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INHIBITION OF GUANIDINOBENZOATASE: EVIDENCE FOR MULTIPLE FORMS OF THIS PROTEASE ON DIFFERENT TUMOUR CELLS

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Guanidinobenzoatase is a proteolytic enzyme capable of degrading fibronectin and is a tumour associated enzyme. 9-Aminoacridine is a competitive inhibitor of this enzyme and has been used to locate cells possessing this enzyme in wax embedded sections by means of fluorescent microscopy. Naturally occuring inhibitors of guanidinobenzoatase can be extracted from different tissues. These inhibitors show selectivity in their ability to inhibit the binding of 9-aminoacridine to different types of tumour cells which have invaded human liver tissue. Inhibition is non-competitive and reversible. The results indicate that guanidinobenzoatase exists in a number of different forms on the surface of different tumour cells. These different forms of the enzyme were recognised by inhibitors obtained from different organs. It is suggested that these inhibitors may have a regulatory role in tumour cell migration.

KEY WORDS: Guanidinobenzoatase, non-competitive inhibition, tumour protease, iso-enzymes.

INTRODUCTION

We have used fluorescent microscopy to examine fixed sections of tumour tissues employing a fluorescent competitive inhibitor of a cell surface protease. These sections act as test systems for determining whether an inhibitor of this protease is already present as a enzyme-inhibitor complex on the tumour cell surface. The protease, referred to as guanidinobenzoatase,[1] is a trypsin-like protease associated with cells capable of migration and in particular tumour cells.[2,3] The present study is concerned with the inhibition of guanidinobenzoatase on the surface of secondary tumour cells in wax embedded sections of human liver. We have used water soluble extracts obtained from human liver, lung, heart and brain tissue to provide crude protein inhibitors of the tumour cell associated guanidinobenzoatase. These inhibitors have been shown to react at sites distant from the active centre of the enzyme, resulting in inhibition and subsequent failure to bind 9-aminoacridine to the surface of the tumour cells. These studies indicate that the tumour cell surface guanidinobenzoatase exists in multiple forms which may be differentiated by their reactions with different tissue inhibitors of guanidinobenzoatase.

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MATERIALS AND METHODS

4-Methylumbelliferyl-*p*-guanidinobenzoate and 9-aminoacridine were purchased from Sigma Chemical Company, St. Louis, Mo. USA. Homogenates of human lung, liver, brain and heart were prepared in isotonic saline using an Ultra-Turrax homogeniser and collecting the soluble extracts by centrifugation. The tissue extracts were diluted with isotonic saline to provide a final solution containing 1 mg protein/ml and frozen for future use. Wax embedded formaldehyde fixed sections of liver and lung were obtained from the routine pathology laboratory.

Kinetic Experiments

We used mouse Ehrlich ascites tumour fluid as a source of guanidinobenzoatase in true solution.[1,2] The tumour-bearing mice were kindly provided by Dr. S. Itzhaki of

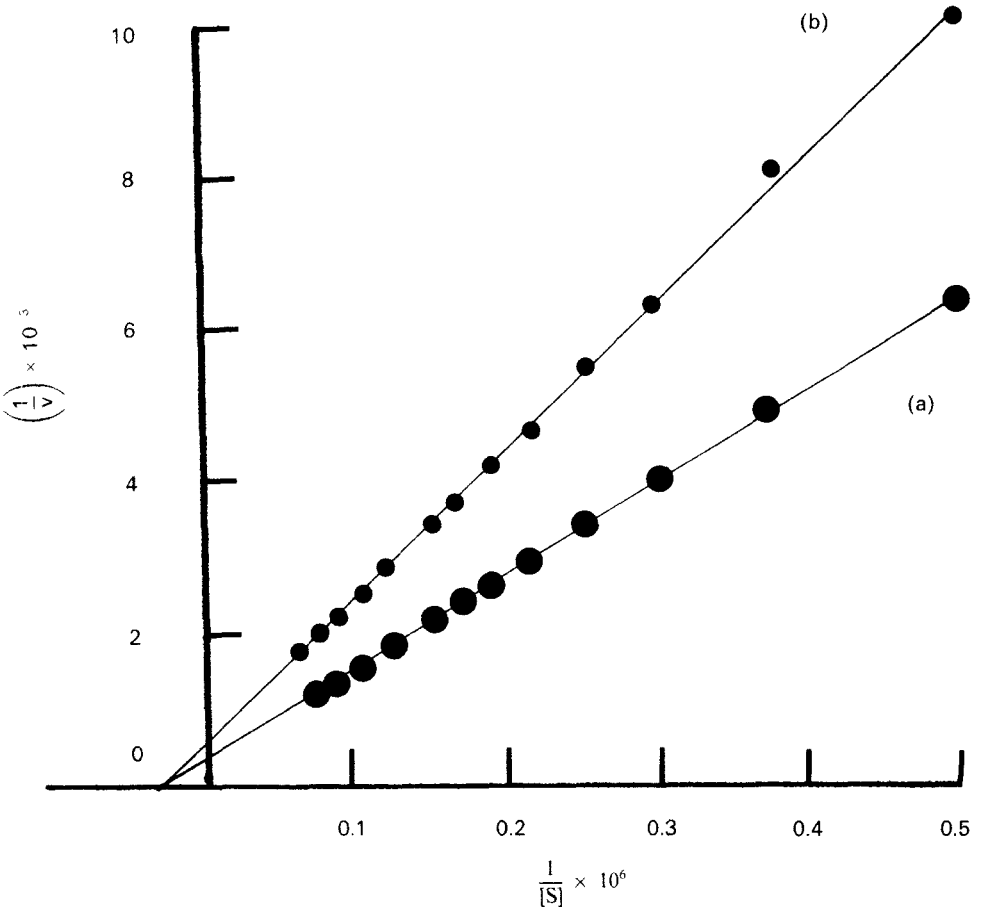

FIGURE 1 Non-competitive inhibition of mouse guanidinobenzoatase, assayed with incremental additions of 4-methylumbelliferyl-*p*-guanidinobenzoate (a) in the absence of inhibitor and (b) in the presence of inhibitor (100 μl lung extract). V represents product formation in terms of change in fluorescence.

the Department of Biochemistry and Molecular Biology. Direct inhibition of guanidinobenzoatase with liver, lung, heart brain extracts employed 4-methylumbelliferyl-*p*-guanidinobenzoate as substrate.[1-2-3] The type of inhibition was defined by double reciprocal plots (as shown in Figure 1).

Location of Cells Containing Guanidinobenzoatase

Wax embedded sections were first dewaxed by passage through tanks containing xylene, cyclohexanone and ethanol. Direct staining of the dewaxed sections was carried out as previously described[2] and is briefly outlined below. The slides were placed in an aqueous solution of 9-aminoacridine (10^{-3}M) for 2 min, drained and washed for 2 min in each of three tanks containing isotonic saline. The sections were mounted with a glass cover slip over a water film and examined by fluorescent microscopy.[2,3] Cells which lacked guanidinobenzoatase activity failed to bind 9-aminoacridine (a competitive inhibitor) and appeared blue-green.[2] Those cells possessing guanidinobenzoatase activity bound this fluorescent inhibitor and appeared yellow.[2,3] Tumour cells are known to possess this enzyme[2,3] and they can be detected by this fluorescent staining technique.[2,4] The background of connective tissue and intercellular matrix appeared blue under these fluorescent microscopic conditions.

The tissue extracts were used in inhibition experiments in the following manner. A drop (*ca* 200 μl) of the tissue extract was spread over the surface of the tumour bearing section and allowed to equilibrate for 18 h in a wet box. Excess tissue extract was

Formaldehyde Fixed Section. (E)

↓

Stain with 9-aminoacridine

↓

Tumour cells fluoresce (yellow) (E)

↓

Add inhibitor (I) of guandinobenzoatase and equilibrate

↓

Stain with 9-aminoacridine

↓

Tumour cells fail to stain (blue)
due to enzyme-inhibitor complex. (E-I)

↓

Displace inhibitor with formaldehyde

↓

(E) + (I) lost in wash

↓

Stain with 9-aminoacridine

↓

Tumour cells fluoresce (yellow). (E)

SCHEME 1

washed off the surface of the tissue and the section stained with 9-aminoacridine as described above. Tumour cells which bound inhibitor to their cell surface guanidinobenzoatase failed to bind 9-aminoacridine and appeared blue-green rather than yellow. These inhibited cells could subsequently be shown to regain their ability to bind the fluorescent probe after the inhibitor had been displaced from the enzyme-inhibitor complex by treatment with 4% formaldehyde.[5] This procedure is outlined in the flow diagram of Scheme 1.

Techniques for demonstrating the presence of inhibitor (I) on tumour cell surface guanidinobenzoatase using a fluorescent probe (9-aminoacridine) for the active centre of the protease. E represents active enzyme, I, inhibitor and E-I enzyme inhibitor complex.

RESULTS AND DISCUSSION

Kinetic Analysis

Extracts from human lung, liver, heart and brain inhibited the cleavage of guanidinobenzoate from the synthetic substrate 4-methylumbelliferyl-*p*-guanidinobenzoate[1] by the crude enzyme present in mouse Ehrlich ascites plasma. Double reciprocal plots indicated that the inhibition was non-competitive and was caused by the binding of the inhibitor to a site distant from the active centre but which modified the function of the active centre. This type of inhibition is shown in Figure 1, employing the lung extract. Similar results have been reported for a liver inhibitor of guanidinobenzoatase; this inhibitor was a protein having molecular weight[5] 67000.

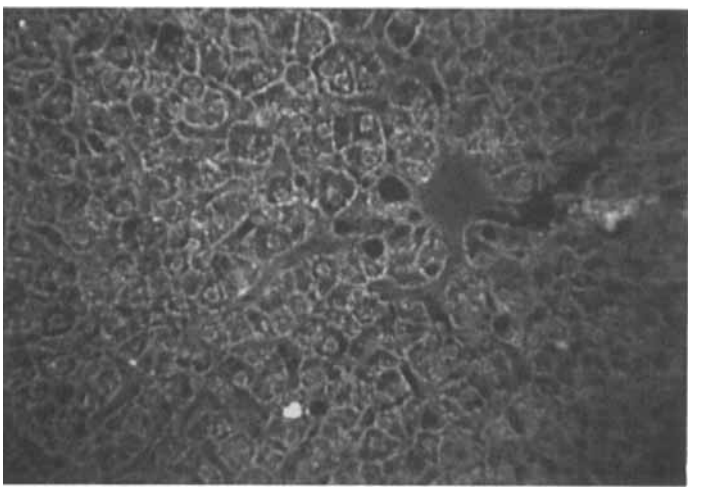

FIGURE 2 Hepatocytes in a normal liver biopsy. Magnification × 250. (See Colour Plate I).

FIGURE 2–10 Fluorescent micrographs of formaldehyde fixed wax embedded human tissue sections stained with 9-aminoacridine and photographed through yellow interference filter. Cells possessing active guanidinobenzoatase have a yellow fluorescence (see Colour Plates I–IX at back of issue).

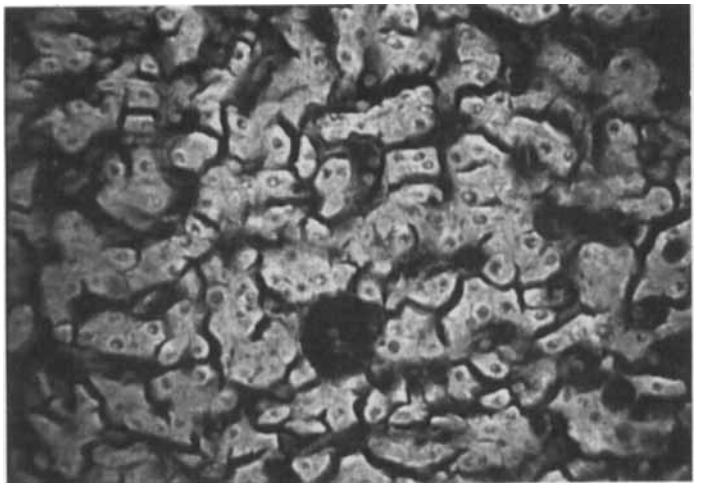

FIGURE 3 Hepatocytes in a liver invaded by tumour cells. Magnification × 250. (See Colour Plate II).

Fluorescent Microscopy of Normal Liver

Biopsies of normal liver when stained with 9-aminoacridine, exhibited very weak staining of the hepatocytes. This staining was associated with cytoplasmic granules which gave the stained hepatocytes a stippled appearance. We could only record this stippled effect on ASA 400 Kodak colour film by employing a yellow interference filter (no. K 490) in our Leitz orthoplan fluorescent microscope (Figure 2). The staining of these cytoplasmic granules may be due to the binding of 9-aminoacridine to cytoplasmic RNA[6] which is particularly concentrated in hepatocytes. In the absence of this interference filter the hepatocytes would appear blue on the colour prints.

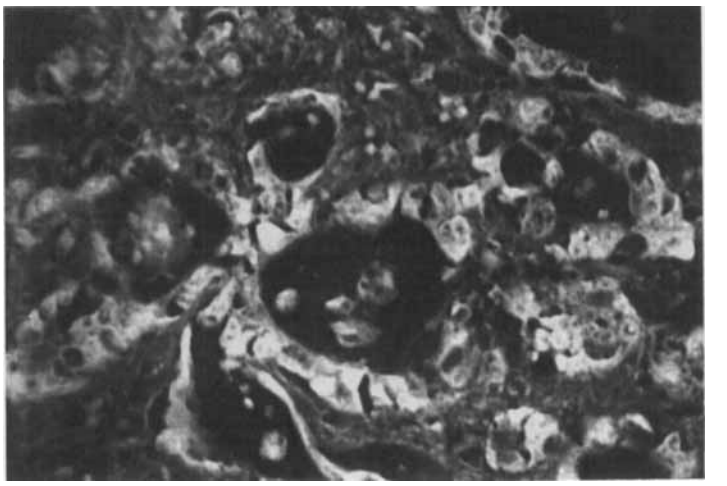

FIGURE 4 Liver invaded by carcinoma of the gall bladder. Magnification × 250. (See Colour Plate III).

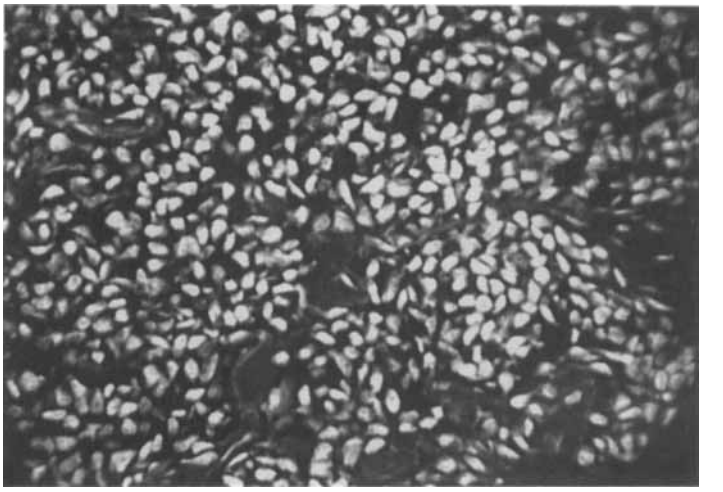

FIGURE 5 Liver invaded by carcinoma of the bladder. Magnification × 250. (See Colour Plate IV).

Fluorescent Microscopy of Secondary Tumours in Liver

The staining of hepatocytes in fixed liver sections bearing secondary tumours was much more intense (Figure 3) than the weak stippling seen in normal liver biopsies (Figure 2). In the tumour-bearing livers the hepatocytes exhibited intense staining throughout the cell; it was impossible to decide whether the binding of 9-aminoacridine was confined to the cell surface or to the cytoplasm. It would appear that the staining properties of the hepatocytes have been remarkably changed, possibly as a consequence of tumour cell invasion. We observed that Kupfer cells in these livers were not able to bind 9-aminoacridine but that infiltrating lymphocytes were able to bind 9-aminoacridine.

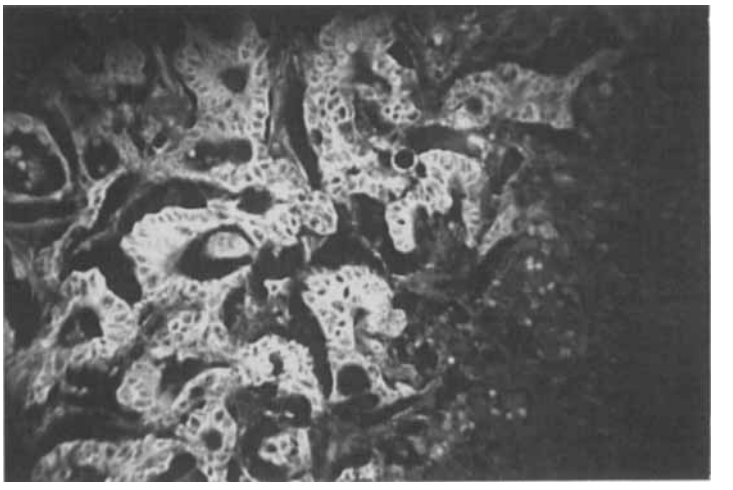

FIGURE 6 Liver invaded by carcinoma of the rectum. Magnification × 160. (See Colour Plate V).

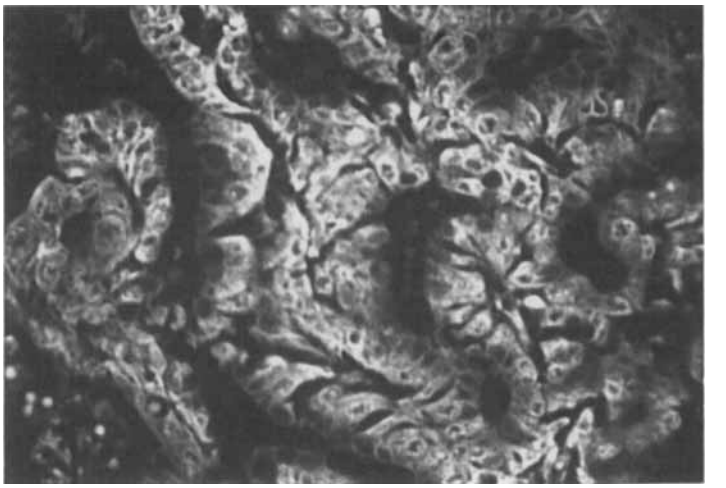

FIGURE 7 Liver invaded by carcinoma of the pancreas. Magnification × 250. (See Colour Plate VI).

Secondary tumour cells were strongly stained in these livers and could be recognised from the hepatocytes and infiltrating lymphocytes by their organisation and morphology (Figures 4–8). The characteristics of these secondary tumour cells are presented in the legends for each of these figures. In the present study, we are just employing these sections to provide different tumour cell types; each of which carries cell surface guanidinobenzoatase in an active form for binding 9-aminoacridine.

The tumour cells of the non-Hodgkins lymphoma, although clearly stained with 9-aminoacridine, were less intensively stained than the other four types of tumour (Table I) in the wax embedded sections in liver. After treatment of the sections with formaldehyde, to displace inhibitors,[5] intense staining of the non-Hodgkins lym-

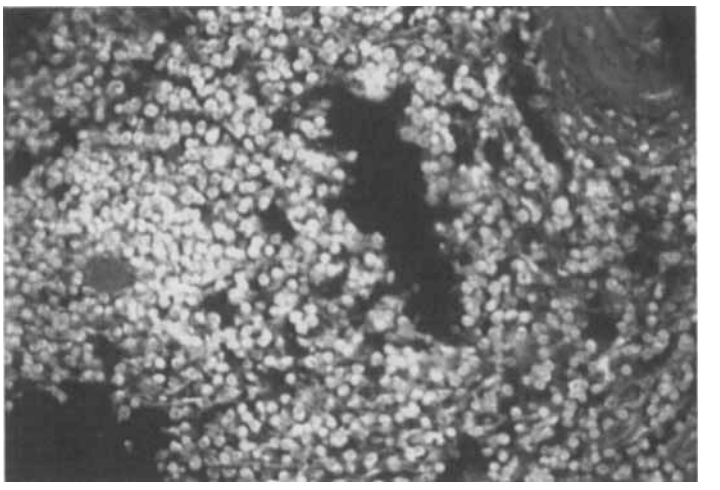

FIGURE 8 Liver invaded by non-Hodgkins lymphoma after treatment of the section in formaldehyde. Magnification × 250. (See Colour Plate VII).

TABLE I
Inhibition of tumour cell surface guanidinobenzoatase by tissue extracts as defined by the enzyme's ability to bind 9-aminoacridine

Liver Secondary Tumour	9-aminoacridine Direct Stain	Extract applied to liver sections			
		Lung	Liver	Heart	Brain
No. 1 Carcinoma of gall bladder	Fig. 4 (Positive)	Inhibition (Negative)	Inhibition (Negative)	No effect (Positive)	No effect (Positive)
No. 2 Carcinoma of bladder	Fig. 5 (Positive)	Inhibition (Negative)	No effect (Positive)	Inhibition (Negative)	No effect (Positive)
No. 3 Carcinoma of rectum	Fig. 6 (Positive)	Inhibition (Negative)	No effect (Positive)	No effect (Positive)	No effect (Positive)
No. 4 Carcinoma of pancreas	Fig. 7 (Positive)	Inhibition (Negative)	No effect (Positive)	No effect (Positive)	No effect (Positive)
No. 5 Non-Hodgkins lymphoma	Fig. 8 (Positive)	Inhibition (Negative)	Inhibition (Negative)	No effect (Positive)	No effect (Positive)

Binding of 9-aminoacridine to the cell surface guanidinobenzoatase resulted in intense yellow fluorescence (Figure 4–10) and is indicated above as (Positive). Reversible inhibition by a tissue extract resulted in failure of the cell surface to bind 9-aminoacridine and is indicated above as (Negative).

phoma cells was observed. This observation implied that an inhibitor was firmly attached to most of the surface enzyme molecules and that this inhibitor was probably derived from the liver tissue. Extracts of normal liver were later shown to be able to inhibit guanidinobenzoatase on the non-Hodgkins lymphoma cells (Table I).

Fluorescent Microscopy of Enzyme-inhibitor Complex Formation on Tumour Cell Surfaces.

We used five different secondary tumours in human liver sections and attempted to inhibit the cell surface guanidinobenzoatase with inhibitors present in the extracts of lung, liver, brain and heart. We first of all demonstrated that the tumour cells contained guanindinobenzoatase capable of binding 9-aminoacridine (see Figures 4–8). We then treated these sections with the extracts (Scheme 1) and restained with 9-aminoacridine to determine whether or not the tumour cells now bound this fluorescent probe. Failure to bind the probe indicated that the active centre had been modified by the binding of an inhibitor at a distant site. Inhibition was confirmed by displacing the previously bound inhibitor with formaldehyde,[5] and subsequently demonstrating the cells' ability to bind 9-aminoacridine again. Inhibition could be carried out equally well on fresh sections and those which had previously been stained with 9-aminoacridine in an earlier experiment.

Rather than present colour prints which failed to show fluorescent yellow tumour cells, the results of this study are presented in the form of a table, indicating which extracts had an inhibitory action on the guanidinobenzoatase of each type of tumour cell. Colour prints are presented in references 5 and 6, showing typical 9-amino-acridine staining of cells possessing active enzyme and inhibited enzyme.

The lung extract was the most successful inhibitor of the tumour cell surface guanidinobenzoatase (Table I), since it prevented the approach of 9-aminoacridine to

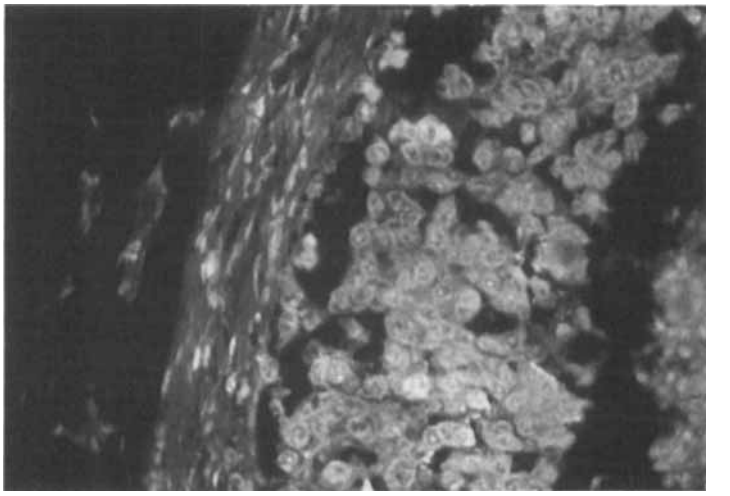

FIGURE 9 Lung tumour in asbestos worker. Magnification × 250. (See Colour Plate VIII).

the active centre of the enzyme on all five secondary tumours. The lung contains the general protease inhibitor aprotinin, ("trasylol", Bayer & Co, Germany). The evidence presented in Table I might suggest that aprotinin was the effective inhibitor against guanindinobenzoatase extracted from the lung. Independent studies with aprotinin demonstrated that this protease inhibitor had no action against guanidinobenzoatase in solution or on the surface of the secondary tumour cell in liver tissue studied in this project using the technique which we have described above. We therefore concluded that the lung extract contained an inhibitor (or inhibitors) distinct from aprotinin which blocked the approach of 9-aminoacridine to the active centre of guanidinobenzoatase on the surface of the secondary tumour cells in the liver sections. On the other hand, brain extract had no inhibitory action on this

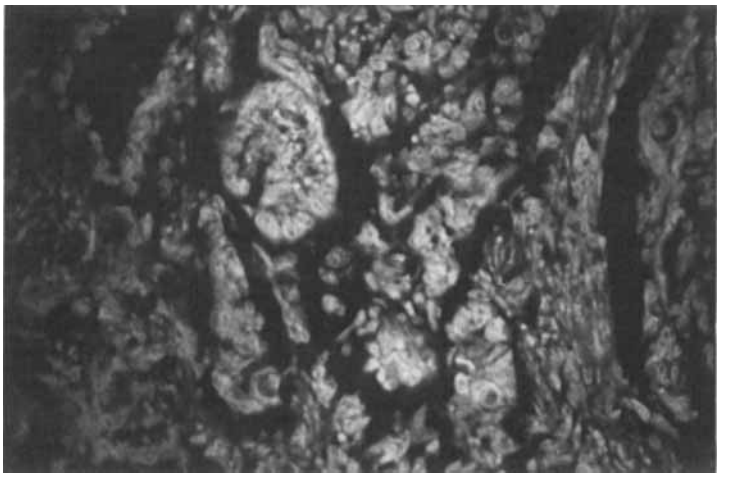

FIGURE 10 Lung tumour after treatment with human lung extract. Magnification × 160. (See Colour Plate IX).

enzyme on these tumour cells. The heart extract inhibited guanidinobenzoatase on a bladder tumour invading the liver (Figure 5) whilst the liver extract had no inhibitory action on this enzyme but was inhibitory to the enzyme associated with a carcinoma of the gall bladder (Figure 4) and non-Hodgkins lymphoma (Figure 8).

It could be argued that these tissue extracts caused destruction of guanindinobenzoatase rather than an inhibition of this enzyme. In each case, however, we were able to regain the ability to bind 9-aminoacridine to the tumour cell surface after displacing the inhibitor with formaldehyde and subsequently restaining with 9-aminoacridine. Clearly, the cell surface enzyme had been reversibly inhibited rather than irreversibly destroyed by these tissue extracts. This conclusion could be confirmed by adding further inhibitor in the form of the tissue extract and repeating this cycle of exchange reactions.

The results presented above demonstrate that the four tissue extracts all possessed the ability to inhibit guanidinobenzoatase in solution yet these extracts show some degree of selectivity in their ability to inhibit guanidinobenzoatase on the surface of tumour cells in wax embedded sections of liver. All five types of tumour present in these liver sections were able to bind 9-aminoacridine to the active centre of guanidinobenzoatase on their cell surfaces with consequent fluorescence of the tumour cells. It is suggested that multiple forms of the protease guanidinobenzoatase exist, each form having a common binding site for 9-aminoacridine and also possessing tertiary structural differences within the membrane-bound enzyme at a site distant from the active centre. This postulate would account for the selective blocking of 9-aminoacridine binding on some secondary tumour cells but no inhibition of 9-aminoacridine binding by other types of tumour cells described above, when exposed to tissue extracts. It could be argued that the lung extract contained an inhibitor (or inhibitors) which were capable of inhibiting guanidinobenzoatase on the surface of all tumour cells. We examined this possibility by exposing a section of human primary lung tumour (obtained from an asbestos worker) to the lung extract. The lung extract had no ability to inhibit the binding of 9-aminoacridine to the surface of the lung tumour cells (Figures 9, 10). It is also worth mentioning that the lung extract was able to inhibit guanidinobenzoatase on the surface of primary tumour cells of the colon, rectum and pancreas.

We conclude that the cell surface guanidinobenzoatase associated with tumour cells in liver tissue exists in a number of distinct forms. These structural differences probably occur at sites distant from the active centre as shown by kinetic analysis with a soluble form of guanidinobenzoatase. These studies are similar to those used to define some of the structural differences observed with alkaline phosphatase isoenzymes.[7] These multiple forms of the enzyme guanidinobenzoatase can be inhibited by tissue extracts which must also contain a variety of inhibitors capable of inhibiting different forms of guanidinobenzoatase. Inhibition must be dependent upon the affinity of part of the inhibitor for a distinct site or locus on the cell surface enzyme in much the same manner as an antibody recognises a specific antigenic determinant group. The biological significance of multiple forms of guanidinobenzoatase and corresponding multiple forms of inhibitors in different tissues may be concerned with the regulation of cell migration. This could become particularly significant in the case of tumour cells with known poetential for metastasis of certain target tissues.

Acknowledgements

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