Antimalarial effects of purified and α-tocopherol–fortified n-3 polyunsaturated fatty acids

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A vitamin E-deficient diet containing fish oil is known to afford a protection against malaria in mice. In the present study we examined whether the dietary supplementation with α-tocopherol–fortified n-3 polyunsaturated fatty acid (PUFA) ethyl esters (eicosapentaenoic acid (EPA) ethyl ester and docosahexaenoic acid (DHA) ethyl ester) had a protective effect against malaria. Four groups of mice were each given one of the following diets: (1). powdered standard chow (REF group); (2). 90% (wt) lipid-free diet with 8% lard and 2% safflower oil (SAF group); (3). 90% lipid-free diet with 8% lard and 2% EPA ethyl ester (EPA group); and (4). 90% lipid-free diet with 8% lard and 2% DHA ethyl ester (DHA group). Safflower oil, EPA, and DHA were fortified with 0.2% α-tocopherol before mixing dietary components. After 6 weeks on these diets, mice were inoculated with Plasmodium yoelii, *and the same diets were continued until the end of the experiment. The survival rates of the four groups 46 days after the inoculation were 1/12, 0/12, 5/12, and 4/10 in the REF, SAF, EPA, and DHA groups, respectively. There was a significant difference in the survival rates ($P < 0.025$) among the groups. It may be possible to conduct a clinical trial with malarial patients using EPA or DHA ethyl ester fortified with α-tocopherol.*

Keywords: malaria; n-3 polyunsaturated fatty acids; α-tocopherol; eicosapentaenoic acid; docosahexaenoic acid

Introduction

The final and effective means of controlling malaria would probably be immunization. However, the progress of developing vaccines is slow, and their efficacy seems rather limited.[1-3] Chloroquine has been the very reliable anti-malarial drug until drug-resistant strains of the parasite *Plasmodium falciparum* appeared in most of the endemic zone.[4] Controlling the malarial vector is hindered by its high cost and by the fear of possible world-wide insecticide pollution.[5] Insecticide-resistant strains are also appearing.[5] In view of some 110 million new clinical cases each year,[4] other effective means of controlling malaria, probably new anti-malarial drugs, should be developed while the vaccine development is still under way and not available. In addition, the development of such means should be rapidly achieved because of the appearance of insecticide-resistant mosquitoes[5] and of the greenhouse effects of increasing CO_2 in the atmosphere, which probably widen the territory of the malarial vector both to the north and south.

In 1957, Godfrey reported that a diet containing cod liver oil markedly suppressed the infection of red blood cells (RBCs) of mice by malarial parasites, but that supplementation with α-tocopherol deprived the cod liver oil diet of its anti-malarial effects completely.[6] This finding had long been forgotten until the more recent rediscovery by Levander et al.[7,8] that a vitamin E-deficient diet containing cod liver oil afforded a significant protection against *Plasmodium yoelii* in mice. The pro-oxidant stress induced by this diet might generate free radicals and thus destroy parasites within RBCs without extensive hemolysis of host RBCs.[9] However, the deprivation of vitamin E is not practical, and this regimen could hardly be applied to humans.

The symptoms of malaria, fever, anorexia, malaise, and finally cachexia causing death, can be simulated by administration of tumor necrosis factor (TNF).[10]

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Malarial infection probably induces TNF release from monocytes/macrophages and causes these symptoms because the prior administration of anti-TNF antibody to rats does reduce malarial symptoms (cerebral malaria) due to its infection.[11] Dietary supplementation with fish oil (vitamin E not deprived) has been shown to reduce TNF release from monocytes in volunteers.[12] Consequently, it is possible that the dietary supplementation with fish oil or with its probable active ingredients, n-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), reduces the cerebral pathology of malaria without vitamin E deprivation. In the present study we fed mice diets containing pure EPA- or DHA-ethyl ester, which had been fortified with α-tocopherol, for 6 weeks before the infection with parasites and thereafter. We investigated the percentage of infected RBCs and survival rates as compared with mice on mouse chow or a safflower oil-supplemented diet also fortified with α-tocopherol.

Materials and methods

Experimental design

Three-week-old female BALB/c mice were purchased from Sankyo Labo Service (Tokyo, Japan), randomly assigned to one of four experimental diet groups of 12 (except for a group for DHA [n = 10]), and housed in groups of four or five per cage. The mice were fed experimental diets for 6 weeks. The mice were then inoculated by intraperitoneal administration of 5×10^4 parasitized erythrocytes suspended in 0.2 mL citrated saline. A malarial parasite, *Plasmodium yoelii nigeriensis* (N-67 strain), was obtained through the courtesy of Dr. Ryo Arakawa, Department of Parasitology, Toyama Medical and Pharmaceutical University, Toyama, Japan. After the inoculation, mice were fed the same diets continuously until the termination of the experiment. The antimalarial effects of various lipids were assessed by observing survival of mice and parasitemia in Giemsa stained blood film made 7 days after the inoculation and every 3 days thereafter. The experiments were terminated when parasites became undetectable in any surviving mice three times in a row.

Diets

Four groups of mice were each given one of the following diets: (1). REF diet (a reference diet): powdered standard chow (CE-2, Nihon Clea, Tokyo); (2). SAF diet (a safflower oil-supplemented diet): 90% (wt) lipid-free powder diet (Funabashi Farm, Chiba, Japan) with 8% lard and 2% safflower oil; (3). EPA diet: 90% lipid-free powder diet with 8% lard and 2% EPA ethyl ester; and (4). DHA diet: 90% lipid-free powder diet with 8% lard and 2% DHA ethyl ester. The EPA and DHA ethyl esters contained more than 96% EPA and 95% DHA, respectively. These two oils were gifts from Nippon Oil and Fats, Tokyo, Japan. Safflower oil was purchased from a local supermarket. Before mixing the dietary components, DL-α-tocopherol (Wako, Tokyo, Japan) was added to the EPA ethyl ester, the DHA ethyl ester, and the safflower oil at a concentration of 0.2% to prevent peroxidation of oils and possible vitamin E deficiency in mice. The fatty acid contents of the diets used are shown in *Table 1*. The lipid-free powder diet was mixed with lard and oil every other day and used on the same day or the next. If the mixed diet was not used on the same day, it was stored at −20° C for not more than 1 day. Lipid peroxides in diet did not increase appreciably during storage (data not shown). Diet was given in clean serving cups every afternoon and old cups were removed to take precaution against peroxidation of diets and contamination with urine and feces. Mice had free access to water and their respective diets in both pre- and post-inoculation periods.

Lipid analysis

A separate experiment was performed for lipid analysis without inoculation. Another four groups of female BALB/c mice ($n = 5$/group), which had been fed the same diets exactly the same way as described above for 6 weeks from the age of 3 weeks, were analyzed for the plasma α-tocopherol concen-

Table 1 Fatty acid (g) and α-tocopherol (mg) contents in 100 g of diets used in the study

	Diets			
	REF*	SAF	EPA	DHA
	Fatty acid contents			
16 : 0	0.58	1.72	1.60	1.60
16 : 1	—	0.24	0.24	0.24
18 : 0	0.09	0.66	0.64	0.64
18 : 1 n-9	1.03	3.98	3.84	3.84
18 : 2 n-6	1.99	2.96	1.28	1.28
18 : 3 n-3	0.22	0.08	0.08	0.08
20 : 5 n-3	0.04	—	1.92[E]	—
22 : 5 n-3	—	—	—	0.08[E]
22 : 6 n-3	0.05	—	—	1.90[E]
	α-tocopherol contents			
α-tocopherol	4.7	13.0	13.0	13.0

90 g of the lipid-free powder diet used for the SAF, EPA, and DHA diets contained 21.8 g casein, 50.9 g corn starch, 9.5 g sucrose, 2.8 g cellulose powder, 0.3 g DL-methionine, 0.9 g vitamine mixture containing 9 mg α-tocopherol, and 3.8 g mineral mixture.
*According to the manufacturer's information.
E, Provided in the form of ethyl ester.

trations ($n = 5$/group) and the fatty acid composition of the total phospholipids in RBCs ($n = 4$/group). These mice were not inoculated with malarial parasite. Under ether anesthesia, a blood sample was drawn from the heart, anticoagulated with heparin, and centrifuged to separate plasma and packed RBCs. Plasma was frozen at −20° C until the measurement of α-tocopherol concentrations, which was done within 4 weeks after plasma sampling. Packed RBCs were washed twice with saline and frozen at −20° C until the fatty acid analysis, which was done within 2 weeks after sampling. The α-tocopherol concentrations of five mice of each diet group were analyzed by high-performance liquid chromatography according to the method of Bieri et al.[13] with a slight modification. After the total lipid extraction from RBCs according to Folch et al.,[14] the total phospholipids of RBCs were isolated by thin-layer chromatography on silica gel 60-plates (Merck, Darmstadt, Germany) using petroleum ether/diethyl ether/acetic acid (80:20:1) as solvent. Methanolysis of phospholipids was done at 70° C for 45 min with 6% sulfuric acid in anhydrous methanol. Methylated fatty acids were analyzed by gas chromatography using a GC-14A (Shimadzu, Kyoto, Japan) equipped with a 30-m SP-2330 column (Supelco, Bellefonte, PA USA).[15]

Statistical analysis

Data are expressed as means ± SD. Significant difference among dietary groups was detected by analysis of variance, and differences between two groups was analyzed according to Scheffé. Survival rates among different groups were analyzed by chi-square test. $P < 0.05$ was determined as significant.

Results

At inoculation there were no significant differences in body weight among the four groups (the REF group: 21.8 ± 0.5 g; the SAF group: 21.4 ± 0.2 g; the EPA group: 21.5 ± 0.3 g; and the DHA group: 21.8 ± 0.3 g).

As shown in *Figure 1*, the survival rates were 1/12, 0/12, 5/12, and 4/10 in the REF, SAF, EPA, and DHA groups, respectively. There was a significant difference in survival rates among the four groups according to chi-square test used for 4 × 2 analysis ($P < 0.025$).

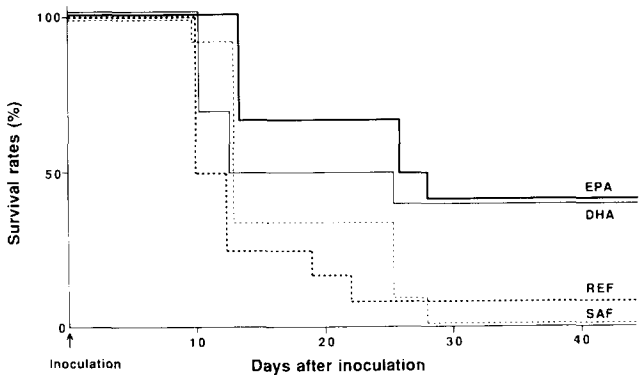

Figure 1 Survival rates of malarial mice fed different diets. Mice were fed one of the four diets for 6 weeks before inoculation and until the termination of the experiment. ——: the EPA group; ——: the DHA group; - - - - : the REF group; - - - - : the SAF group. There was a significant difference in survival rates among the groups ($P < 0.025$).

Statistical analysis of the degrees of parasitemia was performed only for the data of day 7, when all the mice were still alive. Parasitemia in the REF group on day 7 (33 ± 25%) was more severe than in any other groups (11 ± 3, 4 ± 2, and 10 ± 7% in the SAF, EPA, and DHA groups, respectively, $P < 0.05$). The degree of parasitemia at day 7 of infection was highly associated with the survival of mice thereafter. All the mice with less than 2.5% as infected RBCs on day 7 survived until the termination of the experiment, whereas those with more than 2.5% as infected RBCs had a higher mortality rate (data not shown).

The fatty acid composition of the total phospholipids of RBCs is shown in *Table 2*. In the EPA group the contents of EPA and docosapentaenoic acid (22:5n-3) were significantly increased compared with the other groups; in the DHA group the content of DHA was significantly increased compared with the other groups.

Table 2 The fatty acid composition (mol %) of the phospholipid fraction of RBCs and the plasma α-tocopherol contents (μg/mL)

	Diets			
	REF	SAF	EPA	DHA
	Fatty acid composition (n-4/group)			
16 : 0	38.2 ± 1.3[a]	32.7 ± 0.9[b]	37.3 ± 1.0[a]	33.5 ± 2.1[b]
18 : 0	13.0 ± 0.7[ab]	13.5 ± 0.3[a]	13.9 ± 0.4[a]	12.5 ± 0.4[b]
18 : 1n-9	10.1 ± 0.3[a]	12.6 ± 0.1[b]	14.9 ± 0.3[c]	14.3 ± 0.2[c]
18 : 2n-6	9.4 ± 0.4[a]	7.1 ± 0.2[b]	4.3 ± 0.2[c]	6.3 ± 0.1[d]
20 : 4n-6	9.2 ± 1.1[a]	13.6 ± 0.8[b]	4.1 ± 0.2[c]	6.6 ± 0.8[d]
20 : 5n-3 (EPA)	0.42 ± 0.06[a]	0.02 ± 0.02[b]	5.7 ± 0.6[c]	1.6 ± 0.2[d]
22 : 5n-3	0.94 ± 0.04[a]	0.59 ± 0.01[b]	2.93 ± 0.26[c]	0.85 ± 0.05[a]
22 : 6n-3 (DHA)	4.3 ± 0.7[a]	2.9 ± 0.3[b]	3.9 ± 0.2[a]	11.3 ± 2.0[c]
	α-tocopherol contents (n = 5/group)			
α-tocopherol	2.2 ± 0.3[a]	5.7 ± 0.6[b]	4.7 ± 0.3[c]	4.0 ± 0.4[c]

Mice were fed one of the four diets for 6 weeks and killed without inoculation. The fatty acid composition of RBC phospholipids and the plasma α-tocopherol contents were measured by gas chromatography and high-performance liquid chromatography, respectively. Values without common superscripts are different at $P < 0.05$.

In general, the contents of linoleic (18:2n-6) and arachidonic acids (20:4n-6) were lower in the EPA and DHA groups than the other two groups.

The plasma α-tocopherol concentrations are shown in *Table 2*. The concentrations were roughly proportional to dietary concentrations of α-tocopherol. The plasma concentrations were lower in the REF group, whose dietary concentration was 4.7 mg/100 g diet, than in the other dietary groups, in which dietary concentration was 13.0 mg/100 g diet. When the dietary concentrations of α-tocopherol were the same (the SAF, EPA, and DHA groups), plasma concentrations were inversely correlated to the unsaturation degree of supplemented oils, other than lard which was common to the three diets, namely safflower oil (approximately two double bonds per fatty acid), EPA ethyl ester (five double bonds per fatty acid), and DHA ethyl ester (six double bonds per fatty acid).

Discussion

In the present study we showed that the pre- and postinoculational supplementation with the purified n-3 PUFA ethyl esters, which had been fortified with α-tocopherol, increased the survival rates of mice inoculated with *Plasmodium yoelii*. This finding cannot be explained by the plasma α-tocopherol concentrations because those of the REF group were lower than the EPA and DHA groups, and those of the SAF group were higher than the EPA and DHA groups.

At first we considered that the present study seemed inconsistent with the findings of Levander et al.[7,8] who found that a vitamin E-deficient diet containing fish oil afforded a protection against malaria in mice, but that the protective effects of fish oil diets disappeared if the diets were fortified with α-tocopherol. Actually, there were some differences in experimental protocols between the present study and theirs. The parasites used were different (*Plasmodium yoelii nigeriensis* versus *Plasmodium yoelii yoelii*). Supplemental n-3 fatty acids were different; they used fish oils and ours were purified n-3 PUFA ethyl esters. However, difference in molecular forms of n-3 PUFA ethyl esters does not appear to be an important factor because Levander et al. recently showed that the ethyl ester concentrate prepared from menhaden oil was as effective as menhaden oil itself when diets containing either oil were deprived of vitamin E.[16] Interestingly, a closer examination of the work of Levander et al.[7] suggests that there was a difference in antimalarial effects between a corn oil diet with vitamin E and a cod liver oil diet with vitamin E, with the 60-day survival rates of mice fed these two diets being almost 0% and 25%, respectively. They did not stress this finding, probably because this difference was not significant and because the cod liver oil diet without vitamin E was much more effective than the cod liver oil diet with vitamin E.

More recently, Levander et al. observed partial protective effects of fish oil ethyl esters against malaria, which varied in a stepwise manner depending on the vitamin E content of the diet.* Moreover, they also reported a partial protective effect of menhaden oil when mixed directly into a ground chow diet with normal tocopherol nutriture.† Taking these facts into account, we now consider that the present study is in agreement with theirs.

From our experimental design it is impossible to decide whether vitamin E-deficient diets containing n-3 PUFAs are superior to α-tocopherol–fortified diets containing n-3 PUFAs in terms of antimalarial power. However, we do not think it is practical to put patients in the vitamin E-deficient state. Because vitamin E is contained in many foods, the only way to induce such a condition is to institute the total parenteral nutrition without lipid emulsion, which has more or less vitamin E. In addition, it would take a long time to deprive a body of vitamin E because of a large vitamin E reserve in fat tissue. Consequently, our findings indicate a very practical way for the application of n-3 PUFAs as an antimalarial nutritional support.

It is not clear why the n-3 PUFA supplementation improved the survival rate of mice infected with malarial parasites. N-3 PUFA-rich membranes of RBC (see *Table 2*) may have a pro-oxidant and thus antimalarial character,[9] even in the presence of normal plasma concentrations of α-tocopherol. Changes in immune function due to n-3 PUFA supplementation[17] may have something to do with the antimalarial effects; in fact we found that the antigen presenting cell activity was reduced by dietary EPA.[18] As described in the introduction, TNF appears to be an important factor of malarial symptoms,[10] and TNF production by monocytes is reduced by the dietary supplementation with fish oil in volunteers.[12] This may be another mechanism of action. Although Lokesh et al. recently found that TNF production by mouse macrophages was enhanced by dietary n-3 PUFAs,[19] the significance of TNF may be reduced by n-3 PUFAs because a number of effects of TNF are prevented by cyclooxygenase inhibition.[20] It is well known that EPA and DHA inhibit cyclooxygenase.[21,22]

In conclusion, the present study indicated that the administration of α-tocopherol–fortified n-3 PUFAs improved survival rates of mice inoculated with malarial parasites. It may be possible to conduct a clinical trial with malarial patients using the aforementioned oils.

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*Levander OA, Ager AL, Morris VC, May RG. (1991). Antimalarial activity of a marine omega-3 free fatty acid concentrate (FFAC) in mice fed graded dietary levels of vitamine E (abstract). In *Health effects of omega 3 polyunsaturated fatty acids in seafoods*, (A.P. Simopoulos, R.R. Kifer, and R.E. Martin, eds.), p. 535–536, Karger Publ. Co., Basel, Switzerland

†Levander OA, Ager AL, Morris VC, Fontela R, and May RG. (1992). Menhaden oil (MO) protects against malaria in mice fed ground chow (abstract). *FASEB J* **6**, A1212

References

1 Ballou, W.R., Hoffman, S.L., Sherwood, J.A., Hollingdale, M.R., Neva, F.A., Hockmeyer, W.T., Gordon, D.M., Schneider, I., Wirtz, R.A., Young, J.F., Wasserman, G.F., Reeve, P., Diggs, C.L., and Chulay, J.D. (1987). Safety and efficiency of a recombinant DNA *Plasmodium falciparum sporozoite* vaccine. *Lancet* **1,** 1277–1280

2 Herrington, D.A., Clyde, D.F., Losonsky, G., Cortesia, M., Murphy, J.R., Davis, J., Baqar, S., Felix, A.M., Heimer, E.P., Gillessen, D., Nardin, E., Nussenzweig, R.S., Nussenzweig, V., Hollingdale, M.R., and Levine, M.M. (1987). Safety and immunogenicity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum sporozoites*. *Nature* **328,** 257–259

3 Playfair, J.H.L., Taverne, J., Bate, C.A.W., and de Souza, J.B. (1990). The malaria vaccine : anti-parasite or anti-disease? *Immunol Today* **11,** 25–27

4 World Health Organization (1990). World malaria situation 1988 Part I. *Wkly. Epidem. Rec.* **65,** 189–196

5 Gratz, N.G. (1989). The present status and future prospects of malaria control. *Japan J. Trop. Med. Hyg.* **17,** 131–147

6 Godfrey, D.G. (1957). Antiparasitic action of dietary cod-liver oil upon *Plasmodium berghei* and its reversal by vitamin E. *Exp. Parasitol.* **6,** 555–565

7 Levander, O.A., Ager, A.L. Jr., Morris, V.C., and May, R.G. (1989). Qinghaosu dietary vitamin E, selenium, and cod-liver oil: effect on the suseptibility of mice to the material parasite *Plasmodium yoelii*. *Am. J. Clin. Nutr.* **50,** 346–352

8 Levander, O.A., Ager, A.L. Jr., Morris, V.C., and May, R.G. (1989). Menhaden-fish oil in a vitamin E-deficient diet: protection against chlorquine-resistant malaria in mice. *Am. J. Clin. Nutr.* **50,** 1237–1239

9 Clark, I.A. and Hunt, N.H. (1983). Evidence for reactive oxygen intermediates causing hemolysis and parasite death in malaria. *Infect. Immun.* **39,** 1–6

10 Phillips, R.E. and Solmon, T. (1990). Cerebral malaria in children. *Lancet* **336,** 1355–1360

11 Grau, G.E., Fajardo, L.F., Piguet, P-F., Allet, B., Lambert, P-H., and Vassalli, P. (1987). Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. *Science* **237,** 1210–1212

12 Endres, S., Ghorbani, R., Kelley, V., Georgilis, K., Lonnemann, G., van der Meer, J.W.M., Cannon, J.G., Rogers, T.S., Klempner, M.S., Weber, P.C., Schaefer, E.J., Wolff, S.M., and Dinarello, C.A. (1989). The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *N. Engl. J. Med.* **320,** 265–271

13 Bieri, J.G., Tolliver, T.J., and Catignani, G.L. (1979). Simultaneous determination of α-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. *Am. J. Clin. Nutr.* **32,** 2143–2149

14 Folch, J., Lees, M., and Sloanestanley, G.H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226,** 497–509

15 Yamazaki, K., Hamazaki, T., Yano, S., Funada, T., and Ibuki, F. (1991). Changes in fatty acid composition in rat blood and organs after infusion of docosahexaenoic acid ethyl ester. *Am. J. Clin. Nutr.* **53,** 620–627

16 Levander, O.A., Ager, A.L. Jr., Morris, V.C., and May, R.G. (1990). *Plasmodium yoelii* : comparative antimalarial activities of dietary fish oils and fish oil concentrates in vitamin E-dificient mice. *Exp. Parasitol.* **70,** 323–329

17 Kinsella, J.E., Lokesh, B., Broughton, S., and Whelan, J. (1990). Dietary polyunsaturated fatty acids and eiosanoids: potential effects on the modulation of inflammatory and immune cells: an overview. *Nutrition* **6,** 24–44

18 Fujikawa, M., Yamashita, N., Yamazaki, K., Sugiyama, E., Suzuki, H., and Hamazaki, T. (1992). Eicosapentaenoic acid inhibits antigen-presenting cell function of murine splenocytes. *Immunology* **75,** 330–335.

19 Lokesh, B.R., Sayers, T.J., and Kinsella, J.E. (1989/1990). Interleukin-1 and tumor necrosis factor synthesis by mouse peritoneal macrophages is enhanced by dietary n-3 polyunsaturated fatty acids. *Immunol. Lett.* **23,** 281–286

20 Okusawa, S., Gellfand, J.A., Ikejima, T., Connolly, R.J., and Dinarello, C.A. (1988). Interleukin-1 induces a shock-like state in rabbits. *J. Clin. Invest.* **81,** 1162–1172

21 Corey, E.J., Shin, C., and Cashman, J.R. (1983). Docosahexaenoic acid is a strong inhibitor of prostaglandin but not leukotriene biosynthesis. *Proc. Natl. Acad. Sci. USA* **80,** 3581–3584

22 Hamberg, M. (1980). Transformations of 5, 8, 11, 14, 17-eicosapentaenoic acid in human platelets. *Biochim. Biophys. Acta* **618,** 389–398