Biochemical Pharmacology, Vol. 35, No. 5, pp. 779–785, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

EICOSAPENTAENOIC ACID AS A MODULATOR OF INFLAMMATION

EFFECT ON PROSTAGLANDIN AND LEUKOTRIENE SYNTHESIS

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(*Received* 17 *June* 1985; *accepted* 6 *August* 1985)

Abstract—Products derived from arachidonic acid (AA) via both the cyclo-oxygenase and lipoxygenase pathways play a role in inflammation: prostaglandins (PGs), particularly PGE_2, contribute to the formation of oedema, erythema and hyperalgesia whereas leukotriene B_4 (LTB_4), a product of the 5′ lipoxygenase, may modulate the recruitment of leukocytes. We have previously reported that supplementation of a standard rat diet with eicosapentaenoic acid (EPA) caused a significant increase in the formation of LTB_5, which is less active biologically than LTB_4, and a decrease in the synthesis of LTB_4 by stimulated leukocytes. Now we have assessed the effects of administration of highly purified EPA ethyl ester (79% pure), in two models of acute inflammation. Supplementation of a standard rat diet with 240 mg/kg/day EPA for 4 weeks significantly decreased the concentration of PGE_2 and TXB_2 in inflammatory exudate derived from implantation of carrageenin impregnated sponges: neither the concentration of LTB_4 nor the cell number were reduced significantly. Triene prostaglandins were not detected in the exudate, however, significant levels of LTB_5 were present. In the second model, oedema induced by injection of carrageenin into rat paws was significantly reduced in animals fed an EPA-rich diet. Supplementation of the diet with EPA could, by mainly reducing the synthesis of prostaglandins, offer a novel and non-toxic approach to the modulation of an inflammatory response.

The evidence that PGs, particularly PGE_2, mediate some cardinal signs of inflammation (erythema, oedema and hyperalgesia) is convincing [1]. Other eicosanoids formed from AA via the lipoxygenase pathway may also modulate the inflammatory response; in particular, LTB_4 which is a potent chemotactic and chemokinetic agent *in vitro* and *in vivo*, could recruit leukocytes to sites of inflammation [2–4].

EPA is a poor substrate for the cyclo-oxygenase under normal conditions [5, 6] and competitively inhibits the formation of dienoic prostanoids [5, 7]. On the other hand EPA is a relatively good substrate for lipoxygenases and is converted efficiently to LTB_5 [8–10]. LTB_5 is far less active than LTB_4 in inducing aggregation, degranulation and chemokinesis of human neutrophils *in vitro* and at least 10 times less active than LTB_4 in potentiating bradykinin induced plasma exudation [10–14]. We recently reported that supplementation of a normal rat diet with EPA caused a significant increase in the formation of LTB_5 and a decrease in the synthesis of LTB_4 by stimulated leukocytes [15]. Thus, an EPA-rich diet may have an anti-inflammatory effect by reducing the production of dienoic prostaglandins and LTB_4. Indeed, Eskimos, who consume an EPA-rich diet have a low incidence of chronic degenerative disease, including ulcerative colitis and rheumatoid arthritis [16]. Also, Prickett *et al.* [17] reported that feeding fish oil prevents proteinuria and prolongs the survival of mice in a model of human systemic lupus erythmatosis.

We have now evaluated whether administration of EPA to rats has any effect on eicosanoid synthesis and/or the biological response in two animal models of acute inflammation.

MATERIALS

All *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) purified from sardine oil was provided by the Central Research Laboratory, Nippon Suisan Kaisha, Tokyo, Japan; the ethyl ester of EPA (EPA-E) which was used for administration to animals was 79% pure and contained 0.2% α-tocopherol as antioxidant.

Chemically synthesized LTB_4 was obtained from Professor E. J. Corey, Harvard University, Cambridge, MA, U.S.A. LTB_5 was biosynthesized from EPA (96% pure) by rabbit PMN stimulated with the calcium ionophore, A23187; it was purified by high pressure liquid chromatography (HPLC), characterised by gas chromatography-mass spectrometry (GC-MS) and quantified by u.v. spectrophotometry as previously described [10]. Prostaglandin E_3 (PGE_3) was a gift from Upjohn Co., Kalamazoo, MI, U.S.A. PGB_3 was prepared by treatment of PGE_3 with 1M methanolic potassium hydroxide; the product was extracted into ethyl acetate, purified by HPLC and then quantified by u.v. spectrophotometry.

Heparin (Pularin), and polyester sponge (thickness 0.5 cm) were purchased from Duncan Flockhart & Co (London U.K) and Transatlantic Plastic Ltd

(Surbiton U.K.), respectively. Hank's balanced salt solution was obtained from Wellcome Diagnostics (Dartford, Kent, U.K.). γ-carrageenin, HEPES, and silver nitrate were purchased from Sigma Chemical Co. (St. Louis, MO). Reverse-phase silica cartridges (C18-Sep-Pak) were obtained from Waters Associates, Northwich, Cheshire, England. Analytical grade and HPLC grade solvents were obtained from BDH (Poole, U.K.) and Rathburn Chemicals Ltd (Walkerburn, U.K.), respectively.

METHODS

(1) Feeding protocol

Male Wistar rats (approx. 150 g) were maintained for at least 7 days on standard rat diet (rat and mouse feed no. 1, SDS, Essex, U.K.) prior to commencing the supplementary diet. The standard rat diet contained 2.6% (w/w) total fat and this consisted of the following relevant fatty acids with proportions (mole % of total fatty acid) in parentheses; C16:0 (17); C18:1 (23), C18:2 (45), C20:1 (6), C20:4 + C22:1 (1), C20:5 (1); C22:6 (2). One group of animals (40 rats) were given EPA (240 mg/kg/day, i.e. 304 mg/kg/day of fatty acid ethyl ester mixture) as an oil in water (1:20) emulsion (1 ml) through a gastric tube every morning for 4 weeks. Vehicle only (1 ml water p.o.) was administered to another group of animals (control group : 40 rats). Rat standard diet and water were given *ad libitum* to both groups. Body weight was measured weekly.

In order to establish whether the effects observed after supplementing the diet with EPA could be caused non-specifically by the increased intake of fat a further group of rats was monitored. Oleic acid (C18:1, ω-9; ethyl ester) was added to the standard diet (240 mg/kg/day for 4 weeks).

(2) Measurement of eicosanoids and leukocytes in inflammatory exudates

(a) Collection of inflammatory exudate. Sterile polyester sponges (3.5 × 1 × 0.5 cm) soaked in either 1% or 2% carrageenin (w/v sterile saline) were implanted subcutaneously in 25 rats from each group as previously described [18, 19]. Animals were killed at either 4 hr ($n = 5$) or 6 hr ($n = 20$) after sponge implantation. An aliquot (40 μl) of each exudate was removed immediately for determination of the leukocyte number using a model ZBI coulter counter (Coulter Electronics Limited, Herts, U.K.). A differential cell count was also performed. The remainder of the exudate was immediately centrifuged at 12,000 *g* for 30 sec to precipitate cells. The cells were removed as quickly as possible to reduce the possible metabolism of LTB by the leukocytes.

(b) Determination of eicosanoids by specific radioimmunoassay (RIA). LTB_4, PGE_2 and TXB_2 in each exudate supernatant were measured by specific RIA [21, 22] without extraction or chromatography. These determinations have been validated previously [23].

(c) Measurement of LTB_4 and LTB_5. Cell free exudate (15 ml) obtained from 10 animals from each group 6 hr after implanting sponges soaked in 1% carrageenin were pooled separately. LTB in the exudate was extracted by an ODS silica minicolumn (C18-Sep-Pak) and analysed by reverse phase high pressure liquid chromatography (RP-HPLC) using PGB_3 as an internal standard as previously described [15]. The minicolumn was finally eluted with spectroscopic grade ethyl acetate (6 ml) and this extract was taken to dryness under nitrogen, redissolved in methanol–water (30:70, v/v) and injected onto a spherisorb ODS 5 μm colum (250 × 4.5 mm: Laboratory Data Control, Stone, Staffs, U.K.) via a model 6k injector. HPLC solvent (methanol water acetic acid; 70:30:0.01 v/v/v adjusted to an apparent pH 5.7 with aqueous ammonia) was pumped at 1 ml/min through the column using a model 6000 A delivery system (Waters Associates). The ultraviolet absorbance of the column eluate at 270 nm was continuously monitored with a variable wavelength detector (Spectromonitor III, Laboratory Data Control). The eluate was collected every 20 sec and the immunoreactive LTB_4 in an aliquot (100 μl) was determined by RIA. Previous studies have shown that cross-reaction of LTB_5 with the LTB_4 anti-serum was 17.1% [10]. The retention times of LTB_4, LTB_5 and PGB_3 were established using authentic standards. The average overall recovery of [^{3}H]LTB was approximately 70%.

(d) Measurement of triene prostaglandins. Prostaglandins in cell free exudate (15 ml) were extracted as described above (see 2c). The ethyl acetate fraction from the mini-column was evaporated to dryness under nitrogen, reconstituted in chloroform : ethanol (2:1 v/v) and then analysed by thin layer chromatography (TLC) using silver nitrate (5%) impregnated silica gel plates (Whatman, LK5D, Whatman Chemical Separation Ltd., Maidstone, Kent, U.K.) which were developed in the organic phase of ethyl acetate–iso–octane–acetic acid–water (180:50:20:100 v/v/v/v). The R_fs of authentic PGE_3, TXB_2 and PGE_2 in this TLC system were 0.22, 0.30 and 0.36, respectively. TXB_3 was not available but by analogy to the difference of R_f observed between PGE_2 and PGE_3, the R_f of TXB_3 was calculated to be approximately 0.18. Areas (0.5 cm) of silica gel were scraped off and the prostanoids eluted with 1 ml 50 mM Tris buffer pH 7.4. containing 0.1% gelatin and the content of immunoreactive TXB_2 and PGE_2 in aliquots of this solution were determined by specific RIA. PGE_3 cross-reacts with the anti-PGE_2 serum (approximately 10%, data not shown) and although the cross-reaction of TXB_3 with the anti-TXB_2 serum has not been determined, it is assumed that it would be in the order of 10–20%. The mean recovery of standards from silver nitrate impregnated TLC was 79% using [^{3}H]PGE_2 and [^{3}H]-TXB_2.

(3) Carrageenin-induced oedema

The effect of the feeding regime on oedema formation was assessed in 15 animals from each group 4 weeks after commencing the supplementary diet. Oedema was induced by sub-plantar injection of 0.1 ml 2% carrageenin in saline (w/v) into the hind paws of rats [24]. Foot pad thickness was measured hourly using reverse spring dial calipers (Pocotest; Carobronze Ltd., London W4, U.K.) [25]. Data was expressed as the increase in paw thick-

ness (mm) from the reading taken prior to injection of carrageenin.

(4) Fatty acid composition of leukocyte phospholipds

The lipids in peritoneal leukocytes (80% PMN, 20% mononuclear cells), which were obtained 17 hr after i.p. injection of 0.2% oyster glycogen, were extracted and separated by TLC as previously described [15]. The fatty acids in the phospholipid fraction were methylated and analysed by gas liquid chromatography.

(5) Statistical analysis

Significance of difference was assessed using Student's unpaired *t*-test.

RESULTS

There was no significant difference in body weight between the control group of rats and the rats given a supplementary diet of EPA, (347 ± 7.0 g in control 348 ± 5.9 g after EPA: $n = 40$ mean ± S.E.M). Each group consumed approximately 20 g standard diet per day.

Supplementation of the normal diet with EPA (240 mg/kg/day) for 4 weeks caused a significant increase in the EPA content of leukocyte phospholipids (0.55 ± 0.05 and 1.71 ± 0.14 mole %, control and EPA fed group, respectively, $n = 8$–16, $P < 0.001$) without affecting the content of AA, linoleic acid and DHA as reported previously [15].

In inflammatory exudate derived from implanting carrageenin impregnated sponges, more than 95% of the cells were polymorphonuclear leukocytes in both groups of rats. The concentrations of PGE_2 and TXB_2 in the exudate obtained 6 hr after implanting sponges impregnated with 1% carrageenin were significantly reduced (24 and 30% lower than control, respectively) (Fig. 1). Both the concentration of LTB_4 and the number of leukocytes in the exudate were reduced also but not significantly.

Supplementing the standard diet with EPA also reduced significantly the levels of PGE_2 and TXB_2 in exudate obtained 4 hr after implanting sponges impregnated with 2% carrageenin (see Table 1). The concentration of LTB_4 and the number of leukocytes were reduced but, as above, these effects were not significant (Table 1).

If LTB_5 was present in the exudate, it would have contributed to the concentration of LTB_4 determined by RIA since LTB_5 cross-reacts with the antibody used in the assay (17.1%) [10]. In order to determine

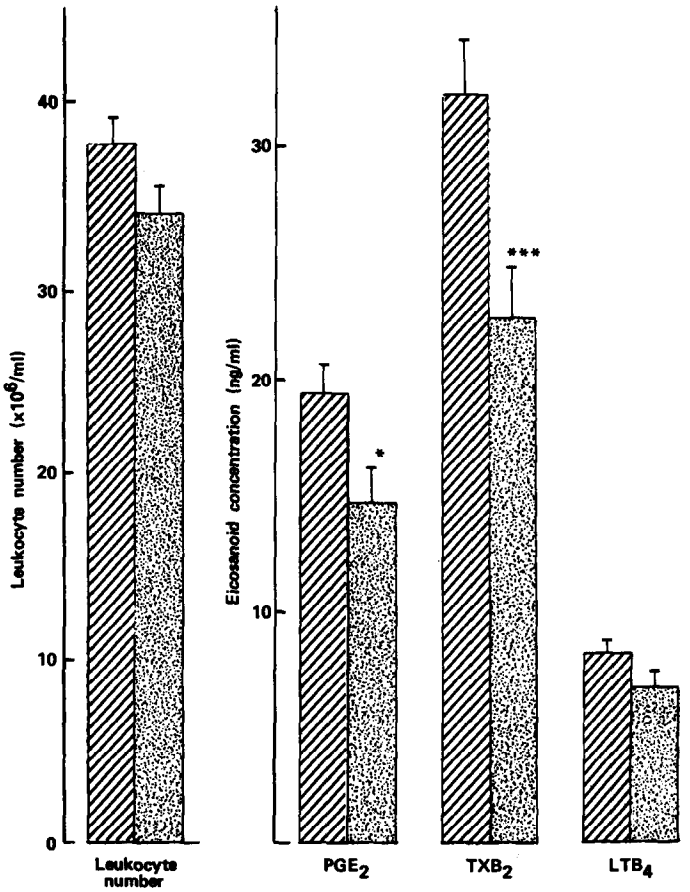

Fig. 1. Eicosanoid concentrations and leukocyte number in inflammatory exudate obtained 6 hr after implanting sponges impregnated with 1% carrageenin; control (▨) and EPA-fed rat (▩). Values given are mean ± S.E.M. of the mean ($n = 20$). *$P < 0.05$, ***$P < 0.01$. Since LTB_5 exhibits a significant cross-reaction in the assay for LTB_4 (see text) the concentration of the latter should be considered as total immunoreactive LTB (i.e. LTB_4 plus LTB_5).

Table 1. Effect of supplementing a normal rat diet with EPA on eicosanoid concentrations and leukocyte numbers in inflammatory exudate obtained by s/c implant of sponges impregnated with 2% carrageenin for 4 hr

Diet	Concentration of eicosanoids (ng/ml)			Leukocyte count ($\times 10^6$/ml)
	PGE_2	TXB_2	LTB_4	
Control	13.9 ± 1.9	9.8 ± 2.4	4.1 ± 1.2	9.5 ± 2.1
EPA-enriched	5.9 ± 1.9***	2.3 ± 0.28*	1.9 ± 0.3	7.1 ± 1.1

Values are the mean ± S.E.M. of data from five animals.
*$P < 0.05$, ***$P < 0.01$.

the concentration of LTB_4 and LTB_5 in exudate separately, RP-HPLC was employed. Immunoreactive LTB_4 in every 20 sec HPLC fraction was determined using RIA for LTB_4 (Fig. 2). Three immunoreactive peaks were detected: by comparison with authentic standards these were provisionally identified as (i) LTB_5, (ii) 6-*trans*-LTB_4 isomer 1 (this cross-reacts approximately 3.3% in the RIA [17]) and (iii) LTB_4. Clearly there is more LTB_5 present in the exudates derived from the animals given EPA. After correcting for the lower cross-reaction in the RIA, (dotted line in Fig 2) the concentration of LTB_5 was approximately 10% of LTB_4.

After separation of dienoic and trienoic prostanoids by silver nitrate impregnated TLC followed by RIA, only dienoic PGE and TXB were detected (Fig. 3). These data demonstrate that EPA is not or very poorly converted to cyclooxygenase products.

EPA-fed rats produced significantly less oedema (20% reduction, $p < 0.01$) at 2, 3 and 4 hr after injection of carrageenin into the hind paws compared with the response in control rats (Fig. 4). The reduction in oedema by EPA administration was maintained for at least 6 hr.

DISCUSSION

Supplementation of a normal rat diet with EPA (240 mg/kg for 4 weeks) resulted in a significant reduction of the concentrations of both PGE_2 and TXB_2 in inflammatory exudate derived 4 and 6 hr after implanting carrageenin-impregnated sponges. There is strong evidence that PGE_2 is involved in mediating the oedema response as well as erythema and hyperalgesia [1]. Therefore, the reduced synthesis of PGE_2 probably plays a role in the decreased

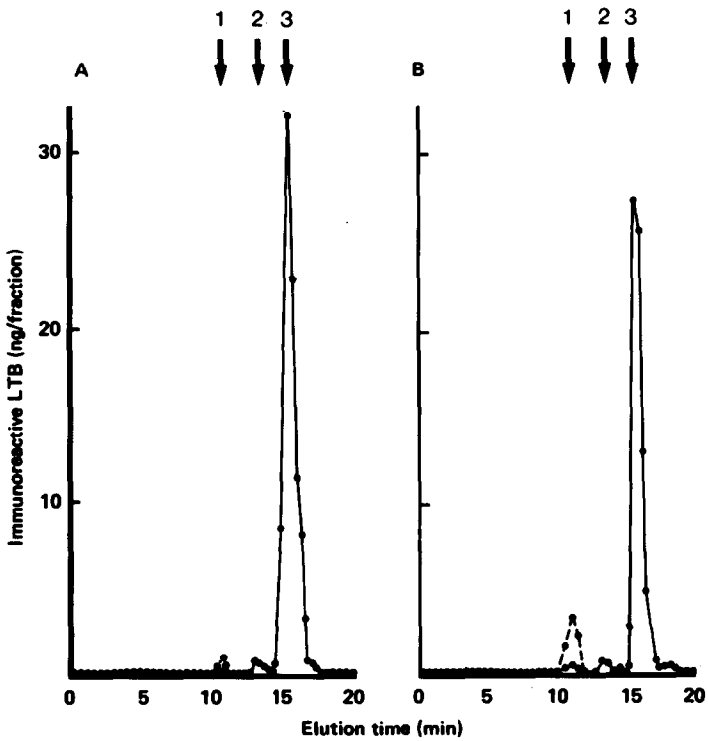

Fig. 2. HPLC separation of LTB_4 and LTB_5 in inflammatory exudate induced by implantation of carrageenin soaked sponges. Metabolites were separated by RP-HPLC and measured by specific RIA for LTB. Peak 1 is LTB_5 (dotted line: corrected for the lower cross-reaction in the RIA). Peak 2 is 6-*trans*-LTB_4 isomer 1. Peak 3 is LTB_4. A, control rat; B, EPA fed rat.

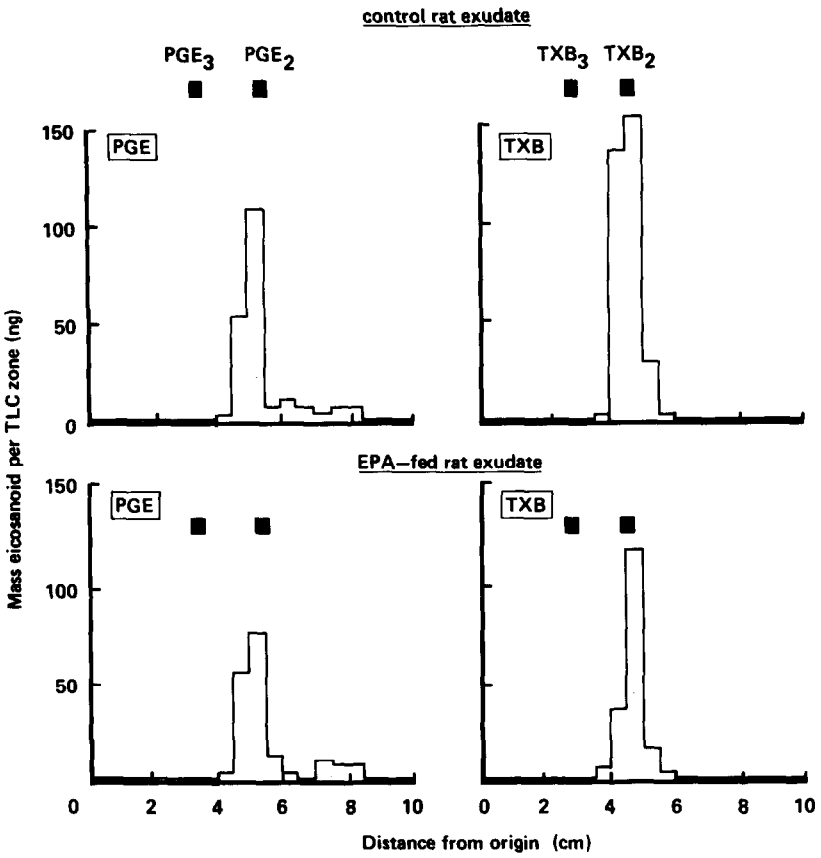

Fig. 3. TLC separation of PGE and TXB produced in inflammatory exudate induced by implantation of carrageenin soaked sponges. Metabolites were separated by silver nitrate TLC and measured by specific RIA as described in Methods.

formation of oedema observed in EPA-fed animals when evaluated in a second model of acute inflammation (discussed further below). There are reports that TXA_2 enhances polymorphonuclear leukocyte adhesion [26] and that TXB_2 is chemotactic [27] but the role of thromboxanes in inflammation is unclear since thromboxane synthetase inhibitors do not appear to modify oedema formation or cell accumulation [28]. Therefore the significance of the observed lowering of TXB_2 in the inflammatory exudate after EPA is not clear. The reduction of both PGE_2 and TXB_2 in the exudate is probably due to competitive inhibition of AA metabolism via the cyclo-oxygenase pathway by EPA; formation of trienoic cyclo-oxygenase products was not observed.

Leukotriene B_5 was detected in the inflammatory exudates from animals fed an EPA-rich diet. Gas chromatographic analysis confirmed that EPA was incorporated into leukocyte phospholipids using the described feeding protocol. It is important to note that the increase of EPA in the phospholipids was achieved without significantly altering the content of AA, linoleic acid and DHA and therefore the anti-inflammatory effects observed in this study can be more confidently related to the change in EPA content. Other studies have invariably used EPA of low purity (MaxEpa, Menhaden oil or unprocessed fish) and this greatly influences the composition of the fatty acids in the phospholipid fraction. The presence of LTB_5 in the exudate confirms that EPA is efficiently released from membrane phospholipids and that it is a good substrate for the 5′ lipoxygenase.

The observed reduction of LTB_4 synthesis after EPA-supplementation, although not significant in this study, may also contribute to the reduction of oedema since LTB_4 in combination with vasodilators, such as PGE_2 and bradykinin, increase plasma extravasation [29, 30]. Decreased formation of the chemotactic principle, LTB_4, may also account for the lower, but not significant, cell count in the exudate. It should be noted that most determinations of LTB_4 were by direct RIA but since LTB_5 cross reacts with the antiserum the data include a contribution by LTB_5. Thus, the actual reduction of LTB_4 could be more marked than indicated by the total immunoreactive LTB data presented. LTB_5 is considerably less active biologically than LTB_4 [10] and consequently will have less influence on the inflammatory response.

In the second model of acute inflammation supplementation of a standard rat diet with EPA for 4 weeks decreased signficantly oedema formation 2–6 hr after injection of carrageenin into the hind paws. Oedema formation during the first hour is attributed to the release of vasoactive amines, such as histamine and serotonin, whereas the increased swelling

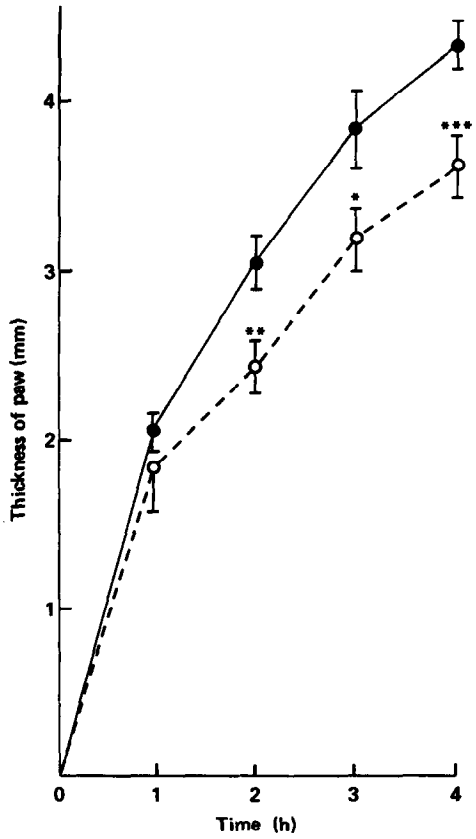

Fig. 4. Comparison between control (—●—) and EPA-fed rats (—○—) in paw oedema response induced by subplantar injection of carrageenin. Each point is the mean ± S.E.M. of the mean ($n = 15$), *$P < 0.05$, **$P < 002$, ***$P < 0.01$.

between 2 and 4 hr is considered to be due to the presence of prostaglandins particularly PGE_2 [33]. This hypothesis is supported by the evidence that inhibitors of prostaglandin synthesis such as indomethacin are not effective at 1 hr but do reduce swelling at 4 hr [34]. Generally there is a good correlation between the effectiveness of anti-inflammatory drugs in the rat paw oedema test and in human arthritis [35]. Our present data suggest that EPA feeding affects the prostaglandin phase of the oedema formation and this is supported by the observed reduction of PGE_2 in exudate obtained after implanting carrageenin-impregnated sponges as discussed above. The suppression of LTB_4 synthesis after EPA could also contribute to the lower oedema formation. Also, it is possible that increased content of EPA in the membrane lipids could increase membrane fluidity [31, 32] and alter cell functions, thereby modifying the inflammatory response.

Oleic acid (C18:1, ω-9; ethyl ester) added to the standard rat diet (240 mg/kg/day for 4 weeks) did not cause a significant change from control in the concentration of eicosanoids (PGE_2, TXB_2 and LTB_4 in the exudate (data not shown) and therefore the effects observed with EPA cannot be attributed non-specifically to increased intake of fat.

Thus, supplementation of a normal diet with EPA appears to reduce acute inflammatory symptoms and this can probably be attributed to reduced synthesis of prostaglandins, particularly PGE_2 and possibly LTB_4. Therefore, increased EPA intake could offer a novel and non-toxic alternative to conventional anti-inflammatory therapy as well as being of benefit in the treatment of thrombosis [37–40]. It is possible that reduced synthesis of PGE_2 is a disadvantage in chronic inflammation as this prostanoid may be immunosuppressive. Indeed, Prickett *et al.* [41] recently reported that feeding rats a diet rich in fish oil (not purified EPA) increased the incidence, but not the severity, of collagen (type II) induced arthritis. Therefore more studies, particularly those in the clinical environment, are required to establish the value of EPA as an adjunct to anti-inflammatory therapy.

Acknowledgement—The authors wish to thank Miss L. C. Tilling for her excellent technical assistance.

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