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Effects of γ-linolenic acid and its positional isomer pinolenic acid on immune parameters of Brown-Norway rats

N. Matsuo[1], K. Osada[2], T. Kodama[1], B. O. Lim[1], A. Nakao[1], K. Yamada[1], M. Sugano[1]

[1]Laboratory of Food Science, Kyushu University School of Agriculture, Fukuoka 812-81, Japan.
[2]Faculty of Agriculture, Hirosaki University, Hirosaki, Aomori 036, Japan.

Summary Male Brown-Norway rats given purified diets containing safflower oil (SFO, linoleic acid, 18:2 n-6), evening primrose oil (EPO, γ-linolenic acid, 6,9,12- 18:3 n-6) or Korean pine seed oil (PSO, 5,9,12- 18:3) at the 10% level were immunized twice with intraperitoneal ovalbumin, on days 14 and 35 of the feeding diets, and killed one day after the second booster. The relative population of $CD4^+$ T-lymphocytes in the spleen was significantly lower in rats fed SFO than in those fed EPO or PSO, while that of $CD8^+$ subsets remained unchanged. There was a significant increase in the splenic production of IgG and IgE in the PSO group compared to the SFO group, while EPO significantly increased IgE. The periodical response patterns of the serum levels of IgG and IgE varied depending on the source of dietary fats, and the initial rise of total immunoglobulins tended to be higher in the EPO group. The release by peritoneal exudate cells of histamine was comparable among three groups irrespective of saturation by calcium ionophore A23187, while PSO significantly increased leukotriene B_4 production. These observations not only indicate specific roles of γ-linolenic acid but also diverse influences of different octadecatrienoic acids in various immune measurements.

INTRODUCTION

The type of dietary fatty acids, in particular polyunsaturated fatty acids (PUFA), crucially influences various parameters of the immune functions.[1–6] In general, PUFA of the n-3 family suppress hypersensitivity while those of the n-6 family tend to exaggerate the response when ingested excessively.[7,8] However, not all the n-6 PUFA cause similar effects, and γ-linolenic acid appears to reduce hypersensitivity.[9,10]

Korean pine seed (*Pinus orientalis*) contains the peculiar octadecatrienoic acid, 5,9,12- 18:3 called pinolenic acid (PNO), and this acid exerts characteristic effects on various lipid parameters including tissue lipid levels, PUFA metabolism and eicosanoid production.[11,12] Considering the specificity of the enzymes involved in the metabolism of PUFA and, hence, the production of eicosanoids, it seems likely that the existence of a double bond at the 5-position, instead of the 6-position as in γ-linolenic acid (GLA), potentially influences various aspects of lipid metabolism. These effects in turn modify the diverse parameters of the immune functions.

The present study deals with the effects of GLA and PNO, as compared to linoleic acid, on the production of immunoglobulins and chemical mediators in immune sensitive Brown-Norway rats while immunized with ovalbumin.

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Correspondence to: N. Matsou, Tel. 92-641-1101; Fax. 92-632-1962.

MATERIAL AND METHODS

Animals and diets

Male 4 weeks old Brown-Norway rats were purchased from Seiwa Experimental Animals, Fukuoka, and given commercial pellet (type NMF, Oriental Yeast Co., Tokyo). After acclimatization for 7 days, the animals were fed

Table 1 Fatty acid compositions of dietary fats

Fatty acid	Safflower oil	Evening primrose oil (weight %)	Pine seed oil
16:0	13.8	17.0	4.9
18:0	2.7	2.5	2.2
18:1	18.8	18.1	28.7
18:2 n-6	64.6	55.8	47.1
18:3 n-3	–	6.5	–
18:3 (PO)*	–	–	17.1

*Pinoleic acid (*cis*-5,*cis*-9,*cis*-12-octadecatrienoic acid).

one of the three AIN-76 type purified diets containing different fats throughout. The diets composition was, by weight percent, casein, 20; fat, 10; vitamin mixture, 1.0; mineral mixture, 3.5; choline bitartrate, 0.2, DL-methionine, 0.3, cellulose, 5; corn starch, 15; and sucrose to 100. The dietary fats used were edible grade safflower oil (Linol Oil Co., Nagoya), evening primrose oil (Eisai Co., Tokyo), and Korean pine seed oil (Tama Co., Nagoya) (Table 1). Rats were immunized on days 14 and 35 of the experiment diets with 10 mg of intravenous ovalbumin (Sigma Chemical Co., St Louis, MO) and blood was withdrawn from the tail vein every week for measurements of serum immunoglobulin antibodies. 1 day after the second booster, peritoneal exudate cells (PEC) were collected under light diethyl ether anesthesia as described below. The spleen and liver were then excised immediately.

Preparation of rat spleen lymphocyte and cell culture

Spleen lymphocytes were squeezed out into the RPMI1640 medium.[13] After incubating the cells at 37°C for 30 min to remove fibroblasts, 5 ml of the cell suspension was layered on 4 ml of Lymphocyte-Rat (Cedarlane, Hornby, Canada) and centrifuged at 300 × *g* for 30 min. The lymphocyte band at the interface was removed, and the cells were rinsed three times with the RPMI1640 medium. The lymphocytes were cultured in 10% FBS/RPMI 1640 medium for 6 h (IgE) or 24 h (IgG), and the Ig contents of the culture supernatants were measured by ELISA.

Measurements of immunoglobulins

Measurements of total and specific Igs were executed using sandwich ELISA methods.[13] To measure total Igs, goat anti-rat IgE, rabbit anti-rat IgG $(Fab')_2$, (all from Biosoft, Paris) were used to fix respective Igs. These antibodies were diluted 1000 times with 50 mM carbonate-bicarbonate buffer (pH 9.6), and each well of 96-well plates was treated with 100 µl of the solution for 1 h at 37°C. To measure specific Igs, ovalbumin was dissolved at the concentration of 50 mg/ml in 50 mM carbonate-bicarbonate buffer and each well was treated with 150 µl of the solution for 1 h at 37°C. After blocking with 300 µl of the blocking solution for 1 h at 37°C, wells were treated with 100 µl of the culture supernatant or rat serum (10 × 6 times diluted for IgG and 10 times diluted for IgE) for 1 h at 37°C. Bound IgE was then detected by reacting with biotin-conjugated mouse anti-rat IgE (2000 times diluted, Betyl, Montgomery, TX) followed by POD-conjugated avidin (5000 times diluted, Dakopatts) for 1 h at 4°C. Bound IgG was detected by reacting stepwise with 100 µl of POD-conjugated rabbit anti-rat IgG$(Fab')_2$ (2000 times diluted, Biosoft) for 1 h at 37°C. Wells were rinsed four times with 0.05% Tween 20 in PBS between each step. After incubating at 37°C for 15 min with 100 µl of the substrate solution, the reaction was stopped by adding 100 µl of 1.5% oxalic acid, and A_{415} was measured with a MPR-A4i ELISA reader (Tohso, Tokyo).

Isolation of splenic T-lymphocyte subsets

To the spleen lymphocytes suspended at 1 × 10^6 cells/100 ml 10% FBS/PBS was added 5 ml of either CD4-FITC or CD8-PE monoclonal antibodies (Serotec, Kidlington, Oxford), and incubated at 4°C for 30 min. The lymphocytes were rinsed three times with PBS containing 10% FBS and centrifuged at 1200 rpm for 5 min. The stained lymphocytes were fixed by 2% paraformaldehyde and were counted by flow cytometry (Epics Profile II, Coulter Electronics, Luton, Beds).[14]

Preparation of rat peritoneal exudate cells (PEC)

Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5.6 mM glucose, pH 7.2) containing 0.1% (w/v) BSA (Boehringer Mannheim GmbH) was injected into the peritoneal cavity and the abdomen was gently massaged for 2 min. The abdomen was then opened, and the fluid containing the PEC was collected with a Pasteur pipette. Cells were gently washed with Tyrode buffer and centrifuged at 200 × *g* for 10 min at 4°C. The cell pellets were resuspended in a modified ammonium chloride buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 10 mM EDTA-2Na, pH 7.4) and then incubated for 5 min at 4°C. The cell suspension was centrifuged at 200 × *g* for 5 min at 4°C and the cells were resuspended in the Tyrode buffer. Cell viability was measured by trypan blue staining and mast cells were identified by toluidine blue staining. Viability of this preparation was more than 95% and mast cells occupied 5–10% of the total cells.

Measurement of histamine

PEC (1 × 10^6 cells) were suspended in 2.25 ml of Tyrode

buffer containing 0.9 mM $CaCl_2$ in polypropylene tubes at 4°C. The cell suspension was mixed with 0.25 ml of 50 μM A23187, and incubated for 20 min at 37°C. The reaction was terminated by incubating for 15 min at 4°C. The cell suspension was then centrifuged at 300 × *g* for 10 min and the content of histamine in the supernatants was measured by fluorometric assay.[15] To the mixture of 2 ml of sample solution, 0.75 g of NaCl and 0.5 ml of 1N NaOH was added 5 ml of the mixture of *n*-butanol and chloroform (3:2, v/v) and mixed for 5 min. After centrifugation at 270 × *g* for 5 min, 4 ml of the organic solvent layer was recovered and mixed with 2 ml *n*-heptane and 1.5 ml 0.1N HCl for 5 min. After centrifugation at 270 × *g* for 5 min, 1 ml of the HCl layer was recovered, mixed with 0.15 ml of 1N NaOH and 0.1 ml of 0.2% *o*-phthalaldehyde (OPT), and stood for 5 min at room temperature. The reaction was terminated by adding 0.14 ml of 0.5N H_2SO_4, and the fluorescence intensity was measured using a spectrofluorophotometer (RF500, Simadzu Co., Kyoto) with the excitation at 360 nm and the emission at 450 nm.

Measurement of leukotriene B_4 (LTB_4)

PEC (2×10^6 cells) were suspended in 45 μl of Tyrode buffer containing 0.9 mM $CaCl_2$ in polypropylene tubes at 4°C. The cell suspensions were mixed with 5 μl of 50 μM A23187 or 10 μg/ml compound 48/80, and incubated for 20 min at 37°C. The reaction was terminated by adding 50 μl of the mixture of acetonitrile:methanol (30:25, v/v) and kept at −30°C for 15 min. To measure LTB_4, the internal standard, 50 ng of PGB_2 (Sigma Chemical Co., St Louis, MO) was added to the cell suspensions and centrifuged at 300 × *g* for 10 min. The supernatant was filtrated through a 0.22 μm filter (Millipore, Tokyo), and LTB_4 was measured by reversed-phase HPLC (SCL-10A, Simadzu Co., Kyoto) as described previously.[16] Briefly, 20 μl of the sample was injected on a ODS-A column (150 × 6.0 mm, 5 μm particle size). A mixture of acetonitrile:methanol:H_2O (30:25:45, v/v/v), 5 mM CH_3COONH_4 and 1 mM EDTA-2Na (pH 5.6) was used as a mobile phase with a flow rate of 1.1 ml/min. LTB_4 and PGB_2 were detected by absorbance at 280 nm (SPD-10A, Simadzu Co., Kyoto). Quantification was made by comparing the LTB_4 peak areas with that of the known amount of the standard and correction for recovery.

Analyses of liver fatty acid compositions

Liver lipids were extracted by a mixture of chloroform–methanol[17] and phospholipids were separated into phosphatidylcholine and phosphatidylethanolamine by thin-layer chromatography.[18] The fatty acid compositions of these phospholipids were analyzed by gas–liquid chromatography.[19]

Statistical analysis

Results were expressed as mean ± SE. To establish the exact nature of the differences between the groups, one-way analysis of variance was followed by Duncan's new multiple range test.[20]

RESULTS

Food intake, growth and liver weight

On average, a rat weighing 82 g was fed 8.1 g per day and gained 140 g body weight over 7 weeks. There were no significant differences in these indices among the three groups. The average relative liver weight was the same in all groups, 3.8 g per 100 g body weight.

Splenic T-lymphocyte subsets

As shown in Table 2, there was a significant reduction of the relative population of $CD4^+$ T cell in rats fed SFO compared to those fed EPO and PSO. However, the proportion of $CD8^+$ remained unchanged. Consequently, the ratio of $CD4^+/CD8^+$ in the EPO and PSO groups tended to be higher than in the SFO group.

Serum immunoglobulin levels

The time courses of the serum immunoglobulin levels are depicted in Figure 1. In the EPO and SAF groups total IgG levels increased 1 week after immunization, and decreased thereafter. The peak level for IgG was highest in the EPO group followed by SFO, while in rats fed PSO it did not increase. The contours of specific IgG were, however, the same among the three groups and it increased after the second booster. The contours of total IgE levels resembled those of IgG, but the peak level of the PSO group was between the EPO and SFO groups after 1 week, and decreased to a low level 2 weeks after immunization. The specific IgE levels of rats fed EPO reached a peak level 1 week after the immunization and kept at the high level until the second booster. The SFO and PSO groups depicted similar response patterns in which the specific IgE levels remained unchanged until 1 week after the ovalbumin administration, particularly

Table 2 Effect of dietary fats on splenic T-lymphocyte subsets

Dietary fat	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
Safflower oil	36.1 ± 0.7[a]	7.73 ± 0.63	4.72 ±0.22
Evening primrose oil	43.1 ± 0.7[b]	7.78 ± 0.48	5.66 ± 0.46
Pine seed oil	39.2 ± 2.7[b]	6.88 ± 0.24	5.74 ± 0.49

Means ± SE for 4 rats. [ab]Values not sharing a common letter are significantly different at $P < 0.05$

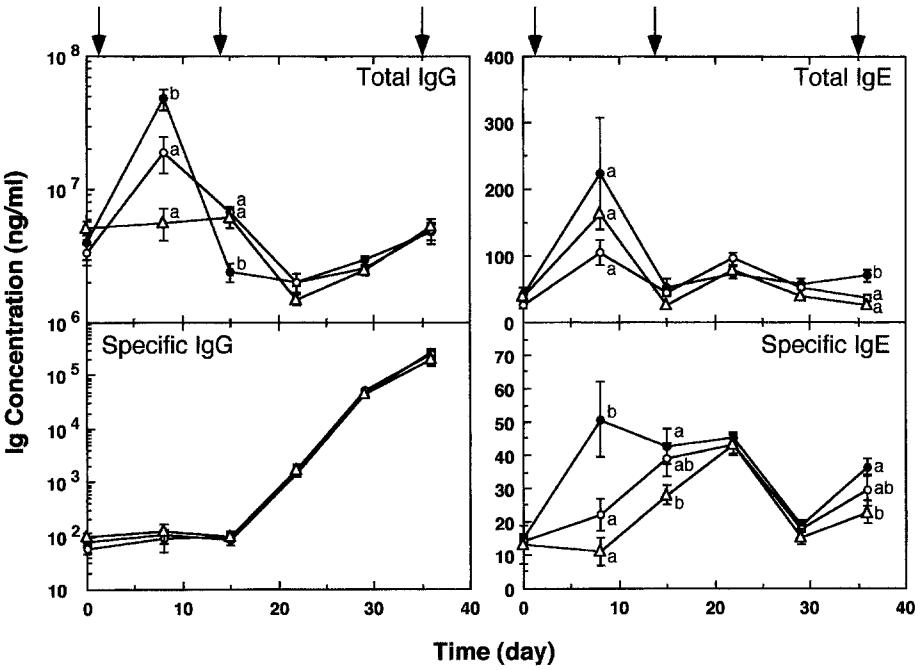

Fig. 1 The effect of various dietary fats on serum immunoglobulin levels in Brown-Norway rats. Measurements of total and specific Igs in rat serum were executed using sandwich ELISA methods as described in Materials and Methods. ○, safflower oil; ●, evening primrose oil; Δ, pine seed oil. Data are presented as means ± SE for 4 rats. [ab]Values without a common superscript letter are significantly different at $P < 0.05$

in the PSO group, and reached the peak values after 3 weeks. The levels then decreased at 4 weeks.

Ig production by spleen lymphocytes

Spleen lymphocytes were cultured in 10% FBS/RPMI1640 medium for 6 h (IgE) or 24 h (IgG), and Ig contents of culture supernatants were measured by ELISA As shown in Figure 2, the content of total IgG in the PSO group was significantly higher than in the other two groups. However, the specific IgG content in the PSO group was similar to the SFO group, and was significantly higher than in the EPO group. On the other hand, the total and specific IgE contents were higher in the EPO and PSO groups than in the SFO group although there was no difference in the two former groups.

Fatty acid compositions of liver phospholipids

As shown in Table 3, the percentage of linoleic acid in liver phosphatidylcholine was significantly lower and that of arachidonic acid was significantly higher in rats fed EPO than in those fed SFO. Consequently, the desaturation index for linoleic acid, the ratio of (20:3 n-6 + 20:4 n-6)/18:2 n-6, was significantly higher in the EPO group. Although the percentage of arachidonic acid was comparable between SFO and PSO, the desaturation index was also significantly higher in the former due to a lower percentage of linoleic acid. A similar response pattern was observed in the desaturation index of phosphatidylethanolamine.

Production of chemical mediators by peritoneal exudate cells

As shown in Figure 3, the peritoneal exudate cells stimulated by calcium ionophore A23187 released a similar amount of histamine in three groups. In contrast, LTB_4 production tended to reduce in the EPO groups, and significantly increased in the PSO group compared to the SFO group.

DISCUSSION

Recent studies have stressed a role of dietary PUFA in diverse immunological functions.[1–6] In general, PUFAs of the n-3 family improve hyperreactivity compared to n-6 PUFA.[7,8] Eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) in fish oil inhibit the 5-lipoxygenase pathway and LTB_4 generation in neutrophils and monocytes in vitro,[21] resulting in alleviation of rheumatoid arthritis.[22] The effect of linoleic acid, the

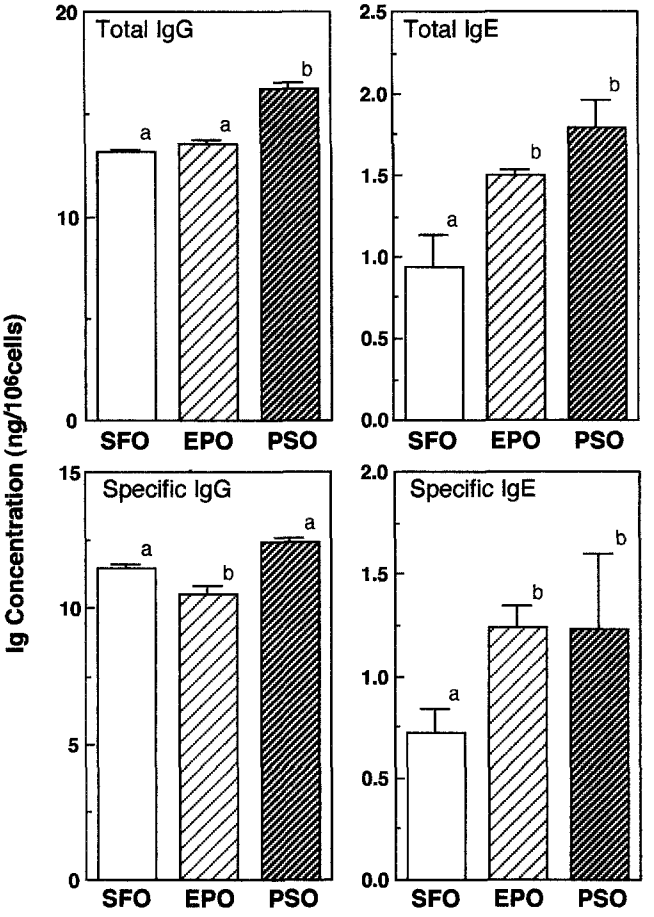

Fig. 2 The effect of various dietary fats on Ig production from rat spleen lymphocytes. Rat spleen lymphocytes were cultured in 10% FBS/RPMI 1640 medium for 6 h (IgE) or 24 h (IgG). The Ig contents of the culture supernatants were measured by ELISA methods as described in Materials and Methods. SFA, safflower oil; EPO, evening primrose oil; PSO, pineseed oil. Data are presented as means ± SE for 3 dishes. [abc]Values without a common superscript letter are significantly different at $P < 0.05$

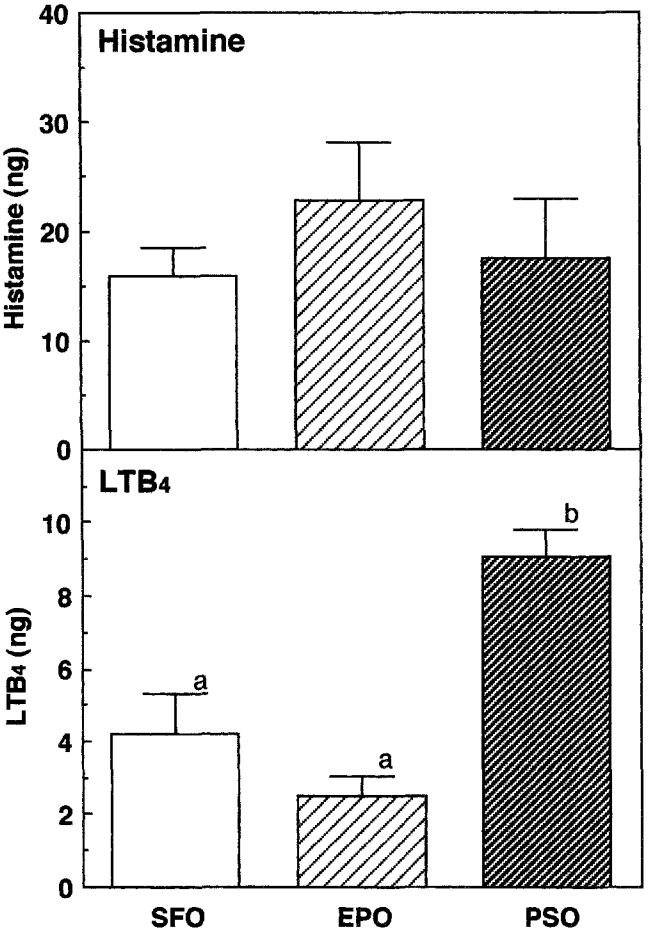

Fig. 3 Effect of various dietary fats on histamine and LTB_4 release from rat peritoneal exudate cells. Cells were incubated at 37°C for 20 min. Histamine content of supernatant was measured by a fluorometric assay and LTB_4 content of supernatant was measured by reversed-phase HPLC assay, as described in Materials and Methods. SFA, safflower oil; EPO, evening primrose oil; PSO, pine seed oil. Data are presented as means ± SE for 4 rats. [ab]Values without a common superscript letter are significantly different at $P < 0.05$.

Table 3 Fatty acids composition of liver phospholipids

Dietary fat	Fatty acids (weight %)										Ratio (20:3 + 20:4) /18:2
	16:0	16:1	18:0	18:1	18:2 n-6	18:3 (PO)*	20:3 n-6	20:4 n-6	22:5 n-6	22:6 n-3	
Phosphatidylcholine											
SFO	19.5 ± 0.2[a]	0.7 ± 0.0[a]	23.0 ± 0.4	4.2 ± 0.2[a]	17.5 ± 0.5[a]	–	0.6 ± 0.1[a]	29.8 ± 0.4[a]	1.1 ± 0.1	1.9 ± 0.2	1.7 ± 0.1[a]
EPO	19.6 ± 0.4[a]	0.6 ± 0.0[a]	23.3 ± 0.4	4.0 ± 0.1[a]	14.8 ± 0.4[b]	–	0.9 ± 0.1[a]	32.0 ± 0.3[b]	1.0 ± 0.1	1.6 ± 0.1	2.2 ± 0.1[b]
PSO	17.5 ± 0.4[b]	1.2 ± 0.0[b]	22.9 ± 0.4	6.0 ± 0.2[b]	14.2 ± 0.5[b]	3.7 ± 0.3	1.7 ± 0.1[b]	28.5 ± 0.9[a]	1.2 ± 0.1	1.9 ± 0.2	2.2 ± 0.1[b]
Phosphatidylethanolamine											
SFO	14.7 ± 0.2[a]	0.2 ± 0.0[a]	26.6 ± 0.2	3.5 ± 0.1[a]	9.2 ± 0.2	–	0.4 ± 0.0[a]	32.5 ± 0.2	3.4 ± 0.3	6.4 ± 0.2[a]	3.6 ± 0.1[a]
EPO	14.8 ± 0.5[a]	0.2 ± 0.0[a]	27.3 ± 0.8	3.6 ± 0.1[a]	7.7 ± 0.4[b]	–	0.6 ± 0.0[b]	32.0 ± 0.72	3.2 ± 0.3	5.5 ± 0.4[b]	4.1 ± 0.2[b]
PSO	12.9 ± 0.4[b]	0.5 ± 0.0[b]	26.7 ± 0.2	4.9 ± 0.1[b]	7.6 ± 0.2[b]	1.6 ± 0.1	1.1 ± 0.1[c]	32.4 ± 0.4	3.6 ± 0.3	6.0 ± 0.2[a]	4.5 ± 0.1[b]

Data are presented as means ± SE for 8 rats. SFO, safflower oil; EPO, evening primrose oil; PSO, pine seed oil. [abc]Values without a common superscript letter are significantly different at $P < 0.05$. *Pinoleic acid (5, 9, 12-octadecatrienoic acid).

most abundantly occurring n-6 PUFA in the diet, rather tends to stimulate these responses.[7,23]

On the other hand, evening primrose oil (EPO), a rare source of GLA (18:3 n-6), improves atopic eczema after oral administration,[9,10] although it simultaneously contains a relatively large amount of linoleic acid. EPO increases tissue levels of prostaglandin E_1 (PGE_1) and suppresses chronical inflammation.[24,25] In rats, PGE_1 suppresses the inflammation of adjuvant arthritis,[26] and immune complex vasculitis.[27] Different GLA pools appear to occur in the body between endogenously formed GLA and exogenously absorbed GLA.[28] Thus, the supplementation of EPO increases the level of dihomo-γ-linolenic acid (DGLA, 20:3 n-6), which is the precursor of the putative anti-inflammatory substance, PGE_1, in neutrophil and epidermal phospholipids,[29] and consequently reduces LTB_4 release from human polymorphonuclear neutrophil.[30] These observations suggest a possible role of GLA in the immune regulation.

The present study indicated that LTB_4 production in the exudate cells tended to be suppressed by feeding EPO, while PSO significantly elevated the production. On the other hand, histamine release from peritoneal exudate cells was not significantly different among three dietary groups. Both histamine and LTB_4 are chemical mediators from mast cells and cause an inflammatory reaction.[31] Since the source of the precursor arachidonic acid for LTB_4 is the membrane phospholipids and since the fatty acid composition of membrane phospholipids is readily modified by the type of dietary fats, it is likely that the production of LTB_4 can be modulated by dietary fat. In fact, n-3 PUFA decrease LTB_4 release from mast cells but do not inhibit histamine release in the case of n-6 PUFA.[7]

Immunoglobulin production is regulated by T lymphocytes, and T cells are classified CD4 and CD8 positive T cells having helper and suppressive functions, respectively.[32] In the present study, the relative proportion of $CD4^+$ T cells in the spleen lymphocytes significantly increased in rats fed EPO and PSO compared to SFO, but the $CD8^+$ T cell population remained unchanged. The ratio of $CD4^+/CD8^+$ T cells in the EPO and PSO groups was therefore higher than in the SFO group. On the other hand, the total IgG production by spleen lymphocytes was significantly higher in the PSO group than in the other two groups, although the specific IgG content in the PSO group was significantly higher than in the EPO group. In addition, total and specific IgE productions by spleen lymphocytes were significantly higher in the EPO and PSO groups than in the SFO group. These results suggest that the increase of the $CD4^+$ T cell population and the $CD4^+/CD8^+$ ratio affects the IgE production by spleen lymphocytes. Kemeny et al[33] reported that the decrease of the $CD8^+$ T cell population and the $CD4^+/CD8^+$ ratio enhanced IgE production.

The regulation of T lymphocytes by dietary fatty acids is related to the development of immune functions. In human, EPO supplemented in atopic eczema reduced the ratio of $CD4^+/CD8^+$ T cells.[34] This ratio in children with atopic eczema was reported to be high.[35] The present study, however, indicated that dietary GLA and its positional isomer, pinolenic acid, tended to elevate the ratio of $CD4^+/CD8^+$.

Dietary fatty acids also exert potent and pervasive effects on both specific and non-specific immune reactions. PUFA inhibited proliferation of T lymphocytes[3] or enhanced proliferation of T and B lymphocytes.[4] Dietary fatty acids modulated immune responses.[5,6] Our observations indicated that concentrations in serum and production by spleen lymphocytes of Igs were different between the GLA and PNO as dietary GLA increased serum Igs in an early phase after immunization, while PNO increased the concentration only a little or left it unchanged. In contrast, Ig production by spleen lymphocytes was increased by the PNO supplementation. These results suggest that dietary GLA and its isomer cause different immune responses including immunoglobulin and inflammatory mediator production. The mechanisms causing these different responses deserve further study.

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