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KINETICS OF THE INHIBITION OF TUMOUR GROWTH IN MICE BY EICOSAPENTAENOIC ACID-REVERSAL BY LINOLEIC ACID

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Abstract—Oral administration of eicosapentaenoic acid (EPA) (2.0 g/kg) by gavage to female NMRI mice bearing the MAC16 colon adenocarcinoma and with weight loss, prevented further loss in body weight and produced a delay in the growth of the tumour. Cell production and loss were determined by the [^{125}I]5-iodo-2′-deoxyuridine method during the stationary and growth phase of the tumour in animals treated with EPA. Tumour stasis appeared to arise from an increase in the rate of cell loss from 38 to 71% without a significant change in the potential doubling time. During the subsequent growth phase the cell loss factor was reduced to 52% and this was combined with a reduced potential doubling time from 32 to 26 hr. The antiproliferative, but not the anticachectic effect of EPA could be reversed by oral administration of pure linoleic acid (LA), (1.9 g/kg) which acted to increase tumour growth by reducing the cell loss factor to 45%. Despite this reversal, incorporation of EPA into tumour cell lipids was not significantly different in animals administered with either EPA alone or combined with LA. This suggests that the antiproliferative effect of EPA in this system may arise from an indirect effect through the blocking of the catabolic effect of the tumour on host adipose tissue, which normally supplies fatty acids essential for tumour growth. This suggests that LA may be required by some tumours to prevent cell loss and that the catabolism of adipose tissue, which accompanies cancer cachexia effectively supplies this fatty acid to the tumour.

Recent data suggest that polyunsaturated fatty acids (PUFAs†) of the n-6 and n-3 series have disparate effects on the growth of solid tumours in experimental animals. Thus while linoleic acid (18:2, n-6) (LA) administered as a 10% corn oil diet has been shown to significantly enhance the growth of transplantable mammary adenocarcinomas in mice [1], fish oil rich in the n-3 fatty acids, eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), has been shown to inhibit the growth of both syngenic [2] and xenografted [3] mammary and colon [4, 5] tumours in rodents. Stimulation of tumour growth in adult rats after acute starvation appears to be due to uptake of linoleic and arachidonic acids present in hyperlipemic blood and it was concluded that these PUFAs are rate limiting for tumour growth *in vivo* [6].

We have recently shown [7] that the n-3 PUFA, EPA, specifically inhibits cachexia and tumour growth in a murine colonic adenocarcinoma model (MAC16). Other fatty acids such as DHA and γ-linoleic acid (GLA) were ineffective in this model, suggesting a strict structural specificity for the effect. The anticachectic effect of EPA appears to be due to an inhibition of the action of a tumour-produced catabolic factor at the level of the adipocyte by directly preventing an increase in cyclic AMP levels [7]. We have hypothesized that the subsequent inhibition of tumour growth may be secondary to the inhibition of cachexia, by depriving the tumour of fatty acids essential for tumour growth *in vivo*, rather than a direct antiproliferative effect, since both EPA and DHA are effective inhibitors of tumour growth *in vitro*, but only EPA is effective *in vivo*.

The present investigation determines the effect of EPA on the kinetics of growth in the MAC16 model and the interaction with other fatty acids.

MATERIALS AND METHODS

Animals. Pure strain NMRI mice were obtained from our own inbred colony and were fed a rat and mouse breeding diet (Pilsbury Ltd, Birmingham, U.K.) and water *ad lib.* Female mice (average body weight 20 g) were transplanted with fragments of the MAC16 tumour into the flank by means of a trocar as described previously [8] and were fed on the rat and mouse breeding diet for 10–12 days after transplantation when the tumours became palpable and weight loss had started to occur. This point was chosen to ensure complete tumour take and weight loss prior to initiation of therapy.

Chemicals. [^{125}I]5-Iodo-2′-deoxyuridine (sp. act. 2000 Ci/mmol) was purchased from Amersham International (Amersham, U.K.). Linoleic acid (LA 76.7%, oleic acid 21.6%) and EPA (80%), (57%) (expressed as a percentage of the fatty acid methyl esters prepared) were kindly donated by Dr D. Horrobin, Scotia Pharmaceuticals Ltd (Guildford, U.K.). DHA (90%) was obtained from the Sigma Chemical Co. Ltd (Poole, U.K.). The EPA (80%)

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† Abbreviations: EPA, eicosapentaenoic acid; LA, linoleic acid; AA, arachidonic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; GLA, γ-linoleic acid; ϕ, tumour cell loss factor.

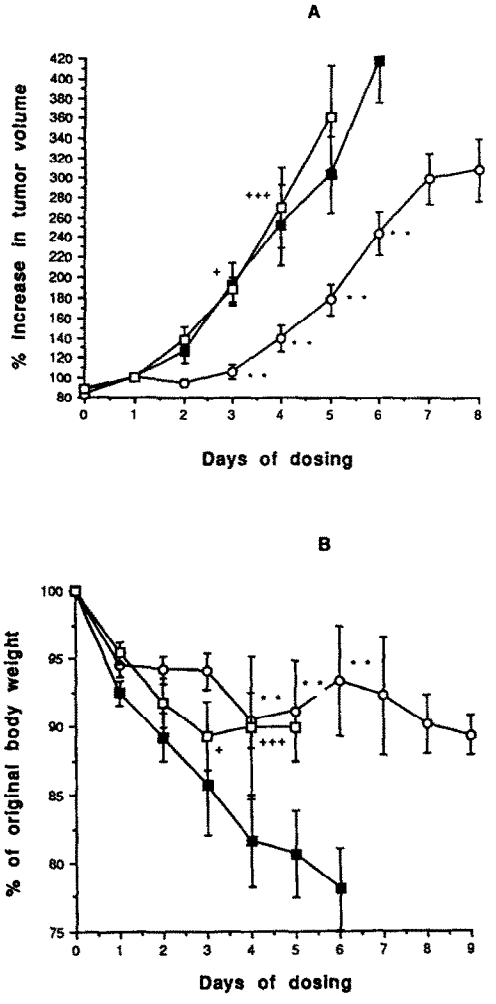

Fig. 1. Effect of oral dosing by gavage of female NMRI mice bearing the MAC16 tumour with water (■) or 80% EPA (○) (50 μL per mouse equivalent to 2.0 g/kg EPA) or 57% EPA (□) (75 μL per mouse equivalent to 2.1 g/kg EPA) on tumour volume (A) and host body weight (B). The average tumour volume on initiation of therapy was 87 ± 11 mm³ which has been normalized to 100% to facilitate comparison between groups. Therapy was initiated on day 1. Values of both tumour volume and host body weight 1 day prior to therapy have been given to show that all animals were losing weight and had growing tumours at the time of initiation of therapy. Tumour volumes were measured daily by calipers and were recorded as a percentage of the initial tumour volume. Host body weight was also measured daily and recorded as a percentage of the body weight prior to oral dosing. Results are expressed as mean ± SEM for eight animals per group. Differences between controls and EPA-treated groups were determined by two way ANOVA followed by Tuckey's test and are: $^{**}P < 0.01$, + indicates the death of an animal.

was diluted to 57% by the addition of DHA 6.7%, palmitoleic acid 13.5%, oleic acid 9.3%, stearic acid 4.4% and palmitic acid 2.1%. No products contained anti-oxidants and all were sealed under nitrogen.

Tumour growth and cell loss. Tumour dimensions were measured daily by means of calipers and the volume was calculated from the formula:

$$\frac{\text{length} \times (\text{width})^2}{2}.$$

The doubling time of the tumours was determined during logarithmic growth from daily changes in volume as well as from the corresponding changes in weight. The protocol to generate the data required for the measurement of cell loss was similar to that previously described [9, 10]. Tumour-bearing mice were given drinking water containing 0.1% KI 4 days prior to an i.p. injection of 4 μCi of [^{125}I]-iododeoxyuridine in 0.1 mL of sterile saline and one group of mice [11] was administered EPA (80%, 2.0 g/kg) orally by gavage, while a second group [11] was administered water as a control. Four animals from each group were killed 2 hr later and then at 24 hr intervals for a further 5 days. To determine the tumour cell loss during the second phase, a third group was administered EPA (80%, 2.0 g/kg) on day 1, but, no [^{125}I]iododeoxyuridine until day 4 and four animals were killed 2 hr later and then at 24-hr intervals for a further 4 days. To determine the radioactivity in tumour cell DNA, the tissues were minced into pieces 1–2 mm³, fixed in a solution of methanol:acetic acid (3:1, v/v) and washed three times with 2 mL of the same solution over the next 72 hr. This washing procedure was effective in removing all of the acid-soluble material from the tissues pieces. Radioactivity in the extracted tissue pieces was determined using a Packard Tri-Carb γ-scintillation spectrometer. The values for cpm per gram of tumour were plotted on semi-logarithmic graph paper, and the ($T_{1/2}$) of the decline in specific activity was determined.

Fatty acid analysis. Total lipids were extracted from tumour and adipose tissue by the method of Folch *et al.* [11]. Tissues were homogenized in 20 vol. of chloroform:methanol (2:1 v/v) and 50 μg of margaric acid was added to measure recovery. The organic phase containing the lipid extract, after addition of 0.01 vol. of 2% butylated hydroxytoluene in ethanol was saponified by the addition of 5% sodium hydroxide in 50% methanol and heating at 100° for 45–60 min in an argon atmosphere. The solution was acidified to pH 2 by the addition of concentrated hydrochloric acid, and the fatty acids were methylated with the use of BF_3–methanol and extracted with hexane:chloroform (4:1 v/v). The fatty acid methyl esters were analysed using a Pye Unicam gas-liquid chromatograph, series 204,

Table 1. Effect of EPA and LA on kinetic parameters of the MAC16 tumour

Group	T_D (hr)	T_P (hr)	ϕ (%)
Control	48	30	38
LA	53	31	40
EPA phase 1	110	32	71
EPA phase 2	55	26	52
EPA + LA	48	31	45

T_D, tumour doubling time; T_P, tumour potential doubling time and ϕ, tumour cell loss factor.

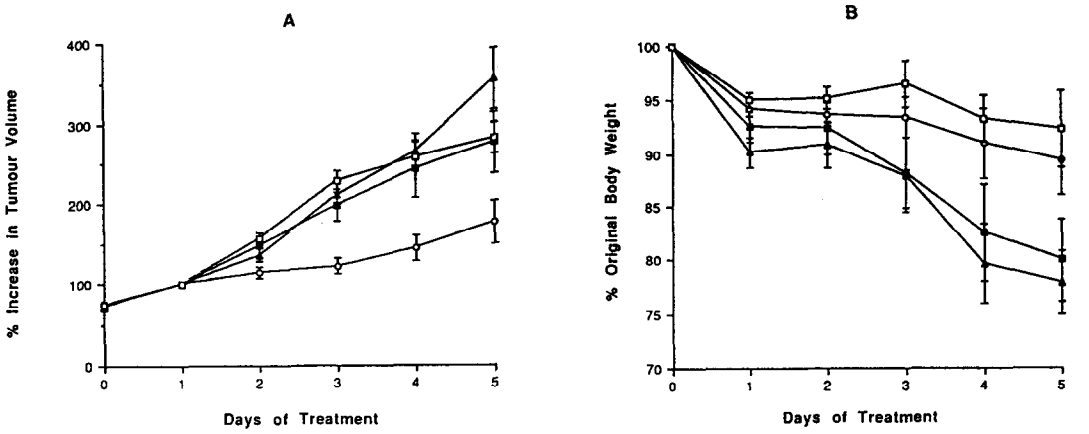

Fig. 2. Effect of oral dosing by gavage of female NMRI mice bearing the MAC16 tumour with water alone (■), 2.0 g/kg EPA (○), 1.9 g/kg LA (▲) or 2.0 g/kg EPA plus 1.9 g/kg LA (□) on tumour volume (A) and host body weight (B). The protocol for the experiment was as described in Fig. 1 with therapy initiated on day 1. Results are expressed as mean ± SEM for six animals per group.

Table 2. Effect of EPA and LA on food intake and water intake in animals bearing the MAC16 tumour*

Group	Food intake (cal/mouse/day)	Water intake (mL/mouse/day)
Control	9.6 ± 1.0	4.00 ± 0.18
LA	9.6 ± 1.5	4.00 ± 0.28
EPA	10.5 ± 1.9	4.17 ± 0.40
EPA + LA	9.5 ± 1.5	3.75 ± 0.53

* Results are expressed as mean ± SEM for five animals per group.

equipped with a flame ionization detector, with a 2 m long column of 2 mm internal diameter packed with GP3% SP-2310/2% SP-2300 on 100/120 Chromosorb WAW (Supelco, U.K.). The column was operated under a temperature programme at 150° initially, but later increasing to 220°, at a rate of 2° min^{-1} with a gas flow of 25 mL/min of nitrogen through the column. The peaks were identified by comparison of the retention times with those of authentic standards.

RESULTS

Growth of the MAC16 tumour in animals administered EPA displayed two phases (Fig. 1A), a stationary phase where tumour growth was almost completely suppressed and an exponential phase where tumour growth continued at a rate similar to that in non-treated control animals. Cell cycle kinetics were investigated during the two phases using a direct method ([^{125}I]iododeoxyuridine) in which the $T_{1/2}$ of the decline in specific activity was measured. The tumour cell loss factor (ϕ) was then calculated using the formula

$$\phi = 1 - (T_P/T_D)$$

[12] where T_P is the tumour potential doubling time and T_D is the tumour doubling time with the values of $T_{1/2}$ substituted for T_P in the formula [13]. The results presented in Table 1 show that during the first phase of treatment with EPA the increased doubling time of the tumours was accounted for solely by an increase in the cell loss factor (ϕ) (from 38 to 71%) without a significant change in the potential doubling time. During the second phase the tumour doubling time was substantially reduced

Table 3. Effect of the tumour-bearing state (TB) and fatty acid treatment on the fatty acid composition of total plasma lipids*

Fatty acid	Non TB	TB	TB + EPA	TB + LA	TB + LA + EPA	TB + DHA
16:0	0.189 ± 0.014†	0.135 ± 0.009	0.136 ± 0.014	0.136 ± 0.005	0.128 ± 0.014	0.133 ± 0.009
18:0	0.049 ± 0.010	0.061 ± 0.010	0.035 ± 0.014	0.047 ± 0.008	0.054 ± 0.008	0.063 ± 0.005
18:1 (n-9)	0.075 ± 0.013	0.046 ± 0.001	0.037 ± 0.013	0.036 ± 0.017	0.036 ± 0.005	0.040 ± 0.003
18:2 (n-6)	0.220 ± 0.014†	0.149 ± 0.026	0.112 ± 0.027	0.365 ± 0.041†	0.224 ± 0.031	0.147 ± 0.015
20:4 (n-6)	0.025 ± 0.004†	0.044 ± 0.007	0.011 ± 0.003†	0.034 ± 0.007	0.033 ± 0.005	0.016 ± 0.001†
20:5 (n-3)	0.001 ± 0.001	0 ± 0	0.082 ± 0.017†	0.005 ± 0.007	0.057 ± 0.015†	0.004 ± 0.0005
22:6 (n-3)	0.008 ± 0.004	0.017 ± 0.002	0.004 ± 0.004	0.010 ± 0.006	0.006 ± 0.003	0.039 ± 0.003†

* Results are expressed as mean ± SEM as mg of fatty acid/mL serum.
† $P < 0.01$ compared with TB by one-way ANOVA followed by Tuckey's test.

so that it was only slightly greater than in the controls, and this arose from a decrease in the cell loss factor (to 52%) combined with a reduced potential doubling time from 32 to 26 hr.

During the initial studies on the efficacy of EPA as an anticachectic and antitumour agent in the MAC16 model it was noted that at least an 80% pure preparation was required for anti-tumour activity. Thus when the EPA was diluted with other fatty acids to 57% and was administered to animals at the same concentration of EPA as the 80% pure material, it was found to be more toxic and without anti-tumour activity (Fig. 1A), although it still appeared to protect effectively against the weight loss (Fig. 1B). This suggested either that the anti-tumour and anti-cachectic effect of EPA were exerted by two different mechanisms, or that in preventing catabolism of adipose tissue by the tumour, EPA effectively blocked uptake by the tumour of an essential fatty acid and that this was supplied by the impure preparation.

To test these alternatives 80% pure EPA (2.0 g/kg) was administered to MAC16 tumour bearing mice with weight loss both alone and in combination with LA (1.9 g/kg). The results presented in Fig. 2 show that while EPA and the combination were equally effective in preventing weight loss, the antiproliferative effect of EPA was reversed so that it was the same as that in non-treated animals, although it was lower than that in animals administered LA alone. The stimulatory effect of LA on tumour growth suggests that this fatty acid is essential for tumour growth and is possibly a limiting factor when supplied from the diet alone. There was no significant difference in food or water intake between the various groups (Table 2), thus confirming that the effects of EPA on host body weight are not mediated by an increased calorie consumption.

The effect of the combination of EPA and LA on the growth kinetic parameters of the MAC16 tumour are shown in Table 1. The effect of the combination was to reduce the cell loss parameter (to 45%) compared with EPA alone, while having no effect on the potential doubling time. This suggests that either LA or a metabolite of LA is required to prevent cell death in the MAC16 tumour.

The effect of treatment with EPA, LA or the combination on the fatty acid composition of plasma, tumour and liver is shown in Tables 3, 4 and 5, respectively. There was no significant difference in the level of EPA in plasma or tumour in animals treated with either EPA alone, or the combination of EPA and LA. This suggests that the lack of effect on tumour growth in the combination is not due to insufficient EPA being absorbed or incorporated into the tumour.

Weight losing animals bearing the MAC16 tumour show major differences in their total plasma lipid fatty acid composition from that found in non tumour-bearing animals (Table 3). Thus the level of stearic acid (16:0) and LA (18:2, n-6) were significantly decreased while the level of arachidonic acid (AA) (20:4, n-6) was significantly increased. The major change in fatty acid composition induced by EPA was a reduction in the 20:4, n-6 to below that found in non tumour-bearing animals and a concomitant increase in the level of 20:5, n-3. When LA was administered in combination with EPA the plasma level of 20:4, n-6 was not significantly different from that found in non-treated cachectic mice. However, changes in 20:4, n-6 are probably not related to tumour growth inhibition since a similar effect is produced by DHA as was observed with EPA (Table 3), although DHA has no effect on the growth rate of the MAC16 tumour or host body weight loss (Fig. 3). The major change in the plasma profile of fatty acids specific to tumour growth inhibition appears to be the stearic:oleic acid ratio which is increased from 0.65 in non tumour-bearing animals to 1.33 in cachectic tumour-bearing animals. This value is reduced to 0.95 in animals treated with EPA while the values for animals treated with DHA (1.58), LA (1.30) and LA + EPA (1.50) are all similar to that in non-treated tumour-bearing mice. The tumour level of 20:4, n-6 was significantly reduced in animals treated with either EPA or DHA (Table 4). The only other major change in tumour fatty acids was a large increase in 18:1, n-9 in animals treated with DHA (Table 4). Unlike the tumour neither EPA or DHA produced any change in the liver content of 20:4, n-6 (Table 5).

Table 4. Effect of the tumour-bearing state (TB) and fatty acid treatment on the fatty acid composition of the major tumour lipids*

Fatty acid	TB	TB + EPA	TB + LA	TB + EPA + LA	TB + DHA
16:0	0.892 ± 0.047	0.783 ± 0.039	0.770 ± 0.160	0.786 ± 0.051	0.971 ± 0.070
18:0	0.821 ± 0.061	0.826 ± 0.043	0.867 ± 0.087	0.758 ± 0.039	0.845 ± 0.067
18:1 (n-9)	0.920 ± 0.019	0.840 ± 0.087	0.945 ± 0.087	1.013 ± 0.010	1.515 ± 0.127†
18:2 (n-6)	0.63 ± 0.08	0.56 ± 0.04	0.63 ± 0.04	0.60 ± 0.01	0.75 ± 0.09
20:4 (n-6)	0.427 ± 0.005	0.310 ± 0.005†	0.051 ± 0.016†	0.340 ± 0.025	0.275 ± 0.029†
20:5 (n-3)	0.019 ± 0.001	0.049 ± 0.009†	0.019 ± 0.001	0.040 ± 0.003†	0.026 ± 0.005
22:6 (n-3)	0.263 ± 0.028	0.266 ± 0.010	0.242 ± 0.009	0.255 ± 0.017	0.530 ± 0.057

* Results are expressed as mean ± SEM as mg of fatty acid/g of tumour.
† $P < 0.01$ from TB by one-way ANOVA followed by Tuckey's test.

Table 5. Effect of the tumour-bearing state (TB) and fatty acid treatment on the fatty acid composition of the major liver lipids*

Fatty acid	Non TB	TB	TB + EPA	TB + LA	TB + LA + EPA	TB + DHA
16:0	0.31 ± 0.03†	0.54 ± 0.04	0.44 ± 0.009	0.28 ± 0.02	0.36 ± 0.03	0.58 ± 0.07
18:0	0.22 ± 0.03	0.38 ± 0.04	0.36 ± 0.07	0.21 ± 0.01	0.24 ± 0.02	0.47 ± 0.04
18:1 (n-9)	0.10 ± 0.02	0.18 ± 0.02	0.19 ± 0.04	0.11 ± 0.01	0.14 ± 0.01	0.23 ± 0.04
18:2 (n-6)	0.28 ± 0.02	0.33 ± 0.06	0.31 ± 0.08	0.21 ± 0.01	0.28 ± 0.02	0.50 ± 0.04
20:4 (n-6)	0.17 ± 0.01	0.19 ± 0.04	0.19 ± 0.04	0.14 ± 0.003	0.12 ± 0.004	0.15 ± 0.01
20:5 (n-3)	0.002 ± 0.002	0.019 ± 0.004	0.031 ± 0.008†	0.004 ± 0.001	0.016 ± 0.002	0.019 ± 0.009
22:6 (n-3)	0.148 ± 0.019	0.301 ± 0.022	0.272 ± 0.062	0.157 ± 0.021	0.198 ± 0.012	0.504 ± 0.021†

* Results are expressed as mean ± SEM as mg of fatty acid/g of liver.
† $P < 0.01$ from TB by one-way ANOVA followed by Tuckey's test.

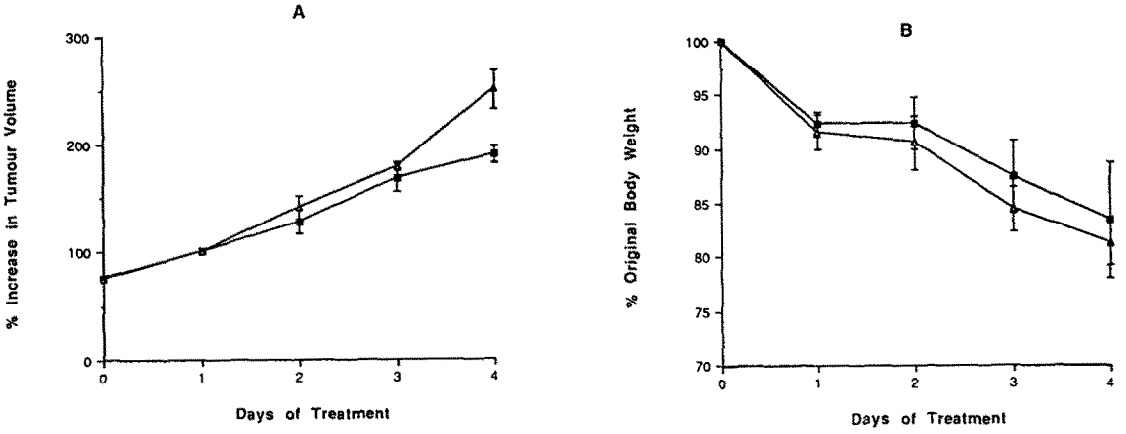

Fig. 3. Effect of oral dosing by gavage of female NMRI mice bearing the MAC16 tumour with water alone (■) or 2.3 g/kg DHA (△) on tumour volume (A) and host body weight (B). The protocol for the experiment was as described in Fig. 1. Results are expressed as mean ± SEM for six animals per group.

DISCUSSION

Feeding a linoleic acid-rich diet (10% corn oil) has been shown to significantly enhance the growth rate of mammary adenocarcinomas in BALB/c mice, when compared with animals fed 10% hydrogenated cottonseed oil, which is lacking in linoleate [9]. The increase in tumour mass in the corn oil fed animals arose solely from a reduced rate of tumour cell loss, without affecting the proliferative compartment of the tumour. The stimulation of tumour growth by corn oil was effectively blocked by the prostaglandin synthesis inhibitor indomethacin, which increased the rate of tumour cell loss. A similar effect was shown in animals fed menhaden oil, which is rich in the n-3 PUFAs EPA and DHA, where the rate of cell loss in the mammary adenocarcinomas was 2.5 times that of corn oil fed animals [10].

Although fish oils are generally used as a supply of n-3 PUFAs, there have been few investigations into the nature of the active principles, and it is generally considered that it is the total n-3 lipids which is important. Using the MAC16 model we have previously shown [7] that only EPA is an effective anticachectic and antitumour agent and that DHA is without biological effects in this model. In the present investigation we have extended these studies and have shown EPA to be effective in inhibiting tumour growth by increasing the rate of cell loss, although the type of cell loss, either necrosis or apoptosis is not known.

The growth of a tumour is a balance between the rate of cell production and the rate of cell loss [12]. For a tumour to increase mass, the rate of cell production must exceed the rate of cell loss and for the MAC16 tumour the cell loss parameter (ϕ) (38%) is close to that reported previously [9] for mouse mammary adenocarcinomas (31%). Since the MAC16 tumour is highly refractory to antitumour agents which interfere with cell production [14] one possible mechanism to inhibit tumour growth rate would be to increase the cell loss parameter. While EPA is partially effective in this respect, tumour growth eventually resumes due to a reduction in the cell loss parameter combined with an increased rate of cell production. It is possible that the tumour may

be more sensitive to agents which interfere with cell production at this point.

The antitumour, but not the anticachectic effect of EPA in the MAC16 model can be effectively reversed by the concurrent administration of LA. The effect of LA on the tumour appears to result from a reduction in the rate of cell loss when compared with the group treated with EPA alone. The reversal by LA is not accompanied by a reduction in the level of EPA in the plasma or the tumour, suggesting that the effect does not arise from simple competition between these two fatty acids. The ability of LA to reverse the antitumour effect of EPA may be important in the clinical investigation of this agent, particularly since patients may be consuming high levels of n-6 fatty acids.

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