Modulation of Adjuvant-Induced Arthritis by Dietary Arachidonic Acid in Essential Fatty Acid-Deficient Rats

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ABSTRACT: Controlled feeding of linoleic acid (LA) or arachidonic acid (AA) to essential fatty acid-deficient (EFAD) rats was used to define the relationship between dietary AA and the inflammatory response evoked during adjuvant-induced arthritis. Based on energy percentage, EFAD rats were fed AA at the human daily equivalent (1×; 5.5 mg/day) or 10 times that amount (10×; 55 mg/day) or, alternatively, 0.5× of LA (273 mg/day). Feeding of 0.5× LA restored the plasma level of AA to that in chow-fed controls. In contrast, feeding of 1× AA only partially restored the plasma level of AA; 10× AA was required to fully replete AA. In parallel to the degree of repletion of AA in plasma, there were accompanying decreases in the levels of palmitoleic acid, oleic acid, and Mead acid. Compared to rats fed the standard laboratory chow diet (Control), edema in the primary hind footpads was decreased by 87% in EFAD, 71% in EFAD + 1× AA, 45% in EFAD + 10× AA, and 30% in EFAD + 0.5× LA. The decrease in edema in the footpads of EFAD rats was nearly identical to the decrease in edema in the footpads of Control rats dosed with indomethacin. Hind footpad edema correlated with the final AA plasma level and eicosanoid levels extracted from hind footpad tissue, but not with neutrophil infiltration. The data showed that 0.5× LA and 10× AA, but not 1× AA, could quickly replete AA, accompanied by the synthesis of AA-derived eicosanoids and restoration of edema. These results suggest that in humans consumption of the average daily amount of AA without concurrent ingestion of LA would not alleviate an EFAD state.

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Modifications of dietary fatty acids have been shown to exert antiinflammatory effects in both acute and chronic models of inflammation (reviewed, 1–3). Such dietary modifications have included supplementation with fish oils that are enriched with n-3 polyunsaturated fatty acids (reviewed, 4,5) and induction of an essential fatty acid-deficient (EFAD) state by deprivation of n-6 polyunsaturated fatty acids (reviewed, 6–8). Effects have included the amelioration of glomerulonephritis in a murine model of the human disease, systemic lupus erythematosus (9,10), ulcerative colitis (11,12), nephrotoxic nephritis (13,14), myocardial infarction (15,16), psoriasis (17), microcirculatory manifestations of ischemia–reperfusion injury (18), diabetes (19–21), and rheumatoid arthritis (reviewed, 22). In rheumatoid arthritis and other chronic inflammatory diseases, evidence has been accumulating which indicates that the quantity and quality of fat in the diet can affect the etiological course of chronic inflammatory diseases (reviewed, 23). Populations in Western countries consume a relatively high amount of arachidonic acid (AA) (approx. 200 mg/day) and an even higher amount of its precursor, linoleic acid (LA) (approx. 20 g/day), both of which are essential, polyunsaturated fatty acids of the n-6 series (reviewed, 2). Eicosanoid production and ensuing inflammation have been correlated with the amount of AA consumed in the diet (24–26).

Essential fatty acid deficiency has been used as an extreme dietary means to deplete AA in key lipid pools, resulting in an antiinflammatory effect that, in part, is due to decreased synthesis of proinflammatory eicosanoids (reviewed, 7,8). Notable ramifications are decreased synthesis of prostaglandins and leukotrienes, a decreased number of resident macrophages in tissues, and a decreased influx of neutrophils and macrophages into sites of inflammation (27–29). The correlative factor associated with compromised macrophage or neutrophil function appears to be partial depletion and accompanying unavailability of AA in key lipid pools.

Adjuvant-induced arthritis (AIA) in the rat is a chronic inflammatory disease model that has many facets in common with human rheumatoid arthritis (reviewed, 30). As such, the model has been used for several decades as a predictor of clinical efficacy of antiinflammatory drugs. The disease is initiated by the injection of a mycobacterium/mineral oil suspension (complete Freund's adjuvant) into the hind footpad of Lewis rats. Progression of the disease proceeds quickly. Acute inflammation occurs in the primary footpad within 24 h. After a plateau period, severity increases dramatically between days 12–15, at which time symptoms occur in the noninjected, secondary hind footpad. Maximal severity is

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Abbreviations: AA, arachidonic acid; AIA, adjuvant-induced arthritis; bid, twice a day dosing; COX, cyclooxygenase; EFAD, essential fatty acid-deficient; ig, intragastric; INDO, indomethacin; LA, linoleic acid; mpk, milligram per kilogram; MPO, myeloperoxidase; NSAIDs, nonsteroidal antiinflammatory drugs; qd, once a day dosing.

achieved by day 25, while joint deformity occurs by day 35. It is well established that inflammation in AIA is prostaglandin-mediated; nonsteroidal antiinflammatory drugs (NSAIDs) which inhibit cyclooxygenase (COX) activity, notably inducible COX-2 activity, mitigate the inflammatory response, principally owing to inhibition of synthesis of proinflammatory eicosanoids derived from AA (31). The literature is replete with reports on attempts to improve the arthritic index in humans by decreasing the level of AA by dietary means, mainly by fish oil supplementation (reviewed, 22). However, there is a paucity of reports describing the effect that dietary manipulations have on the severity of AIA, especially the effect of dietary AA. Lawrence (32) reported that diets enriched with n-6 polyunsaturated fatty acids exacerbated AIA. Denko (33) reported decreased hind footpad swelling in EFAD rats; edema was restored upon supplemental feeding of corn oil, suggesting a role of n-6 polyunsaturated fatty acids.

The purpose of this study was to examine whether correlations existed in AIA between the level of dietary AA, the ensuing level of plasma AA, and the levels of hind footpad inflammatory markers [i.e., edema, eicosanoids, and myeloperoxidase (MPO) activity]. EFAD rats were utilized because they are severely depleted of both LA and AA. Dietary LA was evaluated along with dietary AA because AA is synthesized from LA *in vivo* by sequential Δ6 desaturation, elongation, and Δ5 desaturation (reviewed, 34). The amount of LA or AA fed to rats was calculated by allometric scaling based on caloric intake from humans to rats, assuming the same intake of fatty acids per kcal per day. Daily feeding of LA (0.5× the human daily equivalent) or AA (1× or 10× the human daily equivalent) to EFAD rats was employed to control repletion of LA and/or AA and to define the relationship between dietary AA and the inflammatory response evoked during AIA. By assuming the validity of allometric scaling based on caloric intake between rodents and humans, the data suggest that, in humans, consumption of the average daily amount of AA would not exacerbate the severity of prostaglandin-mediated inflammation.

MATERIALS AND METHODS

Materials and reagents. Authentic fatty acids and fatty acid methyl esters were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). The vehicle used for oral dosing consisted of 0.5% methylcellulose + 0.025% Tween-20 (polyoxyethylene-sorbital monolaurate), both purchased from Sigma Chemical Co. (St. Louis, MO). The ethyl ester of LA or AA was emulsified in vehicle by sonication and administered orally (0.6 mL/dosing), qd (once a day dosing) (p.m.), starting on day 0 and ending on day 25. Control groups not receiving the fatty acid ethyl esters were gavaged with vehicle only. Indomethacin was purchased from Sigma Chemical Co. and was prepared as a suspension in vehicle by sonication. All solvents and reagents were of analytical grade.

Dietary paradigms and induction of arthritis. Male, Lewis rats were received at 3 wk of age (Harlan Sprague-Dawley, Indianapolis, IN) and were maintained on a a standard rodent diet (Certified Rodent Diet #5002; PMI Feeds Inc., St. Louis, MO), herein referred to as the Control diet, or an EFAD diet (#5803C, low essential fatty acid P.D., Purina Test Diets, Richmond, IN). Water was provided *ad libitum* throughout. The composition of the two diets was as follows:

(i) Control diet (w/w): 55.0% carbohydrates, 20.1% protein, and 4.5% fat (LA, 2.15%; AA, <0.01%). Specific ingredients are ground yellow corn, soybean meal, ground wheat, wheat middlings, fish meal, wheat germ meal, brewer's dried yeast, cane molasses, dried beet pulp, ground oats, alfalfa meal, dried whey, ground soybean hulls, soybean oil, casein, calcium carbonate, dicalcium phosphate, salt, DL-methionine, choline chloride, vitamin A acetate, cholecalciferol, cyanocobalamin, calcium pantothenate, folic acid, riboflavin, thiamine mononitrate, nicotinic acid, pyridoxine hydrochloride, di-alpha tocopheryl acetate, calcium iodate, cobalt carbonate, copper sulfate, manganous oxide, zinc oxide, ferrous carbonate, and zinc sulfate.

(ii) EFAD diet: 21% vitamin-free casein, 69% sucrose, 3% solka floc, 2% PMI vitamin mix, 5% PMI mineral mix #10, 0.15% DL-methionine, and 0.2% choline chloride. Fat, 0.1% (w/w), was composed principally of saturated and monounsaturated fatty acids.

The fatty acid composition of the Control and EFAD diets is shown in Table 1.

Allometric scaling based on caloric intake (percentage energy) was used to calculate the approximate human daily equivalent of LA (545 mg/day) or AA (5.5 mg/day) administered to the rat (Lab Diet™, The Richmond Standard™, *Animal Diet Reference Guide,* PMI Feeds, Inc.) (Table 2).

A state of essential fatty acid deficiency was confirmed by fatty acid composition analysis of plasma; the ratio of Mead acid (20:3n-9)/AA(20:4n-6) was approximately 6 at the end of the study, much higher than the defined minimal value of 0.4 for essential fatty acid deficiency (6). Refer to Scheme 1 for a summary of the testing scheme.

Arthritis was induced on day 0, at which time all of the rats were nearly 13 wk old, having been fed either the Control or EFAD diet for nearly 10 wk. To induce arthritis, the rats were injected with 1 mg of *Mycobacterium butyricum* (Difco Lab-

TABLE 1
Fatty Acid Composition of the Control and EFAD Diets[a]

Fatty acid	Control diet	EFAD diet
16:0	22.8 ± 0.1	40.6 ± 1.3
16:1n-7	4.0 ± 0.1	5.4 ± 0.3
18:0	6.5 ± 0.1	14.3 ± 0.1
18:1n-9	23.6 ± 0.2	36.3 ± 0.9
18:2n-6	35.6 ± 0.1	3.3 ± 0.8
18:3n-3	5.0 ± 0.1	n.d.
20:4n-6	0.2 ± 0.0	n.d.
20:5n-3	1.4 ± 0.0	n.d.
22:6n-3	1.0 ± 0.1	n.d.

[a]n.d., Not detectable (<0.1%). The amounts are expressed as relative percentage (average ± SEM, $n = 3$ replicate samples). EFAD, essential fatty acid-deficient; Control, rats fed standard laboratory chow diet.

		Measure paw edema	
---------->		DAY 0 ---------->	DAY 25
Rats, 21 d of age	Feed EFAD or Control diet for 68 d	Inject mycogen LA/AA supplementation INDOmethacin treatment	Measure: Paw eicosanoids Paw myeloperoxidase (MPO) Plasma fatty acid

SCHEME 1

oratories, Inc., Detroit, MI) in 25 μL of mineral oil (Mallinckrodt, Paris, KY) into the right-hind footpad (35). The rats were sacrificed on day 25, at which time the arthritic response was maximal. At the start of the experiment (day 0), the rats were divided into nine groups. Groups 1–7 were injected with mycogen (n = 10/group). The specific manipulations of each group were as follows: (i) Control: Rats were maintained on a control diet throughout. This "chow" diet has been used routinely to evaluate NSAIDs in this model and yields very reproducible results. The rats were gavaged with vehicle [intragastric (ig), bid (twice a day dosing)] from days 0–25. (ii) Acute EFAD: Rats were fed the control diet for 68 d, after which they were switched to an EFAD diet (day 0) until the end of the experiment (day 25). (iii) INDO: Rats were fed the control diet throughout and dosed orally with indomethacin [1 milligram per kilogram (mpk), ig, bid], starting on day 0. Dosing with indomethacin was maintained until the end of the experiment (day 25). (iv) CHRONIC EFAD (referred to as EFAD): Rats at 21 d of age were maintained on the EFAD diet for 68 d prior to the start of the experiment (day 0). Feeding of the EFAD diet was continued until the end of the experiment (day 25). (v) EFAD + 1× AA: Chronic EFAD rats were gavaged (ig, qd) with 1× AA (5.5 mg/day) from day 0 through day 25. (vi) EFAD + 10× AA: Chronic EFAD rats were gavaged (ig, qd) with 10× AA (55 mg/day) from day 0 through day 25. (vii) EFAD + 0.5× LA: Chronic EFAD rats were gavaged (ig, qd) with 0.5× LA (273 mg/day) from day 0 through day 25. This dosing of LA was chosen because in previous experiments it was found that bolus dosing with 1× LA (545 mg/day) led to a 20–30% mortality. The dosing of LA was therefore halved. Groups 8–9 served as control groups, not being injected with mycogen (n = 6/group). (viii) Control/(−) Mycogen. (ix) EFAD/(−)Mycogen. Results from these negative control groups are not reported because they were either baseline (e.g., edema and prostaglandin levels) or were no different than the mycogen-treated groups (e.g., fatty acid composition).

For all groups, the rats were weighed and both right- and left-hind paw volumes were measured twice weekly during the course of the experiment, starting just before injection on day 0. Footpad volume was determined using a model 7150 plethysmometer (Ugo Basile, Camerio-Varese, Italy). On day 25, blood was collected from unfasted rats by retro-orbital puncture for analysis of plasma fatty acids.

Eicosanoid extraction and quantification. On day 25, the rats were euthanized and five of the secondary (left) footpads from each group were injected with 400 μL of sterile saline containing 10 μM indomethacin (15 mM stock solution in ethanol; Sigma Chemical Co.). The footpads were amputated and subjected to centrifugation (5000 × g) to collect the footpad fluid. The fluid extracted was clear, indicating that it was devoid of blood. Furthermore, in order to prevent any possible clotting of the fluid, four drops of a solution of heparin (Na salt, 10,000 U/mL in saline; Sigma Chemical Co.) were placed at the bottom of each centrifuge tube, and the solution was allowed to dry before beginning centrifugation. Eicosanoid levels of prostaglandin E_2 (PGE_2), thromboxane B_2 (TxB_2), 6-keto-$PGF_{1\alpha}$ (6-keto), leukotriene C_4 (LTC_4), and LTB_4 were quantified directly in the hind-footpad fluid by standard ELISA (Caymen Chemical Co., Ann Arbor, MI). Eicosanoid content is expressed on a whole-footpad basis in order to correlate it to footpad edema.

MPO assay. Amputated hind footpads were frozen in liquid nitrogen, and the paws were then pulverized into small pieces. The pieces were homogenized in phosphate buffer and subjected to centrifugation at 35,000 × g for 20 min. The supernatant was discarded, and the homogenization was repeated in the presence of 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co.), followed by sonication using

TABLE 2
Allometric Scaling Based on Caloric Intake (energy percentage) Used to Calculate Human Daily Equivalent of Linoleic Acid (LA) or Arachidonic Acid (AA) Given to the Rat

	Actual intake		Extrapolated intake (energy percentage)
	Human[a]	Rat[b]	Rat[c]
LA	20 g/day = 10 mg/kcal/day	290 mg/day = 5.3 mg/kcal/day	545 mg/day
AA	200 mg/day = 0.1 mg/kcal/day	<1.0 mg/day = <0.018 mg/kcal/day	5.5 mg/day

[a]Based on estimated amounts from the literature. Approximations: rat: average body weight: 0.25 kg; Consumption of Control diet—by weight: 13.5 g/day (12–15 g/day); by energy: 54.5 kcal/day (based on 55.0% CHO, 20.1% PRO, 4.5% fat (wt%); gross energy = 4.04 kcal/g). Human: average body weight: 70 kg; caloric consumption: 2000 kcal/day.
[b]Based on amount of LA or AA as stated by the manufacturer, assuming an average of 13.5 g diet consumed per day.
[c]Daily consumption of AA equivalent to that of humans (energy percentage). LA: 10 mg/kcal/day × 54.5 kcal/day = 545 mg/day. AA: 0.1 mg/kcal/day × 54.5 kcal/day = 5.5 mg/day.

a W385 ultrasonic processor (Heat Systems-Ultrasonics, Inc., Farmingdale, NY). The samples were subjected to freeze–thaw three times, followed by sonication and centrifugation. The final assay buffer was a mixture of 16.7 mg *o*-dianisidine HCl (Sigma Chemical Co.) per 10 mL distilled water, 1.6 µL of 30% hydrogen peroxide (Sigma Chemical Co.), and 90 mL of 50 mM phosphate buffer (pH 6.0). Seven µL of sample was added to 200 µL of buffer and then analyzed using an EL 340 Biokinetics Reader (Bio-TEK Instruments, Winooski, VT) (450 nM wavelength). Standard curves were made using MPO purified from human neutrophils (Calbiochem, La Jolla, CA).

Fatty acid composition analysis. Plasma fatty acid composition was determined by gas chromatography using electron capture detection. Twenty µL plasma was spiked with 5 µg heneicosanoic acid (21:0; 0.1 mg/mL stock in hexane) and evaporated to dryness under N_2 gas. Lipids were saponified directly by the addition of 1 mL of 2 N KOH/MeOH (4:1) and incubation of the samples in a 60°C water bath for 1 h. Fatty acids were protonated by the addition of 1 mL 88% formic acid and then extracted into hexane by the addition of 1 mL water + 3 mL hexane followed by thorough mixing. The hexane layer was removed and transferred to a new tube containing a small amount of anhydrous Na_2SO_4 to remove any residual water. The hexane layer was transferred to another new tube and evaported to dryness under N_2 gas. For electron capture detection, the fatty acids were derivatized to pentafluorobenzyl esters by the addition of 10 µL diisopropyl ethylamine (Sigma Chemical Co.) + 20 µL 35% pentafluorobenzylbromide in acetonitrile (Pierce Chemical Co., Rockford, IL) in tightly sealed tubes. The samples were incubated for 15 min in a 50°C water bath, after which the contents were evaporated under N_2 gas. One mL hexane was added, and the samples were washed twice with 1 mL water. The hexane layer was removed and evaporated under N_2 gas. The sample residue was resolubilized in 1 mL hexane and then transferred to a gas chromatographic autosampler vial. Derivatized fatty acids were separated and identified using a Hewlett-Packard model 5880 gas chromatograph (Palo Alto, CA) equipped with a fused-silica capillary column (30 m, 0.32 mm i.d., 0.20 µm film thickness; Supelco, Bellefonte, PA), electron capture detector, and an HP-5880A terminal integrator. Fatty acid pentafluorobenzyl esters were identified by comigration with authentic pentafluorobenzyl ester standards. Results are expressed as relative percentage ± SEM.

Statistical analysis. Primary analysis: The F-test with one-way analysis of variance model was first conducted to test whether the mean endpoint values were identical in the nine groups. If significant ($P = 0.05$), the Dunnett's multiple comparison test was then conducted to compare each group with the Control group ($P = 0.05$). Significant differences are indicated by a "*" (Figs. 1–5). Secondary analysis: If there were significant differences in the means between the EFAD and Control groups in the primary analysis, a secondary analysis was conducted to evaluate the effects of LA or AA fed to EFAD rats. The same methods were used as described for the primary analysis, only EFAD served as the control group. Significant differences are indicated by a "†" (Figs. 2–4).

RESULTS

Effect of diet on weight. The initial weights of the rats in the Chow groups (Fig. 1A) were higher than in the EFAD groups (Fig. 1B). Rats in the Control/(–)Mycogen control group gained weight throughout the experiment. Rats in the Control and Acute EFAD groups lost weight during the course of the experiment, owing to the increasing severity of the arthritis. Rats in the INDO group maintained a relatively constant body weight, owing to mitigation of the arthritis by indomethacin. The weights of the rats fed an EFAD diet remained relatively constant during the experimental period.

Effect of diet on plasma fatty acid content. The fatty acid composition of plasma obtained at day 25, the experimental endpoint, is shown for all the groups in Figure 2 (A–E). Mead acid (20:3n-9), the hallmark fatty acid of essential fatty acid deficiency, is synthesized from oleic acid (18:1n-9) as a compensatory fatty acid for AA when mammals are deprived of

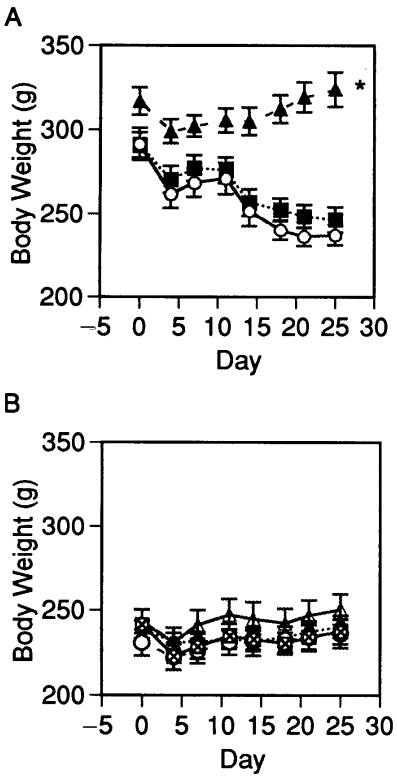

FIG. 1. Body weights of Control (chow groups) (A) or essential fatty acid-deficient (EFAD) (B) groups during the course of adjuvant-induced arthritis. All groups were injected with mycogen on day 0. Body weights (mean ± SEM) were recorded twice weekly for the duration of the experiment. For all groups, $n = 10$, except EFAD + 0.5× LA ($n = 7$). Symbols next to the last data point (day 25) indicate significant differences between the Control (*) or EFAD (†) group ($P < 0.05$). (A) ···■···, Control; —○—, Acute EFAD; -▲--, INDO. (B) ···●···, EFAD; -○--, EFAD + 1× AA; —△—, EFAD + 10× AA; —⊠—, EFAD + 0.5× LA. Abbreviations: INDO, indomethacin; AA, arachidonic acid; LA, linoleic acid.

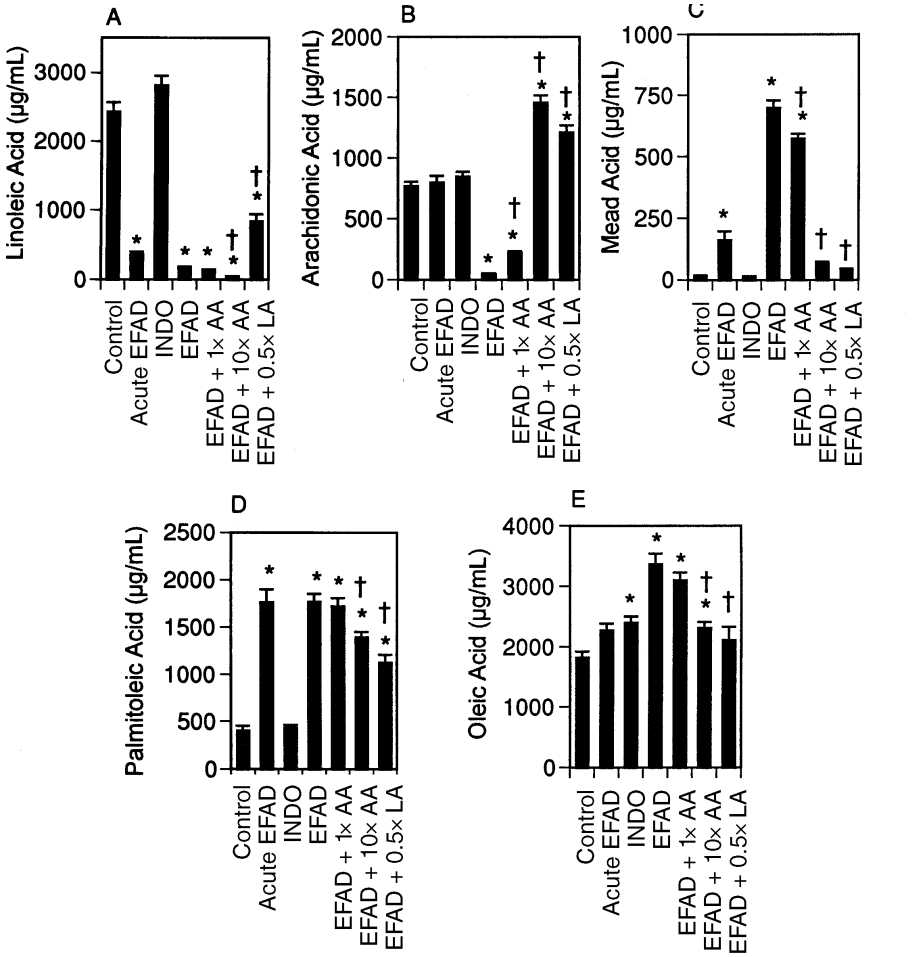

FIG. 2. Plasma fatty acid composition. (A) Linoleic acid (LA) (18:2n-6). (B) Arachidonic acid (AA) (20:4n-6). (C) Mead acid (20:3n-9). (D) Palmitoleic acid (16:1n-7). (E) Oleic acid (18:1n-9). The fatty acid content of plasma was analyzed for each group on day 25, the experimental endpoint. For all groups, $n = 10$, except EFAD + 0.5× LA ($n = 7$). Results are expressed as relative % of total fatty acids (mean ± SEM). Symbols above each bar indicate significant differences between the Control (*) or EFAD (†) group ($P < 0.05$).

n-6 essential fatty acids (reviewed, 6). Palmitoleic (16:1n-7) and oleic acids also accumulate to higher levels in EFAD animals due to induction of the Δ9 desaturase (36).

In the chow-fed groups, the development of arthritis or its mitigation by indomethacin did not cause any alteration in the fatty acid composition; the fatty acid composition remained the same in the Control and INDO groups.

Short-term feeding of the EFAD diet (Acute EFAD) caused changes to occur in some of the fatty acids. LA decreased dramatically (Fig. 2A), whereas AA, the major endproduct of LA metabolism, remained unchanged (Fig. 2B). Mead acid increased (Fig. 2C), as did palmitoleic acid (Fig. 2D) and oleic acid (Fig. 2E).

Chronic feeding of the EFAD diet caused similar changes in fatty acid composition to occur as in Acute EFAD, except that the differences compared to Chow were more extreme; decreases in the n-6 polyunsaturated fatty acids, LA and AA, were compensated for by increases in palmitoleic acid, oleic acid, and Mead acid. The single exception was AA, which was severely depleted in EFAD, but not in Acute EFAD (Fig. 2B). In EFAD rats, the level of AA was only partially restored upon feeding the 1× human daily equivalent of AA (EFAD + 1× AA; Fig. 2B); palmitoleic, oleic, and Mead acids remained relatively high to compensate for the partial depletion of AA. Full repletion of AA occurred only upon feeding the 10× human daily equivalent of AA (EFAD + 10× AA; Fig. 2B); substantive decreases occurred in palmitoleic, oleic, and Mead acids to compensate for the repletion of AA. Feeding the 0.5× human daily equivalent of LA (EFAD + 0.5× LA) gave similar results as did EFAD + 10× AA; there was full repletion of AA and corresponding decreases in the levels of palmitoleic, oleic, and Mead acids. Even though 0.5× LA caused full repletion of AA (Fig. 2B), it caused only partial repletion of LA (Fig. 2A), indicating that LA was quickly metabolized to AA *in vivo*. The saturated fatty acids, stearic (18:0) and palmitic (16:0) acids were unaltered by diet (data not shown).

Effect of diet on hind-footpad edema. The time course of

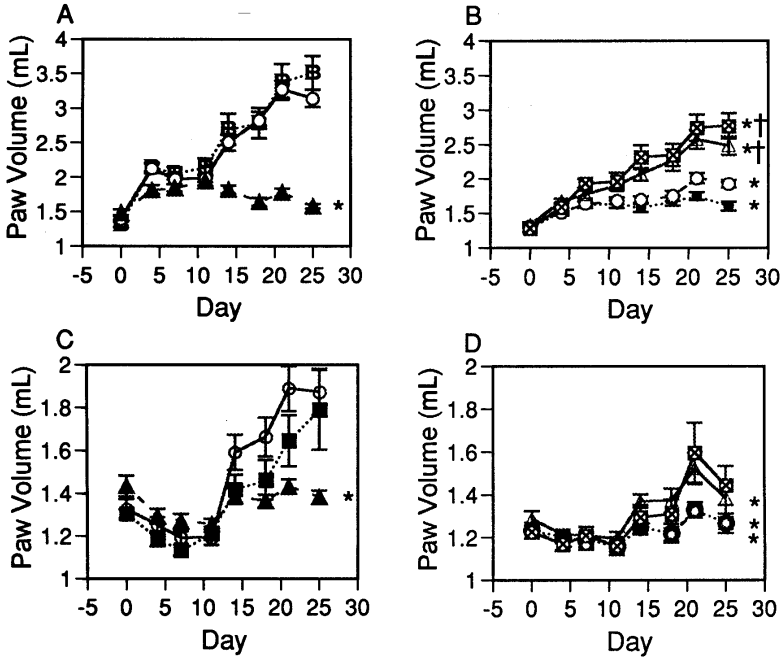

FIG. 3. Time course of mycogen-induced edema in primary and secondary footpads. Mycogen was injected into the right-hind footpads on day 0. Swelling was most apparent in mycogen-injected (primary) footpads, but was also evident in the contralateral, uninjected (secondary) footpads after the second week. For all groups $n = 10$, except EFAD + 0.5× LA ($n = 7$). Results are expressed as paw volume (mL) ± SEM. Symbols next to the last data point (day 25) indicate significant differences between the Control (*) or EFAD (†) group ($P < 0.05$). (A) Control, primary; (B) EFAD, primary; (C) Control, secondary; (D) EFAD, secondary. See Figure 1 for abbreviations. (A) ···■···, Control; —○—, Acute EFAD; -▲-, INDO. (B) ···●···, EFAD; -○-, EFAD + 1× AA; —△—, EFAD + 10× AA; —⊠—, EFAD + 0.5× LA.

mycogen-induced edema is shown for the primary footpads in Figure 3A (Control) and Figure 3B (EFAD) and for the secondary footpads in Figure 3C (Control) and Figure 3D (EFAD). The results were qualitatively the same for the primary and secondary footpads from a given group. The only difference was that the severity of edema was greater in the primary footpads compared to the corresponding secondary footpads. Increases in edema in the primary and secondary footpads in the Control group and mitigation of edema in the INDO group followed the expected progression. Nearly identical increases in edema in the primary and secondary footpads occurred in the Acute EFAD group as in the Control group (Fig. 3A and C), demonstrating that short-term intervention with an EFAD diet was not efficacious. In contrast, edema was mitigated markedly in the chronic EFAD group throughout the time course of the experiment; some initial swelling occurred between days 0–4, after which it plateaued (Fig. 3B and D). On day 25, edema in the EFAD group (Fig. 3B and D) was very similar to that in the INDO group (Fig. 3A and C), demonstrating that EFAD had comparable efficacy as indomethacin. These results are in contrast to the lack of an antiinflammatory response in the Acute EFAD group (Fig. 3A and C). Hence, long-term intervention with an EFAD diet was necessary to evoke an antiinflammatory response.

In the chronic EFAD group, the beneficial effect of EFAD was maintained, even when the rats were dosed with the 1× human daily equivalent of AA (EFAD + 1× AA) (Fig. 3A and B). Compared to the EFAD group, there was perhaps a marginal increase in edema in the EFAD + 1× AA group, but only at the latter time points. In order for dietary AA to evoke a substantial increase in edema, a dosing of the 10× human daily equivalent of AA (EFAD + 10× AA) was necessary (Fig. 3B and D). Significantly, feeding of the 0.5× human daily equivalent of LA was sufficient to increase edema to the same extent as with the 10× human daily equivalent of AA (Fig. 3B and D). It must be kept in mind, however, that the absolute mass of LA at the 0.5× human equivalent (233 mg/day) was still fourfold more than the absolute mass of AA at the 10× human equivalent of AA (57 mg/day). The degree of edema was elevated in animal groups where plasma AA was substantially higher than the EFAD level (i.e., EFAD + 10× AA and EFAD + 0.5× LA).

Effect of diet on hind-footpad eicosanoid levels. The levels of PGE_2, TxB_2, 6-keto-$PGF_{1\alpha}$, and LTC_4 extracted from secondary hind-footpad tissue (Fig. 4A–D) paralleled the level of plasma AA (c.f. Fig. 2B) and the degree of edema (c.f. Fig. 3A–B) in the corresponding Control or EFAD group. LTB_4 was undetectable in any of the control or treatment groups (<20 pg/mL or <2–3 pg/footpad). As predicted from the edema results, eicosanoid levels from the Control and Acute EFAD groups were relatively high. In contrast, the lowest eicosanoid levels were from the INDO and EFAD

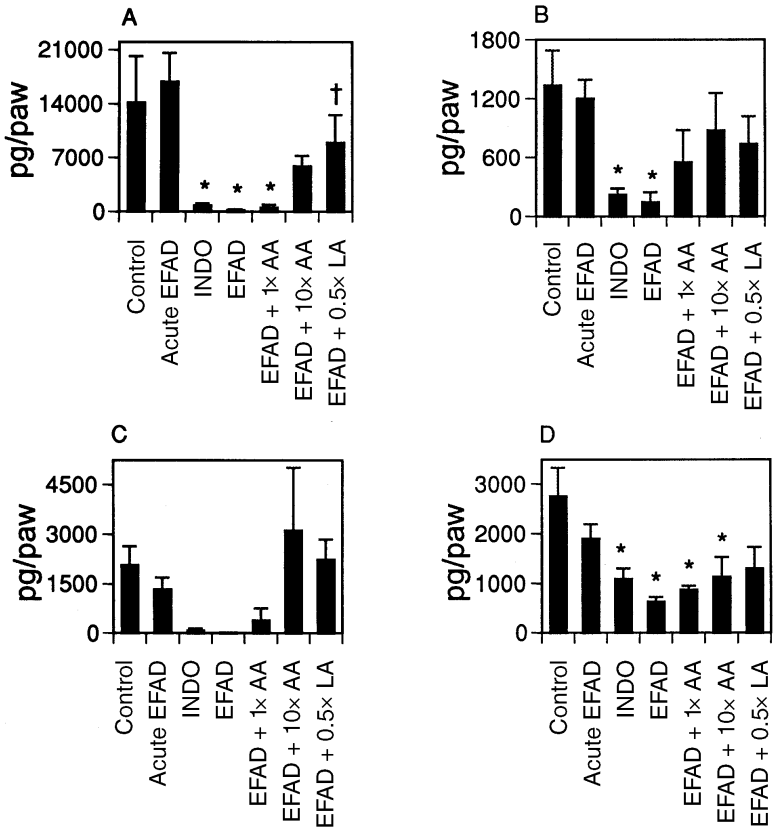

FIG. 4. Eicosanoid levels in hind-footpad extracts. Eicosanoids were quantified by standard ELISA in extracts from secondary hind footpads at day 25, the experimental endpoint. For all groups, $n = 5$, except EFAD + 0.5× LA ($n = 4$). Symbols above each bar indicate significant differences between the Control (*) or EFAD (†) group ($P < 0.05$). (A) PGE_2, (B) TxB_2, (C) 6-keto $PGF_{1\alpha}$, (D) LTC_4. See Figure 1 for abbreviations.

groups. EFAD was just as efficacious in decreasing PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis as in decreasing edema and was comparable to indomethacin. The antiinflammatory properties of EFAD were also observed in unchallenged rats; there were lower basal levels of PGE_2, TxB_2, and 6-keto-$PGF_{1\alpha}$ in the EFAD/(−)Mycogen group compared to the Control/(−)Mycogen group (data not shown). Dietary supplementation with 1× the human daily equivalent of AA (EFAD + 1× AA) only partially restored eicosanoid levels compared to Control. In order to more fully restore eicosanoid levels, but not to completion, the 10× human daily equivalent of AA (EFAD + 10× AA) or the 0.5× human daily equivalent of LA (EFAD + 0.5× LA) had to be administered.

Effect of diet on neutrophil infiltration. MPO activity, an enzyme marker of neutrophils (37), was quantified in secondary hind-footpad extracts as a measurement of neutrophil infiltration. MPO activity was readily detected and was not greatly affected by diet, even when comparing Control vs. EFAD groups (Fig. 5).

DISCUSSION

AA appears to have a unique preference in mammals compared to other polyunsaturated fatty acids; high-level feeding of AA, but not LA, augmented tissue levels of AA and ensuing eicosanoid production beyond control levels obtained in animals fed a normal chow diet (24,25). The relatively high

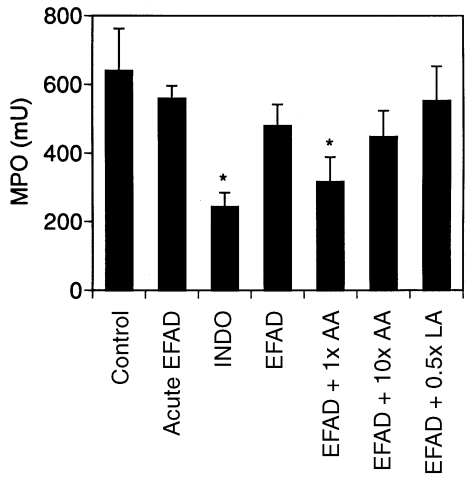

FIG. 5. Myeloperoxidase (MPO) activity in secondary hind-footpad extracts. MPO activity (mU ± SEM) was used as an indicator of neutrophil infiltration at day 25, the experimental endpoint. For all groups, $n = 5$, except EFAD + 0.5× LA ($n = 3$). Symbols above each bar indicate significant differences between the Control (*) or EFAD (†) group ($P < 0.05$).

consumption of n-6 polyunsaturated fatty acids in the Western diet (reviewed, 2), namely, LA (approx. 20 g/day), and, to a much lesser extent, AA (approx. 200 mg/day), raises the possibility that arthritis or other chronic inflammatory diseases could be exacerbated by AA obtained directly in the diet or metabolized *in vivo* from LA. A relatively large clinical effort has attempted to evaluate the role of AA in rheumatoid arthritis, mainly by dietary supplementation with n-3 polyunsaturated fatty acids (20:5n-3 and 22:6n-3) as a means of supplanting existing AA (reviewed, 22). Yet, equivocal results have been obtained in all of these studies, mainly because AA cannot be easily supplanted by 20:5n-3 or 22:6n-3, especially if AA is present in any fish oil-supplemented diet (38,39).

The role of dietary AA in AIA is relevant because proinflammatory eicosanoids derived from AA have been shown to be major contributors of disease severity (31). Essential fatty acid deficiency provides a testable way to evaluate the role of dietary AA in AIA, a rat model of arthritis that mimics many facets of rheumatoid arthritis in humans. Denko (33) showed that EFAD mitigated the severity of arthritis in AIA and that the mitigation was reversed by feeding the animals a corn oil diet. Corn oil is enriched in LA but is totally lacking in AA. Without providing direct evidence, Denko theorized that the LA was metabolized to AA *in vivo* and that proinflammatory eicosanoids derived from AA contributed, in large part, to the inflammatory process. Since EFAD animals are severely depleted of AA, repletion of AA could be titrated precisely by daily feeding of AA. The distribution and metabolism of orally administered AA are similar between EFAD and chow-fed rats (40), lending further validity to the use of the EFAD model as a means to evaluate the effects of dietary AA in AIA. The relevance to the human situation was addressed by feeding rats the daily human equivalent of AA, based on allometric scaling of caloric intake (24). In chronic EFAD rats which were fed either the 1× or 10× human daily equivalent of AA or the 0.5× human daily equivalent of LA, clear correlations were established between the levels of plasma AA (Fig. 2B), hind-footpad edema (Fig. 3B and D), and eicosanoids extracted from edematous hind-footpad tissue (Fig. 4). In contrast, there was no correlation between neutrophil infiltration, as determined by MPO activity, into edematous hind-footpad tissue and the level of plasma AA (Fig. 5). Even though edema and eicosanoid synthesis were markedly decreased in EFAD rats, neutrophil infiltration into secondary hind-footpads occurred unimpeded. This is consistent with data showing that EFAD had no effect on neutrophil infiltration in immune-mediated glomerulonephritis in rats (41).

It was anticipated that acute EFAD would mitigate the severity of arthritis by eliminating AA and its precursor, LA, in the diet. However, acute EFAD did not mitigate edema whatsoever (Fig. 3A and C). Despite the acute EFAD condition, the level of AA remained high in plasma (Fig. 2B) and most likely provided sufficient pools of AA for the synthesis of proinflammatory eicosanoids in hind-footpad tissue (Fig. 4). In contrast to the recalcitrance of AA, rather pronounced changes occurred in other fatty acids during acute EFAD: palmitoleic, oleic, and Mead acids increased, whereas LA decreased. The decrease in LA was striking (Fig. 2A), suggesting that a portion of it was utilized to maintain the level of AA. During the EFAD state, the Δ9 desaturase is induced (36), which would account for the higher levels of palmitoleic, oleic, and Mead acids. These results demonstrated the tenacity with which AA was retained in lipid pools and suggested that long-term curtailment of ingestion of LA or AA might be required to deplete AA sufficiently to manifest an antiinflammatory response. While dietary intervention is probably not feasible, a more acceptable way to decrease AA, besides fish oil supplementation, might be to inhibit Δ6- or Δ5-desaturase activity chronically. Such intervention might result in the antiinflammatory effects associated with EFAD without the accompanying untoward side effects of EFAD (e.g., hair loss, impaired fertility, and psoriatic-type skin) because LA would be present in the diet. Pharmacologic inhibition of the synthesis of AA would allow for LA to be consumed in the diet and, if inhibited at the level of the Δ5-desaturase, for dihomo-γ-linolenic acid (20:3n-6) to serve as the source for PGE_1, thus maintaining gastric and renal function. Also, leukotrienes would not be synthesized from dihomo-γ-linolenic acid because the Δ5 double bond is absent, eliminating another source of proinflammatory mediators. In contrast, no prostaglandins or leukotrienes are synthesized from LA.

The role of dietary AA in modulating the arthritic response in AIA was evaluated by feeding 1× or 10× the human daily equivalent of AA or 0.5× the human daily equivalent of LA to EFAD rats. Since arthritis starts to escalate near day 14, it was reasoned that 2 wk of feeding prior to the arthritic escalation would be sufficient to reach a steady-state level of AA in plasma and inflammatory cells. During the period of maximal arthritic escalation (days 14–25), the AA would then be used as a substrate for the synthesis of proinflammatory eicosanoids. The 1× human daily equivalent of AA was insufficient to restore the plasma level of AA to that of the chow control group (Fig. 2B). In parallel, Mead acid (Fig. 2C), as well as palmitoleic (Fig. 2D) and oleic (Fig. 2E) acids, all products of the Δ9-desaturase, did not decrease in plasma. Based on these results, it was clear why 1× AA was unable to elicit a rebound in edema (Fig. 3) or an increase in eicosanoid production (Fig. 4) in hind footpads. Rather, the 10× human daily equivalent of AA was required to supplant palmitoleic acid (Fig. 2D), oleic acid (Fig. 2E), and, particularly, Mead acid (Fig. 2C) in plasma. Even with full repletion of AA in plasma, there was only a partial rebound in both edema (Fig. 3) and eicosanoid levels in hind footpads (Fig. 4). LA dosed at the 0.5× human daily equivalent was equipotent to 10× AA; AA, synthesized from LA *in vivo* (reviewed, 34), increased to the same level in plasma as in the Control group (Fig. 2B), while palmitoleic (Fig. 2D), oleic (Fig. 2E), and Mead (Fig. 2C) acids decreased. Both edema (Fig. 3) and eicosanoid levels (Fig. 4) rebounded partially in the hind footpads. Even though 0.5× LA resulted in the full repletion of AA in plasma (Fig. 2B), there was only partial repletion of LA in plasma (Fig. 2A). This result, com-

bined with the observation that AA was not depleted during acute EFAD, adds credence to the claim that AA is the most important polyunsaturated fatty acid associated with membrane phospholipids (38).

The combined data suggest strongly that AA obtained directly in the diet or converted from dietary LA by *in vivo* metabolism is an important component of inflammation in AIA; the plasma level of AA (Fig. 2B) correlated with the degree of edema (Fig. 3) and eicosanoid levels extracted from hind footpads (Fig. 4). This would be expected if the progression of AIA depended upon the availability of AA for eicosanoid synthesis. Indeed, prostaglandins derived from AA play a primary role in disease manifestation in AIA (31). Edema and eicosanoid levels were generally highest in the Control and Acute EFAD groups (Figs. 3 and 4). However, the level of plasma AA in those groups was not as high as in the EFAD + 10× AA or EFAD + 0.5× LA groups (Fig. 2B). Thus, while the data indicated that the ready availability of eicosanoid precursor molecules was necessary for disease progression, other factors besides AA, LA, or intermediates between them (i.e., γ-linolenic acid, 18:3n-6, and dihomo-γ-linolenic acid, 20:3n-6) must be involved.

Assuming the validity of allometric scaling based on caloric intake between rodents and humans, the data suggested that in humans the average daily consumption of AA would not exacerbate the severity of prostaglandin-mediated inflammatory diseases. Furthermore, the results suggested that in humans with chronic inflammatory diseases the benefits gained by a low-fat diet would not be compromised by the addition of the average daily amount of AA.

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