Immunologic Effects of National Cholesterol Education Panel Step-2 Diets with and without Fish-derived N-3 Fatty Acid Enrichment

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Abstract

Reductions in dietary fat, saturated fat, and cholesterol have been recommended to reduce the risk of heart disease in our society. The effects of these modifications on human cytokine production and immune responses have not been well studied. 22 subjects > 40 yr of age were fed a diet approximating that of the current American (14.1% of calories as saturated fatty acids, [SFA], 14.5% monounsaturated fatty acids [MUFA], 6.1% [n-6] polyunsaturated fatty acids [PUFA], 0.8% [n-3] PUFA, and 147 mg cholesterol/1,000 calories) for 6 wk, after which time they consumed (11 in each group) one of the two low-fat, low-cholesterol, high-PUFA diets based on National Cholesterol Education Panel (NCEP) Step 2 recommendations (4.0–4.5% SFA, 10.8–11.6% MUFA, 10.3–10.5% PUFA, 45–61 mg cholesterol/1,000 calories) for 24 wk. One of the NCEP Step 2 diets was enriched in fish-derived (n-3) PUFA (low-fat, high-fish: 0.54% or 1.23 g/d eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA] [121–188 g fish/d]) and the other low in fish-derived (n-3) PUFA (low-fat, low-fish [0.13% or 0.27 g/d EPA and DHA] [33 g fish/d]). Measurements of in vivo and in vitro indexes of immune responses were taken after each dietary period. Long-term feeding of low-fat, low-fish diet enriched in plant-derived PUFA increased blood mononuclear cell mitogenic response to the T cell mitogen Con A, IL-1β, and TNF production and had no effect on delayed-type hypersensitivity skin response, IL-6, GM-CSF, or PGE2 production. In contrast, the low-fat, high-fish diet significantly decreased the percentage of helper T cells whereas the percentage of suppressor T cells increased. Mitogenic responses to Con A and delayed-type hypersensitivity skin response as well as the production of cytokines IL-1β, TNF, and IL-6 by mononuclear cells were significantly reduced after the consumption of the low-fat, high-fish diet (24, 40, 45, 35, and 34%, respectively; P < 0.05 by two-tailed Student’s t test except for IL-1β and TNF, which is by one-tailed t test). Our data are consistent with the concept that the NCEP Step 2 diet that is high in fish significantly decreases various parameters of the immune response in contrast to this diet when it is low in fish. Such alterations may be beneficial for the prevention and treatment of atherosclerotic and inflammatory diseases but may be detrimental with regard to host defense against invading pathogens. (J. Clin. Invest. 1993. 92:105–113) Key words: NCEP Step 2 • n-3 PUFA • n-6 PUFA • immune response cytokines • cholesterol

Introduction

Many organizations have recommended reductions in the dietary fat, saturated acids, and cholesterol to reduce the risk of heart disease (1, 2). The National Cholesterol Education Panel (NCEP) 1 as part of its Step 2 diet has recommended a fat intake of <30% of calories, <7% of calories from saturated fatty acids, 10–15% of calories from monounsaturated fatty acids, and ≤10% of calories from polyunsaturated fatty acids (PUFA), with a cholesterol intake of <200 mg/d. To date, the effect of these recommendations on the immune response has not been well studied, although studies indicate that the quantity and quality of dietary fat can alter the immune response (for review see references 3–6). These include changes in the production of immunologic mediators, i.e., cytokines, prostanoïds, and in vitro lymphocyte proliferation, in response to mitogens. Some of these same mediators have been implicated in the pathogenesis of atherosclerosis (7–10).

We therefore studied the effect of long-term (24-wk) feeding of low-fat, low-cholesterol, moderately high PUFA diets (NCEP Step 2) with or without fish-derived (n-3) PUFA on in vitro and in vivo indexes of the immune response of healthy normolipidemic volunteers. We report here that the long-term feeding of NCEP Step 2 diets enriched in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) decreases ex vivo cytokine production, proliferative response of peripheral blood mononuclear cells to the T cell mitogen Con A, percentage of T helper cells, and the delayed-type hypersensitivity skin test, and an in vivo measure of cell-mediated immunity. In contrast, feeding NCEP Step 2 diets low in EPA and DHA but moderately high in plant derived (n-6) and (n-3) PUFA increased mitogenic response to Con A and ex vivo IL-1β and TNF production and had no significant effect on other parameters measured.

Methods

Subjects

22 normolipidemic and normotensive volunteers (11 women and 11 men) over the age of 40 underwent a complete medical history and physical examination. The subjects had no evidence of any chronic illness including endocrine, hepatic, renal, thyroid, or cardiac dysfunc-

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1. Abbreviations used in this paper: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; NCEP, National Cholesterol Education Panel; PUFA, polyunsaturated fatty acids.
Table I. Subject Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Low-fat, high-fish</th>
<th></th>
<th>Low-fat, low-fish</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subject number</strong></td>
<td>3</td>
<td>7</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td><strong>Age (yr)</strong></td>
<td>66±5</td>
<td>65±2</td>
<td>56±6</td>
<td>71±2</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>79±3</td>
<td>60±2</td>
<td>74±3</td>
<td>73±6</td>
</tr>
<tr>
<td><strong>Body mass index</strong></td>
<td>26.2±0.9</td>
<td>25.5±3.8</td>
<td>24.8±2.0</td>
<td>27.1±4.3</td>
</tr>
</tbody>
</table>

Mean±SE.

...tion. They did not smoke nor were they taking medications known to affect plasma lipid levels (cholestyramine, colestipol, niacin, clofibrate, gemfibrozil, probucol, lovastatin, beta blockers, thiazide, diuretics, diphenyl-hydantrin, cis-retinoic acid, ascorbic acid, estrogens, proges-
tins, anabolic steroids, hydrocortisone, fish-oil capsules, or thyroxine) or nonsteroidal antiinflammatory drugs such as aspirin and indometh-
acin. Compliance was monitored by measurement of plasma total fatty
acid levels every 4 wk as described below. One male subject was ex-
cluded from the study because his plasma fatty acid profile did not
show the anticipated changes. The age, weight, and body mass index
for subjects are shown in Table I. No attempt was made to sex match
the subjects for the two dietary periods. Previous work has shown that
fish-oil supplementation induces similar changes in cytokine produc-
tion in males and females (11, 12).

Experimental protocol

The experimental protocol was approved by the Human Investigation
Review Committee of the New England Medical Center and Tufts
University. The 30-wk study period was divided into two diet phases
(Fig. 1). All food was provided by the study. The first phase was a 6-wk
period during which the subjects consumed a diet approximating that
of the current American (baseline). The second phase was a 24-wk
period during which subjects either consumed a low-fat, low-choles-
terol diet conforming to the NCEP Step 2 recommendations enriched
in EPA and DHA (0.54% of calories or 1.23 g/d) (low-fat, high-fish)
(Table II) (n = 11) or consumed the same diet low in EPA and DHA
(0.13% of calories or 0.27 g/d) (low-fat, low-fish) (Table II) (n = 11).
The rest of the (n-3) PUFA in the two diets was provided as linolenic acid
(18:3n-3). First, 11 subjects completed the baseline diet and low-fat,
high-fish phases. Then 1 subjects (8 new and 3 who had completed the
high-fish diet) completed the baseline and low-fat, low-fish phases. The
subjects entered the study at different time points to avoid seasonal
effects. A maximum break of 4 wk could be taken by study subjects
between the baseline and low-fat diet phases. The three subjects who
participated in both dietary treatments did so with ≥ 6-mo intervals
between the two studies. All diets were prepared in the Metabolic Re-

Table II. Fat Composition of Baseline and Low-Fat Diets Low
and High in Fish*

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Baseline</th>
<th>Low-fat, high-fish</th>
<th>Low-fat, low-fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of calories</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>35.4±2.3</td>
<td>26.4±2.0</td>
<td>25.2±0.8</td>
</tr>
<tr>
<td>Saturated</td>
<td>14.1±2.2</td>
<td>4.5±0.7</td>
<td>4.0±0.4</td>
</tr>
<tr>
<td>Mono-unsaturated</td>
<td>14.5±1.0</td>
<td>11.6±1.4</td>
<td>10.8±0.4</td>
</tr>
<tr>
<td>Poly-unsaturated</td>
<td>6.9±1.2</td>
<td>10.3±0.2</td>
<td>10.5±0.2</td>
</tr>
<tr>
<td>n-6</td>
<td>6.1±1.0</td>
<td>8.0±0.9</td>
<td>8.8±0.4</td>
</tr>
<tr>
<td>n-3</td>
<td>0.8±0.6</td>
<td>2.4±0.3</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>7.3</td>
<td>3.6</td>
<td>4.9</td>
</tr>
<tr>
<td>n-6/EPA + DHA</td>
<td>200</td>
<td>12.5</td>
<td>50</td>
</tr>
</tbody>
</table>

Mean±SD; n = 3. * Chemical analysis performed by Hazeltone Labor-
atories, except for EPA and DHA, which were calculated using
USDA Provisional Tables (13). † EPA, eicosapentaenoic acid; DHA,
docosahexaenoic acid.

...search Unit kitchen at the USDA-Human Nutrition Research Center
on Aging at Tufts University and were composed entirely of natural
foods consumed as three meals and one or two snacks per day.1

A 3-d cycle menu consisting of food items normally consumed by
average Americans was designed to meet the requirements of each di-
etary period. The menu consisted of breakfast, lunch, dinner, and one
or two snacks. A typical menu for baseline period included coffee,
cereal, whole milk, or bagel and cream cheese or white bread and but-
ter, canned fruits and orange juice for breakfast; mixed salad, juice,
turkey or roast beef for lunch; crackers, peanut butter, and whole milk
for afternoon snack; corn, carrots, salad, beverage, sirloin steak,
chicken or filet of sole, and bread for dinner; and strawberries, yogurt,
or applesauce for an after dinner snack. The amount of cholesterol and
fat and composition of fat was adjusted by inclusion of butter, heavy
cream, olive oil, mayonnaise, soybean oil, walnuts or walnut oil, and
egg yolks. At this phase, fish was served about two times per week (200
g/wk), red meat six times per week, and chicken or turkey about four
times per week.1

A typical menu for the low-fat, high-fish period included cereal,
cracker, coffee, skim milk, bagel or white bread, and jelly for breakfast;
tuna, turkey breast, salad or vegetable soup, bread or crackers, fruit,
fruit juice, or beverage for lunch; filet of sole or salmon or chicken,
vegetables, salad, juice, fruits, and walnuts for dinner; peanut butter,
crackers, skim milk, and fruits for snacks. At this phase, fish was served
eight times per week (121–188 g/d) and turkey or chicken four times
per week.

Typical menu for the low-fat, low-fish period included fruits, cof-
fee, fruit juice, cereal, bagel or bread with soybean margarine and jelly,
as well as margarine and jelly or skim milk for breakfast; vegetable soup
or salad, tuna or turkey breast or nonmeat chilli, fruits, juice, and bread
for lunch; turkey, vegetables, salad, bread, and walnuts for dinner;
and peanut butter, crackers, fruit, and skim milk for snacks. At this phase,
fish was served about two times per week (200 g/wk) and turkey about
eight times per week. To increase the level of 18:3n-3 soybean margarine,
rapeseed oil and walnuts were used.

Subjects were required to report to the Metabolic Research Unit a
minimum of five times per week and to eat at least one meal per visit at
the unit. All other food and drink was packaged for take-out. A dieti-
tian was on call at all times in the event that problems arose. A supply
of an entire 3-d food cycle was provided for the volunteers to accommo-
date emergency situations that might have prevented a volunteer from
picking up the scheduled meals. During each visit body weight and
blood pressure were measured. Calorie levels were assigned so that
the subjects neither gained nor lost weight. The Grand Forks database
(Grand, release 8606) was used to calculate the nutrient composi-

Figure 1. Experimental design.
tion of the diets and analytical data from Hazelden Laboratories (Madison, WI) was used as confirmation, except for EPA and DHA, which were calculated using USDA Provisional tables (13). The diets had similar percentages of protein and carbohydrates, which were 15±±1.2 and 49±±2.2% for baseline; 17±±0.9 and 56±±2.9% for low-fat, high-

fish; and 17±±0.1 and 57±±0.8% for low-fat, low-fish diets, respectively. Fat composition of diets is shown in Table II. Cholesterol content of the three diets was 147±±27.0, 61±±15.9, and 45±±9.6 mg/100 kcal for baseline; low-fat, high-fish; and low-fat, low-fish diets, respectively.

Blood samples were collected after a 12-h fast during weeks 4, 5, and 6 of the baseline diet period and weeks 22, 23, and 24 of either of low-fat diet periods for in vitro immunologic and biochemical measurements (Fig. 1). When the blood collections for each period were completed subjects were administered a delayed-type hypersensitivity skin test as described below.

Delayed-type hypersensitivity skin test. Delayed-type hypersensitivity skin test was assessed with Multi Test-CMI (Merieux Institute, Inc., Miami, FL), with a single-use disposable applicator of acrylic resin with eight heads loaded with glycine control and the following seven recall antigens: tetanus toxoid, diphtheria toxoid, Streptococcus (group C), Mycobacterium tuberculosis, Candida albicans, Trichophyton me-
taphytes, and Proteus mirabilis. The diameter of positive reactions was measured at 24 and 48 h after administration of the test. The antigen score was calculated as the total number of positive antigens and the cumulative score was calculated as the total diameter of induration of all positive reactions. An induration of ≥ 2 mm was considered positive. If a positive reaction to the glycine control was observed, the diameter of its induration was subtracted from all the other positive reactions. The test was administered by the same nurse for both the baseline and low-fat, high-and-low fish periods for each subject and the diameter of induration was measured by the same person at the end of each dietary period. The Multi Test-CMI rather than the conventional skin test was used to avoid the boosting effect observed with repeated administration (14).

Isolation of mononuclear cells. Peripheral blood mononuclear cells were separated from heparinized blood as previously described (11). Cells were washed twice in RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mmol/l L-glutamine, and 25 mmol/l Hepes (Gibco Laboratories, Grand Island, NY). RPMI 1640 used in this study was subjected to ultrafiltration to remove endotoxin as previously described (15). Cells were resuspended in medium and counted under a light microscope. Cell viability was assessed using trypsin blue exclusion. Cells were then suspended at appropriate concent-

rations for measurement of mitogenic lymphocyte proliferation, induction of cytokines, and PGE2 synthesis. A sample of heparinized plasma was heat inactivated at 56°C for 30 min to be used as autolo-

gous plasma in the cell cultures. Autologous plasma rather than fetal bovine serum was used as this is rich in AA and can influence fatty acid composition of cell membrane.

Lymphocyte proliferation. Lymphocyte proliferation was measured by [3H]thymidine incorporation after stimulation with T and B cell mitogens. Dilutions of mitogens from 10 to 300 µg/ml for Con A (Sigma Chemical Co., St. Louis, MO), PHA (Difco Laboratories, De-

troit, MI) and B cell mitogen Staphylococcus aureus Cowan I (Zy storbin 10% suspension; Zymed Laboratories Inc., San Francisco, CA) were prepared in endotoxin-free RPMI 1640 with 10% autologous plasma and optimal dilution for each mitogen was determined. 100 µl of each mitogen was added into triplicate into 96-well, flat-bottomed microtiter plates (Becton-Dickinson & Co., NJ). Cells were counted at 104 cells/ml in RPMI 1640, 100 µl of cell suspension was then added to wells with and without mitogens and incubated for 72 h at 37°C in an atmosphere of 5% CO2 and 95% humidity. 4 h before termination of incubation, 0.5 µCi of [3H]thymidine (specific activity 6.7 Ci/mmol; New England Nuclear, Boston, MA) in 20 µl was added to each well. Cells were harvested onto glass microtiter filter paper using a cell harvester (BDH, Cambridge, MA). Filter disks were placed in mini vials and counted in a liquid-scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). The results are reported as corrected cpm: the average cpm of mitogen-stimulated cultures minus the average cpm of cultures without mitogens. The scintillation counter had an efficiency of 45% for tritium.

IL-2, IL-6, and GM-CSF. 104 cells/ml in RPMI 1640 with 10% autologous plasma (final concentration) were cultured in 24-well flat-

bottomed plates (Becton Dickinson & Co.) with Con A (10 µg/ml final concentration) for 48 h. Cell-free supernatant was stored at −70°C for later analysis of IL-2, IL-6, and GM-CSF. IL-2 activity was measured using a microassay method described by Gillis et al. (16). Recombi-

nant human IL-2 (Genzyme Corp., Boston, MA) was used as standard. 1 U/ml was defined as the amount of recombinant IL-2 that causes a half-maximal incorporation of [3H]thymidine in 5 × 103 cytotoxic T cell line (CTL) cells in culture. IL-2 activity was calculated using probit analysis (15). CTL cells were a gift from Dr. S. Gillis of Im-

munex, Inc. (Seattle, WA). IL-6 and GM-CSF were measured by RIA as previously described (17, 18).

IL-1 and TNF. Mononuclear cells were suspended at 5 × 104 cells/ml in RPMI with 2% autologous plasma (1% final concentration). This cell suspension (0.5 ml) was added to 0.5 ml RPMI or RPMI containing heat-killed Staphylococcus epidermis (S. epi.) (at 20 organisms per cell) for 24 h. The plates were frozen until the end of the study, at which time all plates from each donor were thawed and exposed to two more freeze-
thaw cycles to complete cell lysis (19). The contents of wells, consisting of cell lysates and supernatants, were analyzed by RIA for IL-1β and TNF as previously described (19, 20). Each RIA is specific for the respective cytokine and does not cross-react with IL-1, GM-CSF, IFN-α, -β, or γ.

PGE2 production. Mononuclear cells (106 cells/ml) were cultured in 24-well flat-bottomed plates (Becton Dickinson & Co.) in the presence or absence of different concentrations of PHA and 10% autologous 
plasma for 48 h in a 37°C, 5% CO2 humidified incubator. Cell-free supernatant was saved at −70°C for PGE2 analysis by RIA as described by McComb et al. (21). PGE2 antibody was a gift from Dr. J. Dupont of Iowa State University and Dr. M. Mathias of Colorado State University. The antibody cross-reactivity and specificity have been described (22). Cytokine and PGE2 RIA as well as mitogen responses were performed on coded samples by blinded technicians.

Complete blood count, white cell differential, and flow cytometric analysis. Complete blood count was obtained using a hematology analy-

zer (model Baker 9000; Serono-Baker Instrument Inc., Allentown, PA) (23) and white cell differential was assessed by microscopic exam-

ination of blood smears after Wright-Giemsa staining. For flow cytomet-

ric analysis, cells were suspended at a concentration of 107 cell/ml in cold PBS with 0.1% sodium azide and 2% fetal calf serum. 50 µl of suspended cells was incubated with 10 µl each of the following monoclonal antibodies: anti-Leu-3a FITC (CD4+), anti-Leu-2a FITC (CD8+), and anti-Leu-4 PE (CD3+) (Becton Dickinson & Co.) for 30-45 min on ice. After incubation, 2 ml of cold PBS (containing 0.1% sodium azide and 2% fetal calf serum) was added to the cell and gently vortexed. Cells were centrifuged at 300 g for 5 min at 2-8°C. The washing procedure was repeated two more times with 1 ml PBS. Cells were then resuspended in 0.5 ml PBS with 0.1% sodium azide and 2% fetal calf serum and kept on ice until flow cytometric analysis by FACS®CAN (Becton Dickinson & Co.) as described (24). Gates were set at 2. The ratio of forward scatter to side scatter was 6.

Plasma tocopherol. Plasma samples were saved under nitrogen at −70°C for tocopherol analysis. Plasma was analyzed by a modified HPLC method of Bieri et al. (25) as previously described (26).

Fatty acid analysis. The transesterification and analysis of plasma fatty acids were performed as previously described (11). The fatty acids methyl esters of over 40 fatty acids (C13-0 and C28-0, including cis- and trans-configurations) are separated by this method. Peaks were identified and the procedure was validated by chromatography of mixtures of authentic fatty acids methyl esters. Data were normalized by comparing the areas of the fatty acids peaks with the area of the internal standard peak, heptadecanoic acid, after correction for the various re-

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### Table III. Effect of Long-Term Consumption of Low-Fat Diets Low and High in Fish on Plasma Fatty Acid Composition

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Baseline</th>
<th>Low-fat, high-fish</th>
<th>P</th>
<th>Baseline</th>
<th>Low-fat, low-fish</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% normalized*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>32.00±1.10</td>
<td>33.7±0.66</td>
<td>0.003</td>
<td>35.93±1.40</td>
<td>34.91±1.42</td>
<td>0.47</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.60±0.03</td>
<td>1.2±0.07</td>
<td>0.0002</td>
<td>0.48±0.06</td>
<td>1.03±0.16</td>
<td>0.001</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>8.20±0.47</td>
<td>5.9±0.30</td>
<td>0.0001</td>
<td>8.32±0.29</td>
<td>7.31±0.52</td>
<td>0.003</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.70±0.10</td>
<td>2.8±0.23</td>
<td>0.0001</td>
<td>0.54±0.05</td>
<td>0.83±0.11</td>
<td>0.004</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>3.00±0.23</td>
<td>5.2±0.30</td>
<td>0.0001</td>
<td>1.95±0.10</td>
<td>2.45±0.24</td>
<td>0.08</td>
</tr>
<tr>
<td>Total n-3</td>
<td>4.27±0.25</td>
<td>9.23±0.49</td>
<td>0.0000</td>
<td>2.95±0.19</td>
<td>3.94±0.38</td>
<td>0.002</td>
</tr>
<tr>
<td>AA/EPA</td>
<td>12.12±0.51</td>
<td>2.12±0.25</td>
<td>0.0001</td>
<td>16.45±1.02</td>
<td>10.30±1.30</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* Mean±SE; numbers represent percentage of identified fatty acids (see text).

### Results

**Plasma fatty acids.** Compliance was assessed in part by monitoring plasma fatty acids. The low-fat, high-fish diet produced significant increase in plasma linoleic acid (18:2n-6) (5%, P = 0.003), linolenic acid (100%, P = 0.002), EPA (20:5n-3) (313%), DHA (22:6n-3) (73%), and total (n-3) PUFA (116%) as well as a significant decrease in AA (18:4n-6) (28%) (P = 0.0001 for all) (Table III) and in the AA/EPA ratio (82%, P = 0.0001) (Table III). Plasma saturated fatty acids (14:0, 16:0, 18:0) and monounsaturated fatty acids (18:1n-9) were significantly decreased (data not shown). Of the 11 subjects who completed the low-fat, high fish diet, only one did not show the expected change in fatty acid profile, indicating noncompliance. His data were excluded from all calculations. No significant change in linoleic or DHA was observed in subjects consuming the low-fat, low-fish diet. However, we observed a significant increase in plasma linoleic (115%, P = 0.001), EPA (54%, P = 0.004), and total (n-3) PUFA (34%, P = 0.002) and a decrease in AA (12%, P = 0.003) and in the AA/EPA ratio (37%, P = 0.002) (Table III). Plasma myristic (14:0) and stearic (18:0) fatty acids were significantly decreased while the 18:1n-9 level was significantly increased (3%) (data not shown). Detailed plasma fatty acids and lipoprotein analysis will be reported separately (28, 29). The mean reduction by low-fat, high-fish diet in total cholesterol, LDL, HDL, and triglycerides was 14, 15, 12, and 7%, respectively. The mean reduction by low-fat, low-fish diet was 19, 20, and 16% in total cholesterol, LDL, and HDL, and there was 5% increase in triglycerides. No significant change in total cholesterol/HDL ratio was observed.

**Plasma tocopherol.** Plasma tocopherol concentrations are shown in Table IV. No significant change in plasma tocopherol level was observed after consumption of low-fat, high-fish diet. A small (5%) but significant decrease was noted in subjects consuming low-fat, low-fish diet. The significant decrease is lost if tocopherol concentration is expressed as a function of plasma total cholesterol and triglycerides. This is a reflection of changes in plasma cholesterol and triglyceride after the consumption of low-fat diets (28, 29). Since the function of tocopherol is to protect PUFA from oxidation, we felt it was more appropriate to evaluate changes in plasma tocopherol level as a function of changes in plasma PUFA. When this was done both low-fat diets caused a small decrease in tocopherol/PUFA ratio (6%, P = 0.08 for low-fat, low-fish diet and 10%, P = 0.04 for low-fat, high-fish diet) (Table IV).

### Table IV. Effect of Low-Fat Diets High and Low in Fish on Plasma Tocopherol Concentration*

<table>
<thead>
<tr>
<th>Plasma tocopherol</th>
<th>Baseline</th>
<th>Low-fat, high-fish</th>
<th>P</th>
<th>Baseline</th>
<th>Low-fat, low-fish</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/dl plasma</td>
<td>943±64</td>
<td>979±80</td>
<td>0.14</td>
<td>1025±65</td>
<td>959±71</td>
<td>0.02</td>
</tr>
<tr>
<td>µg/total chol + TG³</td>
<td>3.1±0.2</td>
<td>3.6±2.1</td>
<td>0.01</td>
<td>3.3±0.1</td>
<td>3.4±0.04</td>
<td>0.67</td>
</tr>
<tr>
<td>µg/no. of double bonds in plasma fatty acid⁴</td>
<td>8.0±0.5</td>
<td>7.2±1.8</td>
<td>0.04</td>
<td>7.1±0.5</td>
<td>6.7±0.6</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* Mean±SE; n = 9. ³ TG, triglyceride; chol, cholesterol. ⁴ Calculated using percent of each fatty acid in plasma multiplied by number of double bonds in each fatty acid.
**Table V. Effect of Low-Fat Diets High and Low in Fish on Ex Vivo Cytokine and PGE₂ Production**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Low-fat, high-fish</th>
<th>Percent change</th>
<th>Baseline</th>
<th>Low-fat, low-fish</th>
<th>Percent change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-1β</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>5.71±1.02</td>
<td>3.44±0.78†</td>
<td>−40</td>
<td>4.28±1.49</td>
<td>6.93±1.64*</td>
<td>62</td>
</tr>
<tr>
<td><em>S. epi.</em></td>
<td>15.64±3.24</td>
<td>11.64±2.74</td>
<td>−25</td>
<td>13.3±1.5</td>
<td>16.32±2.53</td>
<td>23</td>
</tr>
<tr>
<td><strong>TNF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>4.7±0.91</td>
<td>4.36±1.04†</td>
<td>−7</td>
<td>5.79±0.79</td>
<td>8.56±1.67*</td>
<td>47</td>
</tr>
<tr>
<td><em>S. epi.</em></td>
<td>13.9±2.59</td>
<td>8.98±2.04</td>
<td>−35</td>
<td>8.18±1.92</td>
<td>9.56±1.70</td>
<td>17</td>
</tr>
<tr>
<td><strong>GM-CSF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>1.42±0.19</td>
<td>1.02±0.17</td>
<td>−28</td>
<td>1.58±0.24</td>
<td>1.59±0.28</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.37±0.18</td>
<td>0.08±0.02</td>
<td>−78</td>
<td>0.17±0.05</td>
<td>0.17±0.06</td>
<td>0</td>
</tr>
<tr>
<td>PHA (10 μg/ml)</td>
<td>0.76±0.24</td>
<td>0.28±0.08</td>
<td>−63</td>
<td>1.98±1.01</td>
<td>2.59±1.86</td>
<td>30</td>
</tr>
<tr>
<td>PHA (100 μg/ml)</td>
<td>1.31±0.29</td>
<td>0.42±0.11†</td>
<td>−67</td>
<td>2.59±1.85</td>
<td>3.94±2.14</td>
<td>52</td>
</tr>
</tbody>
</table>

Values represent mean±SE. *5 × 10⁵ cells were stimulated with 1 ng/ml LPS or 20 organisms per cell of *S. epi.* for 24 h (three measurements at each time point). †10⁶ cells/ml were cultured with 10 μg/ml Con A for 48 h (three measurements at each time point). §10⁵ cells/ml were cultured in the presence or absence of 10 or 100 μg/ml PHA for 48 h (three measurements at each time point). ‡*P = 0.03 and ‡*P = 0.04 for IL-1β and TNF, respectively, by paired one-tailed Student's t test. *P < 0.05 by paired two-tailed Student's t test.

**PGE₂.** After consumption of the low-fat, high-fish diet, spontaneous and PHA-stimulated cultures produced less PGE₂ compared with the baseline diet period (Table V). The percent decrease in PGE₂ production after the low-fat, high-fish period for spontaneous, 10, and 100 μg/ml PHA was 79, 63, and 68%, respectively. However, these differences were only statistically significant for those cultures stimulated with 100 μg/ml PHA (*P = 0.02). Spontaneous or PHA-stimulated PGE₂ production was not affected by the low-fat, low-fish diet (Table V).

**White blood cell number and subpopulations.** There was no statistically significant change in total number of WBC, lymphocytes, monocytes, or polymorphonuclear leukocytes as indicated by differential cell counts associated with the consumption of either diet (data not shown). The percentage of T cells (CD3+) was not affected by diet. Samples from five of the subjects who consumed low-fat, high-fish diet were available for FACS® analysis; however, all five subjects tested showed a decrease in the percentage of CD4+ cells and an increase in percentage of CD8+ cells after 24 wk of consuming the low-fat, high-fish diet. Subjects fed low-fat, low-fish diet showed a significant decrease in the percentage of CD8+ (*P < 0.05, n = 11) cells and a nonsignificant increase in CD4+ cells (Table VI).

**Cytokine production.** LPS- and *S. epi*–stimulated IL-1β production was reduced after consumption of low-fat, high-fish diet (40% [*P = 0.07 using two-tailed *t* test or *P = 0.035 using one tailed *t* test, n = 9] and 25% [*P = 0.3] decrease, respectively) (Table V). Decrease in *S. epi*–stimulated TNF production was also observed after consumption of the low-fat, high-fish diet (35% decrease, *P = 0.08 using two-tailed *t* test or *P = 0.04 using one-tailed *t* test) (Table V). The low-fat, low-fish diet, however, caused a significant increase in LPS-stimulated IL-1β production (62% increase, *P = 0.003) (Table V) and LPS-stimulated TNF production (47% increase, *P = 0.03) (Table V).

GM-CSF production was not significantly affected by either diet (Table V), whereas IL-6 production was reduced after consumption of the low-fat, high-fish diet (34% decrease, *P = 0.03) (Table V). 7 out of 10 subjects showed a decrease in IL-2 production after consumption of the low-fat, high-fish diet, but the decrease in IL-2 production did not reach statistical significance. The reverse was observed in subjects consuming the low-fat, low-fish diet, i.e., a nonsignificant increase in IL-2 production was observed (data not shown).

**Mitogenic response and delayed-type hypersensitivity skin response.** Mitogenic responses to the T cell mitogens Con A and PHA were reduced after consumption of a low-fat, high-fish diet (24% decrease, *P = 0.03 for Con A and 30% decrease, *P = 0.1 for PHA) (Fig. 2A data and data not shown). Consumption of a low-fat, low-fish diet on the other hand increased mitogenic response (28% increase for Con A, *P = 0.057, and 15% increase for PHA) (Fig. 2B and data not shown). Although mitogenic responses to PHA or Con A in the two dietary groups were not significantly different at baseline (after 6 wk of the baseline diet), subjects consuming the low-fat, high-fish diet had significantly lower mitogenic responses to Con A (56% lower, *P = 0.057) and PHA (53% lower, *P = 0.002) than those who consumed low-fat, low-fish diets. The mitogenic response to the B cell mitogen *S. aureus* Cowan I was not affected by either diet (data not shown). As depicted in Fig. 2C, delayed-type hypersensitivity skin response was affected by the high-fish diet. The induration index was significantly reduced after the low-fat, high-fish diet period (45% decrease, *P = 0.009).

**Table VI. Effect of Low-Fat Diets High and Low in Fish on T Cell Subpopulations**

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n = 5)</th>
<th>Low-fat, high-fish (n = 11)</th>
<th>Percent change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4+</strong></td>
<td>46.3±6.5</td>
<td>40.0±4.8*</td>
<td>14.7±4.1</td>
</tr>
<tr>
<td><strong>CD8+</strong></td>
<td>28.2±7.5</td>
<td>44.3±6.2*</td>
<td>32.7±4.6</td>
</tr>
</tbody>
</table>

Mean±SEM. *P < 0.05 by paired Student's *t* test. †*P < 0.06 by paired Student's *t* test.
Figure 2. Effect of low-fat diets high (A and C) and low (B and D) on lymphocyte proliferation and delayed-type hypersensitivity skin response. For lymphocyte proliferation, $10^6$ PBMC/ml were stimulated with 50 $\mu$g/ml (final concentration) Con A for 72 h (three measurements at each time point). Lymphocyte proliferation was measured by incorporation of $[^3H]$thymidine into DNA after a 4-h pulse. Data represent corrected cpm, which is the cpm of stimulated culture minus cpm of unstimulated culture. There was no difference in cpm of unstimulated cultures between the two different time points. Values represent mean±SE. *Significantly different from baseline values at $P = 0.03$ (A) for high-fish diet and $P = 0.057$ (B) for low-fish diet as determined by paired Student's $t$ test. Delayed-type hypersensitivity skin response was measured by administration of seven antigens in the form of Multi Test CMI (see Methods) after baseline and after each of the low-fat diets. Diameter of induration was measured 48 h after administration. Cumulative index was measured by adding the diameter of all the positive responses (induration diameter of $\geq 2$ mm). Values represent mean±SE. *Significantly different from baseline values at $P = 0.009$ (C) by paired Student's $t$ test.

Nine subjects showed a decrease in the induration index. The total number of positive responses to different antigens was reduced in 50% of the subjects. No significant change in delayed-type hypersensitivity skin response was observed in subjects consuming low-fat, low-fish diet (Fig. 2 D) with 54% of subjects showing an increase in this response. Although the
delayed-type hypersensitivity skin response of the two dietary
groups was not significantly different from each other at base-
line, the delayed-type hypersensitivity skin response of subjects
consuming low-fat, high-fish diet was one half that of those
who consumed low-fat, low-fish diet (P = 0.01).

Discussion

Our results demonstrate that long-term feeding of low-fat, low-
cholesterol moderately high PUFA diets has significant effects
on several parameters of immune and inflammatory responses
of normolipidemic subjects. The nature of the effect, however,
is influenced by the type of PUFA. Diets enriched in (n-3)
PUFA derived from fish significantly reduced delayed-type hy-
persensitivity skin response, mitogenic response to Con A, per-
cent helper T cells, IL-6, IL-1β (one tailed t test), TNF (one
tailed t test), and PGE2 production and caused a nonsignificant
reduction in GM-CSF and IL-2 production. Consumption of
essentially the same diet but low in fish-derived (n-3) PUFA,
however, increased mitogenic response to Con A, IL-1β, and
TNF production, decreased percent cytotoxic/suppressor T
cells, and had no effect on delayed-type hypersensitivity skin
response and percentage of helper T cells, IL-6, GM-CSF, and
PGE2 production.

Low-fat, low-cholesterol moderately high PUFA diets are
recommended to reduce the risk of heart disease due to their
hypercholesterolemic effect (1, 2). It has been suggested that
cytokines secreted by activated monocytes or the cells of the
vessel walls might mediate many of the pathophysiological
changes in the foam cells of vessel wall associated with athero-
genesis. IL-1 and TNF promote adherence of leukocytes to
endothelial cells and stimulate smooth muscle cell prolifera-
tion (7, 8). Furthermore, these cytokines have been shown to
change the hemostatic balance of endothelial cells in vitro so
that their surface changes from one of an antithrombotic to one
of a procoagulant state (30, 31). Recent studies show that ox-
dized lipids stimulate IL-1 production (32). In the present stud-
ies, long-term feeding of diets low in total fat, saturated fat,
and enriched in (n-3) PUFA was associated with decreased pro-
duction of IL-1β, TNF, and IL-6, suggesting that in addition to
their hypolipidemic effects, these diets could reduce the risk of
atherosclerotic diseases by their reduction of the proinflamma-
tory and proliferative cytokines. Epidemiological studies indi-
cate that consumption of fish and fish oil is associated with a
low rate of coronary heart disease (33). Prospective studies and
controlled human trials also indicate that dietary fish protects
against heart disease and reduced mortality after myocardial
infarction (34). Decreased cytokine and PGE2 synthesis in pa-
tients given EPA supplementation are associated with a reduc-
tion in the severity of arthritis and ulcerative colitis (35, 36).
Previous work documenting similar decreases in cytokine pro-
duction in healthy volunteers (11, 12) provided EPA and DHA
in the form of fish-oil supplements. It is noteworthy that the
changes reported in the present studies were the result of con-
sumption of EPA and DHA from natural foods in levels not
considered extreme (121–188 g/d in the form of tuna, fllet of
sole, and salmon). We also observed that long-term intake of
low-fat, high-fish diets is associated with a significant reduction
in the percentage of helper T cells, mitogenic response to T cell
mitogens, and delayed-type hypersensitivity skin response.
These parameters are often used as indicators of host defense
against various diseases. It is not clear whether the decreases
observed in helper T cells, GM-CSF (49%), IL-6 (33%), mito-
genic response (24%), and delayed-type hypersensitivity skin
response (46%) after consumption of low-fat, high-fish diets
are sufficient to compromise the immune response. Published
studies to date have shown that true anergy is associated with
increased mortality risk (37, 38). However, correlations be-
tween a 50% decrease in these parameters and the risks of infec-
tion or cancer are lacking. Animal studies, however, indicate
that these changes might have clinical significance. Fish-oil
feeding decreased the natural resistance of mice to infection
with Salmonella typhimurium (39) and rats fed diets contain-
ing 9% menhadon oil had a shorter life span compared with
those fed corn oil or beef tallow (40). Rabbits given a high fish
diet for 7 d after birth had decreased lung clearance of inspired
Staphylococcus aureus by 50% compared with saline control or
low doses of the oil (41). Fish oil-fed mice had a reduced
capacity for cytotoxicity of mastocytoma cells upon stimulation
with LPS or INF-γ (42). Unfortunately, epidemiological data
from Eskimos on morbidity and mortality due to other
causes than heart diseases are complicated by influences of
other factors such as sanitation. For example, Northern In-

dians and Inuit children are particularly prone to recurrent
persistent chest infections (43). Although the authors sug-
gested weakness in cellular immunity as a contributing factor,
the influence of other factors such as sanitation cannot be ruled
out. Eskimos also have an increased incidence of certain but
not all types of cancers (43). Taken together, however, these
observations suggest that our findings might have clinical
significance. A recent report also provides indirect evidence that
the changes in cell-mediated immunity observed in this study can
have significant effects on incidence of infectious diseases.
Chandra (44) showed that increases in indexes of T cell–me-
diated function in healthy elderly, after dietary supplementa-
tion, were associated with decreased morbidity due to in-
fec
tious
diseases.

It is interesting to note that in the present study feeding
low-fat, low-fish diet enriched in plant-derived (n-6) and (n-3)
PUFA had either no effect on the immunologic parameters
measured (delayed-type hypersensitivity skin response, GM-
CSF, and IL-6) or enhanced them (mitogenic response, IL-1,
and TNF). A significant decrease in the percentage of CD8+ cells
was also observed. The observation that a low-fat, low-fish
diet resulted in a significant increase in mitogenic responses
and cytokine production indicates that, contrary to the pre-
vious speculations, on the basis of animal experiments (3–6),
plant-derived PUFA, when fed in the context of a low-fat diet,
do not suppress the immune response, rather they can have a
modest stimulatory effect.

The low-fat, high-fish diet differed from the baseline in total
fat content, saturated, monounsaturated, polyunsaturated
fatty acids, cholesterol, and carbohydrate. It is unlikely that the
decreased immunologic responses associated with the high-fish
diet are because of a decrease in total fat content since de-
creases in total fat have been shown to enhance the immune
response (5, 45, 46). In our study, consumption of low-fat,
low-fish diet (lower in fat content than the baseline diet as well)
did not reduce the immunologic responses measured, rather, it
was associated with an increase in some parameters. Since the
low-fat, low-fish diet, which contained the same amount of
total PUFA (10%) and (n-6) PUFA (8%) but a lower amount of
fish-derived (n-3) PUFA (0.13% of calories or 0.27 g/d vs.
0.54% of calories or 1.23 g/d EPA and DHA) than low-fat,
high-fish diet, did not decrease immune responses, the effects observed are likely because of fish-derived (n-3) PUFA. This is supported by data from our laboratory as well as that of others, which showed fish-oil supplementation decreases ex vivo cytokine production (11, 12) and mitogenic response (11, 47). Three of the subjects who had completed the high-fish phase of the study also participated in the low-fish phase. The immunologic changes of these subjects reflected each of the two dietary phases. For example, they showed a decrease in delayed-type hypersensitivity skin response after the low-fat, high-fish diet and exhibited no change in delayed-type hypersensitivity skin response after the low-fat, low-fish diet.

In the present study, feeding a low-fat, high-fish diet caused a 313% increase in plasma EPA, 73% increase in plasma DHA, and 100% increase in linolenic, and a 28 and 82% decrease in AA and AA/EPA ratios, respectively. These were associated with a significant decrease in PGE2 production. Furthermore, a significant correlation between changes in EPA and a decrease in delayed-type hypersensitivity skin response was observed (r = 0.66, P = 0.05). No correlation was observed between delayed-type hypersensitivity skin response and 18:2n-6 or 20:4n-6. Consumption of a low-fat, low-fish diet containing 2% 18:3n-3 caused a significant increase in plasma linolenic (115%), a significant but substantially lesser increase in plasma EPA (54%), no significant change in DHA, and significant but small decreases in AA (12%) and in AA/EPA ratios (38%). This suggests that a substantial change in EPA, DHA, and AA/EPA ratio is needed before the decrease in cytokine production, mitogenic response, and delayed-type hypersensitivity skin response are observed. It is interesting to note that, in our previous studies supplementation of EPA and DHA, 4.8 g/d for 6 wk or 2.4 g/d for 12 wk provided in the form of fish-oil capsules, also caused a significant decrease in cytokine production (young and old subjects) and mitogenic response (old subjects), which was associated with 88 and 91% decrease, respectively, in AA/EPA ratio (11, 12). Decreased mitogenic response in healthy subjects after consumption of fish oil was also reported by Kramer et al. (48). However, Payan et al. (49) found an increase in PHA-induced T cell proliferation in asthma patients and Kremer et al. (35) reported enhanced mitogenic response in patients with rheumatoid arthritis after fish-oil supplementation. The different effects observed by Payan et al. (49) and Kremer et al. (35), after consumption of fish oil, on mitogenic response compared with those reported in the present study and that of Kramer et al. (48) might be due to differences in health status of subjects used in these studies.

The observed immunologic changes induced by low-fat, high-fish diet are unlikely due to decreases in PGE2 production, as PGE2 suppresses IL-1, TNF, and IL-2 production as well as proliferation to mitogens (50). We recently showed that PGE2 added in vitro to peripheral blood mononuclear cells in the presence of a T cell mitogen decreases lymphocyte proliferation more than PGE2 (51). Thus the suppressive effects of (n-3) PUFA–enriched diets may be due to the formation of EPA-derived eicosanoids such as PGE2; (n-3) PUFA have been shown to compromize tocopherol status (26), a nutrient needed for normal maintenance of the immune response. Plasma tocopherol levels did not change in our subjects consuming low-fat, high-fish diets, however, the tocopherol/PUFA ratio was significantly reduced. Furthermore, Meydani et al. (52) showed that supplementation with 2.4 g/d of (n-3) PUFA for 3 mo increased plasma lipid peroxide levels despite no significant change in plasma tocopherol levels. Products of lipid peroxidation such as H2O2 were shown to suppress lymphocyte proliferation (53). Therefore, a rise in lipid peroxide level induced by (n-3) PUFA could contribute to the decrease in mitogenic and delayed-type hypersensitvity skin test responses observed in this study. The increase in mitogenic responses and cytokine production by a low-fat, low-fish diet can be due to a decrease in total fat intake or other fatty acid changes. This is supported by the studies of Hebert et al. (45) and Barone et al. (46) who showed increase in natural killer cytotoxicity when subjects were instructed to consume low-fat diets. Further studies are needed to elucidate this.

In conclusion, our results show that long-term feeding of low-fat, low-cholesterol diets enriched in PUFA have significant effects on in vitro and in vivo indexes of immune and inflammatory responses. A low-fat diet enriched in fish-derived (n-3) PUFA decreases several indexes of the immune response whereas a low-fat diet enriched in plant-derived (n-6) and (n-3) PUFA has no effect or increases some indexes of the immune response in human subjects. The decrease in cytokine production by fish-derived (n-3) PUFA may contribute to the antiatherogenic and anti-inflammatory effects of these diets.

The clinical significance of low-fat, high-fish diet-induced decrease in indexes of cell-mediated immunity (delayed-type hypersensitivity skin response, mitogenic response, and percentage of CD4+ cells) or low-fat, low-fish diet-induced modest increase in some indexes of immune response (TNF, IL-1, and mitogenic response) needs to be further investigated. Thus, in addition to hypolipidemic effects of these diets, other changes induced by these diets in immune and inflammatory parameters should be taken into consideration when population-based recommendations are made.

Acknowledgments

The authors are thankful to the staff of the USDA-HNRCA Metabolic Research Unit for their assistance in recruiting, admitting, care of subjects, and diet preparation; Dr. Judith Neral and Ms. Lisa Gualtieri for technical assistance; and Karin Nauth, Angela Park, and Jennifer Munnis for preparation of this manuscript. Special thanks go to our study subjects for their cooperation and dedication.

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References

8. Bevilacqua, M. P., J. S. Pober, M. E. Wheeler, R. S. Cotran, and M. A.
Immunofluorescence staining was performed on paraformaldehyde-fixed and permeabilized cells using a fluorescence microscope. The primary antibodies used were specific for the cytokine of interest. The stained cells were then analyzed using a flow cytometer and the data were analyzed using software designed for this purpose. The results were compared to those obtained from control cells incubated with media alone. The results showed that the cytokine production by the cells was significantly increased upon treatment with the compound of interest. This indicates that the compound has a positive effect on the production of the cytokine in question.

In conclusion, the results of this study suggest that the compound of interest has potential therapeutic applications in the treatment of conditions where the production of cytokines is involved. Further studies are needed to investigate the mechanism of action and the optimal conditions for use of the compound.

References:

National Cholesterol Education Panel Step-2 Diet and Immune Response

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