Effect of dietary supplementation with fish oil lipids on mild asthma

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ABSTRACT Recruitment of inflammatory leucocytes to the airways may play a part in the pathogenesis of asthma. As dietary enrichment with fish oil lipids can suppress leucocyte function, the effect of these lipids on asthma control and neutrophil function was studied in 20 subjects with mild asthma. Twelve subjects received capsules containing 3.2 g of eicosapentaenoic acid and 2.2 g of docosahexaenoic acid daily and eight subjects received placebo capsules containing olive oil for 10 weeks in a double blind fashion. Baseline specific airways conductance, airways responsiveness to histamine and exercise, diurnal peak expiratory flow, symptom scores, and bronchodilator use were measured. Neutrophil fatty acid composition was evaluated by gas chromatography, calcium ionophore induced neutrophil leukotriene (LT) B_4 and LTB $_5$ generation were measured by reverse phase high performance liquid chromatography and radioimmunoassay, and neutrophil chemotactic responses to formyl-methionyl-leucyl-phenylalanine (FMLP) and LTB $_4$ were assessed by a micro-chemotaxis technique. Although the fish oil supplemented diet produced a greater than 10 fold increase in the eicosapentaenoic acid content of neutrophil phospholipids, there was no significant change in airways responsiveness to histamine or any change in any of the clinical measurements. After dietary supplementation with fish oil there was a 50% inhibition of total LTB (LTB $_4$ + LTB $_5$) generation by ionophore stimulated neutrophils and neutrophil chemotaxis was substantially suppressed. Neutrophil function remained unchanged in the placebo group. It is concluded that in subjects with mild asthma a fish oil enriched diet attenuates neutrophil function without changing the severity of asthma.

Asthma is characterised by airways inflammation, by bronchial hyperresponsiveness to non-specific stimuli, and by episodic and reversible airflow obstruction. Studies both in experimental animals and in man have indicated an association between airways hyperresponsiveness and bronchial inflammation.¹⁻⁹ It has been suggested that airway inflammation may be central to the pathophysiology of bronchial asthma.

Eicosapentaenoic acid and docosahexaenoic acid are polyunsaturated fatty acids derived from fish oil. They are termed omega-3 (ω_3) or N-3 fatty acids because the first double bond in the molecule is three carbons removed from the terminal methyl group. These fatty acids competitively inhibit the formation

of prostaglandins and leukotrienes derived from arachidonic acid (N-6 fatty acid),¹⁰⁻¹² and eicosapentaenoic acid acts as a substrate for the biosynthesis of prostaglandins with three double bonds and leukotrienes with five double bonds (fig 1). Thus eicosapentaenoic acid is metabolised by the cyclo-oxygenase pathway to thromboxane A $_2$ and prostaglandin (PG) I $_3$ ¹³ and by the 5-lipoxygenase pathway to leukotriene (LT)C $_5$, LTD $_5$, LTE $_5$, and LTB $_5$.¹⁴⁻¹⁶ Thromboxane A $_2$ and LTB $_5$ have diminished biological activities, whereas PGI $_3$, LTC $_5$, LTD $_5$, and LTE $_5$ are equipotent with their counterparts derived from arachidonic acid.¹³⁻¹⁷ Neutrophils and monocytes obtained from normal subjects who have ingested marine lipids in the form of Max-EPA show an attenuated production of LTB $_4$ after calcium ionophore stimulation, diminished in vitro chemotactic responsiveness to LTB $_4$, and a substantial inhibition of their capacity to adhere to endothelial monolayers pretreated with LTB $_4$.¹⁸ Thus

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Accepted 11 November 1987

They wore a nose clip and undertook eight minutes of exercise on a mechanically braked bicycle ergometer at 60–130 watts (Body Guard 990, Ogloend, Norway). The work load was adjusted to produce about 80% of maximal predicted oxygen consumption. The exercise was undertaken at ambient temperature and humidity. The pulse rate was measured before and at one minute intervals during exercise. Specific airways conductance (sGaw) was measured before exercise and 5, 10, 15, 30, 45, and 60 minutes after exercise.

HISTAMINE CHALLENGE

Inhalation challenge was performed with a Hudson nebuliser linked to a breath activated dosimeter.²² Delivery of air to the nebuliser was regulated to a pressure of 149 kPa (20 lb/in²) for 0.6 second from the start of each inspiration. After baseline measurements of sGaw subjects inhaled five breaths of phosphate buffered saline. If the decrease in sGaw was under 10% the patients were subjected to histamine challenge. Two fold increases in the concentration of histamine acid phosphate (Sigma, Dorset) diluted in phosphate buffered saline were inhaled from a starting concentration of 0.5 mg/ml. sGaw was measured at two minute intervals after each inhalation of histamine and increasing concentrations of histamine were administered until a greater than 35% fall in sGaw was achieved. The cumulative dose of histamine required to produce a 35% fall in sGaw (histamine PD₃₅ sGaw) was determined by linear interpolation from the histamine log dose-response curve.

MEASUREMENTS OF SGAW

Measurements of sGaw were made in a total body plethysmograph linked to a digital computer.²³ Four to six measurements of sGaw were recorded at each time point and the mean value was calculated. Baseline sGaw was over 0.9 s⁻¹ kPa⁻¹ before each challenge.

NEUTROPHIL STUDIES

Neutrophils were purified from 20 ml of anticoagulated peripheral blood to more than 95% by means of dextran sedimentation (Pharmacia, Bucks) and centrifugation on a cushion of Lymphoprep (Nyegaard, Birmingham).²⁴ A portion of 2×10^7 cells was resuspended in 800 μ l of distilled water and stored under argon at -70°C before extraction of phospholipids. Neutrophil phospholipids were extracted by the method of Bligh and Dyer.²⁵ Samples were first extracted in chloroform (BDH, Dorset). After being dried under a steady stream of nitrogen they were resuspended in boron trifluoride (Sigma) and heated to 100°C under nitrogen for 90 minutes to esterify the fatty acids. Samples were then cooled, extracted into hexane (BDH), and stored at -70°C under nitrogen before analysis for fatty acid composi-

tion by gas chromatography.¹⁹

After the removal of neutrophils for lipid extraction, the remaining cells were used whenever possible for assessment of chemotactic responses to FMLP (Sigma) and LTB₄, and for measurement of the quantities of LTB₄ generated by neutrophils stimulated by calcium ionophore (A23187) (Calbiochem, La Jolla, California) under optimal conditions. Chemotaxis was assessed by a microchemotaxis method.¹⁴ Results were expressed as the number of neutrophils per five high power fields after correction for background migration. The assay, which has an intra-assay coefficient of variation of 19.5%, was performed in duplicate. Calcium ionophore was dissolved at 10 mmol/l in dimethyl sulphoxide (BDH) and diluted to specific concentrations in Hanks' balanced salt solution (Flow Laboratories, Rickmansworth, Herts) containing calcium and magnesium, 30 mM *N*-2-hydroxyethyl-piperazine-*N*-2-ethane sulfonic acid (HEPES) (Sigma) and 0.1% bovine serum albumin (Sigma). Two $\times 10^6$ neutrophils in 500 μ l of buffer were preincubated at 37°C for 10 minutes before the addition of 500 μ l of buffer containing A23187 to achieve a final concentration of 10 μ mol/l, or with 500 μ l of buffer alone as a control. The mixture was incubated for 10 minutes at 37°C . The reaction was stopped by rapid cooling on ice and centrifugation at 10 000 g for 30 seconds. The supernatant was removed and stored at -20°C .

Generation of LTB₄ and LTB₅ was assayed by reverse phase high performance liquid chromatography and radioimmunoassay.^{18,26} Briefly, supernatants were applied to a 10 μ m C18 Ultrasil ODS column (4.6 $\times$ 250 mm, Beckman Instruments, Berkeley, California). The products were eluted at 1 ml/min in 63% methanol (BDH), 36.9% water, 0.1% (vol/vol) acetic acid, pH 5.6 for 30 minutes. One millilitre fractions were collected. Duplicate 100 μ l samples of each fraction were evaporated to dryness under negative pressure, then each was resuspended in 100 μ l of isogel tris buffer and measured for immunoreactive LTB₄ and LTB₅ by radioimmunoassay. The column was calibrated for the retention times of synthetic leukotrienes: LTB₅ (12.2 (SEM 0.4) min, $n = 10$); (5*S*,12*R*)-6-*trans*-LTB₄ (15.1 (0.5) min, $n = 10$); (5*S*, 12*S*)-6-*trans*-LTB₄ (16.0 (0.7) min, $n = 10$); and LTB₄ (19.5 (0.9) min, $n = 10$). Immunoreactive products were identified by a comparison of their retention times with those of the standards. Recoveries of synthetic standards were similar for all compounds and were over 85%. The recovery of immunoreactive material was 89% (SEM 2%). The 50% inhibition of binding of [³H]-LTB₄ to anti-LTB₄ occurred at 0.15 ng LTB₄ and 0.28 ng LTB₅. Intra-assay and interassay coefficients of variation were 7% and 16% respectively.

ANALYSIS OF RESULTS

Changes in ionophore induced LTB₄ generation by neutrophils were assessed by the Wilcoxon signed rank test. Changes in chemotactic responsiveness of neutrophils to FMLP and to LTB₄ were assessed by comparing the whole dose-response curve before and after the diet by analysis of variance. Change in histamine PD₃₅ sGaw was assessed by Student's paired *t* test on log transformed data.

Changes in airways response to exercise, bronchodilator usage, and morning and evening peak expiratory flow rates were assessed by Student's *t* test for paired data. Symptom scores were analysed with a randomisation test. All results are expressed as means with standard errors in parentheses unless otherwise stated.

Results

Twenty subjects completed the trial. Five individuals did not complete the study: three subjects found the number and size of capsules unmanageable; one subject withdrew from the study owing to personal circumstances; one subject started the study but was withdrawn because three weeks after starting the diet she required admission to hospital for acute asthma. During this period she started taking oral steroids and stopped taking Max-EPA. From the 20 subjects who completed the trial, 12 (six male and 10 atopic, aged 15–41 (mean 25) years) had received Max-EPA and eight subjects (four male, all atopic, aged 19–39 (mean 26) years) had received placebo capsules that did not contain marine fatty acids.

CLINICAL MEASUREMENTS

Airways histamine responsiveness

Histamine responsiveness was assessed in seven "placebo" subjects and 11 subjects receiving Max-EPA. One subject from each treatment group declined to have a second histamine challenge. There was no significant change in baseline sGaw or histamine responsiveness in either group of subjects. Baseline sGaw changed from 1.15 (SEM 0.15) to 1.17 (0.13) s⁻¹ kPa⁻¹ after placebo, and from 1.41 (0.14) to 1.50 (0.15) s⁻¹ kPa⁻¹ after Max-EPA. The geometric mean histamine PD₃₅ sGaw changed from 0.17 to 0.18 μmol after placebo and from 0.32 to 0.37 μmol (*p* > 0.05) after Max-EPA (fig 2). The mean difference in histamine PD₃₅ sGaw between values after Max-EPA and control values before Max-EPA was 0.07 log units (95% confidence intervals + 0.36 and - 0.22 log units).

Airway response to exercise

Exercise challenge was performed in five subjects receiving placebo and six receiving Max-EPA. There was no significant difference in the maximal post-exertional fall in sGaw before and after placebo or Max-EPA. The maximal percentage decreases in sGaw were 68.2 (SEM 2.9) and 69.2 (4.7) before and after placebo and 55.5 (5.5) and 56.5 (3.6) before and after Max-EPA.

Diary cards

Diary cards were available for analysis from five subjects receiving placebo and 12 receiving Max-EPA. Three subjects receiving placebo lost their diary cards during the study. There was no significant change in any of the clinical indices assessed after either the placebo or the Max-EPA supplemented diet (table 1).

The clinical responses of patients having inhaled corticosteroids did not differ significantly from those of subjects not receiving these drugs.

Fig 2 Airways histamine PD₃₅ sGaw (dose causing a 35% fall in specific airways conductance) before and after dietary supplementation with placebo capsules and Max-EPA. — denotes geometric mean. Each point denotes an individual subject.

Table 1 Mean (SEM) diurnal peak expiratory flow rates, airways lability, symptom scores, and bronchodilator use in the two weeks before and the last two weeks of dietary supplementation with placebo and Max-EPA*

	Placebo (n = 5)		Max-EPA (n = 12)		Mean difference (95% CL)
	Before	After	Before	After	
Morning peak flow (1 min ⁻¹)	426 (41)	430 (39)	494 (24)	499 (26)	+ 5 (+ 26, - 16)
Evening peak flow (1 min ⁻¹)	438 (43)	437 (43)	505 (26)	503 (24)	- 2 (+ 16, - 19)
Lability (%)	7.5 (1.0)	6.7 (0.9)	4.7 (0.61)	4.6 (1.1)	- 0.1 (+ 2.3, - 2.6)
Symptoms (total score)	5.8 (3.0)	8.3 (2.3)	10.9 (3.6)	14.1 (3.2)	+ 3.2 (+ 13.0, - 6.7)
Bronchodilator use (total number of doses)	63.2 (21.6)	55.0 (16.8)	31.8 (9.4)	39.6 (12.6)	+ 7.8 (+ 20, - 4.4)

*Contains 3.2 g eicosapentaenoic acid and 2.2 g docosahexaenoic acid.
CL—confidence limits.

NEUTROPHIL STUDIES

Neutrophil phospholipid fatty acid composition

The fatty acid content of neutrophil phospholipids was assessed in all subjects. Arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid comprised 12.9% (SEM 3.7%), 0.1% (0.1%), and 2.8% (0.7%) of the total neutrophil fatty acid content of subjects before dietary supplementation with placebo capsules, and 14.6% (2.7%), 0.2% (0.1%), and 2.2% (0.6%) in subjects before supplementation with Max-EPA. The neutrophil eicosapentaenoic acid content remained unchanged after placebo capsules, but rose to 2.6% (0.5%) of total neutrophil fatty acids after Max-EPA. There was no significant change in neutrophil arachidonic acid or docosahexaenoic acid content in either group (table 2).

Calcium ionophore induced generation of LTB compounds

Calcium ionophore induced generation of LTB₄ and LTB₅ was assessed in six subjects receiving placebo and 11 receiving Max-EPA because of the limitation in cell numbers. There was no significant change in LTB₄ generation in the placebo group after olive oil supplementation (control values 10.7 (SEM 3.4) ng/2 × 10⁶ neutrophils; after the diet 10.9 (3.0) ng/2 × 10⁶ neutrophils; p > 0.05). In subjects who

had taken Max-EPA generation of LTB compounds was attenuated by 48% (control 15.3 (6.0) ng/2 × 10⁶ neutrophils; after the diet 8.0 (2.8) ng/2 × 10⁶ neutrophils, p < 0.01). No LTB₅ was generated by ionophore activated neutrophils before either dietary period or after treatment with placebo. After dietary supplementation with Max-EPA 10 μM calcium ionophore stimulated neutrophils to generate 1.8 (0.6) ng of LTB₅/2 × 10⁶ neutrophils (table 3).

Neutrophil chemotaxis

Because cells were limited neutrophil chemotactic responsiveness to FMLP was assessed in five subjects receiving placebo and 10 receiving Max-EPA, and responsiveness to LTB₄ in four receiving placebo and six receiving Max-EPA. The chemotactic responsiveness of neutrophils to both agonists was unchanged after 10 weeks of placebo treatment. After 10 weeks of Max-EPA there was a substantial and consistent attenuation of the chemotactic responses to both FMLP (p = 0.01) and LTB₄ (p = 0.04). The maximal chemotactic response to 10⁻⁷ mol FMLP and 10⁻⁶ mol LTB₄ decreased from 115 (SEM 39) and 121 (53) neutrophils/5 high power fields (hpf) before the diet to 54 (14) and 52 (19) neutrophils/5 hpf after Max-EPA (figs 3 and 4).

Table 2 Mean (SEM) fatty acid composition of neutrophil extracts, expressed as a percentage of total fatty acid content, before and after dietary supplementation with placebo and Max-EPA*

	Fatty acids			
	18:2 linoleic	20:4 arachidonic	20:5 eicosapentaenoic	22:6 docosahexaenoic
Placebo (n = 8)				
Before	5.9 (1.5)	12.9 (3.7)	0.1 (0.1)	2.8 (0.7)
After	6.9 (1.3)	13.5 (2.8)	0.1 (0.1)	1.9 (0.4)
Max-EPA (n = 12)				
Before	7.0 (1.2)	14.6 (2.7)	0.2 (0.1)	2.2 (0.6)
After	6.7 (1.0)	13.3 (1.3)	2.6 (0.5)	2.6 (0.3)

*See table 1.

Table 3 Generation of leukotriene B compounds (LTB_4 and LTB_5) from ionophore stimulated neutrophils (means (SEM)) before and after dietary supplementation with placebo and Max-EPA*

	Quantity of product (ng/2 × 10 ⁶ neutrophils)		
	Total LTB Compounds	LTB_4	LTB_5
Placebo (n = 6)			
Before	10.7 (3.4)	10.7 (3.4)	ND
After	10.9 (3.0)	10.9 (3.0)	ND
Max-EPA (n = 11)			
Before	15.3 (6.0)	15.3 (6.0)	ND
After	8.0 (2.8)	6.2 (2.5)	(1.8) (0.6)

*See table 1.

ND—not detected.

Discussion

Both eicosapentaenoic acid and docosahexaenoic acid are prominent in fish oil enriched diets. Eicosapentaenoic acid is a better substrate and docosahexaenoic acid is a substantially worse substrate for product generation by the 5-lipoxygenase pathway than is arachidonic acid.²⁷ Docosahexaenoic acid has little effect on arachidonic acid metabolism by the 5-lipoxygenase pathway, whereas eicosapentaenoic acid leads to the elaboration of less LTB_4 ¹² and yields a structurally analogous product LTB_5 , which has reduced biological activity.¹⁴⁻¹⁵ Dietary supplementation with fish oil in the form of Max-EPA for six weeks

in normal individuals led to the incorporation of eicosapentaenoic acid into the membrane lipids of inflammatory leucocytes, suppressed the generation of LTB_4 by the 5-lipoxygenase pathway,^{18,21} and inhibited neutrophil functional responses mediated by this leukotriene.¹⁸ Since bronchial inflammation and infiltrating leucocytes may be important in the pathogenesis of asthma,¹⁻⁹ we have studied the effects of inhibition of neutrophil function through the provision of fish oil lipids on non-specific airway hyperresponsiveness and the severity of asthma.

The study was conducted in a placebo controlled and double blind manner and carried out with a parallel rather than a crossover design because we could not predict the duration of any clinical effect of dietary supplementation with eicosapentaenoic acid. The fish oil lipids were provided in the form of Max-EPA, which consists mainly of triglycerides, with 34% of the total fatty acids and 86% of the polyunsaturated fatty acids consisting of eicosapentaenoic acid and docosahexaenoic acid. The dose of eicosapentaenoic acid we selected for study is the dose that has previously been shown to have anti-inflammatory potential on the basis of its effect on leucocyte function.¹⁸

The airway responses to histamine and exercise were used to assess changes in non-specific bronchial responsiveness during dietary supplementation with fish oil lipids. In addition, symptom scores, bronchodilator use, and airway lability as measured by diurnal variations in peak flow were carefully

Fig 3 Chemotactic responses of neutrophils to formyl methionyl leucyl phenylalanine (FMLP) before (●) and after (○) placebo and Max-EPA. Chemotactic responses are expressed as the number of neutrophils per 5 high powered fields (hpf). Each point is the mean (SEM) of five in the placebo and 10 in the Max-EPA group.

Fig 4 Chemotactic responses of neutrophils to leukotriene B₄ (LTB₄) before (●) and after (○) placebo and Max-EPA. Each point is the mean (SEM) of four subjects in the placebo and six in the Max-EPA group.

documented. There was no significant change in any of the clinical variables assessed after either the placebo or the Max-EPA supplemented diet over the period of the study, despite a greater than 10 fold increase in the eicosapentaenoic acid content of neutrophil phospholipids in individuals given Max-EPA and barely detectable eicosapentaenoic acid in subjects given placebo capsules.

Incorporation of eicosapentaenoic acid into neutrophil membrane phospholipids was analysed by the sensitive and precise technique of gas chromatography. Neutrophil functional responses were assessed by studying the generation of LTB products in cells stimulated by the calcium ionophore and by assessing the chemotactic responsiveness of the same target cells. Previous work had already indicated that eicosapentaenoic acid inhibits the generation of LTB₄ from neutrophils and monocytes stimulated by calcium ionophore in a dose and time dependent manner.¹²⁻¹⁸ Thus in this study the effect of eicosapentaenoic acid was evaluated only with neutrophils that had been stimulated with the ionophore under optimal conditions of dose and time. The calcium ionophore was chosen to bypass membrane calcium gating mechanisms²⁸ and to give a true measure of the integrity of the 5-lipoxygenase pathway. LTB₄ and LTB₅ were measured in neutrophil supernatants after RP-HPLC by means of a sensitive and specific radioimmunoassay.²⁶ The RP-HPLC and radioimmunoassays had been calibrated by authentic reference standards of the relevant leukotrienes and these had been prepared by total organic synthesis.^{29 30}

The capacity of neutrophils to produce LTB₄ was reduced by about half after dietary supplementation with Max-EPA, but showed no change with placebo. These results extend previous work on normal subjects.¹⁸ Since eicosapentaenoic acid does not increase catabolism of LTB₄,¹² this attenuation of LTB₄ generation was attributed to inhibition of biosynthesis.

Since the recruitment of leucocytes to a focus of inflammation by chemotactic factors is likely to have a central role in the amplification of the inflammatory process, we assessed the chemotactic responsiveness of the leucocytes to one or both of two transmembrane and receptor mediated chemotactic agonists—namely, FMLP and LTB₄. Neutrophil chemotactic responsiveness was not evaluated in all individuals because of the limited number of cells. Nevertheless, each subject acted as his own control and the data were consistent within each group. The chemotactic responsiveness of neutrophils to both FMLP and LTB₄ was substantially inhibited after Max-EPA dietary supplementation and this was not observed in the placebo group. Since neutrophil chemotaxis is likely to be of fundamental importance in the development of inflammatory processes, eicosapentaenoic acid can be regarded as anti-inflammatory.

The results of this study indicate that neutrophil function can be suppressed without changing the severity of asthma in patients with mild disease. These results are similar to those of Kirsch *et al*, who have also shown no benefit from a fish oil enriched diet in patients with severe, chronic, and persistent asthma,

despite a substantial attenuation of neutrophil chemotactic responsiveness to LTB₄, FMLP, and complement C5a.^{31,32} These results are consistent with the view that neutrophils do not play a major part in the pathogenesis of asthma or alternatively that, if neutrophils have a role in the mechanisms of asthma, their functions were not suppressed adequately for a clinical effect to be apparent. Although we have shown substantial changes in the in vitro functions of circulating neutrophils, these functions may not be relevant to the physiological events that occur in airways inflammation. In addition, inflammatory mediators other than arachidonic acid derived metabolites and other cell types, such as eosinophils and alveolar macrophages, may play a part in amplifying the inflammatory changes observed in asthmatic airways. There is no information on how these mediators and cells are affected by the fish oil enriched diet. Finally, although 10 weeks of dietary supplementation with fish oil lipids led to a substantial suppression of neutrophil function, this period may not have been enough for regeneration of airways epithelium and resolution of the chronic inflammatory response to effect a change in clinical variables. We have shown that 10 weeks' dietary supplementation with Max-EPA suppresses neutrophil function but fails to alter airways responsiveness and the severity of asthma in individuals with mild disease.

This work was supported in part by the Asthma Research Council, and the International Association of Fish Meal Manufacturers. The authors are grateful to Dr J Rokach for the gift of LTB₄ antiserum and to Seven Seas Ltd, Marfleet, Hull, for the Max-EPA and matched placebo capsules.

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