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Eicosapentaenoic acid inhibits antigen-presenting cell function of murine splenocytes

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SUMMARY

Recently, many investigators have studied the effects of eicosapentaenoic acid (EPA)-rich fish oil on immune function and immune disease. However, effects of dietary supplementation of fish oil or EPA on the immune system are still unclear. In the present study, the effects of EPA on antigen presentation were investigated. We have used antigen-specific helper T-cell clones that proliferate in the presence of antigen [keyhole limpet haemocyanin (KLH)] and spleen cells as antigen-presenting cells (APC). Mice were divided into two groups and fed an experimental diet or a control diet for 4 weeks *ad libitum*. In mice fed the experimental diet, the arachidonic acid (AA) content of spleen cells was decreased and that of EPA and docosapentaenoic acid was increased markedly compared to those of the control diet. Dietary enrichment with EPA inhibited the ability of accessory cells to present antigen to murine helper T-cell clones. This effect was observed for two distinct helper T-cell clones, Th1 and Th2. We also examined the effects of EPA-TG emulsion on APC function. The direct addition of EPA-TG emulsion to a T-cell proliferation assay system suppressed APC function. The inhibition was proportional to the concentration of EPA-TG emulsion. Pretreatment of splenocytes with EPA-TG emulsion resulted in inhibition of APC function. Inhibition of antigen presentation by dietary supplementation with EPA might depress immune reactivity.

INTRODUCTION

Eicosapentaenoic acid (EPA), termed n – 3 fatty acid to indicate the position of the double bond furthest from the carboxylic acid, is prominent in fish oil-enriched diets. A possible immunosuppressive effect of EPA-rich fish oil and pure EPA has been suggested by experimental and clinical observations. A diet enriched in EPA has been shown to prolong survival in New Zealand Black (NZB) × New Zealand White (NZW) F_1 mice[1] and to suppress autoimmune lupus in MRL-*lpr* mice.[2] Dietary supplementation with EPA has led to improvements in patients with rheumatoid arthritis and psoriasis.[3,4] Virella *et al.*[5] reported that the addition of pure EPA to human peripheral blood mononuclear cell cultures inhibited B-cell responses to mitogenic stimulation and depressed the expression of interleukin-2 (IL-2) receptors in pokeweed mitogen-stimulated lymphocytes. We have already reported infusion of fish oil emulsion into rabbits[6] and infusion of pure EPA emulsion into rabbits[7] and humans.[8,9] Natural killer cell activity of human lymphocytes was markedly decreased after the infusion of EPA-TG emulsion into healthy volunteers. The present study was directed at a clarification of the immunomodulatory mechanisms of EPA. We examined the effects of dietary supplementation with EPA and also *in vitro* use of EPA-TG emulsion on antigen presentation to helper T-cell clones.

Abbreviations: AA, arachidonic acid; APC, antigen-presenting cell; EPA, eicosapentaenoic acid; EPA-EE, EPA ethyl ester; EPA-TG, trieicosapentaenoyl-glycerol; PC, phosphatidylcholine; Th1, T-helper cell type 1; Th2, T-helper cell type 2.

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MATERIALS AND METHODS

Animals

Female C3H/HeN (C3H) and C57BL/6 (B6) mice obtained from Sankyo Labo Service (Tokyo, Japan) were bred in our own facility.

Cell preparations

Spleens from mice, under sterile conditions, were excised and reduced to free cells with a sterile stainless steel mesh. The erythrocytes were removed by hypo-osmotic lysis in ammonium chloride. Splenocytes were washed three times and resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS) (MA Bioproducts, Walkersville, ML), 2 mM L-glutamine, 12·5 mM HEPES, 2-ME (5×10^{-5} M), 100 U/ml penicillin, 60 μg/ml gentamicin (complete medium).

Analysis of the fatty acid composition

The fatty acid composition of total phospholipids in spleen cells was analysed as follows. Spleen cells were washed with 0·9% saline, reconstituted in 0·4 ml of saline and sonicated for 1 min. Total lipids were extracted with 8 ml of chloroform/methanol (2:1, v/v). The phospholipid fraction was obtained as the unmoved fraction following thin-layer chromatography on silica gel plates using petroleum ether/ether/acetic acid (80:20:1, v/v/v) as solvent. Methyl esters of fatty acids of total phospholipids were prepared with 6% H_2SO_4 in methanol, and were analysed by gas chromatography using a GC-14A (Shimadzu, Kyoto, Japan) equipped with a SP-2330 capillary column (Supelco, Bellfonte, PA).

T-cell clones

KLH/I-A^k-specific (23-1-8) and KLH/I-A^b-specific (24-2) T-cell clones were kindly provided by Dr Yoshihiro Asano (University of Tokyo, Tokyo, Japan) and maintained as previously described.[10] The first T-cell clone 23-1-8 was derived from C3H mice. The second T-cell clone 24-2 was derived from B6 mice. The clones were classified as T-helper cell type 1 (Th1) or Th2 according to their ability to secrete interleukin-2 (IL-2) or interleukin-4 (IL-4), respectively, following stimulation with antigen-presenting cells (APC) plus antigen.[11] The two cell types differ also in their dependence on interleukin-1 (IL-1) for proliferation.[12] It has been shown that only Th2 express IL-1 receptor on their surface.[13] Both clones were induced into a resting state by culturing them with relevant APC without their specific antigen (KLH) for 1 week, and they were then used for assays.

T-cell proliferation assay

A total of 2×10^4 T cells of either clone were incubated with various numbers of irradiated (3000 rads) spleen cells and KLH (10 μg/ml) in a final volume of 0·2 ml, in triplicate. After 56 hr 1 μCi [^{3}H]thymidine was added to the cultures. They were harvested 14–16 hr later and counted for [^{3}H]thymidine incorporation in a scintillation counter. In all experiments irradiated spleen cells of H-2 congenic mouse strains were used as APC. Results were expressed as the mean counts per minute (c.p.m.) of [^{3}H]thymidine incorporation, and SE was indicated.

Preparation of emulsion

EPA ethyl ester (EPA-EE) was obtained as described previously.[14] 1,2,3-trieicosapentaenoyl-glycerol (EPA-TG) was synthesized by hydrolysis of EPA-EE. 2-eicosapentaenoil-phosphatidylcholine (EPA-PC) was synthesized by chemical condensation of lysophosphatidylcholine, which was enzymatically prepared from soybean oil phosphatidylcholine with phospholipase A_2, and free EPA. EPA-TG was emulsified with EPA-PC according to the method of Geyer *et al.*[15] One hundred millilitres of the EPA-TG emulsion containing 10 g of EPA-TG, 1·2 g of EPA-PC and 2·5 g of glycerol. Incomplete reactive products of EPA-TG were separated by a silica gel column. The lipid contained α-tocopherol (0·2%,w/w) as an antioxidant and 2·5% glycerol solution. This concentration of α-tocopherol and glycerol had no effect on T-cell proliferation assay (data not shown).

A commercial soybean oil emulsion was obtained from Nikon Pharmaceutical (Osaka, Japan). Egg yolk-PC was used for emulsification of the soybean oil. One hundred millilitres of the soybean oil emulsion contained 10 g of the oil, 1·2 g of egg yolk phospholipids and 2·5 g of glycerol.

Table 1. Fatty acid composition of lipids used in the experiments

	Lipids (mol %)				
		2-EPA-PC			
Fatty acids	EPA-TG	Position 1	Position 2	Soybean oil	Egg yolk
16:0		26		8	34
18:0		7		3	14
18:1 n−9		20		22	32
18:2 n−6		45		59	16
18:3 n−3		2		8	
18:4 n−3	2		2		
20:4 n−6	5		5		3
20:4 n−3	2		2		
20:5 n−3 (EPA)	89		89		

One hundred millilitres of EPA-TG emulsion contained of 10 g of EPA-TG and 1·2 g of EPA-PC. One hundred millilitres of soybean oil emulsion contained 10 g of soybean oil and 1·2 g of egg yolk phospholipids.

The fatty acid compositions of these lipids mentioned above are shown in Table 1.

Experimental design

Weight-, sex-, and age-matched groups of C3H and B6 mice entered the study at 5 weeks of age. They were divided into two groups and fed the following diets for 4 weeks *ad libitum*. Diet 1 (control group): a lipid-free powder diet (90%, w/w) plus lard (8%, w/w) and safflower oil (2%, w/w); diet 2 (experimental group) was the same as diet 1 except that safflower oil was replaced with EPA-EE. After 4–5 weeks on the diet mice were killed and spleen cells were obtained.

Seven-weeks-old female C3H and B6 mice were fed standard chow. They were 9–10 weeks old at the time of use. EPA-TG emulsion was added directly to mixtures of T-cell proliferation assays. Splenocytes were also preincubated with EPA-TG emulsion for 3 hr, washed and tested for APC function.

Data were compared for statistical significance by means of Student's *t*-test and considered significant for $P < 0{\cdot}05$.

RESULTS

Fatty acid composition of splenocyte phospholipids

Changes in arachidonic acid (AA) and EPA of splenocyte phospholipids are shown in Table 2. In the experimental group, the content of AA was decreased and that of EPA and docosapentaenoic acid (22:5 n−3, an elongated product of EPA) was increased markedly compared to those of the control group. The content of docosahexaenoic acid was not altered markedly. There was no significant difference between the two groups in body weights throughout the experiment and in the numbers of splenocytes.

Table 2. Fatty acid composition (mol %) of phospholipids in spleen cells

	C3H/HeN		C57BL/6	
Fatty acids	Diet 1	Diet 2	Diet 1	Diet 2
16:0	24·82	27·01	24·79	26·55
16:1 n−7	0·89	1·48	1·16	1·98
18:0	18·43	18·25	17·60	18·24
18:1 n−9	7·59	9·04	6·51	8·79
18:2 n−6	5·98	5·87	6·55	5·25
20:4 n−6 (AA)	19·49	7·31	19·67	7·21
20:5 n−3 (EPA)	0·00	7·54	0·00	7·32
22:5 n−3	0·57	6·02	0·95	7·91
22:6 n−3	3·03	3·23	3·92	2·25

Mice were divided into two groups and fed diet 1 or diet 2. After 4 weeks on diet mice were killed and spleen cells were obtained. The results are the means of three experiments.

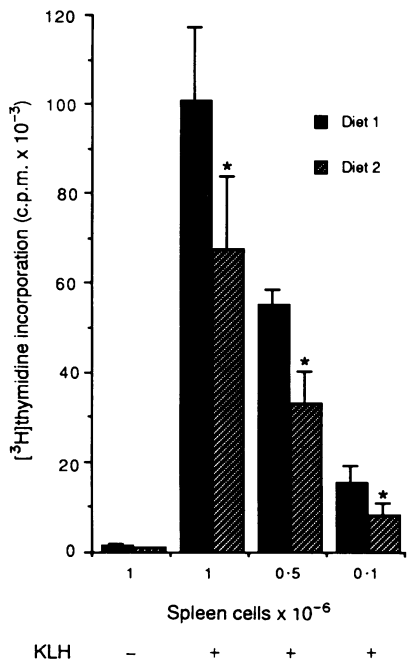

Figure 1. Effect of antigen presentation by dietary supplementation with EPA. Th1 cells (2×10^4) were incubated with 1×10^5 to 1×10^6 irradiated spleen cells in the presence or absence of KLH for 3 days. The results are the means ± SEM of four separate experiments. $*P < 0.05$ compared with control diet.

Effect of antigen presentation by dietary supplementation with EPA

The proliferative responses of the helper T-cell clones were determined with cultures containing irradiated spleen cells and KLH for 3 days. As shown in Fig. 1, the proliferative response of Th1 clone was significantly inhibited in the experimental group compared to the control group at all three amounts of APC. The proliferative response of Th2 clone was also inhibited in the

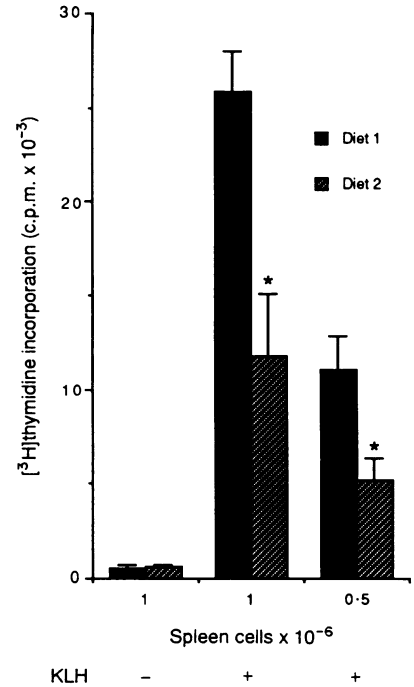

Figure 2. Effect of antigen presentation by dietary supplementation with EPA. Th2 cells (2×10^4) were incubated with 5×10^5 or 1×10^6 irradiated spleen cells in the presence or absence of KLH for 3 days. The results are the means ± SEM of four separate experiments. $*P < 0.05$ compared with control diet.

experimental group compared to the control group at two amounts of APC (Fig. 2). These inhibitions of Th1 and Th2 clones were also apparent over a wide range of spleen cells.

Effect of EPA-TG emulsion or soybean oil emulsion on antigen presentation

When 3×10^{-5} to 3×10^{-7} M (EPA equivalent) EPA-TG emulsion was added to cultures containing Th1 or Th2 clone, antigen and irradiated spleen cells as APC, a significant decrease in the proliferation of both Th clones was seen (Fig. 3). On the other hand, soybean oil emulsion lacking EPA did not inhibit proliferative responses of both clones. The viability of spleen cells was not affected by these emulsions and was comparable with that of the control culture.

Effect of pretreatment of spleen cells with EPA-TG emulsion or soybean oil emulsion

The effect of pretreatment of spleen cells with EPA-TG emulsion on proliferative responses of the Th clones was examined. Spleen cells were preincubated with 3×10^{-3} to 3×10^{-5} M or 3×10^{-3} to 3×10^{-6} M (EPA equivalent) EPA-TG emulsion for 3 hr, washed three times, and started T-cell proliferation assay. Figure 4 shows the dose–response effect of pretreatment of spleen cells with EPA-TG emulsion on proliferative responses of both clones, Th1 and Th2. On the other hand, pretreatment of spleen cells with soybean oil emulsion did not affect proliferative responses of both clones. At the concentrations tested, the viability of spleen cells preincubated with these

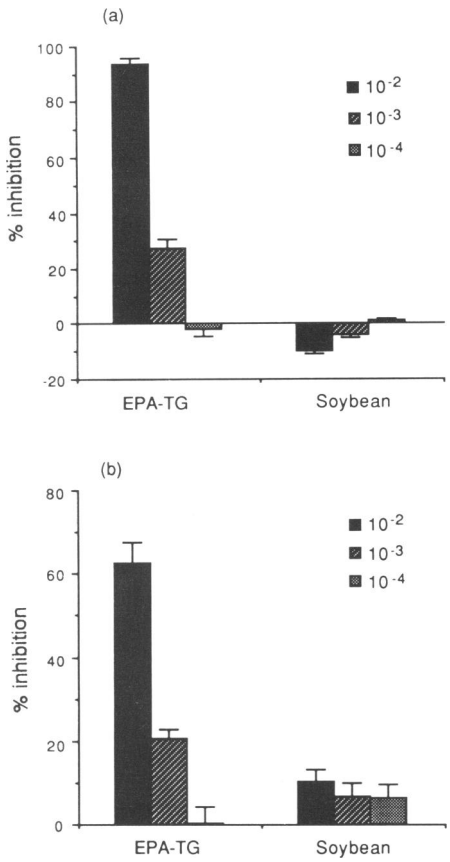

Figure 3. Effect of EPA-TG emulsion or soybean oil emulsion on antigen presentation. (a) Th1 cells (2×10^4) were incubated with 1×10^6 irradiated spleen cells and KLH for 3 days. EPA-TG or soybean oil emulsion, at the concentration indicated (% v/v), was added directly to the culture. (b) Th2 cells (2×10^4) were incubated with 1×10^6 irradiated spleen cells and KLH for 3 days. EPA-TG or soybean oil emulsion, at the concentration indicated (% v/v), was added directly to the culture. The results are expressed as mean percentage inhibition ± SEM of three separate experiments

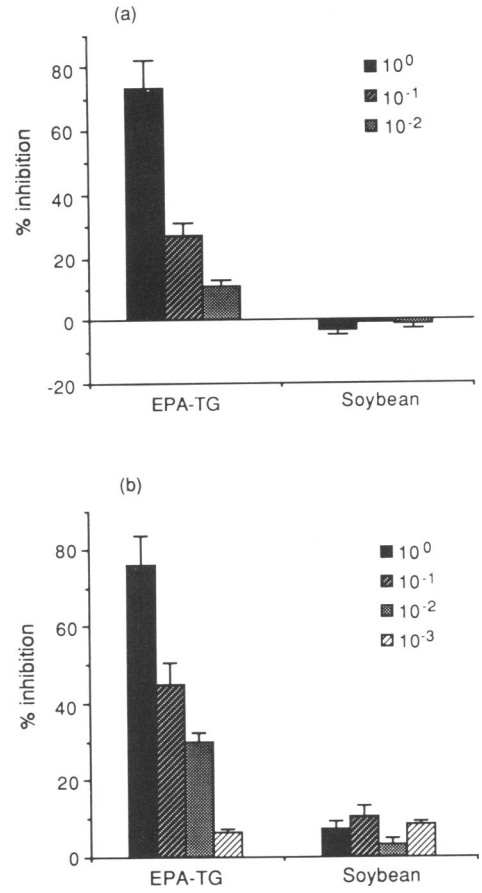

Figure 4. Effect of pretreatment of spleen cells with EPA-TG emulsion or soybean oil emulsion on proliferative responses of Th-cell clones. Spleen cells were preincubated with EPA-TG emulsion or soybean oil emulsion at different concentrations (% v/v) for 3 hr, and started T-cell proliferation assays. (a) Th1 cells (2×10^4) were incubated with 1×10^6 preincubated, irradiated spleen cells and KLH for 3 days. (b) Th2 cells (2×10^4) were incubated with 1×10^6 preincubated, irradiated spleen cells and KLH for 3 days. The results are expressed as mean percentage inhibition ± SEM of three separate experiments.

emulsions was greater than 90% as measured by the trypan blue dye exclusion test.

DISCUSSION

Modulation of many types of cellular functions by alterations of plasma membrane lipid composition has been documented in a variety of cell types. Dietary polyunsaturated fatty acids and eicosanoids have been shown to have highly perturbative effects on a variety of lymphoid cell functions.[16,17] A number of studies have shown EPA to act on monocytes–macrophages and mononuclear cells. Indeed, the generation of leukotrienes, production of IL-1 and tumour necrosis factor were shown to be reduced by dietary enrichment with EPA.[18,19]

In this report we confirmed that dietary supplementation with EPA potently inhibited APC function of murine spleen cells. In the experimental group, the AA content of spleen cells was decreased and that of EPA and docosapentaenoic acid was increased markedly compared to those of the control diet; the content of docosahexaenoic acid was not altered. The possibility was considered that inhibition by dietary supplementation with EPA was due to a direct effect on contaminated T cells rather than an impairment of APC function when the splenic accessory cells were used. We also used macrophage or lymphoid dendritic cells (DC) depleted of lymphocytes by adherence. Antigen presentation by irradiated spleen cells, macrophage, and lymphoid DC was inhibited (data not shown). This suggested that dietary supplementation with EPA did not act on contaminated T cells but on APC.

We have previously demonstrated that the addition of an emulsion of EPA-TG emulsified with EPA-PC to a cytotoxicity assay system resulted in a marked depression of natural killer (NK) cell activity, whereas the addition of soybean oil emulsion lacking EPA resulted in no inhibition.[9] Preincubation of effector cells with EPA-TG emulsion resulted in a significant inhibition of their NK cell activity.[20] In this study, we demonstrated that EPA-TG emulsion inhibited APC function in a dose-dependent manner. The decrease in APC function was not due to direct toxicity of accessory cells, because splenocytes treated with EPA-TG emulsion showed viability comparable to that of untreated control cultures as measured by the trypan blue dye

exclusion test. Preincubation studies indicated that the inhibitory effect of EPA-TG emulsion on APC function was directed against accessory cells rather than against the Th clones. Preincubation of Th clones with EPA-TG emulsion did not cause significant inhibition of proliferation of Th clones when washed Th clones were later incubated with accessory cells (data not shown). On the other hand, preincubation of splenocytes with EPA-TG emulsion resulted in suppression.

The activation of helper T cells requires co-recognition of antigen and class II major histocompatibility complex (MHC) gene products (Ia molecules) on the surface of an APC.[21] Soluble antigens must first be taken up by the accessory cell. For most antigens there is a subsequent requirement for a 'processing step'.[22,23] After this step, antigen is re-expressed on the accessory cell surface.[21,22] Extensive studies have indicated that antigen presentation can be modulated to a great extent by controlling the synthesis and expression of Ia molecules.[24] Recently, Mosquera *et al.*[25] reported that supplementation with fish oil rich in EPA reduced Ia expression in mouse peritoneal macrophages. Reduction of Ia expression in mice fed experimental diet could be associated with a reduction of APC function.

In the induction of immune responses two signals are required to initiate the proliferative response of specific T-helper cells. One is antigen-associated MHC class II molecules at the surface of APC as described above. The other is a co-stimulatory factor such as IL-1.[26] The pattern of proliferation is strikingly different for Th1 and Th2 cells.[27] Th2 clones respond to IL-4 and IL-2. These lines proliferate when cultured with IL-2 alone, and this is generally enhanced by IL-1. In contrast, Th1 clones respond to IL-2 but fail to respond to IL-1. Indeed, the addition of IL-1 to the Th1 (23-1-8) cultures did not alter the inhibitory effect of EPA (data not shown). Although EPA has been noted to reduce the synthesis of IL-1,[19] inhibition of Th1 clone caused by dietary enrichment with EPA or *in vitro* use of EPA-TG emulsion indicates that IL-1 modulation cannot account for the potent inhibition observed. The mechanism underlying the suppression of APC function after dietary supplementation with EPA is, at present, a matter of speculation. Further *in vivo* and *in vitro* investigation into the immunological consequence is required.

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