Postpromotional Effects of Dietary Marine or Safflower Oils on Large Bowel or Pulmonary Implants of CT-26 in Mice

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ABSTRACT

Marine oils containing n-3 fatty acids exhibit variable antineoplastic effects. Diets containing low (11.6% of kcal) or high (46.5% of kcal) levels of marine oils as the exclusive fat source were compared to diets containing identical amounts of safflower oil (n-6) in weanling, male BALB/c ByJ mice. All diets provided approximately 90 kcal/100 g body weight/day, and contained identical quantities of vitamins, minerals, protein, and fiber. The growth of transplantable colon carcinoma, CT-26, (10^6 cells/animal) implanted, subserosally, into the descending colon via laparotomy, was observed weekly over 28 days by necropsy in all dietary groups. At each time period animals fed safflower oil had larger tumors than those fed marine oil. Tumor volumes at 21 days postimplantation were as follows: low fat marine, 55 mm^3 (5-196 mm^3) [median (range)]; high fat marine, 70 (26-194); low fat safflower, 216 (32-800); high fat safflower, 247 (70-1352). Marine oil tumors were smaller than safflower oil tumors (P < 0.005 by analysis of variance; P < 0.01 by Scheffe test). Metastatic potential was assessed by pulmonary colonization. CT-26 was injected i.v. in tail veins (10^6 cells/animal). Mice were sacrificed and counted were collected after 21 days. Mice fed low fat marine, high fat marine, and low fat safflower oil diets, 10-14 colonies; high fat safflower, 55 colonies (P < 0.001 by analysis of variance). Hence, dietary marine oil significantly suppressed growth of this colon carcinoma at all intake levels studied and inhibited pulmonary colonization at higher intakes relative to safflower oil.

INTRODUCTION

Epidemiological investigations indicate that international populations consuming diets high in fat are at a greater risk for colon cancer than those consuming less fat (1, 2). Concordance for these findings has been demonstrated in a variety of laboratory animal models by using several different chemical carcinogens (3-6). The general consensus regarding these observations is that dietary lipid, in some as yet undetermined manner, contributes to bowel tumor promotion. Little experimental data, however, are available regarding the role of nutritional lipids on postpromotional events in colon tumorigenesis. Previous investigations of lipid effects upon tumors have proceeded mainly along two directions, the carcinogen induced and transplantable models. Studies of the carcinogen type (7-9) have provided data on the effects of various lipid diets on tumor incidence and/or multiplicity. These effects derive from a combination of the factors, including carcinogen absorption, trophism, and the rates of carcinogen activation versus inactivation in vivo. The relationship of fat nutrition to the modification of carcinogenesis is greatly obscured by the multiple metabolic processes involved in these models. The utility of these studies lies in establishing dietary fat as a factor able to alter this complex system of “complete carcinogenesis” rather than in describing mechanisms of interventions.

An alternate approach made use of transplantable tumors maintained in culture (10, 11). Suspensions or blocks of these tumors were grown in animals on various fat supplements for a specified time, followed by sacrifice and necropsy. These studies routinely addressed questions of tissue fatty acid content, activity of the cyclooxygenase and lipoxygenase pathways, and concentrations of their products. Thus valuable biochemical information was gathered on the reaction of growing tissues to changes in their lipid milieu. These studies are limited, though, in their utility regarding carcinogenesis, since little attention was given to the postpromotional growth characteristics of the tumor itself in the lipid environments created in vivo by the different diets.

Recently, studies have focused upon the quantity of fat in the diet as well as on the type of fat (saturated versus polyunsaturated) (12, 13). Evidence regarding the latter indicates differential effects on tumor growth, metastasis, prostaglandin synthesis, and membrane lipid composition between the two predominant types of dietary polyunsaturated fats: n-6 versus n-3 (7, 8, 14). The present investigation indicates that marine oils inhibit the postpromotional tumor growth and metastatic propensity of CT-26, a transplantable colon cancer line, and the demise of the host relative to their safflower oil counterparts.

MATERIALS AND METHODS

Tissue Culture Techniques. Frozen CT-26 cells maintained in our bank were thawed, seeded onto 75-cm^2 flasks (Costar or Corning), at 1 x 10^6 cells/flask, and grown in RPMI 1640 medium with 1% glutamine, 1% penicillin/streptomycin combination, and 10% fetal bovine serum (medium changed twice/week). When confluent (7 days), cells were harvested and split to the appropriate number of flasks for the scheduled inoculations (approximately 2 x 10^7 cells/flask). All studies were performed with cells of passage 8.

When again confluent, cells were trypsinized, washed, pelleted at 250 x g, then resuspended in serum-free media. Mice receiving intracolonic inoculations (tumor growth assay and time to death group) were inoculated with 1 x 10^5 cells/animal, and grown in RPMI 1640 medium with 1% glutamine, 1% penicillin/streptomycin combination, and 10% fetal bovine serum (medium changed twice/week). When confluent (7 days), cells were harvested and split to the appropriate number of flasks for the scheduled inoculations (approximately 2 x 10^7 cells/flask). All studies were performed with cells of passage 8.

Received 7/15/88; revised 4/4/89; accepted 5/1/89.

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1 This work was supported by American Cancer Society Grant BC-539, National Large Bowel Project Grant CA-38177, and Oncobiology Training Grant CA9423 of the National Cancer Institute, National Institutes of Health, Bethesda, MD, whom the authors gratefully acknowledge.

2 To whom requests for reprints should be addressed, at: Boston University School of Medicine, Department of Microbiology, 80 East Concord St., Boston, MA 02118.

3 C. C. O'Connor and S. A. Broitman, unpublished results.
animals inoculated at the end of the day received suspensions with the same viability as those inoculated earlier.

Animal Care and Feeding. Weanling BALB/c ByJ mice (The Jackson Laboratory, Bar Harbor, ME) were randomly assigned to one of four diet groups upon arrival. Conditions in the facility are in strict accordance with the guidelines promulgated in the Guiding Principles in the Care and Use of Animals, approved by the American Physiological Society. Temperature and humidity and light-dark cycle are strictly controlled.

After 4 to 7 days of pelleted, commercial mouse chow (food and water ad libitum) the animals began eating experimental diets. All diets conform to the AIN-76 standards (15) with modifications of their fat content to provide identical amounts of energy, protein, vitamins, and minerals to all mice. Further, all diets provided an adequate amount of essential fatty acids to prevent EFA* deficiency (16, 17). The modifications were made by varying the amount of carbohydrate in the diet, since carbohydrate has been shown not to appreciably influence experimental tumorogenesis (18). "Low fat" diets contained 5% fat by weight; this accounted for 11.7% of calories/g. Diets designated "high fat" contained 24.7% fat by weight, accounting for 46.5% of calories/g (Table 1).

The exclusive fat source in each diet was provided as follows. Group 1, low fat safflower oil diets contain 5.0% (w/w) safflower oil (polyunsaturated, high linoleic acid [18:2 n-6] product); Group 2, high fat safflower oil diets contain 24.7% safflower oil; Group 3, low fat marine oil diets contain 5.0% menhaden oil (polyunsaturated, high EPA [20:5 n-3], and DHA [22:6 n-3], fish-derived product); and Group 4, high fat marine oil diets contain 24.7% menhaden oil (food and water ad libitum). The animals were maintained on these diets for 30 days. Mice within each diet group were then randomly divided into the following experimental groups: tumor growth assay (120), colonization assay (90), time to death (60), and controls for observing weight gain (60).

Oils were obtained fresh weekly (and kept sealed under nitrogen at 0°C), at which time new diet was prepared. Diets were maintained refrigerated in sealed plastic bags under nitrogen to prevent lipid peroxidation. The animals were fed every day; all unspent food was removed from the cage, and the cage bedding was changed.

Tumor Volume Assay. Animals in this group were anesthetized with sodium pentobarbital, i.p. (0.25–0.35 ml at 6.5 mg/ml, approximately 78 mg/kg body weight). The abdomen was swabbed with 70% ethanol and the surrounding colon was visualized via the standard 1.5-cm midclavicular, laparotomy incision at the level of the umbilicus. The cell bolus was injected subcutaneously, on the antimesenteric side by using a 27-gauge needle on a 1-ml syringe, being careful not to puncture the lumen. On rare occasions, when leakage from the syringe occurred, it was promptly and completely bled with a swab soaked in 70% ethanol. After implantation, the gut was replaced in the abdomen in the opposite order of removal. Muscle and skin layers were closed together with 9-mm wound clips (Clay Adams, obtained from Harvard Bioscience, South Natick, MA) washed in 70% ethanol. Mice were placed under a heat lamp to prevent postoperative hypothermia until awake and active. At intervals incision scars were observed for signs of infection (pus or necrosis), peritonitis (edema or excessive tenderness), or other abnormal healing. In addition, gross internal and external appearance of the scar was noted on necropsy.

On postoperative days 7, 14, 21, and 28, animals were sacrificed and thoroughly necropsied to ascertain the size of the primary tumor and the number and size of both direct extensions and metastases, as well as the condition of the noncancerous tissue. Primary tumors were measured and their volume was calculated with the formula, width

\[ \times \text{length} \times 0.4 \times (19). \]

The number of animals sacrificed at each time point was predetermined by consideration of the number required for statistically valid comparisons.

Colonization Assay. In the colonization assay, inoculations were administered to animals in this group by i.v. injection into one of the tail veins (tails were washed with 70% ethanol), using a 30-gauge needle on a 1-ml syringe. Animals were observed daily for the 21 days following inoculation, whereupon they were killed, their chests opened, and their tracheas visualized. A blunt 18-gauge needle was introduced into the trachea of each animal and 2 to 3 ml of dye solution (15% India ink in phosphate-buffered saline with 2% ammonia) was infused into the lungs until fully insufflated. The lungs were then excised en bloc and placed in Fakete’s solution. In 24 h, Fakete’s solution bleaches the tumorous tissue white, leaving uninvolved lung black. Tumor foci were counted under magnification independently by three technicians. Intertechnician reliability (multiple correlation analysis) was very high (r = 0.9692, 0.9519, 0.9104 for technician 1 versus 2, technician 1 versus 3, and technician 2 versus 3, respectively; R² = 0.9086 at P < 0.001 by ANOVA).

Random specimens were selected from each group. The tumor foci from these lungs were measured with vernier calipers (Manostat, Switzerland, obtained from Harvard Bioscience) and the diameter of each colony was recorded. The size distribution of the foci was determined in the various diet groups, since it is possible that one group may display lower numbers of tumors due to the coalescence of many small foci into a few large ones; or due to a few, fast-growing lesions engulfing the lung, resulting in a specimen with the same total tumor volume as one with more numerous small, individual tumors.

Time to Death. This group of animals received tumor cell bolus implants exactly as those of the tumor growth assay group. The operative procedure was, in fact, performed in a continuous process by a single individual alternating implantations among the various dietary groups. After implantation and recovery in the warming tray, the animals were returned to their respective cages and observed twice daily for signs of morbidity and for the date of demise.

Statistical Methods. The data on intertechnician reliability for pulmonary colonization assay counts were analyzed by multiple correlation

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**Table 1 Composition of experimental diets**

<table>
<thead>
<tr>
<th>Ingredient*</th>
<th>Low fat (g)</th>
<th>High fat (g)</th>
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<tbody>
<tr>
<td>Oil (marine or safflower)*</td>
<td>5.00</td>
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<tr>
<td>Casein*</td>
<td>20.00</td>
<td>20.00</td>
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<td>Corn starch</td>
<td>15.00</td>
<td>7.17</td>
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<tr>
<td>Sucrose</td>
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<td>23.90</td>
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<tr>
<td>Fiber*</td>
<td>5.00</td>
<td>5.00</td>
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<tr>
<td>DL-methionine</td>
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<td>0.30</td>
</tr>
<tr>
<td>Choline bitartrate</td>
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<td>0.20</td>
</tr>
<tr>
<td>AIN mineral mix</td>
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<td>3.50</td>
</tr>
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<td>AIN vitamin mix</td>
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<td>81.07</td>
</tr>
<tr>
<td>Energy value (kcal/g)*</td>
<td>3.945</td>
<td>4.852</td>
</tr>
<tr>
<td>% of kcal as fat</td>
<td>11.7%</td>
<td>46.8%</td>
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</table>

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<th>Anticipated daily intake for adult BALB/c mice*</th>
<th>Energy (kcal/day)</th>
<th>Food</th>
<th>Protein</th>
<th>Fat</th>
<th>Carbohydrate</th>
<th>Fiber</th>
<th>Choline bitartrate</th>
<th>DL-methionine</th>
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<td>88.00</td>
<td>22.20</td>
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<td>1.11</td>
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<td>0.04</td>
<td>0.07</td>
<td>0.78</td>
<td>0.22</td>
<td></td>
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</tbody>
</table>

* All diets were prepared fresh weekly and kept refrigerated; all animals were given fresh food daily; unspent food was discarded; all cages and bedding were changed daily and the animals were observed regularly for signs of illness or malaise not associated with the study; none was noted by our personnel.

* Diets were formulated according to guidelines set forth in Ref. 15.

* Marine oil contains 5.51% essential fatty acids by weight; safflower oil contains 77.0% essential fatty acids by weight.

* Casein is assumed to be 90% protein.

* Nonnutritive cellulose fiber was used and therefore did not contribute to the tally of absorbed nutrients.

* Assumed to be 4.1 kcal/g for starch, sucrose, and casein, and 9.2 kcal/g for safflower and marine oils.

* For convenience in depicting the smaller quantities of dietary ingredients, anticipated daily intakes are expressed in terms of 100 g of mouse body weight.

* All values are given as g/day of the specific nutrient except otherwise specified.

* The abbreviations used are: EFA, essential fatty acid; LFSO, low fat safflower oil diet; HFSO, high fat safflower oil diet; LFMO, low fat marine oil diet; HFMO, high fat marine oil diet; AOV, analysis of variance; PG, prostaglandin; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.
followed by ANOVA, using the NCSS computer statistical package on our IBM-PC computer (NCSS version 5.01). The data on pulmonary colony size were examined by using a three-level, nested ANOVA after the method given by Sokal and Rohlf (21). No test of multiple comparison was performed since the F values associated with the variance ratios of interest were not significant.

The pulmonary colonization assay data were analyzed by the non-parametric Kruskal-Wallis one-way ANOVA to accommodate the skew of the data; then by the Mann-Whitney U test to identify specific significant differences. The tumor growth assay data were found to be lognormal, and therefore the transformed data for each time point (7, 14, 21, and 28 days) were analyzed by using Gaussian ANOVA and the Scheffe test of multiple comparisons. These statistical procedures were performed with the SPSS Statistical Package on the main frame computer at Boston University.

RESULTS

Tumor Growth Assay. Fig. 1 illustrates that the HFSO diet group had the largest tumors followed by the LFSO group. Tumors from mice fed LFMO and HFMO diets were approximately the same size but were considerably smaller than tumors from either of the safflower oil diet groups at all time points measured.

By the seventh day following implantation, significant differences in tumor sizes between dietary groups as determined by ANOVA ($P < 0.01$) were apparent. Specifically, tumors from the HFMO group (25.8 mm$^3$; 18–70) (median; range) were significantly smaller than those from the LFSO group (131; 25–162) by Scheffe ($P < 0.05$). At day 14 tumors from both safflower oil groups were 2 to 3 times larger than tumors from either marine oil group ($P < 0.001$ by ANOVA). By day 21, tumors from the safflower oil groups were 3 to 5 times larger than those of the marine oil groups ($P < 0.0005$ by ANOVA) (see Fig. 2). Mice fed the LFMO diet versus HFMO diet show no statistical difference in tumor growth at any time point as assessed by Scheffe.

Both the LFMO and HFSO diets were associated with similar growth patterns during the first 2 weeks following implantation; by the third week, mice fed the HFSO diet experienced an accelerated rate of tumor growth. While this was not statistically significant at the 3-week interval, tumors in these mice had extended along the mesenteric lymphatics of the gut to the line of mesenteric attachment of the stomach. Occlusion of the bowel lumen and envelopment of the entire descending colon were also noted frequently. The demise of all the mice in this group by the 28th day after implantation caused a difficulty in quantifying this extensive growth. The mortality rate among HFSO-fed animals was significantly increased over that of the LFMO-fed animals ($\chi^2 = 9.32$; d.f. 1; $P < 0.001$). Thus, mice fed the HFSO diet succumbed to occlusion of bowel a minimum of 1 week to 10 days earlier than mice fed the LFMO diet, indicating a more rapid, aggressive tumor growth in the former group.

Because there were insufficient numbers of animals in some groups, at 28-days the data for the LFMO and LFSO groups were analyzed separately (10 and 6 animals, respectively). The transformed data were examined by using an unpaired $t$ test

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and were found to be significant at the $P < 0.001$ critical level. Thus, it is apparent that tumors from mice fed the LFMO and HFMO diets were significantly smaller than those from mice fed LFSO and HFSO diets throughout the duration of the study.

Pulmonary Colonization Assay. Fig. 3 illustrates triplicate trials of the pulmonary colonization assay. Kruskal-Wallis one-way ANOVAs showed no significant differences between replicates within any of the diet groups. The same statistical analysis showed differences between dietary groups at the $P < 0.001$ significance level. By the Mann-Whitney $U$ test, it was determined that mice in the HFSO group had significantly more tumors than mice in the other dietary groups. The LFSO, LFMO, and HFMO groups did not differ significantly in numbers of tumors. This is demonstrated in Fig. 4, which shows representative lung preparations from each of the four diets used in the study.

The three-level ANOVA performed on the pulmonary colony diameter measurements showed that the frequency distributions of each of the diet groups did not vary significantly from one another ($F = 0.36$; d.f. 16, 120; $P > 0.5$). This means that no diet group favored the growth of disproportionately large or small colonies. Since the lung colony sizes in every group varied within a single frequency distribution, the differences in lung colony counts recorded were a valid measure of differences in total lung tumor load.

Control for Weight Gain. Table 2 contains food consumption and weight gain of BALB/c mice bearing CT-26 in the large bowel over 10 weeks of observation. The actual energy intake is similar to the estimated value set forth in Table 1 and illustrates that regardless of the differing caloric density of the diets, all animals consumed equivalent amounts of kilocalories, protein, vitamins, minerals, and fiber.

**DISCUSSION**

Direct effects of lipid nutriture on tumor growth and pulmonary colonization were studied by using a transplantable tumor. A clear retardation in the rate of growth of CT-26 implanted in colons of mice fed marine oil diets was noted relative to mice fed safflower oil diets. At all time points, both the LFSO- and HFSO-fed animals exhibited larger tumors than the marine oil-fed animals.

The LFMO diet provided $0.62\%$ of total kcal as EFA, marginal but adequate (16), and consistent with findings of virtually identical weight gain among all dietary groups throughout the study. An increase in EFA content of more than 4-fold (HFMO), in excess of the nutritional requirement, resulted in no increase over the LFMO diet in bowel tumor size or in pulmonary colony number. Thus, it is highly unlikely that EFA content per se was the sole inhibiting factor of tumor growth rate and pulmonary colonization in the marine oil diets. The EFA content of the LFSO and HFMO diets was 9 and 36\% of total kcal, respectively, both far in excess of the nutritional requirement. Nevertheless, tumor growth in the bowel and pulmonary colony number increased with increasing fat in these diets. Again, it is unlikely that the EFA content of the diet per se was the determining factor in these findings, since increasing quantities of EFA above the requirements of tumor-bearing hosts would not be expected to enhance tumor growth (22) or

![Fig. 3. Pulmonary colonization assay results. All data from the pulmonary colonization assay are shown. The experiment was performed in triplicate. The safflower oil diets displayed a dose-dependent increase in the number of pulmonary colonies with the marine oil diets. This assay failed to show any dose-dependent differences in pulmonary colonies with the marine oil diets. Rather, these diets proved to be protective at all levels tested.](image-url)

![Fig. 4. Representative gross specimens from pulmonary colonization assay. At 21 days postinoculation, lung blocks were dissected and stained as described in the text. 03-5, low fat marine oil diet animal; 03-20, high fat marine oil diet; 06-5, low fat safflower oil diet; 06-20, high fat oil diet. White spots on each lung represent tumor colonies. Lung blocks 03-5, 03-20, and 06-5 have 10-12 colonies each and lung block 06-20 has 50 colonies.](image-url)
increase the number of pulmonary colonies. Additionally, these findings do not result from varying the quantity of calories, since all animals consumed virtually identical quantities of calories daily regardless of dietary group.

Prostaglandins, derived from membrane fatty acids, influence the growth rate and metastatic potential of various cancers (23, 24). This concept evolved primarily through the administration of known PG inhibitors in animal models of tumorigenesis resulting in inhibition of tumor growth and depression in the PG production in both normal and tumor tissue (23–25). An association between the synthesis of PGs and the growth rates of tumors was thus established. Inhibiting PG production may also inhibit tumor growth through a reversal of tumor-induced immune suppression (25), although there is no evidence to suggest that this system plays a role in postpromotional events. Indeed, Lynch et al. (26), in order to eliminate this possibility, ran immune function tests (anti-sheep RBC direct plaque forming cell assay and phytohemagglutinin response) on all groups in their study and found no difference in immunocompetence between tumor-bearing and non-tumor-bearing animals.

Hillyard and Abraham (27, 28) and Abraham et al. (29) made a strong, circumstantial case for the role of EFA in the growth of normal tissues and tumors. They found certain levels of dietary EFA essential for the growth of carcinomas and that factors that substitute for, or interfere with utilization of EFA (EPA and DHA) will interfere with the growth of cancers. Further, they implicate prostaglandins in the stimulation of tumor growth by dietary EFA, postulating that the mechanism of EPA/DHA-induced growth inhibition lies in a block of cyclooxygenase which metabolizes n-3 fatty acids to largely ineffective 3 series PGs. Studies using synthetic fatty acids (30) or marine oils (10, 11, 31) have the advantage that substances under scrutiny had fewer biological effects than nonsteroidal antiinflammatory drugs previously examined.

Jurkowski and Cave (7) stress the exchange of n-3 fatty acids for n-6 in membrane phospholipids as a mechanism for antitumor effects. They suggest that this substitution affects the structure of hormone receptors on the surface of tumor cells. This has largely been contested by the use of hormone-nonspecific tumors with no change in results (27). This does not rule out the possibility that substitution of n-3 fatty acids for n-6 in the cell surface membrane affects either recognition elements (for lipophilic messenger molecules) or surface membrane/receptor associations (analogous to the grouping of IgE receptors) on tumor cells or target tissue.

Data from the pulmonary colonization experiments are consistent with the tumor growth experiments. Safflower oil enhances tumor colonization proportional to its content in the diet, marine oils show no such dose-dependent effect, exhibiting low, constant colony numbers at all fat levels. Tumor cell aggregates (with or without associated host cells) have been shown to be much more effective in inducing metastatic growth than free cells (32, 33), since individual tumor cells are rapidly killed in the host bloodstream. Further studies have shown that tumor embolus size is proportional to the probability of colony establishment (34). The size of circulating tumor emboli depends on the coagulable state of the donor. This depends, in part, on the balance between the levels of thrombogenic platelet-derived thromboxane A and the antithrombogenic leukocyte-derived prostacyclin, both of which are affected by marine oil diets (34, 35). Karmaili (36) suggests that widespread cyclooxygenase inhibition by marine oil limits production of thromboxanes more than prostacyclin-favoring antithrombogenesis, discouraging metastasis. Metastasis is a multistep process and the effective inhibition of any step will decrease the chance of successful colony formation.

It is concluded from these findings that safflower oil enhances pulmonary colonization and colonic growth and spread in a dose-dependent manner. Further, dietary marine oil, substituted isocalorically, inhibits pulmonary colonization of CT-26 at high levels and growth in the bowel at all levels. These findings are consistent with the theory that EPA and DHA in marine oils substitute for native n-6 lipids in actively metabolizing cells and interfere with the production of substances necessary for the accelerated growth of tumors and survival of early metastatic colonies. These substances are probably prostaglandins, although they might be any lipid–derived element of the cell stimulation cascade, i.e., diacylglycerol or receptor-associated lipids. In all of these locations native n-6 fatty acids can be replaced by n-3, altering the physical and chemical properties of the system due to the dissimilar saturation state of n-3 fats. These potential mechanisms are the subject of ongoing investigation in our laboratory.

ACKNOWLEDGMENTS

We acknowledge the capable assistance of Paul Colon, Ruth Suchodolski, James Giovino, and John Wilkinson IV in the conduct of these studies. We would also like to thank the Boston University Department of Biostatistics for their aid in the data analysis.

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