

## Influence of very low dietary intake of marine oil on some functional aspects of immune cells in healthy elderly people

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Ageing is a multifactorial process involving decreased antioxidant defences and immune functions. *n*-3 Polyunsaturated fatty acids have been associated with human health benefits, especially against inflammatory and autoimmune diseases. However, their immunomodulatory effects were usually observed with high dosages (> 2 g/d) known to increase lipid peroxidation. In contrast, very low doses, that may prevent lipid peroxidation, might affect the immune system differently. To study the latter hypothesis further, we investigated whether the supplementation of healthy elderly people with very low doses of marine oil (MO), a docosahexaenoate (DHA)- and eicosapentaenoate (EPA)-rich triacylglycerol, was able to affect lymphocyte proliferation and biochemical markers known to be altered with age. In a randomized, double-blind design, twenty healthy elderly subjects were assigned to a placebo group (600 mg sunflower oil/d) or to a group consuming 600 mg MO/d providing 150 mg DHA + 30 mg (EPA) for 6 weeks. At day 42, the proliferative responses of lymphocytes to several mitogens were significantly ( $P < 0.01$ ) decreased in the MO group compared with control values. This was accompanied by a slight lowering of their cytosolic cyclic nucleotide phosphodiesterase (PDE) activity, a marked and significant ( $P < 0.05$ ) increase of their particulate PDE activity (+56–57 %) and a slight but significant ( $P < 0.05$ ) increase in cyclic nucleotide intracellular levels. At the same time, the glutathione peroxidase activity was markedly and significantly ( $P < 0.01$ ) depressed in the MO group. None of these modifications could be seen in the placebo group. Collectively, these results demonstrate that even very low doses of *n*-3 fatty acids are sufficient to affect the immune responses of elderly subjects.

***n*-3 Fatty acids: Docosahexaenoic acid: Lymphocyte proliferation: Cyclic nucleotide phosphodiesterase: Glutathione peroxidase: Immune function in human ageing**

Epidemiological observations suggest that *n*-3 polyunsaturated fatty acids (PUFA) from marine oil (MO) have beneficial effects on the prevention and treatment of chronic cardiovascular and inflammatory diseases (Simopoulos, 1991; Endres *et al.* 1995; Meydani, 1996). However, the effects of *n*-3 fatty acids on functional variables of the human immune system remain a focus of research interests. Several studies indicate that the supplementation of human diets with PUFA modulates the immune system with either detrimental or beneficial effects. Thus, dietary lipids have been shown to promote the development and subsequent progression of breast and prostatic cancer (Rose, 1997) and to improve the condition of patients

suffering from disorders involving over-reactive immune responses (Fortin *et al.* 1995; Mohan & Das, 1997). Conversely, dietary supplementation with *n*-3 fatty acids has been shown to prevent trauma-induced immunosuppression (Ayala & Chaudry, 1995). The amount and the nature of lipids are both important for the immunomodulation. In particular, PUFA seem to affect cell-mediated immunity. Indeed, fish oil supplementation of human and animal diets has been shown to decrease interleukin 2 production and T cell mitogen-induced proliferation, indicating an overall decreased T cell-mediated immune function (Endres *et al.* 1993; Calder *et al.* 2002). *In vitro*, PUFA, either from the *n*-6 or the *n*-3 families, inhibit the

**Abbreviations:** DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GSH-Px, glutathione peroxidase; MO, marine oil; PA, phosphatidic acid; PBMN, peripheral blood mononuclear cells; PDE, phosphodiesterase; PE, phosphatidylethanolamine PUFA, polyunsaturated fatty acid; SO, sunflower oil.

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mitogen-induced proliferation of murine or human lymphocytes (Calder & Newsholme, 1992; Joulain *et al.* 1995; Calder *et al.* 2002). Many effects of dietary *n*-3 fatty acids may be explained by their ability to alter arachidonic acid metabolism and eicosanoid synthesis (Miles & Calder, 1998). In addition, they can also alter membrane-associated functions (Murphy, 1990) and cytokine gene expression in cells of the immune system (Miles & Calder, 1998).

Ageing is associated with a decrease in both immune responses and antioxidant defences (Burns & Goodwin, 1997; Beckman & Ames, 1998). Among the age-related functional changes, the depressed proliferative response of lymphocytes to plant lectins and mitogens *in vitro* has been well characterized both in human subjects and rodents (Solana *et al.* 1991; Kariv *et al.* 1992; Burns & Goodwin, 1997). On the other hand, a decrease in the antioxidant defences leading to increased oxidative protein damage and lipid peroxidation has been reported in various tissues of old animals (Tian *et al.* 1998). Meskini *et al.* (1993) have shown that the glutathione peroxidase (GSH-Px) activity of human peripheral blood mononuclear cells (PBMN) decreased with age. This enzymatic defect, which may influence the lymphoproliferative response in several ways, was accompanied by an age-related increase in the oxygenated metabolism of endogenous arachidonic acid by lipoxygenases. Another enzymatic defect that has been associated with ageing was a decrease in lymphocyte cyclic nucleotide phosphodiesterase (PDE) activity (Meskini *et al.* 1990). As cyclic AMP is a potent suppressor of most immune responses (Skalhegg *et al.* 1992), it can be assumed that a defect in lymphocyte cAMP hydrolysing activity would contribute to the impaired lymphoproliferative responses to mitogens observed in elderly people.

Most human nutritional studies have shown that supplementation of the diets with *n*-3 PUFA enhanced cellular lipid hydroperoxide production (Nair *et al.* 1993). These studies generally used high dietary intake (usually >2 g fatty acids/d). However, it has also been shown that very low doses of *n*-3 PUFA can, paradoxically, protect cells from peroxidative stress (Calzada *et al.* 1992). On the other hand, we have also observed that *n*-3 fatty acids, mainly docosahexaenoic acid (DHA), were able to increase both PDE and GSH-Px activities of human PBMN *in vitro* when added to culture medium (Joulain *et al.* 1994, 1995). Taken together, the results discussed here led us to carry out the present nutritional study in elderly people. The aim of the present study was to investigate the possibility of modulating the reactivity of PBMN by the ingestion of low doses of MO (600 mg/d, providing 150 mg DHA + 30 mg eicosapentaenoic acid (EPA)/d). We studied the influence of dietary *n*-3 PUFA

supplementation on the lymphoproliferative response and on the enzymatic targets previously shown to be altered with age.

Thus, cyclic AMP and cyclic GMP-PDE activities, GSH-Px activity, cyclic nucleotide levels (cAMP and cGMP), the proliferative response to the plant lectins concanavalin A and phytohaemagglutinin A and to the anti-CD3 antibody OKT3 were determined in PBMN from elderly people before and after supplementation for 6 weeks with 600 mg MO or 600 mg sunflower oil (SO)/d (placebo).

## Subjects and methods

### Subjects and experimental design

The twenty subjects recruited for the study were healthy and non-institutionalized elderly people. They had no metabolic, malignant or clinically detected cardiovascular diseases. Each of them gave informed consent according to the guidelines of the French Ethical Committee. The mean age of the selected subjects (six men and fourteen women) was 75.6 (SD 4.1) (range 70–83) years. At the beginning of the study each of them had normal blood cell counts, packed cell volume (44.6 (SD 0.9) %, *n* 20), mild hypercholesterolaemia (6.6 (SD 1.4) mM, *n* 20), normal triacylglycerolaemia (1.4 (SD 0.7) mM, *n* 20) and glycaemia (5.5 (SD 0.7) mM, *n* 20). Their mean systolic and diastolic blood pressures were 145 (SD 12) and 81 (SD 2) mmHg respectively (*n* 20). In a double-blind design, subjects were randomly assigned to two groups. The placebo group (four men and six women) ingested 600 mg SO/d for 6 weeks. The treated group (two men and eight women) ingested 600 mg MO (RO-PUFA®; Roche, Basle, Switzerland)/d, for the same period. The fatty acid composition of the oils determined by GC on total lipid extracts is shown in Table 1. This amount of MO provides 150 mg DHA + 30 mg EPA. The dietary supplements were administered in indistinguishable soft gelatine capsules that each contained 600 mg oil and adequate amounts of vitamin E as an antioxidant (0.17 mg  $\alpha$ -tocopherol for the placebo and 0.54 mg for the MO, which represents <5 % of the normal vitamin E intake). The habitual fish intake among elderly people in France is estimated to be about 20 g/d according to the Agence Française de Sécurité Sanitaire des Aliments (Maisons-Alfort, France), providing 0.10 g DHA and 0.05 g EPA/d. Thus the amount of DHA + EPA provided by the supplementation was in the range of the habitual nutritional intake. Each individual was asked not to deviate from their regular habits during the study period and to ingest one capsule per d with their principal meal. The dietary supplements

**Table 1.** Fatty acid composition of the oils\*

Treatment	Fatty acid (mol/100 mol total fatty acids)							
	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Arachidonic	EPA	DHA
Sunflower oil (placebo)	5.9	0.1	3.8	23.0	65.9	0	0	0
Marine oil	19.9	5.6	5.5	13.5	2.0	1.8	5.4	25.6

EPA, eicosapentaenoic; DHA, docosahexaenoic.

\*Values were determined by GC analysis of total lipid extracts.

were manufactured by Imedex (Lyon, France). Blood samples were drawn on day 0 and day 42 for biochemical analyses. The subjects had not taken any drugs at least 10 d before the initial blood sampling and during the test period.

#### *Peripheral blood mononuclear cell isolation*

Venous blood was drawn from an antecubital vein into vacutainers (Becton Dickinson, Meylan, France) containing citric acid–sodium citrate–dextrose anticoagulant. The platelet-rich plasma was removed after initial centrifugation (120 *g* for 18 min) and erythrocytes were passively sedimented for 30 min at 37°C after addition of dextran (final concentration 10 g/l; Sigma-Chimie, L'Isle d'Abeau, France) prepared in buffered saline (9 g NaCl/l), pH 7.4 containing 1 mM-EDTA. The leucocyte-rich supernatant fraction was then removed, layered onto Histo-paque (density 1.077 g/ml Sigma-Chimie) and centrifuged at 600 *g* for 15 min at 20°C. The resulting mononuclear fraction was washed with PBS and then, washed twice with RPMI 1640 (Gibco, Cergy Pontoise, France) by low-speed centrifugation in order to eliminate the contaminating platelets more thoroughly. Cell viability, established by the Trypan Blue exclusion test, was always >95%. PBMN were then adjusted to a concentration of  $20 \times 10^6$  cells/ml RPMI 1640 (with 20 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and bicarbonate) medium.

#### *Biochemical analyses*

**Cell lysis.** For enzymatic activity determinations, pelleted PBMN were washed with Phillips' buffer and disrupted by glycerol lysis as previously described (Meskini *et al.* 1992). After glycerol treatment, the cells were pelleted at 900 *g* for 10 min, resuspended in lysis buffer (20 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 25 mM-sucrose, 0.1 mM-ethylene glycol-*O*,*O'*-bis-(2-amino-ethyl)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.06 mM-phenyl methane sulfonyl fluoride, 10 000 U aprotinin/l, 2 mg pepstatin A/l, pH 7.4) and stored frozen at –30°C. After thawing, the cells were homogenized in a glass–teflon homogenizer (forty strokes at maximal speed) and homogenates were centrifuged at 100 000 *g* for 60 min.

**Cyclic nucleotide phosphodiesterase assays.** PDE activities of supernatant and pellet fractions were measured according to a two-step radioisotopic method lysis as previously described (Meskini *et al.* 1992). Results are expressed as pmol cAMP or cGMP hydrolysed/min per mg protein. Protein was assayed according to Bradford (1976) using bovine serum albumin as a standard.

**Glutathione peroxidase assay.** GSH-Px activity was determined according to the method of Paglia & Valentine (1967) as previously described (Meskini *et al.* 1993). Results are expressed as nmol NADPH oxidized/min per mg protein using an extinction molar coefficient of 0.0622/mm<sup>2</sup> per  $\mu$ mol for NADPH. Inhibition of 90–95% was consistently observed in the presence of 0.2 mM-mercaptosuccinic acid, which indicates a Se-dependent GSH-Px activity.

**Proliferative response of peripheral blood mononuclear cells to concanavalin A, phytohaemagglutinin A and OKT3.** PBMN were cultured in microtitre culture plates at  $2 \times 10^5$  cells per well in a final volume of 200  $\mu$ l RPMI 1640 supplemented with 2 mM-glutamine, 100 mg streptomycin + 100 000 U penicillin/l and decomplexed fetal calf serum (100 ml/l; Gibco, Cergy Pontoise, France). At the initiation of the culture, 5 mg concanavalin A/l or 1 mg phytohaemagglutinin/l (Sigma-Chimie) or 100  $\mu$ g OKT3/l (Cilag Laboratories, Levallois Perret, France) were added to the cell suspensions. Control cells were incubated in the same conditions without mitogen. Cultures were incubated at 37°C in an air–CO<sub>2</sub> (95:5) atmosphere. After 48 h incubation, 18.5 kBq [<sup>3</sup>H]thymidine per well (Amersham, Les Ulis, France) were added to the cultures and the cells were incubated for a further 24 h. The cells were then harvested (Cell-Harvester Autowash, 2000; Dynatech, Fisher-Bioblock Scientific, Illkirch, France) onto glass-fibre filters. Filters were washed and dried. The radioactivity incorporated in the cells was measured by liquid scintillation counting.

**Cyclic nucleotide determinations.** The cAMP and cGMP content of human PBMN was measured by radio-immunoassay (<sup>125</sup>I-labelled cAMP and <sup>125</sup>I-labelled cGMP radioimmunoassay kits; Perkin-Elmer Life Sciences, Les Ulis, France). PBMN ( $20 \times 10^6$  cells/ml) were allowed to rest for 2 h at 37°C in borosilicate glass tubes (Corning, VWR Int., Funtenay-sous-Bois, France). Incubations were terminated by boiling the samples for 2 min. Proteins were removed by centrifugation (4500 *g* for 10 min), supernatant fractions were diluted as required in the immunoassay buffers and cAMP and cGMP were assayed according to the manufacturer's recommendations.

**Lipid analyses.** Plasma was acidified to pH 3–4 and lipids were extracted with chloroform–ethanol (6:3, v/v) in the presence of 50  $\mu$ M-butylhydroxylated toluene, according to the method of Boukhache & Lagarde (1982). The different lipid classes were separated on silica gel G60 plates (Merck, Darmstadt, Germany) with the solvent system hexane–diethyl ether–acetic acid (80:20:1, by vol.). The silica gel areas corresponding to phospholipids, non-esterified fatty acids, triacylglycerols and cholesteryl esters were scraped off and transmethylated. Briefly, 1 vol. H<sub>2</sub>SO<sub>4</sub> (50 ml/l methanol) was added to the scraped silica gel and transmethylation was carried out under an N<sub>2</sub> atmosphere at 100°C for 90 min in screw-capped tubes. The reaction was terminated by the addition of 1.5 vol. K<sub>2</sub>CO<sub>3</sub> (50 g/l) and the fatty acid methyl esters were extracted with isooctane and stored at –30°C under N<sub>2</sub>. The fatty acid methyl esters from cholesteryl esters were purified on TLC with the solvent system hexane–diethyl ether (80:20, v/v) in order to eliminate the contaminating cholesterol. The purified fatty acid methyl esters were scraped off and extracted with diethyl ether–methanol (9:1, v/v), then evaporated and dissolved in isooctane and stored at –30°C under N<sub>2</sub>. The non-esterified fatty acids from plasma were methylated with approximately 250  $\mu$ l diazomethane at room temperature and darkness for 15 min. The diazomethane was evaporated to dryness under reduced pressure and the extracts were dissolved in isooctane for GC analysis. The fatty acid

methyl esters from phospholipids, non-esterified fatty acids, triacylglycerols and cholesteryl esters fractions were analysed using a Perkin-Elmer chromatograph model 5830, equipped with a capillary column (30 m × 0.32 mm; Supelco, Bellefonte, CA, USA) and a flame ionization detection. The column was two-step programmed from 135 to 160°C at 2°C/min and from 160 to 205°C at 1.5°C/min, the detection temperature was maintained at 250°C. The vector gas was He at a pressure of 5.52 kPa (0.8 psi). Peaks were identified using standard fatty acid methyl esters.

### Statistical analyses

Each set of variables measured in the present study had normal distribution according to the Lilliefors' test for normality. All values are expressed as means with their standard errors. Data were analysed by Student's paired *t* test comparing values of the various variables on day 0 and day 42, each subject acting as his own control. This made the calculations independent of possible variations between subjects at day 0.

### Results

Both dietary supplements (SO and MO) were well tolerated and compliance, based on capsule consumption, was excellent in both groups. There were no significant changes in haematological variables and in serum glucose, triacylglycerol and cholesterol after dietary intervention with both oils (results not shown). Mean systolic blood pressure was significantly decreased by MO intake from 145.5 (SEM 5.1) at day 0 to 131.5 (SEM 4.5) mmHg at day 42 (*n* 10, *P*=0.001), whereas no change was observed in the placebo

(SO) group (144.5 (SEM 2.6) at day 0 *v.* 142.5 (SEM 3.8) mmHg at day 42 (*n* 10, NS). In contrast, the diastolic blood pressure was not modified at the end of the treatment either in the placebo (SO) group (80 (SEM 3) at day 0 *v.* 77 (SEM 2) mmHg at day 42 (*n* 10, NS) or in the MO group (82 (SEM 1) at day 0 *v.* 78 (SEM 2) mmHg at day 42 (*n* 10, NS).

The fatty acid composition of phospholipids, triacylglycerols, sterol esters and non-esterified fatty acids in the plasma of the elderly people were analysed before (day 0) and after (day 42) ingestion of 600 mg MO or SO/d. At the end of the supplementation period, no significant modifications in the fatty acid composition of plasma phospholipids, triacylglycerols and free fatty acids were observed either in the MO or in the placebo group (SO; results not shown). The only changes found were in plasma sterol esters (Table 2). An increase in the proportion of DHA was observed at the end of the supplementation period, the largest variation occurring in the MO group (+ 63 %, *P*=0.002) compared with the placebo group (+ 23 %, NS). Thus, the low doses of MO used in the present study proved unable to induce a major enrichment in *n*-3 fatty acids of the main plasma lipids. Although we did not analyse the fatty acid composition of PBMN phospholipids due to an insufficient amount of cells, parallel investigations have been performed on platelets. Interestingly, changes have been observed only in phosphatidylethanolamine (PE) of platelets from subjects ingesting the MO. In this group, the DHA content increased significantly from 2.7 at day 0 to 3.4 mol/100 mol (+26 %, *P*<0.001) at day 42. No other significant change in any other fatty acid could be observed whatever the phospholipid class considered (Véricel *et al.* 1999).

The proliferative responses of PBMN to concanavalin A, phytohaemagglutinin and OKT3 were examined before

**Table 2.** Fatty acid composition of plasma sterol esters (mol/100 mol total fatty acids) at baseline (day 0) and after 6 weeks supplementation (day 42) in the sunflower oil- (placebo) and marine oil-groups†

Fatty acid	Sunflower oil (placebo)				Marine oil			
	Day 0		Day 42		Day 0		Day 42	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
16:0	12.79	0.39	12.56	0.23	12.78	0.36	12.68	0.57
16:1 <i>n</i> -7	3.80	0.48	3.45	0.29	3.70	0.42	3.07	0.35
18:0	0.73	0.07	0.81	0.05	0.71	0.07	0.85	0.07
18:1 <i>n</i> -9	18.66	0.79	17.56	0.44	18.67	1.12	17.34	1.17
18:1 <i>n</i> -7	1.00	0.09	1.06	0.07	0.95	0.06	1.10	0.07
18:2 <i>n</i> -6	53.45	1.52	54.03	0.84	54.60	1.75	55.11	1.84
18:3 <i>n</i> -3	0.31	0.05	0.34	0.04	0.30	0.05	0.39	0.04
20:3 <i>n</i> -6	0.61	0.06	0.68	0.07	0.54	0.04	0.61	0.03
20:4 <i>n</i> -6	7.21	0.46	7.73	0.48	6.49	0.44	7.16	0.44
20:5 <i>n</i> -3	0.86	0.10	1.05	0.18	0.79	0.08	0.88	0.10
22:5 <i>n</i> -3	0.02	0.01	0.04	0.01	0.01	0.01	0.06*	0.01
22:6 <i>n</i> -3	0.56	0.05	0.70	0.05	0.46	0.05	0.75*	0.04
Total saturated	13.52	0.41	13.37	0.24	13.49	0.36	13.53	0.62
Total <i>n</i> -6	61.27	1.60	62.43	0.81	61.62	1.62	62.88	1.76
Total <i>n</i> -3	1.76	0.16	2.14	0.16	1.56	0.15	2.08*	0.16

Mean values were significantly different from those at day 0 for the same treatment group (Student's *t* test): \**P*<0.05.

† Sunflower oil-group (placebo), 600 mg sunflower oil/d for 6 weeks; marine oil-group, 600 mg marine oil for 6 weeks. For details of subjects and procedures, see p. 524.



(day 0) and after (day 42) supplementation of the diets of elderly people with low doses of SO or MO (Fig. 1). The proliferative responses to the three mitogens were significantly decreased in the MO group at day 42, compared with day 0 (concanavalin A  $-45\%$ ,  $P=0.01$ ; phytohaemagglutinin  $-34\%$ ,  $P=0.008$ ; OKT3  $-34\%$ ,  $P=0.01$ ). In the placebo group, a slight and non-significant decrease of the lymphoproliferative responses was observed at day 42, whatever the mitogen used. These results show that the ingestion of 180 mg *n*-3 fatty acids/d for 6 weeks decreased the lymphoproliferative responses of elderly people to mitogens.

No significant variations in the cytosolic cAMP- and cGMP-PDE activities of PBMN were observed after supplementation of the diets of elderly people, either in the MO or in the placebo (SO) group (Fig. 2 (a and b)). However, a slight decrease ( $-12\%$  for cAMP-PDE and  $-17\%$  for cGMP-PDE) was observed in the MO group at day 42. In contrast, both cAMP- and cGMP-PDE activities were significantly increased in the particulate fraction of PBMN after 6 week supplementation of the diet with MO. cAMP-PDE activity significantly increased from 26.51 at day 0 to 41.51 pmol/min per mg protein ( $+57\%$ ,  $P=0.03$ ) at day 42 (Fig. 2(c)), and cGMP-PDE activity

from 17.07 at day 0 to 26.61 pmol/min per mg protein ( $+56\%$ ,  $P=0.02$ ) at day 42 (Fig. 2(d)). In contrast, the particulate cAMP and cGMP-PDE activities were not altered by the SO intake (placebo group).

The ingestion of MO for 6 weeks also induced a slight but significant increase of both cAMP ( $+19\%$ ,  $P=0.03$ ) and cGMP ( $+26\%$ ,  $P=0.05$ ) levels in PBMN, whereas no significant variation was observed in the placebo group (Fig. 3).

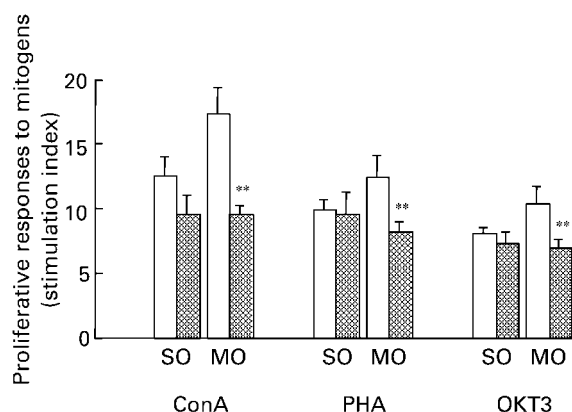
GSH-Px, which catalyses the degradation of a variety of hydroperoxides including lipid hydroperoxides to the corresponding alcohols, may be considered as a marker of the antioxidant status of the cells. MO supplementation of the diet for 6 weeks decreased the GSH-Px activity of PBMN from 602.37 at day 0 to 373.60 nmol NADPH oxidized/min per mg protein at day 42 ( $-38\%$ ,  $P=0.009$ ). No significant alteration was observed in the placebo group (Fig. 4).

## Discussion

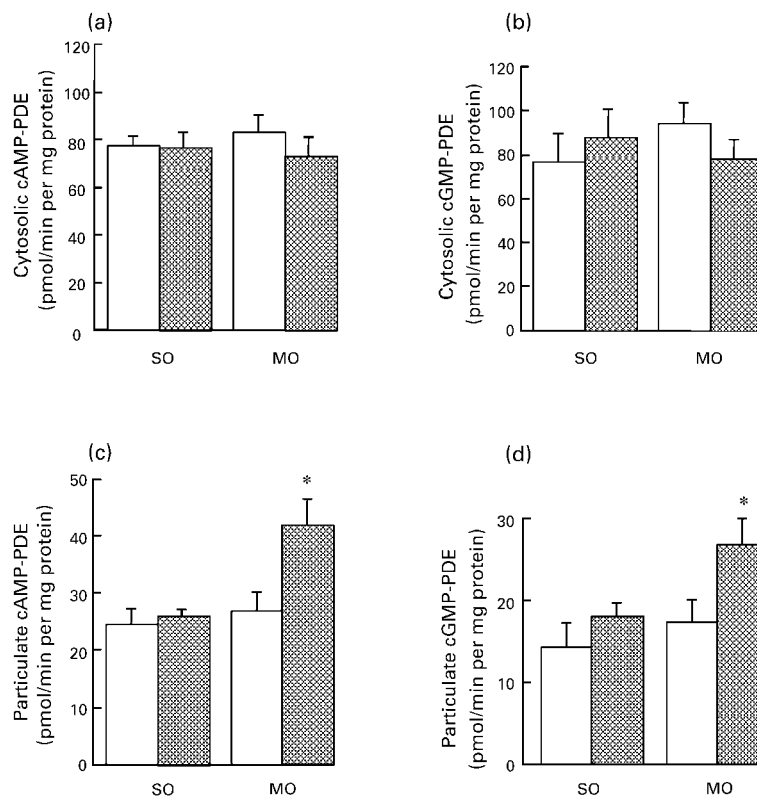
Ageing is associated with a decline in immune function that may be related to the accompanying decrease of cell antioxidant defences (Solana *et al.* 1991; Burns & Goodwin, 1997). Dietary intake of PUFA has also been associated with a decrease of immune cell functions (Calder *et al.* 2002). Most of the nutritional studies reported so far used large amounts of fish oil, usually providing *n*-3 fatty acid dosages  $>2$  g/d. Highly PUFA such as DHA and EPA, known to be very sensitive to peroxidation, have been shown to increase lipid radical formation induced by oxidant stress when incorporated in lipids of cell membranes (Alexander-North *et al.* 1994). However, *in vitro* experiments have also shown that low doses of *n*-3 PUFA may have opposite effects (Calzada *et al.* 1992; Bechoua *et al.* 1999). The present study was undertaken to investigate whether a supplementation of the diet of healthy elderly subjects with low doses of *n*-3 PUFA could affect the activity of enzymes known to be altered with age, such as PDE and GSH-Px, and to examine the possible consequences on lymphocyte proliferation.

The lack of large modification in the fatty acid composition of plasma lipids at the end of the supplementation period, either in the placebo group receiving 600 mg SO/d for 6 weeks or in the MO group supplemented with 600 mg MO/d providing 150 mg DHA + 30 mg EPA/d, clearly indicates that the fatty acid doses used were sufficiently low not to alter drastically cell membrane lipids. The fact that the relative amount of DHA in plasma sterol esters and platelet PE was significantly ( $P<0.002$ ) increased in the MO but not in the placebo (SO) group suggests that compliance obtained in the study was satisfactory. Another finding supporting compliance was the observation of a significant ( $P<0.001$ ) decrease in the systolic arterial blood pressure after 6 weeks of MO supplementation. Although such an effect was usually observed for *n*-3 fatty acid dosages  $>2$  g/d in the mildly hypertensive adult population, about 10-fold lower doses were needed in the elderly population (present study; Croset *et al.* 1990).

It is becoming clear that the consumption of diets that contain large amounts of fish oil-derived *n*-3 PUFA



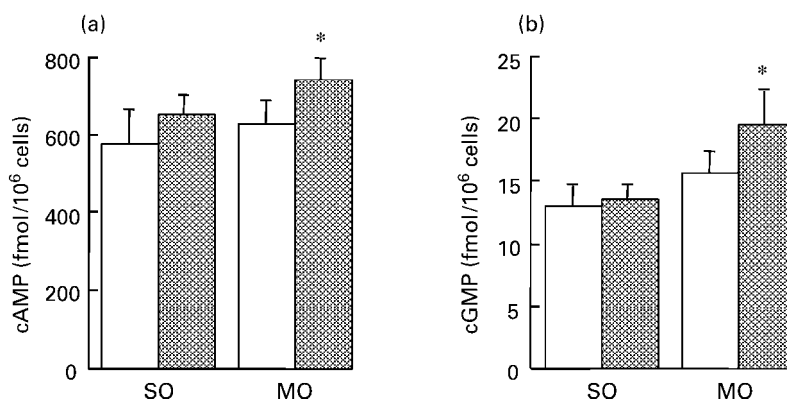
**Fig. 1.** Effects of marine oil (MO, 600 mg/d for 6 weeks) v. sunflower oil (SO (placebo), 600 mg/d for 6 weeks) on lymphocyte proliferation in elderly people. Before (day 0, □) and after (day 42, ▨) supplementation, peripheral blood mononuclear cells were isolated and cultured in RPMI 1640 + fetal calf serum (100 ml/l) in the presence of either concanavalin A (ConA; 5 mg/l), OKT3 (100 µg/l) or phytohaemagglutinin (PHA; 1 mg/l). Control cells were incubated under the same conditions without mitogens. After 48 h, [ $^3$ H]thymidine was added and the cells were incubated for a further 24 h prior to harvesting and radioactivity measurements. Results were expressed as the stimulation index: [ $^3$ H]thymidine incorporated in the presence of mitogen/[ $^3$ H]thymidine incorporated in the absence of mitogen. Values are means with their standard errors represented by vertical bars for ten subjects (six replicates per subject) per group. For details of subjects and procedures, see p. 524. Mean values with their standard errors for [ $^3$ H]thymidine incorporation (cpm) were as follows: unstimulated, SO (day 0) 2673 (SE 568), MO (day 0) 1799 (SE 238), SO (day 42) 2830 (SE 388), MO (day 42) 2091 (SE 278); ConA-stimulated, SO (day 0) 30162 (SE 6808), MO (day 0) 29098 (SE 5156), SO (day 42) 22865 (SE 2691), MO (day 42) 18528 (SE 2253); PHA-stimulated, SO (day 0) 24007 (SE 4732), MO (day 0) 24019 (SE 5156), SO (day 42) 20395 (SE 1475), MO (day 42) 17452 (SE 3371); OKT3-stimulated, SO (day 0) 21259 (SE 4639), MO (day 0) 17785 (SE 3047), SO (day 42) 17083 (SE 1702), MO (day 42) 14201 (SE 2529). Mean values were significantly different from those at day 0 (Student's test): \*\* $P\leq 0.01$ .



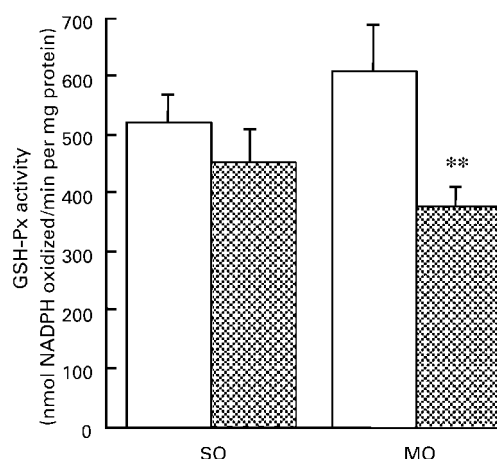
**Fig. 2.** Effects of marine oil (MO, 600 mg/d for 6 weeks) *v.* sunflower oil (SO (placebo), 600 mg/d for 6 weeks) on cAMP and cGMP-phosphodiesterase (PDE) activities of peripheral blood mononuclear cells in elderly subjects. Cytosolic cAMP- (a) and cGMP-PDE (b) activities, and particulate cAMP- (c) and cGMP-PDE (d) activities were assayed in the presence of 0.25  $\mu$ M-cAMP or -cGMP as the substrate. (□), day 0; (▨), day 42). For details of subjects and procedures, see p. 524. Values are means with their standard errors represented by vertical bars for ten subjects per group. Mean values were significantly different from those at day 0 (Student's *t* test): \**P* ≤ 0.05.

(DHA + EPA) causes immunosuppression. However, there is little information about the influence of very low doses of these fatty acids on immune function, especially in human subjects. Results of the present study show that even *n*-3 PUFA doses as low as 180 mg/d for 6 weeks are sufficient to markedly decrease the proliferative response of lymphocytes to mitogenic lectins and antigens. These results are in good agreement with those of a recent animal study showing that diets containing 20–25 % of the level of EPA and DHA found in fish oil exert

immunosuppressive effects similar to those of fish oil (Peterson *et al.* 1998). They are also in agreement with results from Thies *et al.* (2001), who have reported a significant reduction in the mitogenic responses to concanavalin A in subjects consuming 720 mg EPA + 280 mg DHA/d. Because these authors could not observe a significant effect on lymphocyte response with 720 mg of DHA alone, it can be speculated that the effect of *n*-3 fatty acids on immune responses was due to EPA. However, in the experiments reported here, the MO used for the



**Fig. 3.** Effects of marine oil (MO, 600 mg/d for 6 weeks) *v.* sunflower oil (SO (placebo), 600 mg/d for 6 weeks) on the cyclic nucleotide levels of peripheral blood mononuclear cells from elderly subjects. (a), cAMP; (b), cGMP. (□), day 0; (▨), day 42). Values are means with their standard errors represented by vertical bars for ten subjects per group. For details of subjects and procedures, see p. 524. Mean values were significantly different from those at day 0 (Student's *t* test): \**P* ≤ 0.05.



**Fig. 4.** Effects of marine oil (MO, 600mg/d for 6 weeks) v. sunflower oil (SO (placebo), 600mg/d for 6 weeks) on the glutathione peroxidase (GSH-Px) activities of peripheral blood mononuclear cells from elderly subjects. (□), day 0; (▨), day 42). Values are means with their standard errors represented by vertical bars for ten subjects per group. For details of subjects and procedures, see p. 524. Mean values were significantly different from those at day 0 (Student's *t* test): \*\* $P \leq 0.01$ .

supplementation contained 5-fold more DHA than EPA. Furthermore, we only observed significant DHA enrichment in plasma cholesteryl esters and platelet PE ( $P < 0.002$ ) (Véricel *et al.* 1999) with no variation in the EPA content of plasma and platelet lipids. Taken together, these observations suggest that the impairment of the lymphoproliferative response was very probably attributable to DHA, at variance with the study of Thies *et al.* (2001).

Cyclic nucleotides are well recognized as major intracellular regulators of growth especially in lymphocytes, where cAMP potently inhibits proliferation. The decreased proliferative response observed in the MO group may be due to the slight but significant ( $P < 0.05$ ) rise in intracellular cAMP levels. Moreover, the slight lowering of the cytosolic PDE activities might be partially responsible for the cyclic nucleotide level increase. At the same time, the particulate PDE activities were markedly and significantly enhanced in the MO group (+56–57%). Both decreased cytosolic and increased particulate PDE activities suggest that *n*-3 PUFA are able to favour the translocation of the cytosolic enzymes to the membrane, thus leading to a less efficacious hydrolysis of cyclic nucleotides in the cytosol.

Although some of the effects of *n*-3 PUFA on immune cell functions are readily explained by a decrease in membrane arachidonic acid level and a concomitant decrease in eicosanoid production (Miles & Calder, 1998), such a mechanism can be ruled out, as the fatty acid composition of plasma and platelet lipids was only minimally modified.

Alternative hypotheses have correlated the inhibitory effect of PUFA on lymphocyte proliferation with peroxidation. However, contradictory results regarding the influence of the antioxidant vitamins C and E on the immunosuppressive effect of *n*-3 fatty acids have been reported. While some groups have found that the suppressive effect of *n*-3 fatty acids was prevented by vitamin E both *in vivo*

and *in vitro* (Kramer *et al.* 1991; Kumar *et al.* 1992; Meydani, 1996), other studies have shown that antioxidants failed to reverse the inhibitory effect of DHA and EPA on lymphocyte proliferation (Calder & Newsholme, 1992, 1993; Joulain *et al.* 1995). In the present study, both SO and MO supplements contained adequate amounts of vitamin E, as calculated according to Muggli (1989). Thus, it can be concluded that non-specific lipid peroxidation is not likely to be involved in the DHA + EPA suppressive effect.

In most human nutritional studies, the enrichment of cell membranes with *n*-3 fatty acids after fish oil intake has been reported to increase GSH-Px activity, possibly due to increased amount of cellular lipid hydroperoxides (Bellisola *et al.* 1992). Some authors of *in vitro* studies have also reported increased GSH-Px activity after treatment of human PBMN (Joulain *et al.* 1994), platelets (Lemaitre *et al.* 1997) or vascular endothelial cells (Crosby *et al.* 1996) with *n*-3 fatty acids. The results of the present study show that the dietary supplementation of healthy elderly people with very low doses of *n*-3 fatty acids did not increase PBMN GSH-Px activity, but even significantly ( $P < 0.01$ ) reduced it by 38%. Increased GSH-Px activity may be considered as an adaptative response to an enhanced generation of lipid hydroperoxides, as suggested by Bellisola *et al.* (1992), and the present results suggest that the low doses of *n*-3 fatty acids used might prevent lipid peroxidation by a mechanism that remains presently unknown. This proposal is supported by results from Véricel *et al.* (1999), who showed a significant increase of  $\alpha$ - and  $\gamma$ -tocopherol, with a concomitant decrease of malondialdehyde, in platelets of elderly people supplemented for 6 weeks with low doses of MO. We have recently reported (Bechoua *et al.* 1999) that DHA was able to prevent malondialdehyde production induced by low  $H_2O_2$  concentrations in human PBMN. Thus, it is conceivable that DHA also decreased the production of lipid hydroperoxides in PBMN of elderly people after supplementation with MO, leading to a down-regulation of the GSH-Px enzyme. Whether the decreased GSH-Px activity is related to the suppressive effect of DHA + EPA on lymphocyte proliferation or not remains unclear, as both prooxidant and antioxidant states are required sequentially at different period of times during lymphocyte activation (Dröge *et al.* 1991).

Finally, *n*-3 fatty acids may also interfere with signal transduction pathways involved in lymphocyte activation. In a previous *in vitro* study (Bechoua *et al.* 1998), we have observed a decreased production of the phosphatidic acid (PA) mass in DHA-treated cells stimulated by concanavalin A as compared with untreated cells. Because PA is a potent mitogen, a decrease of its intracellular production may be responsible for the inhibition of the lymphoproliferative response observed in the elderly subjects supplemented with MO.

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