

THE ANTIINFLAMMATORY EFFECTS OF ESSENTIAL FATTY ACID DEFICIENCY IN EXPERIMENTAL GLOMERULONEPHRITIS

The Modulation of Macrophage Migration and Eicosanoid Metabolism¹

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Dietary polyunsaturated fatty acid modulation exerts a beneficial effect in immune-mediated glomerulonephritis. To elucidate the mechanisms underlying this phenomenon, the effects of essential fatty acid (EFA) deficiency on the heterologous phase of nephrotoxic nephritis in rats (induced by the injection of a rabbit antglomerular basement membrane antibody) were studied. The heterologous phase of nephrotoxic nephritis was characterized by an invasion of leukocytes into the glomerulus. Polymorphonuclear neutrophils predominated early on (3 h), whereas macrophages predominated at 24 and 72 h. EFA deficiency selectively prevented the influx of macrophages into the glomerulus. The invasion of polymorphonuclear neutrophils, in contrast, was unaffected. The influx of leukocytes into the glomerulus during nephritis was accompanied by a marked enhancement (10- to 40-fold) in glomerular thromboxane and leukotriene B₄ production. EFA deficiency largely attenuated this change. Renal dysfunction during the heterologous phase of nephritis was manifested as azotemia, polyuria, sodium retention, and proteinuria. With EFA deficiency, polyuria, azotemia, and sodium retention were not seen. Proteinuria was reduced by approximately 85%. To address whether the lack of macrophage migration into the glomerulus in the context of nephritis with EFA deficiency might be due to a functional defect in macrophage migration, the chemotactic responsiveness of EFA-deficient macrophages was examined. EFA-deficient macrophages displayed normal chemotactic migration toward activated C. In sum, EFA deficiency prevents the invasion of macrophages into the glomerulus in nephrotoxic nephritis and attenuates the accompanying metabolic and functional alterations, but does not affect macrophage chemotactic responsiveness. Alterations in macrophage elicitation and

lipid mediator generation by inflamed glomeruli thus appear to be central to the salutary effect of dietary polyunsaturated fatty acid modification on glomerulonephritis.

Dietary polyunsaturated fatty acid manipulation exerts a striking beneficial effect on immune-mediated glomerulonephritis. Both EFA³ deficiency and (n-3) fatty acid supplementation have been shown to prevent the lethal glomerulonephritis that occurs in the context of murine models of systemic lupus erythematosus (1-3). The mechanisms underlying this effect, however, are unclear. Suppression of autoantibody formation does not appear to be involved. No unequivocal effects of EFA deficiency on either cellular or humoral immunity have been established (4). Additionally, dietary fatty acid modification exerts a beneficial effect on glomerulonephritis despite the fact that anti-dsDNA antibodies are not consistently suppressed (1, 2).

Recent studies have provided new insights into the salutary effect of dietary polyunsaturated fatty acid modulation on glomerulonephritis. EFAD has been shown to deplete glomeruli of their resident population of macrophages (5). Repletion of EFA-deficient animals with (n-6), but not (n-3) fatty acids, repopulates the glomeruli with macrophages (5). Thus, the presence of these cells within the glomerulus appears to be a specific function of (n-6) fatty acids. EFAD also interferes with glomerular eicosanoid production. EFAD selectively inhibits the production of TxB₂ and PGE₂ by glomeruli in response to the agonist, angiotensin II (5). EFA deficiency additionally decreases the elaboration of LTB₄, the synthesis of which appears to be a function of the presence of the resident mesangial macrophages (6).

In our study, we have examined the effects of EFA deficiency on the cellular, metabolic, and functional alterations that occur in the heterologous phase of nephrotoxic nephritis. The results of this study suggest that a decrease in macrophage elicitation into the glomerulus and an attenuation of glomerular lipid mediator production are critical elements of the beneficial effect of dietary polyunsaturated fatty acid manipulation on glomerulonephritis.

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³ Abbreviations used in this paper: EFA, essential fatty acid; EFAD, essential fatty acid deficiency; LT, leukotriene; Tx, thromboxane; NTS, nephrotoxic serum; PMN, polymorphonuclear neutrophils.

MATERIALS AND METHODS

Materials. Lewis male rats were obtained as weanlings from Harlan Sprague Dawley (Indianapolis, IN). A fat-free diet was purchased from Purina Test Diets (Richmond, IN). The composition and fatty acid content of this diet have been previously published (7). Angiotensin II, shellfish glycogen, zymosan, and DNAase were obtained from Sigma Chemical (St. Louis, MO). Ionomycin was purchased from Calbiochem (La Jolla, CA). Collagenase type II was purchased from Cooper Biochemical (Malvern, PA). PGE₂, Tx_B₂, specific antisera, and iodinated standards were a gift from Dr. Philip Needleman (Department of Pharmacology, Washington University). LTB₄ and specific antiserum was obtained from Merck-Frosst Canada (Pointe Claire-Dorval, Quebec, Canada; gifts of Dr. J. Rokach). [³H]LTB₄ was purchased from New England Nuclear (Boston, MA). Mouse antirat leukocyte common mAb antiserum was obtained from Accurate Chemical and Scientific (Westbury, NY). Fluorescein-conjugated rabbit antimouse antiserum was purchased from Cappel Laboratories (Cochranville, PA).

EFAD. Animals were made EFA-deficient by feeding them a fat-free diet for >8 wk. Hepatic fatty acid composition was periodically monitored during this study using previously published methods (7). Briefly, liver lipids were extracted and the constituent fatty acids transmethylated by the sequential addition of 0.5 N NaOH in methanol and 6 N HCl. Fatty acid methyl esters were extracted, isolated by TLC, and then characterized and quantified by gas chromatography. Authentic standards were used for identification. As shown in Table I, EFA-deficient animals exhibited a decrease in hepatic (n-6) fatty acids and the accumulation of (n-9) fatty acids, particularly 20:3(n-9) which is not present normally. The 20:3(n-9) to arachidonate ratio in liver ranged from 1.8 to 3.8, exceeding by far the minimal criterion for EFA deficiency (i.e., a ratio > 0.4 (8)). Individual experiments were performed with age-matched controls that had been on a control diet an equivalent period to the EFA-deficient animals.

Glomerular isolation and incubation. Glomeruli were isolated from saline-perfused kidneys using a previously detailed sieving protocol (5). Preparations were typically >90% pure. No difference in purity or yield was observed between preparations from normal and EFA-deficient animals. Glomeruli were subsequently permeabilized with collagenase and DNAase as described previously (5). This treatment is necessary to preserve glomerular viability and responses to agonists in vitro.

Glomeruli were incubated in Krebs-Henseleit buffer (approximately 30,000 glomeruli/0.4 ml) for sequential 10-min periods. After two basal periods, glomeruli were washed three times and a basal period of eicosanoid production was performed. Glomeruli were subsequently exposed to angiotensin II 1 μM. Glomeruli were then washed, and a second basal period of eicosanoid production was established. A final incubation using ionomycin 10 μM was performed.

Glomerular supernatants were then assayed for PGE₂, Tx_B₂, and LTB₄ content by specific radioimmunoassays detailed before (9). The values were normalized for the number of glomeruli in the incubation and are expressed as fmol/10³ glomeruli.

Glomerular leukocyte quantification and differential counts. Total leukocyte counts in permeabilized glomeruli were performed using a double antibody fluorescence technique previously detailed (5). Mouse antirat leukocyte common mAb antibody (50 μg/ml) was used as the primary antibody followed by fluorescein-conjugated rabbit antimouse antibody (100 μg/ml). The labeled cell content of isolated glomeruli was evaluated by fluorescence microscopy with a Universal microscope (Carl Zeiss, Thornwood, NY). Cells were quantified by focusing through the glomerulus and counting cells as they appeared in the plane of focus. In each experiment, 50 to 100 glomeruli were counted per point and the results are expressed as

the mean ± SE.

To perform differential counts, glomeruli were dissociated into a single cell suspension by means of a previously described enzymatic protocol (5). Leukocytes were then stained with an immunoperoxidase method using the same primary antibody as above and a Zymed Histostain kit (Zymed Laboratories, San Francisco, CA). Cells were pelleted onto glass slides by cytocentrifuge (Shannon, Astmore, England), fixed in acetone, air-dried, developed for peroxidase, and counterstained with hematoxylin. Positively labeled cells were visualized by light microscopy and categorized by nuclear morphology.

Nephrotoxic nephritis and measurement of renal function. NTS was produced in rabbits by repeated immunization with basement membrane-rich sediment of rat cortex as previously described (10). The NTS was extensively adsorbed with rat IgE and then heated to 56°C for 90 min to deplete IgG. An IgG-enriched fraction was prepared by precipitation of albumin with saturated NH₄SO₄. Albumin was removed to reduce the carry-over of fatty acids by the antiserum. The resulting IgG-enriched fraction was extensively dialyzed against PBS and stored frozen at 28 mg/ml.

Nephritis was induced by injecting the NTS i.v. at a dose of 11 mg/100 g body weight. To assess renal function, the animals were placed in metabolic cages and a 24-h urine collected. A sample of serum was obtained at the time of death for glomerular harvesting. Urine protein was determined using a kit from BioRad (Richmond, CA). Urea, creatinine, sodium, and potassium determinations were performed on urine and serum using a Beckmann Autoanalyzer (Beckman Instruments Inc., Fullerton, CA). Sodium excretion was expressed either as the concentration of sodium in urine (mmol/liter) or total sodium excretion (concentration of sodium × urine volume, μmol). Fractional excretion of sodium was calculated as sodium clearance divided by creatinine clearance ($U_{Na} \times P_{creat}/P_{Na} \times U_{creat}$).

Glomerular antibody binding quantification. Experiments to assess the deposition of the NTS in EFA-deficient and control glomeruli were performed. NTS was injected i.v. as above, and 2 h later glomeruli were perfused and harvested as above. Glomeruli (approximately 20 to 40,000) were then placed in 2% sodium cholate in PBS, rotated for 1 h at 37°C, homogenized, dialyzed against PBS, and placed in a sodium acetate buffer, pH 2.5, for 3 h to elute bound antibody off basement membrane fragments. The dialysate was then microfuged at 10,000 × g for 30 min at 4°C. The supernatants and sediments were subsequently dialyzed against PBS. The concentration of rabbit IgG in supernatants and sediments was then determined by competitive inhibition ELISA according to the following protocol.

Microwell plates were coated with rabbit γ-globulin at a concentration of 500 ng/well in PBS and allowed to remain overnight at 4°C. The plates were then thoroughly washed for 1 h in a solution of PBS containing 1% BSA and 0.05% Tween-80. Goat antirabbit globulin (100 ng) conjugated to horseradish peroxidase was added to each well along with 50 μl of either known concentrations of rabbit γ-globulin, eluate, or sediment (all in 1% BSA in PBS). After 1 h at 4°C, the plates were washed and developed by the addition of o-phenylene diamine (0.01% in distilled water with 0.3% H₂O₂). The reaction was terminated after 30 min by the addition of 10 μl of 1 M H₂SO₄ and read at 492 nm on an automated microplate reader. The assay was linear between 2.5 and 250 ng. Results were normalized for the number of glomeruli in the original homogenate. No inhibition of binding was noted at any dilution of the control glomerular homogenates. Comparison of the acid eluates and sediment in all groups receiving NTS revealed that <1% of the NTS remained bound after acid elution.

Microchemotaxis assay. Chemotaxis was assessed by a modification of the method of Falk et al. (11) using a 48-well microchemotaxis chamber obtained from Neuroprobe (Cabin John, MD). Rat peritoneal macrophages were used as the responder cells. Peritoneal

TABLE I
Validation of EFAD^a

	18:1 (n-9)	18:2 (n-6)	20:3 (n-9)	20:4 (n-6)	20:3 (n-9) 20:4 (n-6)
EFA deficient (n = 5)	36.8 ± 1.0 ^b	1.0 ± 0.1 ^c	6.6 ± 0.6 ^c	2.9 ± 0.4 ^b	2.4 ± 0.4 ^c
Controls (n = 4)	12.8 ± 0.9	9.0 ± 1.7	0.0	10.5 ± 2.4	0.0

^a Livers from EFA-deficient and control animals were removed and the lipids extracted. The fatty acid composition was determined by transmethylating the fatty acids and characterizing them by gas chromatography as detailed in Materials and Methods.

^b Indicates p < 0.01, control vs EFA deficient.
^c Indicates p < 0.05, control vs EFA-deficient.

macrophages from EFA-deficient and normal rats were obtained by lavage after elicitation with 0.5% shellfish glycogen and suspended in a HBSS. Lipid analysis of shellfish glycogen showed no significant content of (n-6) fatty acids (data not shown). Zymosan-activated serum was obtained by incubating fresh rat serum with 25 mg/ml of zymosan for 30 min at 37°C.

The assay was performed as follows: various dilutions of zymosan-activated serum in HBSS in 25-μl aliquots were placed in the bottom wells of the chamber. HBSS was used alone to assess random migration. To the wells in the upper chamber, 25 × 10⁴ macrophages in a volume of 50 μl of HBSS were added. The chamber was then incubated for 3.5 h at 37°C in a humidified atmosphere of 95% air/5% CO₂. Afterward, the polyvinylpyrrolidone-free polycarbonate filter (5-μm pore size, Nucleopore, Pleasanton, CA) separating the upper and lower halves of the chamber was removed, fixed in methanol and stained with Diff-Quick (American Scientific Products, McGaw Park, IL). Cells that had migrated through the filter were quantified by light microscopy with an optical micrometer. Results are expressed as net cells migrating through the filter per well (total cells minus random migration). Assays were performed in triplicate.

Statistical analysis. Statistical computations were made using STATA (Computing Resource Center, Los Angeles, CA). Where replicates were performed in an experiment the mean ± SEM is expressed. When comparisons were made between multiple groups, ANOVA was done before performing individual comparisons to assure the presence of significant differences. Individual comparisons were then made with Student's *t* test and *p* values are indicated in the text.

RESULTS

Macrophage migration and NTS deposition in nephrotoxic nephritis. The heterologous phase of nephrotoxic nephritis is characterized by a C-dependent glomerular influx of polymorphonuclear neutrophils (PMN) in the first 2 to 4 h followed by a C-independent infiltration of macrophages at 24 h which plateaus by 72 h (12). Initial experiments to examine the effects of EFA deficiency on the macrophage migration into the glomerulus in this model of glomerulonephritis focused on the invasion of macrophages seen 72 h after the injection of NTS. As shown in Table II, nonnephritic EFA-deficient rats exhibited a marked basal depletion in the resident glomerular macrophage population as previously reported (5). Nephritic rats on a control diet demonstrated a threefold increase in glomerular macrophages at 72 h after the injection of NTS (Table II). In contrast, EFA-deficient rats experienced no increase in the number of glomerular macrophages after the administration of NTS (Table II).

To investigate whether this difference in macrophage migration was simply due to alterations in the deposition of NTS within the glomerulus, glomerular deposition of

TABLE II
Effects of EFAD on resident and elicited glomerular macrophages and on NTS deposition

		-NTS	+NTS
Mφ/glomerulus	Control	16.6 ± 1.2	59.3 ± 3.7
	EFAD	2.6 ± 0.4	4.0 ± 0.5
NTS (pg/glomerulus)	Control	0.0	499,715
	EFAD	0.0	530,606

^aEFA-deficient and control animals (*n* = 2 for each) were given NTS and killed 72 h later. EFA-deficient and control animals (*n* = 2 for each) not receiving NTS were also studied. The glomeruli from these animals were perfused and isolated as detailed in Materials and Methods. Glomerular macrophages were then quantified using a double antibody fluorescence technique detailed in Methods. All leukocyte common Ag⁺ cells at these time points are macrophages (32, 33). Fifty glomeruli from each animal in each group were evaluated and the results pooled (expressed as mean ± SE). For control glomeruli, -NTS and +NTS were significantly different (*p* < 0.01). EFA-deficient glomeruli were also significantly different from control (-NTS or +NTS, *p* < 0.01). A competitive ELISA was used to determine glomerular NTS deposition in control and EFA-deficient glomeruli at 72 h (see Materials and Methods). Two replicate determinations are shown.

NTS was quantified in control and EFA-deficient animals. As shown in Table II, there was equivalent deposition of the NTS in EFA-deficient and control animals. Our subsequent investigations were therefore broadened to examine the effects of EFAD on the composition of the cellular infiltrate at 3, 24, and 72 h and to establish correlations with glomerular eicosanoid metabolism and renal function.

Effects of EFA deficiency on glomerular leukocyte infiltration in nephrotoxic nephritis. Glomeruli from normal animals contained a resident population of leukocytes (Fig. 1). During the course of glomerulonephritis, an increase in glomerular leukocyte number was apparent at 3 h (Fig. 1). The influx of leukocytes peaked at 24 h (a threefold increase relative to uninflamed glomeruli) and declined somewhat at 72 h to a level twice that seen in uninflamed glomeruli (Fig. 1). As above, EFA-deficient animals manifested a basal depletion of glomerular leukocytes (Fig. 1). However, 3 h after the injection of NTS glomerular leukocytes in EFA-deficient animals were slightly greater than that seen in control animals (Fig. 1). The number of glomerular leukocytes in EFA-deficient animals subsequently declined at 24 and 72 h to levels approximating the level present in uninflamed EFA-deficient glomeruli (Fig. 1).

Differential analysis of glomerular leukocytes during nephrotoxic nephritis was performed at each of the above time points. As shown in Figure 2, the basal population of leukocytes within the glomeruli of control animals was exclusively macrophages. Glomeruli from EFA-deficient animals exhibited a depletion of this basal population of macrophages (Fig. 2). Then 3 h after the injection of NTS, the leukocytes within the glomeruli from control animals were largely PMN (Fig. 2). This influx of PMN was not affected by EFA deficiency. Glomeruli from EFA-deficient animals contained slightly greater numbers of PMN than glomeruli from control animals at 3 h. Glomerular macrophages numbers actually declined in the first 3 h of nephritis in animals on a control diet and then increased threefold over basal levels at 24 h, declining slightly by 72 h (Fig. 2). In marked contrast, glomeruli

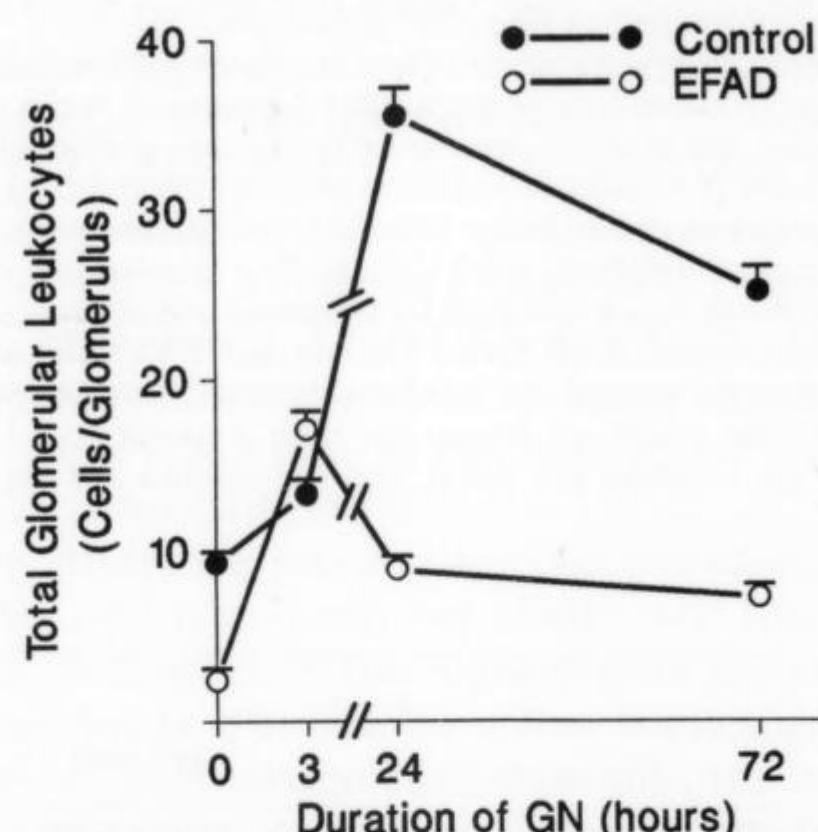


Figure 1. Influx of leukocytes during the heterologous phase of nephrotoxic nephritis: EFAD vs control. The influx of leukocytes during nephrotoxic nephritis was determined at 3, 24, and 72 h using a double antibody fluorescence technique detailed in Materials and Methods. Both control and EFA-deficient animals were studied. Determinations were done on a pool of glomeruli from three animals at each point. EFA-deficient glomeruli were significantly less than control at 0, 24, and 72 h (*p* < 0.01).

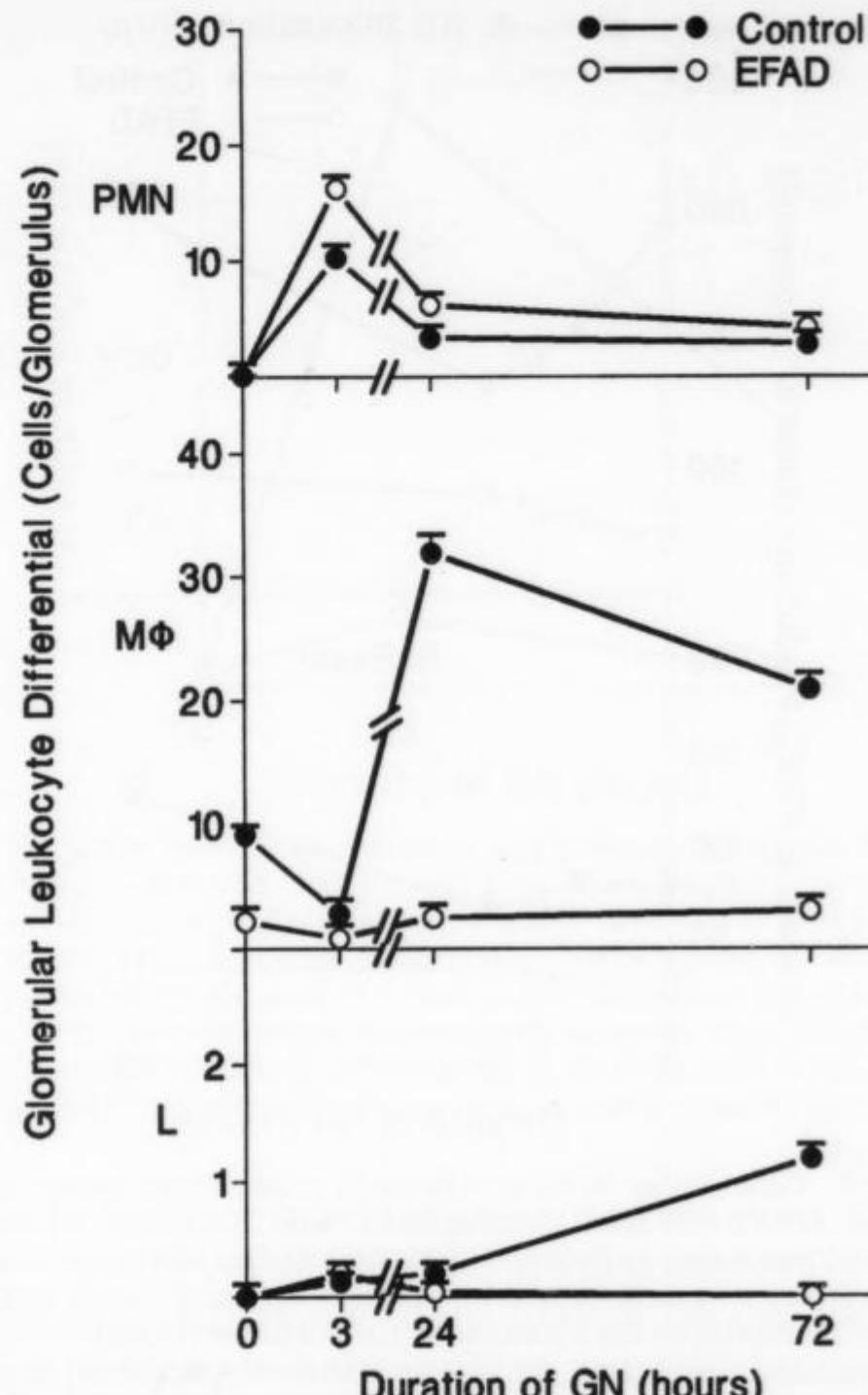


Figure 2. Differential analysis of the leukocyte influx in nephrotoxic nephritis. Differential analysis of leukocytes within the glomerulus during nephrotoxic nephritis was determined by an immunoperoxidase protocol detailed in *Materials and Methods* using a preparation of dissociated cells from glomeruli. Cells were identified by nuclear morphology as either PMN, macrophages ($M\phi$), or lymphocytes (L) and quantified as a percentage of cells expressing the leukocyte common Ag. Total counts were calculated by multiplying the percentage of each cell type by the total glomerular leukocyte count (see Fig. 1). Both control and EFA-deficient animals were studied. Determinations were done on a pool of glomeruli from three animals at each point. For macrophages, EFA-deficient glomeruli were significantly less than control at 0, 24, and 72 h ($p < 0.01$).

from EFA-deficient rats experienced no macrophage infiltration during the acute course of nephrotoxic nephritis (Fig. 2). Levels of macrophages in glomeruli from EFA-deficient rats which received NTS were the same as that seen in EFA-deficient rats which did not receive NTS. Lymphocytes were not a significant component of the heterologous phase of this model of glomerulonephritis (Fig. 2).

The lack of a macrophage infiltrate into the glomeruli of EFA-deficient animals in acute glomerulonephritis was confirmed in four additional experiments performed at 24 h after the injection of NTS. In control animals at this time point there were an average of 22.9 ± 1.6 macrophages per glomerulus whereas in EFA-deficient animals there were 6.1 ± 0.8 macrophages per glomerulus ($p < 0.01$; EFA-deficient vs control). In an additional experiment at 72 h after the injection of NTS, control animals exhibited 20.2 ± 1.3 macrophages per glomerulus relative to 3.2 ± 0.7 macrophages per glomerulus in EFA-deficient animals ($p < 0.01$; EFA-deficient vs control).

Effects of EFA deficiency on enhanced glomerular eicosanoid metabolism of nephrotoxic nephritis. Previous studies have shown that the glomerular inflammation of nephrotoxic nephritis is accompanied by a marked enhancement in glomerular cyclooxygenase (13)

and lipoxygenase (14) metabolism. These studies, however, examined the synthetic capacity of glomeruli when provided with exogenous substrate. These experiments may therefore not be representative of actual synthesis. Additionally, the use of exogenous arachidonate in incubations of EFA-deficient glomeruli may obscure their true synthetic capability as has previously been demonstrated (5). Thus, in the current study we examined basal (constitutive), as well as angiotensin II-elicited, TxB_2 and PGE_2 production by glomeruli. Basal and ionomycin-stimulated LTB_4 production were also determined.

Initial experiments focused on the changes in glomerular arachidonate metabolism present 24 h after the injection of NTS in control diet-fed animals. In five experiments, glomerular basal and angiotensin II-stimulated TxB_2 production increased by an average of 6.6 ± 1.9 -fold and 6.9 ± 2.2 -fold over nonnephritic animals 24 h after the injection of NTS ($p < 0.05$ for both, nephritic vs nonnephritic). LTB_4 production by glomeruli rose 13.4 ± 1.6 -fold after the induction of nephritis ($p < 0.01$, nephritic vs nonnephritic). In contrast to other reports (13), PGE_2 production by glomeruli was relatively unaffected by the injection of NTS. Basal and angiotensin II-stimulated PGE_2 production by glomeruli were 1.6 ± 0.4 - and 1.9 ± 0.8 -fold higher than control ($p = \text{N.S.}$ for both, nephritic vs nonnephritic).

EFA-deficient animals exhibited a markedly attenuated rise in glomerular TxB_2 and LTB_4 production 24 h after the injection of NTS relative to control animals. In two experiments, EFAD attenuated the rise in basal glomerular TxB_2 production by 65 and 83%. The increase in angiotensin II-stimulated TxB_2 production was reduced as well (76 and 88%). The rise in glomerular LTB_4 production was reduced by 61 and 91% by EFA deficiency. Glomerular PGE_2 production, although not elevated 24 h after the injection of NTS in control diet-fed animals, was still attenuated in EFA-deficient animals (basal production by 26 and 59%, and angiotensin II-stimulated production by 40 and 84%).

Subsequent experiments focused on delineating the full time course of changes in glomerular arachidonate metabolism during the course of nephritis. In these experiments, basal and angiotensin II-elicited TxB_2 production by glomeruli from control animals rose roughly 10-fold at 3 h after the injection of NTS (Fig. 3). This increased production of TxB_2 persisted at comparable levels at 24 and 72 h (Fig. 3). The increase in LTB_4 production by glomeruli from control animals was even more dramatic, but followed slightly different kinetics. LTB_4 production by glomeruli from control animals rose over 40-fold at 3 h (Fig. 4). The increase in LTB_4 synthesis was attenuated at 24 and 72 h, but remained 5- to 10-fold over that seen in uninflamed glomeruli from control animals (Fig. 4). At 3 and 24 h glomeruli were also noted to elaborate LTB_4 without stimulation (at approximately $6 \text{ fmol}/10^3$ glomeruli). Uninflamed glomeruli elaborated LTB_4 only in response to ionomycin. As above, glomerular PGE_2 production was not significantly changed during the course of nephrotoxic nephritis. Basal and angiotensin II-stimulated PGE_2 production by glomeruli from control animals was virtually unaltered by nephritis (Fig. 5).

In these experiments on the time course of changes in glomerular arachidonate metabolism, EFA deficiency also markedly attenuated the enhanced glomerular eicos-

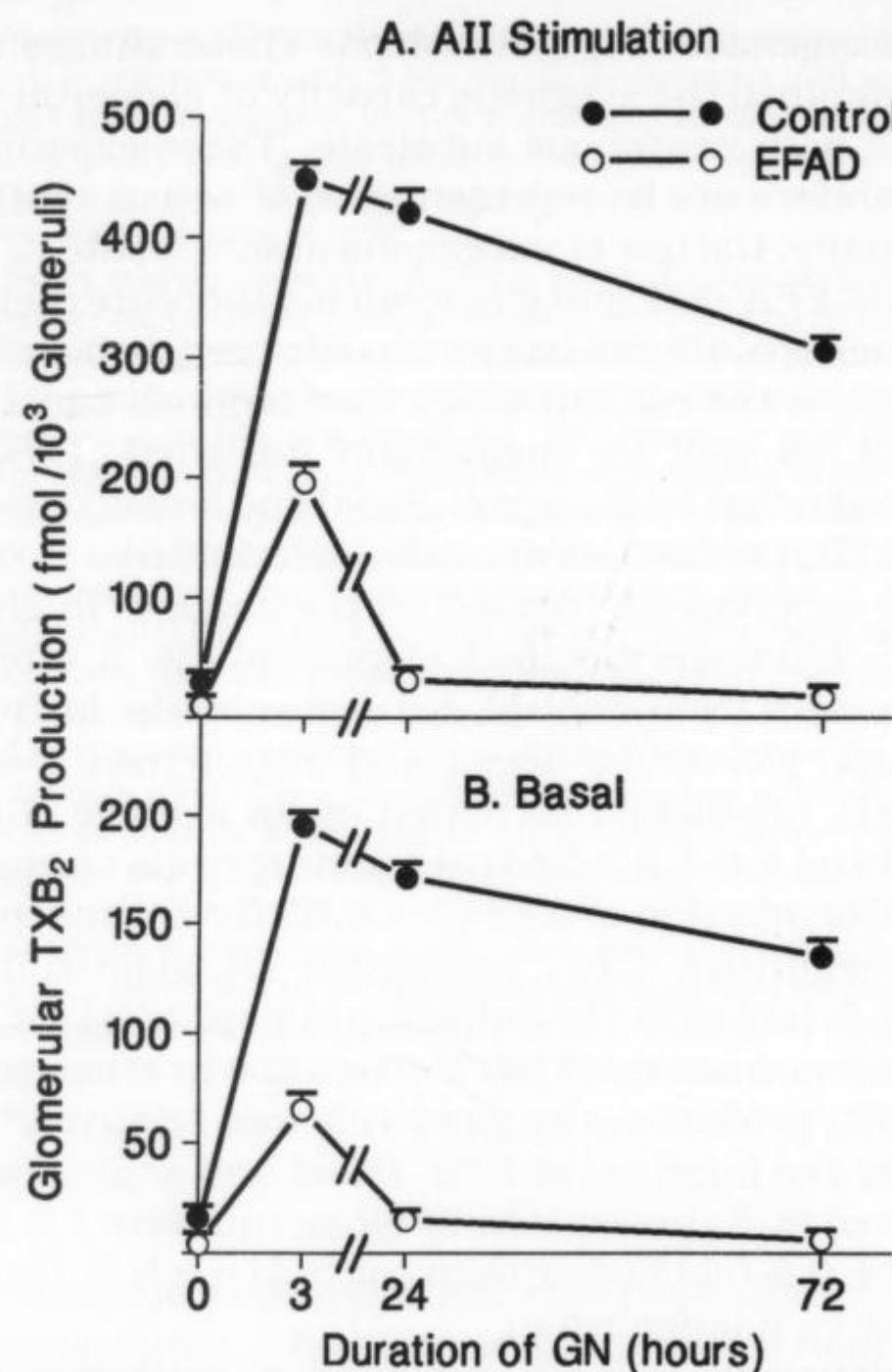


Figure 3. Glomerular TXB₂ synthesis in nephrotoxic nephritis: EFAD vs control. Glomeruli from nonnephritic and nephritic animals were isolated and incubated as detailed in *Materials and Methods*. Glomerular TXB₂ synthesis was determined by specific RIA and normalized for the number of glomeruli in the incubation. Constitutive (basal) and angiotensin II-stimulated production are shown. Both EFA-deficient and control glomeruli were studied. Measurements at each point were performed in triplicate on a pool of glomeruli from three to six animals and means \pm SEM are shown. EFA-deficient glomerular production of TXB₂ (both basal and constitutive) was significantly less than control for all time points shown ($p < 0.01$).

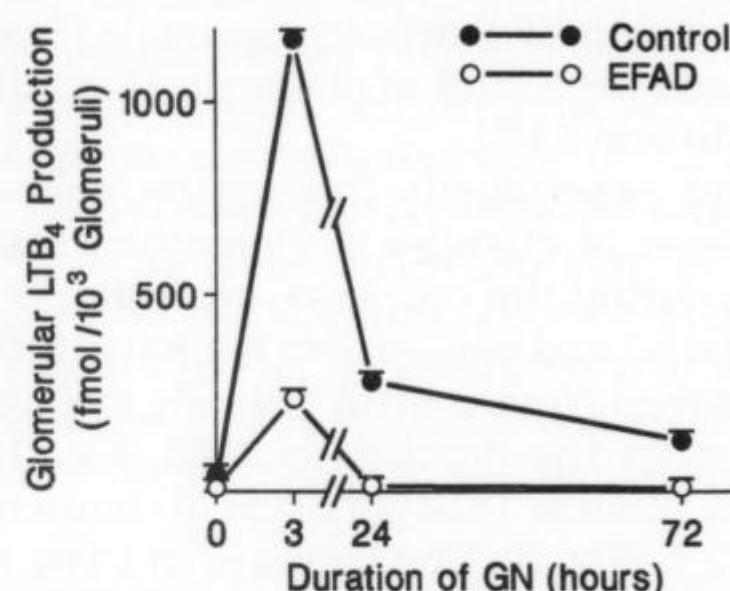


Figure 4. Glomerular LTB₄ synthesis in nephrotoxic nephritis: EFAD vs control. Glomeruli from nonnephritic and nephritic animals were isolated and incubated as detailed in *Materials and Methods*. Ionomycin was used to elicit LTB₄ production. Glomerular LTB₄ synthesis was normalized for the number of glomeruli in the incubation. Both EFA-deficient and control glomeruli were studied. Measurements at each point were performed in triplicate on a pool of glomeruli from three to six animals and means \pm SEM are shown. EFA-deficient glomerular production of LTB₄ was significantly less than control for all time points shown ($p < 0.02$ for 0 h, $p < 0.01$ for 3, 24, and 72 h).

anoid metabolism seen with nephritis. The rise in basal and angiotensin II-stimulated glomerular TXB₂ synthesis at 3 h seen in controls was inhibited by approximately 50 to 70% in EFA-deficient animals (Fig. 3). An even more striking decrease in glomerular TXB₂ production in EFA-deficient animals was seen at 24 and 72 h (approximately a 90% decrease relative to control). LTB₄ production by EFA-deficient glomeruli was also markedly de-

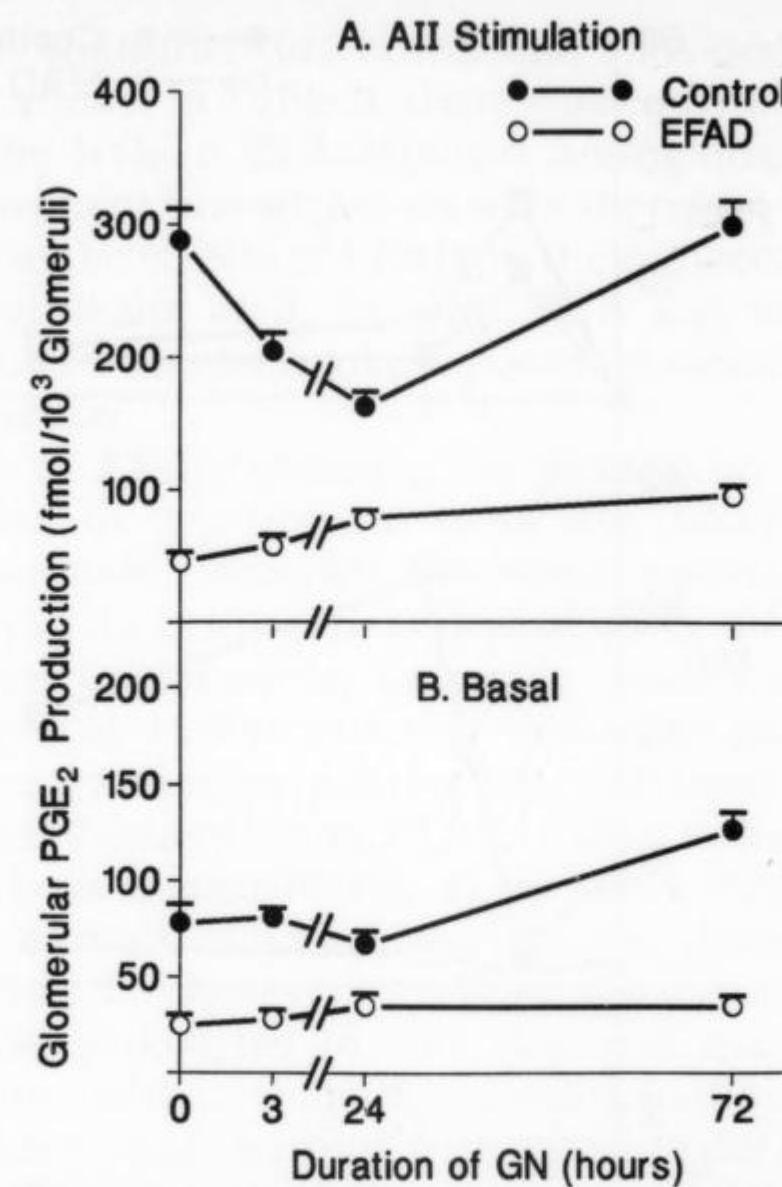


Figure 5. Glomerular PGE₂ synthesis in nephrotoxic nephritis: EFAD vs control. Glomeruli from nonnephritic and nephritic animals were isolated and incubated as detailed in *Materials and Methods*. Glomerular PGE₂ synthesis was determined by specific RIA and normalized for the number of glomeruli in the incubation. Constitutive (basal) and angiotensin II-stimulated production are shown. Both EFA-deficient and control glomeruli were studied. Measurements at each point were performed in triplicate on a pool of glomeruli from three to six animals and means \pm SEM are shown. EFA-deficient glomerular production of PGE₂ (both basal and constitutive) was less than control at all time points ($p < 0.01$).

creased during the heterologous phase of nephritis (Fig. 4). Suppression ranged from 80 to 90% relative to control. Basal and angiotensin II-elicited glomerular PGE₂ production were suppressed in uninflamed EFA-deficient glomeruli (Fig. 5). This diminished synthesis persisted during the inflammatory response.

Effects of EFA deficiency on renal functional changes accompanying nephrotoxic nephritis. Marked proteinuria is the typical concomitant of the glomerular injury of acute nephrotoxic nephritis (12). In our study, urine protein excretion rose dramatically in control animals receiving NTS. The first 24 h of nephritis was characterized by a 40-fold increase in urinary protein excretion (Fig. 6). Between 48 and 72 h urinary protein excretion diminished in control animals, but still remained markedly elevated. EFAD almost completely prevented the proteinuria seen in this model of glomerulonephritis (Fig. 6). Urinary protein excretion rose only modestly during the first 24 h of nephritis in EFA-deficient animals and was almost 90% less than that seen in control animals. Between 48 and 72 h EFA-deficient animals had virtually no proteinuria. These results were confirmed in three subsequent experiments (mean inhibition of proteinuria by EFA deficiency was 76%).

The initial 24 h of nephrotoxic nephritis was also characterized in control animals by polyuria, marked sodium retention, and mild azotemia (Table III). The sodium retention was quite striking with the fractional excretion of sodium falling to 0.01. By 72 h, the polyuria, sodium retention, and azotemia had largely resolved (Table III). No significant changes in urinary potassium, urea, or creatinine excretion were noted during the course of nephritis (data not shown).

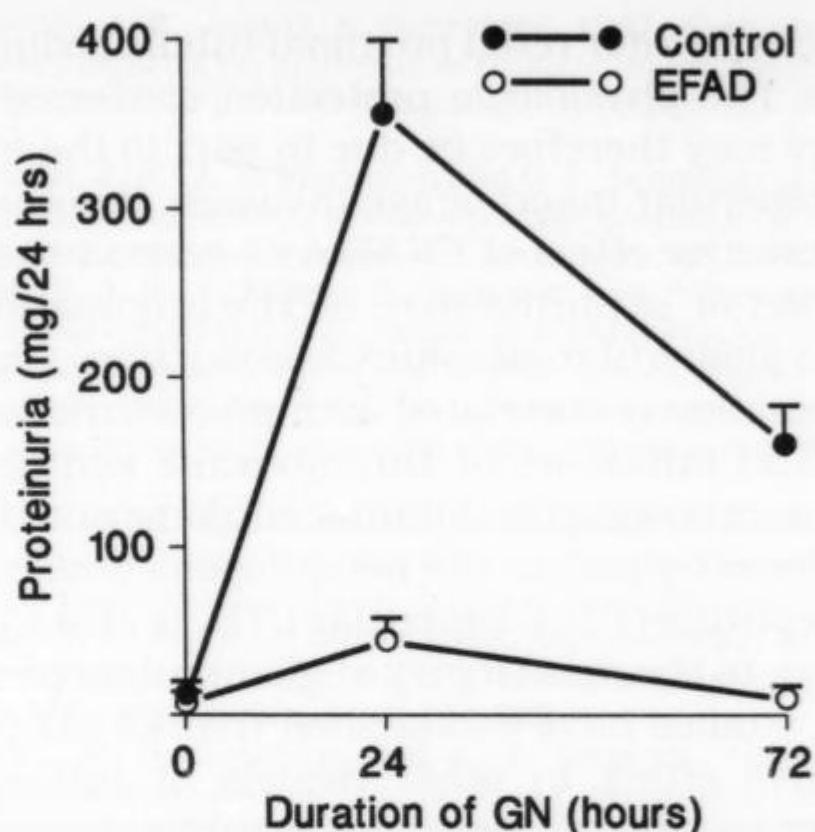


Figure 6. Urine protein excretion in nephrotoxic nephritis: EFAD vs control. Urines were collected for 24 h in nonnephritic and nephritic animals. In the latter, collections were made between 0 and 24 h and between 48 and 72 h. Urine protein was determined as detailed in *Materials and Methods*, and the results are expressed as total protein excretion. Both EFA-deficient and control animals were studied. The number of animals at each point varied from three to nine. EFA-deficient animals exhibited significantly less proteinuria at 24 and 72 h ($p < 0.01$).

EFA deficiency completely prevented the polyuria seen with the injection of NTS into control animals (Table III). Similarly, the deficiency state prevented the profound sodium retention and the mild azotemia also noted in controls (Table III).

Effects of EFA deficiency on macrophage chemotactic responsiveness. Two potential explanations for the marked suppression of the glomerular macrophage invasion of nephritis seen with EFA deficiency were considered at this point. One is that EFA deficiency decreases the circulating pool of monocytes. The second is that the deficiency state impairs the chemotactic responsiveness of macrophages. We and others have demonstrated that the number of circulating leukocytes and monocytes is not affected by EFAD (5, 15). To address the second issue, we compared the chemotactic responses of macrophages obtained from control and EFA-deficient

rats to a standard chemotactic stimulus, zymosan-activated serum. As shown in Figure 7, EFA-deficient and control macrophages exhibited comparable responses over a wide range of serum concentrations. Identical results were seen when serum from EFA-deficient rats was used (data not shown).

DISCUSSION

The ability of dietary polyunsaturated fatty acid modulation to ameliorate glomerulonephritis is striking. EFAD selectively prevents the influx of macrophages into the glomerulus in the context of nephrotoxic nephritis. The enhanced glomerular eicosanoid production which accompanies the inflammatory cell invasion is also largely attenuated by EFA deficiency. The deficiency state further protects against the proteinuria, polyuria, sodium retention, and azotemia which characterize the functional defects of glomerulonephritis.

EFA deficiency appears to provide a unique model of inhibited macrophage elicitation. This diminished influx of macrophages into a focus of inflammation occurs without the use of systemic immunosuppressive manipulations (such as x-irradiation or nitrogen mustard) which have been used in other studies on the abrogation of glomerular inflammation (12, 16). The mechanism by which EFA deficiency interferes with macrophage elicitation in the context of nephritis, however, remains to be clarified. It is clear from previous data that this effect is not a simple function of a depletion of circulating monocytes (5, 15). Our study also suggests that the effect of EFA deficiency on macrophage elicitation is not due to an impairment of macrophage chemotactic responsiveness. EFA-deficient macrophages are attracted to complement fragments comparably to control cells.

One possible explanation for the diminished macrophage influx is that EFA deficiency impairs a signaling mechanism attracting macrophages into the glomerulus during the course of nephritis. A potential mediator of macrophage chemotaxis that has been considered is LTB₄. As shown above and in other studies (17), the

TABLE III
Renal functional parameters in nephrotoxic nephritis: EFAD vs control^a

	Duration of Glomerulonephritis			
	0 h	3 h	24 h	72 h
Urine volume (ml)				
Control	8.3 ± 1.2		20.0 ± 3.9	11.2 ± 1.0
EFAD	6.3 ± 1.1		6.4 ± 0.3 ^b	5.1 ± 0.4 ^b
Urine Na (mmol/liter)				
Control	61 ± 10		3 ± 1	84 ± 5
EFAD	87 ± 15		73 ± 7 ^c	104 ± 26
Total Urine Na (μmol)				
Control	530 ± 152		52 ± 12	924 ± 59
EFAD	512 ± 49		465 ± 41 ^c	535 ± 159
FeNa				
Control	0.19		0.01	0.22
EFAD	0.24		0.14	0.16
Serum creatinine (mg/dl)				
Control	0.65 ± 0.03	0.46 ± 0.03	0.40 ± 0.01	0.40 ± 0.03
EFAD	0.55 ± 0.03	0.43 ± 0.07	0.40 ± 0.05	0.37 ± 0.04
Serum BUN (mg/dl)				
Control	16.4 ± 0.7	26.5 ± 1.3	26.7 ± 3.8	14.2 ± 0.7
EFAD	15.2 ± 0.5	15.9 ± 1.1 ^c	17.8 ± 0.6	15.0 ± 0.6

^a To assess renal function during nephrotoxic nephritis, serum was collected at 0, 3, 24, and 72 h after the injection of NTS. Urine collections were made between 0 and 24 h and between 48 and 72 h. Urine sodium and potassium, and serum creatinine and urea (BUN) were determined on a Beckman autoanalyzer. Both EFA-deficient and normal animals were studied. The number of the animals varied between three and six per determination. Means ± SEM are shown.

^b Indicates $p < 0.05$, control vs EFA-deficient.

^c Indicates $p < 0.01$, control vs EFA-deficient. Abbreviations: FeNa, fractional excretion of Na.

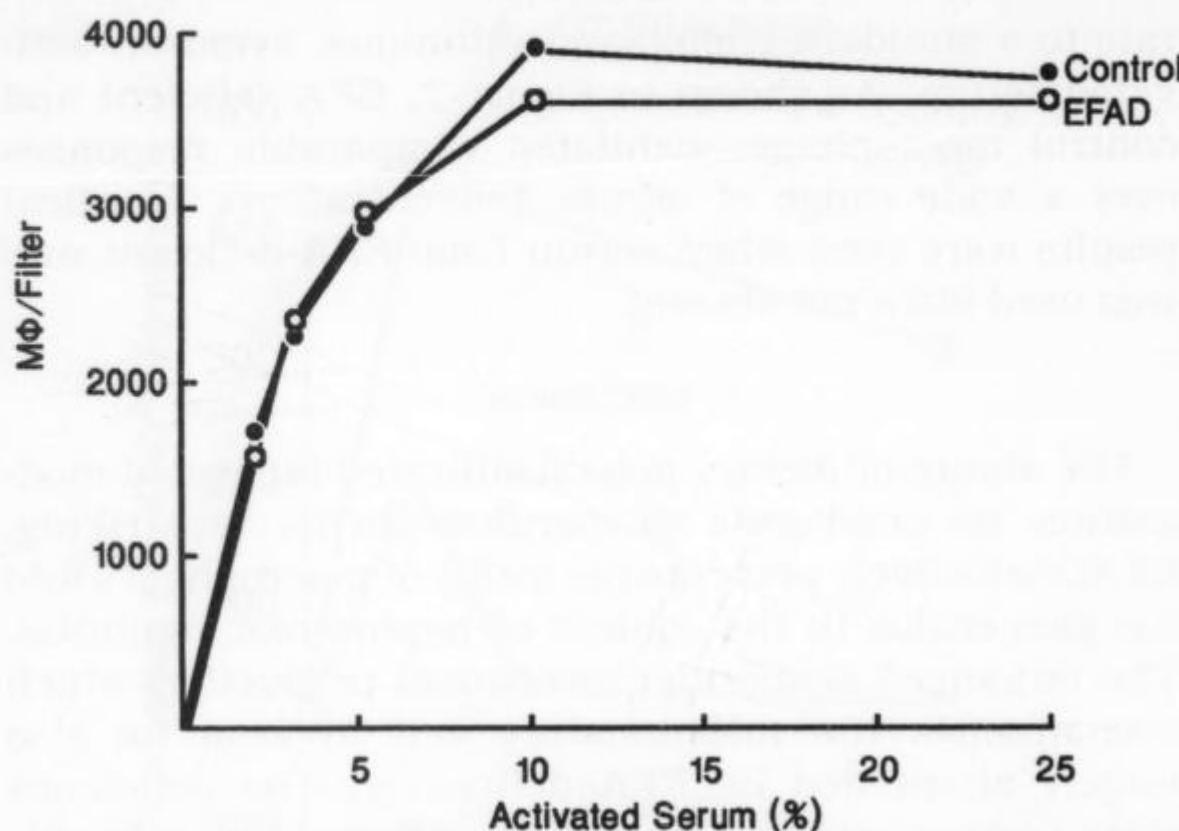


Figure 7. Chemotactic responsiveness of EFA-deficient macrophages. The chemotactic responsiveness of EFA-deficient and control macrophages was assessed using the microchemotaxis assay detailed in Materials and Methods. Responder cells were elicited peritoneal macrophages and the chemotactic stimulus was zymosan-activated serum. Assays were performed in triplicate and the means are shown. SE were <10% of the mean. The curves for EFA-deficient and control macrophages were not significantly different. The entire experiment was repeated three times with comparable results.

elaboration of this eicosanoid is markedly increased in nephritis. Additionally, its elaboration is markedly diminished by EFA deficiency. However, it is unlikely LTB₄ is the relevant macrophage chemoattractant. Studies suggest that LTB₄ is not chemotactic for rat leukocytes (18). We have been unable to observe significant rat macrophage migration toward LTB₄ in our laboratory (G. F. Schreiner, B. Rovin, et al., unpublished observations). Additionally, increased glomerular synthesis of LTB₄ has been noted in models of glomerular immune complex injury which do not involve the influx of leukocytes (19). Thus although LTB₄ may play a role in glomerulonephritis it is not likely to be the agent that elicits macrophages into the glomerulus.

Our study serves to further underscore the intimate relationship between glomerular macrophages and lipid mediator production. Prior studies in normal glomeruli have shown that the presence of resident glomerular macrophages is a specific function of (n-6) fatty acids (5). Additionally, resident macrophages have been implicated as the principle source of LTB₄ within normal glomeruli (6). As noted here and in other studies, a marked increase in glomerular eicosanoid production accompanies the glomerular inflammation in nephritis (13, 14, 17). Furthermore, EFAD inhibits both the leukocyte influx into the glomerulus in nephritis as well as the enhanced production of eicosanoids by glomeruli. One possible connection between glomerular leukocytes and eicosanoid production is the observation that macrophages may produce monokines that may modulate the production of eicosanoids by other cell types (20). In particular, studies have shown that monokines, such as IL-1 (21) and TNF (22), may act to alter the production of eicosanoids by mesangial cells.

The mechanisms underlying the functional defects of nephritis as well as the salutary effects of EFAD with respect to the renal dysfunction remain to be fully elucidated. It is clear from leukocyte depletion studies that macrophages mediate much of the proteinuria accompanying nephrotoxic nephritis (12). Macrophages also have

the capacity to alter renal proximal tubule sodium transport (23). The physiologic protection conferred by EFA deficiency may therefore be due in part to the inhibition of the glomerular macrophage invasion. Another aspect of the protective effect of EFAD with respect to the renal dysfunction of nephritis may be the attenuation of the enhanced glomerular eicosanoid production. Glomerular TXB₂ generation is correlated with proteinuria in nephritis (24), and inhibitors of thromboxane synthetase are effective in reversing the diminished glomerular filtration seen in the early part of the heterologous phase of nephrotoxic nephritis (13). Glomerular LTB₄ is also a potential contributor to the renal injury of glomerulonephritis (19).

Recent studies have established that EFAD can exert a protective effect in other models of inflammation. EFAD completely prevents the insulitis and resultant diabetes seen in a murine model of type I diabetes (25). A similar protective effect has been noted in the BB rat, a strain that is genetically susceptible to the development of autoimmune diabetes (26). In both models, macrophage invasion presages the subsequent development of autoimmune insulitis (27, 28). Additionally, EFAD, which is associated with a depletion of macrophages from glomeruli and renal interstitium, has been shown to alter the immunogenicity of renal allografts and prevent rejection (29). The protective effect of EFAD in inflammatory disease, however, is selective. No beneficial effects of the deficiency state are seen in models of disease in which lymphocytes initially mediate the tissue injury. EFA-deficient animals reject skin grafts normally (30). Additionally, EFAD does not protect against experimental allergic encephalomyelitis (31).

We postulate, therefore, that the common mechanism underlying the beneficial effects of EFAD is the specific inhibition of the lymphocyte-independent localization of macrophages in peripheral tissues. We further propose that as yet uncharacterized lipid mediators whose generation is inhibited by the EFA-deficient state may act as macrophage chemoattractants and play a role in the tissue dysfunction that results upon macrophage invasion. EFAD appears to be remarkably protective in nephrotoxic nephritis. An appreciation of the mechanisms underlying this salutary effect may further understanding of the pathophysiology of glomerulonephritis. Additionally, the information derived from the study of the protective effect of EFAD may yield insights into factors contributing to autoimmune disease in organs other than the kidney.

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