Dietary Fatty Acid Effects on T-Cell–Mediated Immunity in Mice Infected With Mycoplasma pulmonis *or Given Carcinogens by Injection*

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To test whether or not diets enriched in *w*-6 polyunsaturated fatty acids are significantly immunosuppressive, B10.D2, DBA/2, and C3B6F1 mice were fed diets enriched for fatty acids: linoleic (POLY), oleic (MONO), palmitic (SAT), or eicosapentanoic (FISH). The B10.D2 and DBA/2 mice were given injected methylcholanthrene several weeks later, and immune studies were performed several months after carcinogen treatment. In conventional quarters, DBA/2 fed the POLY diet survived poorly, and many were infected with *Mycoplasma pulmonis*, even if given the vehicle, tractinoin, only. B10.D2 mice survived well unless on the POLY diet and given methylcholanthrene. Nevertheless, only mice on the POLY diet were significantly immunosuppressed, and only T-cell–mediated cutaneous sensitivity reactions were affected. Antibody, natural killer cell, and natural cytotoxic cell responses were not influenced by the diets. The C3B6F1 mice were assessed for immune functions prior to carcinogen (ethylnitrosourea) instillation into the trachea, and no immunosuppression was detected. After instillation, mice on the POLY and MONO diets were suppressed for T-cell cutaneous responses. Deliberate infection with *Mycoplasma pulmonis* resulted in suppressed cutaneous T-cell responses in the POLY group of C3B6F1 mice, and aspirin partially reversed the immunosuppression. Mice on the FISH diet were resistant to immunosuppression. It is tentatively concluded that diets rich in *w*-6 polyunsaturated diets, while not directly immunosuppressive, do predispose animals to suppression of certain T-cell–mediated immune responses. This immunosuppression can be "triggered" by infection and/or by exposure to carcinogens. (Am J Pathol 1987, 126:103–113)

THE PATHOGENESIS of both atherosclerosis and neoplastic disease may be affected by the quality and quantity of fatty acids in the diet. Polyunsaturated fatty acids of the *w*-6 variety, when substituted for saturated fatty acids, will lower plasma levels of total cholesterol and low density lipoproteins (LDLs).[1] The *w*-3 polyunsaturated fatty acids cause a lowering of plasma triglycerides.[2] Monounsaturated fatty acids have been considered to be neutral in their action on plasma levels of total cholesterol and LDL;[3] but when they are substituted for saturated fatty acids, plasma LDL levels falls.[4] Therefore, a major shift from saturated to *w*-6 or *w*-3 polyunsaturated or to monounsaturated fatty acids may reduce the risk for atherosclerotic disease.

With respect to neoplastic diseases, diets high in dietary fat are associated with increased incidences of breast and colon cancers.[5,6] The potentially harmful effects of diets enriched with *w*-6 fatty acids have been emphasized. Although factors such as total amount of fat and calories and the presence or absence of other dietary constituents may influence the importance of dietary fats, considerable evidence exists that diets rich in *w*-6 polyunsaturated fatty acids predispose to induction of cancer by chemical carcinogens.[7–14]

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A major mechanism by which w-6 polyunsaturated fatty acids may predispose animals to neoplastic disease is suppression of immunocompetence.[15] These fatty acids can directly suppress immune functions *in vitro*,[16,17] and dietary *w*-6 polyunsaturated fatty acids can result in immunosuppression.[18-20] Serum from mice fed such diets, especially the lipoprotein fraction, can inhibit mitogenic responses of lymphocytes *in vitro*.[10] Since linoleic acid is a precursor of arachidonic acid and therefore prostaglandins, the immunosuppressive effects of prostaglandins have been studied in detail.[21-23] Still, other studies indicate that polyunsaturates are not necessarily immunosuppressive or that other factors may influence any immunosuppression detected.[24-27]

The purpose of the studies reported here was to determine whether or not dietary *w*-6 polyunsaturated fatty acids are more immunosuppressive than *w*-3 polyunsaturated and/or monounsaturated fatty acids, especially after injection of chemical carcinogens. The results of our studies suggest that the *w*-6 polyunsaturated fatty acids are indeed immunosuppressive of T-cell-mediated immune responses *in vivo*, but only under certain conditions. For example, mice apparently are not immunosuppressed unless they are infected with organisms, eg, *Mycoplasma pulmonis*, and/or are given injected carcinogens. We propose that the immunologic effects of dietary constituents cannot be accurately determined when animals are housed in "conventional" animal quarters in long-term studies. Specific pathogen-free (SPF) or germ-free facilities are almost mandatory. Nonetheless, under the influence of infection or chemical carcinogens, *w*-6 polyunsaturates are more immunosuppressive than monounsaturates and *w*-3 polyunsaturates. The mice fed the fish oil appeared to be quite resistant to immunosuppression.

Materials and Methods

Mice

Male (C3H × C57BL/6)F1 (C3B6F1) and female DBA/2 and B10.D2 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). DBA/2 and B10.D2 mice are both *H-2*^{d} types, but DBA/2 mice are resistant and B10.D2 mice are susceptible to induction of fibrosarcomas[28] by methylcholanthrene (MC). The animals were maintained in conventional facilities and were placed on experimental diets at 7 weeks of age. The mice were cared for as prescribed in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health.

Diets

Six diets were employed in two studies. Four diets contained 30% of calories as fat. The first was rich in saturated fatty acids (SAT) and contained palm oil as its only fat. The second was high in monounsaturated fatty acids (MONO) and contained only high-oleic safflower oil. The third contained only high-linoleic safflower oil and thus was rich in *w*-6 polyunsaturates (POLY). The fourth diet contained menhaden oil (FISH), which was rich in eicosapentaenoic acid, *w*-3 fatty acid. In the preparation of these three diets, fat-free diets (ICN Nutritional Biochemicals, Cleveland, Ohio) were enriched with one of the oils. The fifth diet was low in fat (LoFat) and contained 10% of calories as fat with equal quantities (1:1:1) of saturated, monounsaturated, and *w*-6 polyunsaturated fatty acids. The sixth diet was conventional Teklad diet (CHOW), which contains 10% of calories as fats equally divided between vegetables and animal fats. The mice were fed once daily and were allowed to eat *ad libitum*. One group of C3B6F1 mice on the POLY diet received aspirin (acetylsalicylic acid) in the drinking water (50 mg/500 ml). The diets were supplemented with ICN vitamin diet fortification mixture. The contents (in grams per milogram) were: vitamin A acetate, 1.8; vitamin D_2, 0.125; tocophero acetate, 22.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; para-amino benzoic acid, 5.0; niacin, 4.25; riboflavin, 1.0; pyridoxine HCl, 1.0; thamine, 1.0; calcium pantothenate, 3.0; biotin, 0.02; folic acid, 0.09; and vitamin B_{12}, 0.00135.

Experimental Design

The protocols are depicted in Figure 1. In the first experiment with B10.D2 and DBA/2 mice, groups of 50 mice of each strain were placed on the diets 6 weeks prior to injection of 0, 12.5, or 125 μg methylcholanthrene (Sigma Chemicals, St. Louis, Mo) in 0.05 ml trioctanoin (Eastman Kodak Co., Rochester, NY) subcutaneously. In the second experiment, C3B6F1 mice were given intratracheal injections of 600 μg ethyl nitrosourea (Sigma) in 50 μl RPMI 1640 medium or medium only. A separate group of C3B6F1 mice were infected intratracheally with 4×10^8 *Mycoplasma pulmonis* organisms.

Immune Studies

Groups of 4 mice were immunized intraperitoneally with 5×10^8 sheep erythrocytes (SRBCs) and were bled on Days 5, 10, and 18. Serum samples were divided, and one-half were treated with 2-mercaptoeth-

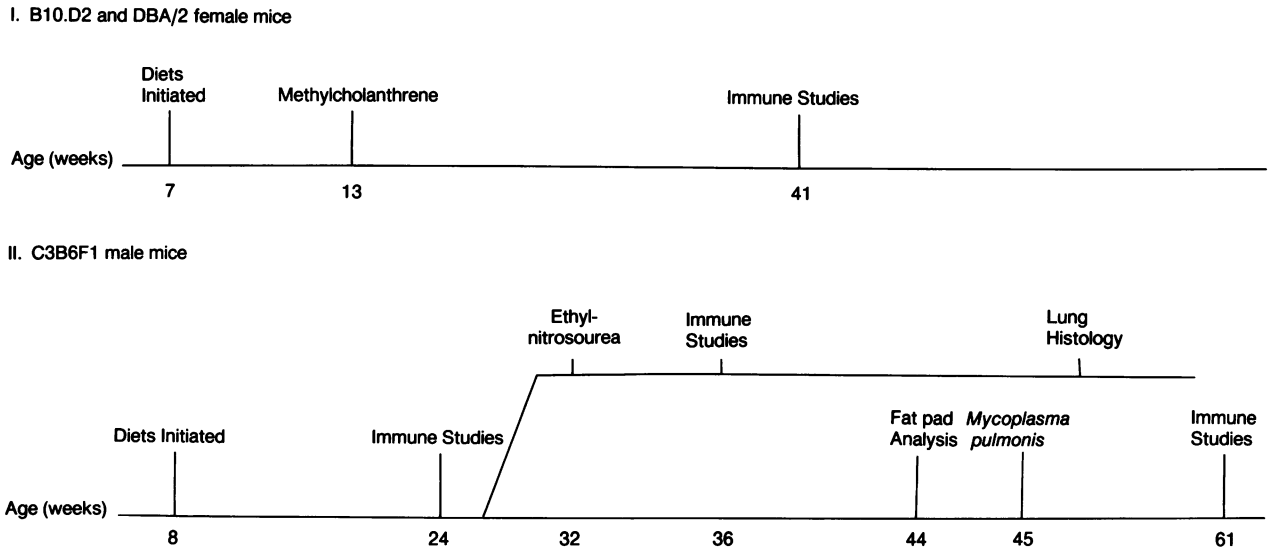

Figure 1 — Experimental protocols.

anol (2ME) to inactivate IgM molecules. Serial two-fold dilutions of antisera, 25 μl, were tested for the ability to agglutinate SRBCs.[29] The values are expressed as $\log_2$ titers of IgG (2ME-resistant) or IgM (2ME-susceptible) agglutinating antibody.

Natural killer (NK) cell activity of spleen cells was determined by culturing various numbers of spleen cells with 2×10^4 ^{51}Cr-labeled YAC-1 lymphoma cells (prototype NK cell target cells) for 4 hours at 37 C in volumes of 0.2 ml in triplicate. The isotope released into the supernatant fluid was counted with a Packard PRIAS gamma scintillation counter.[30] Controls included maximum release (detergent treatment of YAC-1 cells) and spontaneous release (no effector cells). The formula for percent specific cytotoxicity is ^{51}Cr, cpm E - SR/MR - SR×100, where E = experimental, SR = spontaneous, and MR = maximum release, respectively. Since the mice were several months old, some were given intraperitoneal injections of 0.12 mg polyinosinic-polycytidylic acid (pI:pC) 1 day earlier to "boost" NK cell function.[31] The *in vivo* measurement of NK and natural cytotoxic (NC) cell[32] activities was performed by determining the ability of mice (groups of 4) to "clear" radiolabeled YAC-1 (NK) or WEHI-164.1 (prototype NC target) cells from their lungs.[33] Briefly, ^{51}Cr-labeled YAC-1 and ^{125}I-iododeoxyuridine (^{125}IUdR) - labeled WEHI-164.1 cells (5×10^5 each) were infused intravenously, and the lungs were removed 4 hours later. The ^{51}Cr and ^{125}I radioactivities were determined as above. The values are expressed as the percent retention of label (therefore, cells) in the lung. The greater the NK/NC function, the lesser percent retention of label is observed.

Cutaneous sensitivity (CS) to trinitrochlorobenzene (TNCB, Eastman Kodak Company) was one measure of T-cell–mediated immunity. Groups of 6 mice were "painted" with 50 μl 7% TNCB in 4:1 acetone/olive oil on shaved abdominal skin to sensitize them. Five days later, the mice were challenged with 20 μl 1% TNCB on the left pinnae. The right pinnae were painted with the vehicle. An engineer's micrometer was used to measure the thickness of the ears 24, 48, and 72 hours after challenge.[34] The values are expressed as the increase in ear thickness (Δ 10^{-3} inches) from time 0. Controls included mice sensitized but not challenged and mice challenged but not sensitized. A Student *t* test was performed to determine the significance of differences between geometric or arithmetic means. Delayed type hypersensitivity (DTH) to SRBC was determined by immunizing mice with 10^6 SRBCs intravenously. Five days later, the mice were challenged with 10^8 SRBCs/50 μl in the right footpad. A change in footpad thickness was measured 24 hours later. DTH to allogeneic spleen cells was determined by immunizing C3B6F1 mice with 5×10^6 DBA/2 spleen cells in the left footpad. Seven days later, the mice were challenged with 10^7 DBA/2 spleen cells in the same footpad. The change in thickness of the footpad was measured 24 hours later. Controls were similar to the one for CS to TNCB.

The responsiveness of spleen cells to mitogens was determined by incubating 5×10^5 cells for 3 days with

1.0 or 0.5 μg concanvalin A (Con A) or 10 μg lipopolysaccharide (LPS) in volumes of 0.2 ml in flat-bottomed wells of microtiter plates (triplicate samples). Each well was pulsed with 0.5 μCi ^{3}H-thymidine for the final 18 hours to harvest the cells. The values are expressed as the mean ± SEM blastogenesis (cpm ^{3}H-thymidine, mitogen-medium control).

Analysis of Lipids in Fat Pads and Spleen

Intraabdominal mesenteric fat pads and spleens were homogenized with the use of a Dounce homogenizer; lipids were extracted with the use of chloroform/methanol as partition solvent.[35] Lipid samples were analyzed immediately or stored with BHT as antioxidant at −20 C for later chromatographic analysis. We used thin-layer chromatography (TLC) on silica gel G plates with a 250-μ layer thickness with hexane/diethyl ether/methanol/acetic acid (80:20:3:2) as a solvent system to separate triclycerides from adipose tissue and phospholipids from spleen, comparing their migration with known standards. Specific lipid bands from TLC were scraped and fatty acids methylated with the use of the direct transesterification method.[36] Methyl esters of fatty acids were stored under nitrogen at −20 C prior to gas chromatography analysis. We performed separation and quantification of fatty acid methyl esters with flame-ionization detector gas chromatography in duplicate samples, comparing their retention time with authentic standards. A Hewlett Packard Model 5700 GC chromatographer equipped with a 0.75-mm bore 30-m capillary column filled with SP-2330 phase was used for analysis. The relative proportions of individual fatty acids were expressed as the percentage of total fatty acids greater than 12 carbons.

Analysis of Lung Lesions

The C3B6F1 mice given intratracheal injections of ehthylnitrosourea were sacrificed 15 weeks later. The lungs were fixed in 10% buffered formalin, and the paraffin sections were cut so that we could obtain coronal sections of the lung. At least two sections per lung were examined after staining with hematoxylin and eosin.

Results

Studies of B10.D2 and DBA/2 Female Mice

B10.D2 mice given the vehicle or 12.5 μg methylcholanthrene survived well, irrespective of diet (Figure 2). However, B10.D2 mice on the POLY diet and given 125 μg of the carcinogen survived poorly.

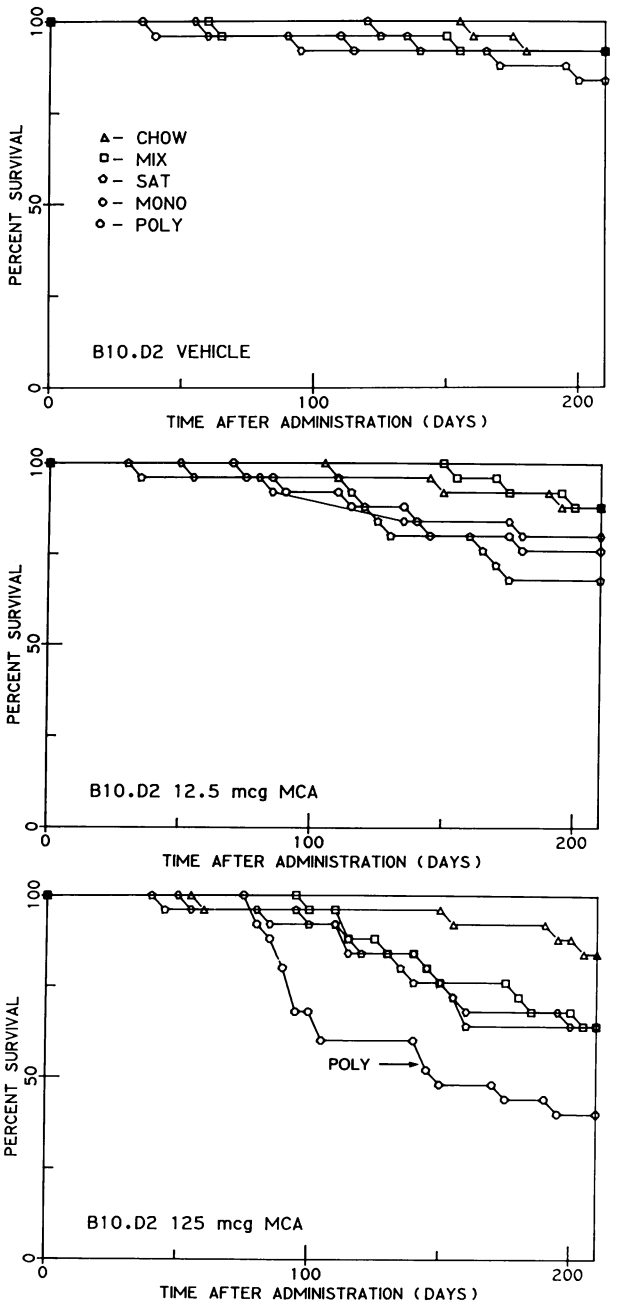

Figure 2— Survival of B10.D2 female mice on the various diets after injection of 0, 12.5, or 125 μg methylcholanthrene in triactanoin subcutaneously. Groups of 25 mice.

DBA/2 mice given the vehicle, tractanoin, survived well unless they were fed the POLY diet (Figure 3). DBA/2 mice on all but the CHOW diet survived poorly if given 125 μg methylcholanthrene. Autopsies of the DBA/2 and the B10.D2 mice revealed histologic evidence of pneumonia consistent with infection with *Mycoplasma pulmonis*. The mice housed in our "conventional" animal facility in the Animal Resource Center (ARC) usually are infected (as indi-

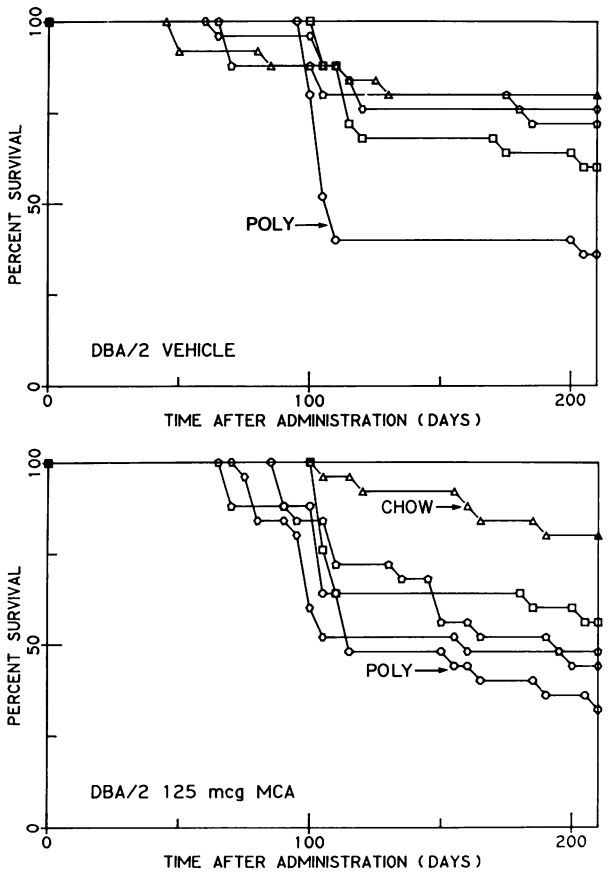

Figure 3 — Survival of DBA/2 female mice on the various diets after injection of 0 or 125 μg methylcholanthrene. The symbols for the dietary groups are the same as in Figure 2.

cated by antibody titers) with *Mycoplasma pulmonis*, Sendai virus, and mouse hepatitis virus. The mice received from the Jackson Laboratory are specific pathogen-free, but they become infected in the ARC at the rate of about 10%/month. Several of the mice were sacrificed, and cultures of lung lavages revealed *Mycoplasma pulmonis*.

None of the experimental diets nor treatment with methylcholanthrene had stimulatory or inhibitory effects on IgM or IgG anti-SRBC agglutinin antibody responses in B10.D2 or DBA/2 mice (Table 1).

The "resting" NK activity of all of the tested 11-month-old B10.D2 and DBA/2 mice was low and was boosted moderately well by pI:pC (data not shown). None of the diets had a consistent inhibitory effect. NK and NC activities, as measured *in vivo* by lung clearance of radiolabeled YAC-1 and WEHI-164.1 cells, were not affected by diet (Table 2). B10.D2 mice did clear WEHI-164.1 cells more efficiently than DBA/2 mice. DBA/2 mice on the MONO diet appeared to clear WEHI-164.1 cells relatively poorly.

When tested for cutaneous sensitivity to trinitrochlorobenzene, all B10.D2 mice on the POLY diet showed a consistent pattern of immunosuppression, whether or not they were given methylcholanthrene (Figure 4). It should be noted that survival was good in the mice given the vehicle or only 12.5 μg methylcholanthrene. Healthy-appearing mice could be immunosuppressed, but even in these "healthy" mice, *Mycoplasma pulmonis* infection was common.

Like the B10.D2 mice, both groups of DBA/2 mice on the POLY diet developed poor cutaneous sensitivity responses to trinitrochlorobenzene (Figure 5). The DBA/2 mice on the MONO and SAT diets were also significantly suppressed ($P<0.05$ versus CHOW diet), but to a much less degree than on the POLY diet. Again, note that survival of DBA/2 mice was poor when they were injected with the carcinogen and good in controls receiving vehicle, with the exception of mice on the POLY diet.

Studies of Male C3B6F1 Mice

The C3B6F1 mice had excellent survival (95%) for the first 4 months and were clinically free from infec-

Table 1 — Antibody Responses of Mice on Diets and Given Injections of Methylcholanthrene (MC)*

	IgM titers (peak)			IgG titers (peak)		
Diet	No MC	12.5 μg MC	125 μg MC	No MC	12.5 μg MC	125 μg MC
I. B10.D2 Mice						
CHOW	38.0	8.6	13.9	9.9	13.2	11.6
LoFat	10.0	15.0	19.0	8.0	4.9	23.9
POLY	25.0	10.9	20.5	2.9	4.0	3.4
MONO	15.0	5.5	10.5	2.9	8.0	3.4
SAT	13.1	33.0	7.9	4.0	3.2	6.6
II. DBA/2 Mice						
CHOW	14.0	—	36.0	8.0	—	8.0
LoFat	6.0	—	91.9	9.4	—	8.0
POLY	10.5	—	13.9	6.6	—	8.0
MONO	15.5	—	13.5	4.6	—	6.4
SAT	3.4	—	6.5	4.6	—	2.9

*Mice were immunized with 5×10^8 SRBCs intraperitoneally, and serum was obtained on Days 5, 10, and 18. The serum was treated or not with 2ME. The peak IgM titers were detected at Day 5, and the peak IgG (2ME-resistant) titers were detected on Day 18.

Table 2—Lung Clearance of Tumor Cells by Mice on Diets and Given Injections of Methylcholanthrene (MC)*

Diet	YAC-1 cells			WEHI-164.1 cells		
	No MC	12.5 μg MC	125 μg MC	No MC	12.5 μg MC	125 μg MC
I. B10.D2 Mice						
CHOW	4.0	2.8	4.2	11.1	5.4	8.4
LoFat	2.0	3.0	4.2	6.5	8.0	10.1
POLY	3.4	2.3	3.9	7.6	4.9	7.3
MONO	3.0	6.7†	2.4	8.6	10.7†	6.6
SAT	3.4	4.6	2.3	8.3	10.4†	8.3
II. DBA/2 Mice						
CHOW	6.3	—	7.8	16.9	—	38.4
LoFat	4.0	—	8.5	7.1	—	42.5
POLY	6.2	—	8.2	10.2	—	22.5
MONO	10.3†	—	10.0	35.6†	—	34.8
SAT	7.4	—	8.6	18.1	—	22.5

*Mice were infused with 5×10^5 ^{51}Cr-labeled YAC-1 cells and 5×10^5 ^{125}IUdR-labeled WEHI-164.1 cells intravenously. The lungs were removed 4 hours later. Values are expressed as geometric mean percent retention of infused cells in the lungs.
†Geometric mean values significantly greater ($P < 0.05$) than those of the CHOW group.

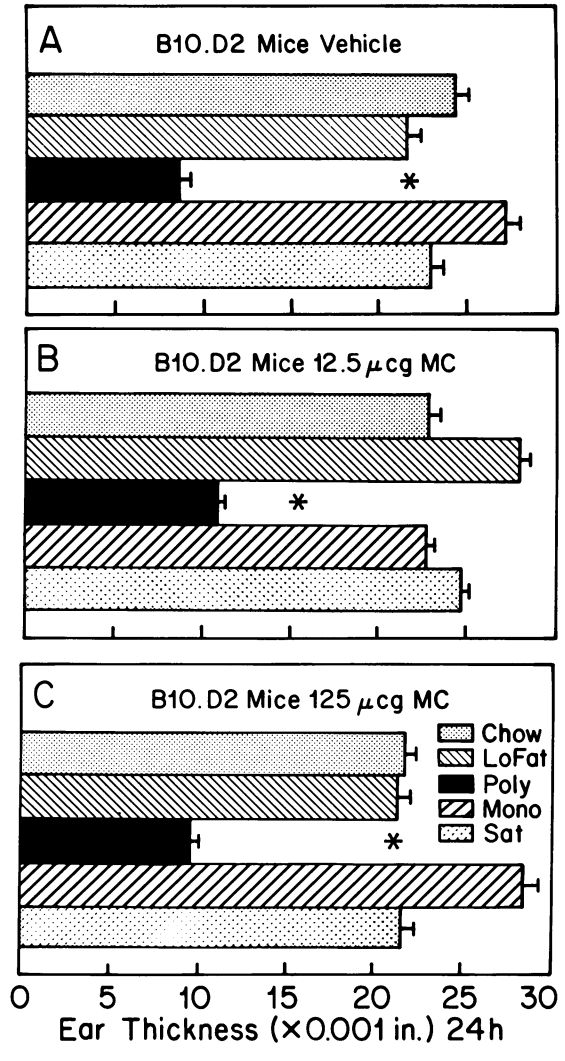

Figure 4—Cutaneous sensitivity to TNCB in B10.D2 female mice on various diets and given vehicle **(A)**, 12.5 μg **(B)**, or 125 μg methylcholanthrene **(C)**. *Significant suppression ($P < 0.05$), compared with CHOW controls.

tion, even though they were housed in the conventional quarters. The fatty acid contents of the mesenteric fat pads reflected the diets quite well (Table 3) and the body weights indicated that the diets were well consumed.

These mice were tested for cutaneous sensitivity to TNCB 4 months after receiving the experimental diets. The data in Table 4 indicate that none of the

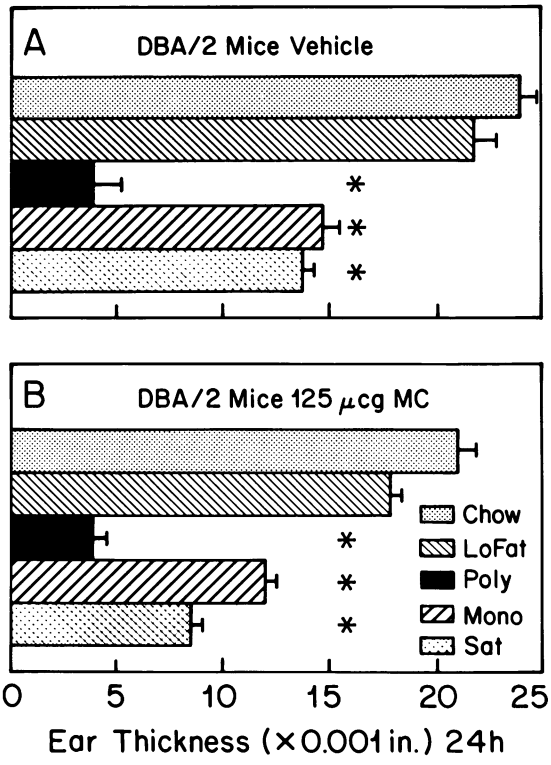

Figure 5—Cutaneous sensitivity to TNCB in DBA/2 female mice on various diets and given vehicle **(A)** or 125 μg methylcholanthrene **(B)**. *See Figure 1.

Table 3—Fatty Acid Contents of the Fat Pads of C3B6F1 Mice on the Various Diets for 7 Months and Mean ± SEM Body Weights at 6 and 11 months*

Fatty acids	CHOW	FISH	MONO	POLY	Poly +ASA	SAT
C12	0	1.0	0	0.3	0.4	0.3
C14	0.8	2.5	0	0	0	0
C16	18.4	35.8	8.8	12.8	17.6	13.2
C16:1	7.4	1.0	0	0	0	7.0
C18	1.6	3.7	0	0	0	64.4
C18:1	46.2	31.1	74.0	19.3	23.5	11.1
C18:2	20.9	15.8	11.8	64.7	55.6	0
C18:2 cis/trans	0.3	0.4	0.8	0	0	0
C18:3	1.0	2.7	1.5	0.8	0.9	1.3
C20:3 *w*-6	1.6	1.2	0.3	0	0	0
C20:4 *w*-6	0	1.4	1.2	0.6	0.5	0
C?	0	0	0	0.5	0.8	0
C20:3 *w*-9	0	0	0	0	0	0.9
C20:5 *w*-3	0.4	1.4	0.4	0.5	0.5	0
C22:6 *w*-3	0	2.4	0.2	0	0	0
Body weights(g)						
At 6 months	30.9 ± 2.7	32.7 ± 2.9	45.7† ± 2.9	40.4† ± 4.5	42.8† ± 7.9	47.9† ± 4.1
At 11 months	34.9 ± 1.8	43.1† ± 1.9	49.8† ± 0.8	45.8† ± 2.2	51.1† ± 1.0	51.9† ± 1.0

*Intraabdominal mesenteric fat pads were removed and frozen immediately. Fatty acid content as a percentage of acids of 12 carbons or more was determined by gas chromatography.

†Mean values significantly greater ($P < 0.05$) than those of CHOW controls.

diets were immunosuppressive. Mice were given injections of ethylnitrosourea (ENU) or vehicle intratracheally and were tested 1 month later. The mice given the vehicle were not immunosuppressed. The mice given ENU and receiving the MONO, POLY, or POLY + ASA diet had significantly depressed cutaneous sensitivity responses to TNCB (Table 4). Mice on the CHOW, FISH, or SAT diet were not suppressed. Survival at this point was excellent. A few mice that had received ENU were wheezing. Their lung lavage grew *Mycoplasma pulmonis*. The mice injected with ENU were sacrified 13 weeks later. The lungs contained histologic foci of Sendai virus pneumonitis and/or alveolar adenomas, which are difficult to distinguish. Mycoplasma pneumonitis, which contains lymphoid cell infiltrates predominantly, was detected to a lesser degree. The frequencies of lung lesions (pneumonitis or adenommas) in the mice on the various diets are presented in Table 4. Mice on the FISH and SAT diets had the lowest incidences of lung lesions.

The preceding data suggested that the immunosuppression was induced in susceptible mice after carcinogen injection and may have been caused, in part, by infection. Therefore, C3B6F1 mice on the various diets were deliberately infected with *Mycoplasma pulmonis* intratracheally. These mice were tested for delayed-type hypersensitivity responsiveness to SRBCs and to H-2 allogeneic spleen cells. Only the mice on the POLY diet were significantly suppressed (Table 5). Aspirin in the drinking water prevented severe suppression of delayed-type hypersensitivity. The mitogenic responses to Con A and to LPS were vigorous by spleen cells from mice on the various diets

Table 4—Cutaneous Sensitivity Reactions to Trinitrochlorobenzene (TNCB) by C3B6F1 Mice Before and After Injection of Ethylnitrosourea (ENU) or Vehicle Intrattracheally*

Dietary group	Mean ± SEM increase in ear thickness at 24 hours (10^{-3} in)			
	Prior to ENU	After ENU's vehicle	After ENU	Frequency of lung lesions‡
CHOW	7.3 ± 0.4	7.3 ± 0.3	6.1 ± 0.6	8/25
FISH	9.7 ± 0.8	9.9 ± 0.7	7.7 ± 0.7	2/26
MONO	7.8 ± 0.1	7.1 ± 0.3	3.6 ± 0.2†	10/28
POLY	7.2 ± 0.3	8.8 ± 0.7	2.8 ± 0.7†	11/17
POLY + ASA	9.0 ± 0.7	7.9 ± 0.3	2.9 ± 0.3†	9/27
SAT	11.2 ± 0.5	9.3 ± 0.6	5.0 ± 0.3	4/22

*Mice were sensitized to TNCB 8 weeks before the instillation of 600 μg ENU (or the vehicle, RPMI 1640 medium) intratracheally or were sensitized 4 weeks later. The mice were challenged 5 days after sensitization.

†Mean value significantly less than that of CHOW controls $P<0.05$).

‡Mice given ENU were sacrificed 13 weeks later, and the lungs were examined histologically. Areas of consolidation with pneumocyte proliferation were scored as positive "lesions." Alveolar cell adenomas and Sendai virus pneumonitis are very difficult to distinguish.

Table 5—Delayed-Type Hypersensitivity Responses to RBCs or to Allogeneic Spleen Cells in C3B6F1 Mice on Various Diets and Deliberately Infected With *Mycoplasma pulmonis**

Experiment	Group	Mean ± SEM footpad swelling ($\times 10^{-3}$ in)
1. SRBCs	CHOW	35.1 ± 3.7
	FISH	27.6 ± 3.1
	POLY	23.5 ± 2.9†
	POLY + ASA	26.8 ± 2.6
	MONO	38.6 ± 1.8
	SAT	37.5 ± 3.6
2. Allogeneic spleen cells	CHOW	7.4 ± 1.2
	FISH	8.2 ± 1.2
	POLY	0.4 ± 0.4†
	POLY + ASA	6.4 ± 2.1
	MONO	9.6 + 1.2
	SAT	9.0 ± 1.4

*C3B6F1 mice on the various diets were infected with 4×10^8 *Mycoplasma* organisms intratracheally. Four months later, the mice in Experiment 1 were immunized intravenously with 10^8 SRBCs. Five days later, the mice were challenged with 10^8 SRBCs in the right footpad. The change in footpad thickness was measured 24 hours later. In Experiment 2, the mice were immunized with 5×10^6 DBA/2 spleen cells in the left footpad. Seven days later, the mice were challenged with 10^7 DBA/2 spleen cells in the left footpad. The change in footpad thickness was determined 24 hours later. Controls were mice "immunized" with saline and/or "challenged" with saline.
†Mean values significantly less ($P < 0.05$) than those of the CHOW group.

4 months after infection (Table 6). Mice fed the SAT diet had the lowest responses to Con A, whereas mice on the FISH diet had the highest responses. The spleen cells from mice on the CHOW diet had the lowest B-cell responses to LPS. The C3B6F1 *(H-2^k/H-2^b)* mice were immunized with allogeneic *(H-2^d)* P815 tumor cells 10 days before testing the spleen cells for cytolytic T-lymphocyte function. The abilities of spleen cells from mice on the various diets to lyse P815 cells were remarkably similar (Table 7). The spleen cells of similar mice also were able to generate cytolytic T lymphocytes *in vitro* (Table 7). Finally, infected C3B6F1 mice were tested for natural killer and natural cytotoxic cell functions. Mice on the FISH diet had the lowest natural killer cell function, and mice on the POLY + ASA diet had slightly depressed natural cytotoxic cell function (Table 8).

Discussion

The results of these experiments indicate that diets enriched in *w*-6 polyunsaturated fatty acids suppress two types of T-cell–mediated immune responses, namely, cutaneous sensitivity to trinitrochlorobenzene and delayed-type hypersensitivity to SRBCs or to alloantigens (Figures 4 and 5, Tables 4 and 5). Other T-cell functions were not suppressed, including the T-dependent antibody responses to SRBCs (Table 1), mitogenic responses to Con A (Table 6), and cytolytic T-lymphocyte responses to allogeneic cells (Table 7). The lack of immunosuppression in C3B6F1 mice not given the carcinogen (Table 4) indicates that the diets rich in linoleic acid were not directly immunosuppressive, but rather rendered mice susceptible to immunosupppression by carcinogens or infectious organisms. The B10.D2, and particularly the DBA/2 mice, were clinically ill (Figures 2 and 3) after injection of the carcinogen, and many were infected with *Mycoplasma pulmonis*. Even the C3B6F1 mice developed *Mycoplasma pulmonis* and Sendai virus infections after instillation of ENU intratracheally. These diseases were confirmed by culture of tracheal washes for bacteria on PPLO broth and histologic examination of the lungs. C3B6F1 mice deliberately infected with *Mycoplasma pulmonis* became immunosuppressed if they were on the POLY or MONO diet (Table 5). In that experiment, the addition of aspirin to the drinking water of mice on the POLY diet did lessen the degree of immunosuppression. This observation suggests that synthesis of prostaglandins of the 2-series, derived from arachidonate,[38] may contribute to the immunosuppression detected. Mice on the FISH and SAT diets were the least affected (immunosuppressed) by the injection of carcinogens or by infection.

Antibody synthesis was not significantly affected by the various diets (Table 1). Diets lacking essential fatty acids resulted in defective antibody responses in young mice,[38] and a similar diet inhibited anti-DNA

Table 6—Mitogenic Responses of Spleen Lymphocytes in Mice on Various Diets Deliberately Infected With *Mycoplasma pulmonis**

Group	Blastogenesis (^{3}H-TdR incorporation $\times 10^3$)		
	Con A 1.0 μg	Con A 0.5 μg	LPS 10 μg
CHOW	90.3 ± 23.2	41.3 ± 6.0	44.2 ± 3.9
FISH	137.6 ± 0.8	115.7 ± 20.4	78.6 ± 3.9
POLY	67.5 ± 3.8†	76.3 ± 7.9	58.3 ± 4.2
POLY + ASA	113.9 ± 0.2	87.0 ± 12.9	70.8 ± 10.7
MONO	104.2 ± 8.7	82.2 ± 14.4	91.4 ± 3.3
SAT	52.9 ± 5.9†	30.7 ± 3.6†	84.2 ± 11.4

*C3B6F1 mice on the various diets were infected with 5×10^8 *Mycoplasma pulmonis* organisms intratracheally. Four months later, their spleen cells were incubated with medium only, Con A or LPS, for 3 days. ^{3}H-Thymidine, 0.5 μCi, was added 18 hours prior to harvesting the cells. The values are expressed as the mean ± SEM Δ blastogenesis (cpm ^{3}H-TdR, mitogen - medium control).
†Mean values significantly less ($P < 0.05$) than those of the CHOW group.

Table 7—Generation of Cytolytic Lymphocytes *in Vivo* and *in Vitro* by Spleen Cells of C3B6F1 Mice on the Various Diets and Deliberately Infected With *Mycoplasm pulmonis**

Experiment	Group	Mean % Specific Cytotoxicity of E:T of			
		100:1	50:1	25:1	12.5:1
1. *In vivo*	CHOW	14.9	9.5	7.1	5.5
	FISH	16.9	12.3	9.7	5.0
	POLY	18.2	9.3	7.1	4.6
	POLY + ASA	18.5	12.1	7.1	4.7
	MONO	ND†	ND	ND	ND
	SAT	8.6	6.5	7.9	5.2
2. *In vitro*	CHOW		52.5	53.1	42.1
	FISH		53.9	57.4	33.1
	POLY		ND	ND	ND
	POLY + ASA		65.1	54.8	49.8
	MONO		ND	ND	ND
	SAT		53.7	44.4	31.8

*C3B6F1 mice on the various diets were infected with 5×10^8 *Mycoplasma pulmonis* organisms intratracheally. Four months later, some mice were given intratracheal injections of 30×10^6 P815 tumor cells. Their spleen cells were harvested 10 days later (Experiment 1). Other mice were used as donors of "responder" spleen cells (5×10^6/ml) stimulated with irradiated DBA/2 spleen cells (5×10^6/ml) for 5 days in *in vitro*. The effector cells were harvested and incubated for 4 hours with ^{51}Cr-labeled P815 cells.

†ND, not done.

antibody synthesis and nephritis in "lupus" (NZB × NZW)F1 mice.[39] Conversely, diets enriched with *w*-6 polyunsaturated fatty acid enhanced anti-DNA antibodies in these "lupus" mice.[20]

Both *in vivo* and *in vitro* NK and NC cell functions were intact in mice on the various diets whether or not they were given carcinogens (Table 2) or deliberately infected with *Mycoplasma pulmonis* (Table 8). Therefore, dietary fatty acids may not be important in influencing the function of these cytotoxic effector cells.

The mitogenic responses of C3B6F1 spleen cells to Con A (T-cell mitogen) or to lipopolysaccharide (B-cell mitogen) were not significantly affected by dietary fats after infection with *Mycoplasma pulmonis* (Table 6) or after injection of ENU (data not shown). Since the cells were removed from the animal and washed prior to testing, suppression of mitogen responses may require circulating immunosuppressive lipoproteins.[10]

The *w*-6 polyunsaturated fatty acids were clearly immunosuppressive in mice given either carcinogens or *Mycoplasma pulmonis*. The instances of impaired function in mice on the MONO diet were 1) DBA/2 mice given the vehicle, tractinoin, and challenged with labeled YAC-1 and WEHI-164.1 *in vivo* (NK/NC functions) (Table 2) and 2) C3B6F1 mice given ENU intratracheally (Table 4). The significance of these observations is questionable, since similar functions were not suppressed in all three types of mice used. The mice on the *w*-3 polyunsaturated fatty acid diet (FISH) showed a significant suppression of only

Table 8—Natural killer (NK) and Natural Cytotoxic (NC) Cell Activities of Spleen Cells off C3B6F1 Mice on Various Diets and Deliberately Infected With *Mycoplasma pulmonis**

Experiment	Group	Mean specific cytotoxicity at E:T of			
		100:1	*50:1*	*25:1*	*12.5:1*
1. NK cells	CHOW	56.8	34.0	26.1	18.3
	FISH	30.1	22.9	15.9	11.6
	POLY	65.6	51.7	35.9	25.4
	POLY + ASA	43.3	32.1	23.1	15.8
	MONO	66.2	47.8	32.9	19.7
	SAT	41.1	28.3	18.3	14.1
		100:1	*25:1*	*6.2:1*	*1.6:1*
2. NC cells	CHOW	31.4	13.2	12.9	6.4
	FISH	25.8	10.2	2.0	2.8
	POLY	28.9	11.7	6.2	0
	POLY + ASA	18.2	9.6	3.8	0
	MONO	28.2	17.4	8.2	0
	SAT	22.8	11.3	7.6	0

*C3B6F1 mice on the various diets were infected with 5×10^8 *Mycoplasma pulmonis* organisms intratracheally. Fifteen weeks later, mice in Experiment 1 were given injections of 0.12 mg polyinosinic: polycytidylic acid intraperitoneally to boost NK cell function. Their spleen cells were tested 1 day later for the ability to lyse YAC-1 lymphoma cells in a 4-hour ^{51}Cr-release assay. Another set of mice were not boosted, and their spleen cells were tested for NC activity, as judged by the ability to lyse WEHI-164.1 fibrosarcoma cells in an 18-hour ^{51}-Cr-release assay.

one immune function, namely, NK cell function in C3B6F1 mice after infection with *Mycoplasma pulmonis* (Table 8). Therefore, neither oleic acid nor eicosapentanoic acid in the diet appears to predispose mice to immunosuppression.

There is currently great interest in fish oil diets in relation to cardiovascular disease. Experimentally, fish oil prevented intimal hyperplasia of autogenous vein grafts used for arterial by-pass.[40] Moreover, dietary fish oils decreased blood viscosity and increased erythrocyte deformability.[41] The ability of eicosapentaenoic acid to inhibit the production of the cyclooxygenase arachidonate metabolite, thromboxane A_2, may help to prevent thrombosis and atherosclerosis.[42] Feeding fish oils may suppress "lupus" in MRL-lpr mice by inhibiting prostaglandin synthesis.[43,44]

In our review of the literature on this subject, we noted that all studies were done with animals (mice or rats) housed in "conventional" quarters. The animal husbandry in various institutions may be excellent, as it is in ours; nevertheless, *Mycoplasma pulmonis*, Sendai virus, and mouse hepatitis virus frequently do contaminate conventional facilities. We tentatively conclude that long-term experiments with mice on experimental diets, at least on diets enriched in various fatty acids, should be performed only in facilities that maintain animals free of pathogenic microorganisms.[45] Until experiments are conducted under such strict conditions, we suggest it is premature to conclude that *w*-6 polyunsaturated fatty acids in the diet are immunosuppressive *per se*. Additional insults, such as infection and/or chemical or drug injections, may be required to significantly immunosuppress animals. Nonetheless, the data do support the idea that *w*-6 polyunsaturates at least predispose to immunosuppression. In contrast, diets enriched in monounsaturated (oleic) or *w*-3 polyunsaturated (eicosapentaenoic) fatty acids appear to be much less immunosuppressive than these enriched in *w*-6 polyunsaturated fatty acids in mice exposed to carcinogens or infectious agents.

References

1. Grundy SM, Ahrens EH Jr: The effects of unsaturated dietary fats on absorption, excretion, synthesis, and distribution of cholesterol in man. J Clin Invest 1970, 49:1135–1152
2. Phillipson BE, Rothrock DW, Conner WE, Harris WS, Illingworth DK: Reduction of lipids, lipoproteins and apoproteins by dietary fish oils in patients with hypertriglyceridems. N Engl J Med 1985, 312:1210–1216
3. Keys A, Anderson JT, Grande F: Serum cholesterol response to changes in diet. Metabolism 1965, 14:747–787
4. Mattson FH, Grundy SM: Comparison of effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids and lipoproteins in man. J Lipid Res 1985, 26:194–202
5. Wynder E: Nutrition and cancer. Fed Proc 1976, 35:1309–1315
6. Carrol KK, Khor HT: Dietary fat in relationship to tumorigenesis, Progress in Biochemical Pharmacology. Edited by KK Carroll: Vol 10, Lipids and Tumors. New York, Karger, 1975, pp 308–345
7. Ip C, Carter CA, Ip MM: Requirement of essential fatty acid for mammary tumorigenesis in the rat. Cancer Res 1985, 45:1997–2001
8. Bansal BR, Rhoads Jr JE, Bansal SC: Effect of diet on colon carcinogenesis and the immune system in rats treated with 1,2-dimethyl hydraxine. Cancer Res 1978, 38:3293–3303
9. Broitman SA, Vitale JJ, Jakuba EV, Gottlieb LS: Polyunsaturated fat, cholesterol and bowel tumorigenesis. Cancer 1977, 40:2455–2463
10. Sakagudi M, Hiramatsu Y, Takada H, Yamamura M, Hioki K, Saito K, Yamamoto M: Effect of dietary unsaturated and saturated fats on azomethane-induced colon carcinogenesis in rats. Cancer Res 1984, 44:1472–1477
11. Schneider EL, Reed JD Jr: Life extension. N Engl J Med 1985, 312:1159–1168
12. Hillyard LA, Abraham S: Effect of dietary polyunsaturated fatty acids on growth of mammary adenocarcinomas in mice and rats. Cancer Res 1979, 39:4430–4447
13. Carter CA, Miholland RH, Shea W, Ip MM: Effect of the prostaglandin synthetase inhibitor indomethacin on 7,12-dimethylbenz(a)anthracene-induced mammary tumorigeneis in rats fed different levels of fat. Cancer Res 1983, 43:3559–3562
14. Carroll KK, Khor HT: Effects of level and type of dietary fat on incidence of mammary tumors induced in female Sprague-Dawley rats by 7,12-dimethylbenz (a) anthracene. Lipids 1971, 6:415–420
15. Meade CJ, Merten J: The mechanism of immunoinhibition by arachidonic and linoleic acid. Effects on the lymphoid and RE systems. Int Arch Allergy Appl Immunol 1976, 51:2–24
16. Merten J, Hughes D, Shenton BK, Dickinson JP: In vitro inhibition by unsaturated fatty acids of the PPD- and PHA- induced lymphocyte response. Klin Wochenschr 1974, 52:248–250
17. Weyman C, Berlin J, Smith AD, Thompson RSH: Linoleic acid as an immunosuppressive agent. Lancet 1975, 2:33–34
18. Kollmorgen CM, Sansing WA, Lehman AA, Fischer G, Longley RE, Alexander SS Jr, King MM, McCay PB: Inhibition of lymphocyte function in rats fed high-fat diets. Cancer Res 1979, 39:3458–3462
19. Erickson KL, McNeill CJ, Gershwin ME, Ossman JB: Influences of dietary fat concentration and saturation on immune ontogeny in mice. J Nutrition 1980, 110:1555–1572
20. Levy JA, Ibrahim AB, Shirai T, Ohta K, Nagasamia R, Yoshida H, Estes J, Gardner M: Dietary fat affects immune response, production of antiviral factors, and immune complex disease in NZB/NZW mice. Proc Natl Acad Sci USA 1982, 79:1974–1978
21. Pelus LM, Strauseen HR: Prostaglandins and the immune response. Life Sci 1977, 20:903–913
22. Goodwin JS, Webb DR: Regulation of the immune response by prostaglandins. Clin Immunol Immunopathol 1980, 15:106–122
23. Rappaport RS, Dodge GR: Prostaglandin E inhibits the production of human interleukin 2. J Exp Med 1982, 155:943–948
24. Gross RL, Newberne PM: Role of nutrition in immunologic function. Physiol Rev 1980, 60:188–302

25. Lochiskar M, Nauss KM, Newberne PM: The effect of quality and quantity of dietary fat on the immune system. J Nutr 1983, 113:951-961
26. Nauss KM, Lochiskar M, Newberne PM: Effect of alterations in the quality and quantity of dietary fat on 1,2-dimethylhydrasine-induced colon tumorigenesis in rats. Cancer Res 1983, 43:4083-4090
27. Clifford CK, Smith LM, Erickson KL, Hamblin CL, Creveling RK, Clifford AJ: Effect of dietary triglycerides on lymphocyte transformation in rats. J Nutr 1983, 113:669-679
28. Kouri RE, Ratrie H, Whitmire CE: Evidence of a genetic relationship between susceptibility to 3-methylcholanthrene-induced subcutaneous tumors and inducibility of aryl hydrocarbon hydroxylase. J Natl Cancer Inst 1973, 51:197-200
29. Sever JL: Application of a microtechnique to visual serologic investigations. J Immunol 1962, 88:320-329
30. Lust JA, Kumar V, Burton RC, Bartlett S, Bennett M: Heterogeneity of natural killer cells in the mouse. J Exp Med 1981, 154:306-317
31. Kumar V, Ben-Ezra J, Bennett M: Natural killer cells in mice treated with ^{89}Sr; normal target-binding cell numbers but inability to kill even after interferon administration. J Immunol 1979, 123:1832-1838
32. Lattime EC, Pecoraro GA, Stutman O: The activity of natural cytotoxic cells as augmented by interleukin 2 and interleukin 3. J Exp Med 1983, 157:1070-1074
33. Riccardi C, Puccetti P, Santoni A: Rapid in vivo assay of mouse natural killer cell activity. J Natl Cancer Inst 1979, 63:1041-1045
34. Stein-Streilein J: Allergic contact dermatitis induced by intratracheal administration of hapten. J Immunol 1983, 131:1748-1753
35. Folch J, Lees M, Stanley GHS: A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957, 226:497-509
36. Lepage G, Roy CC: Direct transesterification of all classes of lipids in a one-step reaction. J Lipid Res 1986, 27:114-120
37. Vane JR: Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 1971, 231:232-235
38. DeWille JW, Fraker PJ, Romsos DR: Effects of essential fatty acid deficiency, and various levels of dietary polyunsaturated fatty acids, on humoral immunity in mice. J Nutr 1979, 109:1018-1027
39. Hurd ER, Johnston JW, Okita JR, MacDonald PC, Ziff M, Gilliam JN: Prevention of glomerulo-nephritis and prolonged survival in New Zealand black/New Zealand white F1 hybrid mice fed on essential fatty acid - deficient diet. J Clin Invest 1981, 67:476-485
40. Landymore RW, Kinley CE, Cooper JH, MacAulay, M. Sheridan B, Cameron CJ: Cod-liver oil in the prevention of intimal hyperplasic in autogenous vein grafts used for arterial bypass. Thorac Cardiovasc Surg 1985, 89:351-357
41. Cartwright IJ, Pockley AG, Calloway JH, Greaves M, Preston FE: The effects of dietary w-3 polyunsaturated fatty acids on erythrocyte membrane phospholipids, erythrocyte deformability and blood viscosity in health volunteers. Atherosclerosis 1985, 55:264-281
42. Dyerberg J, Bang HO, Stoffersen E, Moncada S, Vane JR: Eicosa pentaenoic acid and the prevention of thrombosis and atherosclerosis. Lancet 1978, 2:117-119
43. Culp BR, Titus BC, Lands WEM: Inhibition of prostanglandin biosynthesis by eicoseapentaenoic acid. Prostaglandins Med 1979, 3:269-278
44. Kelly VE, Ferretti A, Izui S, Strom TB: A fish oil diet rich in eicosapentaenoic acid reduces cyclo oxygenase metabolites, and suppresses lupus in MRL-1pr mice. J Immunol 1985, 134:1914-1919
45. Yu BP, Masoro EJ, Murata I, Bertrand HA, Lynd FT: Life span study of SPF Fischer 344 male rats fed *ad libitum* or restricted diets: Longevity, growth, lead body mass and disease. J Gerontol 1982, 37:130-141

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