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Arachidonic Acid Supplementation Enhances Synthesis of Eicosanoids Without Suppressing Immune Functions in Young Healthy Men

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ABSTRACT: This study was conducted to determine the effects of arachidonic acid (AA) supplementation on human immune response (IR) and on the secretion of prostaglandin E2 (PGE2) and leukotriene B4 (LTB4). Ten healthy men (20–38 yr) participated in the study and lived at the Metabolic Suite of the Western Human Nutrition Research Center. They were fed a basal diet (57, 27, and 16 energy percentage from carbohydrate, fat, and protein, respectively, and AA 200 mg/d) for the first 15 d of the study. Additional AA (1.5 g/d) was added to the diet of six men from day 16 to 65, while the remaining four subjects remained on the basal diet. The diets of the two groups were crossed-over from day 66 to 115. *In vitro* indices of IR were examined using blood drawn on days 15, 58, 65, 108, and 115. Influenza antibody titers were determined in the sera prepared from blood drawn on days 92 and 115 (23 d postimmunization). AA supplementation caused significant increases in the *in vitro* secretion of LTB4, and PGE2, but it did not alter the *in vitro* secretion of tumor necrosis factor α; interleukins 1β, 2, 6; and the receptor for interleukin 2. Nor did it change the number of circulating lymphocytes bearing markers for specific subsets (B, T, helper, suppressor, natural killer) and the serum antibody titers against influenza vaccine. The opposing effects of PGE2 and LTB4 may have led to the lack of change in immune functions tested.
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Studies conducted in cultured cells and animal models show that n-6 polyunsaturated fatty acids (PUFA) inhibit immune cell functions (1–10). The results from studies in humans are mixed. A moderate increase in dietary linoleic acid (18:2n-6, LA) did not affect human immune response (IR) when dietary fat was held constant (11–13). But a negative association between plasma LA concentration and the number of circulating natural killer (NK) cells has been reported in a group of elderly individuals (14).

LA is the most abundant dietary n-6 PUFA for humans. It can be converted to arachidonic acid (20:4n-6, AA) by humans and several other animal species. AA is the major n-6 fatty acid of the membranes and it is a precursor of the eicosanoids. It is metabolized by two distinct oxygenase pathways. The cyclooxygenase pathway converts AA to prostaglandins, thromboxanes, and prostacyclins; the lipoygenase pathway converts it to leukotrienes.

In vitro studies show that AA and its metabolic products can alter a number of lymphocyte and monocyte functions, including the maturation of immature T cells into helper and suppressor cells, lymphocyte proliferation, cytokine and antibody secretion (15–21). Prostaglandin E2 (PGE2) inhibited a number of lymphocyte functions in a dose-dependent manner, whereas the leukotriene B4 (LTB4) was stimulatory at low concentrations and inhibitory at higher concentrations (22). When eicosanoid synthesis was blocked with inhibitors of cyclooxygenase and lipooxygenase pathways, addition of AA still inhibited immune cell functions *in vitro* (23–27). Thus, it is not clear if n-6 PUFA inhibit immune cell functions through eicosanoids or through other mechanisms.

The effects of AA supplementation on both immune cell functions and eicosanoid synthesis have not been examined previously in humans, because of concerns regarding its safety and the nonavailability of natural triglycerides of AA. An algal source of natural AA triglycerides recently became available, making human studies of AA supplementation feasible. Use of natural triglycerides of AA permits one to study its effects on human IR while the total amounts of other dietary fatty acids are held constant.

The purpose of this study therefore was to measure eicosanoid secretion and immune functions in humans given a supplement of AA while consuming a constant diet. We examined *in vivo* antibody production in response to influenza vaccine, lymphocyte subsets, and *in vitro* secretion of PGE2, LTB4, interleukin (IL) 1β, IL 2, IL 2R, IL 6, and tumor necrosis factor α using peripheral blood mononuclear cells (PBMNC).

SUBJECTS AND METHODS

Subjects, protocol, and diets. Ten healthy men (20–38 yr) were selected to participate in the study. All subjects were nonsmokers, had no history of alcohol or drug abuse, had moderate exercise levels, had body weights within −10 to

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Abbreviations: AA, arachidonic acid; IL, interleukin; IR, immune response; LA, linoleic acid; LPS, lipopolysaccharide; LTB4, leukotriene B4; NK, natural killer; PBMNC, peripheral blood mononuclear cells; PGE, prostaglandin E; PHA, phytohemagglutinin; PUFA, polyunsaturated fatty acids.

+20% of ideal body weight, and had chemical and hematological measurements within normal ranges. The participants lived and ate all meals at the Metabolic Unit of the Western Human Nutrition Research Center for 130 d (August 14–December 22, 1994). A crossover design was used. All subjects consumed the basal, low-AA, diet during the first 15 d. For the next 50 d six subjects (group 1) consumed the diet supplemented with AA (1.5 g/d) and the other four (group 2) continued to consume the basal diet. Diets of the two groups were switched on day 66, and the new diets were fed for 50 d (days 66–115). Both groups were fed the basal diet for the last 15 d (days 116–130). Body weights of the subjects were maintained constant throughout the study by adjusting the energy intake and by maintaining a constant physical activity schedule. All subjects walked two miles twice every day. Blood samples were collected on days 16, 58, 65, 108, and 115.

Basal diet consisted of natural foods and was adequate in all nutrients. A 5-d rotating menu with three meals and two snacks every day was used. The proportion of energy from carbohydrate, fat, and protein was 57, 27, and 16%, respectively (28). The energy was equally distributed among the saturated, monounsaturated, and n-6 PUFA. The basal diet provided 200 mg AA and 250 mg cholesterol per day. The calculated amount of vitamin E in the basal diet was two times the Recommended Daily Allowance. Three grams ARASCO® oil (a gift from Martek Biosciences Corporation, Columbia, MD) was added to the diet to provide 1.5 g AA/d. The total fat content of the two diets was held constant by replacing an equivalent amount of monounsaturated fat with AA.

Laboratory procedures. Blood was collected by antecubital venipuncture into evacuated tubes containing heparin for cell culture experiments, or EDTA for lymphocyte phenotypic analysis, or without anticoagulants for preparation of sera. Blood samples for all subjects were collected after an overnight fast, between 0700 and 0800 h.

Secretion of cytokines and eicosanoids. PBMNC were isolated using Histopaque-1077 as previously reported (29). The culture medium used was RPMI-1640 (Gibco, Grand Island, NY) containing 10% autologous serum and L-glutamine (2 mmoI/L), penicillin (100 KU/L), streptomycin (100 mg/L), and gentamicin (20 mg/L). Five hundred μL of the culture medium containing 5×10^5 PBMNC was inoculated in each well of a 24-well flat-bottom culture plate. An additional 500 μL of the culture medium with or without the mitogens was added to each well.

The production of IL 1β, IL 6, tumor necrosis factor, PGE2, and LTB4 was stimulated by the addition of lipopolysaccharide (LPS, 0.5 mg/L), and that of IL 2 and IL 2R was stimulated by adding phytohemagglutinin (PHA, 10 mg/L). Tissue culture media were collected by centrifugation 24 h after stimulation with LPS, and 48 h after PHA. The media were stored frozen at −70°C until the cytokine and eicosanoid concentrations were determined.

ELISA kits for cytokine assays were purchased from T Cell Diagnostics, Inc. (Cambridge, MA), and those for eicosanoids from Cayman Chemical Company (Ann Arbor, MI).

Influenza vaccination and serum antibody titers. All subjects were immunized on study day 92 with the trivalent influenza virus vaccine (Fluzone, 1993–1994 Formula) purchased from Connaught Laboratories Inc. (Swiftwater, PA). Preimmunization sera were prepared from the blood drawn on day 92, and the postimmunization sera from the blood drawn on day 115. The antibody titers were determined at the Centers for Disease Control using the hemagglutination inhibition assay (30) and the viral strains A/TEXAS/36/91, A/BEIJING/32/92, and B/PANAMA/45/90ET. Results for antibody titers are expressed as the geometric means of the antibody titer, the geometric mean transformations, and the percentage of subjects with an antibody titer ≥40 or 160.

Lymphocyte phenotypic analysis. Lymphocyte subsets, including B ($CD19^+$), T ($CD3^+$), helper ($CD3^+$, $CD4^+$), suppressor ($CD3^+$, $CD8^+$), and NK ($CD3^-$, $CD16^+$, $CD56^+$) cells, in peripheral blood were determined using the Becton-Dickinson Flowcytometer as previously reported (29).

Data analysis. The data were analyzed by an analysis of variance model using SAS/STAT PROC GLM (31). The cross-over design model included effects of order, subject (order), period, and diet, using subject (order) as an error term for order. The significance of the difference between with and without AA supplementation was assessed from the *P* values for the diet main effects. The data from the two groups were analyzed separately using a paired *t*-test when the treatment was given only during one period, such as immunization with influenza vaccine. Changes in the variables examined are considered significant for $P < 0.05$ or otherwise stated.

RESULTS

The mean ± SEM for age (yr), weight (kg), height (cm), and body mass index (kg/m^2) for subjects in groups 1 ($n = 6$) and 2 ($n = 4$) were 31.2 ± 3.2 and 32.2 ± 2.9, 73.8 ± 2.4 and 71.0 ± 5.4, 177.4 ± 3.6 and 175.5 ± 1.8, 23.8 ± 1.8 and 23.0 ± 1.3, respectively. The composition of the diets and the average daily intake of nutrients have been previously reported (28). None of these parameters was different between the two groups.

Eicosanoid secretion. PGE2 and LTB4 secretion into the tissue culture medium within 24 h of PBMNC stimulation with LPS is shown in Table 1. AA supplementation significantly ($P < 0.05$) increased the secretion of both of these eicosanoids, compared to the values at the end of stabilization period (day 16). The percentage stimulation of LTB4 secretion with AA supplementation was greater (200–400%) than that of PGE2 secretion (50–100%). In the group receiving AA supplementation first (days 16–65), eicosanoid secretions remained elevated 50 d after the discontinuation of the AA supplementation. The amount of PGE2 secreted into the medium in the absence of LPS (unstimulated cultures) was about 25% of that secreted in the presence of LPS and was not altered by AA supplementation (not shown). The amount of LTB4 secreted in unstimulated cultures was about 50% of the amount secreted in the presence of LPS, and it increased significantly ($P < 0.05$) with AA supplementation (not shown).

TABLE 1
Dietary Arachidonic Acid Increases *in vitro* Secretion of Eicosanoids by PBMNC Stimulated with LPS[a]

	Group 1 (n = 6)			Group 2 (n = 4)		
Eicosanoid	Day 15	Day 65	Day 115	Day 15	Day 65	Day 115
PGE2 (ng/mL)	0.81 ± 0.06[a]	1.25 ± 0.12[b]	1.11 ± 0.10[b]	0.53 ± 0.05[c]	0.47 ± 0.01[c]	0.87 ± 0.06[d]
LTB4 (ng/mL)	0.20 ± 0.02[a]	0.43 ± 0.04[b]	0.39 ± 0.04[b]	0.14 ± 0.01[c]	0.19 ± 0.02[c]	0.85 ± 0.14[d]

[a]Data shown are the mean ± SEM for the number of subjects shown. Diet of subjects in Group 1 was supplemented with arachidonic acid from days 16 to 65, and that of Group 2 from days 66 to 115. For each eicosanoid, comparisons were made within each group using a paired *t*-test, and the numbers bearing different superscripts within each group are significantly different ($P < 0.05$). PBMNC, peripheral blood mononuclear cells; LPS, lipopolysaccharide; PGE2, prostaglandin E2; LTB4, leukotriene B4.

Cytokine secretion. The effects of AA supplementation on cytokine secretion are presented in Table 2. The concentrations of IL 1β, IL 6, and tumor necrosis factor α secreted into the tissue culture media by the PBMNC are in response to stimulation with LPS, and that of IL 2 and IL 2R are in response to stimulation with PHA. Mitogens caused a several-fold increase in the release of all cytokines tested, compared to the corresponding cytokine concentrations in the media from unstimulated cultures, which were below the detection limits of the assay systems (not shown). AA supplementation did not alter the secretion of all the cytokines at any time in the study.

Influenza antibody titers. The pre- and postimmunization antibody titers for the A/Texas, A/Beijing, and B/Panama strains of influenza are shown in Table 3. Immunization caused an approximately 10-fold increase in the serum titer for B/Panama and 30-fold increase in A/Texas in both the AA-supplemented and -nonsupplemented groups. The amount of AA in the diet did not influence the antibody titer for these two strains. Immunization caused a 19-fold increase in the antibody titer for A/Beijing in Group 1 (fed low-AA diet at the time of immunization) and a 9-fold increase in Group 2 (fed high-AA diet). The apparent difference in the antibody titers between the two groups for this viral strain was also evident if the results for antibody titers were expressed as percentage of subjects with titers ≥40 or 160. These results suggest an inhibition of A/Bejing antibody production by AA supplementation, however, the differences between the two groups did not attain statistical significance.

Lymphocyte subsets. The percentage and the absolute numbers for the B, T, helper, suppressor, and NK cells at study days 16, 65, and 115 are shown in Table 4. AA supplementation did not alter the absolute numbers or the percentage of these lymphocytes in circulation. Nor was there an effect of AA supplementation on the ratio between the helper and suppressor T lymphocytes.

DISCUSSION

AA feeding caused significant increase in the *in vitro* secretion of both PGE2 and LTB4, without any change in a number of indices of IR tested. These results suggest that either AA has no effect on IR or it may alter immune functions independently of the changes in eicosanoid production. The second interpretation is consistent with the results from previous studies with inhibitors of lipoxygenase and cyclooxygenase (23–27), in which AA inhibited cytokine production *in vitro*, even when there was no change in eicosanoid synthesis. This inhibition could be due to changes in membrane fluidity or receptor expression. The lack of inhibition of cytokine secretion by AA in our study may be a function of AA concentration, or some other differences in experimental conditions.

Results obtained from *in vitro* studies have demonstrated that PGE2 inhibited (17,20,25) and LTB4 stimulated cytokine production. These opposing effects of PGE2 and LTB4 on cytokine production can also explain our results regarding immune functions. Our study design cannot distinguish between this and the possibility discussed above. We are not aware of any studies where the effects on immune cell functions of adding PGE2 and LTB4 concurrently have been tested. Evaluation of such interaction between these two cytokines is important and needs to be monitored in future studies.

Our results showing increases in eicosanoid production *in vitro* are consistent with the results from *in vivo* studies show-

TABLE 2
Dietary Arachidonic Acid Does Not Alter *in vitro* Secretion of Cytokines by PBMNC[a]

	Group 1 (n = 6)			Group 2 (n = 4)		
Cytokine	Day 15	Day 65	Day 115	Day 15	Day 65	Day 115
TNF α (pg/mL)	309 ± 93	296 ± 79	309 ± 51	299 ± 45	335 ± 85	503 ± 101
IL 1β (ng/mL)	2.99 ± 1.03	4.10 ± 0.87	3.27 ± 0.63	2.99 ± 0.78	3.55 ± 0.98	3.65 ± 0.67
IL 6 (ng/mL)	1.85 ± 0.19	2.07 ± 0.19	2.17 ± 0.15	2.01 ± 0.15	2.19 ± 0.14	2.56 ± 0.25
IL 2 (pg/mL)	376 ± 45	349 ± 27	313 ± 74	272 ± 38	453 ± 14	472 ± 98
IL 2R (nM/mL)	1.34 ± 0.15	1.47 ± 0.01	1.60 ± 0.01	1.37 ± 0.18	1.51 ± 0.10	1.65 ± 0.27

[a]Data shown are the mean ± SEM for the number of subjects shown for each group. Abbreviations: TNF, tumor necrosis factor; IL, interleukin; R, receptor. Synthesis of IL 2 and IL 2R were stimulated with phytohemagglutinin, while that of the other three cytokines with lipopolysaccharide. Arachidonic acid supplementation did not alter the secretion of all cytokines tested.

TABLE 3
Dietary Arachidonic Acid Does Not Alter Serum Influenza Antibody Titers[a]

		Group 1 (n = 6)		Group 2 (n = 4)	
Strain	Index	Pre-immun.	Post-immun.	Pre-immun.	Post-immun.
A/Texas	GM	0.95 ± 0.20[a]	2.45 ± 0.09[b]	0.92 ± 0.15[c]	2.35 ± 0.31[d]
	GMT	9	285	8	226
	% ≥40	17	100	0	100
	% ≥160	0	100	0	75
A/Beijing	GM	0.80 ± 0.10	2.05 ± 0.32	0.70 ± 0.10	1.68 ± 0.56
	GMT	6	113	5	48
	% ≥40	0	83	0	50
	% ≥160	0	33	0	25
B/Panama	GM	1.40 ± 0.48	2.40 ± 0.22	1.08 ± 0.14	2.05 ± 0.15
	GMT	25	254	12	113
	% ≥40	17	100	0	100
	% ≥160	17	83	0	25

[a]Data shown are mean ± SEM for the number of subjects shown for each group. Abbreviations: GM, geometric mean; GMT, geometric mean transformation. Immunization significantly increased the antibody titers for all three viral strains in both groups. There was no effect of arachidonic acid supplementation on the antibody titers for all three strains.

ing an increase in AA metabolites in the urine of subjects supplementing their diets with n-6 PUFA (32–35). Results regarding immune functions are also consistent with our previous report from the same study in which we found no changes in lymphocyte proliferation in response to PHA and Concanavalin A, NK cell activity, and delayed hypersensitivity skin response (28). These results, however, differ from those of several *in vitro* studies, which showed inhibition of cytokine production with PGE2 (20,25) and stimulation with LTB4 (18). As stated earlier, the opposing effects of the two eicosanoids may have canceled each other.

The lack of an AA effect on cytokine secretion in our study could also result from the failure of the mitogens to increase the synthesis of the receptors for the cytokines. This is unlikely, because the mitogens and the experimental conditions used in this study have previously been shown (18,20,29) to increase the secretion of the cytokines examined in this report. Even in the present study the cytokine secretion was severalfold higher in the cultures treated with mitogens, compared to the parallel cultures without mitogens. Together these data indicate that cytokine secretion was increased by the mitogens, but it was not altered by AA feeding.

AA feeding did not alter the concentration of serum antibody production against the A/Texas and B/Panama strains. There was a trend toward reduction in antibody produced in response to A/Beijing strain by AA feeding, but it did not attain significance. This is perhaps due to the small number of subjects (six in Group 1, and four in Group 2), or the crossover study design. We believe a 50-d wash-out period should have removed most of the AA in Group 1; however, the amount of eicosanoids produced in this group at day 115 suggests that the wash-out was not complete. Our study design cannot distinguish whether the residual AA leads to the lack of difference in antibody production between the two groups, or AA has no effect on antibody production. There was no carryover effect for other immune functions tested in the six subjects in Group 1. Thus, it is safe to assume that AA feeding did not alter cytokine production and the number of phenotypically distinct lymphocytes in circulation.

Our data indicate that the amount, form, and duration of AA fed had no adverse health effects, as long as the total fat and vitamin E intakes were maintained constant. Further-

TABLE 4
Dietary Arachidonic Acid Does Not Alter Number of Phenotypically Distinct Lymphocytes in Circulation[a]

		Group 1 (n = 6)			Group 2 (n = 4)		
Cell type		Day 16	Day 65	Day 115	Day 16	Day 65	Day 115
B	10^9/L	218 ± 36	280 ± 38	260 ± 30	185 ± 93	198 ± 54	208 ± 51
	%	12.0 ± 1.3	14.6 ± 1.4	14.3 ± 1.4	10.0 ± 2.3	9.9 ± 2.2	11.5 ± 1.9
T	10^{12}/L	1.3 ± 0.2	1.4 ± 0.2	1.3 ± 0.2	1.4 ± 0.1	1.5 ± 0.1	1.3 ± 0.1
	%	73.1 ± 3.4	72.8 ± 3.3	69.3 ± 2.9	77.1 ± 4.1	79.1 ± 4.3	74.5 ± 3.9
Helper	10^9/L	840 ± 118	897 ± 126	788 ± 87	788 ± 65	801 ± 112	690 ± 36
	%	46.3 ± 2.7	45.9 ± 1.6	41.8 ± 1.6	42.5 ± 4.1	42.3 ± 3.8	40.3 ± 3.3
Suppressor	10^9/L	418 ± 73	497 ± 63	448 ± 71	596 ± 91	657 ± 82	555 ± 78
	%	24.5 ± 1.9	25.7 ± 1.3	25.7 ± 1.9	32.5 ± 5.3	36.0 ± 6.0	32.3 ± 4.3
NK	10^9/L	241 ± 44	223 ± 36	287 ± 51	239 ± 69	190 ± 39	240 ± 75
	%	14.7 ± 3.2	12.1 ± 2.0	14.7 ± 2.4	12.4 ± 2.9	10.0 ± 1.8	14.5 ± 3.9

[a]Data shown are the mean ± SEM for the number of subjects shown for each group. Arachidonic acid supplementation did not alter the number or the percentage of lymphocytes within each subset.

more, the concurrent increase in PGE2, and LTB4 secretion had no effect on immune cell functions. These results are consistent with the results from our LA studies, in which several indices of IR were not inhibited by increase in LA intake as long as the total fat intake was maintained constant (8,9). Together these studies show that moderate intake of n-6 PUFA do not inhibit human IR if the diet is adequate in antioxidant nutrients and the total dietary fat is held constant.

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