Breast Cancer Research and Treatment **73:** 145–160, 2002.

Report

The role of dietary long-chain n-3 fatty acids in anti-cancer immune defense and R3230AC mammary tumor growth in rats: influence of diet fat composition *

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Key words: diet, immune, interleukin-2, long-chain n-3 fatty acids, natural killer cell, phospholipids, polyunsaturated/saturated fat ratio, R3230AC mammary tumor, rats

Summary

We determined if long-chain n-3 fatty acids fed as part of a: (1) high polyunsaturated fat diet (currently recommended by several health agencies) or (2) low polyunsaturated fat diet (representative of that consumed by a large segment of the North American population) improved antitumor immune defense and inhibited tumor growth. Rats were fed one of four semi-purified diets (20% w/w fat) for 21 days pre- and 17 days post- R3230AC mammary tumor implantation. The polyunsaturated to saturated fatty acid (P/S) ratio was either 1 (high P/S diet) or 0.35 (low P/S diet). At each P/S ratio, diets provided long-chain n-3 fatty acids at 0 or 5% w/w of total fat. Long-chain n-3 fatty acids fed in a high P/S diet did not affect tumor growth or host immune responses. In contrast, feeding long-chain n-3 fatty acids in a low P/S diet increased natural killer cell cytotoxicity, splenocyte nitric oxide and interleukin-2 production, and the proportion of activated ($CD25^+$) $CD8^+$ and $CD28^+$ cells, but did not significantly inhibit tumor growth. For both P/S diets, tumor cells from rats fed long-chain n-3 fatty acids had a higher n-3 content and n-3/n-6 ratio in phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. Furthermore, the magnitude of increase in n-3 fatty acid incorporation into tumor phospholipids was greater when fed in a low P/S diet. We demonstrated that the dietary P/S ratio significantly influences the effect of long-chain n-3 fatty acids on host immune responses and n-3 fatty acid incorporation into tumor cells. These findings warrant further consideration when designing dietary recommendations.

Abbreviations: Con A: Concanavalin A; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; IL: interleukin; NK: natural killer; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; P/S ratio: polyunsaturated to saturated fatty acid ratio

Introduction

High levels of fish oil-derived long-chain n-3 fatty acids, eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids, inhibit the growth of certain carcinogen-induced and transplantable animal tumors [1]. It has been suggested that the ratio of n-3 to n-6 fatty acids in the diet, not the absolute levels of fatty acids, is critical for this effect [2–5]. A dietary polyunsaturated to saturated fatty acid (P/S) ratio of 1 is currently recommended by several health agencies [6–8]. However, the observation that high levels of n-6 polyunsaturated fatty acids promote tumor growth in animals [1] has likely contributed to the lack of a similar recommendation by cancer agencies [9].

The mechanism for the putative anticancer benefits of dietary long-chain n-3 fatty acids is not known. We investigated whether the tumor suppressor effect

*Presented in part at Experimental Biology in San Francisco, CA (April 1998). [Robinson, L. E. and Field, C. J. Long-chain n-3 fatty acids fed in a low polyunsaturated diet improve membrane-mediated anti-tumor immune defenses and reduce tumor growth] and Experimental Biology in Washington, DC (April 1999) [Robinson, L. E. and Field, C. J. Influence of diet fat composition on incorporation of long-chain n-3 fatty acids into R3230AC tumor cell phospholipids].

of long chain n-3 fatty acids may occur, in part, via alterations in the host immune system. Diets rich in EPA and DHA can affect both the innate (nonspecific) and acquired (specific) cell-mediated immune systems [10]. Cellular components of both of these immune systems, including natural killer (NK) cells, macrophages, T helper ($CD4^+$) and T suppressor/cytotoxic ($CD8^+$) cells, are involved in anticancer immunity [11–13]. We have previously shown that feeding healthy rats long-chain n-3 fatty acids at a level which could potentially be achieved in the human diet (3.3% w/w of total fat) significantly enhanced NK cell cytotoxicity and the proportion of activated immune cells [14].

A direct effect of dietary lipids on modulation of tumor cell membrane composition may also play a role in the ability of EPA and DHA to inhibit tumor growth [15]. It is well established that changes in diet fat composition can induce significant alterations in the composition and function of membranes in a variety of human and animal tissues [16–17], including tumors [5, 18–20]. For example, incorporation of long-chain n-3 fatty acids into tumor cell membranes may alter membrane-mediated functions involved in tumor cell proliferation or apoptosis [5], susceptibility to attack by the host immune system [19] and response to chemotherapy [21].

In the present study we hypothesized that a fixed dose (5% w/w) of fish oil-derived long-chain n-3 fatty acids would be more effective at improving immunity and inhibiting tumor growth when fed in a low P/S, compared with a high P/S, diet. To address this we performed two separate experiments using the R3230AC transplantable mammary adenocarcinoma in rats. The objective of Experiment 1 was to determine if a fixed dose of long-chain n-3 fatty acids would improve antitumor immune defense and inhibit tumor growth when supplemented in a high polyunsaturated fat diet (i.e., a high P/S diet), representative of the fat composition that is currently recommended by several health agencies for the human population. Experiment 2 was conducted to determine if the same dose (5% w/w) of long-chain n-3 fatty acids supplemented in a low polyunsaturated fat diet (i.e., a low P/S diet), representative of that currently consumed by a large segment of the North American population, would enhance immune defense and suppress tumor growth. Since this study consisted of two separate experiments, the purpose was to compare unsupplemented (low n-3) and supplemented (high n-3) diets within one P/S ratio diet. The effect of dietary EPA and DHA on individual phospholipid fractions in tumor cell membranes was also studied.

Materials and methods

Materials

RPMI 1640 culture media, fetal calf serum, glutamine, antimycotic-antibiotic solution [penicillin (1×10^5 U/l), streptomycin (100 mg/l), amphotericin B (25 mg/l)] and HEPES were purchased from Gibco BRL (Burlington, ON, Canada). Bovine serum albumin, 2-mercaptoethanol, Griess reagent chemicals, hyaluronidase, 8-anilino-1-naphthalene-sulfonic acid, and phospholipid standards were obtained from Sigma Chemical (St. Louis, MO). Concanavalin A (Con A) was purchased from ICN (Montréal, QB, Canada), and collagenase (type II) was obtained from Worthington Biochemical Corporation (Freehold, NJ). The OX19, w3/25, OX8, OX12, and OX42 monoclonal antibodies were kindly provided by A. Rabinovitch (University of Alberta, Edmonton, AB, Canada). All monoclonal antibodies were mouse anti-rat IgG. Phycoerythrin-conjugated goat anti-mouse IgG and all other antibodies (except JJ319) were purchased from Cedarlane Laboratories Ltd. (Hornby, ON, Canada). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was obtained from Organon Teknika Inc. (Scarborough, ON, Canada). Antibody JJ319 and IL-2 assay components were purchased from PharMingen (Mississauga, ON, Canada). Immulon® high binding flat-bottom microtiter plates for the IL-2 assay were obtained from Dynex Technologies, Inc. (Chantilly, VA). An NK cell-sensitive murine lymphoma cell line (YAC-1) was purchased from the American Type Culture Collection (Rockville, MD) and sodium ^{51}Cr was obtained from Amersham (Oakville, ON, Canada).

Animals, diets, and tumor implantation

Experiments were reviewed and approved by the Faculty of Agriculture and Forestry Animal Policy and Welfare Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines. Forty-seven female Fischer 344 rats (143 ± 1 g) were obtained from a colony maintained at the University of Alberta and were housed in individual wire-mesh cages in a temperature controlled room (23°C) maintained on a 12 h light/dark cycle. Body weight and

Table 1. Fatty acid composition of experimental diets[a]

Fatty acid	High P/S diet		Low P/S diet	
	Low n-3 diet	High n-3 diet	Low n-3 diet	High n-3 diet
Percentage of w/w				
C14:0	1.3	2.0	2.2	2.8
C16:0	15.7	15.7	21.1	21.0
C18:0	26.9	25.5	44.4	42.6
C18:1 n-9	8.4	8.2	4.6	4.5
C18:2 n-6	44.3	39.3	23.4	18.8
C18:3 n-3	1.2	0.2	1.1	0.2
C20:5 n-3	nd	3.2	nd	3.2
C22:5 n-3	nd	0.2	nd	0.2
C22:6 n-3	nd	0.8	nd	0.8
SFA	45.6	44.8	70.2	69.0
MUFA	8.7	10.4	4.8	6.6
PUFA	45.3	44.5	24.5	24.0
n-6 PUFA	44.3	39.6	23.4	19.1
n-3 PUFA	1.2	5.1	1.1	5.0
n-3/n-6 ratio	0.03	0.13	0.05	0.26
P/S ratio	1.0	1.0	0.35	0.35

[a]Values are g/100 g of total fat, except for ratios. Diets contained 200 g/kg of fat from a mixture of sources [safflower oil, beef tallow, linseed oil (low n-3 diets only), and fish oil (high n-3 diets only]. Minor fatty acids are not reported, therefore totals do not add up to 100%. Abbreviations: nd: not detectable; SFA: sum of saturated fatty acids; MUFA: sum of monounsaturated fatty acids; PUFA: sum of polyunsaturated fatty acids; P/S ratio: ratio of polyunsaturated to saturated fatty acids.

food intake were recorded every third day throughout the study. Rats were randomly assigned to be fed nutritionally complete, semi-purified diets (Teklad Test Diets, Madison, WI) containing (per kg) 270 g high protein casein, 408 g carbohydrate, and 200 g fat. Both diets met the n-6 and n-3 fatty acid requirements of a growing rat. The complete nutrient composition of the diets has been reported [14]. In Experiment 1, diets had a P/S ratio of 1 (high P/S diet), while in Experiment 2 the P/S ratio was 0.35 (low P/S diet) as determined by gas-liquid chromatography [17]. At each P/S ratio there were two diets which differed only in the composition of fat, providing two different levels of long-chain n-3 fatty acids from a mixed fish oil source (P-28 Nisshin lot # 28020, Nisshin Flour Milling Co., Ltd., Tokyo, Japan): low (0 g/kg of total fat) or high (50 g/kg of total fat). The only source of n-3 fatty acids in the low n-3 diet was α-linolenic acid (C18:3n-3), provided by linseed oil (Galaxy Enterprises, Edmonton, Canada). The fatty acid composition of the diets is presented in Table 1. All animals were given free access to food and water. R3230AC mammary adenocarcinoma cells were obtained from the DCT Tumor Repository (National Cancer Institute, Frederick, MD) and were maintained in our laboratory as frozen stocks. Previously frozen R3230AC tumor cells were transplanted into animals used in the study after three passages in recipient animals of the same age, sex, and strain. Specifically, after 21 days of feeding, a freshly harvested R3230AC mammary tumor from a rat implanted 2–3 week earlier was finely chopped under sterile conditions to prepare a tumor brei and 50 μl was injected subcutaneously in the inguinal region of experimental rats. Rats were killed by CO_2 asphyxiation and cervical dislocation 17 days following tumor implantation. At necropsy, tumor and spleen were removed and weighed to be used for the measurements described below. In Experiment 1 (high P/S diet), 14 rats (7/diet) were implanted with the tumor. In Experiment 2 (low P/S diet), there were 14 tumor-bearing rats (7/diet) and 19 healthy (control) rats (9 rats were fed the low n-3 diet and 10 rats were fed the high n-3 diet). Due to the number of variables being measured, this study was carried out in a series of replicate experiments, such that all measurements were not performed

on individual animals. For each assay, the number of animals in each treatment group is provided in the text, table, or figure legend.

Immune variables

A number of different tumor models in various inbred strains of rats are used for studying antitumor immune defense. We chose to study the R3230AC rat mammary tumor, an estrogen-responsive, transplantable adenocarcinoma that shares many similarities with the human disease [22] and whose growth has been shown to be modulated by dietary fat [18]. Although there is limited data on the effect of the R3230AC mammary tumor on host immune function, previous work in our lab has shown that anticancer immunity declines progressively with growth of other transplantable tumors [23]. To measure antitumor immune defense, we used several well characterized *ex vivo* immune assays as substitutes for *in vivo* immune assessment. Standard target cell lines (e.g., YAC) are used extensively by immunological researchers to assess immune cell cytotoxicity or cytostasis in both humans and animals. Their use facilitates comparison of our results with those in the published literature. *In vitro* stimulation with mitogens such as Con A provides a useful tool for studying immune cells in an activated state. Con A is a lectin which binds specifically to certain sugar residues on T cell surface glycoproteins, including the T cell receptor and CD3, thereby stimulating T cells [24].

Splenocyte isolation and activation

Splenocytes were isolated aseptically as previously described [25] in Krebs-Ringer HEPES buffer (KRH, pH 7.4) supplemented with bovine serum albumin (5 g/l). Isolated splenocytes (3.0×10^9 cells/l) in complete culture media [RPMI 1640 supplemented with 4% (v/v) heat-inactivated fetal calf serum, 1% (v/v) antimycotic-antibiotic solution, glutamine (4 mmol/l), HEPES (25 mmol/l), and 2-mercaptoethanol (2.5 μmol/l)] were incubated in 24-well sterile plates for 48 h in a humidified atmosphere at 37°C in the presence of 5% CO_2. The cell culture media contained either no mitogen (unstimulated cells) or was supplemented with Con A (5 mg/l). After 48 h, splenocyte culture supernatants were collected and stored at −70°C for subsequent nitric oxide and cytokine analyses. Con A-stimulated splenocytes were washed twice in KRH (pH 7.4) supplemented with bovine serum albumin (5 g/l) in preparation for indirect immunofluorescence analyses.

Indirect immunofluorescence (phenotype) assay

Immune cell subsets in freshly isolated splenocytes and Con A-stimulated splenocytes were identified by indirect immunofluorescence assay as previously described [14]. The following monoclonal antibodies were used: OX19 (CD5), w3/25 (CD4), OX8 (CD8α), OX12 (B cells), OX42 (CD11b/c), 3.2.3 (NKR-P1), OX39 (CD25), and JJ319 (CD28). Since the monoclonal antibodies were not pre-labeled with a fluorescent marker, they were incubated with either fluorescein isothiocyanate-conjugated goat anti-mouse IgG (FITC) or phycoerythrin-conjugated goat anti-mouse IgG. The percentage of cells expressing each marker was determined by flow cytometry (FACScan, Becton Dickinson, Sunnyvale, CA) and were corrected for background fluorescence using the analysis of cells incubated with FITC or phycoerythrin alone. FITC and phycoerythrin background fluorescence were 5 and 0%, respectively (data not shown). Unwanted events (dead cells and debris) were detected on the basis of forward scatter and side scatter and were excluded from subsequent phenotype analyses by electronic gating of the viable splenocyte population. We have expressed the Con A-stimulated phenotyping data as '% of live cells remaining after culture'.

NK cell cytotoxicity assay

A 4 h sodium chromate (^{51}Cr) release assay was performed using NK cell-sensitive YAC-1 cells as targets and freshly isolated splenocytes as effector cells [14]. The percentage lysis of the target cells was calculated as: Specific lysis (%) = 100 × [mean experimental ^{51}Cr release (cpm)-mean spontaneous ^{51}Cr release (cpm)]/[mean maximum ^{51}Cr release (cpm)-mean spontaneous ^{51}Cr release (cpm)]. Results were also expressed as lytic units with one lytic unit being equal to the number of effector cells ($\times 10^{-3}$) required to cause 20% lysis of target cells.

Nitric oxide production

Nitric oxide production was determined by analyzing nitrite (NO_2^-, a product of the L-arginine-dependent nitric oxide pathway) concentration in splenocyte culture supernatants using a colorimetric assay based on the Griess reaction [26].

Cytokine production

The concentrations of IL-2 in culture supernatants collected from unstimulated and Con A-stimulated splenocytes were determined by enzyme-linked immunosorbent assay. Briefly, flat-bottom microtiter plates were coated overnight with appropriately diluted purified rabbit anti-rat IL-2. After washing, plates were blocked with PBS + 10% (v/v) fetal calf serum to prevent nonspecific binding. Recombinant IL-2 standards (15–2000 pg/ml) and appropriately diluted splenocyte culture supernatants were then added in triplicate at 100 µl per well, incubated for 4 h at room temperature, washed, and further incubated with appropriately diluted biotinylated mouse anti-rat IL-2 for 1 h. After extensive washing, the plates were incubated for 30 min with horseradish peroxidase-conjugated avidin D and the absorbance was measured at 405 nm.

Tumor cell isolation

R3230AC mammary tumor cells were isolated by a modification of the method described by Harmon and Hilf [22]. Non-necrotic tumor tissue was finely chopped, suspended in 10 ml of cold KRH (pH 7.4) supplemented with bovine serum albumin (5 g/l), hyaluronidase (1 g/l) and collagenase (Type II, 0.5 g/l), and incubated in an orbital shaker (50 cycles/min) at 37 °C. After 60 min, the suspension was filtered through a wire strainer, pelleted, resuspended in a fresh 10 ml of enzyme solution and incubated for an additional 60 min. After incubation, the suspension was filtered through a mesh screen and pelleted. The pellet was resuspended in KRH (pH 7.4) supplemented with bovine serum albumin (5 g/l) and separated by density gradient centrifugation using a 40% percoll gradient (400 g for 30 min). The cell interface was removed and washed three times. Cell viability was determined by trypan blue exclusion and was > 90% for all treatment groups. Cells were frozen at −70°C until fatty acid analysis was performed.

Tumor cell fatty acid analysis

Lipids were extracted from R3230AC mammary tumor cells by a modified Folch procedure [17]. Individual phospholipids were separated on thin layer chromatography plates (HPK silica gel 60 Å10 × 10 cm, Whatman, Clifton, NJ) as previously described [27]. Separated phospholipids were visualized with 8-anilino-1-naphthalene-sulfonic acid and identified under ultraviolet light with appropriate standards. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) fatty acid methyl esters were prepared from the scraped silica band using 14% (w/v) BF_3/methanol reagent [28] and separated by automated gas liquid chromatography (Vista 6010, Varian Instruments, Georgetown, ON) on a fused silica BP20 capillary column (25 m × 0.25 mm internal diameter, Varian Instruments) as described [17].

Statistical analysis

Results are presented as means ± SEM. All statistical analyses were conducted using the SAS statistical package (Version 6.11, SAS Institute, Cary, NC). In Experiment 1, the effect of diet was analyzed by Student's *t*-test. In Experiment 2, the main effects of diet and tumor were determined by two-way ANOVA followed by a Duncan's multiple range test to identify significant ($p \leq 0.05$) differences between individual treatments [29]. Body weight changes, food intake, and NK cell cytotoxicity were compared among groups by a split-plot (repeated measures) ANOVA [29]. Paired *t*-tests were used to compare NO_2^- and IL-2 production by immune cells with or without Con A. All tumor cell fatty acid analysis was done at the same time and the effects of long-chain n-3 fatty acids and P/S ratio on phospholipid composition were compared by ANOVA and a Duncan's multiple range test.

Results

Food intake, body weight, spleen weight

In Experiment 1 (high P/S diet) dietary long-chain n-3 fatty acids did not significantly affect food intake (overall mean = 57 ± 1 g/kg body weight/d, $n = 14$), final body weight (overall mean = 154 ± 3 g, $n = 14$), weight increase (overall mean = 16 ± 2 g, $n = 14$), relative spleen weight (overall mean = 3.2 ± 0.1 g/kg body weight, $n = 14$), or the number of spleen cells ($\times 10^6$) per gram of spleen (overall mean = 401 ± 27, $n = 14$). In Experiment 2 (low P/S diet), neither dietary long-chain n-3 fatty acids nor the tumor significantly affected food intake (overall mean = 63 ± 1 g/kg body weight/d, $n = 29$), final body weight (overall mean = 158 ± 2 g, $n = 33$), weight increase (overall mean = 13 ± 1 g, $n = 33$), relative spleen weight (overall mean = 2.8 ± 0.1 g/kg body weight, $n = 33$),

or the number of spleen cells ($\times 10^6$) per gram of spleen (overall mean = 433 ± 13, $n = 32$).

R3230AC mammary tumor weight

Long-chain n-3 fatty acids fed in the high P/S diet did not significantly affect final tumor weight (high n-3 diet, 1.2 ± 0.2; low n-3 diet, 1.2 ± 0.2 g/100 g body weight; $p = 1$, $n = 7$/diet). In the low P/S diet group, final tumor weight was lower when rats were fed long-chain n-3 fatty acids (high n-3 diet, 0.9 ± 0.1; low n-3 diet, 1.3 ± 0.2 g/100 g body weight, $n = 7$/diet), but this did not reach statistical significance (−31%, $p = 0.1$).

Immune cell phenotypes in freshly isolated splenocytes

(a) *High P/S diet*

Long-chain n-3 fatty acids fed in the high P/S diet did not significantly affect the proportion (% of total) of $CD5^+$ T cells (overall mean = 51 ± 1%), $CD4^+$ T helper cells (overall mean = 30 ± 1%), $CD8^+$ T suppressor/cytotoxic cells (overall mean = 18 ± 1%), B cells (overall mean = 30 ± 1%), macrophages (overall mean = 4.0 ± 0.4%), or the CD4/CD8 ratio (overall mean = 1.8 ± 0.1) in freshly isolated splenocytes ($n = 14$). However, rats fed the high n-3 diet had a lower ($p < 0.01$) proportion of NK cells compared with those fed the low n-3 diet (low n-3 diet, 4.1 ± 0.4; high n-3 diet, 2.6 ± 0.2, $n = 7$/diet).

(b) *Low P/S diet*

The presence of a tumor did not significantly affect immune cell phenotypes in freshly isolated splenocytes with the following exception: tumor-bearing rats had a small, yet significantly higher proportion of $CD8^+$ T cells and a significantly lower proportion of B cells compared with healthy rats (Table 2). Feeding long-chain n-3 fatty acids in the low P/S diet did not significantly affect immune cell phenotypes in freshly isolated splenocytes with the following exception: healthy and tumor-bearing rats fed the high n-3 diet had a significantly lower proportion of $CD5^+$ and $CD4^+$ T cells compared with those fed the low n-3 diet (Table 2). The relative proportion of freshly isolated splenocytes expressing either the IL-2 receptor (CD25) or CD28 was low (<2%) and was not significantly different between treatment groups (results not shown).

Immune cell phenotypes after 48 h Con A stimulation

Immune cell phenotypes and IL-2 receptor expression after Con A stimulation were only analyzed in the low P/S diet, and not the high P/S diet, experiment. Phenotype data from the high P/S diet experiment (in which there was clearly no effect of n-3 fatty acid supplementation on tumor growth) would have aided in understanding whether or not diet-induced immune changes were associated with tumor growth inhibitory effects of long-chain n-3 fatty acids in this model. However, since we found no other changes in host immune response in rats fed the high P/S diet, it did not seem justified to conduct these immune measures.

Low P/S diet

In both the low and high n-3 diet groups, tumor-bearing rats had a significantly higher proportion of $CD4^+$ T helper cells, $CD8^+$ T suppressor/cytotoxic cells and $CD28^+$ cells and a lower proportion of B cells following Con A stimulation compared with healthy rats (Figure 1). In addition, tumor-bearing rats fed the high n-3 diet had a significantly higher proportion of $CD4^+$, $CD8^+$, and $CD28^+$ cells and lower proportion of B cells compared with tumor-bearing rats fed the low n-3 diet (Figure 1). Neither diet nor tumor significantly affected the relative proportion of macrophages (overall mean = 3.6 ± 0.3%, $n = 28$) or the CD4/CD8 ratio (overall mean = 2.2 ± 0.1, $n = 29$) after Con A stimulation.

IL-2 receptor (CD25) expression after 48 h Con A stimulation

Low P/S diet

There was a significant interaction between long-chain n-3 fatty acids and the tumor on IL-2 receptor (CD25) expression after splenocytes were stimulated with Con A (Table 3). Tumor-bearing rats fed the high n-3 diet had a significantly higher proportion of $CD25^+$ $CD5^+$ T cells compared with those fed the low n-3 diet, while diet did not affect the proportion of these cells in healthy rats (Table 3). In the high n-3 diet group, tumor-bearing rats had a significantly higher proportion of $CD5^+$ T cells expressing CD25 compared with healthy rats, while the tumor did not affect the proportion of these cells in the low n-3 diet group (Table 3). In both the low and high n-3 diet groups, tumor-bearing rats had a significantly higher proportion of $CD25^+$ $CD4^+$ T helper cells and $CD25^+$ $CD28^+$ cells and a significantly lower proportion of B cells and

Table 2. Immune cell phenotypes in fresh spleen from healthy and tumor-bearing rats fed a low P/S diet either unsupplemented (low n-3) or supplemented with long-chain n-3 fatty acids (high n-3 diet)[1]

Immune cell phenotype	Healthy		Tumor-bearing		Significance, p[2]	
	Low n-3 diet	High n-3 diet	Low n-3 diet	High n-3 diet	Diet	Tumor
Percentage of total immune cells in spleen						
$CD5^+$ T cells	53 ± 1	50 ± 1	54 ± 1	49 ± 1	0.0003	NS
$CD4^+$ T helper cells	31 ± 1	28 ± 1	32 ± 2	26 ± 2	0.005	NS
$CD8^+$ T suppressor cytotoxic cells	13 ± 1	14 ± 1	18 ± 2	15 ± 2	NS	0.04
B cells	26 ± 1	28 ± 1	25 ± 1	25 ± 1	NS	0.03
Macrophages	2.9 ± 0.4	3.6 ± 0.5	3.4 ± 0.4	2.6 ± 0.5	NS	NS
Natural killer cells	5.3 ± 0.7	5.0 ± 0.5	4.0 ± 0.5	4.6 ± 0.8	NS	NS
CD4/CD8	2.3 ± 0.2	2.1 ± 0.2	1.9 ± 0.2	2.0 ± 0.4	NS	NS

[1] Values are means $\pm$ SEM ($n \geq 6$/group).
[2] Significant main effects (diet and tumor) as determined by two-way ANOVA, NS = $p > 0.05$. No significant interactions were found.

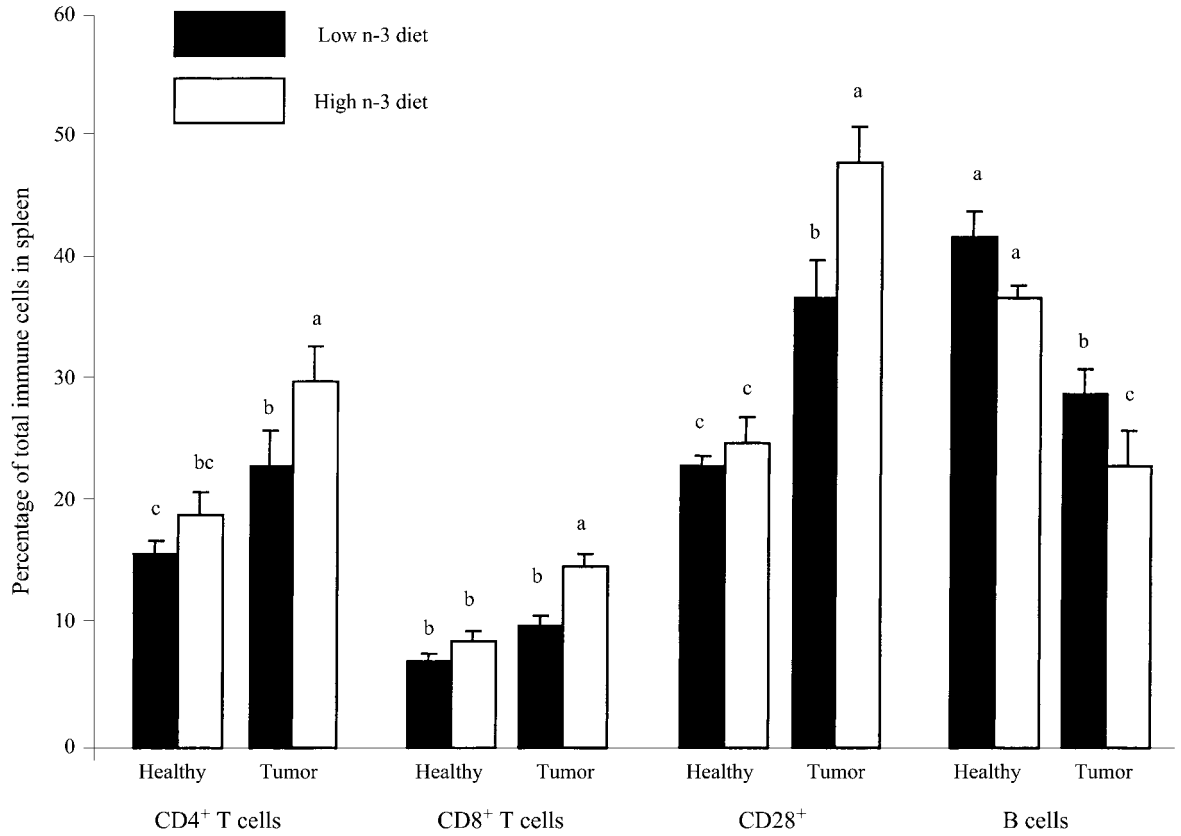

Figure 1. Effect of long-chain n-3 fatty acids fed in a low P/S diet on the proportion (% of total cells) of immune phenotypes in spleen after 48 h Con A stimulation. Splenocytes were isolated from healthy (control) or tumor-bearing rats fed a low P/S diet, with or without long-chain n-3 fatty acids. Isolated splenocytes were stimulated for 48 h with Con A (5 mg/l) and immune phenotypes were identified by indirect immunofluorescence assay and flow cytometry. Bars represent means $\pm$ SEM ($n \geq 7$/group). The effects of diet and tumor were analyzed by two-way ANOVA. For each immune cell phenotype, bars that do not share a common letter are significantly different ($p < 0.05$) as identified by a Duncan's multiple range test.

Table 3. IL-2 receptor (CD25) expression in splenocytes from healthy and tumor-bearing rats fed a low P/S diet either unsupplemented (low n-3) or supplemented with long-chain n-3 fatty acids (high n-3 diet) after splenocytes were stimulated with con A for 48 h[1]

Immune cell phenotype	Healthy		Tumor-bearing		Significance, p[2]	
	Low n-3 diet	High n-3 diet	Low n-3 diet	High n-3 diet	Diet	Tumor
Percentage of live cells remaining after culture						
$CD25^+CD5^+$ T cells	25 ± 2^b	26 ± 2^b	30 ± 3^b	43 ± 4^a	0.02	0.0004
$CD25^+CD4^+$ T helper cells	19 ± 2	21 ± 2	23 ± 2	29 ± 3	NS	0.01
$CD25^+$ $CD8^+$ T suppressor/cytotoxic cells	10 ± 1	12 ± 1	11 ± 1	15 ± 2	0.03	NS
$CD25^+$B cells	34 ± 4	33 ± 3	22 ± 1	18 ± 2	NS	0.0003
$CD25^+$ macrophages	6.7 ± 0.4	6.2 ± 0.7	5.8 ± 0.7	4.5 ± 0.4	NS	0.04
$CD25^+CD28^+$ cells	19 ± 1	24 ± 2	30 ± 3	42 ± 3	0.03	0.0008

[1]Values are means ± SEM ($n \geq 5$/group).
[2]Significant effect of diet and tumor as determined by two-way ANOVA, NS = $p > 0.05$. When a significant interaction was found, means within a row without a common superscript (a, b) are statistically different ($p \leq 0.05$) as identified by a Duncan's multiple range test.

macrophages that were $CD25^+$ compared with healthy rats (Table 3). Both healthy and tumor-bearing rats fed the high n-3 diet had a significantly increased proportion of $CD25^+$ $CD8^+$ T suppressor/cytotoxic cells and $CD25^+$ $CD28^+$ cells compared with those fed the low n-3 diet (Table 3).

NK cell cytotoxicity

(a) *High P/S diet*

Feeding tumor-bearing rats long-chain n-3 fatty acids in the high P/S diet did not significantly affect NK cell cytotoxic activity at the 25 : 1 (overall mean = 19 ± 2%), 50 : 1 (overall mean = 29 ± 2%), and 100 : 1 (overall mean = 43 ± 2%) effector:target cell ratios ($n = 14$). However, at the 12.5 : 1 effector:target cell ratio, the % specific lysis of target YAC-1 cells was greater ($p < 0.01$) for splenocytes from rats fed the high n-3 diet relative to splenocytes from those fed the low n-3 diet (high n-3 diet, 13 ± 1%; low n-3 diet, 9.5 ± 0.4%, $n = 6$/diet). Long-chain n-3 fatty acids fed in the high P/S diet did not significantly affect lytic activity (overall mean = 13 ± 2 lytic units, $n = 14$), the number of splenocytes ($\times 10^{-3}$) required to induce 20% lysis of YAC-1 tumor cells.

(b) *Low P/S diet*

There was a significant effect of long-chain n-3 fatty acids, tumor, and their interaction on NK cell cytotoxic activity when rats were fed the low P/S diet (Figure 2). At each effector:target cell ratios, both healthy and tumor-bearing rats fed the high n-3 diet had a higher ($p < 0.001$) % specific lysis compared with rats fed the low n-3 diet (Figure 2). In the low n-3 diet group, the tumor resulted in decreased ($p < 0.05$) NK cell cytotoxicity, while the tumor did not significantly affect NK cell cytotoxic activity when rats were fed the high n-3 diet (Figure 2). Lytic activity was not significantly different between treatment groups (overall mean = 24 ± 2 lytic units, $n = 32$).

Nitric oxide production

(a) *High P/S diet*

There was no effect of long-chain n-3 fatty acids in the high P/S diet on nitrite (NO_2^-) production by unstimulated splenocytes (overall mean = 7.2 ± 0.5 nmol/ml per 10^6 cells, $n = 14$).

(b) *Low P/S diet*

Compared with rats fed the low n-3 diet, healthy and tumor-bearing rats fed the high n-3 diet had increased ($p < 0.04$) splenocyte NO_2^- production by unstimulated splenocytes. As well, in both the low and high n-3 diet groups, splenocytes from tumor-bearing rats produced higher ($p \leq 0.01$) levels of NO_2^- compared with splenocytes from healthy rats (low n-3 diet, healthy = 2.9 ± 0.3; low n-3 diet, tumor-bearing = 4.5 ± 0.5; high n-3 diet, healthy = 4.7 ± 0.4; high n-3 diet, tumor-bearing = 6.2 ± 0.8 nmol/ml per 10^6 cells).

IL-2 production

For all groups, splenocytes cultured for 48 h without mitogen (unstimulated) produced undetectable levels of IL-2 in the culture supernatants (results not shown).

(a) *High P/S diet*

There was no effect of long-chain n-3 fatty acids in the high P/S diet on IL-2 production by splen-

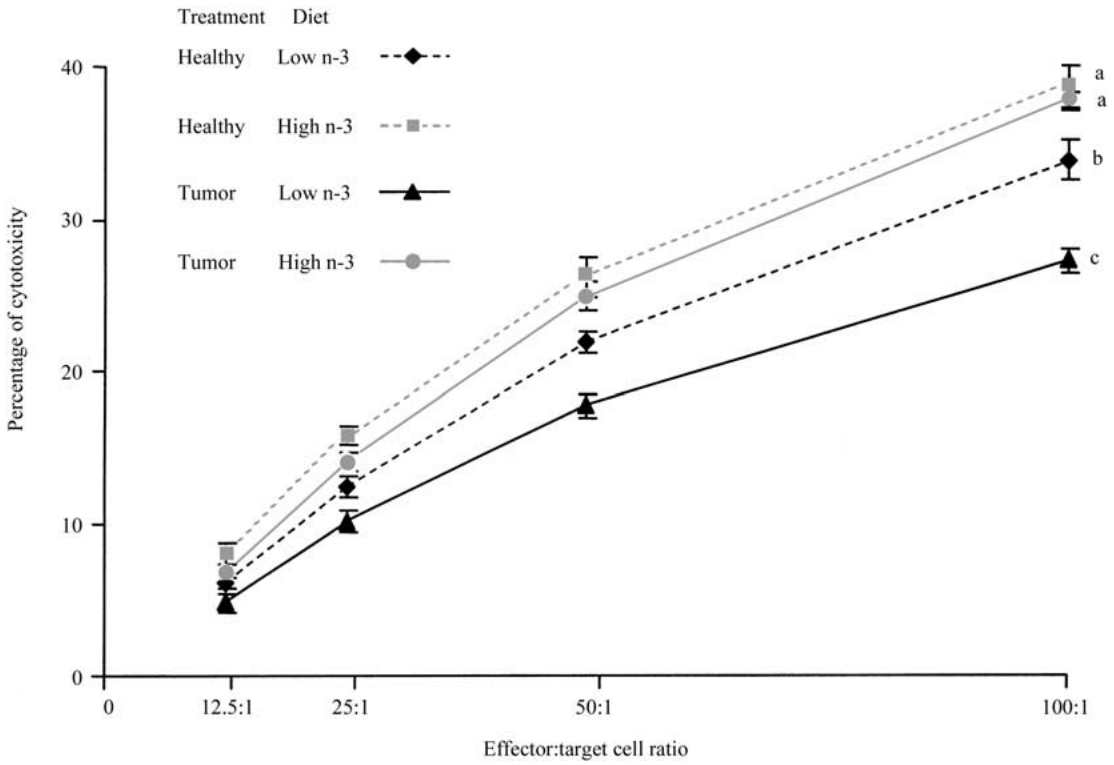

Figure 2. Effect of long-chain n-3 fatty acids fed in a low P/S diet on NK cell cytotoxic activity in healthy and tumor-bearing rats. NK cell cytotoxic activity is expressed as percent specific lysis, which is equal to 100 × (mean experimental ^{51}Cr release from labeled YAC-1 cells – mean spontaneous ^{51}Cr release)/(mean maximum ^{51}Cr release – mean spontaneous ^{51}Cr release). Points represent means ± SEM ($n \geq 7$/group). Lines that do not share a common letter are significantly different ($p < 0.05$) as determined by a two-way split-plot (repeated measures) ANOVA.

ocytes stimulated for 48 h with Con A (overall mean = 1468 ± 75 pg/ml per 10^6 cells, $n = 13$).

(b) *Low P/S diet*

There was a significant interaction between long-chain n-3 fatty acids and the tumor on splenocyte IL-2 production after Con A stimulation (Figure 3). For healthy rats, splenocytes from high n-3-fed rats produced higher levels of IL-2 when stimulated with Con A compared with splenocytes from low n-3-fed rats (Figure 3). Diet did not significantly affect Con A-stimulated IL-2 production in tumor-bearing rats (Figure 3).

Fatty acid composition of R3230AC tumor cell phospholipids

Dietary long-chain n-3 fatty acids and P/S ratio significantly altered the fatty acid composition of PC, PE, and PI of isolated R3230AC tumor cells. The proportions of fatty acids from C14:0 to C24:1(9) in PC, PE, and PI were measured, but only fatty acids which are relevant to the diets fed are reported (Tables 4–6).

(a) *Effect of dietary long-chain n-3 fatty acids*

For both the high and low P/S diets, tumor cells from rats fed long-chain n-3 fatty acids had a significantly higher total n-3 fatty acid content and n-3/n-6 ratio in PC, PE, and PI (Tables 4–6). Specifically, in PC, PE, and PI the percentage of C20:5(3), C22:5(3), and C22:6(3) were increased, while the percentage of C22:4(6) and C22:5(6) were decreased when rats were fed long-chain n-3 fatty acids in either the high or the low P/S diets (Tables 4–6). For both P/S diets, tumor cells from rats fed long-chain n-3 fatty acids had a significantly decreased the percentage of C20:4(6) in PC and PE (Tables 4–5), but not PI (Table 6). However, the total n-6 fatty acid content in PC, PE, and PI was significantly decreased in the high n-3 diet group (Tables 4–6). There was no effect of dietary long-chain n-3 fatty acids on the percentage of C18 : 2(6),

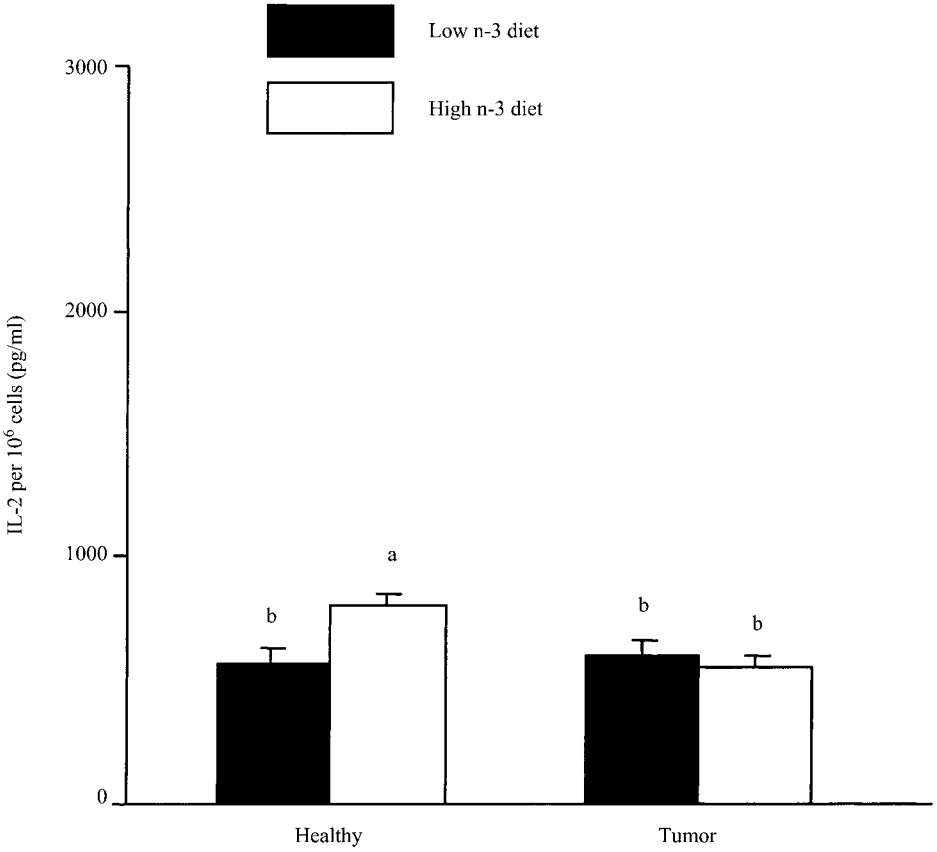

Figure 3. Effect of long-chain n-3 fatty acids fed in a low P/S diet on splenocyte IL-2 production. Splenocytes were cultured without mitogen (unstimulated) or with Con A (5 mg/l) for 48 h. The concentrations of IL-2 in splenocyte culture supernatants were determined by enzyme-linked immunosorbent assay as described in Materials and methods. For all groups, unstimulated splenocytes produced undectable levels of IL-2 in the culture supernatant (results not shown). Bars represent means ± SEM ($n \geq 7$/group). The effects of diet and tumor were analyzed by two-way ANOVA. Bars that do not share a common letter are significantly different ($p < 0.05$) as identified by a Duncan's multiple range test.

total saturated or monounsaturated fat content or unsaturation index (total number of unsaturated bonds) in PC, PE, and PI (Tables 4–6). Dietary long-chain n-3 fatty acids significantly decreased the P/S ratio in PC (Table 4).

(b) *Effect of dietary P/S ratio*

Long-chain n-3 fatty acid incorporation into tumor cell PC, PE, and PI was further affected by the P/S ratio of the diet. Specifically, when rats were fed long-chain n-3 fatty acids in the low P/S diet, total n-3 content and the n-3/n-6 ratio were significantly increased in PC, PE, and PI compared with long-chain n-3 fatty acids fed in the high P/S diet (Tables 4–6). Specifically, the percentage of C20 : 5(3) and C22 : 5(3) were increased in PC, PE, and PI when rats were fed long-chain n-3 fatty acids in the low P/S diet compared with the high P/S diet (Tables 4–6). In contrast, dietary P/S ratio did not affect total n-3 content or the n-3/n-6 ratio in PC, PE, and PI when rats were fed the low n-3 diet (Tables 4–6). Long-chain n-3 fatty acids fed in the low P/S diet resulted in an increased P/S ratio and unsaturation index in tumor cell PE (Table 5). In PC the percentage of C18 : 2(6) was significantly increased when rats were fed the low P/S diet compared with the high P/S diet (Table 4). The percentage of C20 : 4(6) and C22 : 4(6) in PC, PE, and PI were not affected by dietary P/S ratio (Tables 4–6). However, the percentage of C22 : 5(6) was significantly decreased in PC and PE when rats were the low P/S diet compared with the high P/S diet (Tables 4–5). There was no effect of dietary P/S ratio on total saturated, monounsaturated or n-6 fatty acid content in PC, PE, and PI (Tables 4–6).

Table 4. Fatty acid composition of phosphatidylcholine of R3230AC mammary tumor cells isolated from rats fed different diets[1]

Fatty acid	High P/S diet (P/S = 1)		Low P/S diet (P/S = 0.35)		Significance[2]	
	Low n-3 diet	High n-3 diet	Low n-3 diet	High n-3 diet	n-3, $p \leq$	P/S, $p \leq$
Percentage of (w/w) of total fatty acids						
C18:2(6)	3.6 ± 0.2	4.0 ± 0.4	4.7 ± 0.2	5.0 ± 0.4	NS	0.004
C20:4(6)	15.1 ± 0.4	12.5 ± 0.8	15.4 ± 0.3	11.7 ± 0.1	0.0001	NS
C20:5(3)	0.09 ± 0.02^{c}	0.24 ± 0.02^{b}	0.06 ± 0.01^{c}	0.61 ± 0.02^{a}	0.0001	0.0001
C22:4(6)	2.6 ± 0.1	1.4 ± 0.1	2.9 ± 0.2	1.4 ± 0.1	0.0001	NS
C22:5(6)	0.78 ± 0.05	0.37 ± 0.02	0.70 ± 0.03	0.27 ± 0.01	0.0001	0.02
C22:5(3)	0.63 ± 0.04^{c}	1.3 ± 0.1^{b}	0.50 ± 0.02^{c}	1.9 ± 0.1^{a}	0.0001	0.006
C22:6(3)	1.4 ± 0.1	2.2 ± 0.1	1.5 ± 0.1	2.6 ± 0.1	0.0001	0.03
SFA	44 ± 1	46 ± 1	44 ± 1	44 ± 1	NS	NS
MUFA	28 ± 1	29 ± 1	27 ± 1	29 ± 1	NS	NS
n-6 PUFA	23 ± 1	20 ± 1	24 ± 1	19 ± 1	0.0001	NS
n-3 PUFA	2.3 ± 0.1^{c}	4.0 ± 0.3^{b}	2.1 ± 0.1^{c}	5.1 ± 0.2^{a}	0.0001	0.02
n-3/n-6 ratio	0.10 ± 0.01^{c}	0.22 ± 0.01^{b}	0.09 ± 0.00^{c}	0.27 ± 0.02^{a}	0.0001	0.04
P/S ratio	0.61 ± 0.03	0.51 ± 0.01	0.61 ± 0.02	0.58 ± 0.01	0.01	NS
U. I.	128 ± 3	119 ± 3	128 ± 3	126 ± 2	NS	NS

[1]Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 5$ per diet). SFA: sum of saturated fatty acids; MUFA: sum of monounsaturated fatty acids; n-6 PUFA: sum of n-6 polyunsaturated fatty acids; n-3 PUFA: sum of n-3 polyunsaturated fatty acids; P/S ratio: ratio of polyunsaturated to saturated fatty acids; U. I.: unsaturation index (total number of unsaturated bonds).
[2]Significant effect of dietary n-3 fatty acids and P/S ratio as determined by two-way ANOVA, NS = $p > 0.05$. When a significant interaction was found, values within a row without a common superscript (a, b, c) are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test.

Table 5. Fatty acid composition of phosphatidylethanolamine of R3230AC mammary tumor cells isolated from rats fed different diets[1]

Fatty acid	High P/S diet (P/S = 1)		Low P/S diet (P/S = 0.35)		Significance[2]	
	Low n-3 diet	High n-3 diet	Low n-3 diet	High n-3 diet	n-3, $p \leq$	P/S, $p \leq$
Percentage of (w/w) of total fatty acids						
C18:2(6)	1.9 ± 0.1	2.5 ± 0.3	1.7 ± 0.2	1.9 ± 0.1	NS	NS
C20:4(6)	29 ± 1	24 ± 2	29 ± 1	25 ± 1	0.005	NS
C20:5(3)	0.18 ± 0.02^{c}	0.49 ± 0.03^{b}	0.16 ± 0.03^{c}	1.4 ± 0.1^{a}	0.0001	0.0001
C22:4(6)	6.4 ± 0.2	2.8 ± 0.1	6.4 ± 0.5	2.7 ± 0.2	0.0001	NS
C22:5(6)	2.1 ± 0.1	1.1 ± 0.1	2.0 ± 0.1	0.67 ± 0.03	0.0001	0.003
C22:5(3)	1.4 ± 0.2^{c}	2.8 ± 0.4^{b}	1.3 ± 0.1^{c}	5.1 ± 0.4^{a}	0.0001	0.002
C22:6(3)	2.6 ± 0.2	4.6 ± 0.5	3.5 ± 0.2	6.8 ± 0.5	0.0001	0.001
SFA	27 ± 1	33 ± 4	25 ± 1	24 ± 1	NS	NS
MUFA	16 ± 1	16 ± 1	16 ± 1	17 ± 1	NS	NS
n-6 PUFA	40 ± 1	32 ± 2	39 ± 1	31 ± 1	0.0001	NS
n-3 PUFA	4.4 ± 0.4^{c}	7.2 ± 0.5^{b}	5.3 ± 0.1^{c}	14 ± 1^{a}	0.0001	0.0001
n-3/n-6 ratio	0.11 ± 0.01^{c}	0.25 ± 0.02^{b}	0.13 ± 0.01^{c}	0.44 ± 0.03^{a}	0.0001	0.0001
P/S ratio	1.7 ± 0.1^{ab}	1.1 ± 0.2^{b}	1.8 ± 0.1^{a}	1.9 ± 0.1^{a}	NS	0.002
U. I.	202 ± 2	183 ± 11	206 ± 3	214 ± 5	NS	0.02

[1]Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 5$ per diet). SFA: sum of saturated fatty acids; MUFA: sum of monounsaturated fatty acids; n-6 PUFA: sum of n-6 polyunsaturated fatty acids; n-3 PUFA: sum of n-3 polyunsaturated fatty acids; P/S ratio: ratio of polyunsaturated to saturated fatty acids; U. I.: unsaturation index (total number of unsaturated bonds).
[2]Significant effect of dietary n-3 fatty acids and P/S ratio as determined by two-way ANOVA, NS = $p > 0.05$. When a significant interaction was found, values within a row without a common superscript (a, b, c) are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test.

Table 6. Fatty acid composition of phosphatidylinositol of R3230AC mammary tumor cells isolated from rats fed different diets[1]

Fatty acid	High P/S diet (P/S = 1)		Low P/S diet (P/S = 0.35)		Significance[2]	
	Low n-3 diet	High n-3 diet	Low n-3 diet	High n-3 diet	n-3, $p \leq$	P/S, $p \leq$
Percentage (w/w) of total fatty acids						
C18:2(6)	2.0 ± 0.2	2.3 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	NS	NS
C20:4(6)	28 ± 2	27 ± 2	27 ± 1	26 ± 1	NS	NS
C20:5(3)	nd^{c}	0.17 ± 0.07^{b}	nd^{b,c}	0.60 ± 0.05^{a}	0.0001	0.0003
C22:4(6)	2.8 ± 0.3	1.6 ± 0.1	3.0 ± 0.4	1.8 ± 0.1	0.0004	NS
C22:5(6)	0.64 ± 0.11	0.29 ± 0.07	0.65 ± 0.10	0.35 ± 0.03	0.002	NS
C22:5(3)	0.32 ± 0.08^{c}	1.1 ± 0.1^{b}	0.60 ± 0.08^{c}	2.0 ± 0.2^{a}	0.0001	0.0001
C22:6(3)	0.80 ± 0.05	1.3 ± 0.1	1.1 ± 0.1	1.9 ± 0.2	0.0001	0.0008
SFA	50 ± 1	51 ± 2	49 ± 1	48 ± 1	NS	NS
MUFA	10 ± 1^{b}	12 ± 1^{a}	$11 \pm 1^{a,b}$	$11 \pm 1^{a,b}$	NS	NS
n-6 PUFA	35 ± 1	31 ± 2	34 ± 1	32 ± 1	0.04	NS
n-3 PUFA	1.3 ± 0.1^{c}	2.5 ± 0.1^{b}	1.8 ± 0.2^{c}	4.6 ± 0.5^{a}	0.0001	0.0001
n-3/n-6 ratio	0.04 ± 0.00^{c}	0.08 ± 0.00^{b}	0.05 ± 0.01^{c}	0.14 ± 0.02^{a}	0.0001	0.0002
P/S ratio	0.79 ± 0.06	0.70 ± 0.07	0.80 ± 0.04	0.85 ± 0.05	NS	NS
U. I.	159 ± 8	157 ± 6	162 ± 5	169 ± 6	NS	NS

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 4$ per diet). nd: not detectable; SFA: sum of saturated fatty acids; MUFA: sum of monounsaturated fatty acids; n-6 PUFA: sum of n-6 polyunsaturated fatty acids; n-3 PUFA: sum of n-3 polyunsaturated fatty acids; P/S ratio: ratio of polyunsaturated to saturated fatty acids; U. I.: unsaturation index (total number of unsaturated bonds).

[2] Significant effect of dietary n-3 fatty acids and P/S ratio as determined by two-way ANOVA, NS = $p > 0.05$. When a significant interaction was found, values within a row without a common superscript (a, b, c) are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test.

Discussion

Diet composition and tumor growth

Although experimental tumors are not identical to spontaneously occurring human tumors, they can provide valuable models to examine therapeutic principles, such as the impact of potential anticancer nutrients. We designed two separate experiments to determine if supplementation of a fixed dose (5% w/w of total fat) of fish oil-derived long-chain n-3 fatty acids in a diet containing a level of n-6 polyunsaturated fatty acids representative of what many North American's are currently consuming (i.e., a low P/S diet) has the same biological effect when it is supplemented in a diet more closely resembling current dietary recommendations (i.e., a high P/S diet). The results showed that feeding a fixed dose of long-chain n-3 fatty acids in a high polyunsaturated fat diet did not inhibit R3230AC mammary tumor growth. When rats were fed the same fixed dose of long-chain n-3 fatty acids in a low polyunsaturated fat diet, tumor growth was suppressed −31% compared to the unsupplemented, low P/S diet group, but this effect did not reach statistical significance. This lack of a statistically significant effect may have been due to the small number of rats used in the experiment and we recognize this as a limitation of our study. Our experimental diets contained ∼40% of energy from fat from a mixture of sources and were designed to reflect the level and composition of fat in the North American diet [30] with n-3/n-6 fatty acid ratios of 0.13 and 0.26 in the long-chain n-3 fatty acid supplemented high and low P/S diets, respectively. Other studies have reported that n-3/n-6 fatty acid ratios from 0.5 to 2 are optimal for anticancer benefits [2–5]. However, since it is difficult to achieve an n-3/n-6 ratio greater than 0.5 in the North American diet, the practical relevance of such dietary designs for cancer prevention studies is not clear. Although we did not find a statistically significant effect of long-chain n-3 fatty acid supplementation on R3220AC tumor growth at 17 days post-implantation, this does not preclude benefits of these fatty acids on later cancer stages, such as improved response to chemotherapy [21], reduction of tumor metastasis [20], or prevention of cancer cachexia [31], or on earlier stages, such as cancer prevention [32]. For example, Noguchi et al.

[32] showed that low-dose EPA and DHA inhibit the incidence of rat mammary tumors, but not their growth.

Diet and tumor cell membrane composition

Our study confirms previous findings that changing diet fat composition significantly alters tumor cell membrane composition [5, 18–20, 32]. However, the effect of dietary fatty acids on individual membrane phospholipid fractions in tumor cells has not been extensively studied. Such a separation is important because different phospholipid fractions may respond to dietary fat manipulation to varying degrees [33]. As expected, tumor cells from rats fed long-chain n-3 fatty acids had a higher total n-3 content and n-3/n-6 ratio in PC, PE, and PI. Furthermore, the magnitude of increase in incorporation of long-chain n-3 fatty acids, especially EPA and docosapentaenoic acid (C22 : 5n-3), into tumor phospholipids was significantly greater when long-chain n-3 fatty acids were supplemented in a low P/S diet (with a 2-fold higher n-3/n-6 fatty acid ratio, making this the highest ratio of all diets) compared with a high P/S diet. Although feeding long-chain n-3 fatty acids did not affect the amount of linoleic acid in tumor cell phospholipids, the amount of arachidonic acid (C20 : 4n-6) was decreased in PC and PE when rats were fed EPA and DHA. Rose et al. [20] also demonstrated that the accumulation of n-3 fatty acids in human breast cancer cell phospholipids was at the expense of arachidonic acid, but not linoleic acid. It is not known whether diet fat-induced alterations in tumor cell lipid composition are directly associated with changes in eicosanoid production, receptor, or enzyme function, cell permeability, or second messenger pathways involved in tumor growth in this model. Dietary fat modulation of eicosanoids [2] and intracellular second messengers [34] has been reported in other studies. Additionally, incorporation of dietary n-3 fatty acids into tumor cell membranes is associated with increased susceptibility of tumors to cell-mediated lysis [19] and increased sensitivity to chemotherapy [21]. Although the length of time that diets were fed in the present study (17 days post-implantation) was sufficient to alter tumor cell phospholipid composition, it was perhaps not long enough for these mechanisms to impact on tumor growth. We did not continue our study over a longer time period after tumor implantation because we wanted to avoid the onset of complicating factors, such as malnutrition and cachexia which could have influenced both tumor growth and immune function.

R3230AC tumor and host immune function

Components of both the innate (non-specific) and acquired (specific) immune systems are important contributors to host defense against tumor cells [12–13]. There are many putative mechanisms of immune cell cytotoxicity, including production, by $CD4^+$ Th1 cells, of IL-2 which activates NK cells [13]. Nitric oxide, produced by various activated immune cells, has also been identified as a key molecule in anticancer defense and a potential mediator of NK cell cytotoxicity [35]. Although there is limited data on the effect of the R3230AC mammary tumor on host immune function, previous work in our lab has shown that anticancer immunity declines progressively with growth of other transplantable tumors, such as the Morris Hepatoma (MH) 7777 [23]. In the present study, the effect of the R3230AC tumor on host immunity was evaluated in the low P/S diet experiment which included both healthy and tumor-bearing rats. At 17 days post-implantation, tumor burden had a suppressive effect on NK cell cytotoxicity, but only when rats were fed the low n-3 diet without long-chain n-3 fatty acids. A major focus of current research in immunology and oncology is the development of methods to augment host antitumor immune defense. Dietary long-chain n-3 fatty acids increased NK cell cytotoxicity in both healthy and tumor-bearing rats, suggesting that long-chain n-3 fatty acid supplementation may prevent tumor-induced suppression of NK cell cytotoxicity. This upregulation of a component of anticancer immune function with dietary long-chain n-3 fatty acids warrants further investigation in the clinical setting. Healthy rats fed long-chain n-3 fatty acids also had higher splenocyte nitric oxide and IL-2 production. Both of these immune mediators have been proposed to play roles in the tumoricidal capacity of NK cells [35–36], but their precise role in our model requires further work. However, the increased cytotoxicity of NK cells from tumor-bearing rats fed the high n-3 diet was associated with elevated nitric oxide, but not IL-2 production. It is not currently known if such host immune responses can be directed at the R3230AC mammary tumor. Further investigation is needed to determine if diet-induced immune enhancements measured *ex vivo* are important in the growth of this tumor model *in vivo*.

Not all immune parameters were diminished in tumor-bearing animals. For example, compared with healthy rats, tumor-bearing rats had a higher proportion of $CD4^+$T helper cells, which produce IL-2 [13], $CD8^+$ T suppressor/cytotoxic cells, which can generate a population of cytotoxic T-lymphocytes [12], and cells expressing CD28, an important co-stimulatory signal for T cell proliferation, cytokine production and cytokine receptor expression [37]. In addition, tumor-bearing rats had a higher proportion of $CD4^+$ T helper cells and $CD28^+$ cells that were activated after Con A stimulation, as determined by expression of the IL-2 receptor (CD25). Although certain observed changes in immune cell phenotypes were found to be 'statistically significant', they were sometimes very small and thus should be interpreted with caution as such small changes may be of limited biological or clinical significance. It is important to note, however, that we do not currently understand exactly what constitutes a 'clinically relevant' effect of tumor burden on host immunity due, in part, to the vast complexity and variety of tumor types. Overall, tumor-bearing animals had increased CD25 expression on T cells after Con A stimulation, suggesting that cells from tumor-bearing rats were better able to respond to an immune challenge. However, it is not currently known if this host immune response can be directed at the tumor. Since various host immune responses were either up-or down-regulated in tumor-bearing rats when compared with healthy rats, we suggest that various assays of anticancer immunity need to be performed in order to thoroughly evaluate host immunocompetence in rats bearing the R3230AC tumor. In addition, growth of the current transplantable tumor model did not induce the same degree of host immune suppression as we had previously observed when studying the MH 7777 [23]. This finding is not surprising and further emphasizes the complexity of animal models of cancer available for study in this field.

Diet and host immune function

The current study was not designed to statistically compare host immune responses between the long-chain n-3 supplemented high and low P/S diets, as these constituted two separate experiments. Thus, although it may seem that certain immune parameters, such as splenocyte IL-2 production, were affected by the level of polyunsaturated fat in the diet (P/S or n-3/n-6 ratio) the findings should be interpreted with caution as the current study was not designed to directly test this comparison.

We have previously shown that feeding healthy rats long-chain n-3 fatty acids in a high P/S diet upregulated the immune system [14]. However, in contrast to our previous study using healthy rats, in the current study feeding long-chain n-3 fatty acids in a high P/S diet did not significantly alter immune function in rats bearing mammary tumors. It is not known why immune responses to long-chain n-3 fatty acid supplementation in a high P/S diet differed between healthy and tumor-bearing rats. Our results suggest that the presence of a tumor interferes with the ability of long-chain n-3 fatty acids to enhance immune function when fed in a high P/S diet. When compared with the unsupplemented (low n-3), low P/S diet, supplementation of long-chain n-3 fatty acids in a low P/S diet improved certain immune defenses in tumor-bearing rats. For example, tumor-bearing rats fed long-chain n-3 fatty acids in a low P/S diet had significantly increased NK cell cytotoxicity, a higher proportion of activated $CD8^+$ and $CD28^+$ cells and increased nitric oxide production after Con A stimulation compared with those fed the unsupplemented (low n-3), low P/S diet. Our results contradict many previous studies which have concluded that consumption of very large (with respect to the human diet and in comparison to the diets fed in the current study) amounts of long-chain n-3 fatty acids is immunosuppressive [10]. However, a study by Sasaki et al. [38] also found that diets rich in DHA exert some of their immunomodulatory effects by an upregulation of the CD28-mediated costimulatory signal.

We have now shown that long-chain n-3 fatty acids improve aspects of immunity in healthy rats when supplemented in a high P/S diet, as previously described [14], or in a low P/S diet, as observed in the present study. Interestingly, the same diet effect is not observed in tumor-bearing animals since our results demonstrate an immunoenhancing effect of long-chain n-3 fatty acids in tumor-bearing rats only when supplemented in a low P/S, but not a high P/S, diet. The 2-fold difference in the n-3/n-6 fatty acid ratio between the two high n-3 supplemented diets may provide a possible explanation for the observed differences in diet-induced immune effects between the high and low P/S diet experiments. Certainly it has been suggested that n-3 fatty acids are more biologically effective when the proportion of n-3 fatty acids is much greater than that of n-6 fatty acids [1]. Although the current study was not designed to directly examine this

question, this is an important consideration for future studies in this area.

Dietary long-chain n-3 fatty acids fed in a low P/S diet had further enhancing effects when fed to tumor-bearing, as evidenced by an increase in activated $CD25^+$ T cells after mitogen stimulation compared with those fed the low n-3 diet. Since tumor growth was not significantly affected by long-chain n-3 fatty acid supplementation, the potential importance of diet-associated changes in the measured immune variables is not clear. However, the ability of dietary long-chain n-3 fatty acids to enhance the immune system should not be overlooked, since improved host immunity may have other benefits during cancer, such as better response to chemotherapy, prevention of disease recurrence and metastasis, or infectious complications. It remains possible that the R3230AC mammary tumor might respond to dietary long-chain n-3 fatty acids in other experimental situations, especially given that many of the host antitumor immune parameters enhanced by dietary long-chain n-3 fatty acids are important for prevention of cancer recurrence. Future experiments should test the potential immune benefits of feeding long-chain n-3 fatty acids in a low P/S diet to tumor-bearing rats using different models to reflect different human cancer situations.

Conclusions

The present study demonstrated that the polyunsaturated fat content of the diet significantly influences the effect of dietary long-chain n-3 fatty acids on immune parameters assayed in tumor-bearing animals. Long-chain n-3 fatty acids supplemented in a high P/S diet did not affect tumor growth or host immune responses. In contrast, compared with a low n-3 (unsupplemented), low P/S diet, long-chain n-3 fatty acid supplementation in a low P/S diet enhanced antitumor immune defense in both healthy and tumor-bearing rats, increased n-3 fatty acid incorporation into tumor cell phospholipids, but did not significantly reduce tumor growth in this model. Current recommendations for the North American population are to increase consumption of polyunsaturated fat (with a decrease in saturated fat intake) to achieve a P/S ratio of 1 [6–8], which is the same ratio fed in the high P/S diet in the present study. Although cancer agencies have established dietary guidelines to reduce cancer risk [9], they do not currently have a recommendation for dietary P/S ratio. Our results, in an animal model of breast cancer, suggest that a lower level of polyunsaturated fat than is currently recommended by several health agencies may be required to enhance the immune benefits of long-chain n-3 fatty acids, particularly during cancer.

Acknowledgements

The authors gratefully acknowledge J. Aldrich for animal care as well as S. Goruk and A. Wierzbicki for excellent technical assistance. Financial support was provided by a grant from the Natural Sciences and Engineering Research Council of Canada to C. J. Field. L. E. Robinson is a recipient of a Natural Sciences and Engineering Research Council of Canada scholarship and an Alberta Heritage Foundation for Medical Research studentship.

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