

Dietary Ω -3 Polyunsaturated Fatty Acids Promote Colon Carcinoma Metastasis in Rat Liver¹

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ABSTRACT

The effects of Ω -3 polyunsaturated fatty acids (PUFAs) and Ω -6 PUFAs on the development of experimentally induced colon carcinoma metastasis in rat liver were investigated quantitatively *in vivo*. Rats were kept on either a low-fat diet or on a fish oil (Ω -3 PUFAs) or safflower oil (Ω -6 PUFAs) diet for 3 weeks before the administration of colon cancer cells to the portal vein, until they were sacrificed at 1 or 3 weeks after tumor transplantation. At 1 week after transplantation, the fish oil diet had induced 7-fold more metastases (in terms of number and size) than had the low-fat diet, whereas the safflower oil diet had not affected the number and total volume of metastases. At 3 weeks after tumor transplantation, the fish oil diet and the safflower oil diet had induced, respectively, 10- and 4-fold more metastases (number) and over 1000- and 500-fold more metastases (size) than were found in the livers of rats on the low-fat diet. These differences were sex independent. Immunohistochemical analysis revealed that the immune system in the liver (Kupffer cells, pit cells, T cells, newly recruited macrophages, and the activation state of macrophages) did not play a significant role in this diet-dependent outgrowth of tumors. In conclusion, Ω -3 and Ω -6 PUFAs promote colon cancer metastasis in the liver without down-regulating the immune system. This finding has serious implications for the treatment of cancer patients with fish oil diet to fight cachexia.

INTRODUCTION

During the last 30 years, epidemiological studies have focused on the effects of dietary fats on tumorigenesis, demonstrating that high-fat diets increase the incidence of primary tumors (1–3). It has been suggested that this phenomenon is related to differences in caloric consumption rather than in the fatty acid composition of the diet. However, epidemiological and experimental studies have shown that Ω -3 PUFAs⁴ that are found in fish oil not only prevent the development and progression of several types of cancer (4–9), especially those of colorectal cancer (10), but also reduce tumor-induced cachexia (11–13). In contrast, Ω -6 PUFAs that are present in corn oil and safflower oil, for example, promote tumor growth (14, 15) and increase carcinogenesis in animal models (16). The mechanisms of the antiproliferative effects of Ω -3 PUFAs as observed in humans (17, 18) and animals (19, 20) are not clear yet. Begin *et al.* (21) suggested that cancer cells are killed by lipid peroxidation products that damage DNA, particularly when it is uncovered during the cell cycle. Lipid peroxidation products are generated in large amounts in cells that

contain high levels of PUFAs (22, 23). The antiproliferative effect of lipid peroxidation products has been recently demonstrated *in vivo* after a partial hepatectomy in rats on a fish oil diet (24). The different effects of Ω -3 and Ω -6 PUFAs on PG synthesis have also been suggested to be involved in the inhibition or stimulation of tumor growth, respectively (25). PGs, especially of the E series, enhance cell proliferation, and high levels of PGE₂ have been found in patients with cancer (25, 26). Ω -3 PUFAs inhibit and Ω -6 PUFAs stimulate PGE₂ synthesis (27). This hypothesis was confirmed recently by a cell culture study (28).

The effects of Ω -3 and Ω -6 PUFAs on primary tumorigenesis have been investigated frequently (18, 29), but little is known about their effects on metastasis. A few studies that focused on the effects of Ω -3 and Ω -6 PUFAs on the development of lung metastases showed conflicting results. Ω -3 PUFA diets either reduced (9, 30–32) or increased (33, 34) the number of lung metastases or had no effect at all (35–37). Recently, Coulombe *et al.* (38) demonstrated that a Ω -3 PUFA diet induced more hepatic metastases from 3LL Lewis lung carcinoma than did a Ω -6 PUFA diet or a saturated fat diet. Because metastasis is the major cause of death of cancer patients, and because of the increasing clinical application of Ω -3 PUFA supplementation in the treatment of cancer patients, we tested the effects of diets enriched with Ω -3 PUFAs and Ω -6 PUFAs on the development of colon carcinoma metastasis in rat liver in an *in vivo* model.

PUFAs are incorporated into the membranes of both cancer cells and normal cells, altering their physical and functional properties (30, 39), which may interfere seriously with immunological surveillance against cancer cells. Furthermore, Ω -3 PUFA supplementation decreases cytokine production (40) and MHC class II expression on the cell surface of antigen-presenting macrophages (41, 42), thus interfering with the immune response (43, 44). Therefore, in the present study, particular attention was given to the effects of different diets on both interactions between cancer cells and immune cells in the liver such as Kupffer cells, pit cells (natural killer cells), T cells, and newly recruited macrophages and the activation state of macrophages in terms of antigen presentation.

MATERIALS AND METHODS

Animals. Adult male and female Wag-Rij rats (3 months old at the start of the experiments; Broekman, Someren, the Netherlands) were maintained for 2 weeks under constant environmental conditions with free access to food and water. Afterwards, the animals were kept in one of three diet groups. One group was kept on a low-fat diet; one group was kept on a fish oil diet, and one group was kept on a safflower oil diet. Each group contained 17 female and 6 male rats. At first, we used female rats only, but to exclude sex-dependent effects on the results, we also included small groups of male rats. The animals were kept on the respective diets for 3 weeks before administration of the cancer cells. The animals were housed individually at the Academic Medical Center animal facility in accordance with the guidelines for animal care of the University of Amsterdam. All animals were weighed once every 3 days during the entire experiment.

Diets. Diets were prepared by Hope Farms (Woerden, the Netherlands). Fish oil containing 70% Ω -3 PUFAs (38% eicosapentaenoic acid, 22% doco-

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⁴ The abbreviations used are: PUFA, polyunsaturated fatty acid; PG, prostaglandin; BrdUrd, bromodeoxyuridine; UEA-Z, *Europaeus agglutinin*-I.

Table 1 Antibodies and lectin used for the identification of cancer cells, different types of liver cells, and proliferating cells in rat liver containing colon cancer metastases

Antibody	Dilution	Cell type/antigen
ED1 ^a	1:500	Newly recruited macrophages and Kupffer cells
ED2 ^a	1:500	Kupffer cells
OX3 ^b	1:500	Activated macrophages
OX8 ^c	1:100	CTLs/suppressor T lymphocytes and natural killer cells
3.2.3 ^d	Undiluted	Liver natural killer cells (pit cells)
UEA-I ^e	1:100	Colon cancer cells
BrdUrd ^f	1:10	Proliferating cells

^a Ref. 60.

^b Ref. 61.

^c Ref. 62.

^d Ref. 63.

^e Ref. 64.

^f Ref. 65.

sahexanoic acid, and 10% other Ω-3 PUFAs; Pronova Biocare, Sandefjord, Norway) and safflower oil (Sigma Chemical Co., St. Louis, MO) were added to the food each day immediately before it was given to the animals. Fish oil and safflower oil were kept at 4°C under nitrogen to avoid autooxidation of PUFAs. Vitamin E levels in the food were kept at a minimum of 35 mg/kg of the low-fat diet [5% (v/w) soybean oil] and at 75 mg/kg of the Ω-3 and Ω-6 PUFA diets [20% (v/w) fish oil or safflower oil, respectively] to avoid vitamin E deficiency. Other vitamins, minerals, essential fats, and proteins were present at sufficiently high concentrations in the food to provide adequate growth (45). Complete composition of the diets is presented in Van Noorden (24).

Cancer Cells. An established colon carcinoma cell line (CC531) was obtained from a moderately differentiated and weakly immunogenic colon adenocarcinoma after experimental induction in Wag-Rij rats by treatment with 1,2-dimethylhydrazine (46). Cells were cultured at 37°C as monolayers in DMEM (ICN Biomedicals, Irvine, Ayrshire, United Kingdom) supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, penicillin (100 IU/ml), and streptomycin (100 µg/ml). Cells were washed with PBS, and after detachment with 0.05% trypsin in PBS and centrifugation (250 × g, room temperature, 10 min), a single-cell suspension of 1 × 10⁶ cancer cells in 0.05 ml of PBS was obtained with a viability of at least 95%.

Treatment. After 3 weeks on the respective diets, small midline incisions were made in the abdominal walls of the rats, and a suspension containing 1 × 10⁶ cancer cells in 50 µl of PBS was injected into the portal vein while the animals were under sodium pentobarbital anesthesia, using a 27-gauge needle as described previously in detail (47, 48). The portal vein was closed by treating the incision with an aqueous solution of thrombin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands) using a cotton bud. The injection site was covered by Spongostan (Medical Workshop, Groningen, the Netherlands) to prevent peritoneal seeding. The rats were kept on the respective diets until they were sacrificed. As controls, three female Wag-Rij rats were used for each diet group to establish the effects of the diets on profiles of immunocompetent cells in normal livers. These animals did not receive cancer cells. After 1 week and 3 weeks, seven female and three male rats from each group were sacrificed by an overdose of sodium pentobarbital, and livers were immediately removed, separated into lobes, and cut into small pieces (1–5-mm thick). The origin of all pieces of tissue was registered. One part of the pieces was immediately frozen in liquid nitrogen and stored at -80°C until used. The other part was chemically fixed for ultrastructural procedures. One h before sacrifice, the animals received 50 µg of BrdUrd i.p. (Serva, Heidelberg, Germany) per kilogram of body weight. All experiments were carried out in accordance with the guidelines for animal care of the University of Amsterdam.

Immunohistochemistry. Immunohistochemical procedures were carried out on 8-µm-thick cryostat sections as described previously (48). The following monoclonal antibodies and lectin were used (Table 1): (a) Ulex Europaeus agglutinin-I (UEA-I; DAKO, Glostrup, Denmark); (b) OX8, OX3, ED2, and ED1 (all from Serotec, Oxford, United Kingdom); (c) 3.2.3 (kindly provided by Dr. P. J. K. Kuppen, Laboratory of Pathology, University of Leiden, the Netherlands); and (d) anti-BrdUrd (Eurodiagnostics, Apeldoorn, the Netherlands). The following secondary antibodies conjugated with horseradish peroxidase (DAKO) were used: (a) rabbit anti-UEA-I diluted 1:50 in PBS con-

taining 10% human AB serum; (b) rabbit antimouse IgG diluted 1:200 (OX3, ED2, ED1, and anti-BrdUrd) or 1:100 (OX8) in PBS containing 0.2% BSA and 2% normal rat serum. Between the different incubation steps, the sections were rinsed three times in PBS. Peroxidase activity was visualized using a medium containing 20 µg of 3-amino-9-ethylcarbazole (Sigma) dissolved in 5 ml of N,N-dimethylformamide and 95 ml of 50 mM acetate buffer (pH 4.9) and

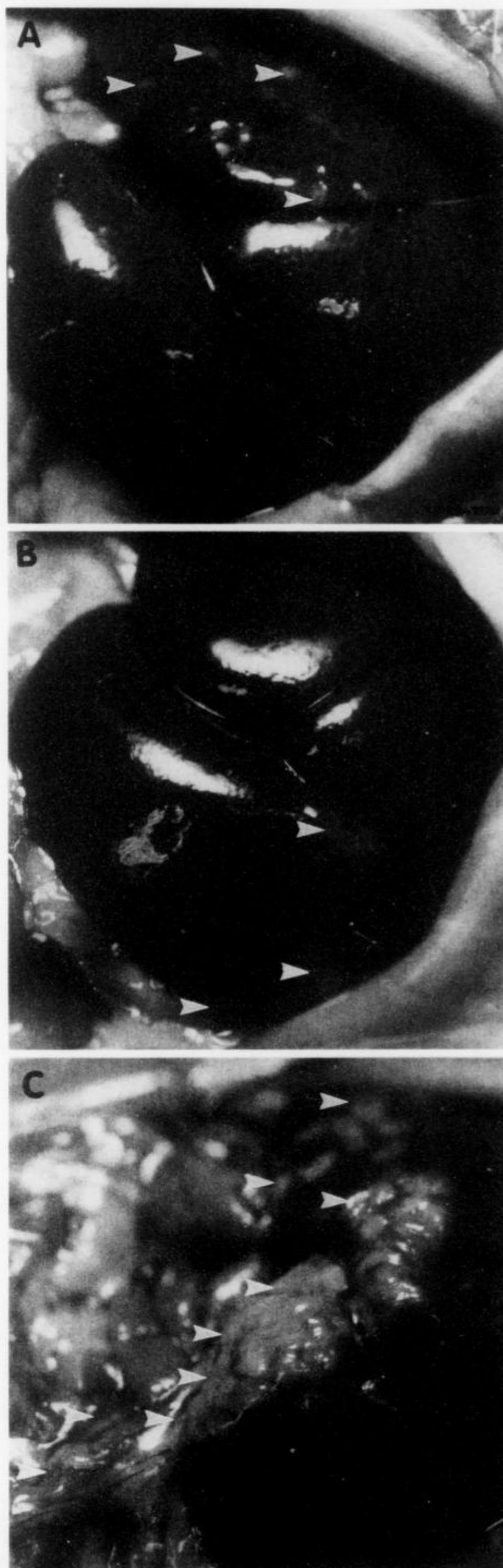


Fig. 1. Livers of rats on the low-fat diet (A), the safflower oil diet (B), and the fish oil diet (C) *in situ*, showing tumor tissue (arrowheads). Bar, 1 cm.

hydrogen peroxide in a final concentration of 0.01% (v/v). The nuclei were counterstained with hematoxylin. Control incubations were performed in the absence of primary antibodies (49). To detect proliferation of Kupffer cells, an immunoenzyme double staining was performed using two complete immunoenzymatic staining methods (48, 50) for ED2 and anti-BrdUrd, respectively.

Morphometric Analysis. The amount and volume of metastases as detected with lectin UEA-I, a selective marker for colon cancer cells, were analyzed quantitatively by morphometry in 22 cryostat sections of each liver obtained from randomly selected sites in all lobes according to a rigid scheme as described previously (51). The number of metastases was expressed per unit volume of liver tissue \pm SE. The volume of cancer cells in the tumors was determined by measuring the areas in the sections occupied by the cancer cells. Images of the sections were captured using an Olympus Vanox-T microscope (Tokyo, Japan) at a magnification of $\times 6.25$ and $\times 2.5$ attached to a charge-coupled-device camera with an 8-bit resolution (Cohu 4910; San Diego, CA), a frame grabber (LG-3; Scion, Frederick, MD), and a Power Macintosh 8100/110 computer (Apple, Cupertino, CA) using the public domain NIH Imaging software program (version 1.57; written by W. Rassband and available via internet from <http://rsb.info.nih.gov>). Settings of the camera and frame grabber were adjusted according to Jonker *et al.* (52). The percentage area of cancer cells was expressed as the ratio of the area of cancer cells:total area of liver tissue including cancer cells ($\times 100$) \pm SE. Significance of differences between the diet groups was determined using ANOVA; $P \leq 0.05$ was considered significant.

Cell Counting. For each liver lobe, eight sections were used after immunohistochemical staining, and three periportal and three pericentral zones were randomly selected in each section to determine the number of immunocompetent cells. ED1-, ED2-, OX3-, and OX8-positive cells and BrdUrd-positive nuclei per unit area were quantified in a standardized microscopic field using

Table 2 Liver and body weight of rats on different diets as determined at 3 weeks after administration of CC531 colon cancer cells

Diet	Body weight ^a	Liver weight ^a	Ratio of liver:body weight ($\times 100$)
Low fat	263.0 \pm 4.6	8.0 \pm 0.7	3.0 \pm 0.3
Safflower oil	272.8 \pm 6.6	9.5 \pm 0.5	3.5 \pm 0.2
Fish oil	238.8 \pm 6.9	12.9 \pm 1.7 ^b	5.4 \pm 0.8 ^b

^a Data are given in grams as mean \pm SE of seven female and three male rats.

^b Significant difference with other diet groups.

Table 3 Number of metastases and volume (expressed as percentage area) of cancer cells at 3 weeks after administration of CC531 colon cancer cells as detected with the lectin UEA-I in livers of rats on different diets

Diet	No. of metastases ^a	Percentage area ^b
Low fat	4.1 \pm 4.6	0.01 \pm 0.03
Safflower oil	18.4 \pm 23.5 ^c	6.97 \pm 9.30 ^c
Fish oil	49.7 \pm 26.3 ^c	17.82 \pm 16.09 ^c

^a Data are expressed as the number of metastases/unit volume of liver tissue \pm SE in each of seven female and three male rats on the low-fat diet and on the diets containing safflower oil and fish oil.

^b Percentage area is expressed as the ratio of the area of cancer cells:total area of liver tissue sections including cancer cells \pm SE in each of seven female and three male rats on the low-fat diet and on the diets containing safflower oil and fish oil.

^c Significant difference with other diet groups.

Table 4 Number of metastases and volume (expressed as percentage area) of cancer cells at 1 week after administration of CC531 colon cancer cells as detected with the lectin UEA-I in livers of rats on different diets

Diet	No. of metastases ^a	Percentage area ^b
Low fat	46.5 \pm 3.5	0.44 \pm 0.17 ^c
Safflower oil	46.0 \pm 64.2	0.51 \pm 0.43 ^c
Fish oil	324.0 \pm 164.6 ^d	2.92 \pm 1.58 ^{c,d}

^a Data are expressed as the number of metastases/unit volume of liver tissue \pm SE in each of seven female and three male rats on the low-fat diet and on the diets containing safflower oil and fish oil.

^b Percentage area is expressed as the ratio of the area of cancer cells:total area of liver tissue sections including cancer cells \pm SE in each of seven female and three male rats on the low-fat diet and on the diets containing safflower oil and fish oil.

^c These values should be divided by 10³.

^d Significant difference with other diet groups.

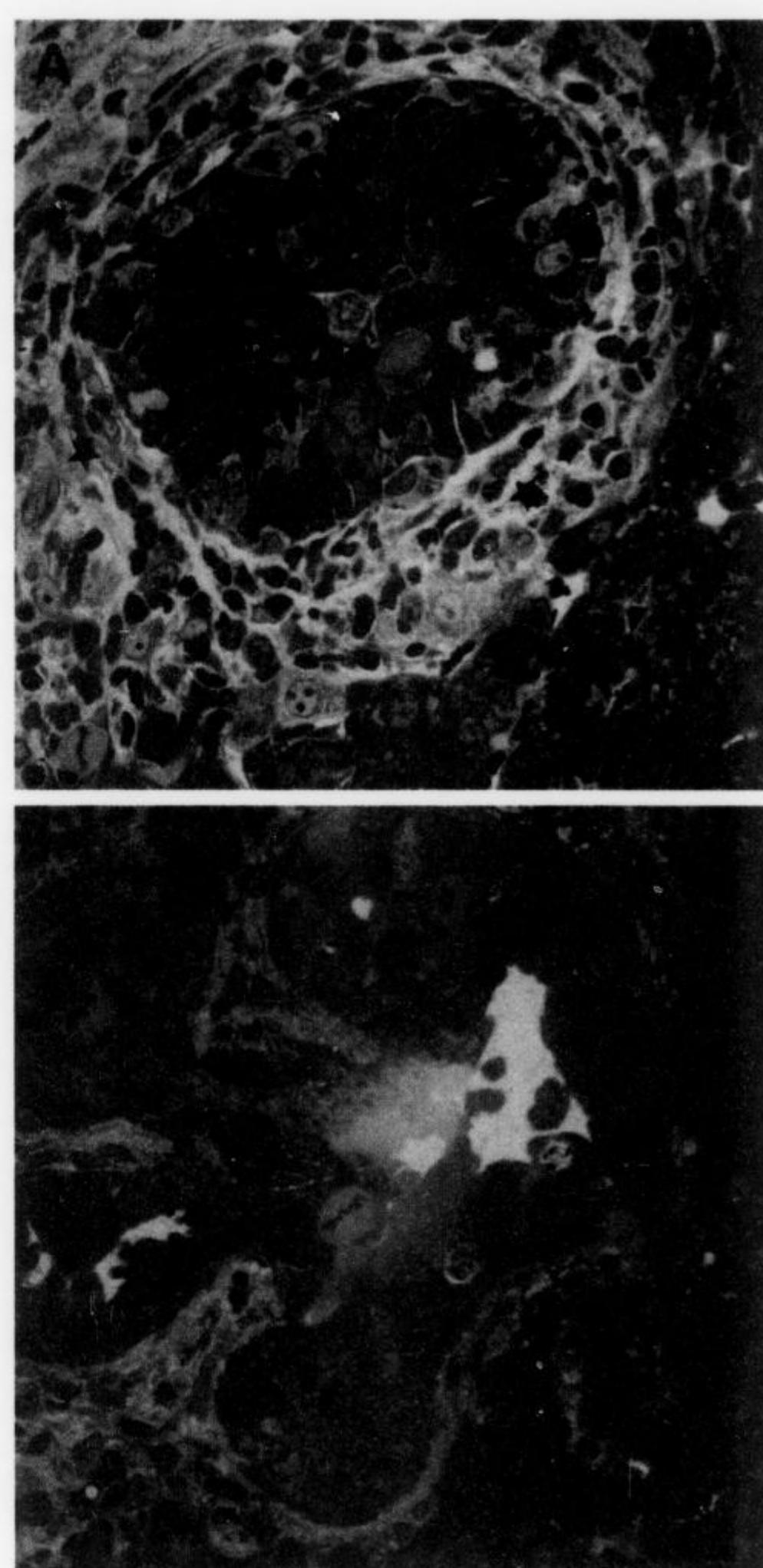


Fig. 2. Semithin sections of the livers of rats on the low-fat diet (A) and the fish oil diet (B) at 1 week after tumor transplantation. Note the high number of colon cancer cells (c) in mitosis (arrowheads) in the livers of rats on the fish oil diet. h, hepatocytes; *, stroma. Bar, 40 μ m.

a Zeiss microscope and a Neofluar objective $\times 40$. Data were expressed as the mean \pm SE of the number of cells or nuclei in the periportal and pericentral zones. The significance of the differences was determined using ANOVA; $P \leq 0.05$ was considered significant.

Semithin Sections. Small liver pieces (1-mm thick) were fixed in McDowell fixative containing 4% formaldehyde and 1% glutaraldehyde in 100 mM cacodylate buffer (pH 7.4) and stored in this fixative for at least 24 h. Postfixation was performed using 1% osmium tetroxide (Sigma). After dehydration, tissue blocks were embedded in LX 112 epoxy resin. Semithin sections were stained with a solution of 0.1% (w/v) toluidine blue.

RESULTS

Effect of Diet on Cell Proliferation. Three weeks after transplantation, tumor mass in the liver was significantly larger in rats of either sex on the fish oil diet and the safflower oil diet than in rats on the low-fat diet (Fig. 1). Liver weight was increased in animals on the fish oil diet; the ratio of liver:body weight increased significantly (Table 2). Morphometric analysis showed that the number of tumors was over 10-fold higher, and the volume of

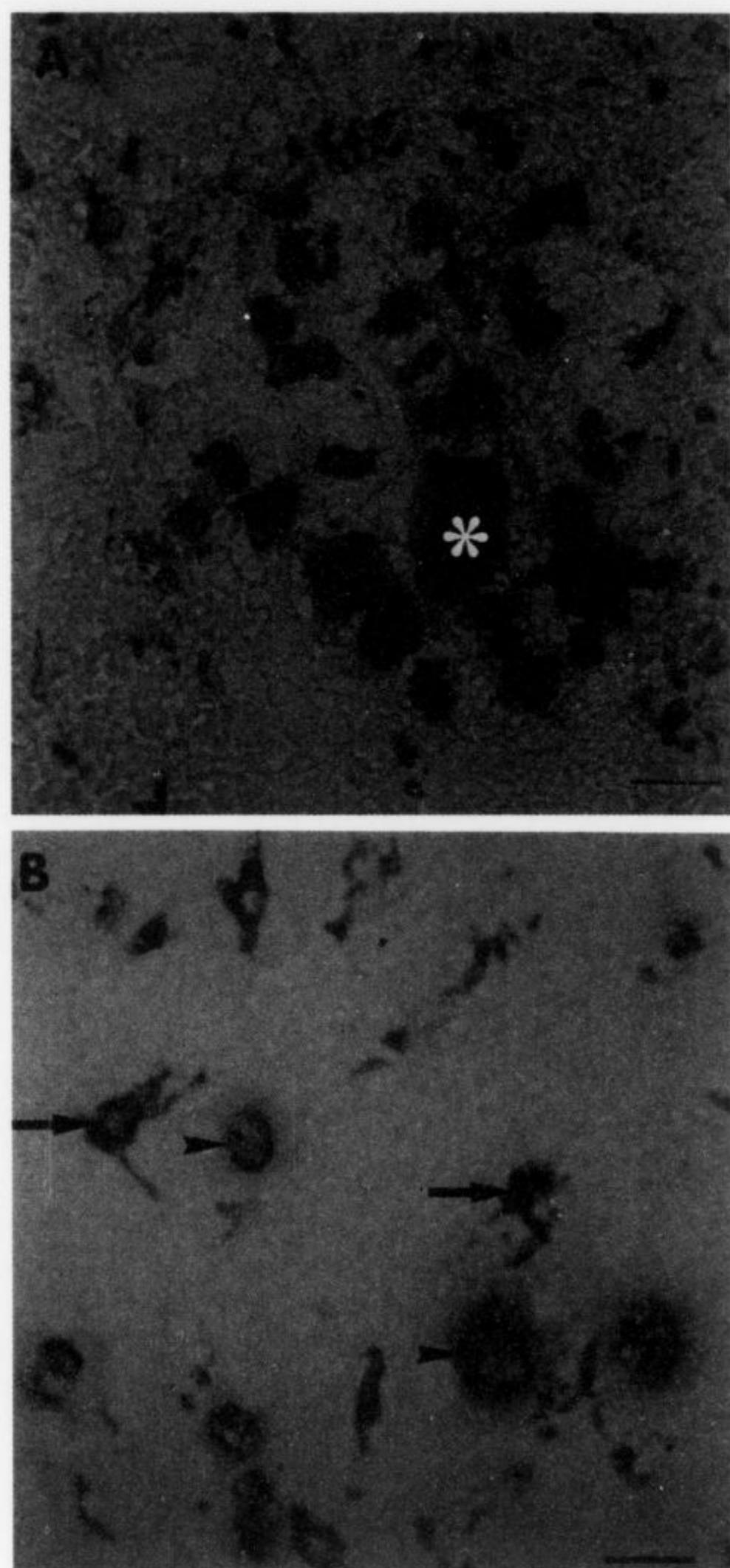


Fig. 3. Double immunostaining of Kupffer cells (ED2; brown) and BrdUrd (blue) in a liver tissue section of a rat on the fish oil diet at 1 week (A) and at 3 weeks (B) after tumor transplantation. Proliferating Kupffer cells (arrows), hepatocytes (arrowheads), and cancer cells (*) are present in the liver parenchyma (B) and the tumor (A), respectively. Bar, 16 μ m in A and 20 μ m in B.

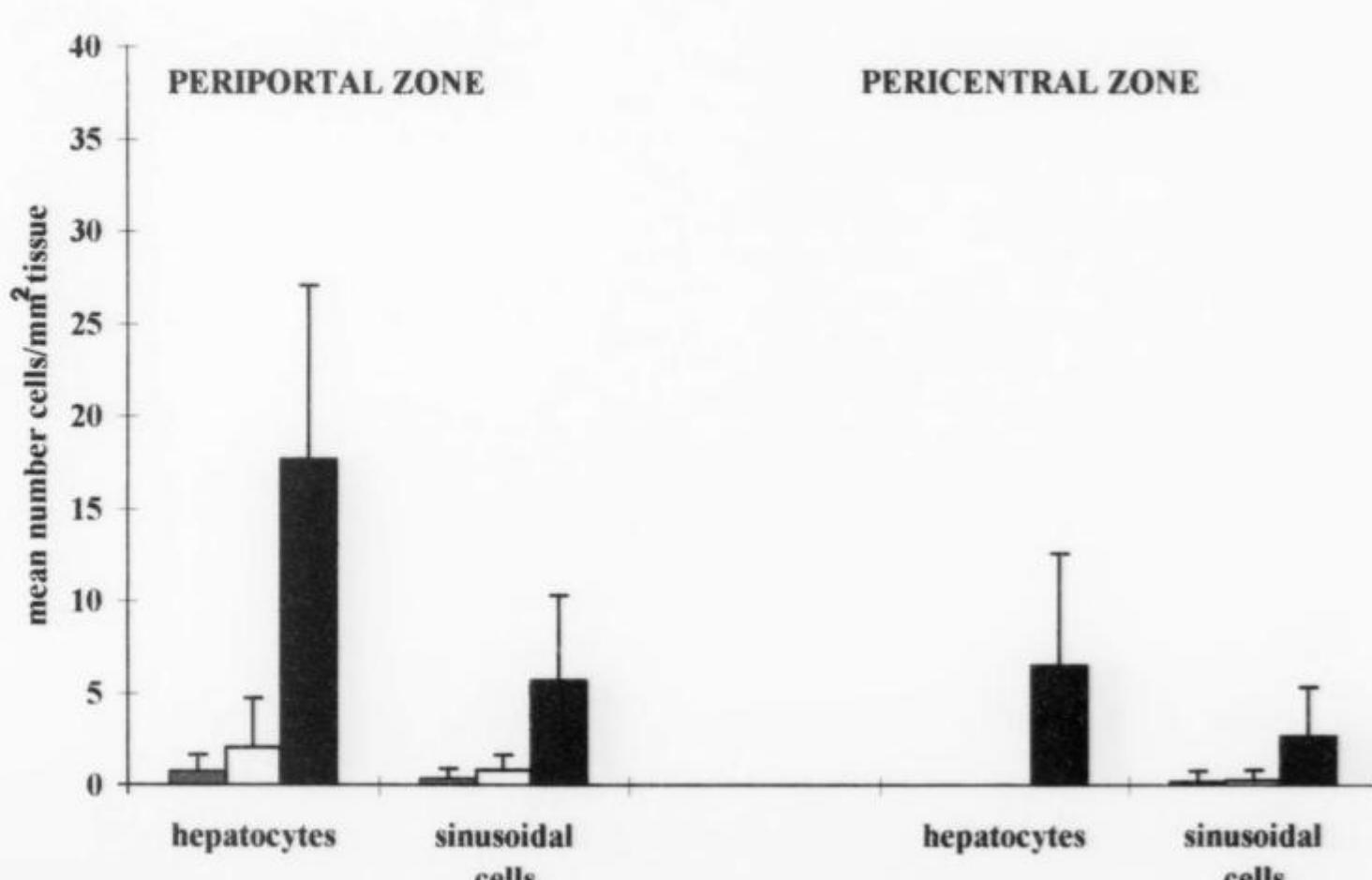
Fig. 4. The number of cells incorporating BrdUrd in the periportal and pericentral zones of the liver lobules of three male and three female rats on different diets at 3 weeks after administration of CC531 colon cancer cells. Rats were on the low-fat diet (■), the safflower oil diet (□), or the fish oil diet (■). Data are expressed as the mean numbers of cells/mm² of tissue in the sections \pm SE ($n = 6$).

cancer cells was over 1000-fold larger than those in animals on the low-fat diet (Table 3). The number of tumors in animals on the safflower oil diet was over 4-fold higher, and the volume of cancer cells was over 500-fold larger than those in animals on the low-fat diet (Table 3). Metastases in livers of rats on the low-fat diet were mainly located at the periphery of the liver lobes, as described previously (53), whereas metastases in the livers of rats on the safflower oil diet and on the fish oil diet were randomly distributed all over the liver lobes. These tumor growth patterns were similar in male and female rats.

Morphometric analysis showed that at 1 week after tumor transplantation, there was already a 7-fold increase in the number and size of metastases in the livers of rats on the fish oil diet when compared with that in the livers of rats on the safflower oil diet and on the low-fat diet (Table 4). These differences were directly correlated with the high proliferation rate of the cancer cells, because a distinctly higher proportion of colon cancer cells in the livers of rats on the fish oil diet were found to be in mitosis when compared with colon cancer cells in the livers of rats kept on the other diets (Fig. 2). Furthermore, the number of liver cells incorporating BrdUrd was elevated in rats on the fish oil diet (Figs. 3 and 4). These numbers were significantly higher than those in rats on the safflower oil and low-fat diets, respectively (Fig. 4).

Distribution Patterns of Immunocompetent Cells. The different diets did not affect the number or distribution patterns of ED1-positive cells (newly recruited macrophages and Kupffer cells), ED2-positive cells (Kupffer cells), and OX8-positive cells (natural killer cells and CTLs/suppressor T lymphocytes) in the livers of animals that did not receive cancer cells (Fig. 5A). One week after tumor transplantation, the number of newly recruited macrophages and Kupffer cells was increased in a similar way in the livers of all rats, irrespective of the diet (Fig. 5B). The number of cytotoxic OX8-positive cells was slightly increased, but the number of OX3-positive cells (activated macrophages) remained unchanged in comparison with those in livers without tumors. Distribution patterns of immunocompetent cells infiltrating or surrounding the tumors were not diet dependent. The immunocompetent cells infiltrating tumors were mainly activated newly recruited macrophages and, to a lesser extent, natural killer cells and CTLs/suppressor T lymphocytes (Fig. 6). Sex-dependent differences in the number or distribution patterns of immunocompetent cells were not found.

Three weeks after tumor transplantation, the numbers of ED1-,



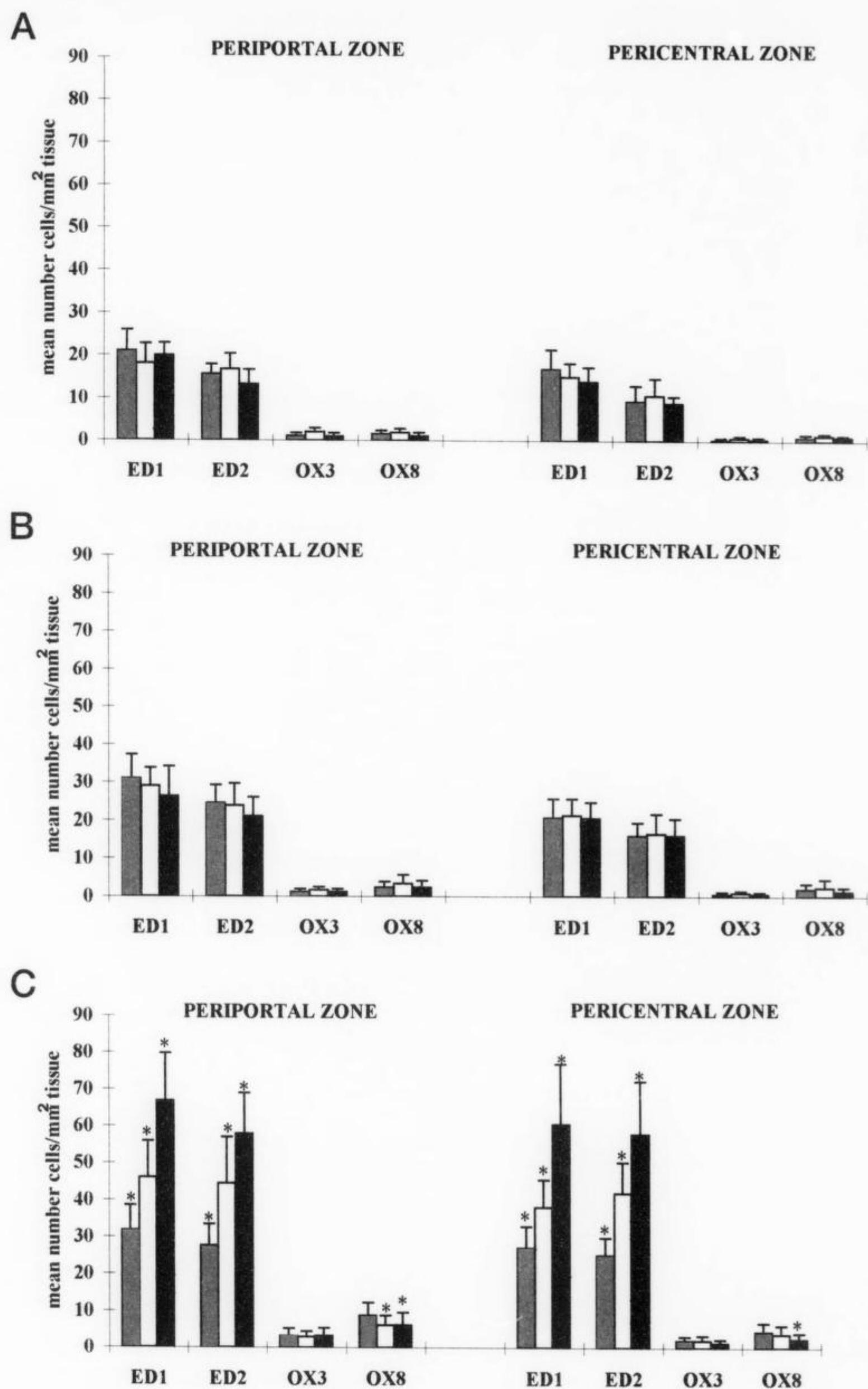


Fig. 5. Total population of Kupffer cells, newly recruited macrophages, and monocytes as detected with ED1, the number of Kupffer cells as demonstrated with ED2, the number of activated macrophages as detected with OX3, and the total population of CTLs/suppressor T lymphocytes and natural killer cells as detected with OX8 in the periportal and pericentral zones of liver lobules of three male and three female rats on different diets before administration of CC531 colon cancer cells (A) and at 1 week (B) and 3 weeks (C) after administration. Rats were on low-fat diet (■), the safflower oil diet (□), or the fish oil diet (■). Data are expressed as the mean numbers of cells/mm² of tissue in the sections ± SE ($n = 6$); *, statistically significant differences between the diet groups; +, statistically significant differences between 0 and 1 week or 3 weeks of metastasis.

ED2-, and OX8-positive cells were increased significantly in all diet groups when compared with those in livers without tumors (Fig. 5C). The enlargement of the Kupffer cell population was partly due to local proliferation, as demonstrated by ED2-positive cells incorporating BrdUrd (Fig. 3). After 3 weeks, the numbers of ED1- and ED2-positive cells were significantly higher in the livers of rats on the fish oil diet than in those of rats on the other diets. The number of OX3-positive cells (activated macrophages) increased only marginally. This marginal increase was diet independent (Fig. 5C). ED1- and ED2-positive cells were abundant in liver parenchyma around metastases and at the edge of metastases, where only few OX3- and OX8-positive cells were detected (Fig. 7). Distribution patterns of 3.2.3-positive pit cells were similar to those of OX8-positive cells (data not shown).

DISCUSSION

The present study shows that diets enriched with Ω-3 PUFAs (fish oil) and Ω-6 PUFAs (safflower oil) enhance the development of colon carcinoma metastases in rat liver in comparison with a low-fat diet. Ω-3 PUFAs already affected both the number and size of the tumors in the early stages of tumor development (1 week after transplantation) whereas Ω-6 PUFAs only affected the number and size of the tumors in the late stages of tumor development (3 weeks after transplantation; Tables 3 and 4). The effects of Ω-3 PUFAs on the proliferation of colon cancer cells and on the growth of metastases in the liver are stronger than the effects of Ω-6 PUFAs. It should be noted here that we studied only effects on the late stages of the metastatic cascade in this animal model. Early stages in metastasis

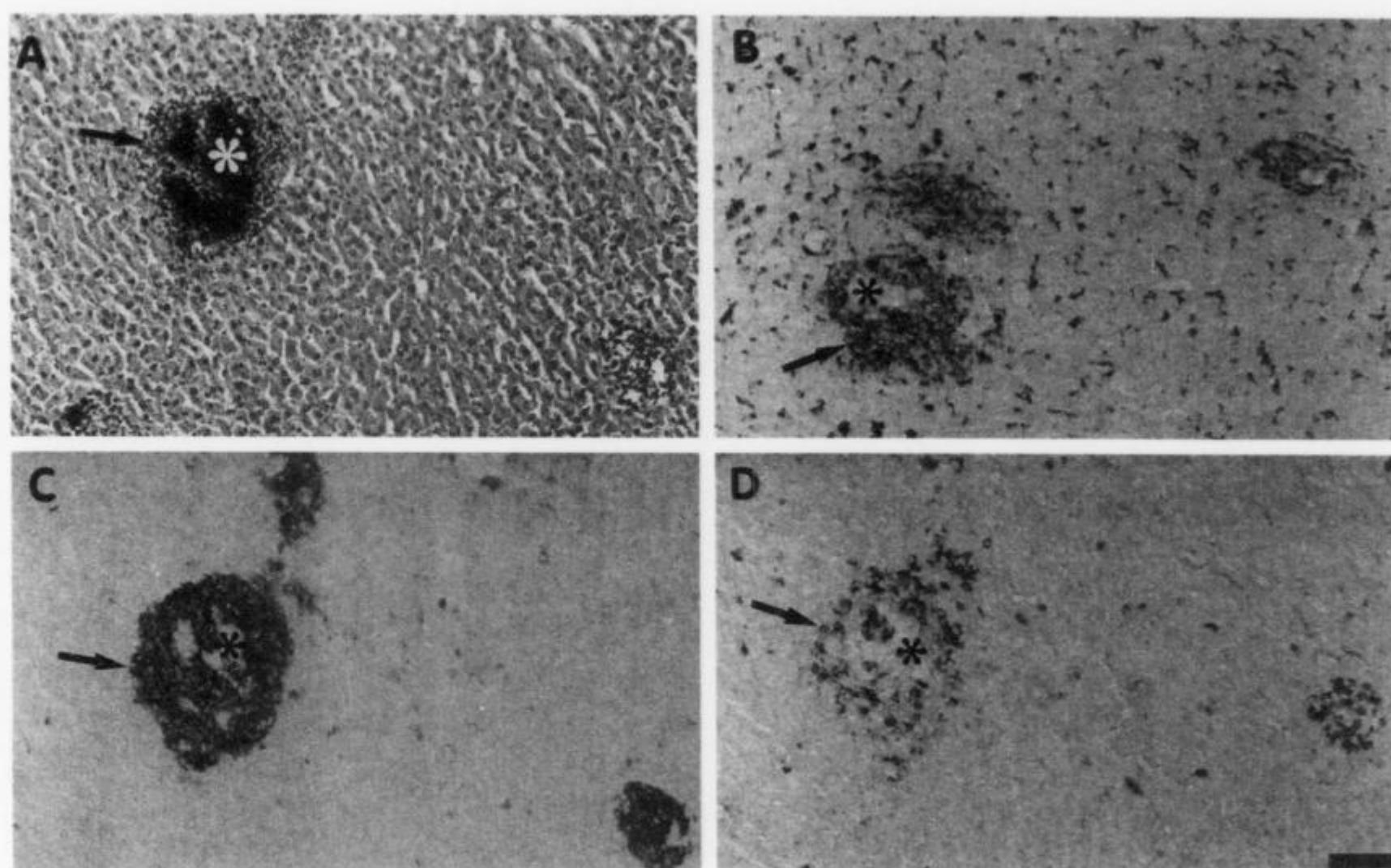


Fig. 6. Immunostaining for UEA-I (A), newly recruited macrophages (B), activated macrophages (C), and natural killer cells and CTLs/suppressor T lymphocytes (D) in the liver tissue sections of a rat on the fish oil diet at 1 week after transplantation of colon cancer cells. *, tumors; arrow, inflammatory cells infiltrating the tumor. Bar, 20 μ m.

may well be affected differently by PUFAs. The proliferation of liver cells was also positively and not negatively affected by Ω-3 PUFA, as demonstrated by the large number of liver cells that incorporated BrdUrd (Figs. 3 and 4). These data are in contrast with findings that Ω-3 PUFAs have antiproliferative effects on primary tumor growth (17–20) and hepatocytes after partial hepatectomy (24), whereas Ω-6 PUFAs promote tumor growth (14, 15). Therefore, the effects of Ω-3 PUFAs described here may well be liver-specific. In agreement with our data are the findings of Young and Young (34) and Coloumbe *et al.* (38) showing an increase in the number of metastases in the lung and liver when using Lewis lung carcinoma cell lines in animals on fish oil diets and on corn oil diets. On the basis of these consider-

ations, we conclude that Ω-3 inhibits the growth of primary tumors and promotes the growth of colon cancer tumors in the liver. These aspects have to be considered and investigated further, because of direct implications for the treatment of patients with primary tumors, such as colon cancer, with a fish oil diet. This treatment has been introduced because of its antiproliferative effect on primary tumors, and because of its efficacy in reducing tumor-induced cachexia (54, 55).

Dagnelie *et al.* (56) demonstrated that a fish oil-enriched diet inhibited tumor-induced cachexia in rats with prostate tumors but did not affect tumor growth. However, they used the MAT-LyLu variant of Dunning prostate tumor that does not metastasize to the liver, so

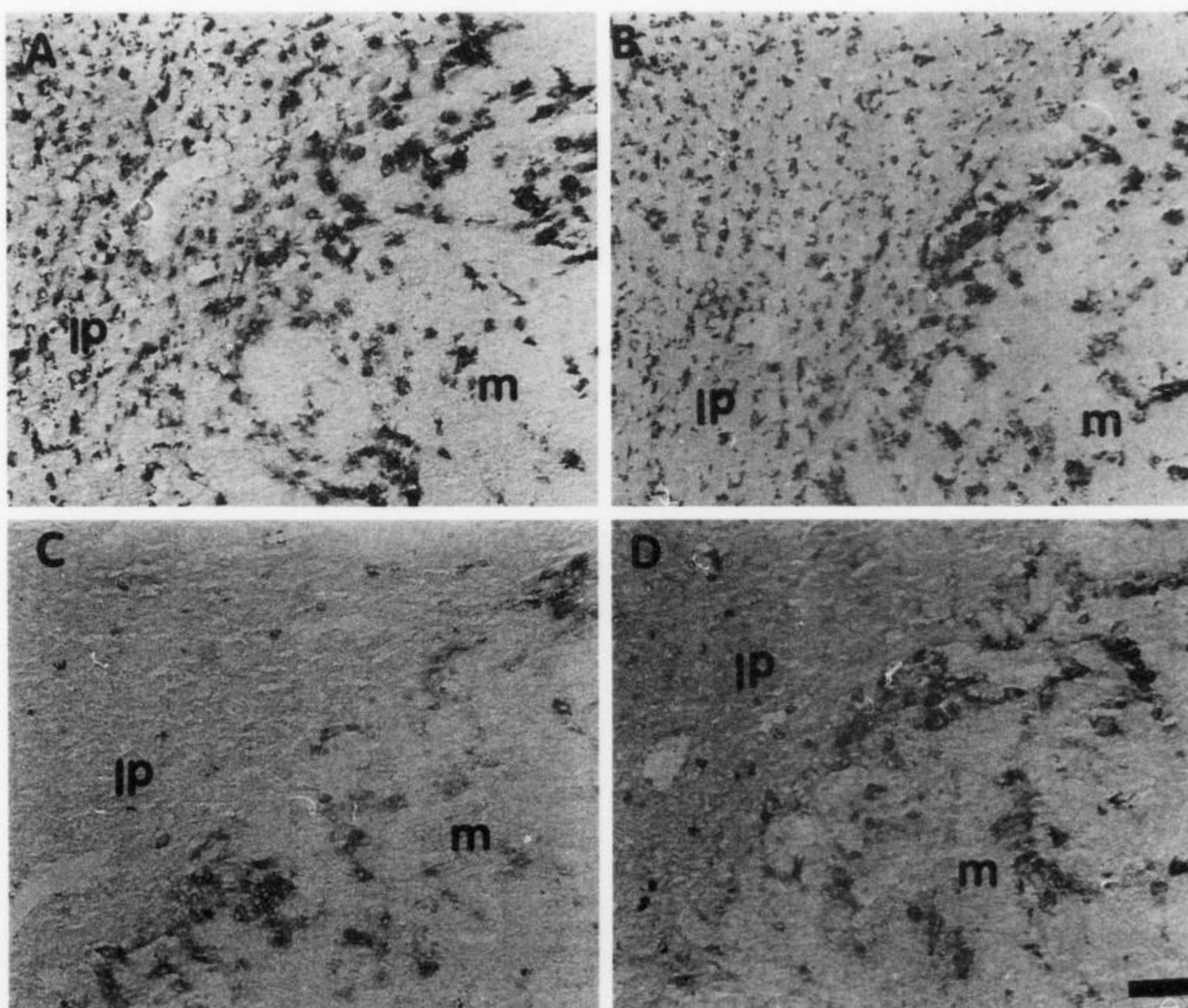


Fig. 7. Immunostaining of Kupffer cells (A), newly recruited macrophages (B), activated macrophages (C), and natural killer cells and CTLs/suppressor T lymphocytes (D) in a liver tissue section of a rat on the fish oil diet at 3 weeks after transplantation of colon cancer cells. IP, liver parenchyma; m, tumor. Bar, 20 μ m.

any effects on metastasis could not be evaluated. Although the anticachectic and antitumor effects of fish oil have been demonstrated in mice bearing colon adenocarcinoma, no data were given with respect to liver metastases in these experimental studies (11, 13). On the other hand, Beck *et al.* (13) demonstrated in the same experimental model that the anticachectic effect of fish oil was more pronounced than the antitumor effect, because the initial reduction of the tumor growth rate observed during the first 3 days of fish oil administration was immediately followed by enhanced growth of the tumors.

To explain our dramatic findings, we investigated whether the overgrowth of metastases in the livers of rats on the fish oil diet was dependent on suppression of the immune system by Ω-3 PUFAs. It is known that cytokine profiles are negatively affected by PUFAs (6, 40, 44, 57). Furthermore, Ω-3 PUFAs decrease granulocyte-macrophage colony-stimulating factor production (40), T-cell and B-cell proliferation (44), natural killer cell activity (58), and the expression of MHC class II antigen on macrophages (41, 42). However, diet did not affect either the expression of MHC class II antigen on the surface of macrophages that infiltrated the small metastases or the infiltration of OX8-positive cells (Fig. 6). Three weeks after tumor transplantation, the livers of rats on the fish oil diet contained not only the largest number of metastases but also the largest numbers of Kupffer cells and newly recruited macrophages (Figs. 5 and 7), indicating that the recruitment and proliferation of macrophages were not negatively affected by Ω-3 PUFA. The number of activated macrophages as antigen-presenting cells was low, irrespective of the diet (Fig. 5), and this fact also indicates that the activation state of these cells was not affected by PUFAs. In a previous *in vivo* study using the same animal model, we found that macrophages and natural killer cells do not play a significant role in the defense against advanced stages of metastasis (48). Therefore, we conclude that the enormous effect of Ω-3 PUFA on colon cancer metastasis in the liver is not mediated via alterations of the immune system.

Thus far, we do not have an explanation for the effects of the fish oil diet on metastasis, but it seems to be related to the proliferation and aggressiveness of the colon cancer cells. *In vitro* studies showed that treatment of cancer cells with Ω-3 PUFA strongly increases the rate of adenosine and guanosine uptake (59). Because both nucleosides are nutrients required for proliferation, we suggest that an increase in purine uptake may be partly responsible for the rapid growth of colon cancer cells in the livers of rats on the fish oil diet.

In conclusion, a diet containing PUFAs such as the fish oil diet has profound effects on the development of secondary tumors such as colon cancer metastasis in the liver of the rat. This finding has serious implications for the treatment of cancer patients with fish oil to fight cachexia.

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REFERENCES

- Lea, A. J. Dietary factors associated with death rates from certain neoplasms in man. *Lancet*, 2: 322–333, 1966.
- Stubbs, R. S. The aetiology of colorectal cancer. *Br. J. Surg.*, 70: 313–316, 1983.
- Wynder, E. L. Dietary habits and cancer epidemiology. *Cancer (Phila.)*, 43: 1955–1961, 1979.
- Reddy, B. S., Buril, C., and Rigotti, J. Effects of diets high in ω-3 and ω-6 fatty acids on initiation and postinitiation stages of colon carcinogenesis. *Cancer Res.*, 51: 487–491, 1991.
- Reddy, B. S., and Maeura, Y. Tumor promotion by dietary fat in azoxymethane-induced female F344 rats: influence of amount and source of dietary fat. *J. Natl. Cancer Inst.*, 72: 745–750, 1984.
- Kanner, J., German, J. B., and Kinsella, J. E. Initiation of lipid peroxidation in biological systems. *World Rev. Nutr. Diet.*, 50: 186–191, 1987.
- Wan, J. M. F., Kanders, B. S., Kowalchuk, M., Knapp, H., Szeluga, D. J., Bagley, J., and Blackburn, G. L. Ω-3 fatty acids and cancer metastasis in humans. *World Rev. Nutr. Diet.*, 66: 477–487, 1991.
- Hardman, W. E., Barnes, C. J., Knight, C. W., and Cameron, I. L. Effects of iron supplementation and ET-18-OCH₃ on MDA-MB 231 breast carcinomas in nude mice consuming a fish oil diet. *Br. J. Cancer*, 76: 347–354, 1997.
- Rose, D. P., Connolly, J. M., Rayburn, J., and Coleman, M. Influence of diets containing eicosapentaenoic or docosahexaenoic acid on growth and metastasis of breast cancer cells in nude mice. *J. Natl. Cancer Inst.*, 87: 587–592, 1995.
- Willett, W. C., Stampfer, M. J., Colditz, G. A., Rosner, B. A., and Speizer, F. E. Relation of meat, fat and fiber intake to the risk of colon cancer in a prospective study among women. *N. Engl. J. Med.*, 323: 1664–1672, 1990.
- Hudson, E. A., and Tisdale, M. J. Comparison of the effectiveness of eicosapentaenoic acid administered as either the free acid or ethyl ester as an anticachectic and antitumor agent. *Prostaglandins Leukot. Essent. Fatty Acids*, 51: 141–145, 1994.
- Tisdale, M. J., and Dhesi, J. K. Inhibition of weight loss by ω-3 fatty acids in an experimental cachexia model. *Cancer Res.*, 50: 5022–5026, 1990.
- Beck, S. A., Smith, K. L., and Tisdale, M. J. Anticachectic and antitumor effect of eicosapentaenoic acid and its effect on protein turnover. *Cancer Res.*, 51: 6089–6093, 1991.
- Jurowski, J. J., and Cave, W. T. Dietary effect of menhaden oil on the growth and membrane composition of rat mammary tumors. *J. Natl. Cancer Inst.*, 74: 1145–1150, 1985.
- Cannizzo, F. L., and Broitman, S. A. Postpromotional effect of dietary safflower oils on large bowel or pulmonary implants of CT-26 in mice. *Cancer Res.*, 49: 4289–4294, 1989.
- Yetiv, J. Z. Clinical applications of fish oils. *J. Am. Med. Assoc.*, 260: 665–670, 1988.
- Anti, M., Marra, G., Armelao, F., Bartoli, G. M., Ficarelli, R., Percesepe, A., De Vitis, I., Maria, G., Sofo, L., Rapaccini, G. L., Gentiloni, N., Piccioni, E., and Miggiano, G. Effect of ω-3 fatty acids on rectal mucosal cell proliferation in subjects at risk for colon cancer. *Gastroenterology*, 103: 883–891, 1992.
- Bartram, H. P., Gostner, A., Scheppach, W., Reddy, B. S., Rao, C. V., Dusel, G., Richter, F., Richter, A., and Kasper, H. Effects of fish oil on colorectal cell proliferation, mucosal fatty acids, and prostaglandin E₂ release in healthy subjects. *Gastroenterology*, 105: 1317–1322, 1993.
- Lindner, M. A. A fish oil diet inhibits colon cancer in mice. *Nutr. Cancer*, 15: 1–11, 1991.
- Karmali, R. A. Eicosanoids in neoplasia. *Prev. Med.*, 16: 493–502, 1987.
- Begin, M. F., Das, V. N., and Horrobin, D. F. Selective killing of human cancer cells by PUFAs. *Prostaglandins Leukot. Med.*, 19: 177–186, 1985.
- Gonzalez, M. J. Lipid peroxidation and tumor growth: an inverse relationship. *Med. Hypotheses*, 38: 106–110, 1992.
- De Vries, C. E. E., and Van Noorden, C. J. F. Effects of dietary fatty acid composition on tumor growth and metastasis. *Anticancer Res.*, 12: 1513–1522, 1992.
- Van Noorden, C. J. F. Effects of ω-3 and ω-6 polyunsaturated fatty acid-enriched diets on lipid metabolism in periportal and pericentral compartments of female rat liver lobules and the consequences for cell proliferation after partial hepatectomy. *J. Lipid Res.*, 36: 1708–1720, 1995.
- Minoura, T., Takata, T., Sakaguchi, M., Takada, H., Yamamura, M., Hioto, K., and Yamamoto, M. Effect of dietary eicosapentaenoic acid on azoxymethane-induced colon carcinogenesis in rats. *Cancer Res.*, 48: 4790–4794, 1988.
- Bennett, A., Del Tacca, M., Stamford, I. F., and Zebro, T. Prostaglandins from tumors of human large bowel. *Br. J. Cancer*, 35: 881–884, 1977.
- Corey, E. J., Shih, C., and Cashman, J. R. Docosahexaenoic acid is a strong inhibitor of prostaglandin but not leukotriene biosynthesis. *Proc. Natl. Acad. Sci. USA*, 80: 3581–3584, 1983.
- Danesch, U., Weber, P. C., and Sellmayer, A. Differential effects of ω-6 and ω-3 polyunsaturated fatty acids on cell growth and early gene expression in Swiss 3T3 fibroblasts. *J. Cell. Physiol.*, 168: 618–624, 1996.
- Anti, M., Armelao, F., Marra, G., Percesepe, A., Bartoli, G. M., Palozza, P., Parrella, P., Canetta, C., Gentiloni, N., De Vitis, I., and Gasbarrini, G. Effects of different doses of fish oil on rectal cell proliferation in patients with sporadic colonic adenomas. *Gastroenterology*, 107: 1709–1718, 1994.
- Abbot, W. G. H., Tezabwala, B., Bennett, M., and Grundy, S. M. Melanoma lung metastases and cytolytic effector cells in mice fed antioxidant-balanced corn oil or fish oil diets. *Nat. Immun.*, 13: 15–28, 1994.
- Suzuki, I., Iigo, M., Ishikawa, C., Kuhara, T., Asamoto, M., Kunimoto, T., Moore, M. A., Yazawa, K., Araki, E., and Tsuda, H. Inhibitory effects of oleic and docosahexaenoic acids on lung metastasis by colon-carcinoma-26 cells are associated with reduced matrix metalloproteinase-2 and -9 activities. *Int. J. Cancer*, 73: 607–612, 1997.
- Iigo, M., Nakagawa, T., Ishikawa, C., Iwahori, Y., Asamoto, M., Yazawa, K., Araki, E., and Tsuda, H. Inhibitory effects of docosahexaenoic acid on colon carcinoma 26 metastasis to the lung. *Br. J. Cancer*, 75: 650–655, 1997.
- Honn, K. V., Onada, J. M., Menter, D. J., Taylor, J. D., and Sloane, B. F. Prostaglandin/thromboxanes and tumor cell metastasis. In: K. V. Honn and B. F. Sloane (eds.), *Hemostatic Mechanisms and Metastasis*, pp. 207–231. Boston: Nijhoff, 1984.

34. Young, M. R. I., and Young, M. E. Effects of fish oil and corn oil diets on prostaglandin-dependent and myelopoiesis-associated immune suppressor mechanisms of mice bearing metastatic lung carcinoma tumors. *Cancer Res.*, **49**: 1931–1936, 1989.
35. Kort, W. J., Weijma, I. M., Stehmann, T. E. M., Vergroesen, A. J., and Westbroek, D. L. Diets rich in fish oil cannot control tumor cell metastasis. *Ann. Nutr. Metab.*, **31**: 342–348, 1987.
36. Drago, J. R., and Al-Mondhir, H. A. B. The effect of prostaglandin modulators on prostate tumor growth and metastasis. *Anticancer Res.*, **4**: 391–394, 1984.
37. Bando, H., Yamashita, T., and Tsubura, E. Effects of antiplatelet agents on pulmonary metastases. *Gann*, **75**: 284–291, 1984.
38. Coulombe, J., Pelletier, G., Tremblay, P., Mercier, G., and Oth, D. Influence of lipid diets on the number of metastases and ganglioside content of H59 variant tumors. *Clin. Exp. Metastasis*, **15**: 410–417, 1997.
39. Baronzio, G. F., Freitas, I., Griffini, P., Bertone, V., Pacini, F., Mascaro, G., Razzini, E., and Gramaglia, A. Ω-3 fatty acids can improve radioresponse modifying tumor interstitial pressure, blood rheology and membrane peroxidability. *Anticancer Res.*, **14**: 1145–1154, 1994.
40. Meydani, S. N. Effect of (ω-3) polyunsaturated fatty acids on cytokine production and their biologic function. *Nutrition*, **12**: S8–S14, 1996.
41. Eicher, S. D., and Scott McVey, D. Dietary modulation of Kupffer cell and splenocyte function during a *Salmonella typhimurium* challenge in mice. *J. Leukoc. Biol.*, **58**: 32–39, 1995.
42. Sherrington, E. J., Sanderson, P., and Calder, P. The effect of dietary lipid manipulation on macrophage cell surface molecule expression. *Biochem. Soc. Trans.*, **23**: 272S, 1995.
43. Calder, P. C., Bond, J. A., Harvey, D. J., Gordon, S., and Newsholme, E. A. Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochem. J.*, **269**: 807–814, 1990.
44. Virella, G., Kilpatrick, J. M., Rugeles, M. T., Hyman, B., and Russel, R. Depression of humoral responses and phagocytic functions *in vivo* and *in vitro* by fish oil and eicosapentaenoic acid. *Clin. Immunol. Immunopathol.*, **52**: 257–270, 1989.
45. Sakaguchi, M., Rowley, S., Kane, N., Imray, C., Davies, A., Jones, C., Newbold, M., Keighley, M. R. B., Baker, P., and Neoptolemus, J. P. Reduced tumour growth of the human colonic cancer cell lines COLO-320 and HT-29 *in vivo* by dietary ω-3 lipids. *Br. J. Cancer*, **62**: 742–747, 1990.
46. Marquet, R. L., Westbroek, D. L., and Jeekel, J. Interferon treatment of transplantable rat colon adenocarcinoma: importance of tumor site. *Int. J. Cancer*, **33**: 689–692, 1984.
47. Martin, M., Chauffert, B., Caignard, A., Pelletier, H., Hammann, A., and Martin, F. Histoimmunological characterization of the cellular reaction to liver metastases induced by colon cancer cells in syngeneic rats. *Invasion Metastasis*, **9**: 216–230, 1989.
48. Griffini, P., Smorenburg, S. M., Vogels, I. M. C., Tigchelaar, W., and Van Noorden, C. J. F. Kupffer cells and pit cells are not effective in the defense against experimentally induced colon carcinoma metastasis in rat liver. *Clin. Exp. Metastasis*, **14**: 367–380, 1996.
49. Polak, J. M., and Van Noorden, S. An Introduction to Immunocytochemistry, 2nd ed. Oxford, United Kingdom: Bios, 1997.
50. Van der Loos, C. M., Becker, A. E., and Van den Oord, J. J. Practical suggestions for successful immunoenzyme double-staining experiments. *Histochem. J.*, **25**: 1–13, 1993.
51. Van Noorden, C. J. F., Jonges, T. G. N., Van Marle, J., Bissel, E. R., Griffini, P., Jans, M., Snel, J., and Smith, R. E. Heterogeneous suppression of experimentally induced colon cancer metastasis in rat liver lobes by inhibition of extracellular cathepsin B. *Clin. Exp. Metastasis*, **16**: 159–167, 1998.
52. Jonker, A., Geerts, W. J. C., Chieco, P., Moorman, A. F. M., Lamers, W. H., and Van Noorden, C. J. F. Basic strategies for valid cytometry using image analysis. *Histochem. J.*, **29**: 347–364, 1997.
53. Griffini, P., Smorenburg, S. M., Verbeek, F. J., and Van Noorden, C. J. F. Three-dimensional reconstruction of colon carcinoma metastases in liver. *J. Microsc. (Oxf.)*, **187**: 12–21, 1997.
54. Wigmore, S. J., Ross, J. A., Falconer, J. S., Plester, C. E., Tisdale, M. J., Carter, D. C., and Fearon, K. C. The effect of polyunsaturated fatty acids on the progress of cachexia in patients with pancreatic cancer. *Nutrition*, **12**: S27–S30, 1996.
55. Giacosa, A., Frascio, F., Sukkar, S. G., and Roncella, S. Food intake and body composition in cancer cachexia. *Nutrition*, **12**: S20–S23, 1996.
56. Dagnelie, P. C., Bell, J. D., Williams, S. C. R., Bates, T. E., Abel, P. D., and Foster, C. S. Effect of fish oil on cancer cachexia and host liver metabolism in rats with prostate tumors. *Lipids*, **29**: 195–203, 1994.
57. Hwang, D. Essential fatty acids and immune response. *FASEB J.*, **3**: 2052–2061, 1989.
58. Yamashita, N., Sugiyama, E., Hamazaki, T., and Yano, S. Inhibition of natural killer cell activity by eicosapentaenoic acid *in vivo* and *in vitro*. *Biochem. Biophys. Res. Commun.*, **150**: 497–505, 1988.
59. Martin, D., and Meckling-Gill, K. Ω-3 polyunsaturated fatty acids increase purine but not pyrimidine transport in L1210 leukaemia cells. *Biochem. J.*, **315**: 329–333, 1996.
60. Dijkstra, C. D., Döpp, E. A., Joling, P., and Kraal, G. The heterogeneity of mono-nuclear phagocytes in lymphoid organs: distinct macrophages subpopulations in rats recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology*, **54**: 589–599, 1984.
61. McMaster, W. R., and Williams, A. F. Identification of Ia glycoproteins in rat thymus and purification from rat spleen. *Eur. J. Immunol.*, **9**: 426–433, 1979.
62. Bouwens, L., and Wisse, E. Pit cells in the liver. *Liver*, **12**: 3–9, 1992.
63. Van den Brink, M. R. M., Palomba, M. L., Basse, P. H., and Hiserodt, J. C. *In situ* localization of 3.2.3+ natural killer cells in tissue from normal and tumor-bearing rats. *Cancer Res.*, **51**: 4931–4936, 1991.
64. Caldero, J., Campo, E., Viñas, J., and Cardesa, A. Lectin-binding sites in neoplastic and non-neoplastic colonic mucosa of 1,2-dimethylhydrazine-treated rats. *Lab. Investig.*, **61**: 670–676, 1989.
65. Frederiks, W. M., Marx, F., Chamuleau, R. A. F. M., Van Noorden, C. J. F., and James, J. Immunocytochemical determination of ploidy class-dependent bromodeoxyuridine incorporation in rat liver parenchymal cells after partial hepatectomy. *Histochemistry*, **93**: 627–630, 1990.