 120 and 180 min relative to AA feeding. Mitogen-induced DAG mass was significantly ($P < 0.05$) reduced at all time points in DHA-fed mice compared to SAF feeding, except at 5 min. Furthermore, EPA feeding significantly ($P < 0.05$) suppressed DAG mass at 2, 20, 45 and 180 min relative to SAF feeding. EPA and DHA-fed mice did not differ significantly ($P > 0.05$) from each other in Con A-induced DAG mass at any of the time points measured.

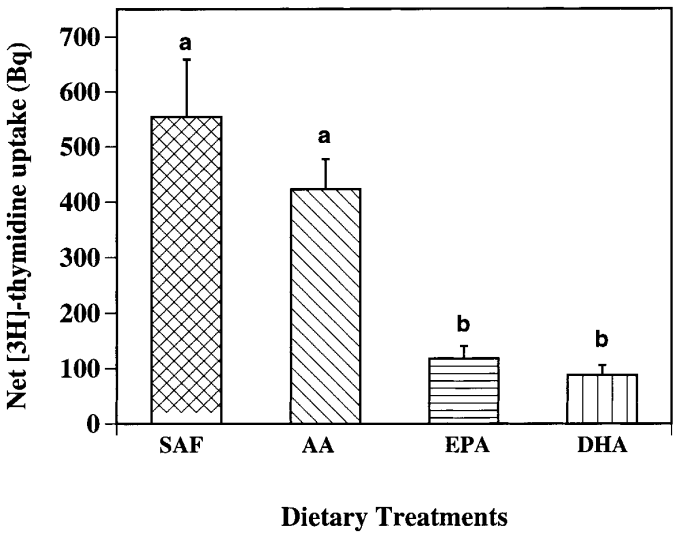

FIGURE 1 Dietary eicosapentaenoic acid and docosahexaenoic acid suppress concanavalin A (Con A)-induced lymphoproliferation in murine splenocytes. Mice were fed diets enriched in safflower oil ester esters (SAF, containing linoleic acid), arachidonic acid triglyceride (AA), eicosapentaenoic acid ethyl ester (EPA) or docosahexaenoic acid ethyl ester (DHA) for 10 d. Splenic lymphocytes were isolated and stimulated with 5 mg/L Con A as described in Materials and Methods. Values ($n = 7$ mice) represent the mean ± SEM of net thymidine uptake from two independent diet studies. Different letters denote significant differences ($P < 0.05$).

Effect of diet on splenocyte ceramide kinetics in response to Con A. Ceramide is a novel intracellular lipid second messenger that plays a positive role in the T-lymphocyte proliferative response (Chan and Ochi 1995). Con A-activated murine splenic lymphocytes produced ceramide in a single prolonged transient peak (Jolly et al. 1996). Figure 3*B* illustrates the effects of dietary lipid on Con A-induced ceramide production in splenic lymphocyte cultures. Basal ($t = 0$) ceramide mass was not significantly ($P > 0.05$) different among the four diets. The AA-fed mice showed a significant increase ($P < 0.05$) in ceramide mass at 2 min (23.4 pmol/10^6 cells) compared to basal levels (11.4 pmol/10^6 cells) which remained significantly elevated for 180 min. A similar kinetic pattern was observed with SAF feeding in that Con A induced a significant ($P < 0.05$) increase in ceramide mass at 2 min (22.3 pmol/10^6 cells) relative to basal (15.0 pmol/10^6 cells), and remained significantly elevated through the end of the 180 min experiment. Ceramide mass did not significantly ($P > 0.05$) differ between SAF- and AA-fed mice or between EPA- and DHA-fed mice at any of the time points measured. In contrast, Con A did not induce an increase in ceramide mass in EPA- or DHA-fed mice. Both EPA and DHA feeding were associated with

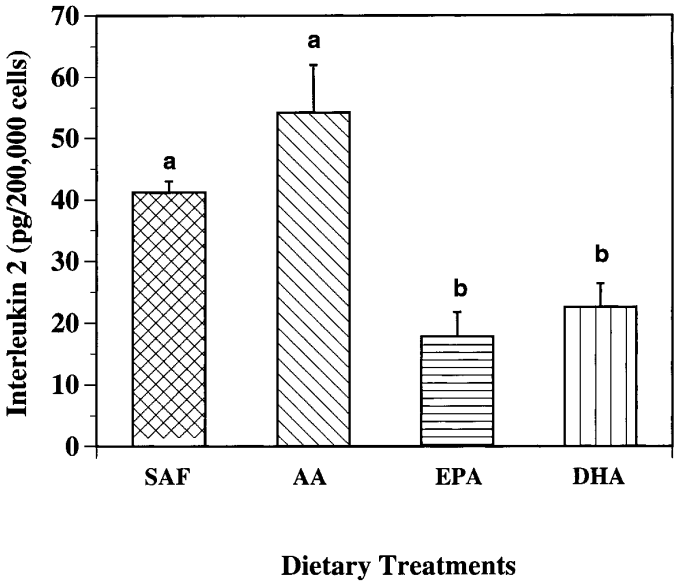

FIGURE 2 Down-regulation of mitogen-induced murine splenocyte interleukin-2 secretion in vitro by dietary eicosapentaenoic acid and docosahexaenoic acid. Mice were fed for 10 d and splenic lymphocytes were stimulated as described in Figure 1. IL-2 in the culture supernatant was quantitated by ELISA as described in Materials and Methods. Values (n = 5 mice) represent the mean ± SEM in pg/200,000 cells from two independent diet studies. Different letters denote significant ($P < 0.05$) differences. SAF = safflower oil ethyl esters, AA = arachidonic acid triglyceride, EPA = eicosapentaenoic acid ethyl ester, DHA = docosahexaenoic acid ethyl ester.

significant ($P < 0.05$) reductions in mitogen-induced ceramide mass at all the time points measured relative to AA- and SAF-fed mice.

DISCUSSION

We have previously shown that low dose (10 g/kg), short term (10 d) feeding of highly purified EPA and DHA to mice suppresses the DTH reponse to vaccination with *M. bovis* BCG in vivo (Hosack-Fowler et al. 1993b). Furthermore, EPA and DHA were significantly incorporated into the cellular lipids of T-lymphocytes, key immune cells in propagating the DTH response (Hosack-Fowler et al. 1993b). To extend these observations, we report here the effect of dietary EPA and DHA on T-lymphocyte function in vitro. Both EPA and DHA significantly suppressed IL-2 production (Fig. 2) and the subsequent proliferative response (Fig. 1). This effect was apparently not due to major alterations in the proportions of CD4+ or CD8+ T-lymphocyte subsets since there was no significant dietary effect on the distribution of these two phenotypes in the spleen (Table 1). Therefore, the data indicate that dietary EPA and DHA affect the process of T-lymphocyte activation. This is corroborated by the observation that dietary EPA and DHA blunted mitogen-induced DAG (Fig. 3A) and ceramide (Fig. 3B) production within T-cells, strongly suggesting that lymphocyte function was altered by dietary influences on cell signalling pathways. The effect of low doses (10 g/kg) of dietary EPA and DHA on T-cell functions most likely is not due to alterations in membrane fluidity because cells can alter their membrane lipid composition to maintain a relatively constant fluidity in response to at least moderate levels of dietary lipid manipulation (Hagve 1988). In addition, the observed effects are likely the result of EPA and DHA substitution and not essential fatty acid deficiency because sufficient quantities of linoleic acid (in the form of SAF) were present in all the experimental diets.

We report here that highly purified EPA and DHA, when fed separately, suppress T-lymphocyte proliferation (Fig. 1). To our knowledge, only one other study has examined the separate effects of dietary EPA and DHA. In that study, investigators showed that 2% supplementation with either DHA or EPA ethyl ester for 6 wk significantly increased survival following malaria infection in mice (Fujikawa et al. 1993). Furthermore, our data show that the intake of a low fat (3% of total energy intake), EPA or DHA enriched diet consumed for only 10 d can have a profound effect on T-lymphocyte proliferation. This novel demonstration of the sensitivity of lymphocyte function to even modest dietary intervention has important implications for the therapeutic application of EPA and DHA. The dietary AA effect did not significantly differ from SAF fed mice, showing that the suppressed proliferative response was due to EPA and DHA substitution, and not the reduction in linoleic acid content in the diet.

The suppressed T-lymphocyte proliferative response in EPA- and DHA-fed mice is consistent with the results of other investigators using fish oil. Dietary fish oil has reduced the T-lymphocyte proliferative response to mitogenic stimulation in healthy humans when eaten for up to 3 months at 1.5% energy in a diet deriving 35-40% of total energy from fat (Meydani et al. 1991). That observation has been extended by showing that a low fat (approximately 25% of energy), fish [(n-3) fatty acid] enriched diet consumed by human subjects for 24 wk led to reduced Con A-induced T-lymphocyte proliferation as well as a suppressed DTH response in vivo (Meydani et al. 1993). However, the patterns of T-lymphocyte proliferation were different from our previously published observations (Hosack-Fowler et al. 1993b). This may be due to the overall difference in the magnitude of activation as reflected by major differences in the control (SAF) group proliferative responses in the two studies.

Interleukin-2 is a potent polyclonal autocrine and paracrine T-lymphocyte growth factor. Interference with IL-2 production or IL-2 receptor-mediated signal transduction would be expected to have a major suppressive effect on T-cell proliferation (Mills et al. 1993, Minami and Taniguchi 1995). Therefore, we examined the effect of dietary fatty acids on Con A-induced IL-2 secretion into the culture medium. Both EPA

TABLE 1

Effect of dietary lipid on the distribution of splenic CD4+ and CD8+ T-lymphocytes in mice[1]

	T-lymphocytes	
Diet	CD4+	CD8+
	% nucleated cells	
SAF	24.0 ± 1.2[a]	16.0 ± 1.8[b]
AA	22.7 ± 2.5[a]	13.8 ± 2.2[b,c]
EPA	24.0 ± 2.9[a]	14.5 ± 2.6[b]
DHA	23.8 ± 1.0[a]	11.3 ± 0.5[c]

[1] Mice were fed SAF (containing primarily linoleic acid), arachidonic acid (AA), eicosapentaenoic acid (EPA) or docasahexaenoic acid (DHA) enriched diets for 10 days. Splenic lymphocytes were isolated and the percentage of CD4+ and CD8+ T-lymphocytes determined by flow cytometry as described in Materials and Methods. Values (n = 4) are expressed as a percent of total nucleated cells and represent the mean ± SEM. Different letters denote significant ($P < 0.05$) differences.

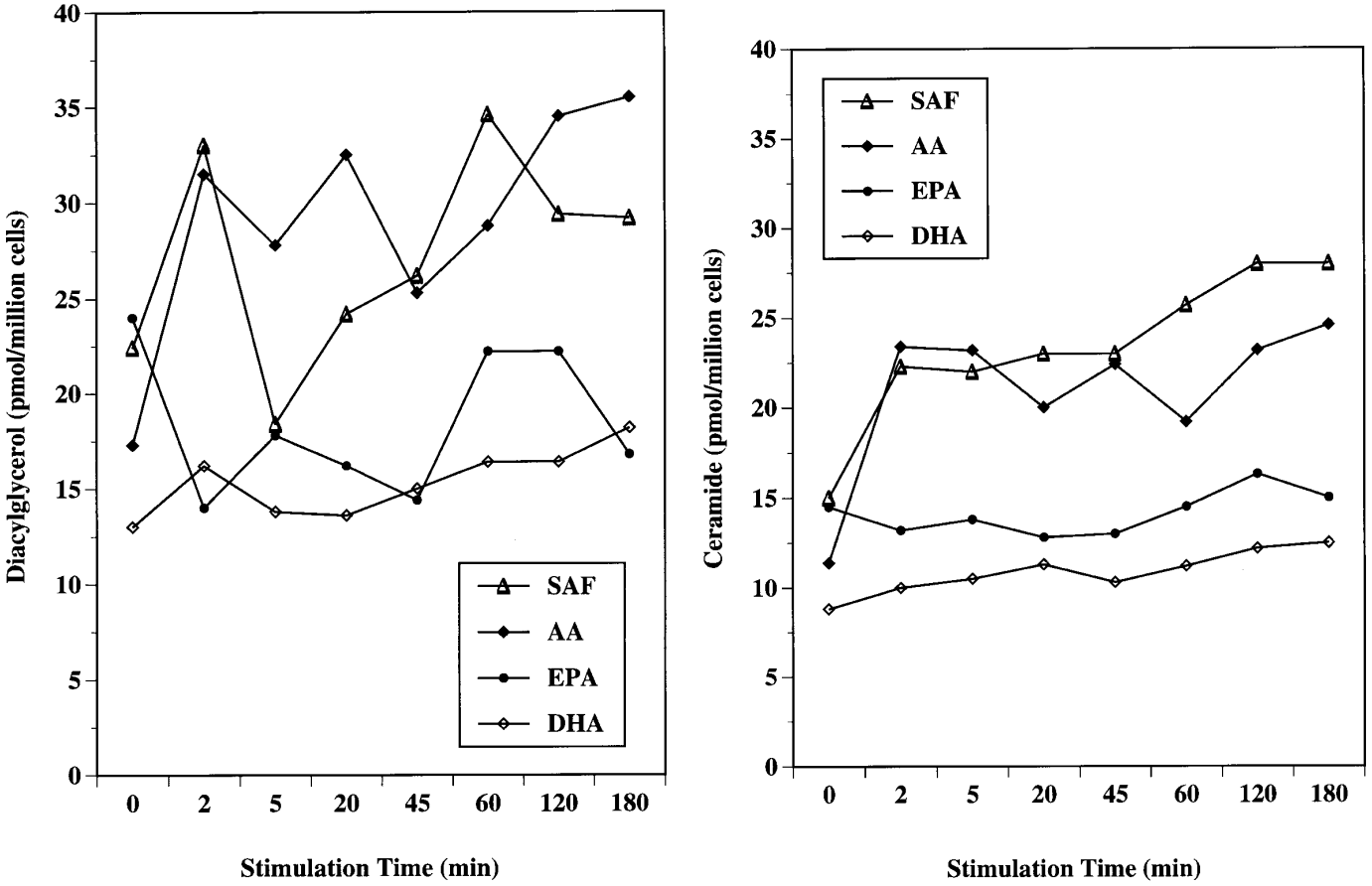

FIGURE 3 Con A-induced ceramide and diacylglycerol kinetics are blunted in response to dietary (n-3) polyunsaturated fatty acids. Mice were fed and splenic lymphocytes stimulated with 10 mg/L Con A. Cellular lipids were extracted at 2, 5, 20, 45, 60, 120 and 180 min after Con A addition, and DAG (*A*) and ceramide (*B*) mass were quantitated using the DAG kinase assay as described in Materials and Methods. Values (n = 4-5) represent the mean in pmol/10^6 cells from two independent diet studies. *A* No significant difference ($P > 0.05$) in basal DAG mass ($t = 0$). Con A induced a significant ($P < 0.05$) increase in DAG mass at 2, 60, 120 and 180 min compared to basal levels at $t = 0$ in SAF-fed mice. AA, Con A induced a significant ($P < 0.05$) increase in DAG mass at 2, 5, 20, 60, 120 and 180 min compared to basal levels. AA vs. SAF, no significant ($P > 0.05$) difference in mitogen-induced DAG mass except at 5 min. AA vs. DHA, Con A-induced DAG mass was significantly ($P < 0.05$) suppressed at all the time points in DHA. AA vs. EPA, EPA feeding significantly ($P < 0.05$) reduced DAG mass at 2, 5, 20, 45, 120 and 180 min. DHA vs. SAF, DAG mass was significantly ($P < 0.05$) reduced at all time points in DHA-fed mice, except at 5 min. EPA vs. SAF, EPA feeding significantly ($P < 0.05$) suppressed DAG mass at 2, 20, 45 and 180 min. EPA vs. DHA, not significantly ($P > 0.05$) different. *B* Basal ($t = 0$) ceramide mass was not significantly ($P > 0.05$) different. AA, significant increase ($P < 0.05$) in ceramide mass at 2 min compared to basal levels which remained significantly elevated for 180 min. SAF, Con A-induced a significant ($P < 0.05$) increase in ceramide mass at 2 min relative to basal and remained significantly elevated for 180 min. AA vs. SAF, no significant ($P > 0.05$) difference. EPA vs. DHA, no significant ($P > 0.05$) difference. Con A did not induce an increase in ceramide mass in EPA- and DHA-fed mice. EPA and DHA vs. AA and SAF, significant ($P < 0.05$) reductions in mitogen-induced ceramide mass at all time points.

and DHA feeding reduced IL-2 secretion (Fig. 2) suggesting that the suppressed lymphoproliferation we observed is due, at least in part, to a reduction in IL-2. Fish oil feeding has previously been shown to reduce IL-2 production in humans, in association with suppressed T-lymphocyte proliferation (Endres et al. 1993, Meydani et al. 1991). Our results extend these observations by showing that both highly purified EPA and DHA can attenuate IL-2 production.

It was not unreasonable to propose that diets containing EPA and DHA might have affected the response of the splenocyte population by altering the relative proportions of T lymphocyte subsets in the spleen. However, our results clearly demonstrate this is not the case (Table 1). Although there was a modest, statistically significant decrease in the proportion of CD8+ T-lymphocytes in DHA-fed mice relative to the EPA and SAF groups (but not to the AA group), this alone cannot explain the suppressed proliferation and IL-2 production in both EPA- and DHA-fed mice. In fact, no dietary effect on the proportions of CD4+ T-cells was observed, and the magnitude of the CD8 effect was only about 30%. Our observations parallel those of others who reported that a 200 g/kg fish oil diet did not affect leucocyte populations in the blood of rats (Yaqoob 1995), and that EPA feeding to humans did not affect T-helper or -suppressor cell numbers (Payan et al. 1986).

To further elucidate the mechanism(s) by which dietary EPA and DHA suppress T-lymphocyte IL-2 secretion and subsequent proliferation, we examined the kinetics of DAG and ceramide production in mitogen-stimulated splenocytes. DAG, the physiologic activator of protein kinase C, is an important intracellular lipid second messenger involved in IL-2 gene transcription in T-lymphocytes (Szamel and Resch 1995). We have previously shown that DAG is produced in a multiphasic fashion in Con A-stimulated murine splenocytes (Jolly et al. 1996). In this study, both EPA and DHA feeding practically abolished mitogen-induced DAG production while AA had no effect relative to the SAF-fed mice (Fig. 3*A*).

While the multiphasic nature of DAG formation in SAF and AA-fed mice is similar to our previous observations (Jolly et al. 1996) the magnitude of the response is different. This most likely is due to the use of mice fed nonpurified diet in our initial study, rather than the semi-purified control (SAF and AA) diets employed here. There were no differences in basal DAG mass between any of the diet groups (Fig. 3*A*) indicating that dietary EPA and DHA modulate receptor mediated DAG production and not basal steady-state levels, ruling out a general metabolic defect in EPA and DHA enriched splenic lymphocytes. These results differ from earlier reports (Hosack-Fowler et al. 1993a and 1993b) possibly because in those studies, splenic lymphocytes were preincubated for 16 h, which can alter the T-cell proliferative response and potentially the generation of intracellular lipid second messengers (unpublished observations, Goodwin et al. 1978). In this work, freshly isolated T-cells were immediately activated, allowing for the correlation between DAG mass and T-cell proliferation. We are the first to directly demonstrate an effect of dietary EPA and DHA on activation-induced DAG production, although a previous study had suggested such an effect by showing alterations in neutrophil PI metabolism in response to fish oil feeding (Sperling et al. 1993).

Ceramide is another intracellular lipid second messenger that has recently been shown to up-regulate T-lymphocyte IL-2 gene levels (Chan and Ochi 1995). Our previous results show that Con A induces a transient prolonged increase of ceramide in murine splenocytes (Jolly et al. 1996). Furthermore, we established that ceramide is a positive effector molecule with respect to T-lymphocyte proliferation in a splenocyte culture system (Jolly et al. 1996). The present results clearly show that both dietary EPA and DHA blunt Con A-induced ceramide generation (Fig. 3*B*) which is positively correlated with the diet effects on lymphoproliferation (Fig. 1) and IL-2 secretion (Fig. 2). While the prolonged ceramide formation seen in SAF- and AA-fed mice is similar to our previous observations (Jolly et al. 1996) the magnitude of the response is different. This may be due to the use of mice fed nonpurified diet in our initial study and not the semi-purified diets employed here. Interestingly, IL-2 stimulation reduces ceramide mass in human T cells (Borchardt et al. 1994). This suggests that ceramide is important only at certain time points in the T-lymphocyte cell cycle, i.e., ceramide is needed for IL-2 synthesis but is no longer required or possibly is inhibitory if present during IL-2 stimulation.

This study clearly indicates that both EPA and DHA play important roles in mediating the immunosuppressive properties of dietary fish oil. Our dietary regimen is unique in that we have examined the effects of highly purified dietary EPA and DHA separately on T-lymphocyte function. This low dose (10 g/kg), short term (10 d) feeding study suppresses the DTH response in vivo (Hosack-Fowler et al. 1993b) which, as would be expected, correlates with suppressed T-lymphocyte IL-2 secretion and subsequent proliferation in vitro (Figs. 1, 2). In an attempt to elucidate the mechanism(s) of action, we demonstrate that both dietary EPA and DHA suppress the time-dependent production of Con A-induced lymphocyte DAG and ceramide mass (Figs. 3*A, B*). Con A was chosen as the activating agent because, as is the case with in vivo responses, it perturbs multiple T-cell plasma membrane receptors. Using this novel feeding regimen, we are the first to show the modulation of DAG and ceramide mass in lymphocytes by dietary EPA and DHA in response to stimulation. This work also complements the hypothesis that DAG and ceramide play positive roles in IL-2 secretion. Additional studies are required to further elucidate the mechanisms of immunomodulation by dietary fish oil to optimize its potential beneficial effects.

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LITERATURE CITED

Ahmed, A. A. & Holub, B. J. (1984) Alteration and recovery of bleeding times, platelet aggregation and fatty acid composition of individual phospholipids in platelets of human subjects receiving a supplement of cod-liver oil. Lipids 19: 617–624.

Asaoka, Y., Oka, M., Yoshida, K. & Nishizuka, Y. (1991) Metabolic rate of membrane-permeant diacylglycerol and its relation to human resting T-lymphocyte activation. Proc. Natl. Acad. Sci. 88: 86871–8685.

Borchardt, R. A., Lee, W. T., Kalen, A., Buckley, R. H., Peters, C., Schiff, S. & Bell, R. M. (1994) Growth-dependent regulation of cellular ceramides in human T-cells. Biochim. Biophys. Acta 1212: 327–336.

Chan, G. & Ochi, A. (1995) Sphingomyelin-ceramide turnover in CD28 costimulatory signaling. Eur. J. Immunol. 25: 1999–2004.

Endres, S., De Caterina, R., Schmidt, E. B. & Kristensen, S. D. (1995) n-3 polyunsaturated fatty acids: update 1995. Eur. J. Clin. Invest. 25: 629–638.

Endres, S., Meydani, S. N., Ghorbani, R., Schindler, R. & Dinarello, C. A. (1993) Dietary supplementation with n-3 fatty acids suppresses interleukin-2 production and mononuclear cell proliferation. J. Leuk. Biol. 54: 599–603.

Fritsche, K. L. & Johnston, P. V. (1990) Effect of dietary omega-3 fatty acids on cell-mediated cytotoxic activity in BALB/C mice. Nutr. Res. 10: 577–588.

Fujikawa, M., Kamitani, T., Tunru, I. S., Yamazaki, K. & Hamazaki, T. (1993) Antimalarial effects of purified and α-tocopherol-fortified n-3 polyunsaturated fatty acids. J. Nutr. Biochem. 4: 153–157.

Goodwin, J. S., Messner, R. P. & Peake, G. T. (1978) Prostaglandin suppression of mitogen-stimulated lymphocytes in vitro. J. Clin. Invest. 62: 753–760.

Hagve, T. A. (1988) Effects of unsaturated fatty acids on cell membrane functions. Scand. J. Clin. Lab Invest. 48: 381–388.

Hosack-Fowler, K., McMuray, D. N., Fan, Y. Y., Aukema, H. A. & Chapkin, R. S. (1993a) Purified dietary n-3 polyunsaturated fatty acids alter diacylglycerol mass and molecular species composition in concanavalin A-stimulated murine splenocytes. Biochim. Biophys. Acta 1210: 89–96.

Hosack-Fowler, K., Chapkin, R. S. & McMurray, D. N. (1993b) Effects of purified dietary n-3 ethyl esters on murine T lymphocyte function. J. Immunol. 151: 5186–5197.

Hwang, D. (1989) Essential fatty acids and immune response. FASEB J. 3: 2052–2061.

Jolly, C. A., Laurenz, J. C., McMurray, D. N. & Chapkin, R. S. (1996) Diacylglycerol and ceramide kinetics in primary cultures of activated T-lymphocytes. Immunol. Lett. 49: 43–48.

Kremer, J. M. (1991) Clinical studies of omega-3 fatty acid supplementation in patients who have rheumatoid arthritis. Rheum. Dis. Clin. North America 17: 391–402.

Mathias, S., Younes, A., Kan, C. C., Orlow, I., Joseph, C. & Kolesnick, R. N. (1993) Activation of the sphingomyelin signaling pathway in intact EL4 cells and in a cell-free system by IL-1b. Science (Washington, DC) 259: 519–522.

Meydani, S. N., Endres, S., Woods, M. M., Goldin, B. R., Soo, C., Morrill-Labrode, A., Dinarello, C. A. & Gorbach, S. L. (1991) Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: Comparison between young and older women. J. Nutr. 121: 547–555.

Meydani, S. N., Lichtenstein, A. H., Cornwall, S., Meydani, M., Goldin, B. R., Rasmussen, H., Dinarello, C. A. & Schaefer, E. J. (1993) Immunologic effects of National Cholesterol Education Panel Step-2 diets with and without fish-derived n-3 fatty acid enrichment. J. Clin. Invest. 92: 105–113.

Mills, G. B., Schmandt, R., Gibson, S., Leung, B., Hill, M., May, C., Shi, Y. F., Branch, D. R., Radvanyi, L., Truitt, K. E. & Imboden, J. (1993) Transmembrane signaling by the interleukin-2 receptor: progress and conundrums. Sem. Immunol. 5: 345–364.

Minami, Y. & Taniguchi, T. (1995) IL-2 signaling: recruitment and activation of multiple protein tyrosine kinases by the components of the IL-2 receptor. Curr. Opin. Cell Biol. 7: 156–162.

Morrow, W.J.W., Homsy, J. & Levy, J. A. (1993) The influence of nutrition on experimental autoimmune disease. In: Nutrient Modulation of the Immune Response, (Cunningham-Rundles, S., ed.), pp. 153–167, Marcel Dekker Inc., New York, NY.

Payan, D. G., Wong, M.Y.S., Chernov-Rogan, T., Valone, F. H., Pickett, W. C., Blake, V. A., Gold, W. M. & Goetzl, E. J. (1986) Alterations in human leukocyte function induced by ingestion of eicosapentaenoic acid. J. Clin. Immunol. 6: 402–410.

SAS Institute Inc. (1985) SAS Users Guide. SAS Institute, Cary, N.C.

Shapiro, A. C., Wu, D., Hayek, M. G., Meydani, M. & Meydani, S. N. (1994) Role of eicosanoids and vitamin E in fish oil-induced changes of splenocyte proliferation to T cell mitogens in mice. Nutr. Res. 14: 1339–1354.

Simopoulos, A. P. (1991) Omega-3 fatty acids in health and disease and in growth and development. Am. J. Clin. Nutr. 54: 438–463.

Sperling, R. I., Benincaso, A. I., Knoell, C. T., Larkin, J. K., Austen, K. F. & Robinson, D. R. (1993) Dietary ω-3 polyunsaturated fatty acids inhibit phosphoinositide formation and chemotaxis in neutrophils. J. Clin. Invest. 91: 651–660.

Surette, M. E., Whelan, J., Lu, G., Hardard'ottir, I. & Kinsella, J. E. (1995) Dietary n-3 polyunsaturated fatty acids modify Syrian hamster platelet and macrophage phospholipid fatty acyl composition and eicosanoid synthesis: a controlled study. Biochim. Biophys. Acta 1255: 185–191.

Szamel, M. & Resch, K. (1995) T-cell antigen receptor-induced signal-transduction pathways: Activation and function of protein kinases C in T lymphocytes. Eur. J. Biochem. 228: 1–15.

Vallette, L., Croset, M., Prigent, A. F., Meskini, N. & LaGarde, M. (1991) Dietary polyunsaturated fatty acids modulate fatty acid composition and early activation steps of concanavalin A-stimulated rat thymocytes. J. Nutr. 121: 1844–1859.

Yaqoob, P., Newsholme, E. A. & Calder, P. C. (1995) The effect of fatty acids on leucocyte subsets and proliferation in rat whole blood. Nutr. Res. 15: 279–287.