

## The influence of different combinations of $\gamma$ -linolenic acid, stearidonic acid and EPA on immune function in healthy young male subjects

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To determine the effects of EPA, stearidonic acid (STA) or  $\gamma$ -linolenic acid (GLA) on immune outcomes, healthy male subjects consumed one of seven oil blends for 12 weeks. EPA consumption increased the EPA content of peripheral blood mononuclear cells (PBMC). Consumption of GLA (2.0 g/d) in the absence of STA or EPA increased di-homo-GLA content in PBMC. Neither STA nor its derivative 20:4n-3 appeared in PBMC when STA (<1.0 g/d) was consumed. However, STA (1.0 g/d), in combination with GLA (0.9 g/d), increased the proportion of EPA in PBMC. None of the treatments altered neutrophil or monocyte phagocytosis or respiratory burst, production of inflammatory cytokines by monocytes, T lymphocyte proliferation or the delayed-type hypersensitivity response. Production of cytokines by T lymphocytes increased in all groups, with no differences among them. The proportion of lymphocytes that were natural killer cells decreased significantly in subjects receiving 2.0 g EPA or GLA/d. There were no other effects on lymphocyte sub-populations. Plasma IgE concentration decreased in most groups, but not in the control group. Plasma IgG<sub>2</sub> concentration increased in the EPA group. Thus, EPA or GLA at a dose of 2.0 g/d have little effect on key functions of neutrophils, monocytes and T lymphocytes, although at this dose these fatty acids decrease the number of natural killer cells. At this dose EPA increases IgG<sub>2</sub> concentrations. STA can increase immune cell EPA status, but at 1.0 g/d does not affect human immune function.

### Cytokine: Immunoglobulin: Phagocytosis: Respiratory burst

In recent years there has been great interest in the anti-inflammatory and immunological effects of the long-chain *n*-3 PUFA EPA (20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Dietary supplementation studies with fish oil in human subjects have shown that providing >2.4 g EPA + DHA/d can decrease respiratory burst by neutrophils and monocytes, decrease production of TNF- $\alpha$ , IL-1 $\beta$ , IL-2, IL-6 and interferon (IFN)- $\gamma$  by stimulated mononuclear cells, and decrease proliferation of stimulated T lymphocytes (for references, see Calder 2001a,b, 2002, 2003). The impact of lower intakes of long-chain *n*-3 PUFA remains unclear, with a variety of effects being reported (Meydani *et al.* 1993; Blok *et al.* 1997; Healy *et al.* 2000; Thies *et al.* 2001a,b,c; Kew *et al.* 2003; Trebble *et al.* 2003a,b; Wallace *et al.* 2003). Very high intakes (14 or 18 g/d) of the precursor *n*-3 PUFA  $\alpha$ -linolenic acid (ALA; 18:3n-3) decreased TNF- $\alpha$  and IL-1 $\beta$  production by lipopolysaccharide (LPS)-stimulated mononuclear

cells (Caughey *et al.* 1996), and decreased mitogen-stimulated T lymphocyte proliferation and the delayed-type hypersensitivity (DTH) response (Kelley *et al.* 1991). However, lower intakes of ALA (2.0–9.5 g/d) appear to have little effect on a range of inflammatory and immune responses in human subjects (Healy *et al.* 2000; Thies *et al.* 2001a,b,c; Kew *et al.* 2003; Wallace *et al.* 2003). One reason for the limited impact of ALA may be that its conversion to the longer-chain active derivatives EPA and DHA is limited in human subjects (Burdge *et al.* 2002; Burdge & Wootton, 2002). This could be due to the low activity of  $\Delta^6$ -desaturase (Huang *et al.* 1991; Yamazaki *et al.* 1992) and/or to the inhibitory effect of a high intake of the *n*-6 PUFA linoleic acid (18:2n-6) on ALA conversion. An alternative strategy for increasing long-chain *n*-3 PUFA status of immune cells may be to provide the product of  $\Delta^6$ -desaturase, stearidonic acid (STA; 18:4n-3). Certain oils, such as that from the seed

**Abbreviations:** ALA,  $\alpha$ -linolenic acid; BO, borage oil; con A, concanavalin A; DGLA, di-homo- $\gamma$ -linolenic acid; DHA, docosahexaenoic acid; DTH, delayed-type hypersensitivity; EO, echium oil; GLA,  $\gamma$ -linolenic acid; ICAM, intercellular adhesion molecule; IFN, interferon; LPS, lipopolysaccharide; MFI, median fluorescence intensity; PBMC, peripheral blood mononuclear cell; SI, stimulation index; STA, stearidonic acid.

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of *Echium plantaginium* (echium oil, EO), include a substantial proportion of STA (approximately 13 g/100 g total fatty acids). The immunological effects of STA are largely unknown, although one animal feeding study showed that dietary STA can decrease TNF- $\alpha$  production by LPS-stimulated whole blood to the same extent as the same amount of dietary ALA or EPA (Ishihara *et al.* 2002). However, apart from a recent report on TNF- $\alpha$  and IL-1 $\beta$  production (James *et al.* 2003), there is currently no information about the immunological effects of dietary STA in human subjects.

One other anti-inflammatory strategy has been to provide  $\gamma$ -linolenic acid (GLA; 18:3n-6), which has been demonstrated in some studies to decrease T lymphocyte proliferation (Rosetti *et al.* 1997) and to decrease production of TNF- $\alpha$ , IL-1 and IL-6 by monocytes (De Luca *et al.* 1999). A good source of GLA is the oil from the seed of *Borage officinalis* (borage oil, BO), in which GLA comprises as much as 25 g/100 g total fatty acids. Again, however, the impact of lower intakes of GLA is not clear (Yaqoob *et al.* 2000; Thies *et al.* 2001a,b,c).

Thus, it seems important to clarify the immunological effects of increased, but achievable, intakes of long-chain n-3 PUFA and of GLA and to identify the immunological effects of STA in human subjects. Therefore, the present study examined the effect of various combinations of GLA, STA and EPA on a range of immune outcomes in healthy volunteers. Outcomes investigated included key functional responses of neutrophils (phagocytosis, respiratory burst), monocytes (phagocytosis, respiratory burst, cytokine production) and T lymphocytes (proliferation, cytokine production), as well as circulating immune cell phenotypes and Ig concentrations and the *in vivo* DTH response.

## Subjects and methods

### Materials

Materials were obtained from sources described elsewhere (Kew *et al.* 2003). In addition, in-house ELISA for measurement of human IgG<sub>2</sub> and IgE used the following materials: streptavidin-conjugated horseradish (*Amoracia rusticana*) peroxidase (Biosource International, Etten-Leur, The Netherlands); 3,3',5,5'-tetramethylbenzidine, dimethylsulfoxide, H<sub>2</sub>SO<sub>4</sub>, H<sub>2</sub>O<sub>2</sub> and Tween-20 (Merck; supplied by VWR International, Amsterdam, The Netherlands); bovine serum albumin fraction V (ICN Biochemicals, Zoetermeer, The Netherlands). The IgG<sub>2</sub> ELISA used mouse anti-human IgG<sub>2</sub> monoclonal antibody as the

capture antibody and biotin-conjugated mouse anti-human IgG monoclonal antibody as the detection antibody; the IgE ELISA used mouse anti-human IgE monoclonal as the capture antibody and biotin-conjugated mouse anti-human IgE monoclonal as the detection antibody (all antibodies from Pharmingen; supplied by Becton Dickinson, Alphen aan de Rijn, The Netherlands). Standards were purified human IgG<sub>2</sub> and IgE (both from Biogenesis; supplied by Nuclilab BV, Ede, The Netherlands).

### Subjects and study design

Ethical permission for all procedures involving human volunteers was obtained from the Southampton and South West Hampshire Joint Ethics Committee. Healthy adult male subjects aged 21–44 years were invited to participate in the study. All volunteers completed a health and lifestyle questionnaire before entering the study. Volunteers were excluded if they: were taking any prescribed medication; were vegetarian; consumed fish oil, evening primrose oil or vitamin supplements; smoked more than ten cigarettes per d; drank more than ten units of alcohol per week; had a BMI > 32 kg/m<sup>2</sup>; consumed more than two portions of oily fish per week. Seventy-four subjects were recruited to the study and seventy completed it. Mean age (32.4 (SE 0.8) years) and BMI (25.2 (SE 0.4) kg/m<sup>2</sup>) did not differ among the treatment groups at study entry.

Subjects were randomly allocated in a double-blind fashion to one of seven intervention groups (eight to twelve subjects per group). Subjects consumed nine 1 g capsules per d for 12 weeks. The capsules were provided by Royal Numico Research, Wageningen, The Netherlands. The control group consumed capsules containing stripped palm oil–sunflower oil (80:20, w/w); this mix has a fatty acid composition that closely resembles that of the average UK diet (British Nutrition Foundation 1992, 1999). The other groups consumed EPA-rich oil–stripped palm oil–sunflower oil, BO, BO–EO, BO–EPA-rich oil–stripped palm oil–sunflower oil or BO–EO–EPA-rich oil–stripped palm oil–sunflower oil (Table 1). This blending was designed in order that the capsules, other than the control, provided a total of approximately 2.0 g GLA + STA + EPA/d. The groups are referred to as control, EPA, BO, EO, blend 1, blend 2 and blend 3 throughout the present paper (Table 1). The fatty acid composition of the capsules is shown in Table 2. Subjects in the EPA group consumed an extra 2.1 g EPA/d, subjects in the BO group consumed an extra 2.0 g GLA/d and subjects in the EO group consumed an

**Table 1.** Blends of oils used to formulate the capsule contents (g/kg total oil)

Treatment group ... Oil	Control	EPA	BO	EO	Blend 1	Blend 2	Blend 3
Palm oil	800	436	–	–	186	151	115
Sunflower oil	200	109	–	–	47	38	29
EPA-rich oil	–	455	–	–	222	178	156
BO	–	–	1000	78	544	267	78
EO	–	–	–	922	–	367	622

BO, borage (*Borage officinalis*) oil; EO, echium (*Echium plantaginium*) oil.

Table 2. Fatty acid composition of the oil in the capsules\*

Treatment group ...	Control		EPA		BO		EO		Blend 1		Blend 2		Blend 3	
	g/100 g total fatty acids		g/100 g total fatty acids		g/100 g total fatty acids		g/100 g total fatty acids		g/100 g total fatty acids		g/100 g total fatty acids		g/100 g total fatty acids	
Fatty acid														
Myristic acid	1.1	0.08	1.0	0.08	0.2	0.02	0.0	0.0	0.5	0.04	0.9	0.07	0.4	0.03
Palmitic acid	33.4	2.7	19.9	1.6	11.9	1.0	5.5	0.5	15.0	1.2	13.6	1.1	10.2	0.8
Stearic acid	4.2	0.34	0.0	0.0	3.4	0.32	2.2	0.18	2.6	0.16	0.0	0.0	2.1	0.17
Oleic acid	38.0	3.1	22.4	1.8	17.0	1.4	11.7	1.0	18.8	1.5	16.4	1.3	14.1	1.1
Linoleic acid	23.0	1.9	14.1	1.1	42.0	3.4	24.8	2.0	27.3	2.2	24.4	2.0	22.1	1.8
GLA	0.0	0.0	0.3	0.02	24.6	2.0	10.7	0.9	12.1	1.0	9.1	0.8	7.8	0.6
ALA	0.2	0.02	0.6	0.05	0.5	0.04	33.4	2.7	0.8	0.06	15.7	1.3	24.2	2.0
STA	0.0	0.0	1.6	0.13	0.3	0.02	11.7	1.0	1.0	0.01	5.4	0.4	8.1	0.7
AA	0.0	0.0	1.8	0.15	0.0	0.0	0.0	0.0	1.0	0.01	0.7	0.06	0.5	0.04
EPA	0.0	0.0	25.7	2.1	0.0	0.0	0.0	0.0	13.9	1.1	9.1	0.8	7.1	0.6
DHA	0.0	0.0	10.9	0.9	0.0	0.0	0.0	0.0	6.1	0.5	4.0	0.3	3.0	0.2

BO, borage (*Borage officinalis*) oil; EO, echium (*Echium plantaginifolium*) oil; GLA,  $\gamma$ -linolenic acid; ALA,  $\alpha$ -linolenic acid; STA, stearidonic acid; AA, arachidonic acid; DHA, docosahexaenoic acid.

\*For details of blends of oils in capsules, see Table 1.

extra 1.0 g STA and 0.9 g GLA/d. Subjects in the blend 1 group consumed an extra 1.1 g EPA and 1.0 g GLA/d. Subjects in the blend 2 group consumed an extra 0.8 g EPA, 0.8 g GLA and 0.4 g STA/d. Finally, subjects in the blend 3 group consumed an extra 0.6 g EPA, 0.6 g GLA and 0.7 g STA/d. Each capsule contained 3 mg  $\alpha$ -tocopherol, so that subjects consumed 27 mg  $\alpha$ -tocopherol/d from the capsules. The daily supply of capsules (nine) was put into a small container and 1 month's supply of these put into a bag. Subjects collected one bag per visit to the laboratory. All treatment groups completed the study in parallel. The study ran from February 2000 (late winter) to June 2000 (early summer). Blood was collected immediately before beginning the interventions and at 12 weeks. Heparinised vacutainer tubes were used for blood collection, which was done between 07.00 and 10.00 hours after a fast of at least 10 h. Plasma was stored at  $-70^{\circ}\text{C}$ .

#### Plasma Ig concentrations

In-house ELISA were used to measure IgG<sub>2</sub> and IgE concentrations in plasma. Well volumes were 100  $\mu\text{l}$  throughout the assays, except during washing where the volume was 300  $\mu\text{l}$ . Polystyrene microtitre plates (Costar; supplied by ICN Biochemicals, Zoetermeer, The Netherlands) were coated with 4  $\mu\text{g}$  capture antibody/ml PBS for 2 h at  $37^{\circ}\text{C}$ . Plates were washed three times with Tween (0.5 ml/l PBS) and then incubated with bovine serum albumin (10 g/l PBS) for 1 h at room temperature. Plates were washed as before and plasma or standard added. Plasma samples were pre-diluted (1:20 000 for IgG<sub>2</sub>; 1:3 for IgE) with bovine serum albumin (10 g/l PBS). Standards were diluted from 2000.0 to 0.3 ng/ml in PBS containing bovine serum albumin (10 g/l). Plasma and standards were incubated for 2 h at room temperature. Plates were washed four times with Tween (0.05 g/l PBS), detection antibody added (2  $\mu\text{g}$ /ml bovine serum albumin (10 g/l PBS)) and plates incubated for 1 h at room temperature. Plates were washed four times with Tween (0.05 ml/l PBS) and streptavidin-conjugated horseradish peroxidase (0.25  $\mu\text{g}$ /ml bovine serum albumin (10 g/l PBS)) added. Plates were incubated for 30 min at room temperature and finally washed five times with Tween (0.05 ml/l PBS). Substrate solution (100  $\mu\text{l}$ ; made by mixing 200  $\mu\text{l}$  3,3',5,5'-tetramethylbenzidine (6 mg/ml dimethylsulfoxide) with 12  $\mu\text{l}$  H<sub>2</sub>O<sub>2</sub> (30 ml/l) and 12 ml 0.11 M-acetate buffer, pH 5.5) was added. Plates were incubated at room temperature for maximal colour development. The reaction stopped with H<sub>2</sub>SO<sub>4</sub> (100 ml/l) when the maximum optical density at 450 nm was still  $<2$ . Optical density was measured at 450 nm on a plate reader.

#### Peripheral blood mononuclear cell subsets

Peripheral blood mononuclear cell (PBMC) subsets in whole blood were identified by flow cytometry following staining with appropriate fluorescently labelled monoclonal antibodies (see Kew *et al.* 2003). Monoclonal antibody combinations used were anti-CD3/anti-CD4 (to distinguish T lymphocytes as CD3<sup>+</sup> and T helper lymphocytes as CD3<sup>+</sup>CD4<sup>+</sup>), anti-CD3/anti-CD8 (to distinguish cytotoxic

T lymphocytes as CD3<sup>+</sup>CD8<sup>+</sup>), anti-CD3/anti-CD16 (to distinguish natural killer cells as CD3<sup>-</sup>CD16<sup>+</sup>), anti-CD3/anti-CD54 (to determine the expression of intercellular adhesion molecule (ICAM; CD54)-1 on T lymphocytes), anti-CD19/anti-CD54 (to distinguish B lymphocytes as CD19<sup>+</sup> and to determine the expression of ICAM-1 on B lymphocytes), and anti-CD14/anti-CD54 (to distinguish monocytes as CD14<sup>+</sup> and to determine the expression of ICAM-1 on monocytes). Erythrocytes were then lysed using 2 ml lysing buffer (Becton Dickinson, Oxford, UK) and leukocytes washed with cell wash (Becton Dickinson) and then fixed with 0.2 ml cell fix (Becton Dickinson). Fluorescence data were collected on  $2 \times 10^4$  cells using a FACSCalibur flow cytometer (Becton Dickinson) and were analysed using CellQuest software (Becton Dickinson).

#### *Measurement of phagocytic activity and respiratory burst*

Phagocytosis of *Escherichia coli* and respiratory burst in response to *E. coli* or phorbol myristyl acetate by neutrophils and monocytes were determined in whole blood using PHAGOTEST and BURSTTEST kits (Becton Dickinson) respectively, as described by Kew *et al.* (2003). Both the percentage of neutrophils or monocytes engaging in phagocytosis or respiratory burst (% positive) and the median fluorescence intensity (MFI; a measure of extent of phagocytosis or respiratory burst per leukocyte) were determined.

#### *Preparation and culture of peripheral blood mononuclear cells*

PBMC were prepared as described by Kew *et al.* (2003). The proliferation of lymphocytes in cultures of PBMC was measured as the incorporation of [<sup>3</sup>H]thymidine over the final 18 h of a 66 h culture period, as described by Kew *et al.* (2003). Cultures contained 50 ml autologous plasma/l and cells were unstimulated or stimulated with 25 µg concanavalin A (con A)/ml; this concentration of con A yields maximal thymidine incorporation under the conditions used. Lymphocyte proliferation was calculated as stimulation index (SI):

$$\text{SI} = \frac{[\text{^3H}]\text{thymidine incorporation in the presence of con A}}{[\text{^3H}]\text{thymidine incorporation in the absence of con A}}$$

PBMC were cultured with either con A (25 µg/ml) or LPS (15 µg/ml) to stimulate cytokine generation as described by Kew *et al.* (2003), except that the culture duration was 48 h. Preliminary experiments indicated that maximal concentrations of all cytokines measured in the present study were achieved under these culture conditions. The concentrations of cytokines were measured by specific EASIA ELISA (Biosource Europe, Nivelles, Belgium). TNF-α and IL-1β were measured in the supernatant fractions of cells stimulated with LPS; IL-2, IFN-γ, IL-10 and IL-4 were measured in the supernatant fractions of cells stimulated with con A. Limits of detection for these assays were 3 pg/ml (TNF-α), 2 pg/ml (IL-1β, IL-4), 1 pg/ml (IL-10),

0.1 U/ml (IL-2) and 0.03 IU/ml (IFN-γ) (data supplied by the manufacturer of the kits). The inter- and intra-assay CV were < 10 % for all cytokine ELISA.

#### *Analysis of peripheral blood mononuclear cell phospholipid fatty acid composition*

Lipid was extracted from PBMC with chloroform–methanol (2:1, v/v) and phospholipids isolated by TLC using hexane–diethyl ether–acetic acid (90:30:1, by vol.) as the elution phase. Fatty acid methyl esters were prepared by incubation with 140 mg boron trifluoride/ml methanol at 80°C for 60 min, and analysed by GC as described by Thies *et al.* (2001b). Fatty acid methyl esters were identified by comparison with standards run previously, apart from the methyl esters of STA and 20:4n-3. For identification of these, authentic methyl esters were purchased from Larodan Fine Chemicals, Malmo, Sweden.

#### *Delayed-type hypersensitivity test*

The DTH skin response was assessed using MultiTest-CMI kits (Merieux Institute, Paris, France). The test consisted of applying a single-use disposable applicator of acrylic resin with eight heads loaded with either glycerine control or with one of seven recall antigens (tetanus, diphtheria, streptococcus, tuberculin, *Candida albicans*, *Tricophyton mentagrophytes*, *Proteus mirabilis*). The test was performed at study entry and after 12 weeks, as described by Kew *et al.* (2003). The diameter of each induration was measured 48 h after application; an induration of ≥ 2 mm diameter was considered positive. If a positive reaction to the glycerol control was observed, the diameter of this induration was subtracted from that of each of the other positive reactions. Results are shown as the total number of positive reactions and the cumulative diameter of induration of all positive reactions.

#### *Presentation of results and statistical analyses*

Apart from SI and the number of positive DTH responses, results are presented as mean values with their standard errors. One-factor ANOVA was used to determine differences among treatment groups at baseline (week 0) and at the end of supplementation (week 12). Student's paired *t* test was used to determine differences within each treatment group across time. Bonferroni's correction for multiple comparisons was used in all statistical analyses. Data for SI and the number of positive DTH responses are expressed as median values. For these data, Kruskal–Wallis one-factor ANOVA was used to determine differences among treatment groups at baseline (week 0) and at the end of supplementation (week 12) and the Wilcoxon matched pairs test was used to determine differences within each treatment group across time. All statistical tests were performed using SPSS, version 11.0 (SPSS Inc., Chicago, IL, USA) and a value of *P* < 0.05 was taken to indicate statistical significance.

## Results

### Fatty acid composition of peripheral blood mononuclear cell phospholipids

The fatty acid composition of PBMC did not differ among the treatment groups at baseline (Table 3). None of the treatments significantly altered the proportions of palmitic (approximately 20.0 g/100 g total fatty acids), stearic (approximately 21.0 g/100 g total fatty acids), oleic (approximately 17.0 g/100 g total fatty acids) or linoleic (approximately 8.5 g/100 g total fatty acids) acids in PBMC (results not shown). Unexpectedly, the proportion of EPA in PBMC was significantly decreased ( $P < 0.05$ ) in the control group (Table 3). Consumption of STA did not result in appearance of either STA or 20:4n-3 in PBMC. The proportions of GLA and ALA in PBMC were low (both approximately 0.1 g/100 g total fatty acids). Increased consumption of ALA (EO, blend 2, blend 3) did not result in significantly increased appearance of ALA in PBMC (results not shown). Likewise, increased consumption of GLA (BO, EO, blend 1, blend 2, blend 3) did not result in significantly increased appearance of GLA in PBMC (results not shown). There was a significant increase ( $P = 0.004$ ; paired  $t$  test) in EPA in PBMC in the EPA group (Table 3); this represented an average 125% increase in EPA content. There was no change in DHA content of PBMC in this group (Table 3). The increase in EPA in the EPA group was accompanied by a small decrease (average about 5%) in the content of arachidonic acid (20:4n-6), but this was not significant (Table 3). EPA also increased in PBMC in the other groups consuming increased amounts of EPA (blend 1, blend 2 and blend 3), although this was not always significant (Table 3). Average percentage increases in EPA in PBMC in these three groups were 160, 60 and 65 respectively. The proportion of EPA was not different between subjects in the EPA group and those in the blend 1, 2 or 3 groups at the end of supplementation (Table 3). Using data from the control, EPA, blend 1, 2 and 3 groups, there was a significant positive linear correlation between the amount of EPA provided in the capsules (g/d) and the increase in EPA in PBMC over the supplementation period ( $r = 0.777$ ;  $P < 0.001$ ). The EPA content of PBMC also tended to

increase (average 125%) in the EO group (Table 3), although this group did not consume EPA in the capsules (Table 3).

The arachidonic acid content of PBMC was not significantly altered in any of the groups, although it tended to decrease in those groups consuming EPA or STA and to increase in the BO group (Table 3). The di-homo- $\gamma$ -linolenic acid (DGLA) content of PBMC was increased ( $P = 0.032$ ; paired  $t$  test) in the BO (average 25%) and blend 2 ( $P = 0.025$ ; paired  $t$  test; average 58%) groups. DGLA also tended to increase in PBMC in the EO (40% increase) and blend 1 (25% increase) groups (Table 3). There was no suggestion of a change in DGLA in the blend 3 group (Table 3).

### Peripheral blood mononuclear cell subsets

There were no differences in the proportions of T lymphocytes (about 56% of PBMC), B lymphocytes (about 7% of PBMC), T helper cells (about 60% of T lymphocytes) or cytotoxic T cells (about 38% of T lymphocytes) among the treatment groups at baseline or at the end of supplementation (results not shown). The proportion of natural killer cells (about 15% of lymphocytes) was not different among the treatment groups at baseline. However, the proportion of lymphocytes as natural killer cells was significantly lower after the EPA ( $P = 0.038$ ; paired  $t$  test) and BO ( $P = 0.008$ ; paired  $t$  test) treatments than at baseline (Fig. 1). The proportions of B lymphocytes or monocytes expressing ICAM-1 (about 70 and 95% respectively) and the level of ICAM-1 expression were not different among the groups at baseline or at the end of intervention (results not shown).

### Plasma Ig concentrations

Plasma IgG<sub>2</sub> and IgE concentrations did not differ significantly among groups at baseline or at the end of supplementation (Table 4). However, there was a significant increase (average 15%;  $P = 0.03$ , paired  $t$  test) in plasma IgG<sub>2</sub> concentration at the end of supplementation in the EPA group (Table 4). IgG<sub>2</sub> concentration was not altered significantly in the other treatment groups (Table 4).

**Table 3.** Fatty acid composition of peripheral blood mononuclear cells in the different treatment groups (g/100 g total fatty acids)† (Mean values with their standard errors)

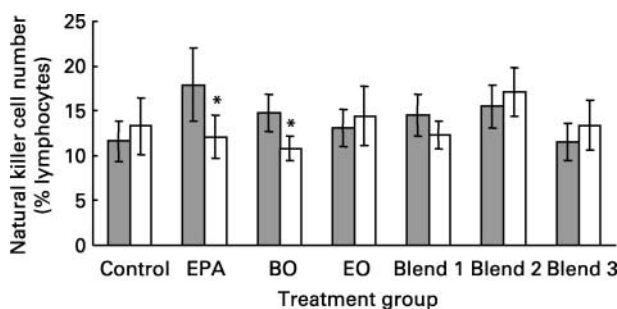
Fatty acid	Time (weeks)	Control		EPA		BO		EO		Blend 1		Blend 2		Blend 3	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
DGLA	0	2.9	0.4	2.5	0.3	2.8	0.2	2.5	0.4	2.9	0.5	2.4	0.2	2.9	0.4
	12	2.6	0.2	2.0	0.1	3.5*	0.4	3.5	0.4	3.9	1.0	3.8*	0.5	2.6	0.3
AA	0	19.3	1.0	17.2	0.8	18.8	1.0	19.8	0.9	20.1	0.6	20.9	1.1	20.9	0.7
	12	20.6	0.9	16.3	0.9	19.5	1.2	19.7	0.6	19.4	0.5	18.2	1.1	20.0	0.9
EPA	0	1.3	0.5	0.7	0.1	0.9	0.3	0.4	0.1	0.5	0.2	1.0	0.4	0.9	0.3
	12	0.5 <sup>b</sup>	0.1	1.6 <sup>a*</sup>	0.3	0.9	0.3	0.9	0.2	1.3*	0.2	1.6	0.4	1.5	0.2
DHA	0	2.8	0.6	2.3	0.3	3.0	0.5	1.8	0.1	2.3	0.3	1.7	0.3	2.3	0.2
	12	2.2	0.1	2.2	0.2	2.4	0.4	2.9*	0.5	2.9*	0.2	3.1*	0.6	2.4	0.3

BO, borage (*Borage officinalis*) oil; EO, echium (*Echium plantaginium*) oil; DGLA, di-homo- $\gamma$ -linolenic acid; AA, arachidonic acid; DHA, docosahexaenoic acid.

<sup>a,b</sup> Mean values within a row with unlike superscript letters were significantly different from each other (one-factor ANOVA,  $P < 0.05$ ).

Mean values were significantly different from those at baseline (paired  $t$  test); \* $P < 0.05$ .

† For details of subjects, treatments and procedures, see Tables 1 and 2 and p. 894.



**Figure 1.** Proportion of natural killer cells in the different treatment groups. The proportion of lymphocytes that were natural killer cells was determined by flow cytometry after staining with anti-CD3 and anti-CD16 monoclonal antibodies. Natural killer cells were defined as CD3<sup>+</sup>CD16<sup>+</sup>. For details of treatments, subjects and procedures, see Tables 1 and 2 and p. 894. BO, borage (*Borage officinalis*) oil; EO, echium (*Echium plantaginium*) oil. ■, Mean baseline values; □, end of supplementation values. Values are means with their standard errors shown by vertical bars. Mean values were significantly different from those at baseline (paired *t* test): *P* < 0.05.

Plasma IgE concentration decreased significantly (by approximately 20%) in the EPA, BO, EO, blend 2 and blend 3 groups (*P* < 0.007 at least; Table 4). The decrease in IgE concentration in the blend 1 group, although similar to that seen in the other groups, was not significant (*P* = 0.068; Table 4).

*Phagocytosis by neutrophils and monocytes*

The percentage of neutrophils and monocytes carrying out phagocytosis of *E. coli* (approximately 90% of neutrophils and approximately 25% of monocytes) and the activity of those cells (MFI) did not differ among the groups at baseline or at the end of intervention (results not shown). The percentage of neutrophils and monocytes undergoing phagocytosis did not change in any of the intervention groups (results not shown). However, MFI decreased by approximately 30% for neutrophils and by approximately 45% for monocytes in all groups during the intervention, but the decrease was not different among the groups (results not shown).

*Respiratory burst by neutrophils and monocytes*

The percentage of neutrophils engaging in respiratory burst in response to *E. coli* or phorbol ester and the activity of those cells (MFI) did not differ among the groups at

baseline or at the end of intervention and were not affected by the treatments (Table 5). The percentage of monocytes engaging in respiratory burst in response to phorbol ester and the activity of those cells (MFI) did not differ among the groups at baseline or at the end of intervention and were not affected by the treatments (Table 5).

*Cytokine production by peripheral blood mononuclear cells*

Production of TNF-α and IL-1β by PBMC stimulated with 15 μg LPS/ml did not differ among the groups at baseline or at the end of intervention (Table 6), although production of IL-1β tended to be lower after supplementation than at baseline in all groups (Table 6). Production of IL-2, IFN-γ, IL-4 and IL-10 by PBMC stimulated with 25 μg con A/ml did not differ among the intervention groups at baseline or at the end of intervention (Table 6). However, production of each of these cytokines was significantly increased in all groups over the duration of the intervention, such that it was higher at 12 weeks than at baseline (Table 6).

*Lymphocyte proliferation*

Thymidine incorporation into unstimulated cells or in response to con A did not differ among the groups at baseline or at the end of intervention (results not shown). Likewise, SI did not differ among the groups at baseline or at the end of intervention (Table 6), although it did tend to increase with time in all groups (Table 6).

*Delayed-type hypersensitivity*

Most subjects responded positively to one or two antigens, most frequently tetanus and tuberculin, with a mean cumulative diameter of response of approximately 6 mm at baseline (Table 7) There were no significant differences among the groups at baseline or at the end of intervention in terms of the number of positive indurations or the cumulative diameter of induration (Table 7). However, the cumulative diameter of the response tended to increase in all groups (Table 7)

**Discussion**

The present study investigated the effect of increased intakes of the long chain *n*-3 PUFA EPA, of its precursor

**Table 4.** Plasma Ig concentrations in the different treatment groups† (Mean values with their standard errors)

	Time (weeks)	Control		EPA		BO		EO		Blend 1		Blend 2		Blend 3	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
IgG <sub>2</sub> (mg/ml)	0	5.7	0.7	6.9	0.9	7.8	1.7	7.4	1.4	5.9	1.2	8.8	1.7	6.1	1.3
	12	5.0	0.6	7.9*	0.9	7.6	1.2	8.1	2.1	5.9	1.3	8.1	1.7	6.0	1.4
IgE (μg/ml)	0	1.17	0.13	1.92	0.36	1.66	0.26	1.44	0.15	1.84	0.46	1.79	0.27	1.51	0.08
	12	0.95	0.09	1.41*	0.28	1.33*	0.24	1.26*	0.18	1.76	0.44	1.44*	0.25	1.16*	0.08

BO, borage (*Borage officinalis*) oil; EO, echium (*Echium plantaginium*) oil. Mean values were significantly different from those at baseline (paired *t*-test): \**P* < 0.05. † For details of treatments, subjects and procedures, see Tables 1 and 2 and p. 894.

**Table 5.** Respiratory burst response of neutrophils and monocytes after incubation with *Escherichia coli* or phorbol myristyl acetate in the different treatment groups\* (Mean values with their standard errors)

Cell	Stimulus	Outcome	Time (weeks)	Control		EPA		BO		EO		Blend 1		Blend 2		Blend 3	
				Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Neutrophil	<i>E. coli</i>	% Positive	0	83.6	6.6	89.8	4.3	94.8	2.2	88.9	6.3	96.7	0.7	90.3	4.2	89.6	5.5
			12	90.3	2.6	91.7	2.0	92.8	1.3	92.4	1.1	93.6	1.4	91.3	2.4	92.9	1.4
<i>E. coli</i>	MFI		0	914	141	805	100	1052	135	769	144	991	29	828	146	784	132
			12	910	148	986	122	862	86	647	64	968	145	796	120	778	74
PMA	% Positive		0	96.8	0.9	94.2	1.4	96.8	0.7	98.6	0.4	98.0	0.7	97.9	0.5	96.9	0.7
			12	96.1	0.6	95.5	1.3	96.1	0.7	94.1	2.4	96.7	0.7	97.2	0.6	97.0	0.8
PMA	MFI		0	2494	410	2002	321	2525	310	2451	325	2966	257	2587	200	2056	352
			12	2438	247	2614	209	2551	169	2271	152	2755	198	2295	210	2337	209
Monocytes	PMA	% Positive	0	78.8	7.2	67.8	8.8	78.4	5.3	87.9	4.0	86.2	6.0	80.8	6.6	77.1	8.4
			12	80.0	7.6	80.2	5.0	76.9	6.5	65.6	9.3	84.3	5.9	80.9	5.0	77.0	3.4
PMA	MFI		0	230	28	209	26	223	24	262	39	297	51	257	30	298	48
			12	199	20	187	21	210	15	183	27	236	33	190	28	234	42

BO, borage (*Borage officinalis*) oil; EO, echium (*Echium plantaginium*) oil; MFI, median fluorescence intensity; PMA, phorbol myristyl acetate.

\* For details of treatments, subjects and procedures, see Tables 1 and 2 and p. 894.

**Table 6.** Cytokine production and lymphocyte proliferation by peripheral blood mononuclear cells in the different treatment groups† (Mean values with their standard errors)

Stimulus	Time (weeks)	Control		EPA		BO		EO		Blend 1		Blend 2		Blend 3	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
IL-1 $\beta$ (pg/ml) LPS	0	7538	2479	5401	881	7365	1856	6800	1565	3971	745	6839	1907	8203	2278
	12	3308	884	3236	1178	4782	1697	4926	2326	3374	470	4058	1194	5202	1938
TNF- $\alpha$ (pg/ml) LPS	0	3076	794	2715	657	2861	482	3094	795	2428	315	2549	525	2653	496
	12	2500	507	2885	885	3064	662	2121	820	2799	316	2317	544	2583	625
IL-2 (U/ml) Con A	0	2.6	0.3	5.7	1.9	2.7	0.4	3.6	0.5	5.0	1.4	5.0	1.2	4.8	1.1
	12	9.6*	2.2	8.9*	2.0	7.8*	1.7	9.4*	2.5	14.2*	4.9	15.2*	4.9	15.6*	3.9
IL-4 (pg/ml) Con A	0	74	23	76	21	62	11	67	24	108	32	80	27	68	18
	12	257*	54	168*	29	180*	44	179*	69	230*	47	196*	51	129*	26
IL-10 (pg/ml) Con A	0	452	157	383	91	403	86	267	37	432	143	289	69	292	45
	12	1260*	214	1254*	221	1369*	273	611*	149	978*	193	944*	176	683*	145
IFN- $\gamma$ (IU/ml) Con A	0	293	144	439	164	235	38	337	128	238	93	242	70	268	92
	12	885*	226	888*	245	1085*	238	595	233	1068*	361	1195*	310	786*	218
Proliferation (SI)‡ Con A	0	57	31, 90	67	33, 111	63	53, 137	51	28, 78	84	24, 138	72	47, 94	70	56, 83
	12	86	42, 160	60	27, 154	82	42, 190	76	55, 85	126	77, 207	56	34, 144	117	56, 137

BO, borage (*Borage officinalis*) oil; EO, echium (*Echium plantaginium*) oil; SI, stimulation index; LPS, lipopolysaccharide; Con A, concanavalin A; IFN, interferon.

Mean values were significantly different from those at baseline (paired *t* test): \* *P* < 0.05.

† For details of treatments, subjects and procedures, see Tables 1 and 2 and p. 894.

‡ Median values and interquartile ranges.

**Table 7.** The delayed type hypersensitivity response of subjects in the different treatment groups\* (Median values and interquartile ranges)

Indurations (n)	Time (weeks)	Control		EPA		BO		EO		Blend 1		Blend 2		Blend 3	
		Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
	0	2.0	1.0, 2.0	1.0	0.5, 2.0	1.0	0.3, 2.0	2.0	1.0, 2.0	0.5	0.0, 2.0	1.5	1.0, 2.3	1.0	1.0, 2.0
	12	2.0	1.3, 3.5	2.0	1.0, 3.0	1.0	1.0, 2.8	1.5	1.0, 4.0	1.5	1.0, 3.0	3.0	1.8, 4.0	2.0	1.0, 3.0
Cumulative diameter (mm)†	0	6.1	1.4	5.9	2.1	5.1	1.2	6.4	1.0	4.3	1.8	7.4	1.7	5.4	1.1
	12	10.3	2.5	8.6	1.3	6.5	1.2	9.1	2.7	8.0	2.4	11.3	1.4	7.8	1.6

BO, borage (*Borago officinalis*) oil; EO, echium (*Echium plantagininum*) oil; IQR, interquartile range.

\* For details of treatments, subjects and procedures, see Tables 1 and 2 and p. 894.

† Mean values with their standard errors.

STA and of the *n*-6 PUFA GLA on circulating immune cell phenotypes and on a range of immune measures representative of key functional activities of neutrophils, monocytes and lymphocytes. The effects of fish oil, which contains EPA, and of GLA on many of these variables have been studied previously (see p. 893). Many of the previous studies of fish oil that report marked immunological effects involved providing fairly large doses of the long-chain *n*-3 PUFA (>2.4 g EPA + DHA/d). However, such doses are not readily achievable and so it is important to identify the effects of more modest doses of long-chain *n*-3 PUFA. The same is true for GLA, where studies reporting significant immunological effects have used >2.4 g/d (Johnson *et al.* 1997; Rosetti *et al.* 1997; de Luca *et al.* 1999). An alternative to providing long-chain *n*-3 PUFA is to provide their precursors that can then be metabolised. Such conversion to EPA may explain the immunological effects of very high intakes of ALA (>14 g/d) as reported by Kelley *et al.* (1991) and Caughey *et al.* (1996). Indeed, the latter study presented the effects of ALA intake on TNF- $\alpha$  and IL-1 $\beta$  production as a function of PBMC EPA content. However, increased consumption of ALA up to 9.5 g/d appears to be without immunological effects (Healy *et al.* 2000; Thies *et al.* 2001a,b,c; James *et al.* 2003; Kew *et al.* 2003; Wallace *et al.* 2003). One reason for this may be that ALA conversion to the longer-chain active derivatives EPA and DHA is limited in human subjects (Burdge *et al.* 2002; Burdge & Wootton, 2002). Therefore, an alternative strategy for increasing the long-chain *n*-3 PUFA status of inflammatory and immune cells may be to provide the product of  $\Delta^6$ -desaturase, i.e. STA. In contrast to the many studies with fish oil and GLA, the effects of dietary STA on human immune functions have not been previously investigated, apart from a recent study by James *et al.* (2003). That study showed no effect of consumption of 0.75 and 1.50 g STA/d for 3 weeks on production of TNF- $\alpha$  and IL-1 $\beta$  by LPS-stimulated whole blood cultures. However, the study of James *et al.* (2003) was not controlled, was of only 3-weeks duration and used STA in ethyl ester form. In contrast, the current study was controlled, was of 12-weeks duration and the fatty acids under investigation (EPA, STA, GLA) were components of triacylglycerols. The present study is the first to examine the effects of dietary STA on human immune outcomes, apart from TNF- $\alpha$  and IL-1 $\beta$  production, which were studied previously by James *et al.* (2003). Furthermore, this is the first study to report in detail the effects of increased intake of STA on the fatty acid composition of human immune cells, although such effects were alluded to, but not shown, by James *et al.* (2003).

One important observation of the current study was that neither STA nor its derivative 20:4*n*-3 appeared in detectable amounts in PBMC, even when STA was added to the diet at the level of 1.0 g/d. This observation is in accordance with the recent report of James *et al.* (2003), that neither STA nor 20:4*n*-3 was detected in phospholipids of erythrocytes, platelets or PBMC after consumption of 0.75 or 1.50 g STA/d for 3 weeks. The lack of appearance of these fatty acids suggests that STA is readily metabolised to EPA, without significant accumulation of 20:4*n*-3. This is supported by the significant increase



in the proportion of EPA in PBMC in the EO group. James *et al.* (2003) also reported an increase in the proportion of EPA in phospholipids of erythrocytes, platelets and PBMC after STA consumption. Given that substantial intakes of ALA are required in order to increase the EPA content of immune cell lipids (Healy *et al.* 2000; Thies *et al.* 2001*b*; Kew *et al.* 2003), STA may offer a plant oil-based option to efficiently increase the EPA status of such cells. The reason why STA may be superior to ALA is that STA bypasses the need for the activity of  $\Delta^6$ -desaturase, which is believed to be low (Burdge *et al.* 2002; Burdge & Wootton, 2002). The lack of appearance of 20:4*n*-3 when STA is consumed indicates that the activity of  $\Delta^5$ -desaturase is sufficient for this fatty acid to be metabolised efficiently.

The significant (125%) increase in the proportion of EPA in PBMC phospholipids in the EPA group is consistent with previous reports of the fatty acid composition of PBMC in subjects given fish oil (Gibney & Hunter, 1993; Yaqoob *et al.* 2000; Thies *et al.* 2001*b*; Kew *et al.* 2003). EPA also increased in PBMC of subjects consuming blends of the EPA-rich oil and the other oils, and this increase was dose-dependently related to the amount of EPA consumed. The proportion of DGLA in PBMC increased in subjects consuming BO or blends that included BO. The extent of the increase in the proportion of DGLA in PBMC when GLA was consumed is consistent with earlier studies (Yaqoob *et al.* 2000; Thies *et al.* 2001*b*). PBMC from subjects in the EO group showed a trend towards an increase in both DGLA and EPA, with a small decrease in arachidonic acid content. The increase in content of both DGLA and EPA may be functionally more effective than increasing either fatty acid alone. Thus, through use of an appropriate combination of GLA and STA, a functionally important increase in content of both DGLA and EPA can occur without an increase in arachidonic acid, which may be undesirable. Therefore, this combination may be superior to the use of GLA alone, since GLA tends to increase arachidonic acid levels in immune cells.

The observed lack of effect of the various PUFA combinations on the proportions of most circulating mononuclear cell populations agrees with earlier findings with fish oil (Yaqoob *et al.* 2000; Thies *et al.* 2001*a,b,c*; Kew *et al.* 2003; Wallace *et al.* 2003;) and GLA (Yaqoob *et al.* 2000; Thies *et al.* 2001*a,b,c*). However, there was a marked effect of EPA and GLA on the proportion of natural killer cells present, an effect not seen with EO or with the various blends. This might be because the amount of EPA or GLA provided in those treatments was insufficient to affect natural killer cell numbers, although it is not clear how this effect occurs. The current study did not determine natural killer cell activity. However, an earlier study indicated that this could be decreased (by an average of 14.3%) by consumption of an extra 1.0 g EPA + DHA/d for 12 weeks in subjects >55 years of age (Thies *et al.* 2001*c*). Interestingly, in that same study consumption of an extra 0.7 g GLA/d also decreased natural killer cell activity (by an average of 6.9%), but this effect was not significant (Thies *et al.* 2001*c*). Taken together, these observations suggest that natural killer cell number and

activity are sensitive to increased intake of some PUFA. There may be some situations in which a reduction in natural killer cell number and/or activity might be useful, for example following transplantation.

The current study did not identify any effects of EPA, GLA, STA or combinations of these fatty acids on phagocytosis or respiratory burst, which are key elements of the innate immune response to bacterial infections. Thus, the present study suggests that increased intake of these fatty acids will not impair these aspects of the innate immune response. This conclusion concurs with other studies of low-dose fish oil (Schmidt *et al.* 1996; Healy *et al.* 2000; Thies *et al.* 2001*a*; Kew *et al.* 2003) and GLA (Thies *et al.* 2001*a*). In addition, there were no significant effects observed upon the production of cytokines (TNF- $\alpha$ , IL-1 $\beta$ ) by monocytes, which also agrees with earlier studies of low- to moderate-dose fish oil (Schmidt *et al.* 1996; Blok *et al.* 1997; Yaqoob *et al.* 2000; Thies *et al.* 2001*a*; Kew *et al.* 2003; Wallace *et al.* 2003) and GLA (Yaqoob *et al.* 2000; Thies *et al.* 2001*a*). Furthermore, James *et al.* (2003) reported that 0.75 or 1.50 g STA/d for 3 weeks did not affect TNF- $\alpha$  or IL-1 $\beta$  production by LPS-stimulated whole-blood cultures. These observations suggest that an intake of EPA >2.1 g/d, of GLA >2.0 g/d and of STA >1.5 g/d are required to affect inflammatory cytokine production by human monocytes, at least under the conditions studied in this present investigation and in those referred to herein. However, it remains possible that immunological effects of these PUFA at the low intakes used in the current study may be observed if different experimental conditions, such as different concentrations of the stimulants con A and LPS or different cell culture time points, are used, or if different populations (e.g. the elderly) are studied.

In the current study we noted a generalised and marked increase in the production of T lymphocyte-derived cytokines (IL-2, IL-4, IL-10, IFN- $\gamma$ ) from baseline to the end of the study. These changes were not expected. There are at least two possible explanations for these time-dependent changes. The first is that they are due to increased consumption of PUFA in all groups, including the control. This raises the question of the most suitable oil or oil blend to use as a control in studies such as this. The blend used here was selected because its fatty acid composition is nearly identical to the fatty acid composition of the average UK diet. In addition, the control blend contained the same amount of total fat and of  $\alpha$ -tocopherol as the other oils and oil blends used. The second explanation is that the changes are due to seasonal factors. These might include such factors as exposure to sunlight, which has been shown to exert a particularly strong effect on T cell functions in both monkeys (Mann *et al.* 2000) and mice (for review, see Nelson & Demas, 1996). However, the different PUFA used did not significantly affect the observed change in cytokine production.

Few studies of PUFA and human immune function have investigated effects on Ig concentrations or production. The current study identifies effects of increased intake of dietary PUFA on circulating Ig concentrations. The generalised decrease in IgE concentration may relate to a seasonal (or other) effect on the T cell phenotypes that regulate

the production of IgE by B cells. Alternatively, it might reflect a seasonal change in exposure to a stimulus that promotes IgE production. A more specific effect observed was the increase in IgG<sub>2</sub> concentration, which occurred only in the EPA group. IgG<sub>2</sub> production by B cells is promoted by Th1-type cytokines and IgG<sub>2</sub> is involved in host defence against bacteria. Nevertheless, it should be noted that the changes in the circulating concentrations of IgE and IgG<sub>2</sub> are not entirely consistent with the observed changes in production of Th1- and Th2-type cytokines by cultured PBMC. However, the observed effect of EPA on plasma IgG<sub>2</sub> concentrations is suggestive of improved immunity at this level of supplementation. IgG<sub>2</sub> concentrations were not altered in the groups consuming STA or combinations of EPA with GLA and STA, suggesting that the intake of *n*-3 PUFA in these groups was below the threshold that affects IgG<sub>2</sub>.

In summary, the current study indicates that intakes of EPA or GLA of 2.0 g/d do not induce widespread effects on human immune function, despite significant changes in the fatty acid composition of the immune cells. Rather, there are specific effects of these fatty acids, with natural killer cells appearing to be particularly sensitive to both EPA and GLA. Increased intake of EPA also increases the concentration of IgG<sub>2</sub>, suggesting an improvement in host defence. This effect of EPA has not been reported previously. Finally, the plant *n*-3 PUFA STA is a suitable precursor for synthesis of EPA *in vivo*, and in this regard appears to be superior to ALA (Thies *et al.* 2001b; James *et al.* 2003; Kew *et al.* 2003). However at the dose used here (1.0 g/d) STA does not alter human immune function.

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