ELSEVIER

Biochimica et Biophysica Acta 1487 (2000) 1–14

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Fish oil diet affects on oxidative senescence of red blood cells linked to degeneration of spleen cells in mice

Motoko Oarada [a], Hiroshi Furukawa [b], Toshiro Majima [c], Teruo Miyazawa [b,*]

[a] *Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba 260-0856, Japan*
[b] *Biodynamic Chemistry Laboratory, Tohoku University Graduate School of Life Science and Agriculture, Sendai 981-8555, Japan*
[c] *Department of Bacteriology, School of Medicine, Tohoku University, Sendai 980-0872, Japan*

Received 3 March 2000; received in revised form 23 May 2000; accepted 24 May 2000

Abstract

The effect of dietary polyunsaturated fatty acids and α-tocopherol supplementation on erythrocyte lipid peroxidation and immunocompetent cells in mice was studied comparatively using seven dietary oils (15% oil/diet, w/w) including fish oil rich in eicosapentaenoic acid (EPA, 20:5, *n*–3) and docosahexaenoic acid (DHA, 22:6, *n*–3). A 43% increase in spleen weight, about twice as many spleen cells and no change in the subpopulations of spleen cells, as well as a significant depression of mitogen-induced blastogenesis of both T and B cells in the spleen were observed in mice fed fish oil for 30 days in comparison with soybean oil diet-fed mice. In the fish oil diet-fed mice, membranous lipid hydroperoxide (hydroperoxides of phosphatidylcholine and phosphatidylethanolamine) accumulation as a marker of oxidative senescence in red blood cells (RBC) was 2.7–3.5 times higher than that in mice fed soybean oil, although there was no difference in the plasma phosphatidylcholine hydroperoxide concentration. In spite of the supplementation of α-tocopherol to up to 10 times the level in the basal diet, the degeneration of spleen cells and the stimulated oxidative senescence of RBC found by the fish oil feeding could not be prevented. The results suggest that oral intake of excess polyunsaturated fatty acids, i.e. EPA and DHA, in a fish oil diet can lead to acceleration of membrane lipid peroxidation resulting in RBC senescence linked to the lowering of immune response of spleen cells, and that supplementation of α-tocopherol as antioxidant does not always effectively prevent such oxidative degeneration as observed in spleen cells and RBC in vivo.

Keywords: Docosahexaenoic acid; Eicosapentaenoic acid; Fish oil; Immunocompetent cell; Lipid peroxidation; Red blood cell; Senescence; α-Tocopherol

1. Introduction

Previous studies have shown that immunocompetent cells such as thymus and spleen cells are affected by the oral intake of oxidized oils in mammals, e.g. depression of DNA synthesis of thymocytes, and suppression of primary antibody response of spleen cells [1–5]. On the other hand, it is not yet clear whether immunocompetent cells are affected by the progress of lipid peroxidation in in vivo systems. The pathophysiological effects of *n*–3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA, 20:5, *n*–3) and docosahexaenoic acid (DHA, 22:6,

Abbreviations: DHA, docosahexaenoic acid (22:6, *n*–3); EPA, eicosapentaenoic acid (20:5, *n*–3); PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide; RBC, red blood cells

* Corresponding author. Fax: +81-22-717-8905;
E-mail: miyazawa@biochem.tohoku.ac.jp

1388-1981/00/$ – see front matter
PII: S1388-1981(00)00076-7

n–3) on mammals have been extensively studied [6–11]. However, dietary polyunsaturated fatty acids incorporated into membrane lipids in tissue organs are apt to be peroxidized if there is a shortage of antioxidants, especially α-tocopherol [12–15]. Therefore, it is of importance to elucidate the combined effects of polyunsaturated intake on immunocompetent cells and oxidative injury of membrane lipids in mammals. Membrane phospholipids of red blood cells (RBC) consist mainly of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), both of which are rich in polyunsaturated acids and thus seem to be highly susceptible to peroxidation, and this peroxidation may directly relate to oxidative senescence of RBC membrane [16–19]. Excess stimulation of oxidative injury of RBC membrane would affect spleen cells, because the spleen is a principal catabolic organ for aged RBC.

In the present study, we examined dietary *n*–3 polyunsaturated fatty acids for their in vivo effect on immunocompetent cells and progress of membrane phospholipid peroxidation in RBC in mice using seven different edible oils including fish oil rich in EPA and DHA, and further investigated the effect of α-tocopherol supplementation in the diet.

2. Materials and methods

2.1. Materials

Fish oil, safflower oil, olive oil, perilla oil, soybean oil, lard and palm oil were kindly supplied by Nippon Oil and Fat Co. (Tokyo, Japan). Casein, α-corn starch, cellulose powder, sucrose, DL-methionine, choline bitartrate and all-*rac*-α-tocopherol were purchased from Wako Pure Chemical Ind. (Osaka, Japan). AIN-76A mineral mixture and AIN-76A vitamin mixture were obtained from Nihon Nosan K.K. (Yokohama, Japan).

2.2. Experiment 1

2.2.1. Animals and diets

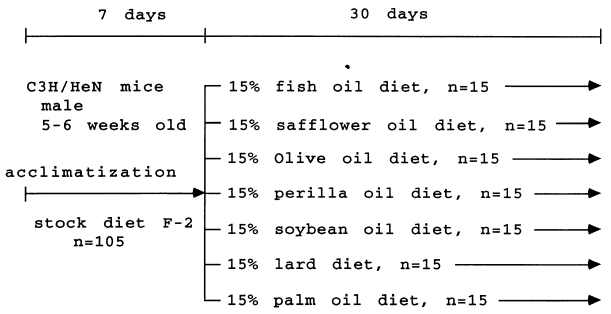
Male C3H/HeN mice, 5–6 weeks old, were purchased from the Mouse Center of the Tohoku University School of Medicine (Sendai, Japan) and were housed in cages in a room controlled for temperature (23 ± 1°C), humidity (55 ± 5%) and lighting (12-h light:dark cycle). During a preliminary period (1 week), mice were allowed free access to conventional stock diet F-2 (Funabashi Farms Co., Chiba, Japan).

Table 1
Fatty acid composition of dietary oils (g/100 g fatty acid)

Fatty acid	Fish[a] oil	Safflower oil	Olive oil	Perilla oil	Soybean oil	Lard	Palm oil
14:0	7.2	–	–	–	–	–	–
16:0	13.5	6.6	10.3	6.2	10.7	24.9	46.5
16:1	9.2	–	0.3	–	–	4.5	–
18:0	3.0	2.3	3.4	1.9	3.2	12.4	3.9
18:1 (*n*–9)	13.0	13.4	80.1	20.4	30.3	46.2	38.8
18:2 (*n*–6)	11.2	77.6	4.6	14.3	50.4	8.5	9.6
18:3 (*n*–3)	–	–	0.6	56.5	5.4	–	–
20:5 (*n*–3)	18.0	–	–	–	–	–	–
22:6 (*n*–3)	11.7	–	–	–	–	–	–
Others	13.2	0.1	0.7	0.7	0.0	3.5	1.2
Total saturated fatty acid	23.7	8.9	13.7	8.1	13.9	37.3	50.4
Total MUFA	22.2	13.4	80.4	20.4	30.3	50.7	38.8
Total *n*–6 PUFA	11.2	77.6	4.6	14.3	50.4	8.5	9.6
Total *n*–3 PUFA	29.7	–	0.6	56.5	5.4	–	–
Total *n*–6+*n*–3 PUFA	40.9	77.6	5.2	70.8	55.8	8.5	9.6
UI	205	169	91	219	147	68	58
PI	142	78	6	127	67	9	10

Abbreviations: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; UI, unsaturation index (sum of percentages of individual fatty acids × number of double bonds); PI, peroxidizability index (sum of percentages of individual fatty acids × number of active methylenes).

[a]Fish oil contained 10% (v/v) safflower oil to avoid linoleic acid deficiency.



Scheme 1. The feeding protocol in Experiment 1.

A total of 105 mice were divided into seven groups of 15 mice each and fed ad libitum for 30 days with test diets containing 15% experimental oils (Scheme 1). The test diet (in wt%) consisted of 45% α-corn starch, 20% casein, 10% sucrose, 5% cellulose powder, 3.5% AIN-76A mineral mixture, 1% AIN-76A vitamin mixture (containing *dl*-α-tocopherol acetate 5 mg/g), 0.3% DL-methionine, 0.2% choline bitartrate, and 15% test oil. The diet was prepared daily by the addition of each kind of oil to the basic fat-free mixture. The peroxide value of the test oils was kept below 2.5 meq/kg throughout the feeding experiment. The fatty acid composition and unsaturation degrees of the test oils are shown in Table 1. Fish oil was added with 10% (v/v) safflower oil to supply linoleic acid. Before the addition of test oils to the basic fat-free mixture, all-*rac*-α-tocopherol was mixed with the oils to attain 50 mg α-tocopherol/100 g oil (Table 2), but the concentrations of γ- and δ-tocopherols were not adjusted.

2.2.2. Mitogen-induced blastogenesis

At the end of the feeding experiment of the seven dietary groups for 30 days, single-cell suspensions of spleen cells were prepared by teasing the spleen with forceps in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (CTCM, complete tissue culture medium). Spleen cell suspensions were treated with Tris-buffered 0.84% ammonium chloride to lyse contaminating RBC. Spleen cells were washed twice with CTCM and then suspended at a density of 5×10^6 cells/ml of CTCM. One hundred microliter of spleen cell suspension (5×10^6 cells/ml) was dispensed in each well of 96-well microtest plates, and 100 μl of concanavalin A (Con A; 5 μg/ml in CTCM; Sigma Chemical, St. Louis, MO, USA) solution, phytohemagglutinin (PHA; 4 μg/ml in CTCM; Difco, Detroit, MI, USA) solution or lipopolysaccharide (LPS; 50 μg/ml in CTCM; Sigma Chemical) solution was added to each well. Cultures were incubated for 60 h at 37°C in 5% CO_2/95% air. After culturing, 0.5 μCi [^{3}H]thymidine (85 Ci/mmol; Amersham Japan, Tokyo, Japan) was added to each well. After 6 h of culturing, the cells were harvested onto glass-filter strips with the aid of an automated multiple-cell culture harvester. Tritium-labeled thymidine was determined with a liquid scintillation counter with toluene-base scintillation fluid. Toluene-base scintillation fluid was prepared by dissolving 2,5-diphenyloxazole (DPO, final concentration 4 g/l; Wako Pure Chemical Ind.) and 1,4-bis[2-(5-phenyloxazolyl)] benzene (POPOP, final concentration 200 mg/l; Kanto Chemical, Tokyo, Japan) in toluene (Wako Pure Chemical Ind.).

2.2.3. Fatty acid analysis

For lipid analysis, single-cell suspensions of spleen cells from the seven dietary groups were prepared by teasing the spleen with forceps in RPMI 1640 medium containing no FCS. Spleen cell suspensions were stored at −80°C until analyzed. The total lipids were extracted from spleen cells with a mixture of chloroform and methanol (2:1, v/v). PC and PE were separated by silica gel thin-layer chromatography using chloroform–methanol–acetic acid–water (25:15:4:2, v/v/v/v) as developing solvent [20]. Fatty acid methyl esters were prepared by refluxing the

Table 2
Tocopherol (Toc, mg/100 g oil) content of test oils in Experiment 1

Test oil	α-Toc	γ-Toc	δ-Toc	Total
Fish oil	50.0[a] (29.1)[b]	197.9	88.9	336.8
Safflower oil	50.0 (48.0)	2.0	–	52.0
Olive oil	50.0 (7.9)	–	–	50.0
Perilla oil	50.0 (5.3)	75.6	–	125.6
Soybean oil	50.0 (10.3)	70.9	19.0	139.9
Lard	50.0 (6.2)	13.4	–	63.4
Palm oil	50.0 (20.8)	–	–	50.0

β-Tocopherol was not detected.
[a]All-*rac*-α-tocopherol was supplemented to the test oil to attain 50 mg α-tocopherol/100 g oil.
[b]Figures in parentheses are the original concentrations of α-tocopherol in the oil.

isolated PC and PE with a 5% HCl–methanol solution and were analyzed with a Shimadzu GC-8A gas chromatograph on a Chromosorb WHP column (2.6 mm×2 m, Gasukuro Kogyo, Tokyo, Japan).

2.2.4. Spleen cell subpopulation analysis

Single-cell suspensions of spleen cells were prepared from mice fed a fish oil diet or a soybean oil diet for 30 days by the same procedure as for the test of blastogenesis. One hundred microliter of spleen cell suspensions (5×10^6 cells/ml) were each dispensed in round bottom tubes (Falcon 2052, Nippon Becton Dickinson Co., Ltd. Tokyo, Japan) and 10 μl of monoclonal antibody solution was added to each tube. Fluorescein isothiocyanate (FITC)-labeled rat monoclonal anti-Thy 1, 2, anti-Lyt 2 and anti-Mac 1 antibodies (Beckman Coulter, Fullerton, CA, USA) and phycoerythrin (PE)-labeled rat monoclonal anti-Lyb 5 and anti-L3/T4 antibodies (Beckman Coulter) were used. Cultures were incubated for 30 min on ice in a dark place. After incubation, cells were washed three times in PBS ($Ca^{2+}Mg^{2+}$-free, pH 7.2), and then resuspended in 200 μl of sheath fluid (FACS Flow, Nippon Becton Dickinson). Samples were stored at 4°C in the dark until analysis, and thereafter analyzed on a flow cytometer (Epics Profile II, Beckman Coulter) equipped with an argon ion laser [21]. A 488-nm light (100 mW of power) from the laser was used to excite FITC and PE.

2.2.5. Prostaglandin E_2 assay

Prostaglandin E_2 (PGE_2) production by spleen cells, prepared from mice fed diets containing either fish oil, soybean oil or lard for 30 days, was determined by enzyme immunoassay using a commercial kit, STAT (Cayman Chemical Co., Detroit, MI, USA). One hundred microliter of single-cell suspensions of spleen cells (5×10^6 cells/ml in CTCM) pooled from three mice and 100 μl of Con A solution (5 μg/ml in CTCM) were dispensed in each well of 96-well microtest plates. Cultures were incubated for 72 h at 37°C in 5% CO_2/95% air. After culturing, supernatants were stored at −80°C until use. Results are expressed in ng of PGE_2 per 5×10^5 cells.

2.2.6. Chemiluminescence assay of peritoneal macrophages

Active oxygen productivity of peritoneal macrophages mediated by the reaction with Fc receptor of macrophages for IgG (IgG-Fc receptor) and IgG-trinitrophenyl (TNP)-coated sheep red blood cells (SRBC) was measured by chemiluminescence assay, as described previously [22]. Briefly, peritoneal cells were collected from the abdominal cavity of each mouse fed a diet containing either fish oil, soybean oil or lard, by repeated lavage with a total of 10 ml of Eagle's minimum essential medium containing heparin (10 U/ml). The peritoneal cells pooled from each of four mice were pelleted by centrifugation ($200\times g$, 10 min) and then suspended at a density of 5×10^5 cells/ml of CTCM. Two hundred microliter of cell suspension (5×10^5 cells/ml in CTCM) was dispensed into chemiluminescence counting tubes. The cells were incubated at 37°C in 5% CO_2/95% air for 2 h, and then non-adherent cells were aspirated and each tube was washed once with CTCM. The number of non-adherent cells was subtracted from the number of incubated peritoneal cells to determine the number of adherent cells. The average number of adherent cells per tube was 5×10^4.

For the preparation of IgG-TNP-coated SRBC, SRBC (Denka Seiken, Tokyo, Japan) were washed three times with PBS and resuspended in PBS (2×10^9 cells/ml). Two milliliter of this suspension was mixed with the same volume of trinitrobenzene sulfonic acid (TNBS, Wako Pure Chemical Ind.) solution (3.5 mg/ml) and incubated for 30 min at 37°C followed by three washes with PBS and resuspended in PBS (2×10^8 cells/ml). Two milliliter of 1:20 dilution of the above suspension was mixed with 17 μl of monoclonal mouse anti-TNP IgG_{2a} antibody (a gift from Dr. Nose, Department of Pathology, Tohoku University) or 50 μl of monoclonal mouse anti-TNP IgG_{2b} antibody and incubated for 30 min at 37°C followed by three washes in PBS and resuspended in PBS (1×10^7 cells/ml).

Solutions of 500 μl of mouse IFN-γ solution (1000 U/ml in CTCM, Shionogi Kenkyusho, Osaka, Japan) were added to each tube (5×10^4 adherent cells in 200 μl of CTCM), and then incubated for 12 h. After incubation, 10 μl of luminol solution (1 mM in PBS) and either 50 μl of IgG-TNP-coated SRBC suspension (1×10^7 cells/ml in PBS) or 50 μl of TNP-coated SRBC suspension (1×10^7 cells/ml in PBS) were added to each tube. The chemiluminescence was measured by LB953 luminometer (Bert-

hold, Germany). Photocounting of individual tubes was recorded every 2 s, after the addition of IgG-TNP-coated SRBC and photocounting was continued for 30 min. For statistical analysis of chemiluminescence measurements, the emission intensity was assessed by the peak height.

2.2.7. Lipid hydroperoxide assay

On day 30 of the fish oil or soybean oil feeding, heparinized blood (heparin concentration 10 U/ml) was collected into sterile tubes. Blood was centrifuged at 1000×*g* for 10 min (4°C), and plasma and RBC were separated. To 0.9 ml of plasma (portions of 0.3 ml plasma were pooled from each mouse and combined), 3.6 ml of chloroform and methanol (2:1, v/v, containing 0.002% butylated hydroxytoluene (BHT) as an antioxidant) was added, followed by vigorous mixing. Mixtures were centrifuged at 1000×*g* for 10 min (4°C) to obtain chloroform layer. The extraction was repeated three times. After dehydration and evaporation of the combined chloroform layer, the plasma total lipids were diluted with 40 μl of chloroform–methanol (2:1, v/v) and then a 20 μl portion was subjected to chemiluminescence detection-high performance liquid chromatography (CL-HPLC). RBC were washed with 5-fold cold PBS (0.2 mM EDTA, 0.002% BHT) and then centrifuged at 2300×*g* for 15 min (4°C). The washing was repeated three times. After the centrifugation, PBS was removed from tubes to obtain packed RBC. Nine tenths milliliter of packed RBC (portions of 0.3 ml RBC were pooled from each mouse and combined) was incubated with 0.9 ml of cold distilled water (0.1 mM EDTA) for 15 min on ice for hemolysis [23]. After incubation, 9 ml of 2-propanol (0.002% BHT) was added and the solution was mixed by shaking and left for 30 min on ice, and then 9 ml of chloroform was added and the solution was further left for 30 min on ice. After that, the hemolyzed RBC solution was submitted to centrifugation (500×*g*, 15 min, 4°C) to obtain a chloroform layer. After dehydration and evaporation of the chloroform layer, the RBC total lipids were diluted with 50 μl of chloroform–methanol (2:1, v/v) and then a 20 μl portion was subjected to CL-HPLC. The analytical system consisting of CL-HPLC was basically the same as described in previous reports [24,25]. The HPLC column was JASCO Finepak SIL NH_2-5 (5 μm, 250×4.6 mm; *n*-propylamine column, Japan Spectroscopic Co., Tokyo, Japan). The column mobile phase was hexane–2-propanol–methanol–water (5:7:2:1, v/v/v/v) and the flow rate was 1.1 ml/min using a JASCO 880-PU pump. After the column eluant passed through a JASCO 875-UV detector set at 234 nm (to monitor conjugated dienes), it was mixed with a luminescent reagent at a post-column mixing joint (Y type; Kyowa Seimitsu Co., Tokyo, Japan) under controlled temperature at 40°C in a JASCO 860-column oven. The luminescent reagent was prepared by dissolving cytochrome *c* (final concentration 10 μg/ml, from horse heart, type VI; Sigma Chemical Co.) and luminol (final concentration 2 μg/ml, 3-aminophthaloyl hydrazine; Wako Pure Chemical Ind.) in 50 mM borate buffer (pH 10) and pumping with a JASCO 880-PU pump. The chemiluminescence generated by reacting hydroperoxide with the luminescent reagent was measured with a CLD-100 chemiluminescence detector (Tohoku Electronic Industries Co., Sendai, Japan).

A calibration curve was made with phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) prepared by photooxidation of egg yolk phosphatidylcholine (PC) and phosphatidylethanolamine (PE) that had been purified by silica column chromatography [26]. Purified PC or PE (each 200 mg) was dissolved in 25 ml of methanol containing 0.1 mM of methylene blue as a photosensitizer. The reaction mixture was placed in a beaker (200 ml vol) cooled with ice-cold water and photoirradiated for 20 min at 10°C. The light source, a 500-W photoreflector lamp (Toshiba Electronics Co., Tokyo, Japan), was held at a 50-cm distance above the surface of the reaction mixture. After the photooxidation, the reaction mixture was passed through a silica column (40×500 mm; Wako gel C-100, Wako Pure Chemical Ind.) with methanol as an eluant. Phospholipid and its oxidized products, including the hydroperoxides, were recovered in the methanol extract, but the methylene blue retained in the column. The methanol extract was then dried under reduced pressure by rotary evaporation, and the hydroperoxide content was determined by KI reduction [27]. In this reduction, 1 mol hydroperoxide quantitatively reacts with 2 mol KI. The hydroperoxide concentrations of the photoirradiated PC and PE were 38.4 μmol hydroperoxide–O_2/g of PC

(29 mmol hydroperoxide–O_2/mol of PC) and 71.8 μmol hydroperoxide–O_2/g of PE (51 mmol hydroperoxide–O_2/mol of PE), respectively. The photooxidized PC and PE were used as standard hydroperoxides, after dilution.

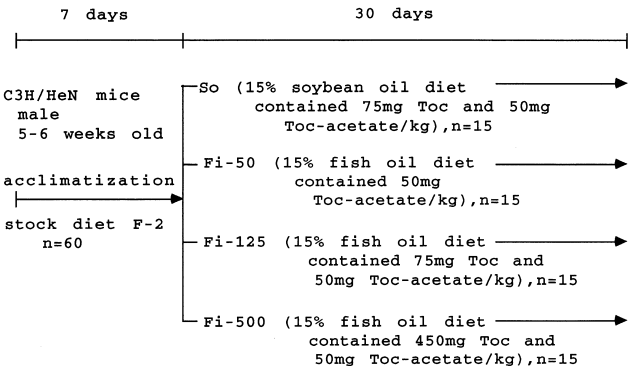
2.3. Experiment 2

2.3.1. Animals and diets

A total of 60 mice (male C3H/HeN, 5–6 weeks old) were divided into four groups of 15 mice each and fed ad libitum for 30 days with test diets after the preliminary feeding (1 week) (Scheme 2). For the diet preparation, all tocopherols (α-, β-, γ- and δ-isomers) in test oils (soybean oil and fish oil) were eliminated by passing the oils (400 g each) twice through a glass column (30×500 mm) packed with dried alumina (Merck, Germany) in vacuo [28]: this removed more than 99% of the tocopherols. Then, graded amounts of all-*rac*-α-tocopherol were added to the soybean oil (50 mg all-*rac*-α-tocopherol/100 g soybean oil) and fish oil (0, 50, 300 mg all-*rac*-α-tocopherol/100 g fish oil). The test diet (in wt%) consisted of 45% α-corn starch, 20% casein, 10% sucrose, 5% cellulose powder, 3.5% AIN-76A mineral mixture, 1% AIN-76A vitamin mixture (containing *dl*-α-tocopherol acetate 5 mg/g), 0.3% DL-methionine, 0.2% choline bitartrate, and 15% test oil. The tocopherol contents in the test diets were: 75 mg all-*rac*-α-tocopherol+50 mg *dl*-α-tocopherol acetate (from AIN-76A vitamin mixture)/kg diet for the soybean oil diet, 50 mg *dl*-α-tocopherol acetate/kg diet for the fish oil-50 diet, 75 mg all-*rac*-α-tocopherol+50 mg *dl*-α-tocopherol acetate/kg diet for the fish oil-125 diet, and 450 mg all-*rac*-α-tocopherol+50 mg *dl*-α-tocopherol acetate/kg diet for the fish oil-500 diet (Scheme 2). The peroxide values of the fish oil and soybean oil supplements were kept below 4.05 meq/kg throughout the feeding experiment.

2.3.2. Mitogen-induced blastogenesis and DNA synthesis

On day 30 of feeding of the four dietary groups (Scheme 2), single-cell suspensions of spleen cells were prepared and mitogen-induced blastogenesis was investigated in the same manner as in Experiment 1. Single-cell suspensions of thymus cells were prepared by teasing the thymus in CTCM. Thymus



Scheme 2. The feeding protocol in Experiment 2.

cells were washed twice with CTCM and then suspended at a density of 2.5×10^6 cells/ml of CTCM. Two hundred microliter of thymus cell suspension (2.5×10^6 cells/ml) was dispensed in each well of 96-well microtest plates. Cultures were incubated for 1 h at 37°C in 5% CO_2/95% air. After culturing, 0.5 μCi [^{3}H]thymidine was added to each well and cultures were then incubated for 6 h. After incubation, the cells were harvested onto glass-filter strips for measurement of DNA synthesis [2].

2.3.3. Tocopherol analysis

Heparinized blood (heparin concentration 10 U/ml) was separated into RBC and plasma by low-speed centrifugation (1000×*g*, 15 min, 4°C). RBC were washed with 5-fold cold PBS three times and resuspended in PBS. Packed RBC were prepared from the resuspended RBC by centrifugation at 1000×*g* for 15 min. Single-cell suspensions of spleen cells and thymus cells were prepared in the same manner as described above. α-Tocopherol in the spleen cells, thymus cells, blood plasma and RBC was analyzed by normal phase HPLC (Finepack SIL column, Japan Spectroscopic Co., Tokyo, Japan) and fluorescence spectrophotometry (Ex. 298 nm, Em. 325 nm), with a mixture of *n*-hexane–2-propanol (99:1, v/v) as column eluant at a 1.3 ml/min flow rate [29,30]. With this method, spleen cells, thymus cells and RBC were added with 2,2,5,7,8-pentamethyl-6-hydroxychroman ethanol solution as an internal standard and saponified with 60% KOH (in the presence of pyrogallol) before being extracted. The saponified cellular samples and blood plasma samples (deproteinized with ethanol) were then extracted by *n*-hexane and evaporated to dryness under

Table 3
Subpopulations (% of total) of spleen cells in mice fed a fish oil diet or a soybean oil diet for 30 days

Diet	Thy 1.2	Lyb 5	Lyt 2	L3/L4	Mac 1
Fish oil	54.1	25.3	20.0	7.6	5.9
Soybean oil	53.3	24.4	20.6	7.6	4.6

Thy 1.2, T cells; Lyb 5, B cells; Lyt 2, killer/supressor T cells; L3/L4, helper/inducer T cells; Mac 1, macrophages.

a nitrogen stream. The residue was redissolved in *n*-hexane and then subjected to normal phase HPLC.

2.3.4. Lipid hydroperoxide assay

Phospholipid hydroperoxides in the packed RBC and blood plasma of the four dietary groups mice were determined by the same manner as described in Experiment 1.

2.3.5. Statistical analysis

Results were expressed as mean ± S.D. The data were evaluated by one- or two-way ANOVA as appropriate, with Tukey's test for comparisons between pairs of means. Statistical significance was accepted at the $P < 0.05$ level. All analyses were performed using SPSS version 6.1 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Experiment 1

3.1.1. Spleen enlargement with fish oil feeding

The final body weights of mice at day 30 of the feeding experiment were not significantly different among the seven test oil diet groups (e.g. 27.1 ± 1.9 g in the soybean oil group, 29.6 ± 2.0 g in the fish oil group, mean ± S.D.). The spleen weight in the fish oil diet group was significantly increased (equivalent to 140% of the value for the soybean oil diet group) as compared with the other six test oil diet groups (Fig. 1A). The number of spleen cells was also increased, about 2-fold, in the fish oil diet group as compared with the other test oil diet groups excepting the olive oil group (Fig. 1B). Although no major differences were observed in the spleen cell subpopulations, there was a slight increase in the macrophage ratio in the fish oil mice as compared with the soybean oil mice (Table 3).

Table 4
Fatty acids (w/w %) of spleen cell phosphatidylcholine in mice fed test diets for 30 days

Fatty acid	Diet						
	Fish oil	Safflower oil	Olive oil	Perilla oil	Soybean oil	Lard	Palm oil
16:0	37.9 ± 1.3	41.7 ± 0.6	36.4 ± 1.0	35.6 ± 1.2	37.8 ± 1.4	36.9 ± 0.3	38.7 ± 0.4
16:1	5.0 ± 0.7	3.2 ± 0.5	3.0 ± 0.1	2.7 ± 0.5	3.6 ± 1.5	2.8 ± 0.1	2.5 ± 0.2
18:0	14.8 ± 1.1	17.9 ± 0.5	14.6 ± 1.2	17.3 ± 0.2	22.5 ± 1.9	19.4 ± 2.9	16.1 ± 1.1
18:1 (*n*–9)	11.0 ± 1.2	8.7 ± 1.2	23.3 ± 0.8	11.8 ± 0.2	9.5 ± 0.7	14.4 ± 2.1	13.2 ± 2.1
18:2 (*n*–6)	6.7 ± 1.1	16.5 ± 3.4	6.1 ± 2.1	13.5 ± 1.9	10.9 ± 0.3	7.1 ± 1.4	5.3 ± 0.2
18:3 (*n*–3)	0.8 ± 0.1	–	–	3.1 ± 0.0	–	–	–
20:4 (*n*–6)	4.8 ± 0.1	9.7 ± 0.6	13.6 ± 0.5	8.0 ± 0.1	11.2 ± 0.5	15.4 ± 2.6	18.1 ± 2.3
20:5 (*n*–3)	6.5 ± 1.0	–	–	3.1 ± 0.0	–	–	–
22:4 (*n*–6)	–	0.5 ± 0.1	0.5 ± 0	–	0.8 ± 0.2	0.8 ± 0.3	1.4 ± 0.1
22:5 (*n*–6)	1.6 ± 0.1	1.1 ± 0.4	0.9 ± 0.1	–	1.9 ± 0.3	1.6 ± 0.9	1.8 ± 0.2
22:5 (*n*–3)	3.2 ± 0.2	–	–	1.7 ± 0.0	–	–	–
22:6 (*n*–3)	7.5 ± 0.4	0.6 ± 0.2	1.6 ± 0.0	3.2 ± 0.3	1.9 ± 0.8	1.6 ± 0.1	2.8 ± 0.3
Total saturated fatty acid	52.7	59.6	51.0	52.9	60.3	56.3	54.8
Total MUFA	16.0	11.9	26.3	14.5	13.1	17.2	15.7
Total *n*–6 PUFA	13.1	27.8	21.1	21.5	24.8	24.9	26.6
Total *n*–3 PUFA	18.0	0.6	1.6	11.1	1.9	1.6	2.8
Total *n*–6+*n*–3 PUFA	31.1	28.4	22.7	32.6	26.7	26.5	29.4
UI	163	95	109	125	104	114	130
PI	101	55	60	79	64	30	85

Data are the mean ± S.D. of three determinations of pooled spleen cells from three mice. For abbreviations see Table 1.

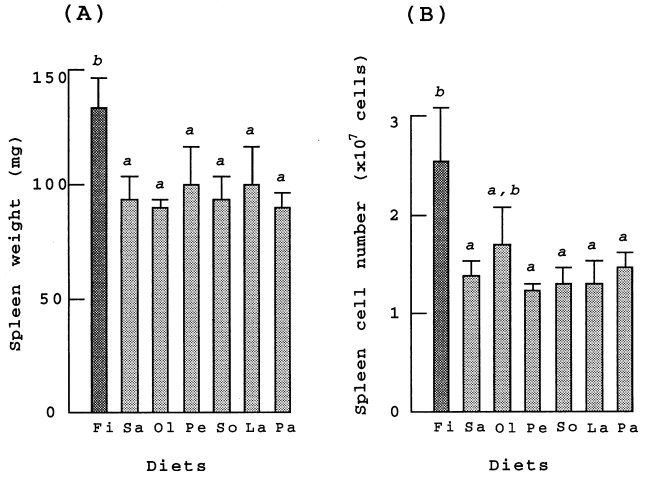


Fig. 1. Spleen weight (A) and spleen cell number (B) in mice fed test diets for 30 days in Experiment 1. Test diet oils: Fi, fish oil; Sa, safflower oil; Ol, olive oil; Pe, perilla oil; So, soybean oil; La, lard; Pa, palm oil. Data are the mean ± S.D. of six mice from each group. a, b: Values with different superscripts are significantly different ($P < 0.05$).

3.1.2. Fatty acids of spleen phospholipids

The most notable difference between fish oil-fed mice and the other test oil-fed mice was the highest concentration of *n*–3 PUFA, EPA (6.5% in PC and 8.6% in PE) and DHA (7.5% in PC, 19.3% in PE) in the spleen phospholipids (Tables 4, 5). The spleen PC and PE of the fish oil diet mice showed the highest UI (unsaturation index) and PI (peroxidizability index) values among the seven dietary groups. Substantial levels of EPA and DHA were also found in spleen PC and PE in mice fed a perilla oil diet rich in *n*–3 α-linolenic acid, although the values were lower than those in mice fed fish oil. Arachidonic acid (20:4, *n*–6) content in spleen PC and PE of fish oil mice was lowest among the seven dietary groups. *n*–6 Docosapentaenoic acid (22:5) typically appeared in spleen PE of mice fed a diet with safflower oil or palm oil. In the palm oil-fed group, 22:4 (*n*–6, 4.8% in PE) also appeared in spleen. Rather higher arachidonic acid concentrations (15.4% and 18.1% in PC, 23.6% and 25.7% in PE) were detected in mice fed a diet with lard or palm oil, respectively. Linoleic acid (18:2, *n*–6) content was high in spleen phospholipids in mice fed safflower oil or perilla oil, and low in mice fed a palm oil diet. The oleic acid concentration was high in the spleen lipids of olive oil-fed mice. No noteworthy differences were observed in the lipid class composition of spleens among these seven dietary oil groups (data not shown).

3.1.3. Decreased blastogenesis of spleen cells

Blastogenesis of spleen cells induced by Con A or

Table 5
Fatty acids (w/w %) of spleen cell phosphatidylethanolamine in mice fed test diets for 30 days

Fatty acid	Fish oil	Safflower oil	Olive oil	Perilla oil	Soybean oil	Lard	Palm oil
16:0	15.8 ± 2.0	23.8 ± 2.3	24.4 ± 3.3	20.7 ± 1.3	28.9 ± 3.8	21.2 ± 6.2	17.2 ± 2.2
16:1	3.2 ± 0.1	3.3 ± 1.1	3.1 ± 1.0	2.2 ± 0.1	1.6 ± 1.0	2.0 ± 1.0	3.7 ± 0.1
18:0	21.7 ± 1.1	24.1 ± 1.6	22.5 ± 2.4	22.9 ± 0.3	28.9 ± 0.9	26.7 ± 0.6	23.7 ± 1.3
18:1 (*n*–9)	7.3 ± 0.2	10.7 ± 1.3	19.5 ± 3.2	9.5 ± 0.7	7.6 ± 1.0	9.2 ± 1.4	8.5 ± 1.1
18:2 (*n*–6)	7.8 ± 0.2	12.4 ± 2.0	4.2 ± 1.2	11.1 ± 1.0	5.9 ± 1.0	6.2 ± 2.1	3.3 ± 0.6
18:3 (*n*–3)	0.6 ± 0.1	–	–	1.3 ± 0.0	–	–	0.5 ± 0.2
20:4 (*n*–6)	9.4 ± 1.0	12.7 ± 2.0	18.4 ± 1.5	11.5 ± 1.1	14.4 ± 0.3	23.6 ± 3.4	25.7 ± 3.0
20:5 (*n*–3)	8.6 ± 0.9	–	–	2.6 ± 0.9	–	–	–
22:4 (*n*–6)	–	2.5 ± 0.4	1.2 ± 0.4	–	1.8 ± 0.4	3.3 ± 1.8	4.8 ± 1.1
22:5 (*n*–6)	–	5.2 ± 0.6	2.3 ± 1.0	2.4 ± 1.1	2.0 ± 0.5	2.3 ± 0.2	5.7 ± 1.2
22:5 (*n*–3)	6.3 ± 1.2	–	–	5.7 ± 1.1	–	–	–
22:6 (*n*–3)	19.3 ± 2.6	2.3 ± 0.5	4.4 ± 0.6	10.2 ± 0.1	8.9 ± 1.2	5.6 ± 1.6	6.9 ± 1.2
Total saturated fatty acid	37.5	47.9	46.9	43.6	57.8	47.9	40.9
Total MUFA	10.5	14.0	22.6	11.7	9.2	11.2	12.2
Total *n*–6 PUFA	17.2	32.8	26.1	25.0	24.1	35.4	39.5
Total *n*–3 PUFA	26.2	2.3	4.4	19.8	8.9	5.6	0.5
Total *n*–6+*n*–3 PUFA	43.4	35.1	30.5	44.8	33.0	41.0	40.0
UI	256	139	147	198	148	176	212
PI	193	90	94	142	107	123	153

Data are the mean ± S.D. of three determinations of pooled spleen cells from three mice. For abbreviations see Table 1.

PHA for T cells and by LPS for B cells was significantly depressed in fish oil-fed mice and was below half the level in mice fed other test oils (Fig. 2). Mice fed perilla oil rich in α-linolenic acid (18:3, *n*–3) showed no such depression in blastogenesis. Olive oil-fed mice showed slight depression in blastogenesis induced by PHA or LPS.

3.1.4. Decrease in prostaglandin E_2 production of spleen cells

PGE_2 production by Con A-activated spleen cells was lower in mice fed a fish oil diet than in mice fed diets containing either soybean oil or lard (Fig. 3).

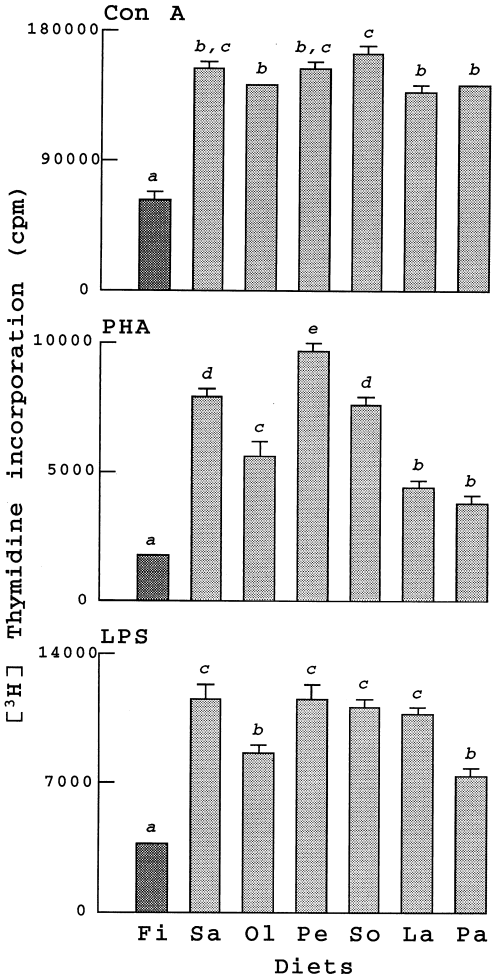


Fig. 2. Mitogen-induced blastogenesis of spleen cells in mice fed test diets for 30 days in Experiment 1. Mitogens: Con A, concanavalin A; PHA, phytohemagglutinin; LPS, lipopolysaccharide. Abbreviations of diets are the same as given in Fig. 1. Data are the mean ± S.D. of three determinations of spleen cells pooled from each of five mice. a, b, c, d, e: Values with different superscripts are significantly different ($P < 0.05$).

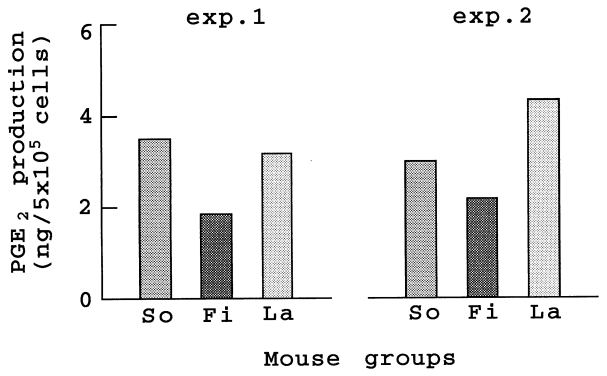


Fig. 3. Prostaglandin E_2 production by spleen cells isolated from mice fed diets containing either fish oil, soybean oil or lard for 30 days in Experiment 1. Values represent mean of duplicate cultures. Experiment was replicated and the results are shown as exp.1 and exp.2. For abbreviations see Fig. 1.

3.1.5. Active oxygen productivity of peritoneal macrophages

Active oxygen productivity of peritoneal macrophages, mediated by the reaction with Fc receptor for IgG and either IgG_{2a}- or IgG_{2b}-TNP-coated SRBC, was higher in mice fed a fish oil diet than in mice fed diets containing either soybean oil or lard (Fig. 4).

3.1.6. Phospholipid peroxidation in RBC and blood plasma

Phospholipid hydroperoxide concentrations of RBC and plasma of mice fed a fish oil diet or a soybean oil diet in Experiment 1 are shown in Table 6. Mice RBC contained PCOOH and PEOOH, and both phospholipid hydroperoxides were significantly higher in fish oil diet mice than in soybean oil diet mice. In plasma, no significant difference was found in PCOOH concentrations between fish oil diet mice and soybean oil diet mice. PCOOH was a predominant phospholipid hydroperoxide present in plasma but PEOOH was detected to a small extent.

3.2. Experiment 2

3.2.1. Effect of α-tocopherol supplementation

In Experiment 2, the effect of graded amounts of α-tocopherol supplementation as antioxidative nutrient in mice was investigated using four test oil

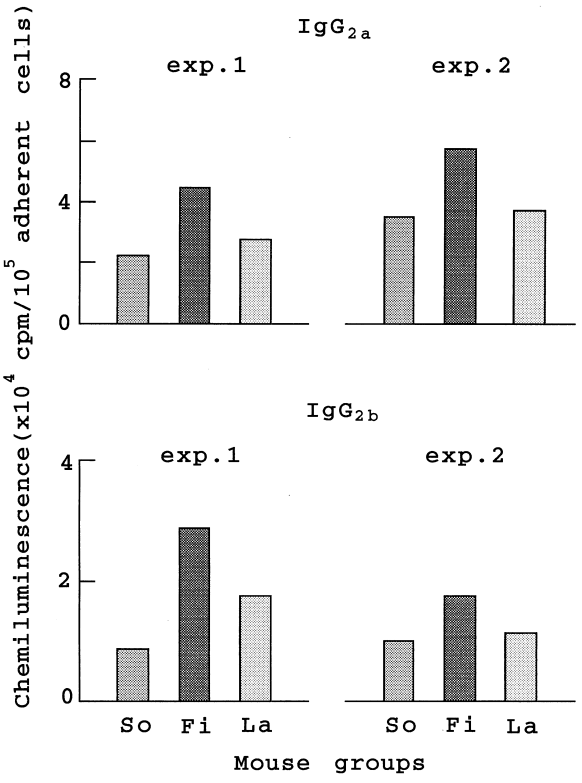


Fig. 4. Active oxygen production of peritoneal macrophages, mediated by the reaction of IgG-coated SRBC and Fc receptor of macrophages for IgG, isolated from mice fed diets containing either fish oil, soybean oil or lard for 30 days in Experiment 1. Values represent mean of duplicate cultures. Experiment was replicated and the results are shown as exp.1 and exp.2. For abbreviations see Fig. 1.

diet groups: the So diet group (containing soybean oil, with 75 mg all-*rac*-α-tocopherol+50 mg *dl*-α-tocopherol acetate/kg diet), and Fi-50, Fi-125 and Fi-500 diet groups (containing fish oil, with 0, 75 or 450 mg all-*rac*-α-tocopherol+50 mg *dl*-α-tocopherol acetate/kg diet, respectively). The Fi-50 mice showed the lowest α-tocopherol levels in tissue samples examined (plasma, RBC, spleen cells, thymus cells) among the four dietary groups (Fig. 5). In the Fi-125 mice, the α-tocopherol concentrations of RBC and spleen cells were almost equivalent to those found in the So diet mice but lower in plasma and thymus cells. In the Fi-500 mice, the RBC α-tocopherol level was significantly higher than that in the So mice. The α-tocopherol contents in spleen cells were almost the same in the So, Fi-125 and Fi-500 groups.

Table 6
Phospholipid hydroperoxide contents of red blood cells (RBC) and blood plasma in mice fed a fish oil diet or a soybean oil diet for 30 days

Diet	RBC (pmol/ml packed RBC)		Plasma (pmol/ml)
	PCOOH	PEOOH	PCOOH
Fish oil	271 ± 45[a]	518 ± 137[a]	74 ± 11
Soybean oil	159 ± 21	106 ± 38	51 ± 26

Data are the mean ± S.D. ($n=4$; each RBC or plasma sample was pooled from three mice). PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide.

[a]Significantly different from soybean oil diet mice ($P<0.05$).

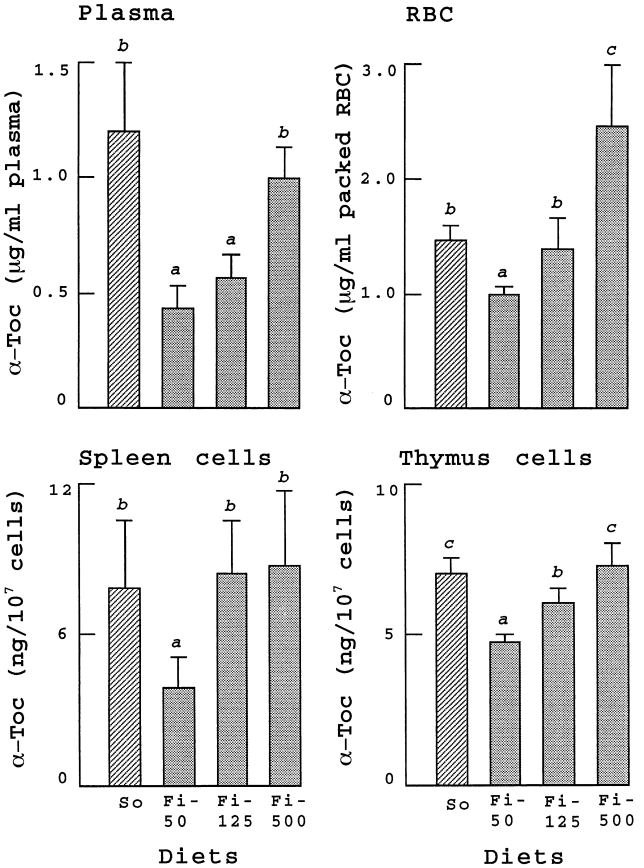


Fig. 5. α-Tocopherol concentration in plasma, red blood cells (RBC), spleen cells and thymus cells in mice fed fish oil diets with graded amounts of α-tocopherol, in Experiment 2. Diets: So, soybean oil; Fi-50, fish oil-50; Fi-125, fish oil-125; Fi-500, fish oil-500. Tocopherol contents in the diet for Experiment 2 are given in Section 2. Data are the mean ± S.D. ($n=4$; each plasma, RBC, spleen cells or thymus cells sample was pooled from three mice). a, b, c: Values with different superscripts are significantly different ($P<0.05$).

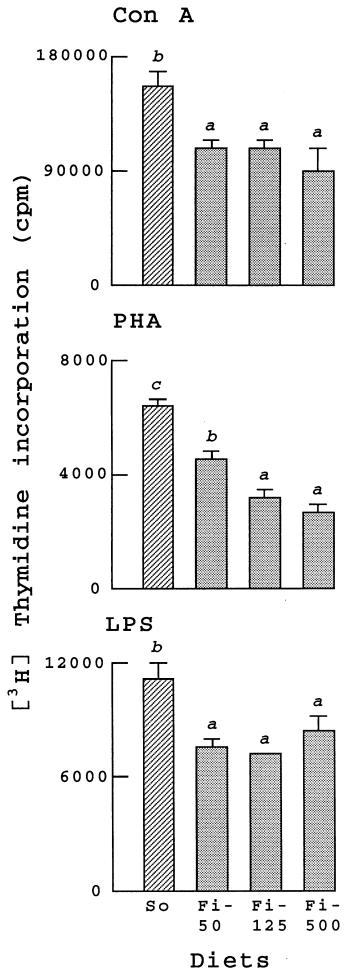


Fig. 6. Mitogen-induced blastogenesis of spleen cells in mice fed fish oil diets with graded amounts of α-tocopherol, in Experiment 2. Diets and abbreviations are the same as those given in Fig. 5. Data are the mean ± S.D. of three determinations of spleen cells pooled from each of five mice. a, b, c: Values with different superscripts are significantly different ($P < 0.05$).

These tocopherol supplementations did not improve the fish oil-induced enlargement of the spleen (69.4 ± 4.6 mg in So group, 84.8 ± 9.9 mg in Fi-50 group, 94.3 ± 16.8 mg in Fi-125 group, 90.0 ± 16.0 mg in Fi-500 group, mean ± S.D. of each six mice) or the fish oil-induced increase in spleen cell number ($0.9 \pm 0.4 \times 10^7$ cells in So group, $1.6 \pm 0.5 \times 10^7$ cells in Fi-50 group, $1.4 \pm 0.3 \times 10^7$ cells in Fi-125 group, $1.6 \pm 0.1 \times 10^7$ cells in Fi-500 group, mean ± S.D.). Furthermore, the profound depression in blastogenesis of T cells and B cells in the spleen induced by fish oil feeding was not prevented by the increase of α-tocopherol supplementation (Fig. 6). No major difference in blastogenesis of spleen cells was observed among the Fi-50, Fi-125 and Fi-500 diet mice. DNA synthesis of thymocytes in mice fed the Fi-50 or Fi-125 diet was significantly ($P < 0.05$) lower than that of the So diet mice ([^{3}H]thymidine incorporation (cpm): 16 600 ± 966 in So group, 13 472 ± 384 in Fi-50 group, 13 720 ± 203 in Fi-125 group (mean ± S.D.), but in Fi-500 mice (19 157 ± 1157 cpm) the level was similar to that in the So diet mice.

3.2.2. Phospholipid hydroperoxides in RBC

The level of phospholipid hydroperoxide, a marker of oxidative injury in membrane lipids in RBC, is shown in Fig. 7. Both PCOOH and PEOOH were detected in RBC, and the hydroperoxide concentrations were significantly higher in fish oil diet-fed mice (Fi-50, Fi-125, Fi-500 groups) than in soybean oil diet-fed mice (So group). The RBC PCOOH in the fish oil-fed mice groups was two times that of the So diet mice, and the RBC PEOOH level of the fish oil diet mice was 3.8–5.2 times that of the So diet mice. The increase in α-tocopherol supplementation to the fish oil diet failed to prevent the hydroperoxide accumulation in RBC observed by the fish oil feeding.

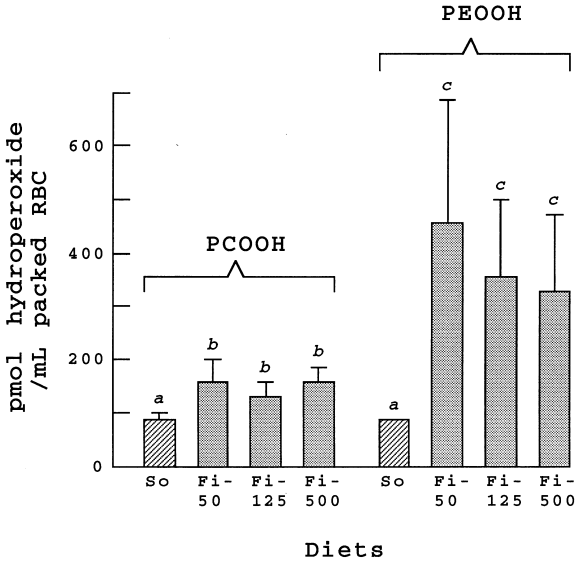


Fig. 7. Phospholipid hydroperoxide concentrations of red blood cells (RBC) in mice fed fish oil diets with graded amounts of α-tocopherol, in Experiment 2. Diets and abbreviations are the same as those given in Fig. 5. Data are the mean ± S.D. ($n = 4$; each RBC sample was pooled from three mice). a, b, c: Values with different superscripts are significantly different ($P < 0.05$).

No significant difference in mice plasma PCOOH concentration was observed among these four dietary groups (77.4 ± 25.8 nM PCOOH in So group, 64.2 ± 9.6 nM in Fi-50 group, 82.6 ± 24.6 nM in Fi-125 group, 99.0 ± 36.4 nM in Fi-500 group, mean ± S.D.).

4. Discussion

The present study evidently showed that only fish oil among seven dietary oils examined is sufficient to affect modification of spleen cell functions linked with acceleration of peroxidation to RBC membrane lipids in mice. We previously observed, with 3-months feeding of partially oxidized soybean oil (peroxide value, 150 meq/kg) to mice, that the DNA synthesis of thymocytes was depressed and mitogen-induced blastogenesis of spleen cells was increased without any increase in the level of plasma thiobarbituric acid reactive substances (TBARS) as a tentative index for lipid peroxidation [4]. When graded amounts of linoleic acid hydroperoxide (8.6, 12.3 and 14.1 g/kg body wt.) were orally administered to mice, necrosis of thymocytes was typically observed together with an increase of plasma TBARS [1]. Following the oral administration of linoleic acid hydroperoxide (2.29 g/kg body wt.) four times to mice, the in vivo primary antibody response against SRBC was significantly suppressed [3]. All of these previous findings, taken together, suggested that immunocompetent cells in mammals have susceptibility for the deleterious effects of lipid peroxidation products in vivo. The objective of the present study was to compare simultaneously the effect of seven different dietary oils on such immunocompetent cells and to evaluate the contribution of in vivo lipid peroxidation to the modification of immunocompetent cells, since almost all previous studies had been carried out with dietary oxidized oils (exogenous lipid peroxides) but not with endogenous lipid peroxides formed in the body of animals.

We have demonstrated here, as shown in Experiment 1, only fish oil diet caused an enlargement of the mouse spleen (Fig. 1A) and resulted in a remarkable decrease of blastogenesis of spleen cells (Fig. 2) with increases in spleen cell number (Fig. 1B) and RBC membranous lipid hydroperoxide accumulation (Table 6). Since safflower, olive, perilla, soybean, lard and palm oils did not cause such changes in the spleen, it is possible that long chain polyunsaturates like EPA (20:5, *n*–3) and DHA (22:6, *n*–3), being typically rich in fish oil (EPA, accounted for 18.0% of total acids; DHA, 11.7% of total acids) (Table 1), are responsible for the modification of immunocompetent cells as observed in the present study. Although perilla oil contained *n*–3 α-linolenic acid (56.5% of total fatty acids) (Table 1), perilla oil diet-fed mice did not show any appreciable symptoms as observed in the fish oil diet-fed mice. The unsaturation index (UI) of dietary oils does not simply correlate with such spleen cell depression, since the UI value of fish oil was lower than that of perilla oil (Table 1). Linoleic acid concentrations in dietary oils are also recognized as having no correlation with the lowered blastogenesis of spleen cells in the present study. Although it is well known that PGE_2 depresses mitogen-induced blastogenesis of spleen cells [31,32], PGE_2 production by Con A-activated spleen cells prepared from fish oil-fed mice was lower than that in soybean oil- or lard-fed mice in the present study (Fig. 3), suggesting that PGE_2 is not responsible for the lowered blastogenesis of spleen cells observed by the fish oil diet.

The significant increase in total spleen cell number as found in fish oil diet mice (Fig. 1B), which could be interpreted as a response activity of the spleen, did not involve any augmentation of certain cell subpopulations (Table 3), although a very slight increase was noticed in the macrophage number. The increase in spleen cell number in fish oil diet-fed mice may be the result of protective adaptation to compensate for failed functions such as the observed lower mitogen-induced blastogenesis of the cells. Although the apparent increase in the number of macrophages could not be confirmed in the present study, the active oxygen production by stimulated macrophages was revealed to have been increased more by a fish oil diet than that by a soybean oil or lard diet (Fig. 4). This enhanced sensitivity of macrophages may be needed to dispose of the accumulated senescent RBC, as have high levels of membranous phospholipid hydroperoxides (Table 6), in mice fed a fish oil diet.

While erythrocyte membranes contained both PCOOH and PEOOH as principal lipid hydroperoxides (Table 6), mouse plasma showed only PCOOH

as a predominant polar lipid hydroperoxide and a small amount of PEOOH. This observation with mouse plasma agrees well with our previous findings regarding rat and human plasma [15,33,34]. The plasma PCOOH level reflects peroxidation of phosphatidylcholine molecules locating on the surface of lipoprotein particles.

The increase in susceptibility to lipid peroxidation may be due to the increase of *n*–3 polyunsaturated fatty acids, i.e. EPA and DHA of membrane phospholipids [35], as shown in Tables 4 and 5. For the fatty acid contents of spleen phosphatidylcholine, fish oil treatment resulted in increases in EPA and DHA concentrations, and the UI and PI values were also highest in the fish oil-treated mice (Table 4). Such high unsaturation was also confirmed for fatty acid content in spleen phosphatidylethanolamine in the fish oil-treated mice (Table 5). A large decrease in arachidonic acid as observed in the fish oil-treated mice may be responsible for the consequent decrease of eicosanoid production (Fig. 3). The modifications of spleen fatty acid composition by fish oil treatment might also alter other functions of membrane phospholipids such as the lipid signalling responsible for the reduction of blastogenesis.

As shown in Experiment 2, the increased supplementation of α-tocopherol, a chain-breaking type antioxidant, failed to prevent either the formation and accumulation of membranous phospholipid hydroperoxides in RBC (Fig. 7) or the decrease in blastogenesis of spleen cells (Fig. 6) mediated by fish oil feeding. These results suggest that α-tocopherol may not work well as an antioxidant in RBC and spleen cells in vivo. The oxidative modification leading to senescence of RBC occurs even in the normal human body, and the oxidative susceptibility of RBC significantly increases with age [19]. Although the α-tocopherol concentrations in RBC, spleen cells and thymus cells of the α-tocopherol-supplemented Fi-125 mice were almost equivalent to those in the soybean oil diet mice (SO mice), the plasma α-tocopherol concentrations in Fi-50 and Fi-125 mice were below half that in So mice (Fig. 3). It has been reported that plasma and liver α-tocopherol concentrations are significantly reduced in *n*–3 polyunsaturated oil-fed rats compared to rats fed lard when menhaden oil, cod liver oil or sardine oil is used as a source of *n*–3 polyunsaturates [36]. In the rat plasma and liver, a reciprocal relation has been observed between α-tocopherol and phospholipid hydroperoxide concentrations when the animals received polyunsaturated oils like safflower oil or fish oil [15].

In conclusion, the present study demonstrates the comparative effects of feeding seven different oils on physical and membrane structural changes of mice spleen cells and erythrocytes. These results further indicate that only fish oil rich in *n*–3 polyunsaturated fatty acids modifies spleen weight, the numbers of spleen cells and blastogenesis of spleen cells, and increases the endogenous content of phospholipid hydroperoxides in red blood cells (RBC). It is likely that these effects are due to an increased oxidative stress in vivo and are not always sufficiently controllable by vitamin E supplementation.

References

[1] M. Oarada, E. Ito, K. Terao, T. Miyazawa, K. Fujimoto, T. Kaneda, The effect of dietary lipid hydroperoxide on lymphoid tissues in mice, Biochim. Biophys. Acta 960 (1988) 229–235.

[2] M. Oarada, K. Terao, Injury of mouse lymphocytes caused by exogenous methyl linoleate hydroperoxides in vitro, Biochim. Biophys. Acta 1165 (1992) 135–140.

[3] M. Oarada, N. Kurita, M. Miyaji, The effect of methyl linoleate hydroperoxides and radical initiator 2,2′-azobis (2-amidinopropane) dihydrochloride on antibody production by mouse spleen cells, J. Nutr. Sci. Vitaminol. 43 (1997) 377–385.

[4] M. Oarada, T. Majima, T. Miyazawa, K. Fujimoto, T. Kaneda, The effect of dietary oils on immunocompetent cells in mice, Biochim. Biophys. Acta 1012 (1989) 156–160.

[5] M. Oarada, T. Miyazawa, K. Fujimoto, E. Ito, K. Terao, T. Kaneda, Degeneration of lymphoid tissues in mice with the oral intake of low molecular weight compounds formed during oil autoxidation, Agric. Biol. Chem. 52 (1988) 2101–2102.

[6] M. Neuningen, G.J. Anderson, W.E. Connor, The essentiality of *n*–3 fatty acids for the development and function of the retina and brain, Annu. Rev. Nutr. 8 (1988) 517–541.

[7] J.M. Bourre, G. Durand, G. Pascal, A. Youyon, Brain cell and tissue recovery in rats made deficient in *n*–3 fatty acids by alteration of dietary fat, J. Nutr. 119 (1989) 15–22.

[8] S.E. Carlson, R.J. Cooke, P.G. Rhodes, J.M. Peeples, S.H. Wekman, Effects of vegetable and marine oils in preterm infant formulas on blood arachidonic and docosahexaenoic acids, J. Pediatr. 120 (1991) S159–S167.

[9] A.P. Simopoulos, Omega-3 fatty acids in health and disease and in growth and development, Am. J. Clin. Nutr. 54 (1991) 438–463.

[10] W.S. Harris, *N*–3 fatty acids and lipoproteins: Comparison of results from human and animal studies, Lipids 31 (1996) 243–252.
[11] H.R. Knapp, *N*–3 fatty acids and human hypertension, Curr. Opin. Lipidol. 7 (1996) 30–33.
[12] K. Mouri, H. Ikesu, T. Esaka, O. Igarashi, The influence of marine oil intake upon levels of lipids, α-tocopherol and lipid peroxidation in serum and liver of rats, J. Nutr. Sci. Vitaminol. 30 (1984) 307–318.
[13] S.N. Maydani, A.C. Shapiro, M. Maydani, J.B. Macauley, J.B. Blumberg, Effect of age and dietary fat (fish, corn and coconut oils) on tocopherol status of C57BL/6Nia mice, Lipids 22 (1987) 345–350.
[14] M. Maydani, F. Natiello, B. Goldin, N. Free, M. Woods, E. Schaefer, J.B. Blumberg, S.L. Gorbach, Effect of long-term fish oil supplementation on vitamin E status and lipid peroxidation in women, J. Nutr. 121 (1991) 484–491.
[15] T. Miyazawa, Membrane phospholipid hydroperoxides as estimated by chemiluminescence: The effect of dietary polyunsaturated fatty acids, in: A. Sinclair, R. Gibson (Eds.), Essential Fatty Acids and Eicosanoids, Am. Oil Chem. Soc., Champaign, 1993, pp. 383–388.
[16] D.E. Mills, M. Muythy, W.R. Galey, Dietary fatty acids, membrane transport, and oxidative sensitivity in human erythrocytes, Lipids 30 (1995) 657–663.
[17] P. Palozza, E. Sgarlata, C. Luberto, E. Picciani, M. Anti, G. Marra, F. Armelao, P. Franceschelli, G.M. Bartoli, *N*–3 fatty acids induce oxidative modifications in human erythrocytes depending on dose and duration of dietary supplementation, Am. J. Clin. Nutr. 64 (1996) 297–304.
[18] M.A.P. Van Den Boom, M.G. Wassink, B. Roelofsen, N.J. De Fouw, J.A.F. Op Den Kamp, The influence of a fish oil-enriched diet on the phospholipid fatty acid turnover in the rabbit red blood cell membrane in vivo, Lipids 31 (1996) 285–293.
[19] T. Miyazawa, T. Suzuki, K. Fujimoto, M. Kinoshita, Age-related change of phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide levels in normal human red blood cells, Mech. Ageing Dev. 86 (1996) 145–150.
[20] F. Parker, N.F. Peterson, Quantitative analysis of phospholipids and phospholipid fatty acids from silica gel thin-layer chromatograms, J. Lipid Res. 6 (1965) 455–460.
[21] D.R. Parks, L.A. Herzenberg, Fluorescence-activated cell sorting: Theory, experimental optimization, and applications in lymphoid cell biology, in: G.D. Sabato, J.J. Langone, H.V. Vunakis (Eds.), Methods in Enzymology, Vol. 108, Academic Press, New York, 1984, pp. 197–241.
[22] T. Majima, K. Itoh, J. Yatsu, O. Yoshie, N. Ishida, Sensitive detection of two IgG Fc receptors of mouse macrophages by chemiluminescence analysis, Immunopharmacol. Immunotoxicol. 11 (1989) 289–307.
[23] H.G. Rose, M. Oklander, Improved procedure for the extraction of lipids from human erythrocytes, J. Lipid Res. 6 (1965) 428–431.
[24] T. Miyazawa, T. Suzuki, K. Fujimoto, K. Yasuda, Chemiluminescent simultaneous determination of phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide in the liver and brain of the rat, J. Lipid Res. 33 (1992) 1051–1058.
[25] T. Miyazawa, K. Fujimoto, T. Suzuki, K. Yasuda, Determination of phospholipid hydroperoxides using luminol chemiluminescence-high performance liquid chromatography, in: L. Packer (Ed.), Methods in Enzymology, Vol. 233, Academic Press, New York, 1994, pp. 324–332.
[26] D.J. Hanahan, J.C. Dittmer, E. Warashina, A column chromatographic separation of classes of phospholipids, J. Biol. Chem. 228 (1957) 685–700.
[27] Official and Tentative Methods of AOCS, American Oil Chemists' Society, 1964, Item 8/53.
[28] G. Kajimoto, H. Yoshida, A. Shibahara, Comparison of nutritive value among thermally oxidized oil and tocopherol-free fresh oil, Yukagaku, J. Jpn. Oil Chem. Soc. 24 (1975) 511–517.
[29] K. Abe, G. Katsui, Determination of tocopherols in serum by high speed liquid chromatography, Vitamins (Japan) 49 (1975) 259–263.
[30] K. Abe, M. Ohmae, G. Katsui, Rapid and micro-method for the determination of tocopherols in liver, Vitamins (Japan) 50 (1976) 453–457.
[31] J.S. Goodwin, A.D. Bankhurst, R.P. Messner, Suppression of human T-cell mitogenesis by prostaglandin, J. Exp. Med. 146 (1977) 1719–1734.
[32] A. Fischer, A. Durandy, C. Griscelli, Role of prostaglandin E_2 in the induction of nonspecific T lymphocyte suppressor activity, J. Immunol. 126 (1981) 1452–1455.
[33] T. Miyazawa, K. Yasuda, K. Fujimoto, T. Kaneda, Presence of phosphatidylcholine hydroperoxide in human plasma, J. Biochem. 103 (1988) 744–746.
[34] T. Miyazawa, Determination of phospholipid hydroperoxides in human blood plasma by a chemiluminescence-HPLC assay, Free Radic. Biol. Med. 7 (1989) 209–217.
[35] J.P. Allard, R. Kurian, E. Aghdassi, R. Muggli, D. Royall, Lipid peroxidation during *n*–3 fatty acid and vitamin E supplementation in humans, Lipids 32 (1997) 535–541.
[36] W.D. Alexander, O.S. Mcguire, A.N. Cassity, L.K. Fritsche, Fish oils lower rat plasma and hepatic, but not immune cell α-tocopherol concentration, J. Nutr. 125 (1995) 2640–2649.