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INHIBITION OF NATURAL KILLER CELL ACTIVITY BY EICOSAPENTAENOIC ACID IN VIVO AND IN VITRO

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To examine the effects of in vivo eicosapentaenoic acid (EPA) on natural killer (NK) cell activity, C3H/He mice each received a single intraperitoneal bolus of an emulsion of trieicosapentaenoyl-glycerol (EPA-TG). Spleen cells were tested for NK activity using ^{51}Chromium-release assays against YAC-1 target cells. Forty eight hours after injection, NK activity was inhibited in a dose-dependent manner. EPA-TG emulsion also inhibited the NK activity of NK-enriched effector cells. Decreased cytotoxicity was first noted 24 hr after injection; it resumed the baseline by 7 days. The addition of EPA-TG emulsion to a cytotoxicity assay system resulted in moderate depression of NK activity. These results demonstrate that EPA has significant immunomodulatory effects on NK activity.

Eicosapentaenoic acid (EPA), termed ω3 fatty acid to indicate the position of the double bond furthest from the carboxylic acid, is prominent in fish-oil-enriched diets. Recently, many investigators have examined the effect of fish oils enriched in EPA in the alleviation of glomerular nephritis, rheumatoid

Abbreviations used in this paper: EPA, eicosapentaenoic acid; EPA-TG, trieicosapentaenoyl-glycerol; EPA-PC, EPA phosphatidylcholine; PBS, phosphate bufferd saline; NK, natural killer; LGL, large granular lymphocytes; AA, arachidonic acid; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid.

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arthritis, and lupus erythematosus and in modulating the immune function and immune disease (1-5).

Natural killer (NK) cells have attracted attention because of their possible role in providing protection against tumor growth, immune regulation, and control of hematopoiesis (6-9). We recently reported that EPA is a potent inhibitor of NK activity of human lymphocytes in vitro. This inhibition of cytotoxicity was not due to direct toxicity to effector cells or decreased target cell binding (10). We now report that an emulsion of trieicosapentaenoyl-glycerol (EPA-TG) suppresses the NK cell activity of mouse spleen cells in vivo and in vitro.

MATERIALS AND METHODS

Mice: Seven wk-old female C3H/He mice were purchased from a pothogen-free barrier colony. They were 9 to 10 wk old at the time of use.

Groups of mice were received i.p. injection of EPA-TG emulsion, soybean oil emulsion or phosphate bufferd saline (PBS). At varying intervals, the mice were sacrificed and the spleens were removed and used in various experiments.

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 hypoosmotic lysis in ammonium chloride. Splenocytes were washed 3 times and resuspended in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal bovine serum (M.A. Bioproducts, Walkersville, ML), 2 mM L-glutamine, 60 $\mu g/ml$ gentamicin (complete medium).

Large granular lymphocytes (LGL) were isolated on Percol (Pharmacia, Sweden) gradients by the technique of Timonen, Ortaldo and Herberman (11).

To assess the in vitro EPA effects, EPA-TG emulsion was added directly to mixtures of target and effector cells in 4-hr ^{51}Cr-release assays.

Cytotoxicity assays: NK activity was assessed by the standard ^{51}Cr-release assay. Briefly, 1×10^4 ^{51}Cr-labeled YAC-1 tumor cells were incubated with effector cells for 4 hr at 37°C in U-bottomed microtitration plates at various effector to target cell ratios. The percentage of specific lysis was calculated as follows;

$$\% \text{ lysis} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximum cpm} - \text{Spontaneous cpm}} \times 100$$

All experiments were performed in triplicate.

TABLE 1

Fatty acid compositions of lipids used in the experiments

Fatty acids	Lipids (mol %)				
	EPA-TG	2-EPA-PC		Soybean Oil	Egg Yolk
		Pos. 1	Pos. 2		
16:0		26		8	34
18:0		7		3	14
18:1		20		22	32
18:2 (ω6)		46		59	16
18:3 (ω3)		2		8	
18:4 (ω3)	2		2		
20:4 (ω6)	5		5		3
20:4 (ω3)	2		2		
20:5 (ω3)*	89		89		

* EPA

One hundred ml of EPA-TG emulsion contained 10 g of EPA-TG and 1.2 g of EPA-PC.

Preparation of emulsion: 1,2,3-trieicosapentaenoyl-glycerol (EPA-TG) was synthesized by chemical condensation of glycerol and free EPA obtained by hydrolysis of 90 % pure EPA ethyl ester (12). 2-eicosapentaenoyl phosphatidylcholin (EPA-PC) was synthesized by the chemical condensation of free EPA and lysophosphatidylcholine enzymatically prepared from soybean oil phosphatidylcholine with phospholipase A_2. EPA-TG was emulsified with EPA-PC according to the method of Geyer et al. (13). One hundred ml of the EPA-TG emulsion contained 10 g of EPA-TG, 1.2 g of EPA-PC and 2.5 g of glycerol. The lipid contained α-tocopherol (0.2 %, w/w) as an antioxidant. This concentration of α-tocopherol had no effect on NK activity (data not shown).

A commercial soybean oil emulsion was obtained from Daigo Nutritive Chemicals, Ltd. (Osaka, Japan). Egg yolk-PC was used for emulsification of the soybean oil. The fatty acid compositions of the above lipids are shown in Table 1.

RESULTS

Inhibition of NK activity of spleen cells by i.p. injection of EPA-TG emulsion: The EPA-TG emulsion inhibited NK activity of spleen cells when 25 ml/kg were injected i.p. into adult C3H mice 48 hr prior to the assay, whereas the soybean oil emulsion

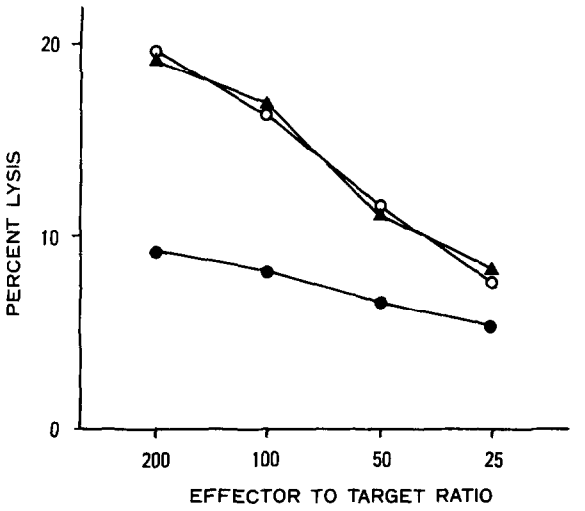


Fig. 1. Effects of the EPA-TG emulsion or soybean oil emulsion on the NK activity of spleen cells. The mice were injected i.p. with 25 ml/kg of PBS (○), 25 ml/kg of EPA-TG emulsion (●) or 25 ml/kg of soybean oil emulsion (▲) and tested for NK activity 48 hr later. Each value is the mean percent lysis of three separate experiments.

lacking EPA failed to do so at the same dose (Fig. 1). At an E:T ratio of 200:1, specific release decreased from 19.5% to 9.2% and at a 50:1 ratio, from 11.4 % to 6.6 % (mean of 3 experiments).

As seen in the replicate experiments, NK activity in spleens of EPA-TG emulsion treated mice was markedly suppressed in a dose-dependent manner (Table 2).

Kinetics of NK activity suppression by EPA-TG emulsion: The suppressive effect on NK activity by the EPA-TG emulsion was noticed 24 hr to 96 hr after a single i.p. injection (Fig. 2). It resumed the baseline preinjection values by 7 days.

NK activity is inhibited by EPA-TG emulsion in the absence of monocytes: Because monocytes are particularly high in lipoxygenase activity, we examined the effects of the EPA-TG emulsion on the cytotoxicity of Percoll-enriched NK effector cells (LGL) depleted of monocytes. As shown in Figure 3, EPA-TG emulsion

TABLE 2

Dose response effects of the EPA-TG emulsion on the NK activity of spleen cells

EPA (ml/kg)	Percent lysis		
	200:1*	100:1	50:1
0	20.4	14.8	8.2
12.5	13.1	8.1	6.2
25	6.4	3.0	3.2
50	2.3	1.8	1.1

* Effector to target ratio

Mice were administered i.p. 0, 12.5, 25 or 50 ml/kg of EPA-TG emulsion and examined for NK activity 48 hr later.

inhibited the NK activity of enriched cells when 25 ml/kg were injected i.p. into mice 48 hr prior to the assay.

NK activity is inhibited by EPA-TG emulsion in vitro: The EPA-TG emulsion inhibited NK activity of spleen cells in a dose-dependent manner when added directly to the standard 4-hr ^{51}Cr-

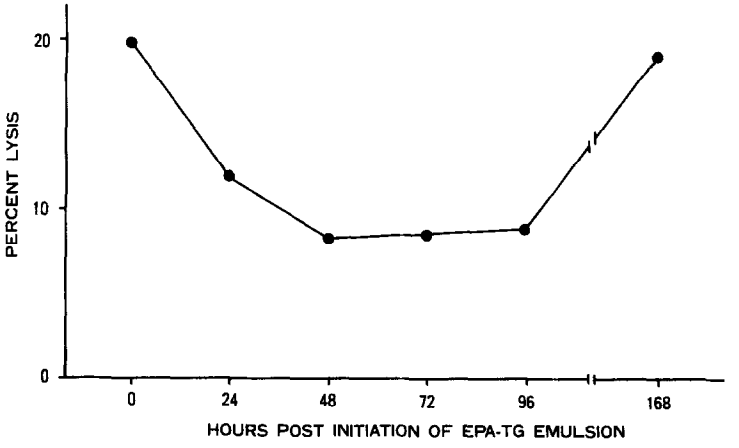
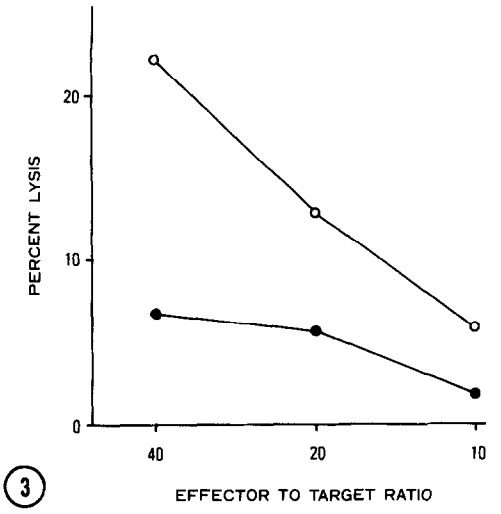


Fig. 2. Kinetics of NK activity by EPA-TG emulsion. The mice were injected i.p. with 25 ml/kg of EPA-TG emulsion and spleen cells obtained prior to (0 hr), or 24, 48, 78, 96, or 168 hr after injection were used as effectors. The results are the mean percent lysis of three separate experiments.



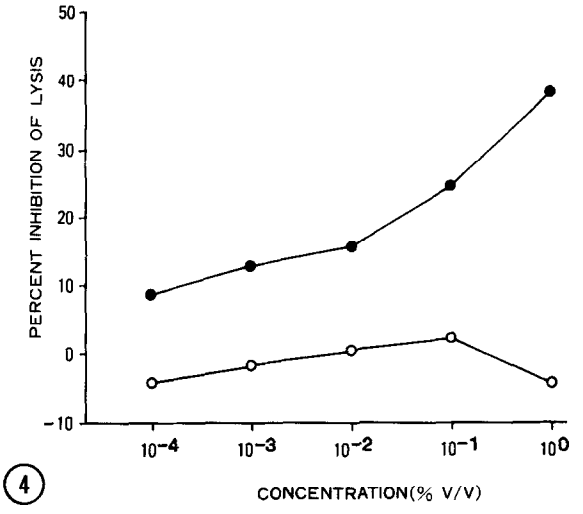


Fig. 3. Effects of the EPA-TG emulsion on the cytotoxicity of Percoll-enriched NK effector cells. The mice were injected i.p. with 25 mg/kg of PBS (○) or 25 mg/kg of EPA-TG emulsion (●) and examined for NK activity 48 hr later.

Fig. 4. Inhibition of NK activity of spleen cells by direct addition of EPA-TG emulsion (●) and soybean oil emulsion (○) to the ^{51}Cr-release assay. The results are expressed as mean percent suppression of three separate experiments.

release assay, whereas the soybean oil emulsion lacking EPA did not (Fig. 4). The viability of spleen cells was not affected by the emulsion and was comparable with that of the control culture.

DISCUSSION

We showed previously that EPA has a marked suppressive effect on the NK activity of human PBMC in vitro (10). Inhibition of cytotoxicity was not due to direct toxicity to effector cells or decreased target cell binding.

The results of this study indicate the injection of EPA-TG emulsion into mice to inhibit NK activity of spleen cells. The decrease in cytotoxic activity was not due to the direct toxicity of the EPA-TG emulsion on the effectors since the EPA-TG emulsion-treated spleen cells showed comparable viability to untreated preparations (data not shown). A number of studies have

shown EPA to act on neutrophils and monocytes-macrophages (14-18). Our report further reveals that EPA exerts an inhibitory effect on Percoll-enriched NK effector cells (LGL).

The findings in this study demonstrate the addition of the EPA-TG emulsion to a cytotoxicity assay system to cause significant inhibition of NK activity. Suppression of NK activity by EPA-TG emulsion was concentration-dependent and observed at various effector to target ratios.

Although the lytic mechanism for natural killing is not fully established, indirect evidence for the role of lipoxygenase products in NK cell functions has been reported recently (19-21). The authors of such studies used inhibitors of lipoxygenation and observed reversible inhibition of NK cell activity. Further support for the role of lipoxygenase products is provided by Bray and Brahmi, who found 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and leukotriene-B_4 to enhance NK activity (22). They reported the significant restoration of NK activity by 5-HPETE in the presence of lipoxygenase inhibitors. It thus appears that lipoxygenase products play a direct role in modulation of NK activity.

Eicosapentaenoic acid, a fatty acid analog of arachidonic acid (AA) containing one additional double bond, constitutes a large proportion of the fatty acids of marine lipids. In comparison to AA, EPA is a preferred substrate for product generation by way of 5-lipoxygenase, and the physiological effects of these EPA products differ from those produced by AA metabolites (14,15,23). Although clarification of the cellular mechanisms of the effects of these EPA products on NK cell and other lymphocytes awaits further study, it seems reasonable to say that the EPA-TG emulsion modifies the contributions of lipoxygenase products to immunological reactions.

The effects of human NK activity after the injection of EPA-TG emulsion in to a healthy volunteer are now being investigated. After the injection of EPA-TG emulsion, the NK activity of human lymphocytes was depressed markedly (24).

Based on the foregoing, the in vivo use of EPA-TG emulsion appears to influence immune reactivity of the host.

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