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LABELLING OF TUMOUR CELLS WITH A BIOTINYLATED INHIBITOR OF A CELL SURFACE PROTEASE

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Our objective has been to prepare a biotinylated affinity probe for the active centre of a protease associated with the surface of tumour cells. We employed three model systems in which easily recognisable tumour cells containing the active protease were used as targets for the biotinylated affinity probe. These were: squamous cell carcinoma, leukaemia cells in muscle and outgrowths of prostate carcinoma cells grown in three dimensional collagen gels. The presence of the bound biotinylated affinity probe was demonstrated by its ability to bind Texas-red labelled streptavidin with the results that the tumour cells exhibited red fluorescence. This binding was shown to be competitive with 9-amino acridine, a compound known to bind to the active centre of the target protease. This technique depends upon the affinity of the active centre of an enzyme for a competitive inhibitor and therefore should be applicable to other enzyme systems employing suitable ligands for their active centres.

KEY WORDS: Competitive inhibition, biotinylated agmatine, protease, tumour cells, Texas-red streptavidin.

INTRODUCTION

The high affinity of avidin and streptavidin for biotin is the basis of most enzyme-linked immunohistological techniques now in use.[1,2,3] One of the objectives of the present study has been to determine whether biotin labelling of specific target proteins could be achieved without the use of a specific antibody directed to this target protein.

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In the present study we have designed a biotinylated ligand which was directed to the active centre of guanidinobenzoatase (GB), a protease[4] associated with the surface of tumour cells.[5,6] In this model system the target protein is an enzyme of known significance to the tumour cells.[7,8] In order to demonstrate the selective binding of the biotinylated ligand to the active centre of GB we located the biotinylated ligand with fluorescent streptavidin (Texas red-streptavidin, TR-S) employing fluorescent microscopic analysis of tumour containing sections. The TR-S locates tumour cells but this can be prevented by prior treatment of the sections with a known competitive inhibitor of GB (i.e. 9-amino acridine, 9-AA). As the binding of 9-AA to GB is concentration dependent and reversible,[5] the 9-AA, which originally blocked the binding of the biotinylated ligand, could be displaced and replaced subsequently by the biotinylated ligand with consequent ability to bind TR-S and fluoresce.

In order to demonstrate the binding of a biotinylated ligand to cell surface GB we required a test system which contained easily recognisable tumour cells in which the GB was known to be active and therefore capable of binding 9-AA or the biotinylated ligand. We chose three model systems to illustrate this interaction of the biotinylated ligand with active GB.

1) Squamous cell carcinoma cells have readily recognisable morphology since they appear as either well differentiated clumps of cells in which the cells at the migrating edge often have more GB than those in central region[9] or as scattered single cells with large nuclei.

2) Rat leukaemia cells[10–12] which have been implanted into the muscle and which have then begun to form a solid tumour within the muscle. In the early stages of this model system, the leukaemia cells can be readily located, spreading between the bundles of muscle fibres. Earlier studies with tissues from the leukaemic rats demonstrated the presence of GB on the surface of the leukaemia cells[6] and this therefore seemed a suitable test system in which the tumour cells were easily recognisable by their intramuscular location and their ability to bind 9-AA.[6,13]

3) We wished to demonstrate whether the ability to locate tumour cells with the biotinylated ligand could be carried out on wax embedded formalin fixed sections, since this is the most common form of section used in pathology. We chose a relatively simple system: the outgrowth of prostate carcinoma cells in three dimensional collagen gels. These tumour cells can easily be recognised by conventional histochemical techniques and also possess GB which binds 9-AA and can therefore be detected by fluorescent microscopy.[5]

The design of the biotinylated ligand is based upon the ability of N-substituted agmatines to bind to the active centre of GB as competitive inhibitors.[14,15] We simply coupled the N-terminal group of agmatine to an extended biotin molecule. It was not necessary to purify the product, since in the test system only the biotinylated ligand linked to the cell surface GB remained attached to the slide, the excess reagents being easily washed from the surface of the slide. When we had labelled the tumour cells on the slide with biotinylated agmatine it was very simple to locate these cells with Texas red-streptavidin (TR-S), which has a great affinity for biotin. The red fluorescence of TR-S on the surface of the tumour cells therefore indicated the binding of the biotinylated agmatine to the active centre of GB on these cells.

MATERIALS AND METHODS

Reagents

9-amino acridine, biotinamidocaproate-N-hydroxysuccinimide ester, streptavidin and Texas red acid chloride were purchased from Sigma Chemical Co., St. Louis, Mo, USA. PD-10 Sephadex G-25M columns were obtained from Pharmacia AB, Uppsala, Sweden. The antiquench agent Citifluor AF/73 was obtained from Citifluor Ltd., Connaught Building, City University, London EC1V 0HB, UK.

Tumour Tissues

Three tumour cell types were studied, serving as examples of this location technique.

1) Squamous cell carcinoma of the oral cavity is one of the most common tumours of the head and neck region. Tumours were collected after removal by surgery in the HNO-Klinik, University of Heidelberg. Dr. Antonio Born of the Department of Pathology, University of Heidelberg, selected the five typical squamous cell carcinomas used in the present study and also supplied multiple frozen sections of each, as well as the original tumours for work in Manchester.

2) The rat leukaemia model used a block of frozen unfixed rat leg muscle, in which the spread of leukaemia cells[10–12] through the muscle was at an early stage. This block was generously provided by Dr. Clive Ramsden of the Department of Experimental Pathology, University of Manchester. Multiple frozen sections were obtained from this block.

3) The prostate carcinoma cells were obtained by explant culture of small chips of prostate tumour tissue within a three dimensional collagen gel (containing all the necessary nutrients for cell growth and proliferation.[16] After a period of 14–17 days, when the carcinoma cells had clearly grown out from the original explant, the gels were fixed in formaldehyde, dehydrated and wax embedded prior to sectioning. The sections were dewaxed, subjected to conventional haematoxylin-eosin staining and prostate specific antigen using the ABC immunoperoidase method. 9-Amino acridine location of cells containing active GB and labelling with biotinylated agmatine was carried out.

Prior Treatment of Sections to Remove Inhibitors of GB

Previous experience had demonstrated the presence of soluble GB-inhibitors in the cytoplasm of tumour cells.[7,8] These inhibitors can be displaced from GB by treatment with formaldehyde.[7] We therefore treated all the sections with formaldehyde (10% v/v or 4% w/v) in isotonic saline for 1 h and then placed these fixed sections in a tank (300 ml) of isotonic saline for 2 h to wash out unbound inhibitor before attempting to bind biotinylated agmatine.

Preparation of Biotinylated Agmatine

Agmatine possesses a single free primary amino group and this should be readily substitutable with an activated carboxyl group attached to biotin, such as

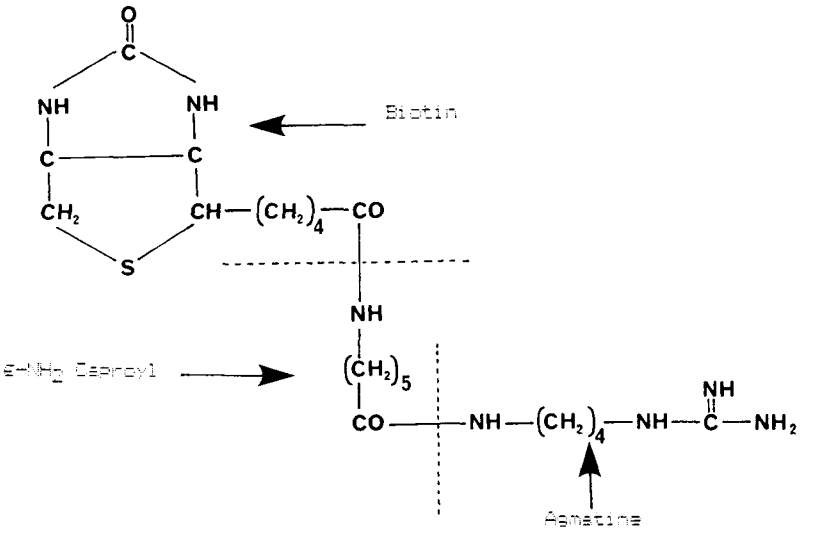


FIGURE 1 Structure of Biotinylated Agmatine (BA).

biotinamidocaproate-N-hydroxysuccinimide ester. The expected product (Figure 1) is the biotinylated agmatine (referred to in this paper as BA). No attempt was made to purify the BA since we wanted to provide an easy and rapid method for selective biotinylation of the target protein, GB, in which the GB was the only molecule capable of capturing the BA. In order to achieve this objective it is necessary to react the biotinamidocaproate-N-hydroxysuccinimide ester with at least 1.5 molar equivalents of agmatine hydrochloride at pH 7–8, to ensure that all of the biotin is linked to agmatine. The condensation proceeded for 6 h at room temperature. On the basis of the completion of the condensation, the final product contained 10^{-3} M BA in isotonic saline, pH 7.0.

Preparation of Texas Red-streptavidin

Affinity purified streptavidin (0.1 mg) was coupled with Texas red acid chloride in 2% $NaHCO_3$ and the derivatised protein purified from excess Texas red reagent on a PD-10 Sephadex column. The final product contained 28 μg Texas red-streptavidin (TR-S)/ml of isotonic saline.

Fluorescent Labelling of Cells containing GB with 9-AA

The sections were placed in a tank containing 100 ml 9-AA (10^{-3} M) in isotoinic saline for 2 min, then drained of excess reagent and finally washed in a tank containing 100 ml of fresh isotonic saline for 1 min. A glass coverslip was placed over the section and the tumour cells located by fluorescent microscopy. A Leitz fluorescent microscope (Diaplan) with filter cube [G] (catalogue reference No. 513602) and barrier filter K490 was employed when recording our results by colour photography. An Olympus OM-2_N camera was used with automatic exposure meter and Kodak ASA 400 colour

film. Under these conditions the cells possessing GB exhibited yellow surface fluorescence[5] which appeared to be cytoplasmic in the sectioned cells used in this study.

Fluorescent Labelling of Cells containing GB with Biotinylated Agmatine linked to Texas Red-streptavidin

The formaldehyde fixed sections were dried in air and 10–15 μl BA (10^{-3} M) placed over the surface of the section. The sections were incubated at room temperature in a wet box for 30 min and then the excess reagent was washed from the surface of the slide with isotonic saline. Excess moisture was dried from the surface of the slide and 10–15 μl TR-S (28 μg/ml) was placed on the surface and allowed to react for 5 min, prior to washing off the excess reagent. The staining procedure is referred to as BA/TR-S. The surface of the tissue was covered with the antiquench reagent Citifluor AF/73; usually 10 μl was sufficient and a glass coverslip was placed on top. The red fluorescence of the Texas red labelled cells was observed by fluorescent miscroscopy employing a Zeiss microscope with the filter cube appropriate for Texas red fluorescence supplied by Zeiss. The same camera and film was used for colour photography as with the Leitz microscope. Those cells possessing GB binding BA fluoresced red on their surfaces, whilst the background was almost black.

Competition of 9-AA and BA for the Active Centre of GB on Tumour Cell Surfaces

In these experiments the formaldehyde treated sections were first treated with 9-AA for 2 min, then the excess fluid was drained from the surface of the slide. Each slide was then treated with BA/TR-S, as described above, prior to examination by fluorescent microscopy and photography. They were then placed in isotonic saline for 6 h, to remove all bound fluorescent ligands, checked by fluorescent microscopy and then resubjected to labelling with BA/TR-S, before being re-examined by fluorescent microscopy for bound TR-S.

RESULTS AND DISCUSSION

All those cells which contained GB bound 9-AA, these cells were also capable of binding TR-S after treatment with BA but did not bind TR-S if BA pretreatment was excluded. We present our evidence in the form of colour prints, the yellow colour corresponding to 9-AA staining and the red corresponding to the TR-S bound to tumour cell surface GB.

Squamous Cell Carcinoma of the Oral Cavity

We have already described the location of squamous cell carcinoma cells by their ability to bind 9-AA due to the presence of active GB on their cell surfaces.[9] Figure 2 shows a typical clump of squamous cell carcinoma cells located by their ability to bind 9-AA. The tumour cells are also present as individual squamous cell carcinoma cells which lie in the connective tissue matrix between the clumps of cells (not shown here). Both types of squamous cell carcinoma cells bind 9-AA and fluoresce yellow when using filter cube [G] in the Leitz Diaplan microscope and orange when using the filter cube for Texas red in the Zeiss microscope. When similar sections were treated with

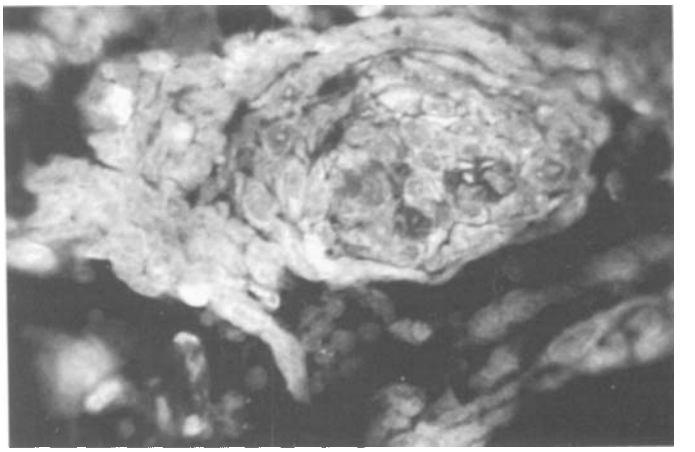


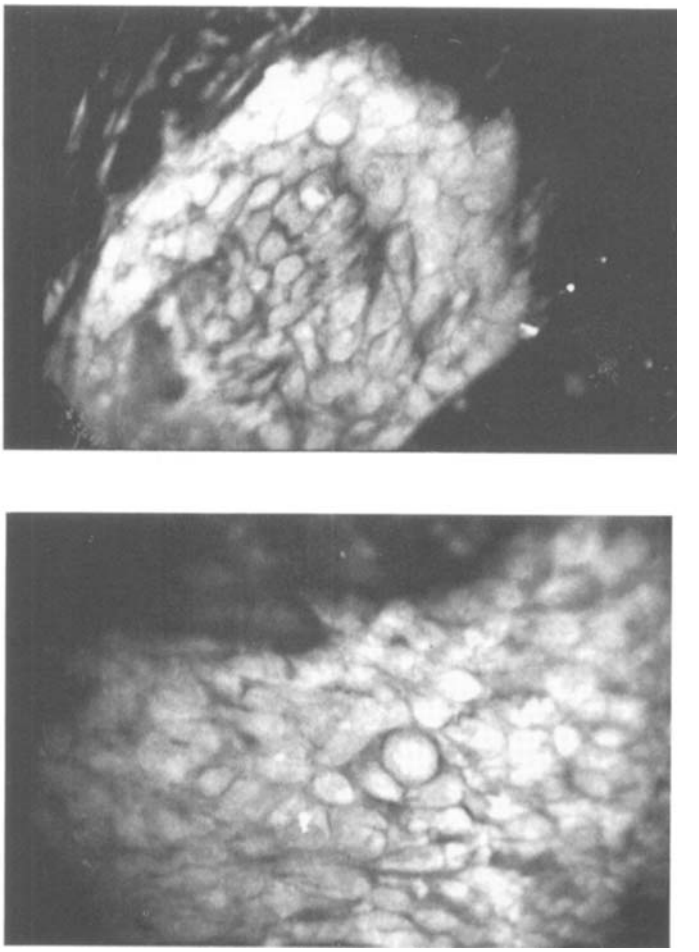
FIGURE 2 Squamous cell carcinoma stained with 9-AA. Cells possessing GB bind 9-AA and fluoresce yellow when viewed with filter cube [G]. Magnification × 500. (See colour plate at back of issue.)

BA/TR-S and examined with the Zeiss microscope, with the Texas red filter cube, these tumour cells fluoresced red in their cytoplasm in both the clumps of cells (Figure 3) and the individual cells (Figure 4). This apparent fluorescence of the tumour cell cytoplasm we believe to be caused by the binding of the fluorescent label to the cell surface[6] which gives the appearance of cytoplasmic staining in sectioned cells. The evidence of Figures 2–4 clearly shows that the tumour cells bind both 9-AA and BA/TR-S and that cells which lack GB do not bind either probe.

Competition between 9-AA binding and BA/TR-S binding indicated that these two markers locate the same binding site on the tumour cells, *viz.* the active centre of GB. When the sections were first treated with 9-AA followed by BA/TR-S no red fluorescence was observed but the tumour cells fluoresced yellow strongly when examined with the Leitz microscope with filter cube [G], similar to Figure 2. This indicated that the tumour cells retained active GB, able to bind 9-AA, but that the presence of 9-AA blocked the binding of BA/TR-S. When the 9-AA had been washed out of the active centre of GB on the fixed tumour cells, BA/TR-S binding was uninhibited and the tumour cells now fluoresced red when examined with the Zeiss microscope with the Texas red filter cube in place (data similar to Figures 3,4). The evidence presented above clearly demonstrates the reversible competitive inhibition of GB by both 9-AA and BA/TR-S. It can therefore be concluded that BA/TR-S binds to and locates the active centre of GB on the surface of these tumour cells. Two other types of tumour cells were employed to demonstrate the fact that the labelling of GB and BA/TR-S is not confined to squamous cell carcinoma cells but is probably a universal property of tumour cells which possess active GB.

Leukaemia Cells implanted into Rat Leg Muscle

The binding of 9-AA to this type of rat leukaemia cell has already been described,[6,13] although in the past we have concentrated on the kidney. It has been shown that the GB is associated with the surface of these leukaemia cells[6] and it usually inhibited *in vivo*. In the present study we examined the leukaemia cells spreading through the muscle tissue after a local injection of leukaemia cells. After formaldehyde displace-



FIGURES 3,4 Squamous cell carcinoma stained with BA/TR-S. Cells possessing GB bind BA; the BA then binds TR-S with the result that these cells fluoresce red when viewed with the Texas red filter. Note in Figure 3 the more intense staining of the cells at the advancing edge of the tumour. Note in Figure 4 the individual cells can be clearly defined within the tumour mass. (See colour plate at back of issue.)

ment of inhibitor, the leukaemia cells bind 9-AA and fluoresce yellow when filter cube [G] was used in the Leitz Diaplan microscope (Figure 5). The location of the tumour cells between the muscle bundles clearly defines these target cells as possessing active GB. Treatment of corresponding sections of this rat leukaemic muscle with BA/TR-S resulted in the leukaemia cells fluorescing red when examined with the Zeiss microscope using the filter cube for Texas red (Figure 6). In Figure 6 the location of the red cells between the muscle bundles is similar to that seen in Figure 5 after 9-AA staining. Clearly, the same type of cells (*viz.* leukaemia cells) are being detected in each case. This would be expected[14,15] since the two probes are both directed to the active centre (see above) of GB.

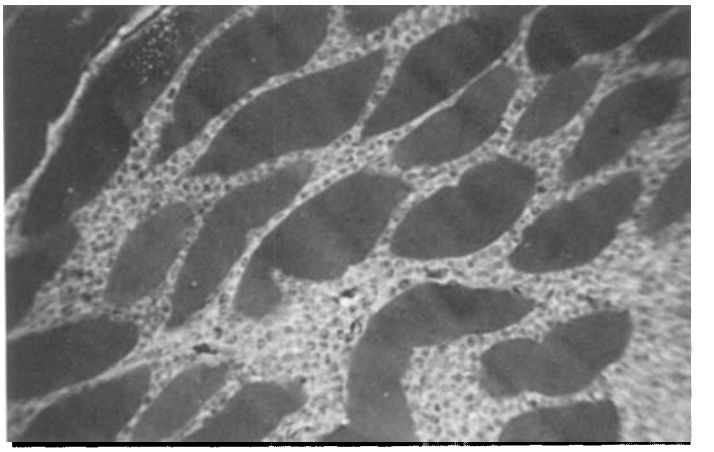


FIGURE 5 Leukaemia cells invading rat muscle tissue, stained with 9-AA. Note the location of the leukaemia cells (yellow fluorescence) between the bundles of non-staining muscle fibres. Magnification × 250. (See colour plate at back of issue.)

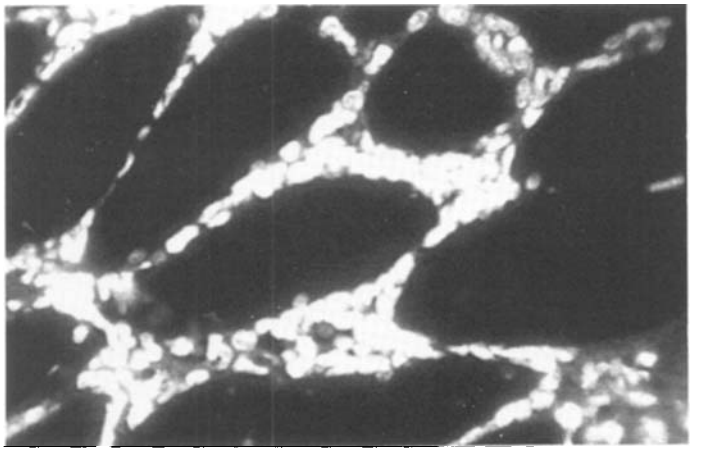


FIGURE 6 Leukaemia cells invading rat muscle tissue, stained with BA/TR-S. The leukaemia cells bind BA/TR-S and fluoresce red when viewed with the Texas red filter. The muscle cells, which do not possess GB, do not bind BA/TR-S and appear black. Magnification × 500. (See colour plate at back of issue.)

Outgrowths of Prostate Carcinoma Cells in vitro

Sections of the three dimensional collagen gel containing outgrowths of carcinoma cells could be located by their ability to bind 9-AA (Figure 7). Occasionally normal cellular outgrowths from the tissue explants were also seen; these appeared green, did not possess GB and failed to bind 9-AA (data not shown). When these sections were treated with BA/TR-S, the tumour cells alone fluoresced red on a black background

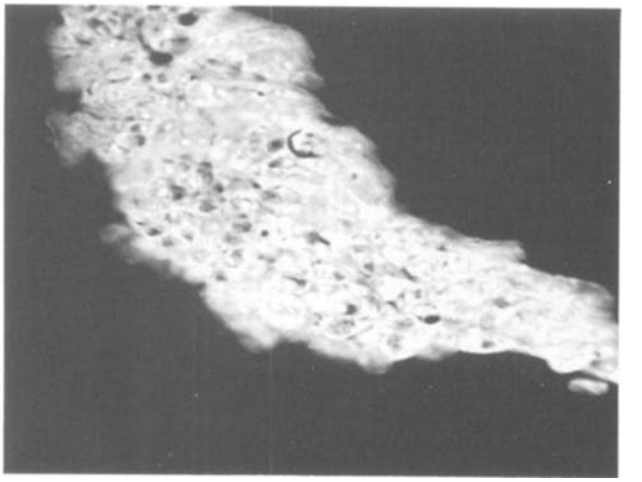


FIGURE 7 Outgrowths of prostate carcinoma cells stained with 9-AA. The tumour cells possess GB, bind 9-AA and fluoresce yellow when viewed with filter cube [G]. The collagen matrix appears black. Magnification × 250. (See colour plate at back of issue.)

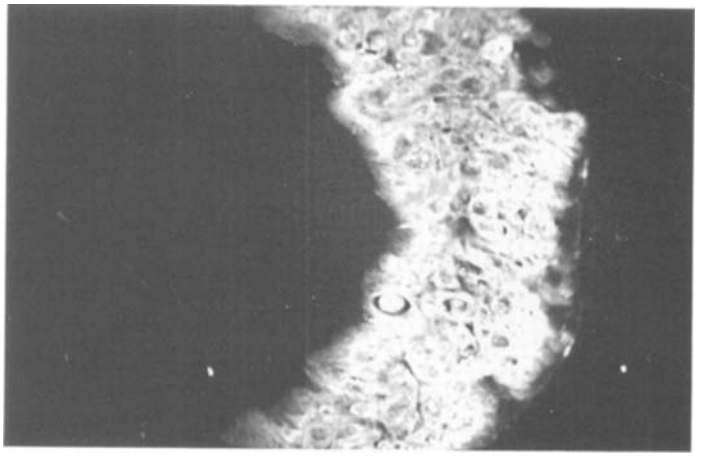


FIGURE 8 Outgrowths of prostate carcinoma cells stained with BA/TR-S. Tumour cells possessing GB bind BA and subsequently TR-S, resulting in red fluorescence when viewed with the Texas red filter. Magnification × 250. (See colour plate at back of issue.)

(Figure 8), when examined with the Zeiss microscope. The binding of BA/TR-S to the prostate carcinoma cells was inhibited by prior exposure to 9-AA, as described above, in a competitive manner. Double labelling with both 9-AA and BA/TR-S was achieved by appropriate modification of the experimental conditions when these probes were in competition for the active centre of GB.

We conclude from the above data that BA is selectively bound to the active centre of GB and that this complex can be made fluorescent by the subsequent binding of

TR-S. The technique can be used for the fluorescent location of cells possessing active GB on their surfaces. The purpose of the present study has been to demonstrate the binding of a biotinylated probe to the active centre of a target enzyme on the surface of tumour cells. The ability to label selected target proteins with low molecular weight affinity probes may offer an alternative to the use of biotinylated antibodies in immunoenzymological techniques, when the approach of the antibody may be impaired, for example in resin sections. In order that the biotinylated affinity probe be really effective in enzyme-linked techniques, the biotinylated probe must also possess the potential for irreversible binding to the target protein. This desirable property will be the subject of further study. At the present time we merely wish to present evidence for the successful binding of a low molecular weight affinity probe containing biotin to the active centre of a proteolytic enzyme associated with tumour cells.

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