J. Enzyme Inhibition, 1993, Vol. 7, pp. 65–76
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AFFINITY PREPARATION OF A PROTEIN INHIBITOR RECOGNISING A CELL SURFACE PROTEASE

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(Received July 12, 1992)

Epithelial cell surfaces possess a trypsin-like protease, referred to as guanidinobenzoatase (GB). The cytoplasm of these cells contains an extractable protein (I) which recognises the cell surface GB by forming an enzyme-inhibitor complex (GB-I). Rhodamine-agmatine (Rh-Agm) was designed as a red fluorescent probe, directed to the active centre of GB, which can be used to locate cells with GB, employing fluorescence microscopy. Rh-Agm has a high affinity for GB and will displace I from GB-I on the surfaces of cells in frozen sections. Rh-Agm has been used to displace I from immobilised GB-I complexes on the surface of cultured colonic carcinoma cells in an affinity procedure aimed at purifying the inhibitors of GB obtained from cultured carcinoma cells. These inhibitors have been tested on *protected* frozen sections of normal colon and carcinoma of the colon, the formation of GB-I complexes being followed by a second yellow fluorescent probe which competes for the active centre of GB.

The study of the protein-protein interactions to form GB-I has been facilitated by employing two synthetic fluorescent inhibitors of GB with differing affinities for GB and different fluorescent properties. The use of sections of tissue in this study has enabled a sequence of reactions to be carried out on the same cell surface GB, such that reversible inhibition reactions can be quickly demonstrated and recorded by fluorescence microscopy.

KEY WORDS: Cell surface protease, inhibitor, colonic carcinoma.

INTRODUCTION

Cell surface proteases play important roles in both normal cells and tumour cells *in vivo*.[1,2] A cell surface protease,[3] referred to as guanidinobenzoatase (GB),[4] has been

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Abbreviations used: Guanidinobenzoatase, GB; Tissue plasminogen activator, t-PA; 9-Amino acridine, 9AA; protein inhibitor, I; GB-inhibitor complex, GB-I; Sodium dodecyl sulphate, SDS; Rhodamine-Agmatine, Rh-Agm; Propidium Iodide, PI.

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shown to be associated with tumour cells and other normal cells capable of migration. GB is functionally similar to tissue type plasminogen activator (t-PA).[5,6]

The cytoplasm of tumour cells contains soluble protein inhibitors of GB which may be readily extracted and transferred to cell-bound GB in *protected* frozen sections.[7] *Protected* frozen sections contain active cell surface GB but lack local cytoplasmic inhibitors.[5] *Protected* sections were prepared by extracting the soluble protein inhibitors from the sections when placed in isotonic saline containing 10^{-3} M 9-aminoacridine (9AA). The enzyme-bound 9AA was then washed from the sections with isotonic saline, leaving the cell surface enzyme in an active state in the absence of local inhibitor proteins. The interaction of GB with the cytoplasmic inhibitor can be followed by the use of the fluorescent probe 9AA, a competitive inhibitor of GB. Cells possessing active GB bind 9AA and fluoresce yellow whilst cells lacking GB or possessing the inhibited form of GB (GB-I) fail to bind 9AA and do not exhibit yellow surface fluorescence.[7] The study of tumour cell surface GB and the interaction with potential inhibitors (extracted from frozen sections) has led to the demonstration of isoenzymic forms of GB recognised by specific isoinhibitor proteins.[8]

We have recently employed carcinoma cells grown in culture, to provide cytoplasmic protein inhibitors which recognise both the isoenzymic forms of GB associated with normal colonic epithelial cells and colonic carcinoma cells but not the GB associated with the surfaces of other types of tumours.[8] The cytoplasm of the carcinoma cells was extracted to provide a solution of inhibitor protein (I) and the washed cell membranes were used to provide a source of immobilised active GB. The cytoplasmic extract (containing I) was allowed to interact with washed cell membranes of carcinoma cells, in order to selectively bind I to membrane bound GB. The newly formed GB-I was dissociated with a fibrin overlay[8] and a soluble inhibitor fraction was shown to inhibit both isoenzymic forms of GB on normal colonic epithelial cells and carcinoma cells in *protected* frozen sections.

We need to purify the inhibitor fraction since there were two isoinhibitors as well as large amounts of fibrin-derived polypeptides present in this fraction (as seen by sodium dodecyl sulphate polyacrylamide gel electrophoresis). Although the fibrin overlay procedure was successful in dissociating GB-I a method was needed for obtaining I without the presence of associated fibrin peptides.

Previous studies[4] have shown that N-substituted agmatines act as competitive inhibitors of GB. In the present study rhodamine bound to agmatine (Rh-Agm) was used as a successful probe for GB and also as a displacing agent for I (in the complex GB-I) linked to cell surfaces. Rh-Agm has a higher affinity for GB than the protein inhibitors and can be used to purify I in an affinity system, similar to the one described for the partial purification of I employing fibrin overlays. Rh-Agm has the advantage that this compound could be used in the automated analysis of cells possessing GB in cervical smears and spreads[9] rather than the use of 9AA and propidium iodide (PI), the latter being a potential health hazard.

MATERIALS AND METHODS

Reagents

Agmatine sulphate (Agm), 9-amino acridine (9AA), rhodamine isothiocyanate (Rh) and sodium dodecyl sulphate (SDS) were all purchased from Sigma Chemicals Co.

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Ltd., St. Louis, Mo, USA. PD-10 columns of sephadex G-25M were obtained from Pharmacia LKB, Uppsala, Sweden.

Preparation of Rhodamine-Agmatine

The conjugation of rhodamine isothiocyanate with agmatine to yield rhodamine-agmatine (Rh-Agm) was carried out in 2% w/v sodium bicarbonate solution, pH 8.0; the excess rhodamine isothiocyanate being removed by repeated ether extraction. The absence of rhodamine isothiocyanate was checked by thin layer chromatography using the solvent mixture methanol:chloroform (60:40) on Cellulose-Avicel plastic TLC plates (Schleicher and Schuell, supplied by Anderman & Co., Kingston-upon Thames, UK). Only one compound was observed; a slow moving spot which fluoresced red under UV light. This is consistent with the formation of Rh-Agm, since the spot also gave a positive reaction with Sakaguchi reagents, indicative of the presence of a guanidino group.

Frozen Sections, Imprints and Cultured Cells

Frozen sections of breast carcinoma were provided by JB and normal colonic and colonic carcinoma sections were provided by ICT. The breast carcinoma imprints were provided by JB. Breast imprints were prepared by touching the surface of a clean polylysine coated slide with a piece of freshly excised breast tissue. The cultured colonic carcinoma cells (LS174T) used in this study were generously provided by Dr D.C. Blakey, ICI Pharmaceuticals, Alderley Park, Macclesfield, UK. These cells were provided as part of another study.[8]

Fluorescent Location of Breast Carcinoma Cells with Rh-Agm

Three types of frozen section were used in this study: (a) frozen sections stained directly, (b) *protected* frozen sections[7] in which the local inhibitor proteins had been removed and the cell surface GB was known to be active by its ability to bind 9AA and (c) sections in which the cells had been treated with inhibitor and the GB was known to be present in the form of GB-I, lacking the ability to bind 9AA.[7]

The surface of these sections was covered with 10 μl Rh-Agm (10^{-4} M) and after 3 min, the excess reagent was washed from the surface of the section in isotonic saline for 5 min (see Figure 1). Fluorescence microscopy employing a Leitz Diaplan microscope with filter cube [N] (Leitz catalogue no. 513609) in place, revealed cells with active GB as red fluorescent cells.

Imprints of breast carcinoma tissue were similarly treated. In most cases, the GB on the carcinoma cells was in the form of GB-I as determined by prior staining with 9AA. The 9AA was removed from the cells by exhaustive washing in isotonic saline prior to Rh-Agm staining (see Figures 2 and 3).

Affinity Purification of Colonic Carcinoma GB Inhibitors

Basically this followed the same procedure described for the fibrin release of I from GB-I[8] except that fibrin was replaced by exposure of the cells to Rh-Agm (10^{-3} M) to release the I.

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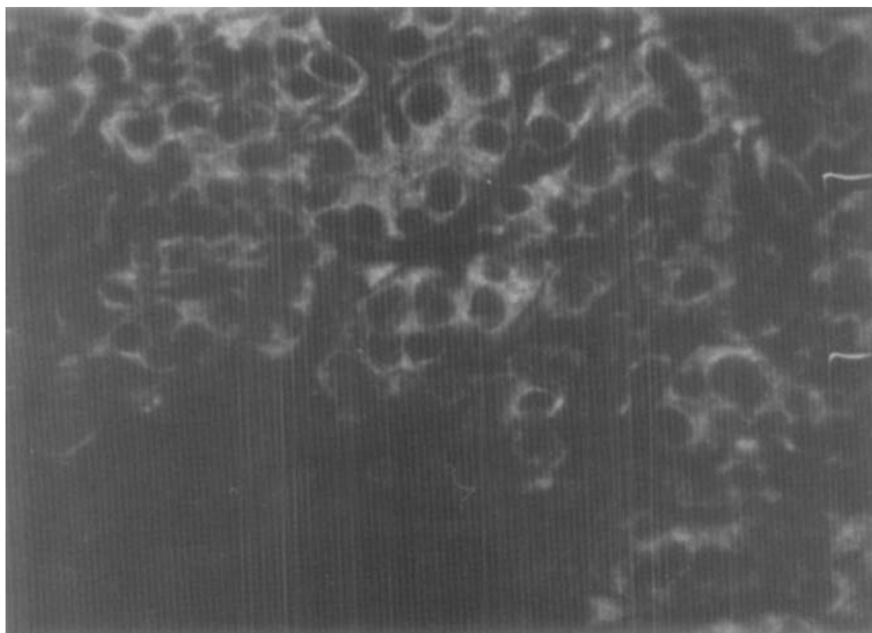

Figure 1 Frozen section of breast carcinoma stained with Rh-Agm. The nuclei of the carcinoma cells appear as dark central regions in each cell; the cell surface GB binds Rh-Agm and appears orange-yellow in these colour photographs, although when viewed down the microscope the cell surfaces fluoresce red. Magnification × 350. (See colour plates at rear).

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Washed cultured colonic carcinoma cells were used as a source of cytoplasmic inhibitors of GB. The washed cells were disrupted and soluble I extracted in isotonic saline. This soluble I was checked for its ability to inhibit GB on colonic carcinoma cells in *protected* sections. Washed carcinoma cells were treated with formaldehyde to fix these to the plastic surface of tissue culture flasks and to remove I from the cell surface,[8] leaving active GB on immobilised cell surfaces. This soluble I was allowed to wash over the immobilised cells for 1 h, then the cells were washed in isotonic saline and finally covered with a layer of Rh-Agm (10^{-3} M) for 1 h to release I. The liquid over the cells was collected, passed over a PD-10 column to separate the inhibitor protein from salts and free Rh-Agm. The protein fraction was freeze dried and redissolved in 200 μl water for testing for colonic GB inhibitors on targets of *protected* sections of colonic tissue.

Testing of Affinity Purified I on Colonic Epithelial Cell GB using 9AA as a Fluorescent Probe for Active GB

Direct staining of *protected* sections of normal and colonic carcinoma tissue with 9AA was carried out with 9AA (10^{-3} M) for 2 min followed by washing for 30 s in isotonic saline. Fluorescent microscopy with the Leitz Diaplan fluorescent microscope and filter cube [G] (Leitz catalogue no. 513602) in place revealed cells with active cell surface GB as yellow fluorescent cells (see Figures 4 and 7). Inhibition of *protected*

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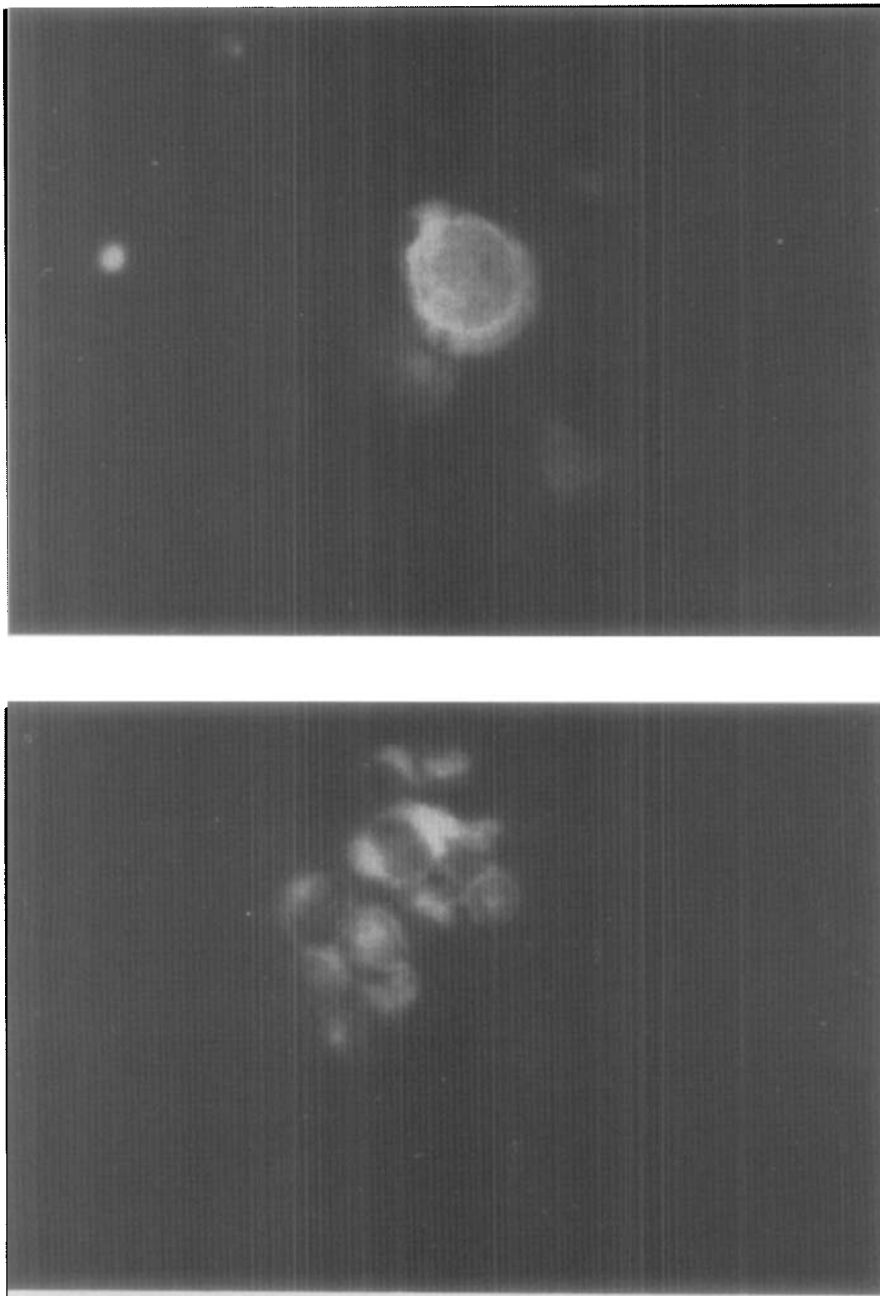

Figures 2 and 3 Breast carcinoma cells prepared as imprints and stained with Rh-Agm. An individual carcinoma cell (Figure 2) and a small clump of carcinoma cells (Figure 3) exhibit cell surface orange-yellow fluorescence. In Figure 2, normal cells do not exhibit cell surface fluorescence. Magnification × 700. (See colour plates at rear).

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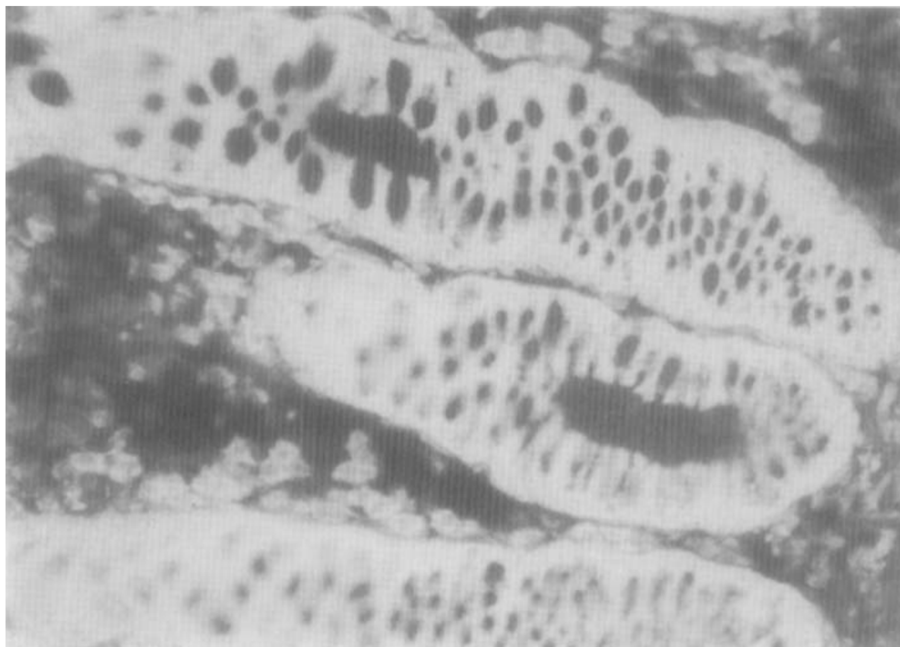

Figure 4 Protected section of normal colonic tissue stained directly with 9AA. Active GB binds 9AA and cells possessing active GB-9AA complex fluoresce yellow. The cell surfaces of normal colonic epithelial cells possess active GB. Magnification × 350. (See colour plates at rear).

sections was carried out by placing 10 μl of the affinity purified inhibitor over the tissue for 10 min followed by washing in isotonic saline and 9AA staining for 2 min. This was followed by a further wash in isotonic saline for 10 s prior to fluorescence microscopic examination (see Figures 5 and 8). The subsequent release of inhibitor was achieved by placing 10 μl SDS (10^{-4} M) over the section for 10 min, followed by washing in isotonic saline for 10 min and "direct" 9AA staining (as above). A regain in the ability to bind 9AA was indicated by the regain of cell surface yellow fluorescence (see Figures 6 and 9).

Colour Photography of Results

Kodak Ektachrome ASA 400 film was used throughout with the Leica MPS 46/52 Photoautomat photomicrographic system.

RESULTS AND DISCUSSION

General

The interaction of cell surface GB with Rh-Agm results in the formation of GB-Agm-Rh, the N-substituted agmatine binding to the active centre of GB. As a consequence, cells with active GB in a frozen section or in a spread fluoresce due to

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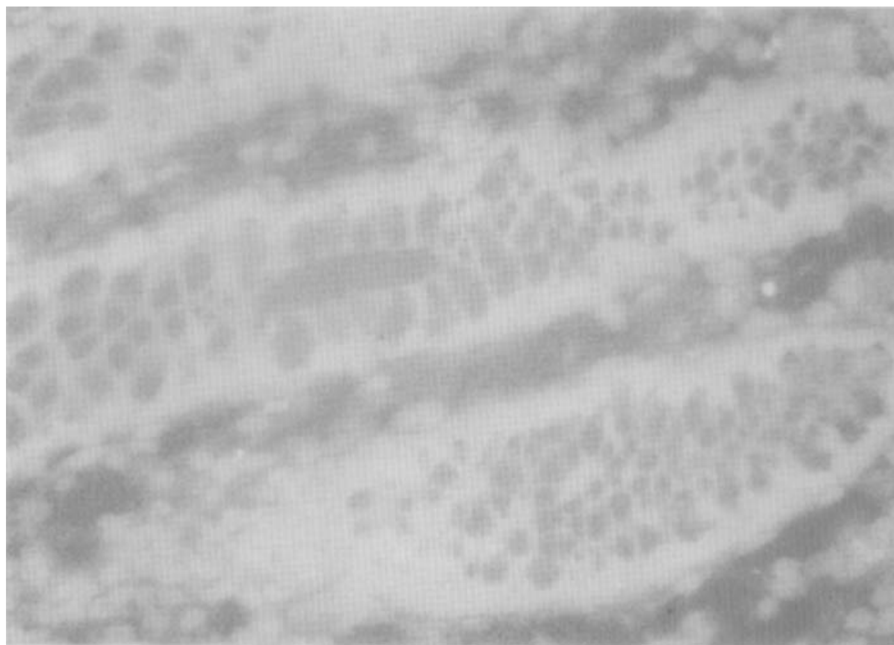

Figure 5 Protected section of normal colonic tissue challenged with affinity purified inhibitor followed by 9AA staining. *The cell surface GB has been recognised by the affinity purified inhibitor with the formation* of GB-I which fails to bind 9AA and the cell surfaces lack yellow fluorescence. Magnification × 350. (See colour plates at rear).

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the bound rhodamine moiety. When viewed with the Leitz Diaplan fluorescence microscope with filter cube [N] in place the cells exhibit red cell surface fluorescence. On filming with Kodak Ektachrome ASA 400 film, this red surface fluorescence is recorded as an orange-yellow colour depending on the intensity of fluorescence and the length of exposure.

Location of GB on Breast Carcinoma Cells with Rh-Agm

Frozen sections of breast carcinoma illustrate the cell surface location of GB after fluorescent staining with Rh-Agm (Figure 1). The central region of each cell carries the nucleus (see as a dark zone) whilst the cell surface exhibits orange-yellow ring staining with the fluroescent probe. It was of particular interest to observe that frozen sections and *protected* sections of breast carcinoma stained identically (the GB being located in all sections) with sections which had previously been exposed to inhibitor and had been shown to possess GB-I and which failed to bind 9AA. In the past we have used 9AA to distinguish between active GB and inactive GB-I on cell surfaces,[7] this distinction was not possible when Rh-Agm was used as a probe. This evidence suggests that Rh-Agm has a much higher affinity for GB than does 9AA and it is also able to dislodge I from GB-I and so form theGB-Agm-Rh complex which exhibits red fluoresence, characteristic of rhodamine.

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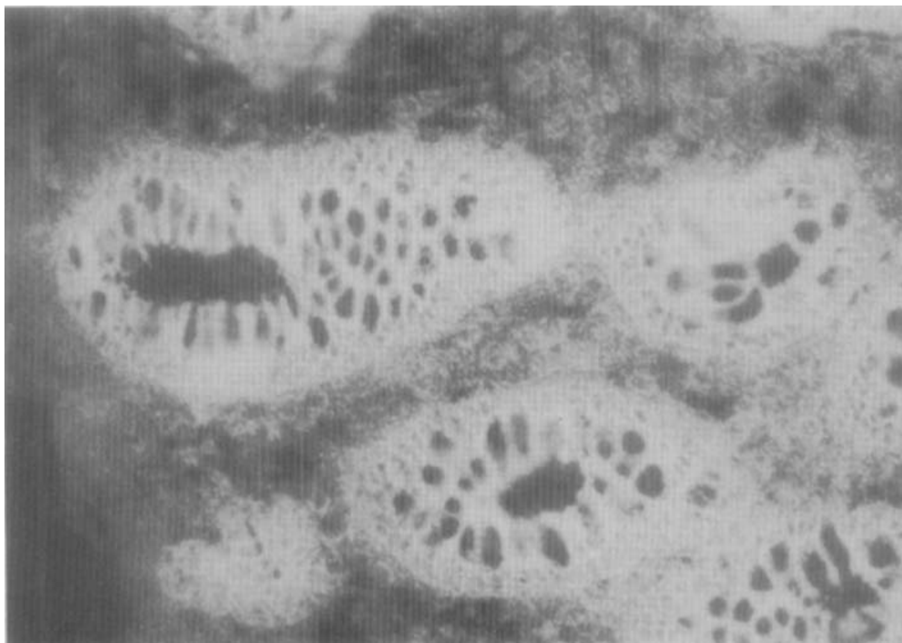

Figure 6 Protected section of normal colonic tissue, challenged with affinity purified inhibitor, treated with SDS and finally 9AA. The section (previously used for Figure 5) was treated with SDS to release I from GB-I and subsequently the reactivated GB was stained with 9AA. Clearly, the normal epithelial cell surface GB has regained activity and can be located with 9AA. This series (Figure 4–6) shows that the Rh-Agm, used in the affinity preparation of I from colonic carcinoma cells, displaced as inhibitor protein capable of recognising GB on normal colonic epithelial cells. Magnification x 350. (See colour plates at rear).

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It was important to determine whether the GB on the surface of intact cells could be recognised by Rh-Agm since we wished to use intact cells, immobilised on plastic surfaces, to capture I and later to release I in the presence of Rh-Agm (see below). Imprints obtained from freshly dissected breast carcinoma tissue were therefore used as a source of known, easily recognisable carcinoma cells as targets for Rh-Agm staining of cell surface GB. Conventional cytology of these imprints revealed the presence of authentic intact carcinoma cells as well as fat and other cells associated with breast tissue. Similar cells were analysed for GB activity in fine needle aspirates obtained from patients with breast carcinoma.[10] The carcinoma cells were easily recognised by their large nuclei and cell surface GB,[10] these cells were present either as individuals or as small clumps of cells.

In the imprints of breast carcinoma, the carcinoma cells were obviously stained with Rh-Agm (Figures 2 and 3) as individuals and as small clumps. The large nuclei of these cells can be seen as the dark central region whilst the cell surfaces exhibit red fluorescence recorded here as orange-yellow. It was confirmed with these imprints that carcinoma cells known to possess GB-I, as demonstrated by the lack of ability to bind 9AA, bound Rh-Agm equally well as those carcinoma cells known to possess active GB.

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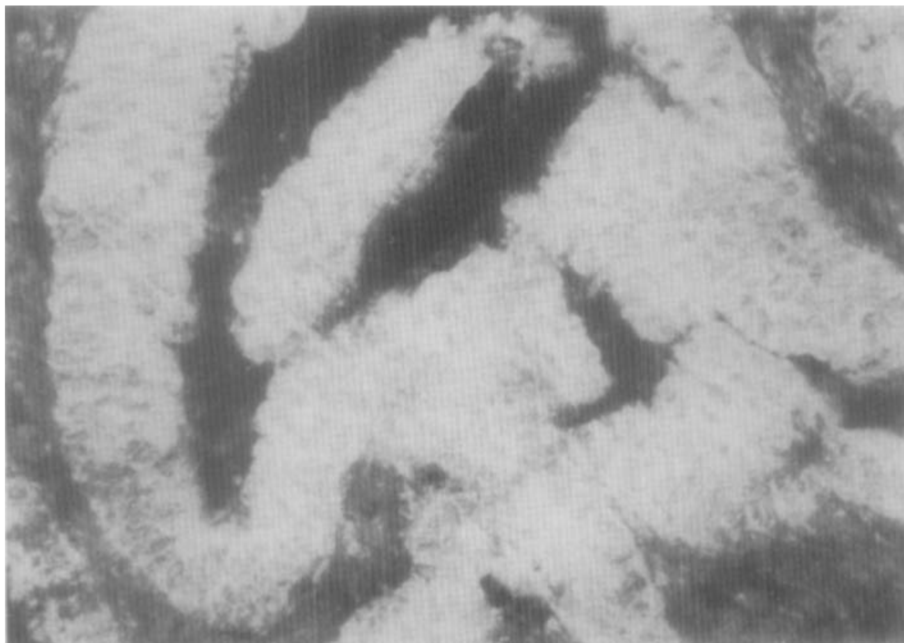

Figure 7 Protected section of colonic carcinoma tissue stained directly with 9AA. The cell surface GB is active, binds 9AA and the cells exhibit yellow fluorescence. Magnification × 350. (See colour plates at rear).

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Clearly, the Rh-Agm recognised cell surface GB on intact cells and was capable of displacing the inhibitor from GB-I associated with these cell surfaces. Rh-Agm should then be capable of dissociating the affinity bound I on the surfaces of immobilised colonic carcinoma cells which had been grown in monoculture conditions.

Testing of Affinity Purified I on Colonic Epithelial Cell GB using 9AA as a Fluorescent Probe for Active GB

GB binds 9AA and as a consequence, cells with GB-9AA fluoresce yellow. We can therefore use *protected* sections of normal and carcinoma colon as targets for the affinity purified inhibitor obtained from the colonic carcinoma cells. These *protected* sections lack local inhibitor proteins but the epithelial cell possess active GB[7] and the inhibition of this GB can be followed by the subsequent loss in ability to bind 9AA if inhibition has taken place. The inhibitor may be displaced from the GB-I complex by treatment with sodium dodecyl sulphate (SDS)[11,12,13] followed by a regain in GB activity and regain in the ability to bind 9AA.[11]

In the first series of experiments *protected* frozen sections of normal human colon were employed (Figures 4, 5 and 6) whilst in the second series *protected* frozen sections of colonic carcinoma were employed (Figures 7, 8 and 9). The GB on normal colonic epithelial cells binds 9AA (Figure 4) and the cell surfaces fluoresce yellow. After exposure of a *protected* section of normal colonic tissue to the Rh-Agm affinity

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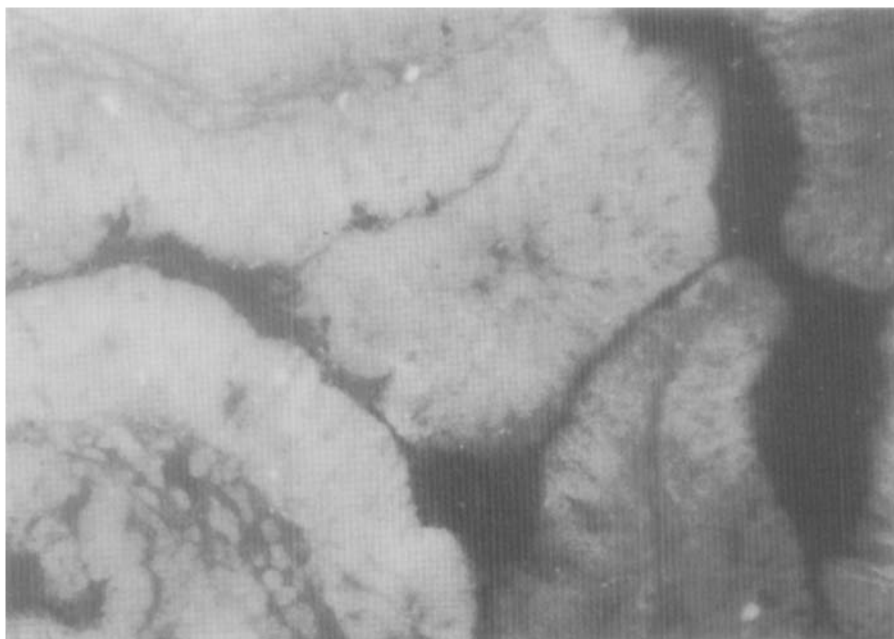

Figure 8 Protected section of colonic carcinoma tissue challenged with affinity purified inhibitor followed by 9AA staining. The inhibitor protein recognised the carcinoma cell surface GB forming a GB-I complex and failed to bind 9AA. The cells therefore lack yellow surface fluorescence. Magnification × 350. (See colour plates at rear).

purified I, the epithelial cells failed to bind 9AA and exhibited no cell surface yellow fluorescence (Figure 5). This would imply that the inhibitors obtained from the colonic carcinoma cells in culture had recognised the GB on normal colonic epithelial cells preventing the GB binding 9AA. This interpretation of the lack of fluorescence was confirmed by treatment of the section with SDS, an agent which releases protein inhibitors from t-PA[12,13] and cell-bound GB[11] with regain in enzymic activity (Figure 6).

The GB on colonic carcinoma cells in *protected* sections (Figure 7) was also regonised by the Rh-Agm affinity purified I resulting in failure of the cell surface GB to bind 9AA (Figure 8). Again this inhibition was reversed by SDS treatment and subsequent exposure to 9AA (Figure 9).

The data presented in Figures 4–9 above clearly demonstrate that the colonic epithelial cell surface GB is inhibited by a protein fraction relased from the affinity bound GB-I on the surface of colonic carcinoma cells. The release of these inhibitors was brought about by the use of Rh-Agm as a competing ligand for the active centre of GB; this displaced the I, later to be tested on the colonic epithelial cell surface GB isoenzymes. Since normal colonic epithelial cell GB is a distinct isoenzymic form from the GB specific for colonic carcinoma cells, the purified inhibitor fraction must have contained two isoinhibitors for GB; one for normal and one for carcinoma specific GB.[8] These colonic specific inhibitors were unable to recognise GB on other types of epithelial carcinoma cell surfaces.[8]

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Figure 9 Protected section of colonic carcinoma tissue challenged with affinity purified inhibitor, treated with SDS and finally with 9AA. The section (previously used for Figure 8) was treated with SDS to release I from GB-I. The reactivated GB then bound 9AA and the cells regained their yellow fluoresence. Magnification × 350. (See colour plates at rear).

In conclusion, Rh-Agm recognises both active GB and latent GB (GB-I) on cell surfaces. The interaction with GB-I results in the fluorescent labelling of the cell surface GB and the release of active I. We can take advantage of both of these events by using Rh-Agm to locate cells possessing GB amongst many cells lacking GB (for example in cervical smear analysis) and in the preparation of cell specific inhibitor proteins from cultured cell lines of cells (such as colonic carcinoma cells).

Acknowledgements

FSS wishes to thank the Wigan and District Cancer Research Committee, Joseph T. Starkey Memorial Fund, as well as the Association for International Cancer Research for their generous funding which allowed this study to be carried out in the University of Manchester.

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