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EFFECTS OF DIETARY FISH OIL SUPPLEMENTATION ON POLYMORPHONUCLEAR LEUKOCYTE INFLAMMATORY POTENTIAL

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Abstract—Polymorphonuclear leukocytes (PMNLs) are an important contributor to inflammation and are thus a part of the pathophysiology of many human diseases. We assessed the effect of fish oil on PMNL inflammatory potential by measuring chemiluminescence and superoxide production before and after six weeks of daily cod liver oil ingestion by healthy volunteers. Phagocytosing PMNLs demonstrated a 27% decrease in chemiluminescence ($P < 0.05$) and a 64% decrease in superoxide production ($P < 0.01$), following the cod liver oil supplementation. Analysis of PMNL and platelet fatty acids revealed the appearance of eicosapentaenoic acid and a significant decrease in arachidonic acid in both types of cells.

INTRODUCTION

The response of polymorphonuclear leukocytes (PMNLs) to inflammatory stimuli is an important contributor to the pathogenesis of several clinical disease states (1–3). Recently, it has been demonstrated that both endogenous and exogenous supplementation with N-3 fatty acids derived from fish oil profoundly suppresses PMNLs chemotactic responsiveness and endothelial cell adherence, at least in part through the suppressed biosynthesis of an important proinflammatory mediator, leukotriene B_4 (LTB_4) (4). We assessed the effect of dietary marine oil supplementation in human volunteers on PMNL inflammatory potential by measuring two parameters of PMNL activation, superoxide produc-

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tion and the appearance of chemiluminescence in response to a phagocytic stimulus, both before and after six weeks of cod liver oil (CLO) ingestion.

MATERIALS AND METHODS

Thirty milliliters of CLO containing 3.6 g of eicosapentaenoic acid was given daily to six healthy volunteers who had abstained from all medication including aspirin for at least one week. Studies were performed on these subjects just prior to CLO and again at six weeks.

Laboratory Techniques. Whole blood (75 ml) was drawn into plastic syringes and then immediately transferred into plastic tubes containing acid–citrate anticoagulant. Leukocytes were prepared as previously described (5, 6). The resulting leukocyte pellet was resuspended in modified JNL buffer (pH 7.35) and adjusted to a final count of 1×10^7 cells/ml. The mean differential count was 66% PMNLs, 29% lymphocytes, and 5% monocytes. Viability of the leukocytes was 92% by trypan blue exclusion. Fifteen milliliters of whole blood was anticoagulated with acid–citrate–dextrose and spun at 200*g* for 10 min at room temperature to yield platelet rich plasma (PRP). The PRP was spun at 2000*g* for 20 min at 4°C. The platelets and an aliquot of the above leukocyte suspension were analyzed for their fatty acid content by gas chromatography as previously described (7).

Measurement of chemiluminescence was performed using only the rear photomultiplier tube of an ambient-temperature liquid scintillation counter (Beckman Instruments, Model LS-100C, Fullerton, California) (8). An aliquot (0.5 ml) of the leukocytes was suspended in modified JNL buffer and stimulated with 0.025 ml latex particle suspension (1.09 μm, Sigma Chemical Co., St. Louis, Missouri). The numbers were expressed as counts $\times 10^3/5$ min/10^3 leukocytes.

Leukocyte samples were assayed for superoxide by measuring superoxide dismutase-inhibitable reduction of cytochrome *c* using a modification of the method of Curnutte and Babior (9). Reaction mixtures containing 0.2 ml of the leukocyte suspension, 0.15 ml cytochrome *c* (horse heart type VI, 18 mg/ml, Sigma) and 2.5 ml modified JNL buffer were stimulated with 0.15 ml of opsonized zymosan A 60 mg/ml (Sigma). A 1.5-ml aliquot of the reaction mixture was immediately removed and maintained on melting ice. Test samples were incubated at 37°C. The reaction was stopped at 30 min by placing test samples on melting ice. Samples were spun at 2200*g* for 20 min at 4°C. Cytochrome *c* reduction was determined by measuring the difference in absorbance between the incubated supernatant and the unincubated supernatant at 550 nm in a Beckman model 25 spectrophotometer (Beckman Instruments). For cytochrome *c*, $E_{550} = 21.1$/mM/cm, and the observed value was equated with the yield of superoxide (10). Control samples with volumes adjusted to a final 3 ml containing no stimulus or stimulus plus superoxide dismutase (15 mg/ml) were run simultaneously. In pilot studies using purified lymphocytes in quantities contaminating our leukocyte preparations, both chemiluminescence and superoxide production were negligible. Thus, the results observed are attributable almost entirely to the PMNLs in the leukocyte preparations.

Data Analysis. Statistical analysis was done using a two-tailed paired *t* test comparing pre- and post-cod liver oil treatment data.

RESULTS

The data in Table 1 demonstrate that PMNL superoxide production and chemiluminescence are both suppressed significantly by six weeks of dietary

Table 1. Chemiluminescence and Superoxide Production in PMNL

Subject	Chemiluminescence (counts × 10^3/5 min/ 10^3 PMNL)			Superoxide Production (nmol/30 min/1 × 10^6 PMNL)		
	Pre-CLO	Post-CLO		Pre-CLO	Post-CLO	
1	22	14		23.0	10.3	
2	23	17		29.6	11.0	
3	23	13		37.1	13.0	
4	38	31		42.7	15.2	
5	27	29		34.4	12.7	
6	48	29		—	—	
Mean ± S.D.	30 ± 11	22 ± 8	$P < 0.05$	33.3 ± 7.5	12.5 ± 1.9	$P < 0.01$

CLO supplementation when compared to baseline values. To assure compliance with the CLO regimen, both PMNLs and platelet fatty acid levels were measured before and after dietary fish oil supplementation. Pretreatment eicosapentaenoic acid (EPA), the major N-3 fatty acid derived from fish oil, was not detectible in either PMNLs or platelets, but after six weeks of CLO it represented 3.3% of total leukocyte fatty acids (Table 2). Leukocyte arachidonic acid levels declined significantly during the treatment. EPA levels also became detectable and arachidonic acid levels declined significantly in the platelets of treated individuals. The sum of the fatty acids presented in Table 2 for both

Table 2. Fatty Acid Composition of PMNLs and Platelets Before and After Cod Liver Oil

Fatty Acids		Pre-CLO	Post-CLO	Significance
PMNL fatty acid composition (% of total)				
Palmitic	(16:0)	14.7 ± 3.0	14.5 ± 1.5	N.S.
Stearic	(18:0)	18.8 ± 2.1	19.0 ± 3.6	N.S.
Oleic	(18:0)	21.5 ± 2.2	20.3 ± 5.0	N.S.
Linoleic	(18:2)	11.8 ± 4.9	10.8 ± 1.9	N.S.
Arachidonic	(20:4)	18.9 ± 3.3	12.2 ± 3.7	$P < 0.005$
Eicosapentaenoic	(20:5)	0.0	3.3 ± 2.1	
Platelet fatty acids (% of total)				
Palmitic	(16:0)	15.9 ± 2.6	15.7 ± 2.3	N.S.
Stearic	(18:0)	19.3 ± 3.0	18.4 ± 4.0	N.S.
Oleic	(18:0)	16.4 ± 2.9	15.4 ± 2.8	N.S.
Linoleic	(18:2)	6.2 ± 2.1	6.6 ± 1.7	N.S.
Arachidonic	(20:4)	23.4 ± 2.2	15.5 ± 4.6	$P < 0.005$
Eicosapentaenoic	(20:5)	0.0	5.7 ± 1.2	

platelets and leukocytes does not reach 100% because minor fatty acid constituents are not included.

DISCUSSION

Our results reveal that dietary fish oil supplementation with CLO reduces PMNL superoxide production and the generation of chemiluminescence by these cells in response to a standard stimulus. Both of these parameters are markers of the nonmitochondrial, oxygen-dependent respiratory burst produced by PMNLs which have been activated by an inflammatory stimulus (11). Superoxide is one of several highly toxic oxygen metabolites produced by PMNLs during inflammation which can damage not only bacteria but also surrounding tissue (12). Chemiluminescence is a measure of the generation of highly excited free radicals by simulated PMNLs (13). Oxygen metabolites such as superoxide, hydrogen peroxide, the hydroxyl radical and singlet excited oxygen are all cytotoxic against invading bacteria and can also damage host cells. The suppressed production of these oxygen metabolites suggested by our studies may therefore indicate a reduced cytotoxic potential of PMNLs in individuals receiving dietary N-3 fatty acid supplements.

Prostaglandins, especially PGE_2, may be important inducers of inflammation, and leukotrienes, primarily leukotriene B_4, are important inducers of PMNL chemotaxis and endothelial cell adherence (14, 15). Dietary fish oil supplementation reduces the production of inflammatory prostaglandins and leukotrienes (4, 16). These fish oil fatty acid effects are primarily related to the N-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid. These fatty acids may inhibit phospholipase-induced release of the prostaglandin and leukotriene substrate, arachidonic acid, from lipid membranes and may also competitively inhibit the enzymes cyclooxygenase and lipoxygenase (4, 16). Additionally, when EPA is metabolized by lipoxygenase the product, leukotriene B_5, has a markedly reduced capacity to stimulate PMNL chemtotaxis when compared to the arachidonate metabolite, leukotriene B_4 (17, 18). It is speculative but quite possible that the suppressant effects upon PMNL inflammatory capability that we observed are related to these effects of dietary fish oil supplementation upon prostaglandin and leukotriene production. Corticosteroids, a class of drugs with potent antiinflammatory capabilities, inhibit prostaglandin and leukotriene production by indirectly reducing phospholipase activity and also suppress the generation of toxic oxygen species by PMNLs (6, 19).

Dietary fish oil supplementation has been shown to reduce clinical manifestations of rheumatoid arthritis without having any significant effect on laboratory parameters of disease activity (20). Leukotriene B_4 is significantly elevated in the synovial fluid of rheumatoid arthritis patients, and PMNLs are

important inflammatory mediators in this disorder (21). Further, PMNLs from synovial fluid of rheumatoid arthritis patients produce increased amounts of oxygen intermediates and have increased chemiluminescence (22). It is possible that the clinical effects of dietary fish oil in rheumatoid arthritis are related to both reduced PMNL inflammatory potential and suppressed leukotriene B_4 production. Leukotriene B_4 and the PMNL inflammatory response also appear to be related to other disease states such as gout, psoriasis, and ulcerative colitis (3, 23, 24). The effect of dietary fish oil supplementation in these disorders has yet to be evaluated, but it is possible that there may be clinical benefit.

The incorporation of EPA into the PMNL fatty acid pool and the decline in arachidonic acid levels during dietary fish oil supplementation were also seen in platelets. These changes in EPA and arachidonic acid levels of platelets have been correlated with reduced activity of various platelet function measurements (25, 26). Our data demonstrate similar PMNL fatty acid changes in association with a suppressant effect upon PMNL inflammatory potential. Therefore, dietary fish oil produces changes in the EPA and arachidonic acid levels of both platelets and PMNLs, and these changes are associated with reduced physiologic activity in both blood cells, not a surprising result given the common stem-cell lineage of these two highly specialized blood cells.

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REFERENCES

1. Howe, G. B., J. N. Fordham, K. A. Brown, H. L. F. Currey. 1982. Polymorphonuclear cell function in rheumatoid arthritis and Felty's syndrome. *Ann. Rheum. Dis.* **40**:370–375.
2. Ginsberg, M., and F. Kozin. 1978. Mechanisms of cellular interaction with monosodium urate crystals. *Arthritis Rheum.* **21**:896–903.
3. Greaves, M. W. 1983. Neutrophil polymorphonuclears, mediators and the pathogenesis of psoriasis. *Br. J. Dermatol.* **109**:115–118.
4. Lee, T. H., R. L. Hoover, J. D. Williams, et al. 1985. Effect of dietary enrichment with eicosapentaenoic and docasahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. *N. Engl. J. Med.* **312**:1217–1224.
5. Levine, P. H., R. S. Weinger, I. Simon, K. L. Scoon and N. I. Krinsky. 1976. Leukocyte-platelet interaction, release of hydrogen peroxide by granulocytes as a modulator of platelet reaction. *J. Clin. Invest.* **55**:955.
6. Levine, P. H., J. C. Hardin, K. L. Scoon, and N. I. Krinsky. 1981. Effect of corticosteroids in the production of superoxide and hydrogen peroxide and the appearance of chemiluminescence by phagocytosing polymorphonuclear leukocytes. *Inflammation* **5**:19–27.
7. Fisher, M., P. H. Levine, B. Weiner, et al. 1986. The effects of vegetarian diets on plasma lipids and platelets. *Arch. Intern. Med.* **146**:1193–97.
8. Krinsky, N. I., K. L. Scoon, J. C. Hardin, and P. H. Levine. 1977. Source of hydrogen peroxide and chemiluminescence observed in activated human platelets. *Blood* **50**:597–602.

9. CURNUTTE, J. T., and B. M. BABIOR. 1974. Biological defense mechanisms. The effect of bacteria and serum on superoxide production of granulocytes. *J. Clin. Invest.* **53**:1662–1674.
10. VANGELDER, B. F., and E. C. SLATER. 1962. The extinction coefficient of cytochrome *c*. *Biochim. Biophys. Acta.* **58**:593.
11. BABIOR, B. M. 1978. Oxygen dependent microbial killing by phagocytes. *N. Engl. J. Med.* **298**:659–669.
12. KLEBANOFF, S. J. 1980. Oxygen metabolism and toxic properties of phagocytes. *Ann. Intern. Med.* **13**:480–489.
13. TRUSH, M. A., M. E. WILSON, and K. VANDYKE. 1978. The generation of chemiluminescence by phagocytic cells. *Methods Enzymol.* **57**:642–645.
14. TRANG, L. E. 1980. Prostaglandins and inflammation. *Semin. Arthritis Rheum.* **9**:153–159.
15. FORD-HUTCHINSON, A. W. 1985. Leukotrienes: Their formation and role as inflammatory mediators. *Fed. Proc.* **44**:25–29.
16. KELLEY, V. I., A. GERRETTI, S. IZUI, and T. B. STROM. 1985. A fish oil diet rich in eicosapentaenoic acid reduces cylcooxygenase metabolites, and suppresses lupus in MRLI-1 pr mice. *J. Immunol.* **123**:1914–1919.
17. GOLDMAN, D. W., W. C. PRICKETT, and E. J. GOETZL. 1983. Human neutrophil chemotactic and degranulating activities of leukotriene B_5 (LTB_5) derived from eicosapentaenoic acid *Biochem. Biophys. Res. Commun.* **117**:282–288.
18. TERANO, T., J. A. SALMON, and S. MONCADA. 1984. Biosynthesis and biologic activity of leukotriene B_5. *Prostaglandins* **27**:217–232.
19. BLACKWELL, G. J., and R. J. FLOWER. 1983. Inhibition of phospholipase. *Br. Med. Bull.* **39**:260–284.
20. KRENNER, J. M., A. V. MICHALEK, L. LINNIGER, et al. 1985. Effects of manipulation of dietary fatty acids on clinical manifestations of rheumatoid arthritis. *Lancet* **1**:184–187.
21. KLICKSTEIN, L. B., C. SHAPLEIGH, and E. J. GOETZL. 1980. Lipoxygenation of arachidonic acid as a source of polymorphonuclear leukocyte chemotactic factors in synovial fluid and tissue in rheumatoid arthritis and spondyloarthritis. *J. Clin. Invest.* **65**:1165–1170.
22. NWIA, Y., T. SAKANE, M. SHINGO, and M. M. YOKOYAMA. 1983. Effect of stimulated neutrophils from synovial fluid of patients with rheumatoid arthritis on lymphocytes—a possible role of increased oxygen radicals generated by neutrophils. *J. Clin. Immunol.* **3**:228–240.
23. RAE, S. A., E. M. DAVIDSON, and M. J. H. SMITH. 1982. Leukotriene B_4, an inflammatory mediator in gout. *Lancet* **2**:1122–1123.
24. SHARON, P., W. F. STENSON. 1983. Production of leukotriene by colonic mucosa from patients with inflammatory bowel disease. *Gastroenterology* **84**:1306.
25. BRADLOW, B. A., N. CHETTY, J. VAN DER WESTHUYZEN, D. MENDELSON, and J. E. GIBSON. 1983. The effect of a mixed fish diet on platelet function, fatty acids and serum lipids. *Thromb. Res.* **20**:561–568.
26. HAY, C. R. M., A. P. DURBER, and R. SAYNOR. 1982. Effect of fish oil on platelet kinetics in patients with ischemic heart disease. *Lancet* **1**:1269–1272.