Biochemical Pharmacology, Vol. 33, No. 19, pp. 3071–3076, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

EFFECT OF ORALLY ADMINISTERED EICOSAPENTAENOIC ACID (EPA) ON THE FORMATION OF LEUKOTRIENE B_4 AND LEUKOTRIENE B_5 BY RAT LEUKOCYTES*

TAKASHI TERANO,† JOHN A. SALMON and SALVADOR MONCADA
Department of Prostaglandin Research, Langley Court, Beckenham, Kent BR3 3BS, U.K.

(*Received* 9 *March* 1984; *accepted* 15 *May* 1984)

Abstract—Eicosapentaenoic acid (EPA) is a poor substrate for the fatty acid cyclo-oxygenase but is a good substrate for lipoxygenase enzymes which catalyse the biosynthesis of hydroperoxy-acids, hydroxy-acids and leukotrienes. Recently, we reported that leukotriene B_5 (LTB_5) was at least 30 times less potent than LTB_4 in causing aggregation, chemokinesis and degranulation of polymorphonuclear leukocytes *in vitro*. In this paper, the effect of oral administration of EPA on LTB_4 and LTB_5 production by rat leukocytes stimulated with the calcium ionophore, A23187, was assessed. The concentration of LTB was determined by radioimmunoassay and also by reverse-phase high pressure liquid chromatography using PGB_3 as internal standard. Supplementation of a normal rat diet with EPA (240 mg/kg per day) for 4 weeks caused a significant increase in the formation of LTB_5 and a decrease in the synthesis of LTB_4 by stimulated leukocytes. The EPA-rich diet significantly increased the EPA content of leukocyte phospholipids without altering the content of arachidonic acid (AA) or linoleic acid. The ratio of EPA/AA in leukocytes correlated ($r = 0.795$, $P < 0.001$) with the LTB_5/LTB_4 ratio produced after stimulation of leukocytes. If LTB_4 has a chemotactic role during inflammation, the present data suggest that an EPA rich diet could decrease the accumulation of leukocytes at sites of inflammation.

Epidemiological evidence and subsequent studies with animals and human volunteers given an eicosapentaenoic acid (EPA) rich diet demonstrate that EPA reduces the incidence of thrombotic disorders and complications of atherosclerosis [1, 2]. Biochemical investigations have focused on EPA's effect on the metabolism of arachidonic acid (AA) via the cyclo-oxygenase pathway in both blood platelets and vessel walls [3–5]. These studies have demonstrated that EPA itself is a poor substrate for the cyclo-oxygenase, only small quantities of trienoic prostaglandins and thromboxanes are biosynthesized [4, 6]. The trienoic cyclo-oxygenase products have different biological activities from those derived from AA and this may be one of the underlying mechanisms explaining the anti-thrombotic activity of EPA [3, 4]. However, EPA also competitively inhibits the metabolism of AA causing a reduced formation of, for example, the pro-thrombotic thromboxane A_2 (TXA_2) in platelets and this is more likely to account for the biological activity of EPA [4, 7].

It is now known that AA is also metabolized by a separate group of enzymes, lipoxygenases, to hydroperoxy derivatives. The arachidonate 5′-lipoxygenase is of particular interest because the product, 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) can be further metabolized to the biologically active leukotrienes [8, 9]. One of these metabolites, leukotriene B_4 (LTB_4) is biosynthesized by leukocytes and causes chemokinesis and chemotaxis of polymorphonuclear leukocytes (PMN) at doses comparable to the complement fragment, C5a and the synthetic chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (FMLP) [10–12]. Biologically active concentrations of LTB_4 are present in exudates from experimentally-induced inflammation [13] and in inflammatory disease [14–16] and, therefore, it has been suggested that LTB_4, in part, mediates the accumulation of leukocytes occurring during an inflammatory response.

Several *in vitro* studies have demonstrated that although EPA is a poor substrate for the fatty acid cyclo-oxygenase, it is a good substrate for the lipoxygenase enzymes [17, 18]. Recently, it was reported that both LTB_5 and another leukotriene, LTC_5, are biosynthesized by mastocytoma cells from mice fed an EPA-rich diet [19]; the conversion of EPA to LTB_5 was as efficient as of AA to LTB_4. We recently reported that LTB_5 (prepared by incubating rabbit PMN with EPA) was at least 30 times less potent than LTB_4 in causing aggregation, chemokinesis and degranulation of PMN *in vitro* [20]. Thus, if supplementation of the diet with EPA causes an increased synthesis of LTB_5 and reduced formation of LTB_4 the net effect could be a reduced chemotactic activity and this in turn may lower leukocyte accumulation during the inflammatory response. In order to investigate whether EPA supplementation of the diet has this potential, we have determined the effect of supplementing the diet with EPA on the formation of LTB_4 and LTB_5 by rat PMN. Also, we have compared the FMLP-induced aggregation of PMN from rats fed a normal and an EPA-rich diet.

* Preliminary data were presented at the Symposium on Prostanoids held in Cracow, Poland during September 1983.

† To whom correspondence should be addressed.

MATERIALS AND METHODS

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), characterized by gas chromatography–mass spectrometry (GC–MS) and quantified by u.v. spectrophotometry as previously described [20]. Prostaglandin B_3 (PGB_3) was prepared by treatment of PGE_3 (a gift from Upjohn Co., Kalamazoo, MI, U.S.A.) with 1 M methanolic potassium hydroxide; the product was extracted into ethyl acetate, purified by HPLC and then quantified by u.v. spectrophotometry (ε_{278}^{MeOH} 28,500; [21]). Heparin, oyster glycogen (grade II), *N*-2-hydroxy-ethylpiperazine-*N*-2-ethane sulphonic acid (HEPES) and FMLP were from Sigma Chemical Co., Poole, Dorset, U.K. The calcium ionophore, A23187, was purchased from Calbiochem, Bishops Stortford, Herts, U.K. Hanks' balanced salt solution (HBS) was provided by Wellcome Diagnostics, Dartford, Kent, U.K. Reverse-phase silica cartridges (C_{18}-Sep-Pak) were obtained from Waters Associates, Northwich, Cheshire, U.K. Analytical grade and HPLC grade solvents were from BDH (Poole, Dorset, U.K.) and Rathburn Chemicals (Walkerburn, Peebleshire, Scotland) 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. A detailed description of the composition of this standard diet is provided by the manufacturer but the fatty acids in the diet were re-examined in our laboratories. The acids of interest with proportions (mole % of total fatty acid) in parentheses are as follows: 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 (20 rats) were given EPA-E (240 mg/kg per day, i.e. 304 mg/kg per day of fatty acid ethyl ester mixture) as an oil in water 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 (20 rats). Rat standard diet and water were given *ad lib.* to both groups. Body weight was measured weekly.

(2) *Preparation of rat leukocytes.* Peritoneal exudate cells were obtained from individual rats 17 hr after intraperitoneal (i.p.) injection of 0.2% oyster glycogen as described previously [20]. Briefly, cells were harvested immediately following i.p. injection of 20 ml HEPES buffered Hanks' balanced salt solution, pH 7.4 (HHBS) containing 20 U/ml heparin. Contaminating erythrocytes were lysed with ammonium chloride. The leukocytes were washed once with HHBS and resuspended in HHBS to give concentrations of 5×10^6, 1×10^7 and 5×10^7 cells/ml. Approximately 80% of the total leukocytes were neutrophils and the remainder were mononuclear cells (monocytes and macrophages) as assessed by differential staining and light microscopy. The concentration of leukocytes was determined using a model ZBI Coulter Counter (Coulter Electronics Limited, Herts, U.K.).

(3) *Radioimmunoassay of LTB_4 synthesized by leukocytes.* After 15 min prewarming, cell suspensions (500 μl; 5×10^6 cells/ml) were incubated in duplicate with 5 μg/ml A23187 for 10 min at 37°. The reaction was terminated by centrifugation at 12000 *g* for 1 min. The concentration of immunoreactive LTB_4 in the cell free supernatant was determined by specific radioimmunoassay (RIA) for LTB_4 as described previously [22].

(4) *Reverse-phase (RP)-HPLC determination of LTB_4 and LTB_5 synthesized by leukocytes.* Leukocyte suspensions (1 ml; 5×10^7 cells) from each rat were pre-warmed at 37° for 15 min and then stimulated with 5 μg/ml A23187 for 10 min at 37°. Incubations were terminated by addition of 2 vol ethanol and then PGB_3 (500 ng) was added as internal standard. Preliminary experiments had shown that PGB_2, which has been used as a standard in assays for LTB_4 [23] was not resolved from LTB_5 during HPLC; also, PGB_1 was not suitable as it was not discriminated from LTB_4. However, PGB_3 was clearly separated from both LTB_4 and LTB_5 (Fig. 1) and was a suitable internal standard in the assays of LTB_4 and LTB_5.

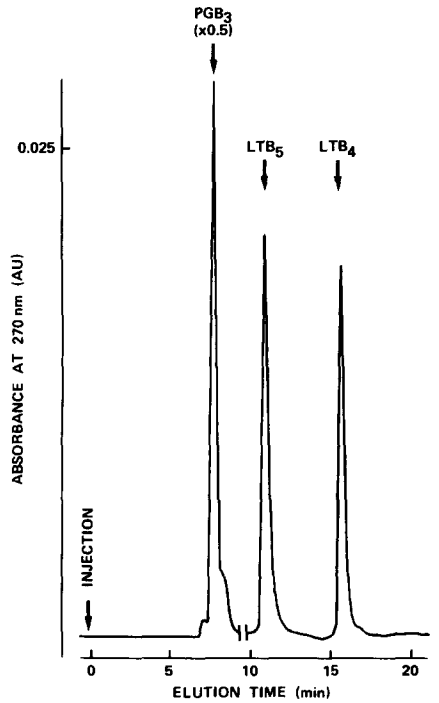

Fig. 1. Reverse-phase HPLC separation of a mixture containing PGB_3 (500 ng), LTB_5 (100 ng) and LTB_4 (100 ng). The HPLC conditions are described in Methods.

The LTB_4, LTB_5, and PGB_3 in each incubation mixture were extracted using ODS silica mini-columns (C_{18}-Sep-Pak) as described previously [20]. Briefly, the ethanolic incubation mixture was centrifuged and the supernatant removed. This aqueous-ethanol mixture was acidified with 1 N HCl to pH 3 and then applied to a pre-wetted (20 ml ethanol then 20 ml water) C_{18}-Sep-Pak. The mini-column was successively washed with 15% aqueous-ethanol (20 ml), water (20 ml), petroleum ether (20 ml) and finally spectroscopic grade ethyl acetate (6 ml). The ethyl acetate fraction was evaporated to dryness under nitrogen and then redissolved in 100 μl HPLC solvent (methanol–water–acetic acid; 70:30:0.01 v/v/v adjusted to an apparent pH of 5.7 with aqueous ammonia). Samples were applied onto a spherisorb ODS 5 μm column (250 × 4.5 mm; Laboratory Data Control, Stone, Staffs, U.K.) via a model 6K injector (Waters Associates). HPLC solvent was pumped through the column at 1 ml/min using a 6000A solvent delivery system (Waters Associates). The absorbance of the column eluant at 270 nm was continuously monitored with a variable wavelength detector (Spectromonitor III; Laboratory Data Control). To obtain standard calibration curves for both LTB_4 and LTB_5, PGB_3 (500 ng) was added as internal standard to aqueous solution of leukotrienes (25–1000 ng) and then the standard solutions were extracted and subjected to HPLC as for the samples (see above). The peak heights ratio LTB_4/PGB_3 and LTB_5/PGB_3 were calculated and plotted against the concentration of the relevant leukotriene (Fig. 2). The concentrations of the leukotrienes in samples were calculated with reference to these standard curves. Eluates from HPLC were collected every 15 sec for 20 min and the immunoreactivity in the fractions was assessed using a RIA for LTB_4 to confirm the presence of LTB_4 and LTB_5.

(5) *Analysis of fatty acid composition in leukocyte phospholipids.* The lipids in residual leukocytes (3×10^7–5×10^7 cells) were extracted with chloroform–methanol (2:1 v/v) containing butylated hydroxytoluene (BHT, 0.2%) at room temperature for 60 min. The lipids were separated by thin layer chromatography on glass plates with a 0.5 mm layer of silica gel 60F 254 (Merck, West Germany) using petroleum ether:ethyl ether:acetic acid (82:18:1, v/v/v) as developing solvent. The fatty acids in the phospholipid fraction were methylated and analysed by GC using C23:0 fatty acid as internal standard as previously described [24, 25].

(6) *Leukocyte aggregation.* Leukocyte aggregation induced by FMLP was assessed using a previously described modification [20] of the method reported by Cunningham *et al.* [26]. Briefly cell suspensions (500 μl; 1×10^7 cells/ml) were warmed to 37° and stirred at 800 rpm in a dual channel optical aggregometer (Payton Associates) for 3 min before the addition of FMLP (10^{-7}–5×10^{-9} M). Aggregation was assessed as the maximum increase in light transmission which occurred after 3 min.

Statistical analysis was performed using Students' unpaired *t*-test.

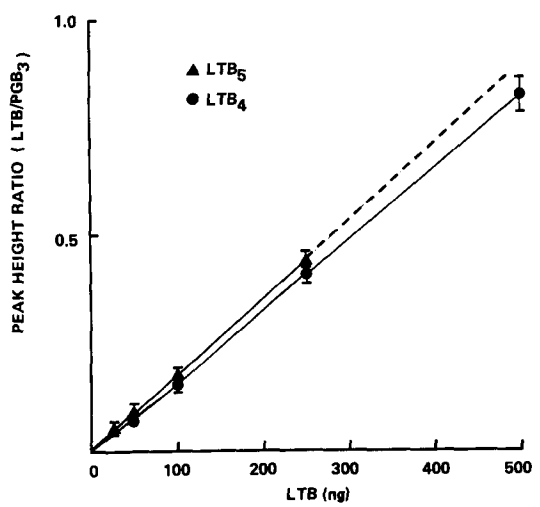

Fig. 2. Standard curves for LTB_4 (●) and LTB_5 (▲), using PGB_3 as internal standard, after extraction and RP-HPLC. Each point is the mean ± S.E. of the mean (N = 3).

RESULTS

There was no significant difference in body weight between the control group of rats and the group given a supplementary diet of EPA (240 mg/kg/day). Rats given EPA consumed a similar amount of standard diet as the control group (approximately 20–25 g/day). Also, the number of leukocytes elicited into the peritoneal cavity with glycogen was not significantly different between the two groups; the total number of cells elicited were $11.2 \pm 0.74 \times 10^7$ and $10.9 \pm 0.56 \times 10^7$ (mean ± S.E.) in the control and EPA-fed groups, respectively. The proportion of neutrophils in the elicited cells was the same in both groups (80%); the majority of the other cells were mononuclear leukocytes.

After 4 weeks supplementation of the diet with EPA the production of LTB_4 by A23187-stimulated leukocytes measured by direct RIA of cell-free supernatant was significantly less than in the control group (42.6 ± 3.25 and 53.50 ± 3.49 ng/5×10^6 cells respectively; $P < 0.05$). If LTB_5 was present in the samples 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%, see Ref. 20).

In order to determine the concentrations of LTB_4 and LTB_5 separately, RP-HPLC was employed using PGB_3 as internal standard. Ultraviolet absorbing peaks were detected at the retention times corresponding to LTB_4 (15.5 min) and LTB_5 (10.75 min) in the incubations of leukocytes from both control and EPA-fed groups of rats (Fig. 3). The amount of LTB_5 formed by cells from the control group was low (Fig. 3) but was significantly higher in cells from the EPA-fed group (see Fig. 3 and Table 1). Also, leukocytes from animals given EPA produced lower amounts of LTB_4 relative to the control group but this was not significant (see Fig. 3 and Table 1). Confirmation that the u.v. peaks corresponding to LTB_4 and LTB_5 were indeed these leukotrienes was obtained by the demonstration that immunoreactive "LTB_4" was detected at the retention time corresponding to LTB_5 as well as LTB_4 (Fig. 4). After

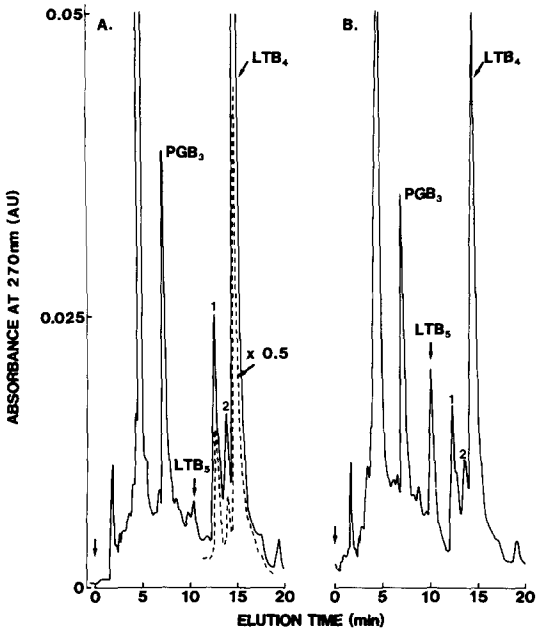

Fig. 3. RP-HPLC chromatograms of products obtained after incubation of rat leukocytes with A23187: (A) leukocytes from a rat fed a normal diet; (B) leukocytes from a rat fed an EPA-rich diet. PGB_3 was used as an internal standard; other experimental conditions are described in Methods. Peak 1 is 5(*S*),12(*R*)-6 *trans*-LTB_4, Peak 2 is 5(*S*),12(*S*)-6 *trans*-LTB_4.

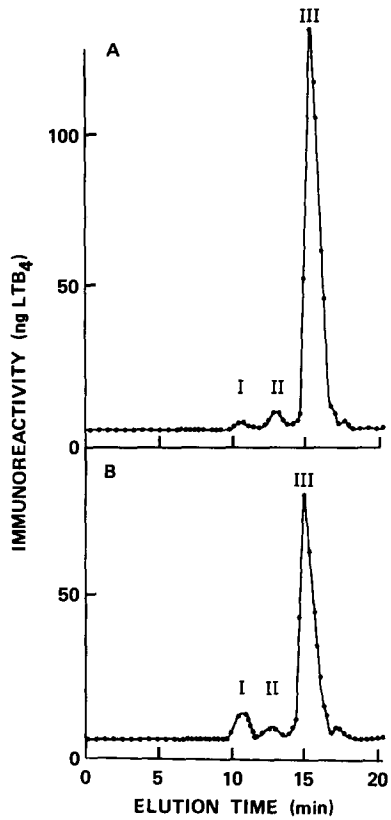

Fig. 4. Assessment of immunoreactivity after RP-HPLC separation of LTB_4 and LTB_5 synthesized during incubation of A23187-stimulated leukocytes from (A) rat fed normal diet, (B) rat given EPA. Fractions of column eluent were collected every 15 sec, the solvent evaporated under nitrogen and the immunoreactivity determined using a RIA for LTB_4 (see Methods). Peak I, Peak II and Peak III are eluted at the exact retention volumes corresponding to LTB_5, 5(*S*),12(*R*)-6-*trans*-LTB_4 and LTB_4, respectively. LTB_5 and 5(*S*),12(*R*)-6-*trans*-LTB_4 cross-react 17.1% [20] and approximately 3.3% [22] respectively.

4 weeks feeding of EPA-E the ratio of LTB_5/LTB_4 increased significantly ($P < 0.001$; Table 2).

The concentrations of LTB_4 measured by RIA and RP-HPLC were similar. Interestingly, the sum of LTB_4 and LTB_5 biosynthesized was approximately the same in both groups of animals. The presence of LTB_5 in the control group is explained by the fact that the standard rat diet employed contained a small quantity of EPA.

Supplementation of the normal diet with EPA-E (240 mg/kg/day) for 4 weeks caused a significant increase in the EPA content of leukocyte phospholipids, however, neither AA nor linoleic acid were significantly affected (Table 2). The ratio EPA/AA in leukocytes phospholipids was increased significantly ($P < 0.001$) after EPA administration and this correlated ($r = 0.795$, $P < 0.001$) with the LTB_5/LTB_4 ratio produced after stimulation of leukocytes with A23187 (Fig. 5).

Leukocytes from EPA-fed rats exhibited a decreased aggregation response to FMLP compared to cells from control animals but this change was not significant ($P < 0.1$) (Table 3).

Table 1. Formation of LTB_4 and LTB_5 by A23187-stimulated leukocytes from rats fed (A) normal diet (B) diet supplemented with EPA

Treatment	Synthesis LTB (ng/5 × 10^6 cells)		Ratio LTB_5/LTB_4
	LTB_4	LTB_5	
Control group (N = 13)	44.0 ± 4.74	2.56 ± 0.51	0.055 ± 0.007
EPA fed group (N = 13)	37.3 ± 6.91	9.22 ± 1.78*	0.259 ± 0.015†

The amounts of LTB_4 and LTB_5 were assayed separately by RP-HPLC using PGB_3 as internal standard. Results are presented as mean ± S.E.
* $P < 0.01$.
† $P < 0.001$.

Table 2. Effect of oral administration of EPA on fatty acid composition of rat leukocyte phospholipids

Fatty acid	Fatty acid composition (mole %)	
	Control group (N = 8)	EPA fed group (N = 16)
16:0	22.85 ± 0.31	21.93 ± 0.62
18:0	22.02 ± 0.52	21.29 ± 0.41
18:1 (ω9)	13.74 ± 0.19	13.86 ± 0.25
18:2 (ω6)	12.38 ± 0.37	12.50 ± 0.28
20:3 (ω6)	1.43 ± 0.11	1.40 ± 0.10
20:4 (ω6)	17.13 ± 0.56	17.47 ± 1.16
20:5 (ω3)	0.55 ± 0.06	1.71 ± 0.14*
22:5 (ω3)	1.18 ± 0.15	1.88 ± 0.09*
22:6 (ω3)	1.46 ± 0.18	1.15 ± 0.07
EPA/AA ratio	0.032 ± 0.005	0.108 ± 0.031*

Values are the mean ± S.E.
* $P < 0.001$.

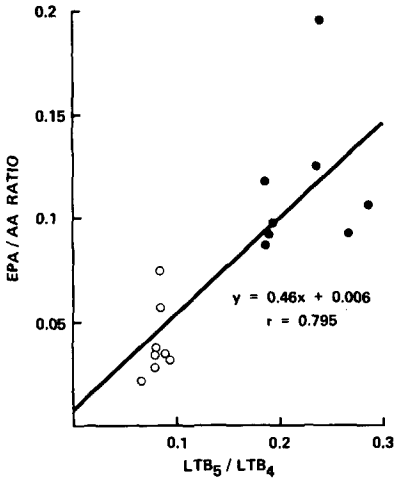

Fig. 5. Correlation between the ratio of EPA/AA present in leukocytes and LTB_5/LTB_4 formation in the cells. EPA and AA content in phospholipids of unstimulated leukocytes was determined by GC; LTB_5 and LTB_4 were measured by RP-HPLC after stimulation of cells with A23187. Data from control group (○) and rats fed EPA (●).

DISCUSSION

Although EPA is a poor substrate for the fatty acid cyclo-oxygenase, other investigators have shown that, *in vitro*, EPA is a good substrate for mammalian lipoxygenases [17, 18] and can be converted to pentaenoic leukotrienes [17, 27]. In this study, we have demonstrated that supplementation of a normal rat diet with EPA (240 mg/kg per day) for 4 weeks, caused a significant increase in the formation of LTB_5 by leukocytes stimulated with A23187, thereby confirming that EPA is a substrate for the 5'-lipoxygenase. The amount of total LTB (i.e. LTB_4 plus LTB_5) was similar in control and EPA-fed rats because synthesis of LTB_4 was decreased after EPA supplementation.

Murphy *et al.* [19] reported that feeding an EPA-rich diet to mice with a neoplastic mast cell tumour caused an increased synthesis of LTB_5 and LTC_5 by tumour cells stimulated with A23187. The latter study also demonstrated that synthesis of LTB_4 was drastically reduced (to approximately 2.5% of control) by feeding mice with EPA. In our study, the reduction of LTB_4 was not so marked probably because of differences in feeding protocols: Murphy *et al.* administered fish oil to supplement a fat-free diet whereas the animals in our study were given a normal diet with additional EPA. In both studies, the concentrations of LTB_4 and LTB_5 formed reflected the relative abundancies of the precursor fatty acids in the cell lipids or phospholipids. The feeding protocol adopted by Murphy *et al.* greatly reduced AA in the tumour cells, in fact, nearly equal amounts of EPA and AA were present in the lipid fraction. The feeding regime used in the present study did not modify the AA content of PMN phospholipids; the significant increase of EPA/AA ratio was due only to the increase in EPA. The significant increase in docosapentaenoic acid (C22:5) observed in leukocyte phospholipids after feeding EPA (Table 2) may be explained by chain elongation of EPA which has been reported to occur in human endothelial cells after incubation with EPA [28].

The reduced formation of LTB_4 observed by Murphy after feeding EPA can be explained by the reduced AA content in the lipids, but our observation that LTB_4 was reduced when AA was not modified requires an alternative explanation. EPA may be an inhibitor of AA metabolism by 5'-lipoxygenase as indicated by recent data [29]. Also, EPA may inhibit the release of AA from PMN membrane phospholipids; this occurs in platelets [25]. Whatever the mechanism, our data indicate that the supplement of EPA to normal diet employed in this study causes a significant effect on the production of LTB_4 and LTB_5 by PMN.

Since LTB_5 is less biologically active than LTB_4 in causing chemokinesis, degranulation and aggregation of PMN [20] the effect of feeding an EPA-

Table 3. FMLP-induced aggregation of rat leukocytes from control and EPA fed animals

Treatment	Concentration of FMLP (M)			
	10^{-7}	2×10^{-8}	10^{-8}	5×10^{-9}
Control group (N = 9)	45.8 ± 5.6	26.9 ± 4.2	18.1 ± 2.9	9.7 ± 1.3
EPA fed group (N = 9)	41.6 ± 3.5	17.8 ± 1.8	12.8 ± 1.6	6.4 ± 0.8

Maximum height of aggregation response (mm) are presented as mean ± S.E.

rich diet could be an overall decrease of chemotactic activity. The decrease of biological activity may be compounded, since we have observed that LTB_5 is also a weak antagonist of LTB_4-induced degranulation (unpublished data). Thus, if LTB_4 does play an imporant role in mediating the accumulation of PMN at sites of inflammation it is tempting to speculate that EPA-feeding could reduce this effect and may, therefore, be of benefit in the prevention and/or treatment of chronic inflammatory disease. Indeed, Eskimos, who consume an EPA-rich diet are almost free from chronic degenerative disease, including ulcerative colitis and rheumatoid arthritis [30]. Also, Prickett and colleagues [31] reported that feeding EPA prevents proteinuria and prolongs survival of mice in a model of human systemic lupus erythematosis.

After feeding EPA, FMLP-induced aggregation of PMN was reduced, but not significantly. Since arachidonate metabolites are probably not involved in FMLP-induced aggregation [32, 33], these data suggest that EPA supplementation could affect the functional response of neutrophils by a separate mechanism.

Leukocyte infiltration occurring in cerebral and myocardial infarction may be mediated partly by a lipoxygenase product (possibly LTB_4) since BW755C, a dual inhibitor of arachidonate cyclooxygenase and lipoxygenase, reduces cell accumulation and this correlates with a decreased size of infarction [34, 35] indicating that cell influx contributes to the tissue damage. If LTB_4 does indeed mediate the cell movement then EPA-feeding may, by decreasing LTB_4-synthesis, reduce the area of damage after infarction. Epidemiological surveys indicate a low incidence of myocardial infarction among Eskimos [1] and Japanese [36] who consume a diet rich in EPA. Also animal studies suggest that the incidence of both experimentally-induced cerebral and myocardial infarction is reduced by the intake of an EPA-rich diet [37, 38].

Acknowledgements—The authors wish to thank Drs. T. Fujita and A. Ozawa for performing the fatty acid analysis and Miss L. C. Tilling for her excellent technical assistance.

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