Effects of Dietary Arachidonic Acid on Human Immune Response

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ABSTRACT: Arachidonic acid (AA) is a precursor of eicosanoids, which influence human health and the *in vitro* activity of immune cells. We therefore examined the effects of dietary AA on the immune response (IR) of 10 healthy men living at our metabolic suite for 130 d. All subjects were fed a basal diet containing 27 energy percentage (en%) fat, 57 en% carbohydrate, and 16 en% protein (AA, 200 mg/d) for the first and last 15 d of the study. Additional AA (1.5 g/d) was incorporated into the diet of six men from day 16 to 65 while the remaining four subjects continued to eat the basal diet. The diets of the two groups were crossed-over from day 66 to 115. *In vitro* indexes of IR were examined using the blood samples drawn on days 15, 58, 65, 108, 115, and 127. The subjects were immunized with the measles/mumps/rubella vaccine on day 35 and with the influenza vaccine on day 92. Dietary AA did not influence many indexes of IR (peripheral blood mononuclear cell proliferation in response to phytohemagglutinin, Concanavalin A, pokeweed, measles/mumps/rubella, and influenza vaccines prior to immunization, and natural killer cell activity). The post-immunization proliferation in response to influenza vaccine was about fourfold higher in the group receiving high-AA diet compared to the group receiving low-AA diet ($P = 0.02$). Analysis of variance of the data pooled from both groups showed that the number of circulating granulocytes was significantly ($P = 0.03$) more when the subjects were fed the high-AA diet than when they were fed the low-AA diet. The small increases in granulocyte count and the *in vitro* proliferation in response to influenza vaccine caused by dietary AA may not be of clinical significance. However, the lack of any adverse effects on IR indicates that supplementation with AA may be done safely when needed for other health reasons.

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Both the concentration and type of dietary fat have been reported to alter immune response (IR) in several human studies. Decreasing the amount of fat in the diet enhanced several indexes of IR, and the converse was true when fat intake was increased (1–3). Supplementing diets with n-3 polyunsaturated fatty acids (PUFA) from flaxseed or fish oils inhibited several indexes of IR (4–7), while a moderate increase in the intake of n-6 PUFA inhibited IR in some but not in other studies(1–3,8). The source of n-6 PUFA used in the above studies was oils rich in linoleic acid (LA), which can be converted to arachidonic acid (AA) in healthy individuals through desaturation and chain elongation. LA can have some direct effects on metabolic functions, but most of its effects are due to its conversion to AA. AA is one of the major fatty acids of the membranes and is a precursor for eicosanoids. Dietary AA is not required for humans, but it has been reported to alter tissue fatty acid composition and the eicosanoids produced in humans (9,10). Results from a study with Syrian hamsters indicate that dietary AA was metabolized differently from the endogenously synthesized AA (11). Addition of AA or its metabolites to cultured immune cells altered their activity and the eicosanoids produced *in vitro* (12–17). These studies suggest that AA and its metabolites may play an important role in modulating IR, and that the metabolism of AA may be altered depending on its source.

There is currently no recommended daily allowance (RDA) for AA or other fatty acids for humans. Diets rich in eggs and organ meats are high in AA. The estimates for the daily intake of AA in the United States for adults consuming eggs and meats range from 200–1000 mg with an average of about 500 mg (18,19). Others, however, have estimated the daily intakes of AA from Western diets to range from 100–500 mg (20–23).

No human or animal studies have examined the effects of dietary AA on IR. The purpose of this study was to examine the effects of dietary AA on several indexes of human IR, particularly the adverse consequences from the increased intake of AA. Considering the safety of the subjects and the minimum amount of AA needed to perturb the tissue fatty acid composition, we modified the diets of 10 healthy men to contain additional AA (1.5 g/d for 50 d) using a crossover design. The indexes of IR examined in this study were those that have previously been shown to be affected by the amount and type of dietary fat; these included lymphocyte

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Abbreviations: AA, arachidonic acid; CD, cluster designation; Con A, concanavalin A; DTH, delayed-type hypersensitivity skin response; en%, energy percentage; FBS, fetal bovine serum; II-2, interleukin-2; INF, interferon; LA, linoleic acid; MMR, measles/mumps/rubella; MP, metabolic period; NK, natural killer; PGE, prostaglandin E; LTB_4, leukotriene B_4; PBMNC, peripheral blood mononuclear cells; PUFA, polyunsaturated fatty acids; PHA, phytohemagglutinin; RDA, recommended daily allowance.

proliferation, phenotypic analysis for B and T cells, helper, suppressor, cytotoxic, and natural killer (NK) cells, NK cell activity, and *in vivo* delayed-type hypersensitivity (DTH) skin response.

MATERIALS AND METHODS

Subjects, protocol, and diets. The research protocol and informed consent form were approved by the Institutional Review Board of the University of California, Davis, and by the Human Studies Review Committee of the Agricultural Research Service, U.S. Department of Agriculture (Washington, D.C.). Twelve healthy men (ages 20–38 yr) were selected to participate in the study after medical and physiological screening. Only subjects that were nonsmokers, had no history of alcohol or drug abuse, had moderate physical activity and body weight within −10 to +20% of ideal body weight, and had hematological and chemical measurements within normal ranges were included in the study. Data from two subjects, who did not complete the study, were not included.

The participants lived and ate all meals at the Western Human Nutrition Research Center (WHNRC) (San Francisco, CA) for 130 d (August 14–December 22, 1994). All subjects consumed the basal/reference (low-AA) diet during the first 15 d (metabolic period 1, MP1). For the next 50 d (MP2), six subjects (Group 1) consumed the high-AA diet 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 between days 66 and 115 (MP3). Both groups were fed the low-AA diet between days 116 and 130 (MP4). Body weights of the subjects were maintained constant throughout the study by adjusting the caloric intake and by maintaining a constant physical activity. All subjects went for two two-mile walks every day. Blood samples from the subjects were collected on days 16, 58, 65, 108, 115, and 127 to study *in vitro* indexes of IR. All subjects were immunized with measles/mumps/rubella (MMR) vaccine on day 35 and with influenza vaccine on day 92. Antigens for DTH skin response were injected into all subjects on days 65 and 115.

Both diets were composed of natural foods and were adequate in all nutrients. Diets were fed on a 5-d rotating menu with three meals and two snacks every day. The planned percentage of energy from carbohydrate, fat, and protein in both diets was 55, 30, and 15% respectively. The 30% calories from fat in the basal diet were intended to come 10% from each of the saturated, monounsaturated, and n-6 PUFA. On an average, the basal diet provided 200 mg AA and 250 mg cholesterol every day. The calculated level of vitamin E in both diets was at twice the RDA for this nutrient. ARASCO® oil, a gift from Martek Biosciences Corporation (Columbia, MD), was the source of additional AA for the high-AA diet. This oil is purified from algae and contains fatty acids as natural triglycerides. Its fatty acid composition by weight percentage is AA 50, palmitic acid 9, stearic acid 14, oleic acid 9, linoleic acid 6, γ-linolenic acid 4, homo-γ-linolenic acid 3, and other fatty acids 5. AA (1.5 g/d from 3 g of ARASCO® oil) was incorporated into the diets by replacing an equivalent amount of the monounsaturated fat from the basal diet. ARASCO® oil was kept at −20°C under nitrogen until ready to be served in cold yogurts or salad dressings. Other details of the study design are given in a preceding paper in this journal volume (24).

Laboratory procedures. Blood samples were collected by antecubital venipuncture into evacuated tubes containing heparin for cell culture experiments, or EDTA for blood cell count and 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.

Blood cell count and lymphocyte phenotypic analysis. For each blood draw, a complete and differential cell count was performed by using a Serono Baker Automated system (model 9000 diff; Allentown, PA). Phenotypic analysis of the various lymphocyte subsets was done using Becton-Dickinson FACStar flow cytometer (San Jose, CA). Whole blood collected in EDTA-containing vacutainer tubes was transferred into tubes containing fluorochrome-labeled monoclonal antibodies against specific cluster differentiation (CD) antigens. After incubation for 30 min, the red cells were lysed with formaldehyde and removed by washing the leukocytes twice with Dulbecco's phosphate buffered saline without calcium and magnesium. The stained lymphocytes were fixed in 1% paraformaldehyde prior to analysis with the flow cytometer. The percentage of lymphocytes of a given phenotype was determined from a total of 10,000 lymphocytes counted.

Isolation and culture of peripheral blood mononuclear cells (PBMNC). The PBMNC were isolated using Histopaque-1077 as previously reported (25). The culture medium used was RPMI-1640, (Gibco, Grand Island, NY) containing 10% autologous serum and L-glutamine (2 mmol/L), penicillin (100 KU/L), streptomycin (100 mg/L), and gentamicin (20 mg/L). One hundred μL of the culture medium containing 1×10^5 PBMNC was inoculated in each well of a 96-well flat-bottom culture plate. An additional 100 μL of the culture medium with or without the mitogens was added to each well. The mitogens used were phytohemagglutinin (PHA), Concanavalin A (Con A), and pokeweed (all from Sigma Chemical Co, St. Louis, MO). Each mitogen was used at two concentrations; final concentrations (mg/L) in the culture media were 10 and 20 for both PHA and Con A, and 0.005 and 0.05 for pokeweed. Separate cultures of PBMNC were also stimulated with influenza and MMR vaccines at three different concentrations (1:250, 1:500, 1:1000 dilutions). Trivalent influenza virus vaccine (Fluzone, 1993–1994 Formula) was from Connaught Laboratories Inc. (Swiftwater, PA) and the MMR live vaccine, MSD, was from Merck, Sharp & Dohme (West Point, PA). PBMNC stimulated with mitogens were cultured for 72 h and those stimulated by antigens for 120 h; [^{3}H] thymidine, 37 K Bq, in 50 μL, was added to each well during the last 24 h. PBMNC were collected on filter strips and the radioactivity was determined using a Packard β-gas counter. The [^{3}H]thymidine incorporation into cellular DNA (Bq/1000 cell) was used as the index of PBMNC proliferation.

Determination of NK cell activity. A fraction of the PBMNC isolated above was suspended in serum-free RPMI to a final concentration of $3–5 \times 10^6$/mL to be fractionated for determining NK cell activity. PBMNC suspension (3 mL) was mixed with 7 mL of RPMI 1640 containing 10% fetal bovine serum (FBS), and incubated in T75 culture flasks for 1 h at 37°C in a 5% CO_2 incubator. Parallel flasks with the addition of interleukin-2 (IL-2) (30,000 IU/L) and interferon γ (INF_γ, $I \times 10^6$ IU/L) to stimulate NK cell activity were also prepared. After 1 h incubation, the flasks were gently shaken and the nonadherent cells were transferred into 15-mL conical tubes and centrifuged for 5 min at $500 \times g$. The pellet was resuspended in 1 mL RPMI containing 10% FBS, and cell concentration determined, and adjusted to 6.6×10^6/mL. ^{51}Cr-labeled K-562 cells were used as the target cells, and their concentration was determined by using the Elzone Particle Counter (Particle Data Inc., Los Angeles, CA). The cell concentration was adjusted to 2×10^5 cells/mL with RPMI 1640 containing 10% FBS. Cell suspension (50 μL) containing 10,000 of K-562 cells was added to each well of the 96-well round-bottom plates. The nonadherent PBMNC isolated above were then added at concentrations of $(0.50, 0.25, 0.125, 0.063, 0.031) \times 10^6$ to each well of the 96-well plate to give effector/target cell ratios of 50:1, 25:1, 12.5:1, 6.2:1, and 3.1:1. Six wells were used for each effector cell concentration and for the spontaneous and maximum release (caused by 3% centrimide) of ^{51}Cr.

Plates were incubated for 4 h at 37°C in 5% CO_2 and then centrifuged for 10 min at $200 \times g$. The supernatant (100 μL) was removed from each well into vials and the radioactivity released was determined by using a Packard gamma counter (Hewlett-Packard, Palo Alto, CA). Percentage lysis was calculated in Equation 1.

$$\% \text{ lysis} = \frac{(\text{experimental cpm} - \text{spontaneous cpm})}{(\text{maximum cpm} - \text{spontaneous cpm})} \times 100 \quad [1]$$

DTH. DTH response to seven recall antigens was assayed by intradermally injecting 0.1 mL of each antigen solution into the forearm. The antigens used were tuberculin purified-protein derivative (one international test unit), mumps (four complement-fixing test units), tetanus toxoid (1:100, vol/vol dilution of a solution containing four flocculation units/0.5 mL), candida (1:100, vol/vol dilution), trichophyton (1:30, vol/vol dilution), streptokinase streptase (100 KU/L), and coccidioidin (bioequivalent to U.S. reference coccidioidin 1:100; provided by the Office of Biologics, Food and Drug Administration, Washington, D.C.). The antigens were diluted with a diluent containing, per liter, 3 mL normal human serum and 9 g sodium chloride. Tuberculin purified-protein derivative, mumps, and tetanus toxoid were supplied by Connaught Laboratories Inc. (Swiftwater, PA). Candida (Dermatophyton O), trichophyton, and the antigen diluent were obtained from Hollister Stier (Spokane, WA). Streptokinase streptase, and coccidioidin were purchased from Behringwereke Ag (Marburg/Lahn, Germany) and Berkeley Biologicals (Berkeley, CA), respectively. Response to these antigens was determined by measuring mean induration diameters (mm) at 48 and 72 h after injections. Induration diameters with less than 4 mm were scored negative. Data are reported as the sum of induration diameters for all positive responses (induration score) and the number of positive responses to the seven antigens (antigen score).

Data analysis. The data were analyzed with analysis of variance by using SAS/STAT PROC GLM (26). The crossover design model included effects of order, subject (order), period, and diet, using subject (order) as an error term for order. If the period effects were not significant, the data from two groups were pooled (e.g., leukocyte counts), otherwise analyzed separately (e.g., DTH skin response, NK cell activity, and PBMNC proliferation in response to mitogens). The significance of the differences between the effects of the two diets 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 MMR or influenza vaccines. Changes in the variables examined are considered significant for $P < 0.05$ or otherwise stated.

RESULTS

As stated in the Materials and Methods section, the 10 subjects included in this study were divided into two groups. The high-AA diet was fed to subjects in Group 1 ($n = 6$) from study days 16 to 65, and to those in Group 2 ($n = 4$) from study days 66 to 115. The mean ± SEM for age (yr), weight (kg), height (cm), and body mass index (kg/m^2) for subjects in Groups 1 and 2 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. None of these parameters was statistically different between the two groups.

Dietary intake data prior to entering the study were not collected; however, all subjects consumed a typical American diet, which provides 35–40% calories from fat. The mean daily intake of nutrients from the low- and high-AA diets are shown in Table 1. These intakes are based on an average daily intake of 2800 kcal, which did not differ between the two diets or two groups. The proximate analysis for the macronutrients showed that the energy percentage (en%) from the protein, fat, and carbohydrate were not different between the two diets. These analyzed values were 27, 57, and 16 en% from fat, carbohydrate, and protein, respectively, which were close to the planned values of 30, 55, and 15% respectively.

The weight percentage fatty acid compositions of the two diets are shown in Table 2. While there are minor differences in the concentration of other fatty acids between the two diets, the major difference is in the concentration of AA, which is nine times higher in the AA diet than in the basal diet. The high-AA diet contained about 2% less oleic acid than that found in the low-AA diet.

White blood cell counts and lymphocyte subsets. The data

TABLE 1
Mean Daily Nutrient Intake[a]

Nutrient	Low-AA diet	High-AA diet
Protein		
(g)	104.6 ± 3.1	106.0 ± 5.3
(% of energy)	15.8 ± 0.5	15.2 ± 0.8
Total fats		
(g)	91.6 ± 4.7	85.2 ± 6.7
(% of energy)	27.1 ± 1.5	27.9 ± 2.2
Carbohydrates		
(g)	388.7 ± 9.8	401.8 ± 11.1
(% of energy)	57.1 ± 1.4	57.0 ± 1.6
Ash (g)	17.0 ± 1.3	19.5 ± 1.6
Copper (mg)	1.27	1.26
Iron (mg)	23.5	22.1

[a]Mean ± SEM (n = 5). Proximate analysis was performed on a composite sample of each diet prepared for each day of the 5-d rotating menu. For copper and iron, analysis was done on pooled samples from the individual composites; AA, arachidonic acid.

for leukocyte counts (Fig. 1) and lymphocyte subsets (not shown) could be pooled from all 10 subjects to determine the effects of the two diets, since the period effects (carryover) for these variables were not significant. The rank transformation was used to stabilize the subject variances among groups and periods, so the difference is in terms of means of ranks. For the diet effect, the crossover analysis of variance model tested the difference between the average of the high-AA diet and the low AA-diet. Figure 1 shows that compared to the baseline values the number of circulating leukocytes increased during the study in both groups, but the difference between the high- and low-AA diets did not attain statistical significance (P = 0.1). This increase in leukocyte numbers was primarily due to the increase in circulating granulocytes (Fig. 1), and the number of circulating monocytes and lymphocytes did not change (not shown). The increase in the number of circulating granulocytes was significantly (P = 0.03) higher when the subjects were fed the high-AA diet than when they were fed the low-AA diet.

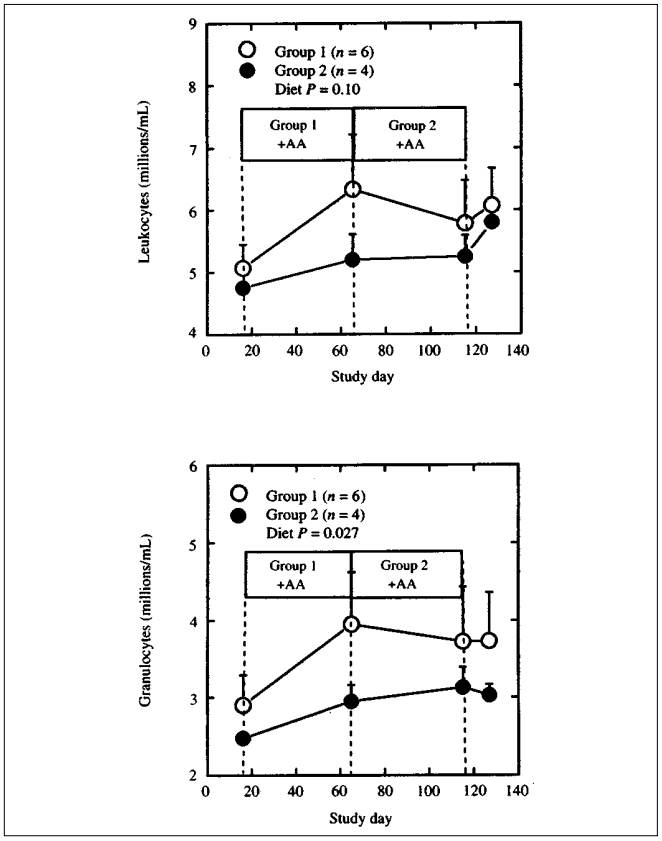

FIG. 1. Effect of low- and high-arachidonic acid-(AA) diets on the number of circulating leukocytes and granulocytes. Mean ± SEM. Group 1 was fed high-AA diet between days 16 and 65 and group 2 between days 66 and 115; low-AA diet was fed for all other days. Crossover analysis of variance model for the rank transformed data showed a significant (P = 0.027) difference between the number of circulating granulocytes when subjects were fed the high- vs. the low-AA diets, while the increase in the number of leukocytes was not significant (P = 0.1).

TABLE 2
Fatty Acid Composition (wt%) of Experimental Diets[a]

Fatty acid	Low-AA diet	High-AA diet
12:0	0.73 ± 0.18	0.68 ± 0.10
14.0	2.43 ± 0.65	2.38 ± 0.60
14:1n-5	n.d.	0.14 ± 0.05
15:0	0.30 ± 0.07	0.29 ± 0.07
16:0	15.11 ± 1.53	14.98 ± 1.50
16:1n-9	0.83 ± 0.13	0.80 ± 0.13
17:0	0.32 ± 0.04	0.31 ± 0.03
18:1n-9 DMA	0.24 ± 0.04	0.23 ± 0.03
18:0	7.43 ± 0.71	7.47 ± 0.64
18:1n-7 *trans*	6.14 ± 0.85	5.92 ± 0.83
18:1n-9	34.64 ± 1.46	31.94 ± 1.40
18:1n-7	1.50 ± 0.07	1.42 ± 0.07
18:1n-5	1.45 ± 0.30	1.44 ± 0.29
18:2 *trans-trans*	0.50 ± 0.06	0.55 ± 0.07
18:2n-6	23.77 ± 2.01	24.07 ± 1.86
18:3n-6	0.37 ± 0.01	0.39 ± 0.01
18:3n-3	1.97 ± 0.29	1.98 ± 0.27
20:1n-11	0.25 ± 0.02	0.25 ± 0.01
22:0	0.26 ± 0.01	0.30 ± 0.02
20:4n-6	0.24 ± 0.02	2.19 ± 0.11
Total	98.50 ± 0.03	97.72 ± 0.17
Unknowns	1.50 ± 0.03	2.28 ± 0.17

[a]Mean ± SEM (n = 5); n.d. = none detected; DMA = dimethyl acetal of (9*Z*)-octadecenal. See Table 1 for abbreviation.

Proliferation of PBMNC cultured with antigens and mitogens. Proliferation of PBMNC cultured with three different dilutions (1:250, 1:500, and 1:1000) of influenza vaccine are shown in Figure 2. This figure shows that prior to *in vivo* immunization the proliferation of PBMNC cultured with all three dilutions of the vaccine did not differ between the two groups, and that it significantly increased in both groups following *in vivo* immunization on day 92. The increases in PBMNC proliferation were, however, different between the two groups. For Group 1 consuming the low-AA diet, the maximum increase in proliferation caused by the influenza vaccine was about 100%, with P values of 0.355, 0.047, and

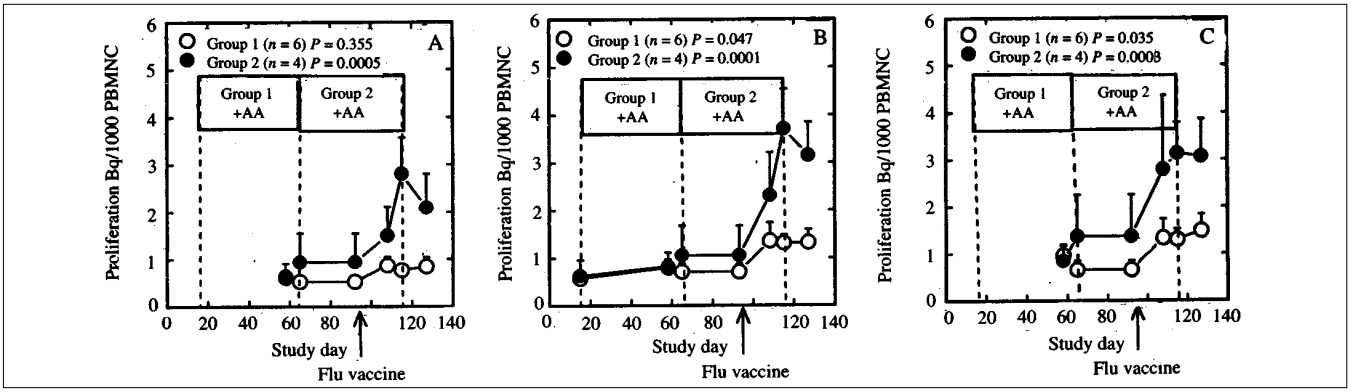

FIG. 2. Effect of low- and high-AA diets on the proliferation of peripheral blood mononuclear cells (PBMNC) cultured with influenza vaccine. X ± SEM. Diet schedule same as in the legend to Figure 1. Analysis of variance showed no difference between the two groups prior to the *in vivo* immunization on day 92. After the *in vivo* immunization, proliferation increased in both groups, and the increase was significantly ($P = 0.02$) higher in group 2 than in group 1. See Figures 1 and 2 for other abbreviations. Dilutions: (A) 1:1000 (B) 1:500 (C) 1:250.

0.035 at the vaccine dilutions of 1:1000, 1:500, and 1:250, respectively. For Group 2, which was consuming the high-AA diet, the maximum increase in PBMNC proliferation in response to influenza vaccine was about 400% and the *P* values for the three dilutions of the vaccine were 0.0005, 0.0001, and 0.0003. The difference between the two groups in PBMNC proliferation *in vitro* after *in vivo* immunization with the influenza vaccine was significant ($P = 0.02$). The three dilutions of MMR vaccine that were tested (1:1000, 1:500, and 1:250) failed to stimulate *in vitro* PBMNC proliferation above the basal level both at pre- and postimmunization with this vaccine (not shown).

Figure 3 contains the data regarding proliferation of PBMNC cultured with three different mitogens: PHA and Con A (20 mg/L) and pokeweed (0.05 mg/L). These concentrations caused the maximum proliferation of PBMNC for each mitogen. This figure shows that PBMNC proliferation in response to PHA and Con A was different in the two groups at the start of the study; however, by the end of the study, the proliferation levels in the two groups were not different. There was no effect of dietary AA on PBMNC proliferation in response to all three mitogens in both groups. The basal diet, however, caused significant increases in PBMNC proliferation in response to Con A and pokeweed in both groups and PHA in Group 2 only. Results obtained with the second concentration of these mitogens (10 mg/L for PHA and Con A, and 0.005 mg/L for pokeweed, not shown) were similar to those shown in Figure 3. The proliferation of PBMNC cultured without mitogens was about 1% of that obtained with pokeweed (not shown). No effect of AA or basal diet could be detected on the proliferation of PBMNC cultured without mitogens.

DTH. All 10 subjects responded to tetanus and mumps, six to candida, three to trichophyton, and two to each of streptokinase, tuberculin, and coccidioidin.The cumulative induration scores determined 48 h after the application of DTH antigens for Group 1 at day 65 and day 115 were 20.1 ± 4.2 and 46.6 ± 13.3 mm (mean ± SEM), and for Group 2 were 31.9 ± 6.1 and 54.2 ± 10.1 mm. The corresponding antigen scores (number of antigens testing positive) for Group 1 were 2.33 ± 0.42 and

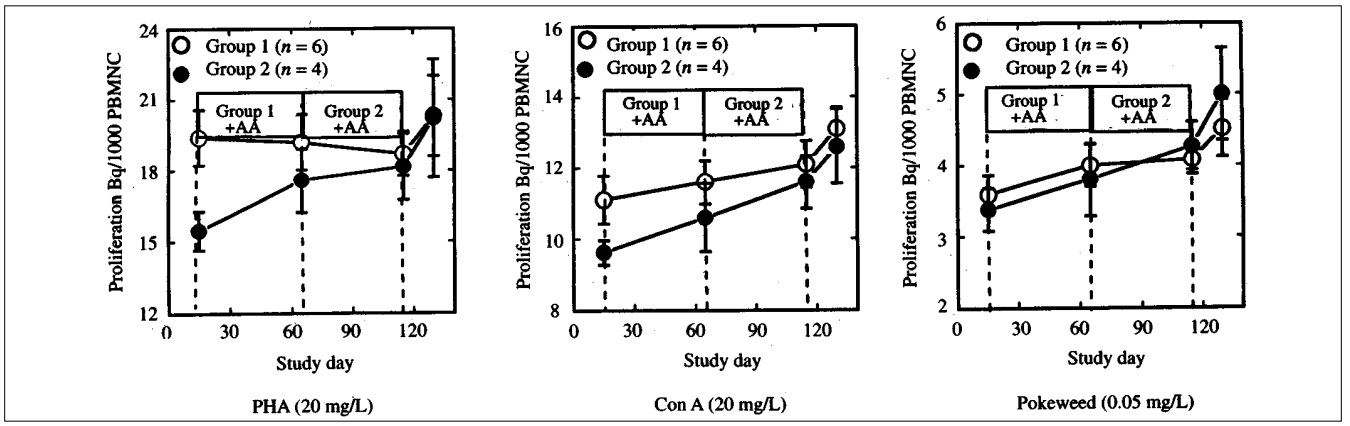

FIG. 3. Effect of low- and high-AA diets on the proliferation of PBMNC cultured with mitogens. X ± SEM. Diet schedule same as given in the legend to Figure 1. Analysis of variance showed no effect of AA on all variables included in this figure; however, there was a significant ($P < 0.05$) increase with time for all variables except the phytohemagglutinin response in group 1.

3.17 ± 0.6, and for Group 2 were 3.25 ± 0.48 and 3.75 ± 0.85, respectively. In both groups, induration and antigen scores on day 115 were higher than those on day 65. This was perhaps caused by the low-fat basal diet. There was no difference in the induration and antigen scores between the subjects fed the high- or low-AA diet. Induration results obtained at 72 h were similar to those at 48 h in Group 1, but in Group 2 the difference between the induration found on days 65 and 115 was not significant (not shown).

NK cell activity. In both groups of subjects, the basal level (unstimulated) NK cell activities determined at effector/target ratios of 50:1, 25:1, 12.5, 6.25:1, and 3.1:1 did not change during the study from their values at study day 16 (not shown). These data suggest that NK cell activities were not affected by the low- and high-AA diets. Incubation of NK cells *in vitro* with IL-2 and INFγ caused a 30–35% increase in the percent lysis caused by them at all effector/ target cell ratios when compared to the corresponding percent lysis caused by nonstimulated cells. However, again there was no effect of the low- or high-AA diet on NK cell activity.

DISCUSSION

In this study, we attempted to define the effect of dietary AA on human IR by feeding a natural triglyceride of AA. We had intended to feed AA at 3.0 g/d for 50 d, but in order to minimize health risk to the subjects, the Human Use Committee approved only 1.5 g/d. The study was designed assuming that there will be no period effect (effect of basal diet or that of the residual AA fed in the previous period), and that data from all subjects could be pooled. However, the statistical analysis of the data showed that for several of the variables there were significant period effects, for which the data from two groups had to be analyzed separately. Our results show that dietary AA significantly increased the number of circulating granulocytes, and the secondary response to influenza vaccine. Several other indexes of IR, including DTH skin response, PBMNC proliferation in response to PHA, Con A, and pokeweed, NK cell activity, number of circulating lymphocytes bearing markers for B and T cells, helper, suppressor, cytotoxic, and NK cells were not affected by the additional intake of AA. The cumulative effect of AA used in this study may actually be lower than that of AA-rich diets based on eggs and meats eaten for years. Lymphocyte proliferation in response to mitogens, and the number of circulating leukocytes were increased by both the low- and high-AA diets when compared to the corresponding values at the start of the study (day 16). The DTH response at day 115 was also significantly higher than the corresponding values on day 65 in both groups of subjects. Some of these changes may be due to the low-fat nutritionally balanced basal diet, as we previously observed (1,2).

We are not aware of any human or animal studies where AA consumption has been reported to increase the number of circulating granulocytes (neutrophils). However, injection of AA metabolites [prostaglandin E (PGEs) and leukotrienes] into human skin induced a dose-dependent erythema by increasing the supply of blood to the inflamed site (27–29). The circulating neutrophils are thought to be in dynamic equilibrium with their marginating pools. Neutropenia can be caused by inflammatory factors like endotoxin and C5a (30,31), and neutrophilia can be caused by exercise or adrenaline infusion (32). Leukotriene B_4 (LTB_4) is a chemoattractant for neutrophils, and addition of AA *in vitro* has been found to enhance neutrophil chemotaxis and diapedesis (33). The increase in the consumption of AA in our study may have increased the circulating levels of LTB_4 and other chemoattractant AA metabolites, which may have led to the increase in the number of circulating neutrophils. There also appears to be an AA-independent increase in the number of circulating neutrophils, which was the difference in the values at the end of the low-AA diet and at the end of the stablization period. This increase could have been caused by several factors, including a latent infection, confinement stress, increased exercise, and the effects of dietary components other than AA. We believe it was most likely due to the daily four-mile walks, as exercise has been reported to increase the number of circulating neutrophils (32).

Prior to immunization with the influenza vaccine, the proliferation of PBMNC stimulated with the same vaccine *in vitro* was comparable between the subjects fed the low- and high-AA diets (Fig. 2). However, following *in vivo* immunization, the *in vitro* proliferation in response to this vaccine was significantly higher in the group fed the high-AA diet than in the group fed the low-AA diet (Fig. 2). Thus, AA effect on PBMNC proliferation in response to influenza vaccine seems to be on the secondary rather than on the primary response of lymphocytes. Whether the increased lymphocyte proliferation will translate into increased antibody production cannot be determined from our data because we did not examine *in vitro* cell differentiation or antibody production. Enhancement of secondary antibody response to keyhole limpet hemocyanin has been reported by the addition of AA to rabbit popliteal lymph node cells *in vitro* (34). Similarly, in humans the secondary antibody response to influenza A-victoria was significantly enhanced by indomethacin, an inhibitor of AA metabolism through the cyclooxygenase pathway (35). These results imply that the secondary antibody response was enhanced by the increased production of lipoxygenase products. We believe the difference in PBMNC proliferation in response to influenza vaccine between the two groups was due to stimulation of this response by AA in Group 2. However, it is also possible that it is due to an inhibition by the residual AA in Group 1. Our study design cannot distinguish between these two possibilities.

The effect of AA on PBMNC proliferation was specific for influenza vaccine, because no stimulation was found when PBMNC were stimulated with MMR, PHA, Con A, or pokeweed. The failure to find an AA effect on MMR vaccine may simply be due to our failure to detect the effect of this vaccine or the AA effect may be antigen-specific. We do not have a specific explanation for the failure of MMR to stimulate PBMNC proliferation *in vitro* after immunization with the same vaccine *in vivo*; it may a defective vaccine or the way

of our testing. The lack of an AA effect on lymphocyte proliferation in response to polyclonal mitogens may be due to a relatively much higher stimulation by the mitogens compared to the stimulation caused by a specific antigen.

Our results showing no effect of dietary AA on proliferation of PBMNC cultured with mitogens are at variance to those results showing stimulation or inhibition of this response by the *in vitro* addition of AA to human lymphocytes and monocytes (36–38). In these studies AA concentrations up to 1.0 μg/mL enhanced the PHA-stimulated proliferation of human PBMNC cultured in lipid-poor medium (36); concentrations higher than 5 μg/mL inhibited their growth (37,38). The discrepancy between our results and those from previous studies may be due to the differences in the effects of free fatty acids (36–38) vs. those of the natural triglycerides (our study) and due to the differences in the concentrations and the metabolism of AA.

Previous studies have examined the effect of LA-rich diets on human IR, but not that of AA-rich diets. In a study with total dietary fat of 25 en%, increasing the LA content to 13 en% for 11 wk had no adverse effects on the IR of healthy men (1). Similar results with LA were obtained in a study with healthy women (2). LA is metabolized rapidly in humans, with only a limited conversion to AA, and thus should not have the same physiologic effects as the dietary AA. Direct comparisons between different studies should be made with caution, because several factors including total fat, chain length of different fatty acids and the ratios between them, antioxidant and other nutrient status, duration of feeding, and nutritional and health status of subjects determine the overall effects of diets on IR. Furthermore, not all indices of IR are affected equally by a given nutrient—as in the present study some indexes were stimulated (PBMNC proliferation with influenza vaccine), and others were unaffected by AA supplementation.

Infections of the upper respiratory tract were found in some individuals during the study; however such symptoms did not show any definite pattern and were randomly distributed in both dietary groups. At no time during the study did the basal (unstimulated) level of PBMNC proliferation for any given subject show more than two-fold variation. These observations indicate that the stimulation of IR was caused by AA and not by any latent viral infection. Furthermore these effects were not seen in the group devoid of AA, which also indicates that those were not caused by the stress of confinement at the metabolic unit. These observations indicate that the feeding of AA for a limited period did not significantly increase their risk for infections, although it may be increased in studies involving longer duration of AA feeding.

Even with the small number of subjects in the study (six in group 1; four in group 2), the results are encouraging, because there were no adverse effects of increased AA consumption as indicated in a previous study where esters of AA were fed (9). In our study, none of the indexes of IR tested were inhibited by AA feeding. Two of the indexes were significantly increased by AA, but the clinical significance of this increase is uncertain. It is possible that some other indexes would have been significantly affected, if AA was supplemented for more than 50 d or if there were more subjects in the study. These issues will be addressed in future studies with AA. Findings presented in this paper show neither any benefit from increased AA consumption by the healthy subjects nor any health risk if increased AA consumption was needed in cases of essential fatty acid deficiency. Further studies are needed before recommendations regarding daily intake of AA can be made.

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