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OBSERVATIONS ON THE INHIBITION OF SERUM AND CELL SURFACE ENZYMES BY EICOSAPENTAENOIC ACID

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The relationship between serum and tumour cell surface proteolytic enzymes and the development of muscle breakdown in cancer cachexia has been studied in a murine model of the condition (MAC16). The surface of the MAC16 tumour cells carried a proteolytic enzyme referred to as guanidinobenzoatase (GB). Serum from mice also contained an enzyme (referred to as MSE) which cleaved the trypsin inhibitor 4-methylumbelliferyl-*p*-guanidinobenzoate as a true substrate, but there was no relationship with weight loss or the presence or absence of tumour and the level of this serum enzyme. Polyunsaturated fatty acids (PUFAs) were shown to be inhibitors of MSE at μM concentrations and one PUFA, eicosapentaenoic acid (EPA) was found to be a non-competitive inhibitor of both MSE and GB. The effect of EPA was specific since other proteolytic enzymes, trypsin, esterase and tissue plasminogen activator were unaffected by concentrations inhibiting GB and MSE. MSE and GB are two different enzymes which possess some common properties. However, GB is likely to be significant for tumour development since MSE is also found in normal mouse serum.

KEY WORDS: Eicosapentaenoic acid, guanidinobenzoatase, tumour cell surface enzyme, MSE.

INTRODUCTION

Certain human and experimental animal tumours are associated with a massive weight loss in the host, referred to as cachexia.[1] In a study of cachexia in mice bearing the MAC16 tumour it was discovered that the polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA) had a pronounced anticachectic and antitumour activity which was specific to this PUFA. This study suggested that proteolytic enzymes played a significant role in muscle degradation in cancer cachexia.[2,3]

Many tumours have been found to be associated with an increased proteolytic activity when compared to their normal counterparts.[4,5] The latter authors indicated that this proteolytic activity was often associated with the cell surface of tumour cells and normal cells concerned with invasion.

Other studies[6] have shown that tumour cells possess a cell surface enzyme similar to plasminogen activator. The tumour cell surface enzyme was originally referred to as guanidinobenzoates (GB),[7,8] because of earlier studies on a soluble form of GB present in the ascitic fluid of Ehrlich ascitic tumour grown in mice.[7] The name *guanidinobenzoatase* was used to describe this soluble enzyme which acted on both

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4-nitrophenyl-*p*-guanidinobenzoate (NPGB) and 4-methyl-umbelliferyl-*p*-guanidinobenzoate (MUGB) as true substrates rather than active site titrants. It was pointed out[7] that normal mice also possessed a similar serum enzyme and consequently further work on this serum enzyme was discontinued. Tozser *et al.*[9] reported a similar serum enzyme which they considered to be a carboxylesterase. The mouse serum enzyme shows some similarity to a proteolytic factor associated with the cachexia-inducing MAC16 tumour[1] in being a serine protease present in serum and not being inhibited by protein inhibitors of trypsin.

The presence of active GB on a cell surface can be detected by the binding of 9-aminoacridine (9AA), a competitive inhibitor of GB. Cells which possess active GB bind 9AA and fluoresce yellow under appropriate microscopic conditions.[8] If a compound interacts with GB in such a manner that the active centre is modified, then 9AA will be unlikely to bind and these cells will no longer be able to exhibit yellow fluorescence, after staining with 9AA. In the present paper evidence is presented for the inhibition of GB on the surface of MAC16 tumour cells which have been treated with EPA *in vitro*.

We refer to the mouse serum enzyme, which cleaves guanidinobenzoate from guanidinobenzoate substrates as MSE. In the present paper we have measured the activity of MSE in normal and cachetic mice and concluded that MSE has no significant role in cachexia. We conclude that MSE is distinct from the GB associated with MAC16 tumour cell surfaces, even though both enzymes are in some way affected by EPA, leading to inhibition in both instances.

MATERIALS AND METHODS

4-Methylumbelliferyl-*p*-guanidinobenzoate, trypsin (bovine pancreas), soya bean trypsin inhibitor, esterase (rabbit liver), tissue-type plasminogen activator, eicosapentaenoic acid (EPA) and all polyunsaturated fatty acids were purchased from Sigma Chemical Co., Poole, Dorset. BZAR was prepared according to the method previously reported.[10]

Male NMRI mice were implanted s.c. with fragments of the MAC16 or MAC13 tumours as previously described.[11] Animals bearing the MAC16 tumour were monitored for weight loss and were used 15 to 20 days after transplantation, when they had lost approximately 2 to 4 g in weight. Animals bearing the MAC13 tumour do not lose weight and were used when the tumour mass was similar to that in animals bearing the MAC16 tumour.

Determination of MSE activity

Blood was removed from animals under anaesthesia, by cardiac puncture. Blood samples were allowed to stand for 10 min to clot and serum was prepared by centrifugation for 30 s in a Beckman microfuge, after removal of clots. Serum samples were kept on ice and used immediately. MSE activity was determined on 5 μl of serum using 4-methylumbelliferyl-*p*-guanidinobenzoate as substrate. Enzyme activity was determined in 0.1 M phosphate buffer, pH 6.0 and release of 4-methylumbelliferone was measured fluorimetrically with excitation and emission wavelengths of 323 and 445 nm respectively. A stock solution of 2 mM 4-methylumbelliferyl-*p*-guanidinobenzoate was prepared in dimethyl formamide and 5 μl (0.25%) was used

in the assay (final concentration 1 μM). The mean value of the spontaneous cleavage of methylumbelliferone was determined separately and deducted from the overall change in fluorescence level. The normal assay was run over a 6 min time course. A standard curve was constructed daily.

Determination of trypsin activity

The chromogenic substrate used in this assay was Nα-benzoyl-L-arginine-7-amido-4-methylcoumarin, which was cleaved by trypsin to form the fluorescent product 7-amino-4-methylcoumarin.[11] The substrate was dissolved at a concentration of 0.1 mM in 50 mM Tris/HCl, pH 8.0, containing 20 mM calcium chloride and 1% DMSO; 0.1 ml of this solution was added to 2 ml of 50 mM Tris, pH 8.0, together with 20 μg of trypsin. The product was determined fluorimetrically with excitation and emission wavelengths of 380 and 440 nm respectively.

Determination of esterase activity

Esterase activity was determined by the release of *o*-nitrophenol, measured by the increase in absorbance at 412 nm, from *o*-nitrophenyl butyrate in 25 mM Tris/HCl, pH 8.0 containing 10 mM calcium chloride.

Determination of tissue-type plasminogen activator (tPA) activity

The method employed to assay tPA uses thiobenzyl benzyloxycarbonyl-L-lysinate (Z-Lys-SBzl) as a substitute for the plasmin substrate, in a two step coupled assay.[12] In the first step, plasminogen (5 μg) is activated by plasminogen activator (10^{-4} plough units, single chain) to form plasmin. In the second step, the Z-Lys-SBzl plus the 5,5′-dithiobis(2-nitrobenzoic acid) reacts with the plasmin to form Z-Lys plus the mixed disulphide and thiophenolate, which is quantified spectrophotometrically by its absorption at 412 nm.

Location of cells in wax embedded sections

This was carried out as previously described.[13] Formaldehyde fixed tumour tissue was wax embedded, sectioned and the sections dewaxed prior to being placed in 10^{-3} M 9-aminoacridine in isotonic saline for 2 min and washed for 1 min in fresh isotonic saline. Cells possessing active GB bound 9-aminoacridine (9AA) and fluoresced yellow. Treatment with EPA (3.7×10^{-4} M) in equimolar lysine was carried out for 30 min followed by 9-aminoacridine as above.

Interaction of mouse serum with GB on protected sections of MAC16 tumour

Protected sections are frozen sections in which the GB is still active on the cell surface but from which all the cytoplasmic soluble protein inhibitors have previously been removed.[14] Such *protected* sections were treated with serum (10–20 μl) for 1 h prior to 9AA staining as above.

Removal of cell surface GB by exposure to fibrin

Two series of experiments were carried out with fibrin fibrils overlaid on: (a) frozen sections of MAC16 tumour with active GB and, (b) frozen sections of MAC16 tumour in which the GB was fully inhibited by prior exposure of the section to 10 μl isotonic saline for 1 h. The technique and mechanisms involved in fibrin binding of GB and GB-Inhibitor complex (GB-I) have been fully described elsewhere.[15]

RESULTS

Mouse serum enzyme (MSE)

MSE in serum from NMRI mice was capable of cleavage of 4-methylumbelliferyl-*p*-guanidinobenzoate (MUGB) with consequent release of methylumbelliferone detected by its fluorescence (Figure 1A). Fluorescence intensity increased with increasing incubation time, with a linear time course over the first 3 min and was proportional to the volume of serum in the assay over the range 0 to 5 μl (Figure 1D). The serum enzyme level was similar in animals bearing the MAC16 tumour

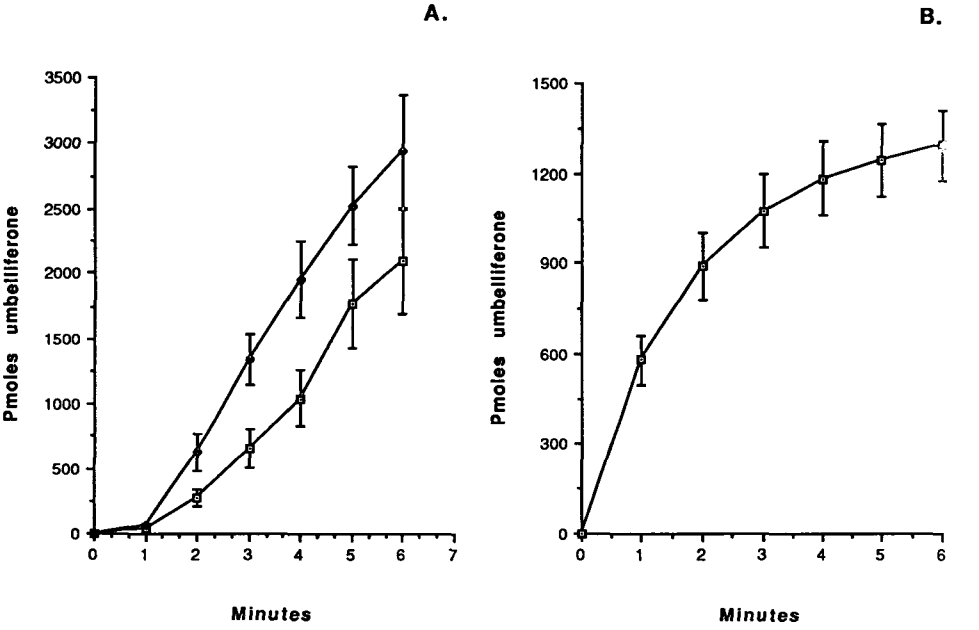

FIGURE 1 Rate of release of methylumbelliferone from 4-methylumbelliferyl-*p*-guanidinobenzoate by 5 μl of serum from a non-tumour-bearing mouse in the absence (□) or presence (●) of soya bean trypsin inhibitor (1 mg ml^{-1} in water) (A) or from a mouse bearing the MAC16 tumour, but without weight loss (B) or from a mouse bearing the MAC16 tumour with a weight loss of 2.4 g (C). The relationship between the release of methylumbelliferone by different concentrations of serum of a non-tumour-bearing mouse is shown in (D).

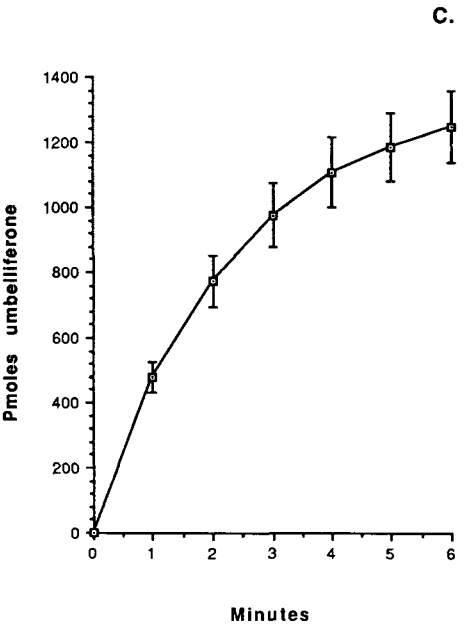

FIGURE 1(C)

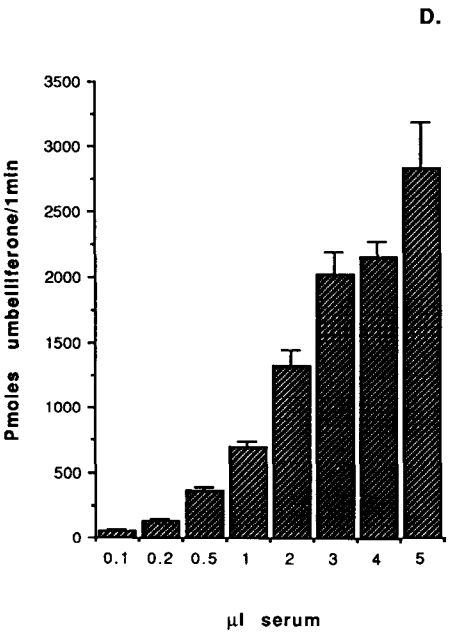

FIGURE 1(D)

and in control non-tumour-bearing animals (Figures 1B and 1C) and was not affected by weight loss (Figure 2). The effect of the addition of trypsin inhibitor is shown in Figure 1A. It was considered that some activity could be attributed to trypsin or trypsin-like enzymes, but the trypsin inhibitor seemed to increase rather than decrease methylumbelliferone formation.

The effect of PUFAs on MSE is shown in Figure 3. All of the PUFAs inhibited MSE at μM concentrations, which were in the same range as that found for bis(carbobenzoxycarbonyl-L-argininamido)-rhodamine (BZAR), a known inhibitor of mouse Ehrlich ascites GB.[16] EPA was a significantly more potent inhibitor of MSE than the other fatty acids. The inhibition of MSE by EPA and BZAR is presented in the form of Lineweaver-Burk plot in Figure 4. Both BZAR and EPA were non-competitive inhibitors of the enzyme with K_i values of 0.29 ± 0.02 μM (mean $\pm$ SEM) and 0.98 ± 0.07 μM, respectively. While BZAR was an effective inhibitor of t-PA at concentrations greater than 10^{-7} M (Figure 5A) no inhibition of t-PA was observed with EPA at concentrations up to 12.8 μM (Figure 5C). Since t-PA is inhibited by ethanol, EPA was dissolved in 0.1 M sodium carbonate for this experiment. EPA dissolved in this solvent was an effective inhibitor of MSE activity (Figure 5B). Since MSE is inhibited by EPA, this suggests that MSE is distinct from tPA.

Interaction of EPA with MAC16 tumour cells in wax embedded sections

In view of the similarity between MSE and GB the effect of EPA on cell surface GB was determined. Formaldehyde fixed wax embedded sections, after dewaxing, were

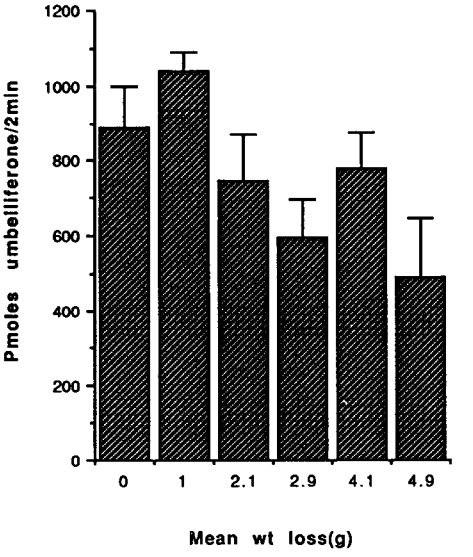

FIGURE 2 Extent of release of methylumbelliferone from 4-methylumbelliferyl-*p*-guanidinobenzoate by serum from male NMRI mice bearing the MAC16 tumour and various extents of weight loss.

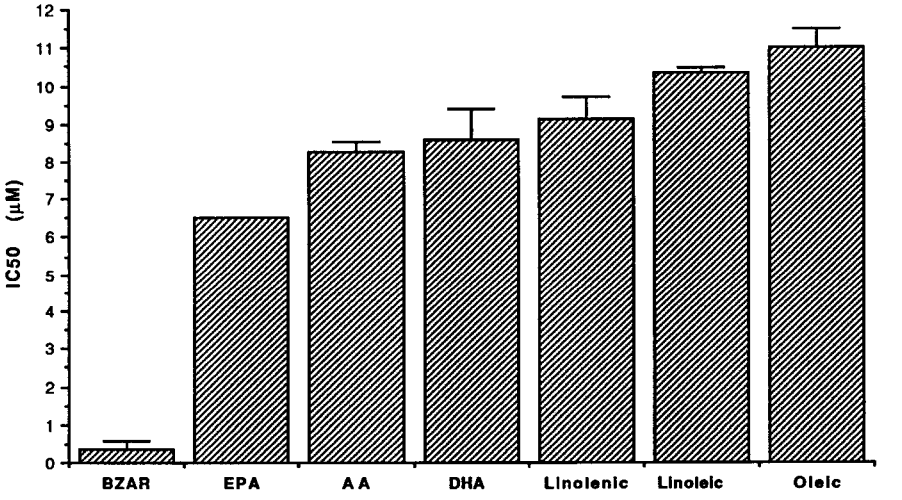

FIGURE 3 Concentrations of BZAR and PUFAs giving 50% inhibition of MSE in the serum of non tumour-bearing animals. The PUFAs were dissolved in ethanol while BZAR was dissolved in 1 mM aspartic acid. The solvents alone gave no inhibition. Results are presented as means ± SEM. The error bars for EPA are too small to be shown. AA = arachidonic acid, DHA = docosahexaenoic acid.

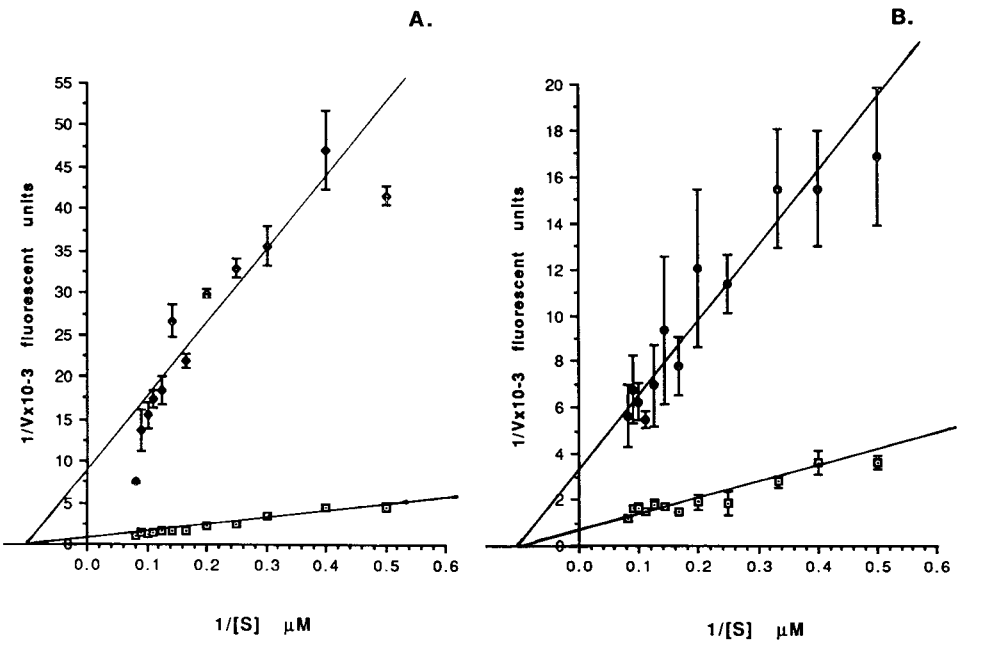

FIGURE 4 Lineweaver-Burk plots for the release of methylumbelliferone from 4-methylymbelliferyl-*p*-guanidinobenzoate in the absence (□) or presence (●) of either BZAR (A) or EPA (B) (both 3.2 μM). Results are presented as means ± SEM.

stained with 9AA. The cell surfaces of the MAC16 cells bound 9AA and fluoresced yellow (Figure 6A), indicating the presence of active GB on these cells. Pretreatment of the sections with EPA (3.7×10^{-4} M) completely blocked the binding of 9AA to the GB on the surface of these tumour cells (Figure 6B). The interaction of EPA with the cell surface GB was demonstrated to be reversible, since the EPA could be washed out of the cell surface GB by placing the slide in a tank of 9AA (300 ml, 10^{-3} M for 1 h). In this case, the concentration of 9AA was maintained whilst the concentration of EPA in the 300 ml of washing fluid was diluted sufficiently to be considered minimal (Figure 6C).

Action of mouse serum on frozen sections of MAC16 tumour

Treatment of *protected* frozen sections of MAC16 mouse tumour with mouse serum for 1 h resulted in the inhibition of the cell surface GB as judged by the inability of the cells to bind 9AA (data similar to Figure 6B).

Removal of cell surface GB from MAC16 tumour cells in frozen sections by fibrin

Both surface bound GB and the GB-I complex were removed from MAC16 tumour cells by exposure to fibrin films with the result that the MAC16 cells failed to bind 9AA and did not fluoresce yellow, even after formaldehyde treatment (data similar to Figure 6B). In the case of GB-I, the released inhibitor was shown to be transferable and capable of inhibiting a second *protected* section of the tumour.

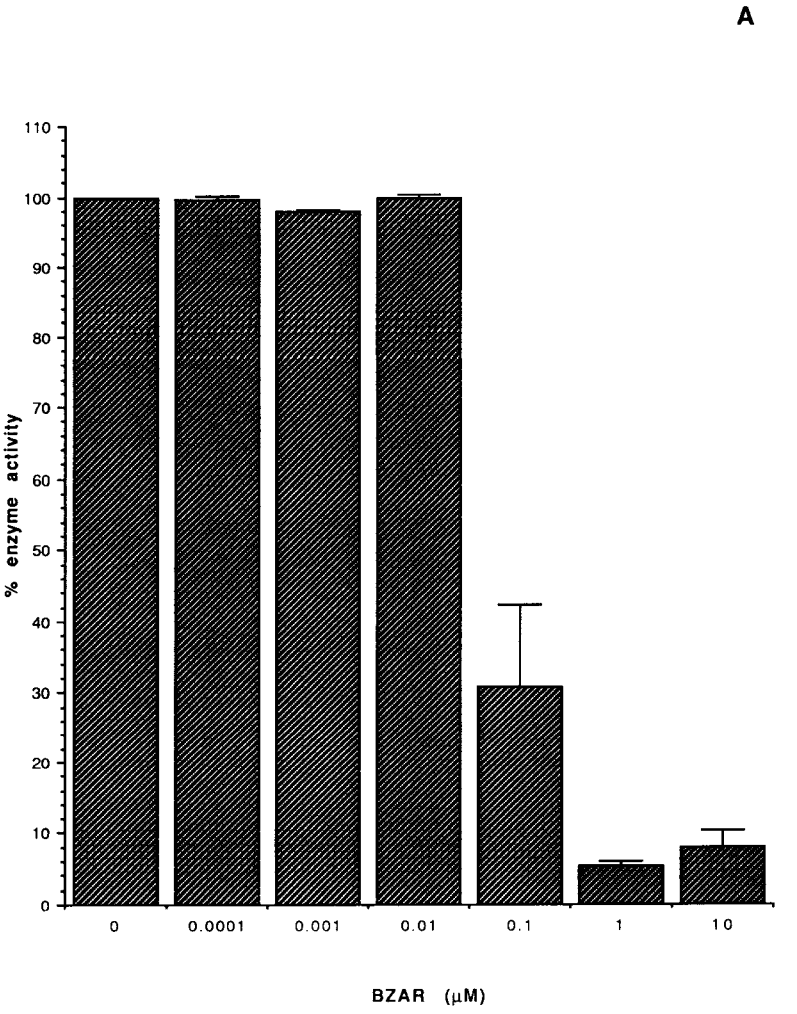

FIGURE 5 Effect of BZAR on t-PA activity (A) and the effect of EPA dissolved in 0.1 M Na_2CO_3 on the activity of MSE (B) and t-PA activity (C). Results are presented as means ± SEM.

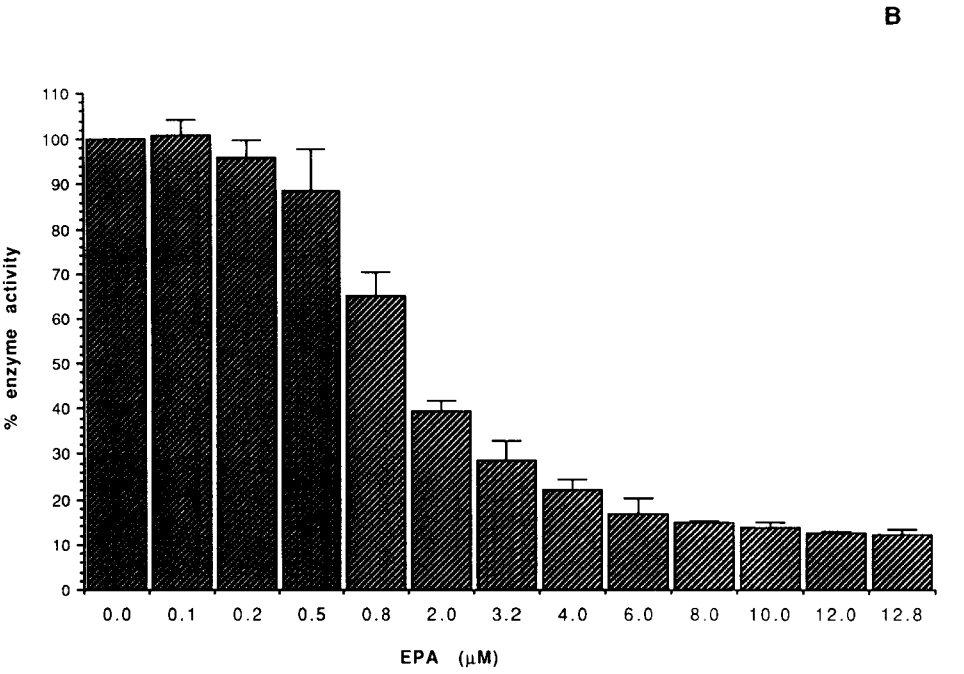

FIGURE 5(B)

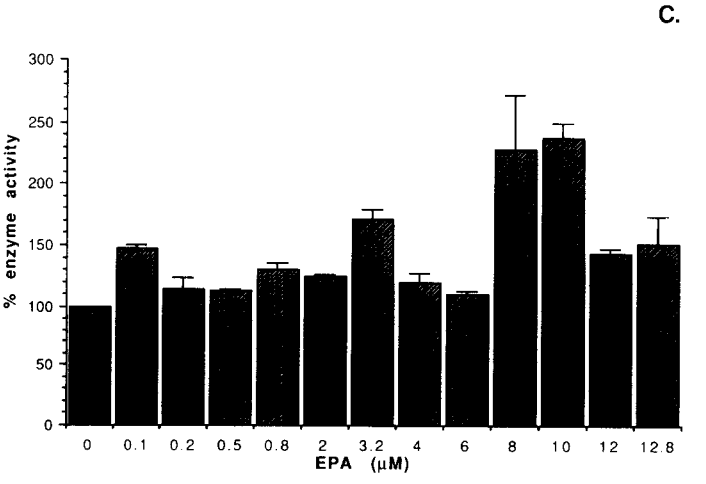

FIGURE 5(C)

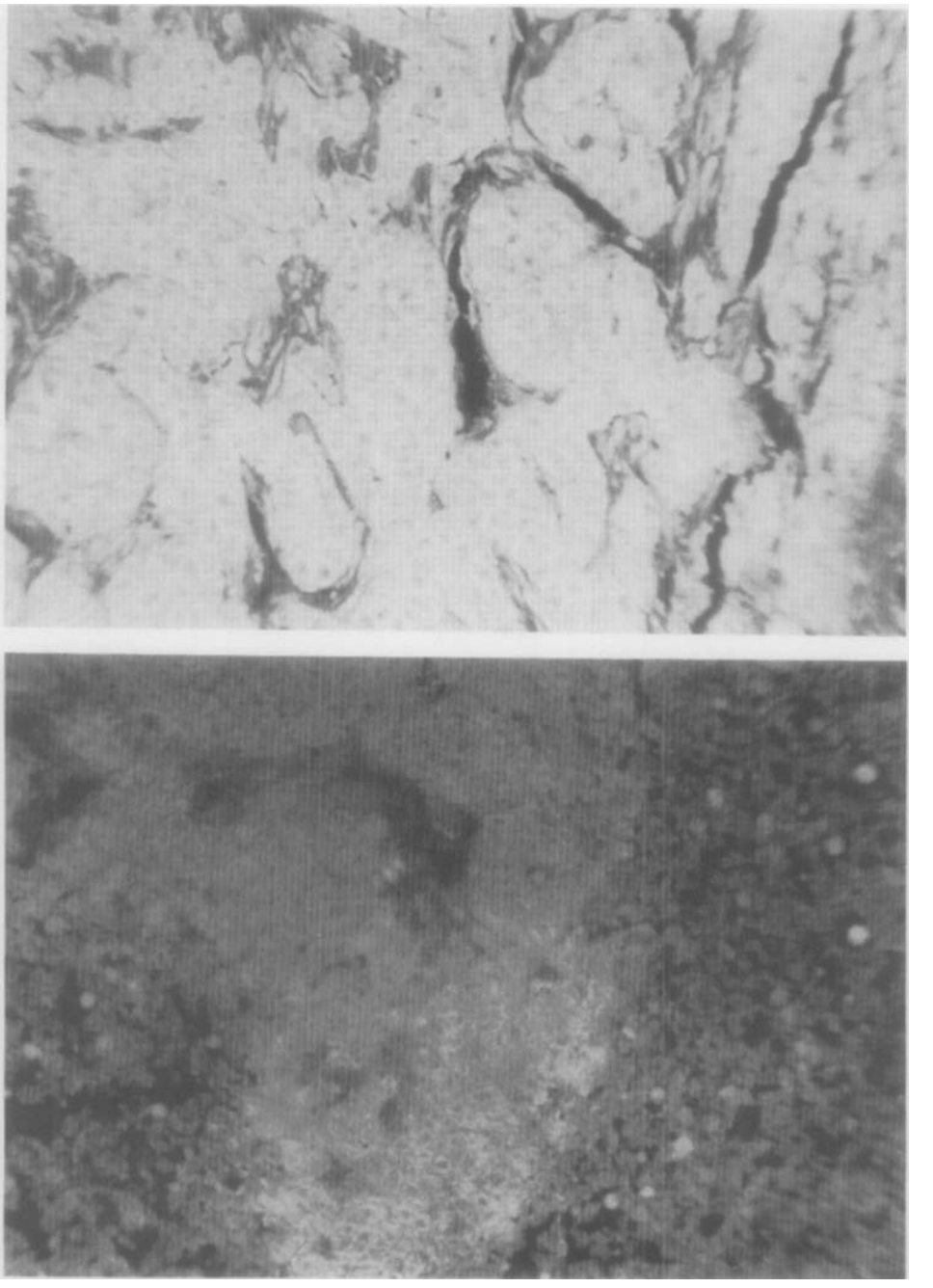

FIGURE 6 A. 9-Aminoacridine staining of a section of the MAC16 tumour. B. With EPA pretreatment for 30 min followed by 9-aminoacridine staining for 2 min. C. With EPA pretreatment for 30 min followed by 1 h in 9-aminoacridine in the absence of EPA.

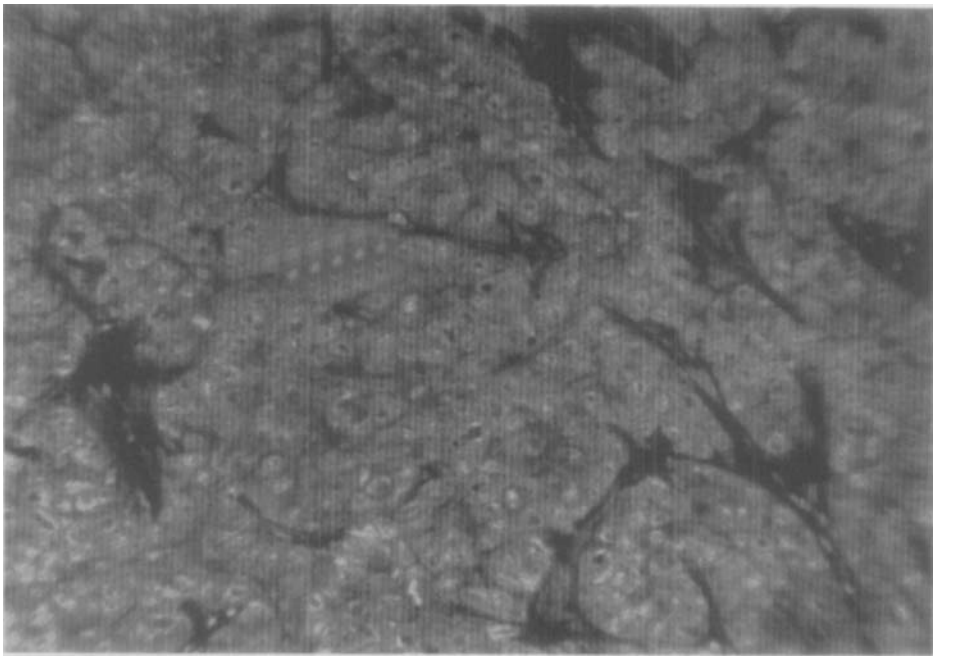

FIGURE 6(C)

DISCUSSION

The mouse serum enzyme (MSE)

This study shows that there is no correlation between the tumour status of the mouse and the MSE activity assayed with 4-methylumbelliferyl-*p*-guanidinobenzoate as substrate. Since MSE activity was not related to tumour status, it is unlikely to be related to the phenomenon of cancer cachexia. The ability of EPA to inhibit MSE (Figure 5) but not other enzymes is of interest; the mechanism of such an observed inhibition is not known. The evidence suggests that MSE differs from the serum enzyme reported as being associated with the plasma of animals bearing MAC16 tumours and exhibiting weight loss,[1] since in that study no activity was measured in the plasma of non-tumour bearing animals.

The presence of an enzyme in the serum capable of degrading 4-methylumbelliferyl-*p*-guanidinobenzoate as a true substrate is difficult to understand. If the enzyme is a protease, it seems unlikely to be active in the presence of the range of protease inhibitors present in normal serum, although Beck and Tisdale[1] have reported an elevated level of proteolytic activity in the serum of animals bearing the MAC16 tumour, which was not completely inhibited by trypsin inhibitor. Two observations indicate that MSE cannot be the same as the cell surface GB present on mouse MAC16 cells. The MAC16 cell surface GB was inhibited by exposure to mouse serum for 1 hour; clearly in contrast to MSE which is active in mouse serum. Secondly, the MAC16 GB was transferred to and bound by fibrin, with release of inhibitor

from GB; yet the moust enzyme MSE was prepared initially from fluid containing a mixture of fibrin and serum.

We therefore believe that MSE and GB of the tumour cell surface are distinct enzymes; with some common properties such as their ability to cleave guanidinobenzoates and to be inhibited by BZAR and EPA. MSE is unlikely to be trypsin, an esterase or t-PA, since these enzymes are not inhibited by EPA. The nature of MSE remains unknown, but in some respects it is more closely related to surface GB than to other enzyme activities.

In view of the above comments and the presence of MSE in normal mouse serum we believe that MSE plays no significant role in cancer cachexia in MAC16 tumour-bearing mice.

MAC16 tumour cell surface GB

The MAC16 tumour cells are directly associated with the state of cancer cahcexia in these mice. It is therefore of considerable interest that EPA causes both protection against cancer cachexia[2,3] and has an inhibitory effect on the tumour cell surface GB (Figure 6), when tested *in vitro* with concentrations similar to those which were effective in causing a reduction in protein loss in these mice. Since single tumour cells capable of metastasis possess uninhibited guanidinobenzoatase,[16] inhibitors of this enzyme might also be expected to have an inhibitory effect on the metastatic process. Indeed culturing murine melanoma cells with pure EPA in ethanol has been shown to cause a dose and time-dependent decrease in invasiveness, collagenase IV production and a reduced ability to metastasize to the lung after i.v. injection.[17] The antimetastatic activity of BZAR also seems worthy of investigation. Cell surface GB has been shown to be functionally similar to tissue plasminogen activator and recognised by antibodies directed against t-PA.[6] The observations that cell surface GB on MAC16 tumour cells is inhibited by EPA (Figure 6B), whilst t-PA in true solution is not inhibited (Figure 5C) is difficult to explain. The mechanism of action of EPA is unknown, but it is likely to be due to the induction of a conformational change in the membrane bound GB leading to the loss of the ability to bind 9AA and therefore loss of enzyme function. This effect appears to be specific for this type of enzyme, since other proteolytic enzymes, trypsin, esterase and t-PA were unaffected suggesting that the effect does not arise solely from the detergent properties of the PUFA.

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