J. Enzyme Inhibition, 1989, Vol. 2, pp. 295-303
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INHIBITION OF TRYPSIN-LIKE ENZYMES ON CELLS WITH RHODAMINE-APROTININ

F.S. STEVEN, M.M. GRIFFIN and L.A. WILLIAMS,

Department of Biochemistry and Molecular Biology, School of Biological Sciences, University of Manchester, Manchester M13 9PT. U.K.

A.J. FREEMONT,

Department of Pathology, University of Manchester, Manchester. M13 9PT, U.K.

H. MAIER and H. WEIDAUER,

E.N.T. Clinic, University of Heidelberg, 6900 Heidelberg, West Germany

(Received 4 May 1988)

Aprotinin, a polypeptide inhibitor of trypsin-like enzymes, has been labelled with rhodamine. Rhodamine-aprotinin inhibits trypsin in free solution in an identical manner to aprotinin. Rhodamine-aprotinin binds to trypsin-like enzymes on cells in formaldehyde fixed wax embedded sections. This technique has been used to locate cells possessing trypsin-like enzymes by means of fluorescent microscopy. In the present study we have used this technique to locate tumour cells.

KEY WORDS: Aprotinin, trypsin-like enzymes, cell surfaces, fluorescent location, inhibition.

INTRODUCTION

Aprotinin is a peptide inhibitor of trypsin-like enzymes.[1] The molecule exists as a dimer in solution with a molecular weight of approximately 11,500 and is rich in lysine residues.[1] Aprotinin would therefore seem to be easily convertible to fluorescent derivatives which could have a number of useful properties. It might be possible to use fluorescent aprotinin to locate cells possessing trypsin-like enzymes in tissue sections, provided that these enzymes are not already inhibited and can be approached by the fluorescent peptide.[2] Tumour cells are known to possess surface proteases, for example the plasminogen activator system,[3] the neutral protease concerned with target-cell cytolysis,[4,5] guanidinobenzoatase[6,7] and probably other proteases. Some of these cell-bound proteases are protected from the approach of high molecular weight inhibitors[2] but might be more accessible to aprotinin which is an inhibitor of relatively low molecular weight.[1] Aprotinin has also been employed therapeutically to depress the level of kallikrein enzymes[1,8] Fluorescent labelled aprotinin might be of value in locating the tissues in which aprotinin, given by injection, has therapeutic activity in man[1,8] and those in which aprotinin is handled for excretion from the body.

Although aprotinin is chiefly known for its inhibitory action on trypsin-like enzymes, it is also known to inhibit chymotrypsin to a lesser extent.[1] We have employed a specific synthetic inhibitor of trypsin-like enzymes,[9] 4-methylumbelliferyl-*p*-guanidinobenzoate, in this study to define the binding sites for aprotinin on tumour cells to be trypsin-like enzymes rather than chymotrypsin-like enzymes. We have examined

the possible role of tissue inhibitors of these trypsin-like enzymes in the control of protease activity on tumour cells *in vivo*.

MATERIALS AND METHODS

Rhodamine-iso-thiocyanate, crystalline bovine trypsin, α-N-benzoyl-DL-arginine-β-naphtlylamide HCl, and 4-methylumbelliferyl-*p*-guanidinobenzoate were obtained from Sigma Chemical Company, St. Louis, Mo. U.S.A. PD-10 columns of Sephadex G-25 medium were obtained from Pharmacia AB, Uppsala, Sweden. Aprotinin was kindly provided by Farbenfabriken Bayer AG, Leverkusen, Germany, as an isotonic saline solution containing 1.4 mg peptide/ml. Soluble extracts were prepared by homogenising fresh rat liver and kidney in isotonic saline. These extracts had a final protein concentration of approximately 1 mg/ml. Wax embedded sections of formaldehyde-fixed tissues were dewaxed by conventional solvent extraction prior to analysis. Frozon sections of tissue were examined directly after staining.

Preparation of rhodamine-aprotinin

Aprotinin solution, 50 ml, containing 70 mg peptide was reacted with 1 mg rhodamine iso-thiocyanate at pH 8.0 in the presence of 2% w/v $NaHCO_3$ for 18 h. The product was divided into suitable aliquots and passed over PD-10 columns. The peptide was eluted with isotonic saline and the elution profile monitored by fluorescence. A single fluorescent peptide fraction was eluted, whilst low molecular weight rhodamine derivatives remained absorbed to the support. The peptide fractions form the PD-10 columns were combined and freeze dried, a suitable concentration of rhodamine aprotinin being made up before use. The peptide content of these solutions was determined by the Folin-Lowry technique[10] using bovine serum albumin as a standard.

Inhibition of trypsin in solution by rhodamine-aprotinin

The conventional trypsin assay with α-*N*-benzoyl-DL-arginine-β-naphthylamide HCl as substrate[5] with 10 μg trypsin in each tube and incremental additions of rhodamine-aprotinin was set up. After 1 h at 30°C the amount of product formed (β-naphthylamine) was determined by fluorescent analysis. The residual trypsin activity was plotted as a percentage of the trypsin activity in the absence of rhodamine-aprotinin (Figure 1), against the final concentration of rhodamine-aprotinin in the tube.

Treatment of frozen sections with fluorescent probes

The sections were covered with an isotonic saline solution (ca, 20 μl) containing 10^{-4} M rhodamine-aprotinin and allowed to stand for 5 min. Then the excess reagent was removed by washing in isotonic saline for 30 s.

Treatment of frozen sections with inhibitors

Sections were treated with 20 μl of an isotonic sodium chloride solution containing 10^{-4} M-methylumbelliferyl-*p*-guanidinobenzoatase for 3 h. The excess reagent was

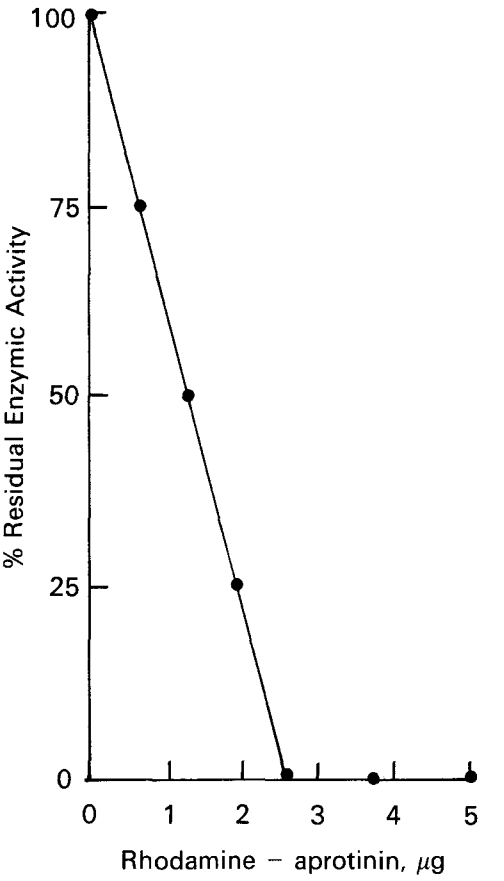


FIGURE 1 Inhibition of trypsin by rhodamine-aprotinin. 10μg cystalline trypsin assayed with α-N benzoyl-DL-arginine-β-naphthylamide HCL in the presence of incremental additions of rhodamine-aprotinin.

washed from the surface of the slide in isotonic saline for 2 min, followed by treatment with fluorescent probe and fluorescent microscopy. Sections were also treated with extracts of rat kidney and rat liver (1 mg protein/ml), overnight, extensively washed in isotonic saline and then prepared for fluorescent microscopy as described above with the fluorescent probes.

Treatment of fomaldehyde fixed wax embedded sections

The sections were dewaxed and treated with rhodamine-aprotinin as described above except that the washing in isotonic saline was extended over 1–2 days in order to remove non-specifically bound probes. The prior application of 4-methylumbelliferyl-*p*-guanidinobenzoatate and tissue extracts to wax sections was identical to that described for frozen section.

Microscopy

We employed a Leitz orthoplan fluorescent microscope with filters set either in positions 4 and 4 or in position 3 and 3 and a Kodak ASA 400 colour film and an automatic Olympus OM-2 camera for filming. Under these conditions, cells binding rhodamine exhibit a red or orange fluorescence.

RESULTS AND DISCUSSION

An excess of rhodamine iso-thiocyanate was employed in the reaction with aprotinin in order to label all molecules of the peptide with the fluorescent tag. The fact that excess rhodamine iso-thiocyanate reacted with the PD-10 Sephadex G-25 confirmed this point. The concentration of rhodamine-aprotinin used in the fluorescent location of cells in this study was 1.3 mg/ml based upon the Folin-Lowry[10] assay. This stock solution of rhodamine-aprotinin was therefore approximately 1×10^4 M with respect to aprotinin dimer of molecular weight[1] 11,500. The data in Fig 1 clearly show that rhodamine-aprotinin retained its ability to combine with trypsin and caused inhibition of this enzyme in free solution. The titration of this enzyme with rhodamine-aprotinin is exactly the same as that obtained with an equivalent weight of unlabelled aprotinin; 2.6 μg aprotinin being the molar equivalent of 10 μg trypsin.

Examination of wax embedded sections

Rhodamine-aprotinin bound to cells can be located with a variety of filter combinations employing the Leitz orthoplan fluorescent microscope. The most brilliant red fluorescence is seen with the filters set in postions 4 and 4. This has the disadvantage with formalin fixed wax embedded sections of exciting background pale red autofluorescence of the tissue. We therefore filmed rhodamine-aprotinin stained wax

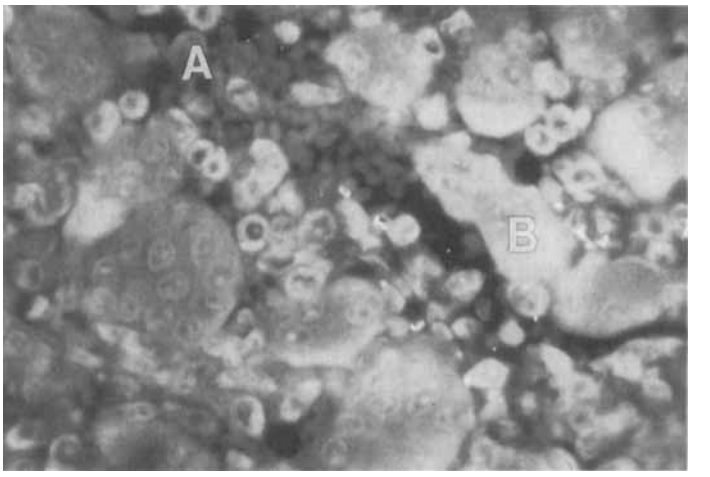


FIGURE 2 Wax embedded bone tumour treated with rhodamine-aprotinin and photographed with filters in positions 3 and 3. Erythrocytes appear blue, osteosarcoma cells labelled (A) exhibit red cytoplasmic staining; the large multi-nucleated osteoclasts are labelled (B). Magnification × 500. (See Colour Plate XVIIa at the back of the Issue).

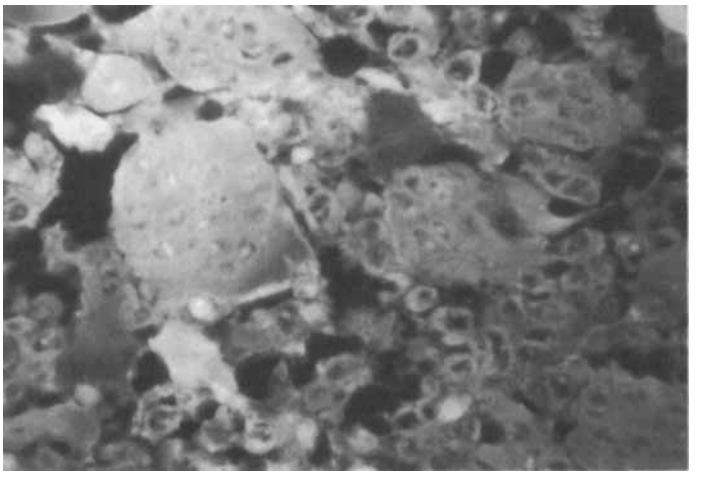
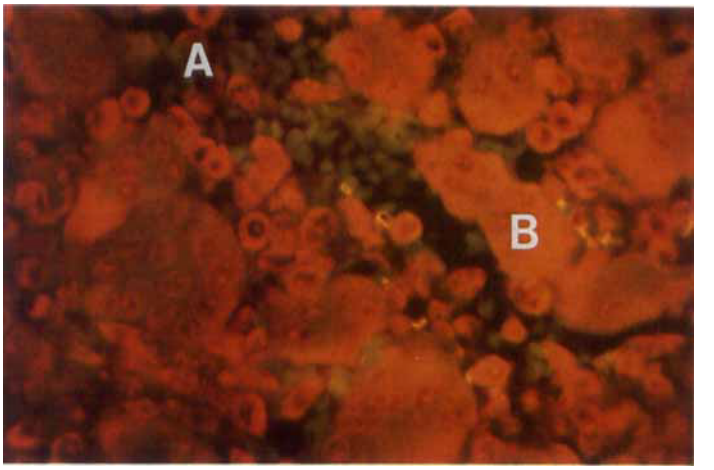


FIGURE 3 Similar section of Figure 2 but pretreated with the specific trypsin inhibitor, 4-methyl-umbelliferyl-*p*-guanidinobenzoate, prior to staining with rhodamine-aprotinin. Magnification × 500. (See Colour Plate XVIIb at the back of the Issue).

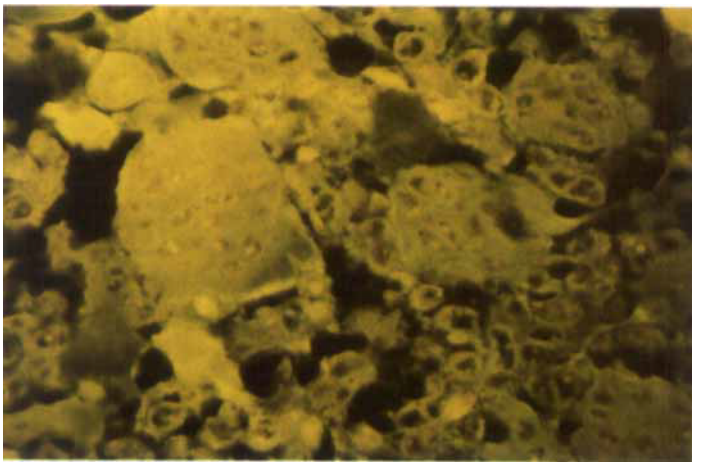
embedded sections with the filters set in positions 3 and 3 where the rhodamine label appeared as orange-red fluorescence on a pale yellow background (Figures 2, 3). We have chosen a tumour of the bone, osteosarcoma, to represent our findings with wax embedded material. In this tissue two invasive cell types predominate, the giant multinucleated osteoclasts and the smaller round cells of the osteosarcoma. Erythrocytes and connective tissue are also present in these sections and these components do not bind the fluorescent probe. In Figure 2, the small cells which appear blue under these microscopic conditions are erythrocytes. The slightly larger round cells (labelled, A) with the intense fluorescent ring staining in their cytoplasm and/or surface membranes are the osteosarcoma cells. These cells have clearly bound rhodamine-aprotinin although it is not clear whether the binding site is extra cellular or cytoplasmic, from this study. In Figure 2 the nuclei and the cytoplasmic surface of the osteoclasts have also bound some rhodamine-aprotinin. Since both the osteoclasts and osteosarcoma cells are highly destructive cells the presence of trypsin-like enzymes associated with these cells would be expected. It is remarkable that the binding site for aprotinin on the formaldehyde fixed wax embedded sections remains functional and it could be argued that this binding has nothing to do with trypsin-like enzymes. Selective inhibitory analysis (see below) makes such an argument invalid.

Pretreatment of the wax embedded sections with 10^{-4} M 4-methyl umbelliferyl-*p*-guanidinobenzoate (a specific active site directed inhibitor of trypsin-like enzymes[9] abolished the binding of rhodamine-aprotinin to both the osteosarcoma cells and to the osteoclasts (Figure 3). The autofluorescence of the section caused the overall yellow appearance of the cells which now lack the red fluorescence seen in Figure 2. The absolute specificity of irreversible inhibition of trypsin-like enzymes by 4-methylumbelliferyl-*p*-guanidinobenzoate[9] defines that the rhodamine-aprotinin was bound to trypsin-like enzymes associated with osteoclasts and osteosarcoma cells (Figure 2) and this binding was not due to an artefact in section preparation.

Trypsin inhibitors are present in serum and can be extracted from homogenates of fresh tissues.[11] We therefore examined the effect of incubating the wax embedded



COLOUR PLATE XVIIa,
Steven *et al.*, Figure 2, p. 298
Wax embedded bone tumour treated with rhodamine-aprotinin and photographed with filters in positions 3 and 3. Erythrocytes appear blue, osteosarcoma cells labelled (A) exhibit red cytoplasmic staining; the large multi-nucleated osteoclasts are labelled (B). Magnifications × 500



COLOUR PLATE XVIIb,
Steven *et al.*, Figure 3, p. 299
Similar section of Figure 2 but pretreated with the specific trypsin inhibitor, 4-methyl-umbelliferyl-*p*-guanidinobenzoate, prior to staining with rhodamine-aprotinin. Magnification × 500

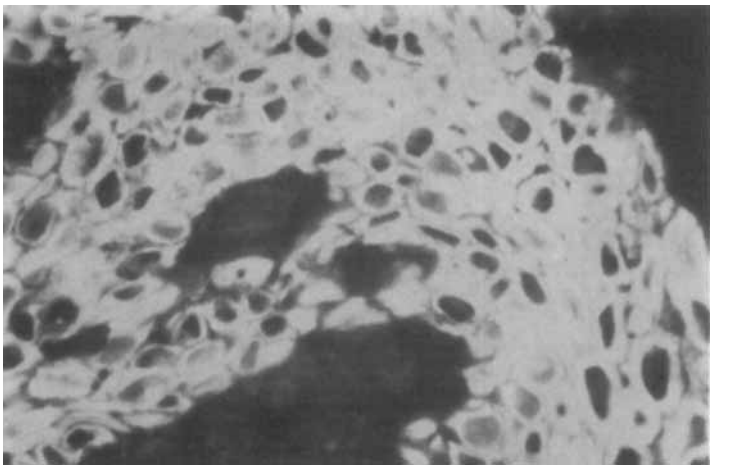


FIGURE 4 Frozen section of carcinoma of the oral cavity treated with rhodamine-aprotinin, photographed with filters in positions 4 and 4. Magnification × 500. (See Colour Plate XVIIIa at the back of the Issue).

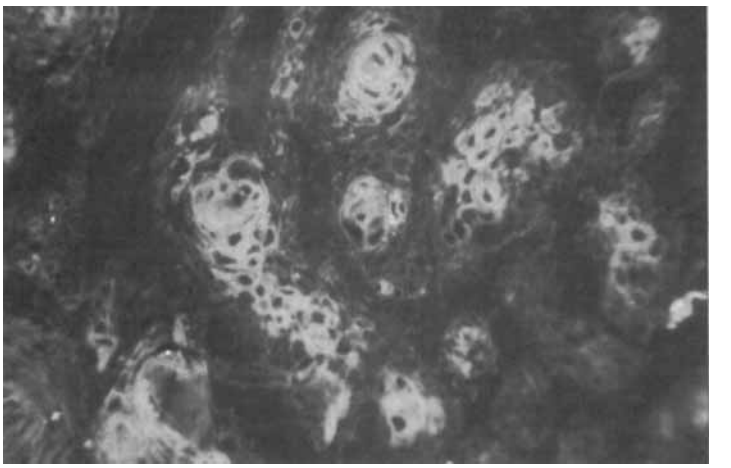
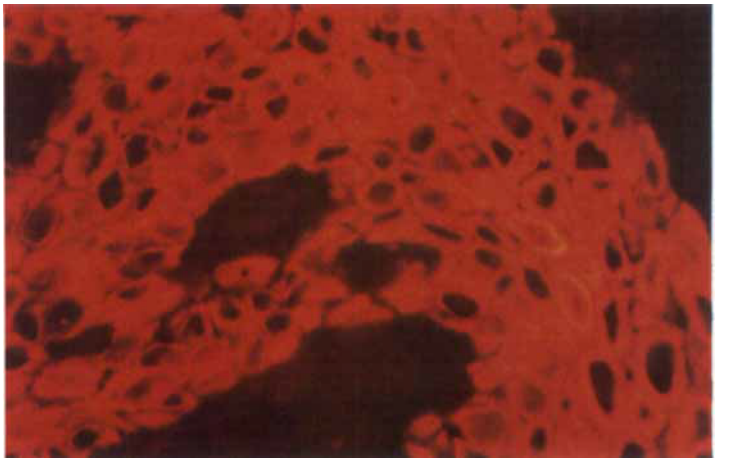


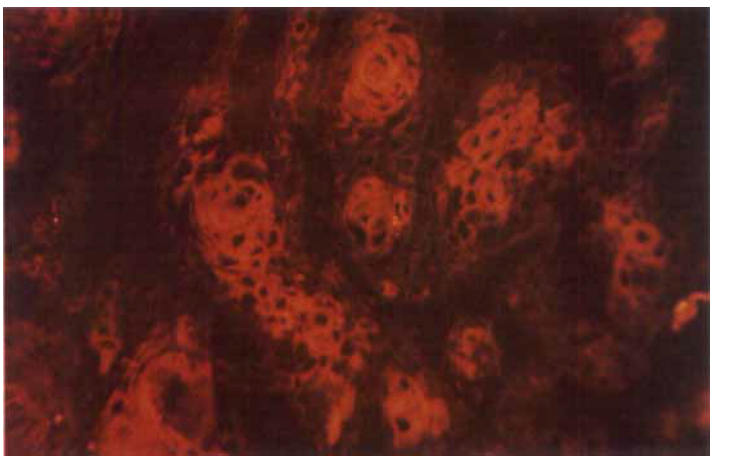
FIGURE 5 Frozen section of well differentiated squamous cell carcinoma of the oral cavity – treated with rhodamine-aprotinin. Magnification × 250. (See Colour Plate XVIIIb at the back of the Issue).

sections with extracts of fresh rat kidney and liver followed by treatment with rhodamine-aprotinin. Both these extracts completely abolished the binding of rhodamine-aprotinin to osteosarcoma cells and osteoclasts, (data similar to Figure 3). This evidence suggests that trypsin-like enzymes on the surface of cells *in vivo* might be inhibited if suitable inhibitors were present in the extracellular fluid surrounding these cells. A similar situation has been described for the inhibition of the cell surface protease guanidinobenzoatase.[12]

We therefore examined frozen sections obtained from a number of patients with tumours of the head and neck region[13] to determine whether the unfixed cells possessed active trypsin-like enzymes capable of binding rhodamine-aprotinin. With frozen



COLOUR PLATE XVIIIa.
Steven *et al.*, Figure 4, p. 300
Frozen section of carcinoma of the oral cavity with rhodamine-aprotinin, photographed with filters in positions 4 and 4. Magnification × 500.



COLOUR PLATE XVIIIb
Steven *et al.*, Figure 5, p. 300
Frozen section of well differentiated squamous cell carcinoma of the oral cavity – treated with rhodamine-aprotinin. Magnification × 250

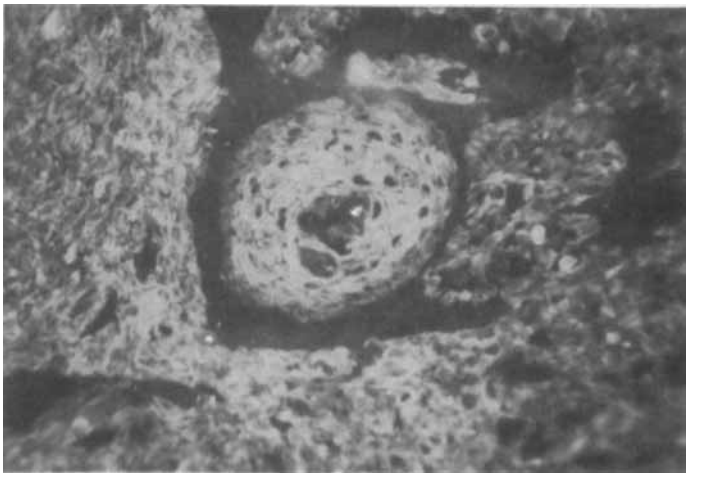


FIGURE 6 Frozen section of a poorly differentiated carcinoma of the hypopharynx. Magnification × 250. (See Colour Plate XIXa at the back of the Issue).

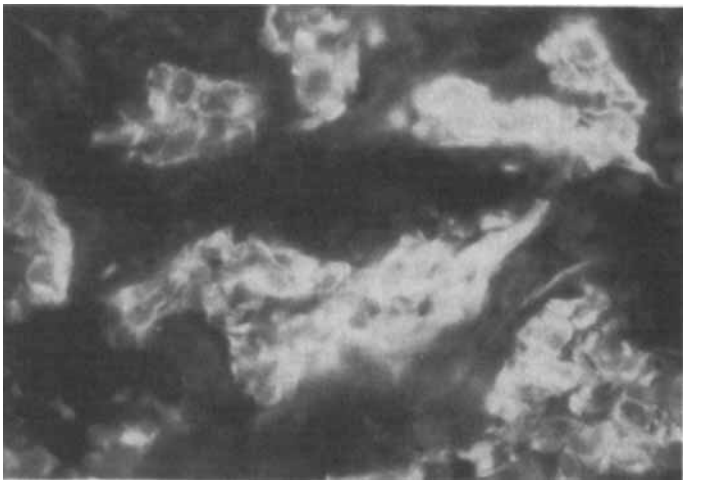


FIGURE 7 Frozen section of cervical lymph node metastasis of a well differentiated carcinoma of unknown origin. Magnification × 500. (See Colour Plate XIXb at the back of the Issue).

sections we employed the filter combination 4 and 4, since there was very little autofluorescence in these unfixed sections. Rhodamine-aprotinin binding resulted in intense red fluorescence on a black background (Figure 4 and 5). This background contained non-fluorescent cells and extracellular matrix. We provide examples, from three different patients, of frozen sections of tumour cells treated with rhodamine-aprotinin.

The first example was taken from a patient with a carcinoma of the oral cavity (Figure 4). The typical ring staining of the cytoplasm/cell surface is shown as red fluorescence. This ability to bind rhodamine-aprotinin was almost completely abolished after a pretreatment with 4-methylumbelliferyl-*p*-guanidinobenzoate and

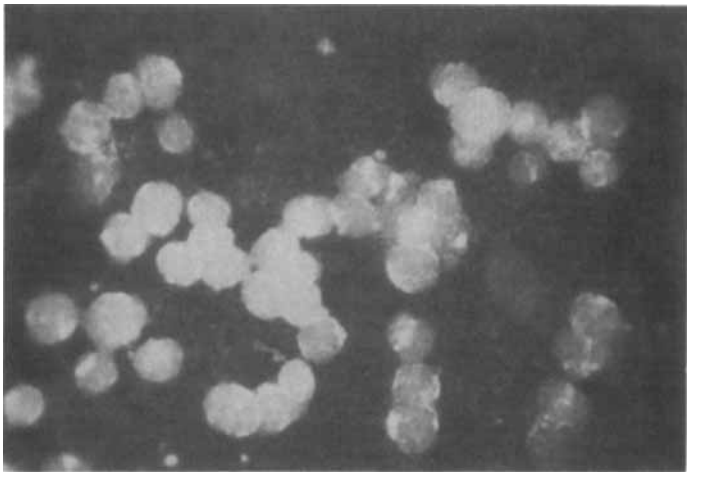
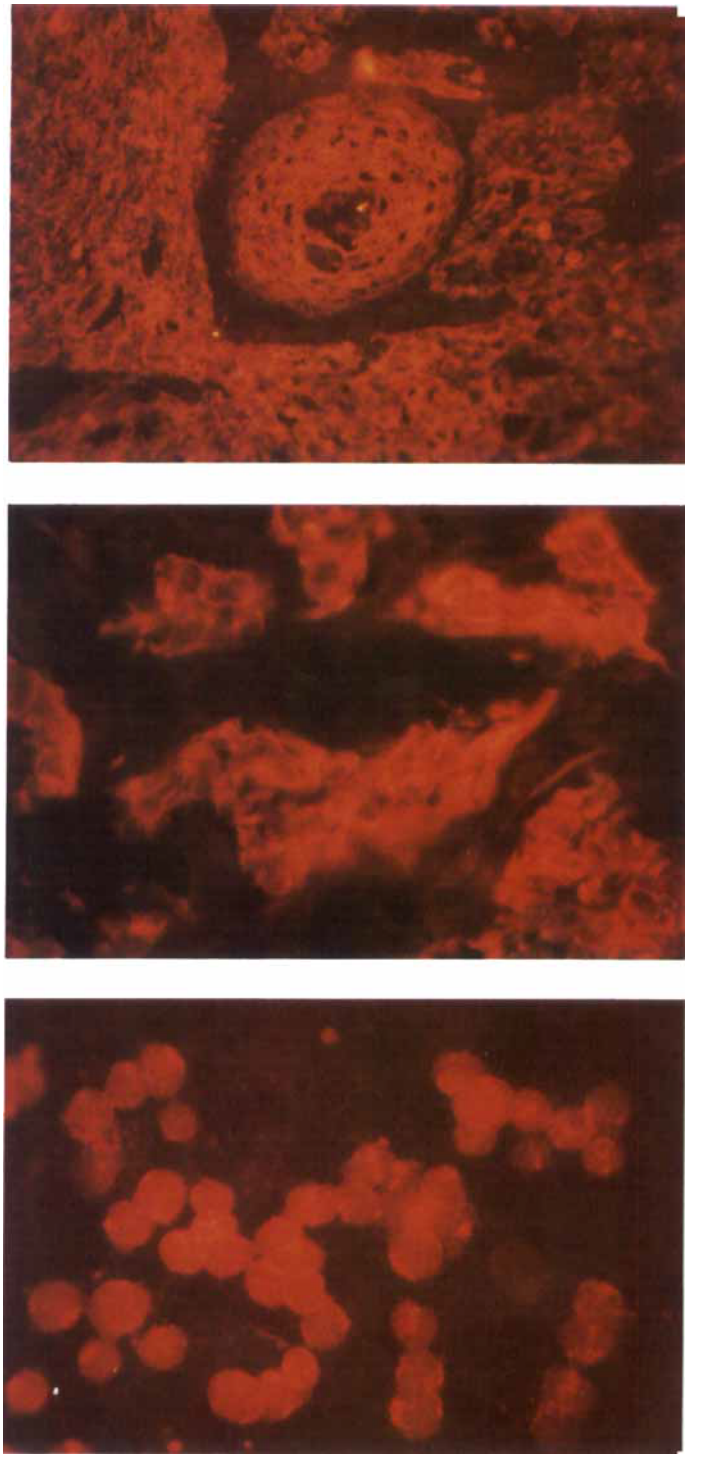


FIGURE 8 Rat T-leukaemia cells mixed with normal human blood and smeared on a slide, dried and fixed in formaldehyde prior to treatment with rhodamine-aprotinin. Note that only these intact leukaemia cells bind the red probe, all other cells lack the trypsin-like enzyme which binds aprotinin. Magnification × 500. (See Colour Plate XIXc at the back of the Issue).

completely inhibited after exposure to each of the tissue extracts. Similar staining and inhibition of staining were observed with tumours from two other patients. These two sections were obtained from a patient with a well developed squamous cell carcinoma of the oral cavity (Figure 5) and a patient with a poorly differentiated carcinoma of the hypopharynx (Figure 6). The section presented in Figure 7 was taken from a cervical lymph node metastasis of a well differentiated carcinoma of unknown origin. It should be noted that many cells do not bind the fluorescent probe and appear to have no fluorescence in these colour prints.

We believe the evidence presented above demonstrates that these tumour cells possess active trypsin — like enzymes *in vivo*, in spite of the fact that serum and tissue extracts contain inhibitors of these cell bound enzymes. This fluorescent location of an enzyme associated with a tumour cell in a tissue section makes it difficult to decide whether the binding of the probe is in the cytoplasm or on the cell surface. This problem can be overcome if intact cells are used rather than sections of tissue. Intact rat T-cell leukaemia cells[14] when prepared as a cell smear and stained with rhodamine-aprotinin fluoresce (Figure 8). The molecular weight of the aprotinin dimer in solution[1] excluded the possibility of the fluorescent aprotinin passing through the cell membrane into the cytoplasm. We therefore suggest that in the case of the leukaemia cell, the trypsin-like enzymes are located on the external surface of the cell membrane. This binding of the fluorescent aprotinin was completely inhibited by exposure to the kidney and liver extracts for 18 h.

We conclude that tumour cells possess an active form of trypsin-like protease which may be selectively located by the fluorescent aprotinin, an inhibitor of these enzymes. This use of fluorescent-aprotinin may be of value as a diagnostic aid in tumour cell location and could possibly be used to transport cytotoxic drugs to tumour cells. We have already developed fluorescent probes for guanidinobenzoatase[7,15] and alkaline phosphatase,[16] two enzymes of particular importance for certain tumours. These enzyme inhibitor probes may play a role in the future in assessing the enzymic activity



COLOUR PLATES XIXa, b, c
Steven *et al.*, Figures 6, 7, 8, pp. 301, 302

of tumour cells *in vivo* just as the lectins are now playing an increasingly important role in locating glycosylated cell surface molecules in modern histochemistry.

Acknowlegements

Dr Frank S. Steven gratefully acknowledges the generous financial support of the Imperial Cancer Research Fund without which this work would not have been carried our in Manchester.

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