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MANIPULATION OF THE ACUTE INFLAMMATORY RESPONSE BY DIETARY POLYUNSATURATED FATTY ACID MODULATION[1]

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Dietary polyunsaturated fatty acid modulation has been used as an anti-inflammatory strategy in experimental models of disease as well as in clinical trials. To elucidate the mechanisms underlying the anti-inflammatory effects of manipulating dietary polyunsaturated fatty acids, the in vivo effects of essential fatty acid (EFA) deficiency and (n-3) fatty acid supplementation were contrasted using a model of acute inflammation induced by the i.p. injection of zymosan into mice. Both diets led to a substantial decrease in tissue (n-6) fatty acid content. EFA deficiency was also characterized by the accumulation of (n-9) fatty acids, particularly 20:3 (n-9), the fatty acid that uniquely characterizes the deficiency state. Dietary (n-3) fatty acid supplementation led instead to marked increases in (n-3) fatty acids, especially 20:5(n-3). With respect to the anti-inflammatory effects of the two diets, EFA deficiency, but not (n-3) fatty acid supplementation, depleted levels of resident peritoneal macrophages. EFA deficiency was also more effective than (n-3) fatty acid supplementation in inhibiting the influx of polymorphonuclear neutrophils in response to zymosan. The effect of the two diets on the in vivo generation of leukotriene(LT)B also differed markedly. EFA deficiency completely inhibited the synthesis of LTB. Dietary (n-3) fatty acid supplementation, in contrast, reduced the production of LTB_4 by only 50%. With (n-3) fatty acid supplementation LTB_5 was produced. The more modest effect of (n-3) fatty acid supplementation in decreasing LTB_4 generation was not due to blockade of the cyclooxygenase pathway. EFA deficiency, but not (n-3) fatty acid supplementation, was associated with the decreased synthesis of thromboxane. Although dietary fatty acid modulation has been shown to diminish platelet activating factor (PAF) synthesis, studies using the PAF receptor blocker, L659989, established that PAF was not a significant factor in the elicitation of leukocytes in this model of inflammation. In summary, the anti-inflammatory effect of EFA deficiency was more marked than that of dietary (n-3) fatty acid supplementation in acute inflammation. This difference in anti-inflammatory potential appeared to be due to either the greater effect of EFA deficiency in decreasing levels of resident peritoneal macrophages or in suppressing the in vivo generation of LTB_4.

Dietary polyunsaturated fatty acid manipulation has been used as an anti-inflammatory manipulation in several models of inflammation. EFA[3] deficiency has been shown to prevent or ameliorate the inflammation and consequent tissue damage in murine lupus glomerulonephritis (1), nephrotoxic nephritis (2), hydronephrosis (3), interstitial nephritis (4), myocardial infarction (5), and immune-mediated diabetes (6, 7). Dietary (n-3) fatty acid supplementation has also been demonstrated to ameliorate a variety of inflammatory conditions such as murine lupus nephritis (8) and collagen-induced arthritis (9). The strategy of dietary polyunsaturated fatty acid manipulation has also been used in human inflammatory disease. Clinical studies have used dietary (n-3) fatty acid supplementation as a means to treat inflammatory disorders such as rheumatoid arthritis (10, 11).

The mechanisms underlying the protective effect of dietary polyunsaturated fatty acid manipulation have not been wholly elucidated. One potential mechanism behind this protective effect, however, is a decrease in the elaboration of certain proinflammatory autacoids such as LTB_4 or PAF (12, 13). In vitro studies have shown that dietary polyunsaturated fatty acid modification may decrease both LTB_4 and PAF production by leukocytes (14–17). Additionally, the LT of the B series synthesized from polyunsaturated fatty acids which substitute for arachidonate such as 20:3(n-9) and 20:5(n-3) (the fatty acids that accumulate in EFA deficiency and dietary (n-3) fatty acid supplementation respectively (18, 19), are relatively weak agonists compared to LTB_4 (20, 21). Thus, a potential common mechanism underlying the protective effect of dietary polyunsaturated fatty acid manipulation in inflammation is that the synthesis of proinflammatory lipid mediators is diminished and/or the lipid mediators that are produced are relatively inactive.

In prior studies on the mechanisms underlying the protective effect of dietary polyunsaturated fatty acid manipulation in inflammation, it was established that EFA deficiency decreases the number of resident peritoneal macrophages and inhibits the acute inflammatory response to i.p. zymosan (22). A critical role for LTB_4 in this model of inflammation was suggested by data that

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[3] Abbreviations used in this paper: EFA, essential fatty acid; LT, leukotriene; PMN, polymorphonuclear neutrophil; PAF, platelet activating factor; Tx, thromboxane.

demonstrated that LTB_4 was synthesized in vivo in concentrations adequate to exert biologic activity (nM or more), that the synthesis of LTB_4 preceded the influx of PMN, and that EFA deficiency and BW755C (both of which completely inhibited the synthesis of LTB_4) both inhibited the influx of PMN (22).

In our study the anti-inflammatory efficacy of EFA deficiency was compared to that of dietary (n-3) fatty acid supplementation using this model of acute inflammation. The results derived from this study suggest that EFA deficiency is relatively more potent in its anti-inflammatory effects in acute inflammation than (n-3) fatty acid supplementation. The reason behind this difference in efficacy appears to be due to the difference in the effect of the diets on resident macrophages and LTB generation.

MATERIALS AND METHODS

Materials. Zymosan, polyunsaturated fatty acid methyl esters, and Giemsa stain were purchased from Sigma Chemical Co. (St. Louis, MO). 20:3(n-9) methyl ester, LTB_4, LTB_5, LTB_4 isomers and metabolites, [$^2H_4$]-TxB_2, and PAF were obtained from Biomol (Plymouth Meeting, PA). TLC plates were purchased from Analabs (Norwalk, CT). [^{3}H]LTB_4 was purchased from New England Nuclear (Wilmington, DE) and a specific antiserum was a gift from Merck-Frosst (Point Claire-Dorval, Canada, gift from Dr. A. Ford-Hutchinson). [^{125}I] TxB_2 and specific antiserum were a gift from Dr. P. Needleman (Washington University). (N-3) ethyl esters for dietary supplements were obtained through the Fish Oil Test Materials Program (Bethesda, MD). L659989 was a gift from Merck Sharp and Dohme (West Point, PA, gift of Dr. S. Hwang). Organic chemicals were gas chromatography/mass spectrometry grade and were obtained from Burdick and Jackson (Muskegon, MI).

Dietary manipulations. C57BL/6J weanling male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). After arrival they were placed on one of three diets: standard Purina rodent Chow, a fat-free diet obtained from Purina Test Diets (Richmond, IN), or a special powdered fat-free diet (Purina Test Diets) with 5% (n-3) fatty acid ethyl esters added. Descriptions of the first two diets have been previously published (23). Briefly the control diet contains 20 mg/g food of linoleate and 0.1 mg/g food of arachidonate. The fat-free diet contains <10 μg/g food of linoleate and no detectible arachidonate. The third diet was a special powdered formulation to be reconstituted with a 5% lipid source so that the final product was isocaloric with the other two diets. This diet contained vitamin-free casein (22.11%), sucrose (76%), solka floc (3.16%), vitamin mix (2.10%), mineral mix (5.26%), *dl*-methionine (0.16%), and choline chloride (0.21%). The diet was made up with the (n-3) ethyl ester preparation noted above. The final mixture contained 25 mg/g diet of 20:5(n-3) and 15 mg/g diet of 22:6(n-3) as determined by gas chromatography. The diet was made weekly and stored at −20°C. Animals were fed daily and leftover food discarded after 1 day at room temperature. Animals were fed each of the diets for >8 wk.

Zymosan-induced acute inflammation. The model of acute inflammation used in the study has been detailed previously (22). Basically, inflammation was induced by the i.p. administration of zymosan 1 mg. Peak LTB_4 production occurs at 2 h and peak cellular influx at 6 h (22). After these time periods animals were killed and the peritoneal cavities washed with 2 ml of saline. Bloody exudates were discarded. Cell counts were performed using a hemocytometer and differentials performed on cell preparations made with a Cytospin (Shandon, Pittsburgh, PA) stained with Giemsa.

RIA were performed directly on the peritoneal exudate (after removing the cells by centrifugation) as the fluid does not interfere with any of the assays used. Radioimmunoassays for LTB_4 and TxB_2 were performed as previously detailed (14). Cross-reactivities for the antibody used in the LTB_4 assay are: 6-trans-LTB_4 6%, 20-hydroxy-LTB_4 2%, 5S,12S-LTB_4 0.25%, other isomers and metabolites of LTB_4 <0.1%, peptidoleukotrienes <0.2%, LTB_5 61.9%, and LTB_3 4.5%. The cross-reactivity of TxB_3 in the TxB_2 assay was undetermined because TxB_3 was not available but was estimated to be <1% from the gas chromatography/mass spectrometry studies detailed below.

Studies on the role of PAF in this model of acute inflammation were performed using the drug, L659989. This drug is a potent and selective competitive antagonist of PAF (23, 24). The parenteral ED_{100} is 0.3 mg/kg (23) and the duration of action in vivo is >6 h (24, 25). In studies using L659989, animals were injected with the drug included in the zymosan that was administered i.p. The dose was 0.3 mg/kg. Inasmuch as stock solutions of the drug (2 mg/ml) were made up in DMSO, an equivalent amount of DMSO was included in the zymosan injected into controls.

HPLC. LTB_4 and LTB_5 were separated by HPLC and quantified by RIA. In these experiments the peritoneal cavities of four mice were extensively lavaged and the fluids pooled. The cells were removed by centrifugation and the pH was adjusted to 6.2. LTB was extracted using a C_{18} column prepared as detailed before (14). The column was eluted with methanol and the eluate was then subjected to HPLC. The system used was a Beckman 338 chromatograph run by System Gold with an Beckman C_{18} column (5 μ particle size, 5 × 250 mm) (San Ramos, CA). The mobile phase was initially methanol:water (0.08% acetic acid, pH 6.2) 50:50. A gradient from 50 to 100% methanol was run over the first 40 min. Fractions (1 ml) were collected with a Gilson model 203 fraction collector (Middleton, WI) and aliquots evaporated for RIA with a Hetovac VR-1 (Heto, Copenhagen, Denmark). Migration of immunoreactivity was compared with the migration of authentic standards monitored by absorbance at 280 nm. Although 5S,12S-LTB_4 does not resolve from LTB_4 under these conditions, this isomer of LTB_4 has only trivial cross-reactivity in the immunoassay as noted above. Recovery of LTB_4 after open bed C_{18} column extraction and HPLC was >90%.

Tissue lipid fatty acid analysis. Tissue fatty acid composition was determined by a combination of TLC and gas chromatography detailed previously (26). Tissue lipids were extracted by the method of Bligh and Dyer (27), and the constituent fatty acids were transmethylated by the sequential addition of 0.5 N NaOH in methanol (10 min at room temperature) and then 6 N HCl. The fatty acid methyl esters were then extracted with hexane:diethyl ether 1:1 (v/v), purified by TLC (hexane:diethyl ether:acetic acid 75:5:1), and analyzed using gas chromatography.

Fatty acid methyl esters were separated and identified using a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) containing an SP-2380 capillary column (25 m, 0.32 mm internal diameter, Supelco, Houston, TX). The carrier gas was He and column head pressure was 15 psi. The injector was maintained at 250°C and the flame ionization detector was maintained at 300°C. The column was operated isothermally at 175°C. Fatty acid methyl esters were identified by comigration with authentic standards. Fatty acid methyl ester retention times and peak integration were computed with a Hewlett-Packard 3323A integrator interfaced with the gas chromatograph. Results are expressed as mol%.

Gas chromatography/mass spectrometry. TxB_2 and TxB_3 were separated and quantified by gas chromatography/mass spectrometry. To 0.5 ml of peritoneal exudate was added 2 ng [$^2H_4$]-TxB_2 and 0.1 g methoxamine HCl (Pierce Chemical Co., Rockford, IL) in 0.3 ml 6 M Na acetate buffer pH 5.0. The sample was allowed to stand at room temperature for 10 min. The sample was then added to a Bond Elut phenylboronic acid column (Analytichem International, Harbor City, CA) which was activated with 2 ml of 0.1 N HCl. The column was rinsed with 2 ml methanol/0.1 N HCl (1:1) followed by 2 ml methanol/0.01 N $(NH_4)SO_4$ (1:1) then 1 ml methanol. TxB_2/TxB_3 was eluted with 4 ml methanol/0.1 N NaOH (1:1), and the eluate was diluted to 15 ml with H_2O and pH adjusted to 3.5 with 100 μl 10% formic acid. The sample was then applied to an activated C_{18} column (Baker, Phillipsburg, NJ), washed with 5 ml H_2O followed by 5 ml H_2O:methanol (3:1), and the sample eluted with 4 ml ethyl acetate. The ethyl acetate fraction was transferred to a reactivial and evaporated to dryness under N_2. A total of 20 μl of 35% pentafluorobenzyl bromide in acetonitrile and 20 μl diisopropyl ethylamine was added and the vial heated to 45°C for 30 min. The contents were then evaporated to dryness under N_2 and 10 μl *N,o*-bis(trimethylsilyl)trifluoroacetamide (with 1% trimethylchlorosilane) and 10 μl pyridine added for 15 min at room temperature. The reagents were evaporated under N_2 and 10 μl heptane added. Gas chromatography/mass spectrometry was performed using a Delsi gas chromatograph with a 15-m OV-1, 0.20-mm internal diameter fused silica column interfaced to a Nermag 1010H mass spectrometer (Delsi, Houston, TX) operating in the negative ion/chemical ionization mode. Reagent gas was CH_4 and carrier gas He. Ions monitored for quantitation were m/z 614 for d_0-TxB_2, m/z 618 for [$^2H_4$]-TxB_2 (internal standard) and m/z 612 for d_0-TxB_3.

Data analysis. Data were analyzed with the aid of Stata (Computing Resources, Los Angeles, CA). Data are expressed as mean ± SEM. Where multiple comparisons were made, a one-way analysis of variance was performed before individual comparison. If the one-way analysis of variance was significant ($p < 0.05$) then individual comparisons were made using an unpaired *t*-test. χ^2 was used for 2 × 2 analysis. Significance levels are indicated in the text.

RESULTS

Effects of dietary polyunsaturated fatty acid manipulation on hepatic and macrophage fatty acid composition. Both EFA deficiency and dietary (n-3) fatty acid supplementation effected substantial alterations in hepatic fatty acid composition. As shown in Table I, both dietary manipulations were characterized by marked decreases in the major hepatic (n-6) fatty acids, linoleate and arachidonate. Linoleate levels were decreased by 95 and 99% by EFA deficiency and (n-3) fatty acid supplementation, respectively. Arachidonate levels were also significantly decreased by both dietary manipulations. Arachidonate was decreased by 90% by EFA deficiency and 75% by (n-3) fatty acid supplementation.

With EFA deficiency, there was a marked accumulation of (n-9) fatty acids concomitantly with the depletion of (n-6) fatty acids. As in Table I, hepatic oleate more than doubled and 20:3(n-9) was detected. The 20:3(n-9) to arachidonate ratio was 4.4 ± 1.3 which is 10 times the minimal criterion for EFA deficiency (20:3(n-9) to arachidonate >0.4 (18)).

Dietary (n-3) fatty acid supplementation, in contrast to EFA deficiency, did not lead to an increase in (n-9) polyunsaturates. Oleate was not increased and 20:3(n-9) was not detected. Instead hepatic levels of both 20:5(n-3) and 22:6(n-3), the principle constituents of the (n-3) fatty acid supplement, were markedly elevated (Table I). 20:5(n-3) increased 50-fold and 22:6(n-3) increased nearly threefold. The ratio of 20:5(n-3) to arachidonate was 2.5 ± 0.4. There was no significant accumulation of 18:3(n-3) (or linolenate) which would have been formed from retroconversion of 20:5(n-3) or 22:6(n-3) (data not shown).

The changes in peritoneal macrophage fatty acid composition paralleled those seen in the liver (Table I). Both diets led to decreases in the major (n-6) polyunsaturated fatty acids, although (n-3) fatty acid supplementation was relatively more effective in decreasing macrophage arachidonate. Macrophage arachidonate was decreased by 80% in cells taken from animals on the (n-3) fatty acid-supplemented diet whereas macrophage arachidonate was decreased by 65% in EFA-deficient cells. EFA-deficient cells, however, accumulated oleate and 20:3(n-9). The 20:3(n-9) to arachidonate ratio in EFA-deficient macrophages was half that seen in the liver, 2.2 ± 0.2 vs 4.4 ± 1.3. Dietary (n-3) supplementation in contrast to EFA deficiency led to a substantial accumulation of 20:5(n-3) and 22:6(n-3) in macrophage lipids. There was no detectible 20:5(n-3) in control macrophages whereas there was 4.2 mol% ml of 20:5(n-3) in cells from animals on an (n-3) fatty acid-supplemented diet. Macrophage 22:6(n-3) increased by nearly fivefold. The ratio of 20:5(n-3) to arachidonate was comparable to the 20:3(n-9) to arachidonate ratio: 2.2 ± 0.6 vs 2.2 ± 0.2, respectively.

Both dietary manipulations thus led to striking alterations in tissue fatty acid composition. Peritoneal macrophages were substantially affected by either dietary manipulation. Both diets also led to comparable ratios of the substituted eicosanoid precursor (20:3(n-9) in EFA deficiency, 20:5(n-3) in the (n-3) supplemented animals) to arachidonate.

Effects of dietary polyunsaturated fatty acid manipulation on acute inflammatory response. The anti-inflammatory effects of EFA deficiency and (n-3) fatty acid supplementation were compared using a model of acute inflammation induced by the i.p. injection of zymosan (22). The inflammatory response in this model is characterized by an acellular phase (0 to 2 h) during which eicosanoids, including LTB_4, can be detected in the peritoneal exudate. This phase is followed by the influx of leukocytes peaking at 6 h and consisting predominantly of PMN.

Before the injection of zymosan, there were 3.53×10^6 peritoneal leukocytes per ml in control animals (Fig. 1). These were >90% macrophages by morphology with small numbers of lymphocytes and mast cells (data not shown). EFA-deficient animals exhibited a decrease in the number of resident cells (Fig. 1) as has been observed before (22). The decrease in resident macrophages in EFA-deficient animals varied from 30 to 60% with different groups of animals (data not shown). In contrast, dietary (n-3) fatty acid supplementation did not affect the number of resident peritoneal leukocytes (Fig. 1).

Six h after the injection of zymosan, the number of peritoneal leukocytes rose nearly fivefold in control animals (Fig. 1). This inflammatory cell infiltrate consisted mostly of PMN as shown in Figure 2. The influx of leukocytes was significantly diminished in EFA-deficient animals (approximately 50% of control levels). This decrease was due predominantly to a decrease in PMN influx although macrophage numbers were decreased as well (Fig. 2). The acute inflammatory response was much

TABLE I

Tissue fatty acid composition of EFA-deficient and (n-3) fatty acid supplemented animals[a]

Experimental group	Hepatic Fatty Acid Analysis					
	18:1 (n-9)	18:2 (n-6)	20:3 (n-9)	20:4 (n-6)	20:5 (n-3)	22:6 (n-3)
Control (n = 3)	23.6 ± 1.5	16.7 ± 0.9	0.0	13.1 ± 1.5	0.2 ± 0.0	6.3 ± 1.0
EFAD (n = 3)	54.9 ± 0.4[b]	0.7 ± 0.0[b]	5.5 ± 0.8[b]	1.4 ± 0.3[b]	0.0[b]	0.3 ± 0.0[b]
(N-3) (n = 3)	15.5 ± 1.0[c]	0.1 ± 0.0[b]	0.0	3.3 ± 0.1[b]	8.0 ± 1.0[b]	18.3 ± 0.7[b]
	Macrophage Fatty Acid Analysis					
Control (n = 3)	16.6 ± 5.3	11.3 ± 2.0	0.0	12.6 ± 2.3	0.0	1.6 ± 0.2
EFAD (n = 3)	37.7 ± 1.4[c]	5.9 ± 0.5[c]	9.8 ± 0.9[b]	4.6 ± 0.2[c]	0.0	1.2 ± 0.1
(N-3) (n = 4)	26.5 ± 2.7	0.6 ± 0.1[b]	0.0	2.4 ± 0.5[b]	4.2 ± 0.6[b]	8.1 ± 1.2[b]

[a] Tissue lipids were extracted and the fatty acid composition determined by TLC and gas chromatography as detailed in *Materials and Methods*. Results for the major unsaturated fatty acids are shown and are expressed as mol% ml. Each replicate to determine macrophage fatty acid composition was performed on a pool of cells from two animals. (N-3) = (n-3) fatty acid supplemented animals.

[b] $p < 0.01$ EFAD or (N-3) vs control.

[c] $p < 0.05$ EFAD or (N-3) vs control.

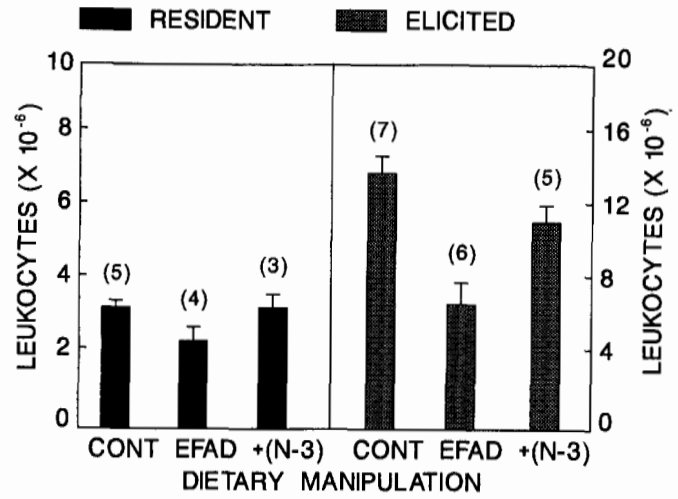

Figure 1. Effect of dietary fatty acid manipulation on resident and elicited leukocyte number. Acute inflammation was induced in control, EFA-deficient (*EFAD*), and (n-3) fatty acid supplemented mice (+(N-3)) by the i.p. injection of zymosan 1 mg. Before and 6 h after the injection of zymosan, the peritoneal cavities were lavaged with 2 ml of PBS. Leukocyte numbers were determined using a hemocytometer and are expressed as leukocytes per ml. Means ± SEM are shown. Numbers of animals are shown in *parentheses*. For resident leukocytes, EFA-deficient mice were significantly different than control ($p < 0.05$). For elicited leukocytes, EFA-deficient and (n-3) fatty acid supplemented mice were different than control ($p < 0.01$ and <0.05, respectively). EFA-deficient mice were also different from (n-3) fatty acid supplemented mice ($p < 0.01$).

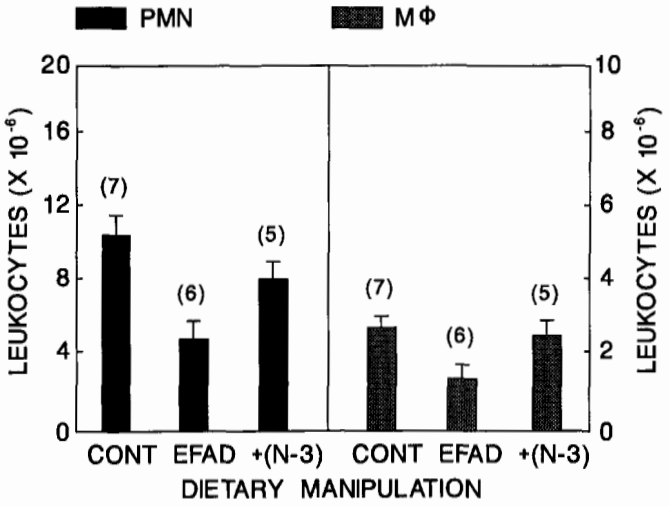

Figure 2. Effect of dietary fatty acid manipulation on elicited PMN and macrophages. Acute inflammation was induced in control, EFA-deficient (*EFAD*), and (n-3) fatty acid supplemented mice (+(N-3)) by the i.p. injection of zymosan 1 mg. Then 6 h after the injection of zymosan, the peritoneal cavities were lavaged with 2 ml of PBS. Total leukocyte counts were determined using a hemocytometer and differential counts were performed on Giemsa-stained slides. Results are expressed as mean ± SEM. Numbers of animals are shown in *parentheses*. For PMN, EFA-deficient animals were different from controls and (n-3) fatty acid supplemented mice ($p < 0.01$ and <0.05, respectively). (N-3) fatty acid supplemented mice were not different from controls although a trend existed ($p = 0.10$). For macrophages EFA-deficient animals were different from controls and (n-3) supplemented mice ($p < 0.01$ for both).

more modestly inhibited in animals fed a diet supplemented with (n-3) fatty acids. The total influx of leukocytes was diminished only 20% (Fig. 1). This decrease was attributable to a 20% decrease in the influx of PMN (Fig. 2). Dietary (n-3) fatty acid supplementation did not affect the number of macrophages present.

Effect of dietary fatty acid manipulation on in vivo synthesis of eicosanoids. Because LTB_4 has been shown to be a significant factor in the elicitation of leukocytes in this model of inflammation (22), the production of LTB in vivo was examined in control animals and animals on the EFA-deficient and (n-3) fatty acid-supplemented diets. Peak production of LTB_4 occurs at 2 h after the injection of zymosan (22). As shown in Table II, LTB_4 was detectible in the peritoneal exudates of control animals 2 h after the injection of zymosan i.p. by direct RIA. This immunoreactivity migrated as a single peak by HPLC using a pool of exudates (Fig. 3, and Table II). Quantitation of this peak by RIA agreed well with estimation by UV spectrometry (17.2 and 13 ng, respectively). No omega

TABLE II

Quantification of in vivo production of LTB and TxB in acute inflammation[a]

Experimental Group	LTB Production	
	RIA (ng/ml)	LTB_5/LTB_4 (HPLC, ng)
Control ($n = 6$)	0.58 ± 0.03	0/17.2
EFAD ($n = 3$)	<0.03[b]	
(N-3) ($n = 5$)	0.52 ± 0.02	13.2/6.7
	Thromboxane Production	
	RIA (ng/ml)	TxB_3/TxB_2 (GC/MS, pg)
Control ($n = 3$)	0.32 ± 0.03	0/195
EFAD ($n = 3$)	0.14 ± 0.01[c]	
(N-3) ($n = 3$)	0.28 ± 0.05	144/190

[a] Inflammation was induced by the i.p. injection of zymosan and 2 h later peritoneal exudates were collected by lavage. RIA was performed on peritoneal fluids directly. To determine the relative amounts of LTB_4 and LTB_5, several exudates were pooled and fractionated by HPLC and quantified by RIA as detailed in *Materials and Methods*. LTB_5 values were corrected for the lesser cross-reactivity of this LTB in the RIA. To determine the relative amounts of TxB_2 and TxB_3 several exudates were pooled and the 2 and 3 series thromboxanes identified and quantified by gas chromatography/mass spectrometry as detailed in *Materials and Methods*.

[b] $p < 0.01$ EFAD vs control.

[c] $p < 0.05$ EFAD vs control.

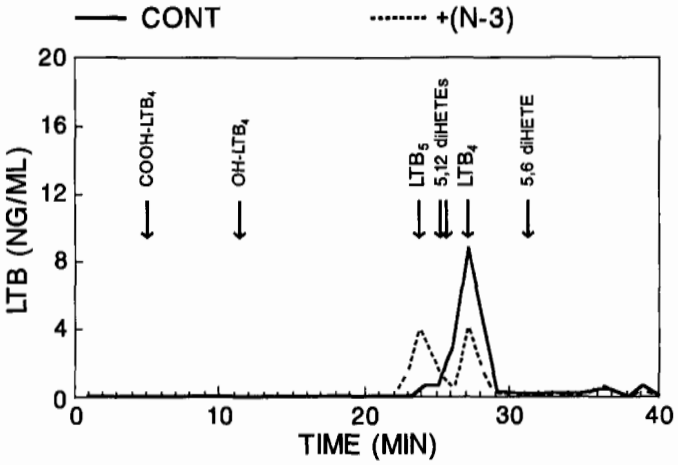

Figure 3. High-pressure liquid chromatographic analysis of peritoneal exudates from control and (n-3) fatty acid supplemented mice. Acute inflammation was induced in control and (n-3) fatty acid supplemented mice by the i.p. injection of zymosan 1 mg. After 6 h, the peritoneal cavities were exhaustively lavaged, and the fluids were pooled. LTB was extracted using an open bed C_{18} column. Column eluates were subsequently subjected to HPLC as detailed in *Materials and Methods*. One-min fractions were collected and analyzed by RIA. Results are expressed as ng/ml. The migration of authentic standards for LTB_4, LTB_5, and the various isomers and metabolites of LTB_4 are shown. Peaks comigrating with LTB_4, and LTB_4 and LTB_5 were also visible by monitoring at 280 nm in samples from control and (n-3) fatty acid-supplemented animals, respectively. Scanning UV spectroscopy of the LTB_4 from the control sample showed a typical triene configuration with a maximum at 270 nm. No peaks comigrating with the isomers or metabolites of LTB_4 were noted by monitoring at 280 nm or by immunoassay of concentrated HPLC fractions.

oxidation products of LTB_4 (20-hydroxy-LTB_4 or 20-carboxy-LTB_4) were detected in the pooled peritoneal exudates either by UV spectrometry or by RIA of concentrated HPLC fractions (<2 ng). These results thus establish the production of LTB_4 in control animals. These data and prior studies (28, 29) also suggest that LTB_4 levels in vivo may be more directly a function of synthesis than omega oxidation.

EFA deficiency completely inhibited the synthesis of LTB. There was no detectible LTB by radioimmunoassay in the peritoneal exudates from EFA-deficient animals (Table II). Previous studies using HPLC have shown that neither LTB_4 nor LTB_3 are present (22). In contrast, LTB generation in the peritoneal exudates from (n-3) fatty acid supplemented animals was comparable to controls by RIA (Table II). When LTB was fractionated into LTB of the 4 and 5 series by HPLC it was seen that half of the immunoreactivity migrated as LTB_5 and the remainder as LTB_4 (Fig. 3). Thus, LTB_4 production was decreased by approximately 50% in (n-3) fatty acid-supplemented animals. Correcting for the lesser cross-reactivity of LTB_5 in the RIA the actual ratio of LTB_4 to LTB_5 was closer to 1:2 (Table II).

One possible explanation of the relative ineffectiveness of (n-3) fatty acid supplementation in suppressing LTB_4 formation is that 20:5(n-3) might act as an inhibitor of cyclooxygenase and cause shunting of arachidonate down the 5-lipoxygenase pathway. Such shunting has been shown to occur in this model of acute inflammation with administration of the cyclooxygenase inhibitor, indomethacin (30). This possibility was addressed in the current study by measuring the production of thromboxane in peritoneal exudates, because cyclooxygenase is the rate-limiting step in the synthesis of this eicosanoid (31). As shown in Table II, thromboxane production at 2 h as determined by RIA was suppressed in EFA-deficient animals by approximately 50%. In contrast, thromboxane production was not decreased in (n-3)-supplemented animals. The relative amounts of TxB_2 and TxB_3 in peritoneal exudates were determined by gas chromatography/mass spectrometry. Using this method of quantitation, only TxB_2 was detected in control samples (Table II). In samples from (n-3) fatty acid supplemented animals, both TxB_2 and TxB_3 were detected in approximately a 1:1 ratio. Thus (n-3) fatty acid supplementation did not substantially block cyclooxygenase metabolism.

Role of PAF in zymosan-induced acute inflammation. Because PAF synthesis has been shown to be diminished by either EFA deficiency (16) or (n-3) fatty acid supplementation (17) and since PAF may stimulate lipoxygenase metabolism (32), the role of PAF in the present model of acute inflammation was investigated by using the PAF receptor blocker, L659989. As shown in Figure 4, administration of L659989 did not significantly decrease the influx of leukocytes in the context of the inflammatory response. Neither PMN nor macrophage elicitation was diminished.

To validate the efficacy of L659989 in vivo, the ability of this drug to block the effects of exogenously administered PAF was examined. After the injection of PAF 250 μg/kg i.p., mortality in controls was five in six with an average survival time of 30 ± 2 min. L659989 reduced the mortality to one in six ($p < 0.02$), with a survival time in the one animal of 54 min. These results contrast with

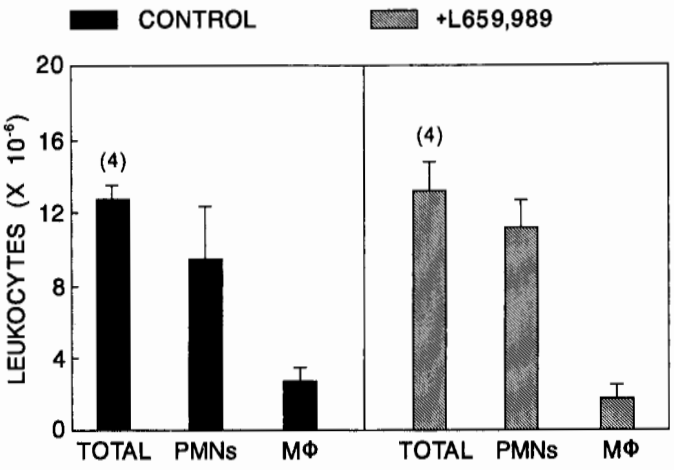

Figure 4. Effect of L659989 on leukocyte elicitation in acute inflammation. Acute inflammation was induced by the i.p. injection of zymosan 1 mg in the presence and absence of the PAF receptor blocker, L659989. Leukocytes were quantified by counting with a hemocytometer and differential counts were performed on Giemsa-stained slides. Results are expressed as mean ± SEM. Numbers of animals are shown in *parentheses*. Animals given L659989 were not significantly different from control.

those seen with 5-lipoxygenase inhibition that does diminish the leukocyte influx in this model (22). Thus PAF, as contrasted with LTB_4, did not appear to be a major contributor to the influx of leukocytes induced by i.p. zymosan.

DISCUSSION

Manipulation of dietary polyunsaturated fatty acids has been used both experimentally and clinically as a means to alter inflammatory disease. In an effort to understand the mechanisms underlying the effectiveness of dietary fatty acid modulation in altering inflammation, the current study compared two different manipulations (EFA deficiency and (n-3) fatty acid supplementation) on a model of acute inflammation. Both dietary manipulations exerted an anti-inflammatory effect in terms of diminishing the influx of PMN into a focus of inflammation, but EFA deficiency was relatively more effective than dietary (n-3) fatty acid supplementation. This difference in efficacy did not appear to be due to the fact that EFA deficiency altered fatty acid composition more substantially than (n-3) fatty acid enrichment. Both diets markedly altered hepatic and macrophage fatty acid composition. In fact, both diets led to comparable ratios of the eicosanoid precursors, 20:3(n-9) and 20:5(n-3), to arachidonate in macrophage lipids. The reason behind the difference in anti-inflammatory efficacy between the diets appeared to be more a function of the effect of the two diets on LTB synthesis. EFA deficiency completely inhibited LTB_4 synthesis whereas dietary (n-3) fatty acid supplementation only partially inhibited LTB_4 synthesis.

One possible mechanism that could explain why EFA deficiency is more effective than (n-3) fatty acid supplementation in suppressing LTB_4 synthesis is that 20:5(n-3), the eicosanoid precursor that accumulates in EFA deficiency, does not inhibit the cyclooxygenase pathway (14) and is only a poor substrate for cyclooxygenase (33). 20:5(n-3), the eicosanoid precursor that accumlates in (n-3) fatty acid supplementation; however, is both a substrate and an inhibitor of cycloxygenase (34). Thus the presence of 20:5 (n-3) might serve to shuttle arachidonate from the cyclooxygenase to the lipoxygenase pathway. In

our study, however, this explanation seems less likely because EFA deficiency was associated with an inhibition in the generation of thromboxane whereas dietary (n-3) fatty acid supplementation did not suppress the generation of thromboxane at all.

A second possibility is based on the relative properties of LTA_3 and LTA_5 as inhibitors and substrates for LTA hydrolase. LTA_3, which is synthesized from 20:3(n-9), has been shown to be a very poor substrate for LTA hydrolase leading to the formation of only trivial amounts of LTB_3 (35). LTA_3, however, is a potent inhibitor of LTA hydrolase (35). LTA_5, the corresponding product from 20:5(n-3), in contrast appears to be a relatively weaker inhibitor of LTA hydrolase and a better substrate (36). Thus one would expect from these in vitro data that EFA deficiency, which is characterized by the accumulation of 20:3(n-9), would be more effective in suppressing LTB_4 production than (n-3) fatty acid supplementation that is characterized by the accumulation of 20:5 (n-3). The data from our study would seem to support this latter possibility.

The mechanism of the resident macrophage depletion seen with EFA deficiency and its role in the anti-inflammatory effect of the deficiency state are unclear. EFA deficiency has previously been shown to deplete resident macrophages in the rat glomerulus (37) and exert a marked anti-inflammatory effect on nephrotoxic nephritis (2). Inasmuch as macrophages are capable of the synthesis of a host of pro-inflammatory mediators in addition to LTB_4, the depletion of resident macrophages may further diminish the ability of EFA-deficient animals to mount an inflammatory response. The decrease in resident macrophage numbers with EFA deficiency may, in fact, explain why EFA deficiency decreases the synthesis of TxB_2 in vivo despite the fact that EFA deficiency does not inhibit the synthesis of TxB_2 by macrophages in vitro (14).

Previous studies have established that EFA deficiency and dietary (n-3) fatty acid supplementation may decrease the synthesis of PAF (16, 17) although the mechanisms behind this decrease and the relative effectiveness of these two diets in decreasing PAF synthesis have not been elucidated. PAF may also stimulate the 5-lipoxygenase pathway (32) and some of its actions can be blocked with lipoxygenase inhibitors (38). Consequently, in the present study the role of PAF in zymosan-induced acute inflammation was assessed using the PAF receptor blocker, L659989. As shown above, PAF did not seem to be a significant factor in the elicitation of leukocytes in this model of acute inflammation.

Not all of the effects of dietary polyunsaturated fatty acid manipulation are necessarily attributable to alterations in LTB synthesis. It has been established that dietary polyunsaturated fatty acid manipulation can decrease the inflammatory response in models of chronic inflammation, such as nephrotoxic nephritis (2), in which the inflammatory cell infiltrate is largely macrophages. In fact, in this model of inflammation the acute infiltration of PMN (which is complement dependent) is unaffected by EFA deficiency whereas the later infiltration of macrophages is completely prevented by EFA deficiency (2). Inasmuch as LTB_4 is a relatively weak chemotactic agent for macrophages relative to PMN (2) it would seem that the mechanism underlying the efficacy of EFA deficiency in this model of inflammatory disease is necessarily different from that involved in acute inflammation. This hypothesis is supported more directly by the finding that BW755C is not effective in reducing the influx of macrophages into the glomerulus in nephrotoxic nephritis (25). Additionally, data suggest that dietary fatty acid manipulation may alter leukocyte function by altering membrane fatty acid composition (14, 39, 40). These studies, in sum, suggest that the beneficial effects of dietary fatty acid manipulation are multifactorial.

The relative ineffectiveness of dietary (n-3) fatty acid supplementation in suppressing inflammation and in inhibiting LTB synthesis has been suggested by other prior investigations. One previous study showed that supplementation of a normal diet with pure 20:5(n-3) did not suppress LTB_4 synthesis or diminish the cellular influx using the carrageenin-impregnated sponge model of inflammation (41). Additionally, in a study on a model of anaphylaxis, dietary (n-3) fatty acid supplementation actually increased the production of LTB_4 (42). The relative lack of efficacy of dietary (n-3) fatty acid supplementation to suppress LTB_4 generation in vivo and prevent PMN influx into a focus of inflammation as seen in these studies as well as in the present one might thus serve to counterbalance enthusiasm for the use of marine oil therapy in man for inflammatory diseases such as rheumatoid arthritis (10, 11). In our study, despite feeding animals a purified diet with (n-3) fatty acids as the sole fatty acid source and effecting substantial changes in tissue fatty acids, the ultimate effects of (n-3) fatty acid supplementation on LTB generation and the inflammatory response were fairly modest. In human studies, where of necessity the amount of (n-3) fatty acid intake will be less and the intake of (n-6) fatty acids from the diet greater, tissue fatty acid changes will be less marked and the effects on LTB production and the inflammatory response even more attenuated. Thus marine oil supplementation as therapy for inflammatory diseases in which LTB is though to play a role may be a relatively ineffective way to ameliorate the inflammatory response.

To recapitulate, EFA deficiency appears to be relatively more effective than dietary (n-3) fatty acid supplementation in diminishing the influx of leukocytes during the acute inflammatory response. This effect appears to result in part from the greater efficiency of the deficiency state in decreasing LTB generation in vivo presumably because of the greater inhibitory potential of LTA_3 relative to LTA_5 with respect to LTA hydrolase. EFA deficiency, but not (n-3) fatty acid supplementation, also leads to a decrease in resident macrophage number that may be responsible for a further decrease in the elaboration of pro-inflammatory mediators. Dietary (n-3) fatty acid supplementation appears to be a relatively ineffective way to manipulate the generation of LTB in vivo and decrease the acute inflammatory response.

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