J. Enzyme Inhibition, 1987, Vol. 1, pp. 275–287
Photocopying permitted by license only

THE ROLE OF INHIBITORS IN THE FLUORESCENT STAINING OF BENIGN NAEVUS AND MALIGNANT MELANOMA CELLS WITH 9-AMINO ACRIDINE AND ACRIDINE ORANGE

FRANK S. STEVEN,[§†] UMA SURESH,[‡] THERESIA L.H. WONG[†] and MARGARET M. GRIFFIN[†]

[†] *Department of Biochemistry and Molecular Biology, School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, UK.* [‡] *Department of Histopathology, Withington Hospital, Nell Lane, West Didsbury, Manchester M20 8LR, UK*

(Received October 31, 1986)

Guanidinobenzoatase is a trypsin-like protease capable of degrading fibronectin. An inactive form of guanidinobenzoatase is present on the surface of benign naevus cells and these cells stain very weakly with 9-aminoacridine, a known competitive inhibitor of guanidinobenzoatase. Malignant melanoma and metastatic malignant melanoma cells exhibit strong surface staining with 9-aminoacridine and also exhibit strong staining of cytoplasmic RNA with acridine orange. These simple fluorescent techniques have been used to distinguish benign naevus cells from malignant melanoma cells in human skin sections. This difference in cell surface staining with 9-aminoacridine has been demonstrated to be caused by the presence or absence of an inhibitor. The inhibitor can be displaced from the cell surface enzyme and then replaced by an affinity purified inhibitor obtained from fresh liver homogenates. It is proposed that the inhibition or control of cell surface guanidinobenzoatase may be one of the regulatory mechanisms by which benign naevus cells are prevented from developing into malignant melanoma cells.

KEY WORDS: Protease, guanidinobenzoatase, cell surface, benign, malignant, inhibitor.

INTRODUCTION

Some authors[1] consider epidermal melanocytes and naevus cells to be identical. Many malignant melanoma contain cells which can be classified histologically as residual benign naevus cells. Muir[2] states that melanocytes migrate into the dermis and proliferate to form pigmented naevi. Those pigmented naevi with junctional activity have the potential to undergo malignant changes becoming malignant melanoma. The varied morphology of malignant melanoma cells may further complicate histological examination and diagnosis. It would seem to be of value to examine the enzymic status and functional potential of benign naevus cells and malignant melanoma cells in the hope of providing additional information to that obtained by classical histological techniques. We have chosen to study the distribution of a protease, guanidinobenzoatase,[3] which is known to be associated with the surface of tumour cells and cells capable of migration.[4–6] This enzyme may be located by the ability of the cell surface to bind the fluorescent probe 9-aminoacridine.[4] Typical malignant melanoma and metastatic malignant melanoma cells possessed cell surface guanidinobenzoatase and

§Correspondence.

fluoresced strongly when treated with 9-aminoacridine. Typical benign naevus cells possessed negligible guanidinobenzoatase activity and remained virtually unstained by 9-aminoacridine. Acridine orange staining of cytoplasmic RNA was employed to locate cells actively synthesising protein according to the method of Bertalanffy *et al.*[7] This RNA staining technique confirmed the results of the 9-aminoacridine and conventional histological analyses. An explanation for the observed difference in enzymic activity found in benign naevus cells and malignant melanoma cells is presented on the basis of inhibition studies.

MATERIALS

Acridine orange and 9-amino acridine were purchased from Sigma Chemical Company, St. Louis, Mo. USA. Ribonuclease (1-A, 5 times crystallised) was also obtained from Sigma. Wax embedded sections were kindly provided by the Histopathology Department, Withington Hospital, Manchester. In all we examined a number of sections from each of 61 formalin fixed, wax embedded blocks of human tissue. These comprised 5 normal skin, 33 benign naevi, 15 malignant melanoma, 5 lymph nodes with metastatic malignant melanoma, 1 gall bladder with metastatic malignant melanoma and 2 normal lymph nodes with reactive changes.

METHODS

Dewaxed sections were first examined by fluorescent microscopy after staining with 9-aminoacridine[4] and then by conventional microscopy of corresponding haematoxylin-eosin stained sections. The results of these two independent analyses were then compared.

Fluorescent staining with 9-aminoacridine was carried out as previously described.[4] This consisted of dipping the dewaxed sections in a tank of 9-aminoacridine (10^{-3} M) plus *N*-tosyl-L-lysine chloromethyl ketone (10^{-5} M) dissolved in water for 2 min. The excess reagent was drained from the surface of the slides and the slides washed for 2 min in a series of three tanks containing isotonic saline. The whole process should take 8 min and is very cheap to carry out.

The stained slides were mounted in water under a cover slip and examined by a Leitz orthoplan fluorescent microscope employing violet illumination and filters set in positions 2 and 2. These filter settings correspond closely to the 513–599 filter system B_2, or 513–596 filter system A, or 513–600 filter system D of the new Leitz microscopes. Sections were stained with acridine orange as described by Bertalanffy *et al.*,[7] and examined under the same microscopic conditions as described above. Digestion with ribonuclease A (1 mg/ml) was carried out for 1 h at 20°C prior to fluorescent staining. The displacement of the inhibitor was carried out with 10% v/v formaldehyde in isotonic saline for 18 h at room temperature followed by 9-amino acridine staining as described above. The replacement of the inhibitor was achieved by incubating the section on a slide in a fresh aqueous homogenate of bovine liver[8] for 18 h, followed by washing the slide and subsequent analysis by 9-amino acridine staining of the section.

An affinity purified inhibitor was prepared from fresh bovine liver.[8] This inhibitor contained 5 μg protein/ml.

RESULTS AND DISCUSSION

9-aminoacridine binds to the surface of cells possessing guanidinobenzoatase and these cells appear to fluoresce yellow when examined under the appropriate conditions.[4] Cells which lack this surface enzyme appear blue, as does the connective tissue.

Past experience with human skin sections enabled us to recognise and ignore normally present cells with the ability to migrate and which bound 9-aminoacridine viz. epithelial cells, sweat glands and hair follicles. These cells exhibited surface fluorescence and could be recognised in all the skin sections examined in this study.

Metastatic Malignant Melanoma Cells

We examined sections from 5 lymph nodes containing metastatic malignant melanoma. In every case the malignant melanoma cells possessed intense surface fluorescence after treatment with 9-aminoacridine (Figure 1). Invasive lymphocytes were also stained, but could easily be distinguished from the melanocytes on the basis of differences in size and intensity of fluorescent staining. We also examined a gall bladder containing metastatic malignant melanoma, again the malignant cells were intensely fluorescent and could be clearly defined (Figure 1). There were no unstained melanocytes in these lymph nodes nor in the gall bladder. It was noticeable that the malignant melanocytes in each of these sections showed no variation in the intensity of fluorescent staining; each cell was intensely stained. Normal lymph nodes exhibited no fluorescent cells with the appearance of typical malignant melanoma cells. This fluorescent study was confirmed later by an independent analysis employing conventional haematoxylin-eosin stained sections from all these tissues.

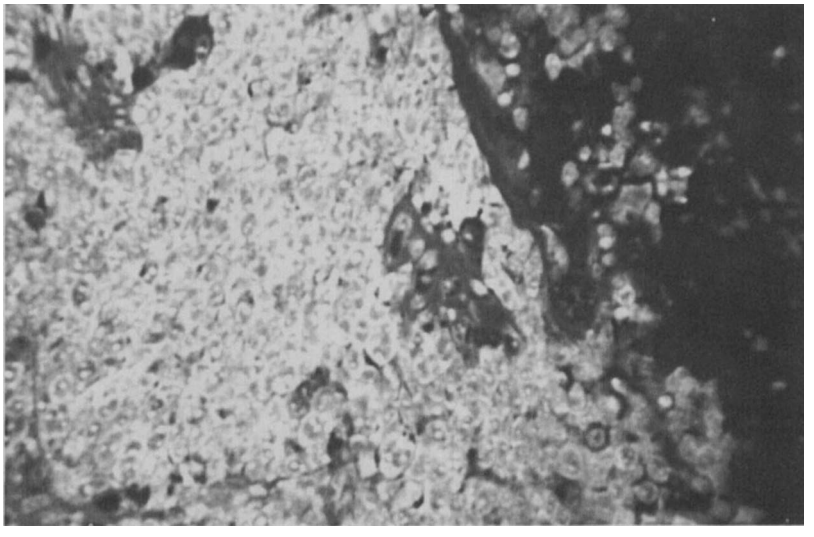

FIGURE 1 9-aminoacridine staining of metastatic malignant melanoma of the gall bladder. Malignant melanoma cells exhibit strong cell surface fluorescent staining. Magnification × 240. (See Colour Plate I).

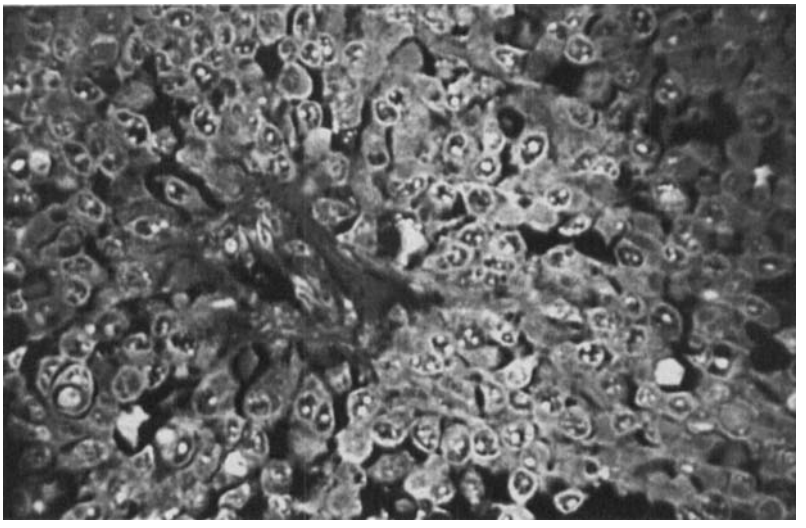

FIGURE 2 9-aminoacridine staining of malignant melanoma of the skin. Malignant melanoma cells exhibit strong cell surface staining and also staining of the nucleolus. Magnification × 240. (See Colour Plate II).

Malignant Melanoma

Sections of malignant melanoma usually exhibited two classes of melanocytes distinguished by their ability to bind 9-aminoacridine and to exhibit fluorescence. Firstly, melanocytes which exhibited intense surface fluorescence when stained with 9-aminoacridine; these cells appeared to behave identically to the metastatic malignant

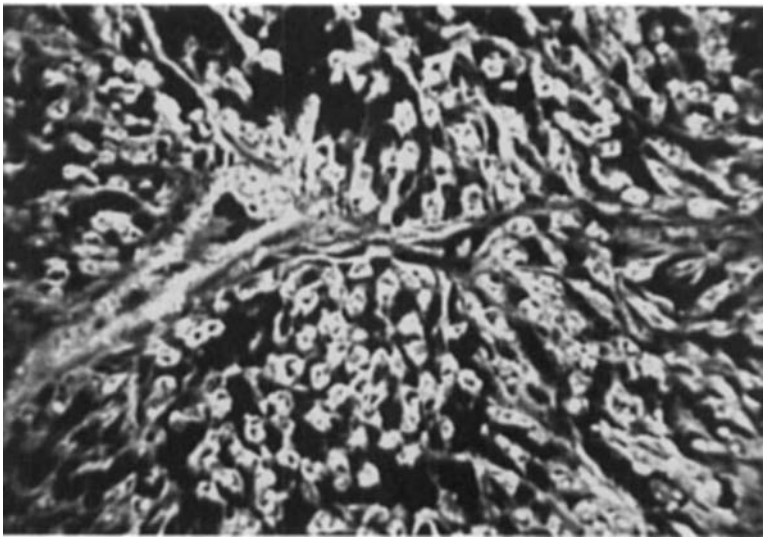

FIGURE 3 9-aminoacridine staining of malignant melanoma of the skin. Malignant melanoma cells exhibit strong surface fluorescent staining – note the difference in shape and size of these melanoma cells and those shown in Figure 2. Magnification × 240. (See Colour Plate III).

melanoma cells observed in the lymph nodes and gall bladder. We consider these melanocytes to be typical of malignant melanoma (Figures 2 and 3), whether they are located in the skin or the lymph nodes. Secondly, melanocytes which were very weakly fluorescent and lacked surface guanidinobenzoatase; in this report we consider these cells to be negative or unstained with 9-aminoacridine in contrast to the obviously stained malignant melanoma cells. The 15 malignant melanoma skin sections were all correctly diagnosed by this fluorescent technique before the conventional analysis with haematoxylin-eosin was known. In reaching our diagnosis, based upon the fluorescent technique, we classified those sections which contained predominantly the intensely staining melanocytes (see above) as malignant melanoma. In some sections there were regions containing only those cells typical of malignant melanoma (Figures 2 and 3).

Benign naevus cells

In three skin sections we observed that all the melanocytes were unstained and therefore lacked guanidinobenzoatase activity. We believe these melanocytes are typical of the benign naevus and lack the ability to metastasise possibly because they lack guanidinobenzoatase (Figures 4 and 5), an enzyme known to degrade fibonectin.[8] We also observed that some of the benign naevi possessed two populations of melanocytes just as we observed in the malignant melanoma. In the case of the benign naevi, however, the *predominant* cell type was the unstained melanocyte lacking guanidinobenzoatase activity. On the basis of this predominance of melanocyte type (viz. benign naevus type of Figures 4 and 5) we correctly classified the 33 benign naevi examined in this study. We use the term "correctly classified" as being in agreement with the independent diagnosis by classical histological examination of the haema-

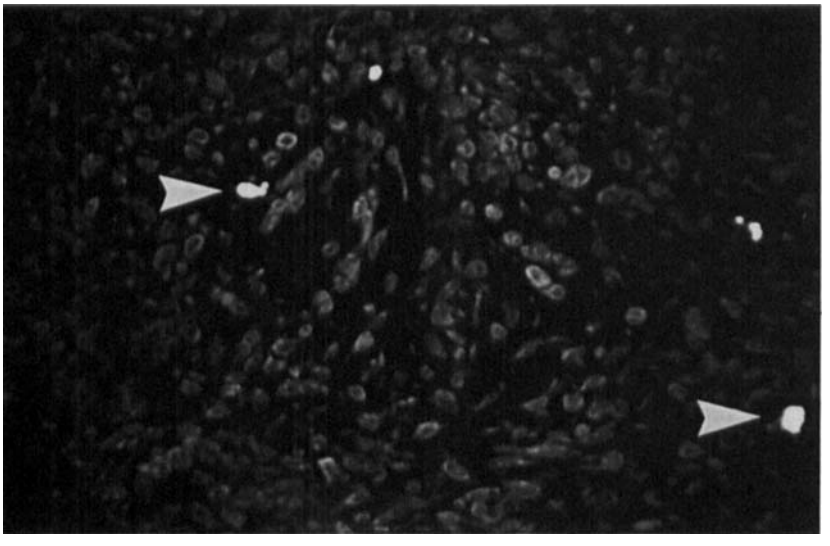

FIGURE 4 9-aminoacridine staining of benign naevus of the skin. The benign naevus cells stain very weakly with 9-aminoacridine and are described as negative in the text. The arrows indicate mast cells. Magnification × 240. (See Colour Plate IV).

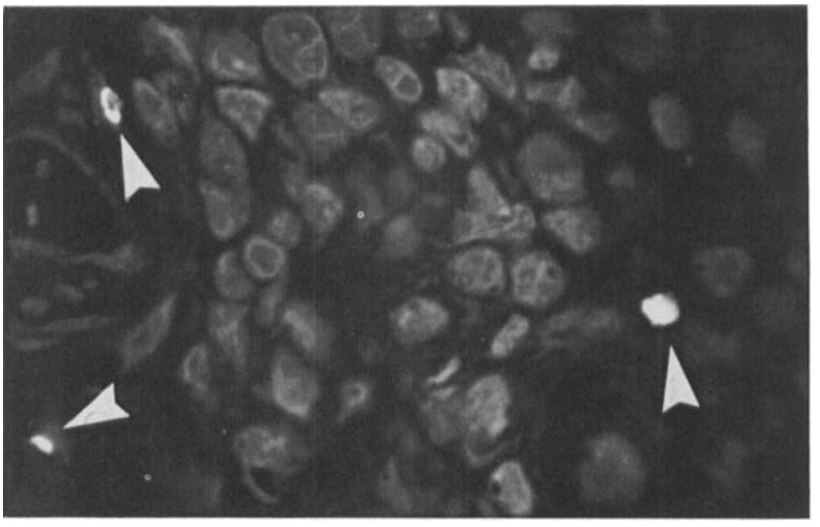

FIGURE 5 9-aminoacridine staining of benign naevus of the skin. The benign naevus cells stain weakly, in spite of the fact that a water immersion lens was used in obtaining this picture at higher magnification. Mast cells can also be seen (arrows) and can be characterised by their failure to stain with propidium iodide. Magnification × 480. (See Colour Plate V).

toxylin-eosin stained sections which was carried out *after* the fluorescent analysis had been completed. We conclude that 9-aminoacridine clearly distinguished typical benign naevus cells from typical malignant melanoma cells.

Acridine Orange Staining

Malignant melanoma cells differ greatly in their size and shape. The large multinucleated malignant melanoma cells exhibited intense red RNA staining in the cytoplasm with acridine orange (Figure 6). The cytoplasm of benign naevus cells (Figure 7) stained yellow–brown. These observations are consistent with those described by Bertalanffy[7] in a series of papers on the fluorescent location of tumour cells based upon the polychromic staining properties of acridine orange. Bertalanffy observed that the cells with large amounts of cytoplasmic RNA were those cells which were very actively engaged in protein synthesis, as seen for example in tumour cells. As the concentration of RNA increased in the cytoplasm the quantity of bound acridine orange increased with a consequent change in fluorescence from yellow to red.

We observed that those skin sections which we had classified as benign by the 9-aminoacridine procedure and by conventional histochemistry, exhibited melanocytes with predominantly yellow-brown cytoplasm as opposed to the malignant melanocytes in which the cytoplasm stained red with acridine orange (compare Figures 6 and 7). We further confirmed that this cytoplasmic binding of acridine orange was related to the RNA content by demonstrating the loss of red staining with acridine orange after 1 h treatment with ribonuclease. Similar findings were reported by Bertalanffy.[7]

We concluded that both the 9-aminoacridine and acridine orange fluorescent

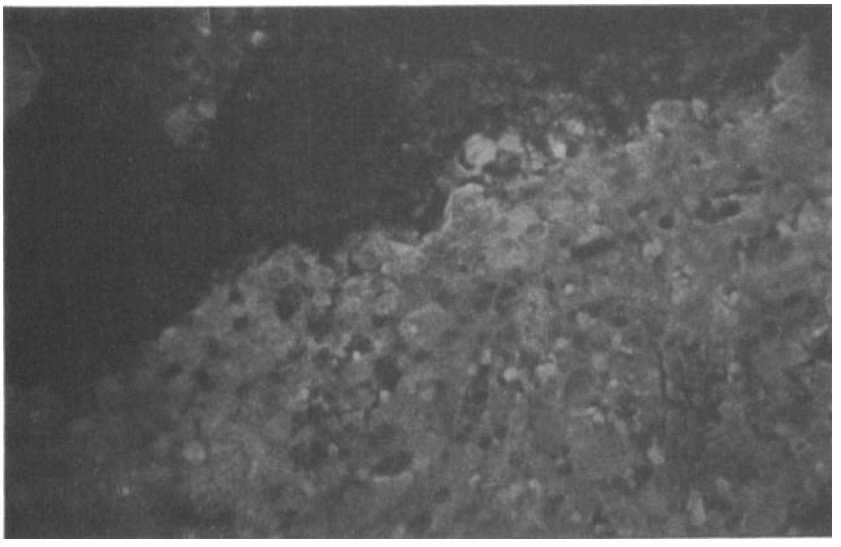

FIGURE 6 Acridine orange staining of malignant melanoma in skin. The cytoplasm of the malignant melanoma cells contained higher concentration of RNA which results in a red fluorescence of bound acridine orange.[7] Magnification × 240. (See Colour Plate VI).

techniques were able to distinguish the typical benign naevus cell from the malignant melanoma cell. Taken together, we believe these two techniques can provide a valuable aid to the differentiation of individual melanocytes in cases of benign naevus and malignant melanoma.

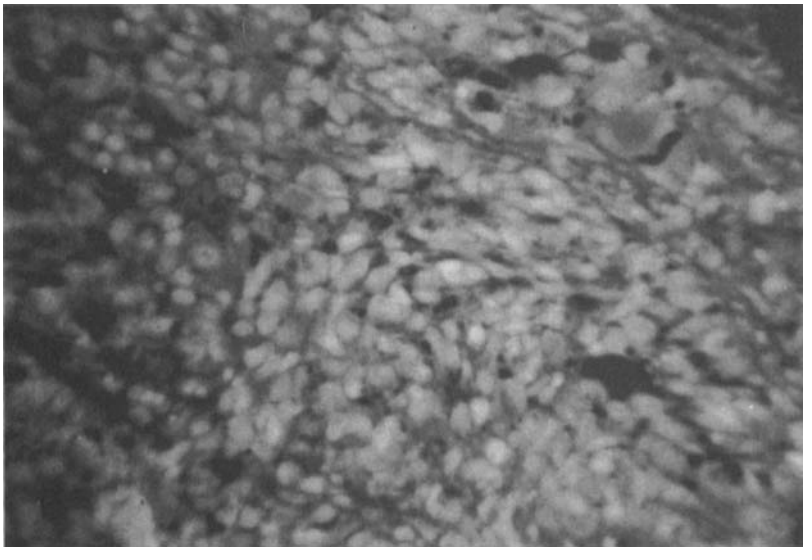

FIGURE 7 Acridine orange staining of benign naevus of the skin. The cytoplasm of the benign naevus cells contained a low concentration of RNA which results in a brown yellow fluorescence of bound acridine orange.[7] Magnification × 240. (See Colour Plate VII).

Acridine orange can be removed from the stained section by placing the slide in ethanol:water (1:1) for 10 min. The destained slide can be subsequently stained with 9-aminoacridine. Those cells which could be classified as malignant melanoma by their cytoplasmic binding of acridine orange to the RNA (red) now appeared to be intensely staining with 9-aminoacridine and again would have been classified as typical malignant melanoma cells by this technique used on its own.

It was observed that after ribonuclease treatment of part of a section containing the large malignant melanoma cells (e.g. Figures 2 and 6) and subsequent 9-aminoacridine staining of the whole section, the cytoplasm of the ribonuclease-treated cells appeared less stained than the cytoplasm of the untreated cells. This suggested that the 9-aminoacridine staining of these large multinucleated cells was in part due to the binding of 9-aminoacridine to cytoplasmic RNA. In this situation, the staining of the cell surface and the cytoplasmic RNA are both indicators of malignant melanoma and this enhances the differentiation of these cells from those of the typical benign naevus after 9-aminoacridine staining.

The ability of 9-aminoacridine staining to distinguish between melanocytes which are typical of benign naevus cells and those cells typical of malignant melanoma depends upon the difference in binding affinity of the fluorescent probe for a cell surface receptor (guanidinobenzoatase). It should be possible to provide a rational explanation in terms of macromolecules for these observed differences.

The lack of 9-aminoacridine binding to benign naevus cells implies either that these cells lack guanidinobenzoatase or that the cells possess an inhibited form of guanidinobenzoatase. An inhibited form of guanidinobenzoatase would further imply that the inhibitor was located on the enzyme *in vivo*, prior to tissue fixation and wax embedding. The presence of an inhibited enzyme could be established if the putative inhibitor was experimentally displaced from the enzyme with the subsequent ability to stain these modified cells with 9-aminoacridine. Previous studies[8] have shown that guanidinobenzoatase can be inhibited by a protein of approximate molecular weight 67000, which may be conveniently obtained in a crude extract of fresh liver. This inhibitor could be displaced from the cell bound enzyme by treatment of the slide with formaldehyde or with guanidine hydrochloride,[8] agents which are known to disturb the conformation of proteins. We therefore took skin sections which had previously been correctly classified as being benign naevi or malignant melanoma by 9-aminoacridine staining and fluorescent microscopy. These sections were placed in isotonic saline containing 10% v/v, formaldehyde at pH 7.5 for 18 h and then restained with 9-aminoacridine as described above. We observed that *all* the melanocytes now showed intense surface fluorescence and exhibited the typical appearance of malignant melanoma cells. A second independent analysis of these treated sections by a colleague (who was unaware of the treatment) resulted in her opinion, that each of the sections represented typical malignant melanoma on the basis of their cell surface fluorescence. This minor deception was used to provide convincing evidence that we could experimentally convert benign naevus cells to cells indistinguishable from malignant melanoma cells, when stained with 9-aminoacridine. We concluded that the typical benign naevus cell possessed masked guanidinobenzoatase which could be unmasked by the displacement of an inhibitor. The ability to displace an inhibitor from cell surface guanidinobenzoatase has previously been shown to be reversible. We therefore exposed the formalin treated skin and gall bladder sections (obtained as described above) to a fresh aqueous extract of bovine liver for 18 h, washed the slides in saline and then restained with 9-aminoacridine. The melanocytes now failed to bind

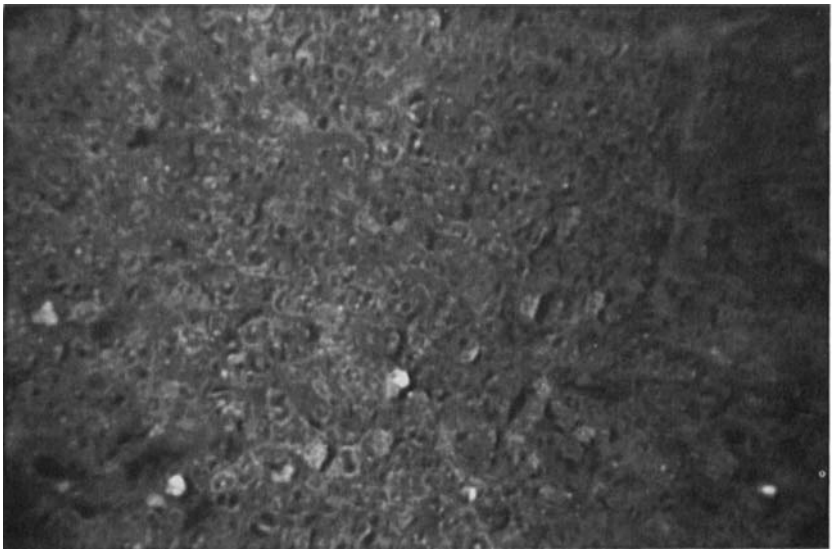

FIGURE 8 9-aminoacridine staining of metastatic malignant melanoma of the gall bladder after treatment with the liver extract containing inhibitor(s) of guanidinobenzoatase. The malignant melanoma cells are now unable to bind 9-aminoacridine due to the presence of the experimentally added liver inhibitor of guanidinobenzoatase (compare Figure 1 and Figure 9). Magnification × 240. (See Colour Plate VIII).

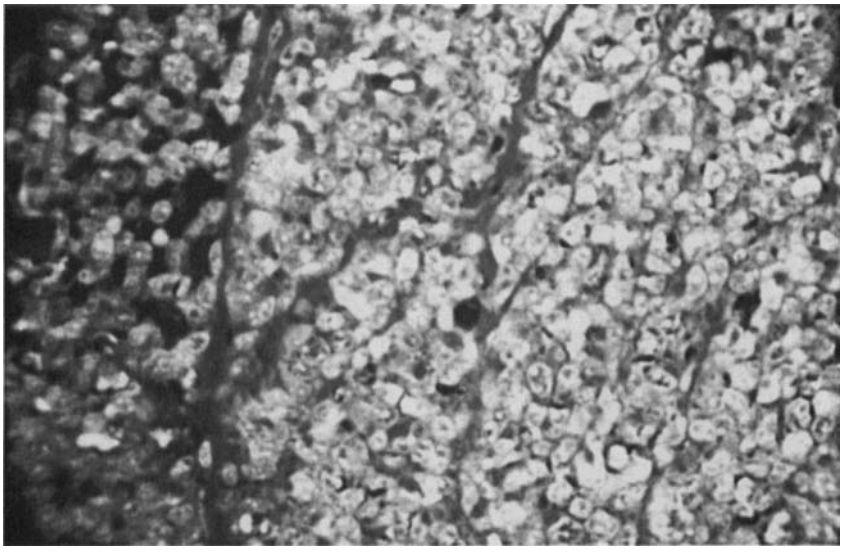

FIGURE 9 Displacement of inhibitor from metastatic malignant melanoma of the gall bladder followed by 9-aminoacridine staining. The same section which was filmed for Figure 8 was treated with formaldehyde to displace inhibitors and restained with 9-aminoacridine. This resulted in a marked recovery of ability to bind 9-aminoacridine and demonstrated the reactivation of guanidinobenzoatase after the displacement of the liver inhibitor (compare Figures 1, 8 and 9). Magnification × 240. (See Colour Plate IX).

9-aminoacridine and appeared to be typical benign naevus cells in all the sections, including those sections which had originally been "correctly classified" as malignant melanoma.

This exchange of inhibitors is illustrated with metastatic malignant melanoma cells (Figures 1, 8 and 9). Direct 9-aminoacridine staining resulted in intense cell surface staining (Figure 1), after overnight exposure to the bovine liver extract, the metastatic malignant melanoma cells failed to exhibit surface staining (Figure 8). It could be argued that the liver extract hydrolysed or dissolved the component binding the fluorescent probe and thus destroyed the ability of these cells to fluoresce. Treatment with formaldehyde (Figure 9) restored this ability to bind the probe and thus excluded the possibility that the liver extract had removed the binding site for the probe. The evidence would indicate the presence of an inhibitor in the liver extract.

In order to be more precise in defining the loss of ability to bind 9-aminoacridine with an inhibitor, we employed an affinity purified liver inhibitor of guanidinobenzoatase[8] instead of the crude liver extract. We used a benign naevus to illustrate this exchange (Figures 4, 10 and 11). The direct staining of the melanocytes of the benign naevus is only weak and has been considered to be "negative" in this report (Figure 4). Treatment with formaldehyde resulted in binding of the fluorescent probe and the cells appearing to stain in the same manner as malignant melanoma cells (Figure 10). Further treatment of this slide with the purified inhibitor resulted in loss of binding ability (Figure 11) which could be regained by a subsequent treatment with formaldehyde (not shown). These experiments with the purified inhibitor confirmed that the

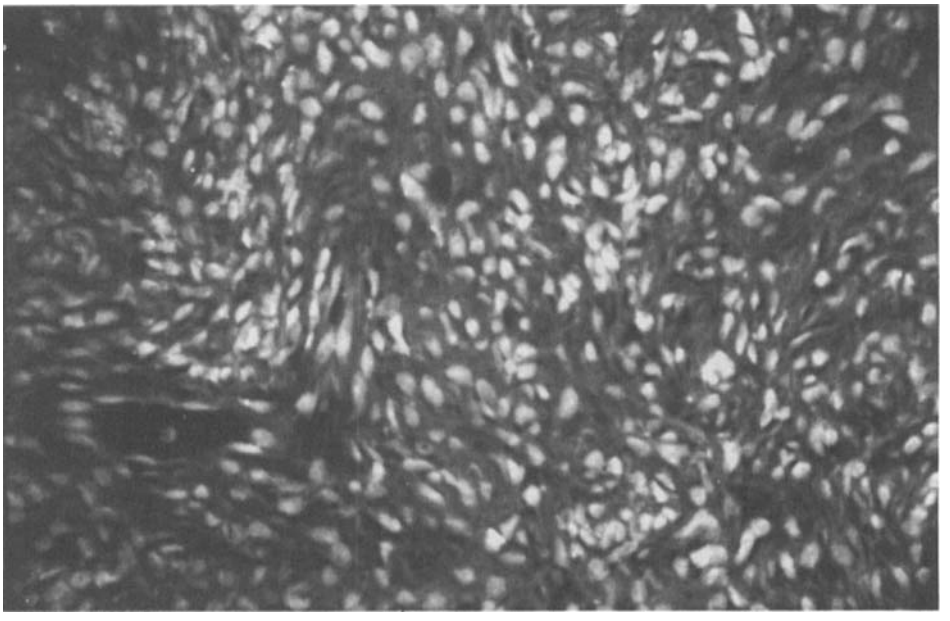

FIGURE 10 Benign naevus of the skin after displacement of the inhibitor and followed by 9-aminoacridine staining. This section was the same as that used to obtain Figure 4 by direct staining with 9-aminoacridine. In Figure 10 the section was treated with formaldehyde prior to 9-aminoacridine staining, which resulted in the displacement of inhibitor from the benign naevus cells; as a result these cells stained strongly and had the same appearance as malignant melanoma cells. Magnification × 240. (See Colour Plate X).

SCHEME I

Reversible exchange of inhibitors on melanocytes

Step	Treatment		Observations on cells	Enzymic status	Fluorescent diagnosis	Comment
Benign Naevi Sections (See Figures 4 and 5)						
1.	None	Stain	No surface staining	−	Benign Naevus	"Correct classification" by H/E
2.	Formaldehyde 18 h	Restain	Intense surface staining	+	Appears identical to malignant melanoma	Loss of inhibitor
3.	Liver extract 18 h	Restain	No surface staining	−	Benign Naevus	Replace inhibitor
Malignant Melanoma Sections (See Figures 2 and 3)						
1.	None	Stain	Intense surface staining	+	Malignant Melanoma	"Correct classification" by H/E
2.	Formaldehyde 18 h	Restain	Intense surface staining	+	Malignant Melanoma	No change
3.	Liver extract 18 h	Restain	No surface staining	−	Appears benign	Inhibitor on enzyme
4.	Formaldehyde 18 h	Restain	Intense surface staining	+	Appears malignant melanoma	Loss of inhibitor

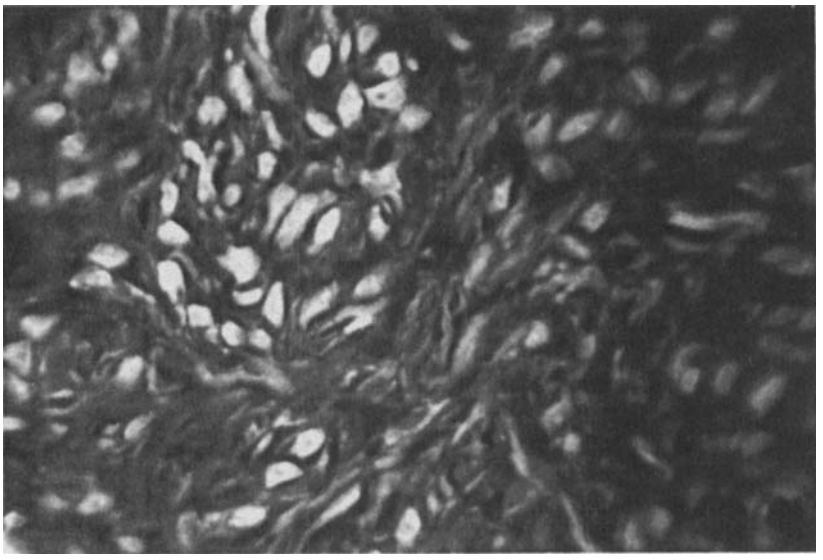

FIGURE 11 Benign naevus cells after displacement of the natural skin inhibitor and replacement with purified rat liver inhibitor for guanidinobenzoatase stained with 9-aminoacridine. The same slide used to film Figure 10 was incubated with affinity purified rat liver inhibitor prior to 9-aminoacridine staining. The binding of fluorescent probe has been inhibited and the benign naevus cells have been returned to their original enzymic status (Figure 4), i.e. enzyme-inhibitor complex. This situation could be reversed by formaldehyde treatment with the result that the benign naevus cells now appeared strongly fluorescent as in Figure 10. Magnification × 240. (See Colour Plate XI).

successful fluorescent diagnosis of benign and malignant cells was dependent upon the activity of guanidinobenzoatase since the inhibitor was purified on an affinity support with guanidinobenzoatase as a ligand. These results are summarised in Scheme 1.

We believe these results can be explained in the following manner. Malignant melanoma cells possess active guanidinobenzoatase which enables these cells to exhibit strong surface staining with 9-aminoacridine, a competitive inhibitor of this enzyme. This ability to bind 9-aminoacridine can be largely prevented by preincubation with the liver inhibitor. On the other hand the typical benign naevus cell does not initially bind 9-aminoacridine due to the presence of an inhibitor. Displacement of this inhibitor results in the naevus cell having identical ability to bind 9-aminoacridine to the malignant melanoma cell; indeed the two types of melanocyte now appear to be indistinguishable by fluorescent microscopy under these experimental conditions. This exchange is reversible.

Since the typical benign naevus cell can be clearly distinguished from the typical malignant melanoma cell by means of 9-aminoacridine fluorescence (compare Figures 4 and 5 with Figures 2 and 3) then this distinction is controlled by the presence (in naevus) or absence (in melanoma) of an inhibitor on the surface enzyme of the respective melanocytes. We know that guanidinobenzoatase is a protease capable of cleaving[8] the linkage peptide, GlyArgGlyAsp, in fibronectin.[19] It would make sense in biochemical terms for the malignant melanoma cells to possess an active form of guanidinobenzoatase whilst the benign naevus cells should possess an inactive enzyme. On the evidence presented above, it would therefore seem that the potential for malignancy may be controlled by the presence or absence of an inhibitor of a cell

surface protease on melanocytes. We recognise of course that this enzyme is only *one* marker for potential malignancy and other control mechanisms may also be present *in vivo*. At present it is possible to employ this enzymic marker as an aid to diagnosis of the benign and malignant melanocyte. The significance of the biological control of this marker enzyme and its inhibition must await further research into how this knowledge can be taken advantage of in the course of possible therapy.

It could be argued that the preparation of the original skin involved fixation in formaldehyde and that this process should displace the inhibitor from the enzyme on the surface of melanocytes. We believe the high molecular weight of the inhibitor (67000 for liver)[8] prevents the free diffusion of the naturally occurring inhibitor from within the mass of tissue during formaldehyde fixation. On the other hand, the diffusion of the inhibitor from the cell surface of the thin section, promoted by formaldehyde treatment, is much easier to accept. This argument is supported by the experimental inhibition of the cell surface enzyme by the liver inhibitor. If we can exchange inhibitors and control the enzymic status of the melanocyte in thin sections at will, it is clear that we can observe the effects of inhibitor diffusion in thin sections. It also follows that a mechanism of biological control is by inhibition which is experimentally reversible.

We conclude that melanocytes of the typical benign naevus and malignant melanoma can be distinguished by both 9-aminoacridine and by acridine orange staining; these fluorescent probes locate a cell surface protease and cytoplasmic RNA respectively. The control of the cell surface protease on melanocytes is exercised by the presence or absence of an inhibitor *in vivo*, which may be exchanged *in vitro* leading to a regain of the cell surfaces' ability to stain with 9-aminoacridine. We believe these techniques could be of value in diagnosis of benign and malignant melanocytes in skin.

Acknowledgements

We wish to thank Dr. N.Y. Haboubi and his staff in the Histopathology Department, Withington Hospital for their generous provision of the clinical material used in this study and also for their continued support and encouragement throughout the project.

FSS wishes to thank the Cancer Research Campaign for financial support, without which this study would not have been attempted.

References

1. W.F. Lever and G. Schaumberg-Lever in *Histopathology of Skin*, 6th Edition p. 868 Lippincot, Philadelphia, USA (1983).
2. Muir's Textbook of Pathology; J.R. Anderson (ed.) 12th Edition pp. 27 Arnold, London (1985).
3. F.S. Steven and R.K. Al-Ahmad, *Eur. J. Biochem.*, **130**, 335, (1983).
4. F.S. Steven, M.M. Griffin and R.K. Al-Ahmad, *Eur. J. Biochem.*, **149**, 35, (1985).
5. F.S. Steven, F.B. Barnett, H. Jackson and N.C. Jackson. *Int. J. Cancer*, **37**, 933, (1986).
6. J. Johnson, F.S. Steven and C.H. Buckley, *J. Tumour Marker Oncology* (in press).
7. L. Bertalanffy, M. Masin and F. Masin. *Cancer*, **II**, 873, (1958).
8. F.S. Steven, M.M. Griffin, T.L.H. Wong, and S. Itzhaki. *J. Enz. Inhibition*. (in press).
9. M.D. Pierschbacher and E. Ruoslahti, *Nature (Lond.)*, **309**, 30, (1984).