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FLUORESCENT LOCATION OF ORNITHINE DECARBOXYLASE EMPLOYING DERIVATIVES OF THE SPECIFIC INHIBITOR α-DIFLUOROMETHYL ORNITHINE

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Two fluorescent derivatives have been made from α-difluoromethyl ornithine by linking the carboxyl group of the ornithine derivatives to fluorescent amines. α-difluoromethyl ornithine is a potent inhibitor of ornithine decarboxylase, an enzyme which plays an essential role in cell division. We have used these fluorescent derivatives as probes for ornithine decarboxylase in frozen sections of skin to locate the epithelial cells which are known to contain ornithine decarboxylase. The probes also located squamous cell carcinoma cells in human skin.

KEY WORDS: Ornithine decarboxylase,Fluorescent inhibitors, Carcinoma.

INTRODUCTION

Polyamine synthesis is known to be associated with cell division[1]. Ornithine decarboxylase (ODC) is the key enzyme in polyamine synthesis[2] and this enzyme has been the subject of extensive research aimed at inhibiting ODC and regulating cell proliferation[3,4]. A large number of ornithine derivatives[3] have been prepared which inhibit ODC either reversibly or irreversibly. In the present study we were concerned with α-difluoromethyl ornithine[5] (DFMO) which has been claimed to inhibit ODC specifically[2] and react with no other protein. This claim was made after *in vivo* labelling with isotopically labelled DFMO and the demonstration that a single DFMO labelled protein extracted from the cells corresponded to pure ODC[2]. DFMO has since been employed to inhibit the proliferation of tumour cells as an additive to cytotoxic agents in chemotherapy[6]. Although all cells must be able to make ODC at some time in their life cycle, cells which are actively dividing possess an elevated level of ODC activity[7]. We have been concerned with the preparation of fluorescent derivatives of ODC which may be used to locate cells possessing this enzyme. We have used the epithelial cells of the skin as an example of cells known to possess ODC and compared the location of such cells with a monoclonal antibody specific for ODC and with the fluorescent derivatives of DFMO. Preliminary studies with DFMO linked to Sepharose indicated that the α-carboxyl group of DFMO was not required for the binding of DFMO to ODC. We therefore coupled the α-carboxyl group to the amino group of fluoresceinamine and Texas red amine to make two fluorescent derivatives of DFMO, each of which was capable of being bound to ODC within epithelial cells.

The affinity of these probes for the active centre of ODC is based upon the absolute specificity of the DFMO moiety for ODC[2] leading to inhibition of this enzyme.

We hope that these fluorescent inhibitors of ODC will prove useful in the location of tumour cells in frozen sections due to the fact that these cells are often rapidly dividing. The ease of preparation and inexpensive components required to make these probes could make them attractive alternatives to monoclonal antibodies which exhibit species specificity, a disadvantage which is not shared by these fluorescent probes.

We required a rapidly dividing cell type which was easily accessible and recognisable to illustrate the location of ODC. We chose the epithelial cells on the surface of skin in freshly sectioned, frozen, normal human and mouse tissue.

The epithelial cells of the skin are all derived from the basal layer of epithelial cells which divide rapidly and migrate to the surface of the skin. These cells at the surface of the skin are matured epithelial cells which contain keratin, these cells are dead and would normally be shed from the surface of the skin. We have concentrated on human tissue, since the epithelial layer is often many cells thick whilst the mouse skin often has only two or three cells in this layer. The mouse skin was used to demonstrate the presence of ODC in these epithelial cells with an antibody against mouse ODC. The mouse skin was merely used to correlate ODC location by the immunological and fluorescent probe techniques. One of the objects of making these probes was the prospect of locating rapidly dividing tumour cells which would be expected to contain enhanced ODC levels. We used sections of squamous cell carcinoma of the skin to illustrate the location of tumour cells with these probes.

MATERIALS AND METHODS

α-difluoromethyl ornithine (DFMO) was very generously provided by Merrell Dow Pharmaceutical Inc., 2110E Galbraith Road, P.O. Box 156300, Cincinnati, Ohio 45215-6300, U.S.A. The acid chloride of Texas red was obtained from Sigma Chemical Company, St. Louis, Mo. U.S.A. and sold as sulforhodamine 101 acid chloride. Activated carbon, fluoresceinamine and 1,1'-carbonyldiimidazole were also obtained from Sigma, Activated AH-Sepharose-4B was purchased from Pharmacia AB, Laboratory Separation Division, Uppsala, Sweden.

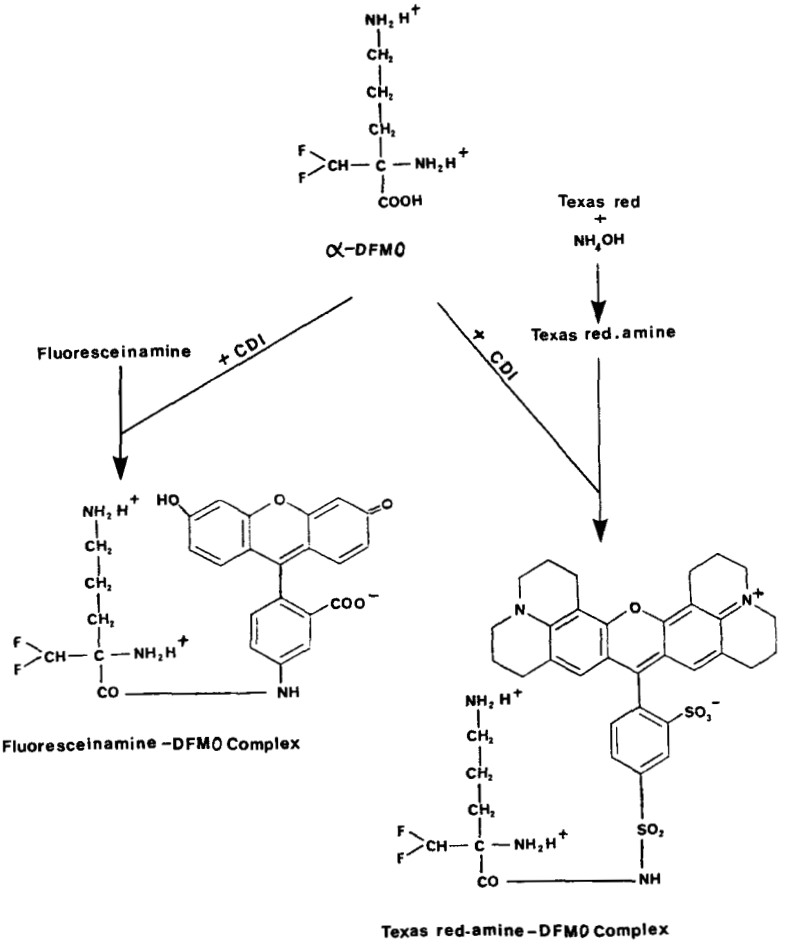
Preparation of DFMO linked to AH-Sepharose-4B and inhibition of ODC

Excess DFMO was reacted with AH-Sepharose-4B and the pH maintained at 5–6 for 6 h in the presence of excess water soluble carbodiimide. The insoluble product was exhaustively washed to remove all traces of DFMO and carbodiimide. A small amount of the washed DFMO-Sepharose complex was then equilibrated for 10 min with a 3 ml aliquot of ODC containing a known activity of ODC. The DFMO-Sepharose was centrifuged and washed twice by further centrifugation and the combined supernatants assayed for ODC activity[8] by the decarboxylation of radiolabelled ornithine. Over 80% of the ODC activity was lost as a result of the equilibration of the enzyme with the insoluble-DFMO-linked-Sepharose.

Preparation of fluorescent derivatives of DFMO

The preliminary studies with DFMO-Sepharose indicated that the α-carboxylic group

of DFMO could be substituted without destroying the ability of DFMO to inhibit ODC. The preparation of fluorescent derivatives of DFMO involved the condensation of a fluorescent amine with the carboxyl group of excess DFMO in the prescence of excess water soluble carbodiimide at pH 5–6. The fluorescent complex was then adsorbed on carbon and exhaustively washed with water to remove excess DFMO, finally the fluorescent DFMO derivative was washed from the carbon with methanol/chloroform (40:60). The organic solvent was removed by vacuum distillation and the fluorescent DFMO derivative dissolved in isotonic saline. The molarity of the fluorescein and Texas red DFMO derivatives was defined by calibration with authentic fluorescein-glycine and Texas red-glycine standards, employing fluorimetry. The fluorescent amines used in this study were fluoresceinamine and Texas red-amine. Texas red-amine was prepared by reacting Texas red sulphonyl chloride with excess ammonium hydroxide for 18 h followed by removal of the excess ammonia by vacuum distillation. The structures of these probes are presented in scheme 1.



Preparation of slides for fluorescent microscopy

Fresh frozen sections were placed in a wet box and 25 μl of fluorescent-DFMO in isotonic saline applied to the surface of the tissue. The concentrations of the fluorescent DFMO ranged from 10^{-6} to 10^{-12}M; we normally employed 10^{-6} to 10^{-8}M for photographic recording of the fluorescent cells. After exposure to the fluorescent DFMO solution for 3–4 min the slides were washed for 10 s in a tank of isotonic saline prior to fluorescent microscopy. We employed a Leitz Diaplan fluorescent microscope with filter cubes [G] or [N] and examined the section immersed in isotonic saline under a cover slip. Inhibition of ODC was achieved by placing the section under 25 μl of DFMO (10^{-5}M) in isotonic saline for 30 min; prior to addition of fluorescenamine-DFMO.

Competition for ODC with fluorescent-DFMO probes and a massive excess of ornithine

In these experiments we had to adjust conditions so that just sufficient time was allowed for Texas red-DFMO to complex with the epithelial cell ODC to enable these cells to be readily located by fluorescent microscopy and photographed. If we used Texas red-DFMO in the presence of a massive molar excess of free L-ornithine, the chances of the fluorescent probe bindig to ODC were greatly reduced. This type of competition between the substrate and the fluorescent probe is initially competitive but becomes progressively non-competitive as more molecules of the fluorescent inhibitor bind *irreversibly*. Clear cut results can only be obtained with rapid exposure to these two competing molecules, otherwise the cumilative build up of the DFMO derivative on the ODC will result in red fluorescence of the epithelial cells. We used 6.2×10^{-8}M Texas red-DFMO in isotonic saline alone or in the presence of 3.9×10^{-2}M ornithine to treat the frozen sections for 2 min followed by washing in isotonic saline for 10 s. The molar ratio of fluorescent probe to free substrate was approximately 1: 6.3×10^{5}.

Attempted displacement of Texas red-DFMO from ODC by subsequent exposure to a massive excess of ornithine

In these experiments the skin epithelial cells were first exposed to 6.2×10^{-8}M Texas red-DFMO for 2 min, then exposed to 3.9×10^{-2}M ornithine for 2 min prior to washing and fluorescent microscopy. These experiments were designed to determine whether excess ornithine could displace Texas red-DFMO which was already bound to cellular ODC.

RESULTS

Fluorescent location of ODC containing cells in human skin

The epithelial layer of cells on the surface of human skin were examined in frozen sections prior to treatment with fluorescent probes. The untreated sections exhibited no autofluorescence of the epithelial cells employing cubes [N] and [G] in the Leitz Diaplan fluorescence microscope. Treatment of frozen sections of human skin with the fluoresceinamine-DFMO complex resulted in the epithelial cells exhibiting yellow fluorescence when viewed with the conventional cube [G] for fluorescein labelling

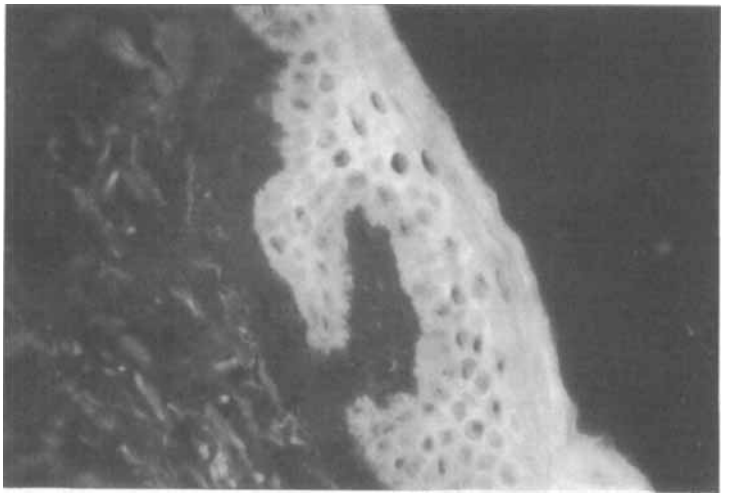


FIGURE 1 Human skin treated with fluoresceinamine-DFMO. The cells containing ODC fluoresce yellow with filter cube [G]; note the cytoplasmic rather than nuclear binding of the probe. Magnification × 500. (See colour plates at rear)

(Figure 1). The location of the fluorescent probe is extranuclear (Figure 1) and since this enzyme is known to occur both in the nucleus and cytoplasm of cells rather than on the cell surface[2] we conclude that the bulk of the enzyme is located in the cytoplasm of these skin epithelial cells. The same section employed to produce Figure 1 was next viewed with filter cube [N] and the cytoplasm of these epithelial cells then fluoresced red (Figure 2).

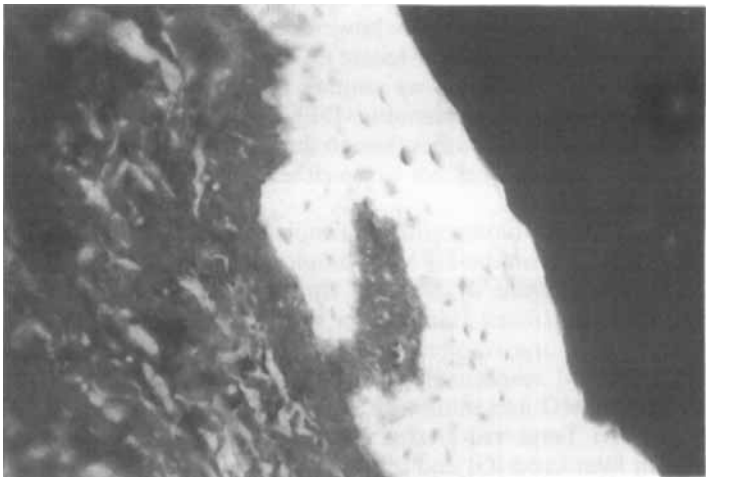


FIGURE 2 Human skin treated with fluoresceinamine-DFMO. The cells containing ODC fluoresce red with filter cube [N]. This is exactly the same field of view as in Figure 1. Magnification × 500. (See colour plates at rear)

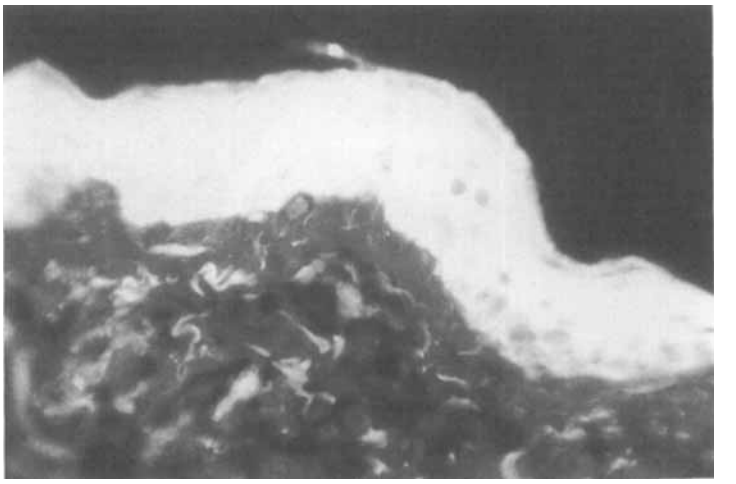


FIGURE 3 Human skin treated with DFMO only. The cells containing ODC fluoresce red with filter cube [N]. Magnification × 500. (See colour plates at rear)

Pretreatment of a corresponding fresh section of human skin with DFMO in saline, followed by washing in saline and then treating with fluoresceinamine-DFMO complex as described above, resulted in the total absence of fluorescence in the cytoplasm of these skin epithelial cells when viewed with filter cube [G]. This observation would be expected since DFMO is a potent inhibitor of ODC. This pretreatment would be expected to block the active centre of ODC and prevent the binding of the yellow fluorescent fluoresceinamine-DFMO complex. DFMO attached to cell- bound ODC does not fluoresce under the microscopic conditions using cube [G]. DFMO bound to the ODC of skin epithelial cells does however fluoresce red when viewed with filter cube [N] (Figure 3). We can therefore locate ODC with pure DFMO without the need to include a fluorescent moiety, if we employ filter cube [N]. The red fluorescence (Figure 2) observed with fluoresceinamine-DFMO complex could well be due to the DFMO moiety in this complex rather than to the fluoresceinamine moiety. Treatment with fluoresceinamine alone does not cause either red or yellow fluorescence in these skin epithelial cells.

When we replaced the fluoresceinamine moiety by the Texas red amine moiety (employing the Texas red amine-DFMO complex) orange fluorescence was observed with filter cube [G] (Figure 4) and red fluorescence with cube [N] (Figure 5). Pretreatment of a fresh frozen skin section with DFMO prior to Texas red amine-DFMO treatment of course resulted in orange and red fluorescence with filter cube [G] and filter cube [N] respectively. This combination cannot be used to define competition with DFMO and inhibition of cell bound ODC. Treatment of the skin sections with 10^{-8}M Texas red-glycine failed to locate the epithelial cells by their fluorescence with filter cube [G] and [N] thus excluding the Texas red moiety as the binding site for ODC when using Texas red amine-DFMO complex.

In frozen sections of human skin containing squamous cell carcinoma (a rapidly proliferating tumour of the skin) the tumour cells bound both fluoresceinamine-

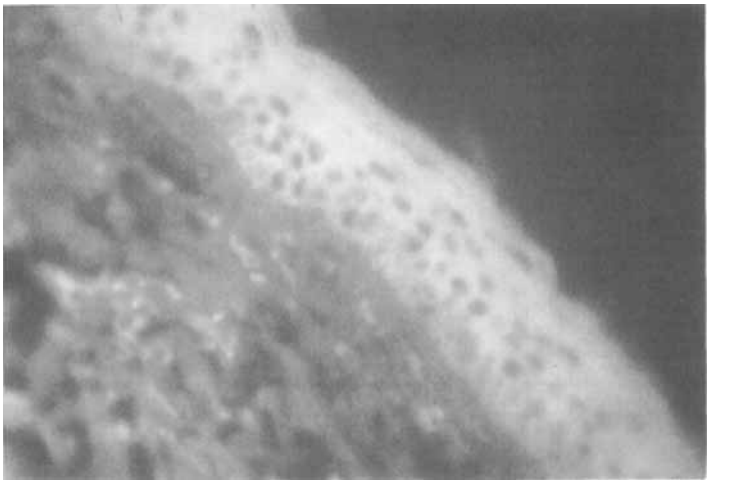


FIGURE 4 Human skin treated with Texas red-DFMO. The cells containing ODC fluoresce orange with filter cube [G]. Magnification × 500. (See colour plates at rear)

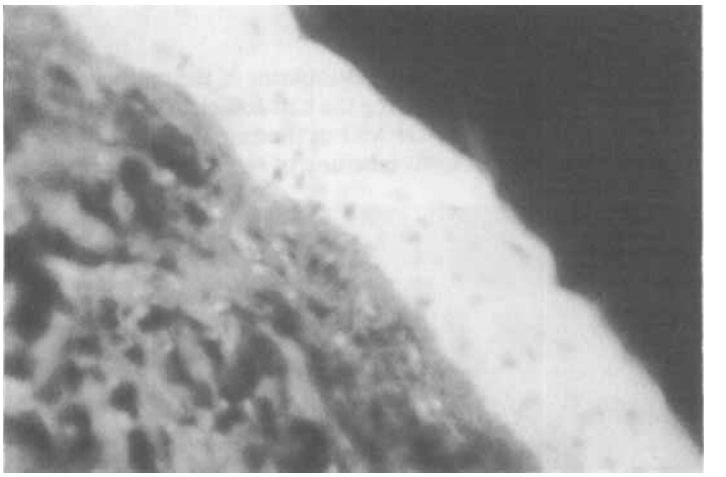


FIGURE 5 Human skin treated with Texas red-DFMO. The cells containing ODC fluoresce red with filter cube [N]. This is exactly the same field of view as Figure 4. Magnification × 500. (See colour plates at rear)

DFMO complex (Figure 6, yellow fluorescence on cube [G]) and Texas red -DFMO complex (Figure 7, red fluorescence on cube [N]).

Fluorescent location of ODC containing cells in mouse skin

Frozen sections of mouse skin treated with these ODC-linked fluorescent probes

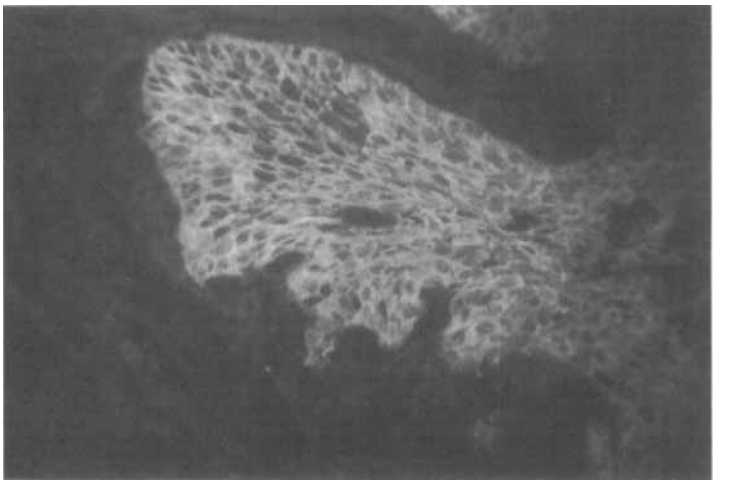


FIGURE 6 Human skin containing squamous cell carcinoma treated with fluoresceinamine-DFMO. This tumour lies beneath the surface epithelial layers. The tumour cells were located by their ability to bind this probe; note the yellow fluorescence of the tumour cell dytoplasm. The dark background contains normal cells and normal tissue. Filter cube [G] was used for this photograph. Magnification × 500. (See colour plates at rear)

exhibited the expected fluorescence in the cytoplasm of the epithelial cells on the skin surface and the epithelial cells surrounding the hair follicles. When these mouse frozen sections were exposed to Texas red-DFMO in the presence of a swamping excess of ornithine for 2 min, no red fluorescent labelling of the epithelial cells took place. The

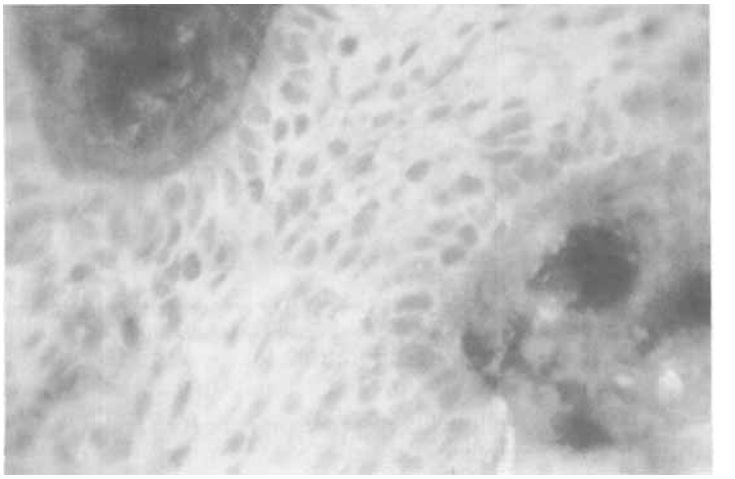


FIGURE 7 Human skin containing squamous cell carcinoma treated with Texas red-DFMO. This tumour lies beneath the surface epithelial layers. The tumour cells bind the red fluorescent probe in their cytoplasm. The black areas were normal tissue. Filter cube [N] was used for this photograph. Magnification × 500. (See colour plates at rear)

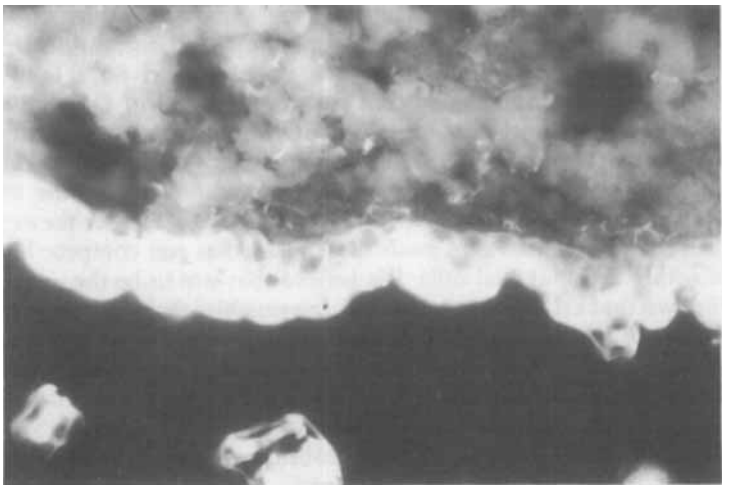


FIGURE 8 Mouse skin treated with Texas red-DFMO. This is similar to the human skin but the number of cells in the epithelial layer is reduced in the mouse. Filter cube [N] was used for this photograph. Magnification × 500. (See colour plates at rear).

whole section appeared black under these conditions of fluorescent microscopy with filter cube [N] in place. Clearly the presence of the excess ornithine blocked the binding of the Texas red-DFMO. On the other hand if the section was first exposed to the Texas red-DFMO and then exposed to the excess ornithine, the epithelial cells, fluoresced red (similar to Figure 8) indicating that the ornithine could not displace the fluorescent probe previously bound to the epithelial cells. These same mouse epithelial cells also showed good immunological location with a peroxidase-linked polyclonal antibody against mouse ODC (data not presented here). The mouse antibody was unable to recognise ODC in human skin whilst the fluorescent ODC probe was able to locate these epithelial cells in man, mouse and rat skin. We only used this polyclonal antibody to confirm that the mouse epithelial cells actually contained ODC as confirmation that the fluorescent ODC derivatives were locating cells containing authentic ODC.

DISCUSSION

We employed human skin sections for the fluorescent location of ODC-containing cells in preference to mouse skin due to the thick layer of epithelial cells on human skin. We believe the correlation of the fluorescent location of ODC, together with the known absolute specificity of DFMO for ODC[2], points to the specificity of the DFMO probes used in this study of human skin ODC. In the preparation of our DFMO-fluorescent probes, all the unsubstituted DFMO is water soluble and has no affinity for carbon, the isolation of fluorescent-DFMO complexes (after washing the carbon exhaustively) excludes the presence of the DFMO in the product. The targeting of these probes to ODC containing cells must therefore be due to the DFMO moiety of the fluorescent probes. The fluorescent labelling of these same epithelial cells with free

DFMO (Figure 3) clearly demonstrates that these cells bind DFMO and that this binding must be due to cellular ODC[2]. The preliminary observation that DFMO linked through its carboxyl group to Sepharose-4B caused 80% inhibition of a standard ODC preparation indicated that the carboxyl end of DFMO would be a suitable site for adding a fluorescent tag to DFMO without loss of inhibitory function. We have therefore used fluorescent inhibitors specific for ODC to locate cells possessing this enzyme. The observation that massive amounts of ornithine blocked the fluorescent labelling of epithelial cells with Texas red-DFMO under the experimental conditions described confirms that both these molecules *can* compete for the same binding site within the epithelial cells. We believe that site to be the active centre of ODC. It is known that DFMO binds to ODC irreversibly[2] therefore we cannot expect a massive molar excess of ornithine to displace prebound Texas red-DFMO from ODC in epithelial cells. This expectation was confirmed experimentally and again supports the claim that Texas red-DFMO locates ODC. An excess of this enzyme is considered to be a prerequisite for cell division due to this enzyme's key role in polyamine biosynthesis. These fluorescent probes may therefore be of value in the location of rapidly dividing cells, eg. tumour cells. Two examples of the fluorescent location of squamous cell carcinoma have been presented (Figures 6,7) to illustrate this point. DFMO has been shown to have only limited use in the treatment of human tumours[8].

Fluorescent derivatives of ODC have been described[9] in which the δ-amino group of ODC has been substituted with a fluorescent tag. It is our belief that the choice of the α-carboxyl group for attaching the fluorescent tag may provide more selective probes for ODC. In our fluorescent experiments we excluded the addition of pyridoxal phosphate from the cytochemical staining procedure, since we observed that pyridoxal phosphate alone caused fluorescence of the cells in the sections. We assumed that the cells in the frozen sections contained sufficient pyridoxal phosphate to act as a cofactor for the binding of fluorescent-DFMO probes.

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