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THE PROTECTIVE ROLE OF A NATURAL INHIBITOR IN THE FLUORESCENT LOCATION OF CELLS POSSESSING A LATENT FORM OF CELL SURFACE PROTEASE

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Leukaemia cells possess a latent form of a cell surface protease referred to as guanidinobenzoatase. Latency is due to complex formation between an inhibitor protein and the cell surface enzyme which is stable under acid conditions but is dissociated with formaldehyde treatment. The latent form of the cell surface protease has been used as a protecting mechanism during a preliminary step to stain all the nuclei of cells with haematoxylin. The enzyme-inhibitor complex was then dissociated and a combination of 9-amino acridine and propidium iodide employed to enable the fluorescent location of cells possessing active guanidinobenzoatase. We were thus able to visualise the nuclei by conventional light microscopy and simultaneously visualise the cell surface of leukaemia cells by fluorescent microscopy. This simple model system has provided technology applicable to the more complex analysis of neoplastic cells in cervical smears.

KEY WORDS: Guanidinobenzoatase, enzyme-inhibitor complex, fluorescent location, neoplastic cells.

INTRODUCTION

The object of the present study was to design a simple procedure for the double labelling of neoplastic cells. In this case, the labelling consisted of a nuclear stain visible with conventional light microscopy and a cell surface fluorescent marker which could be visualised by fluorescent microscopy. A simple system was chosen to act as a model for more complex systems (e.g. the analysis of cervical smears for neoplastic cells). The rat T-cell lymphoblastic leukaemia model was chosen[1] to provide a good source of leukaemia cells. These leukaemia cells possess a cell surface protease[2]. This enzyme is associated with tumour cells[3] and has the ability to bind the fluorescent probe 9-amino acridine at its active centre. The stacked 9-amino acridine (9-AA) allows these cells to be located by fluorescent microscopy[2].

This cell surface protease is very similar to plasminogen activator, being a seryl protease which is not inhibited by 4-methylumbelliferyl-*p*-guanidinobenzoate but which acts on it like a true substrate.[3,4,5] We have described this cell surface protease as guanidinobenzoatase (GB) due to its ability to cleave guanidinobenzoates.[5] Naturally occurring protein inhibitors of GB are present in most tissues.[6,7] These inhibitors are readily extractable in isotonic saline and form reversible enzyme-inhibitor complexes with the cell surface GB.

Leukaemia cells, being passively transported in the blood of the leukaemic rat, possess largely inhibited GB. We obtained leukaemia cells from the enlarged spleen of these leukaemic rats and mixed these cells with normal human blood (in order to

dilute the leukaemia cells) prior to making smears of the mixed cells on polylysine coated slides. The leukaemia cells thus represented neoplastic cells which were known to possess latent GB and could be used as a model for the neoplastic cells of the cervical smear, which also possess latent GB.

Leukaemia cells can be located by nuclear staining with haematoxylin followed by fluorescent labelling of the GB; the cells with latent GB being protected whilst undergoing nuclear staining with haematoxylin.

MATERIALS

On formaldehyde fixation erythrocytes become weakly fluorescent. In order to overcome this problem, the blood smears were first placed in distilled water for 1 min to lyse the erythrocytes prior to processing for fluorescent microscopy. Leukaemia cells were obtained from the spleens of leukaemic rats,[1] kindly supplied by Dr. H. Jackson of the Department of Physiological Sciences. These cells were either diluted with isotonic saline or with normal human blood in isotonic saline and spread as a film on polylysine coated slides. Similar smears were made from human leukaemic blood. The dried slides were kept at $-20°C$ until they were to be used. Poly-D-lysine, 9-amino acridine and propidium iodide were obtained from Sigma Chemical Co., Ohio, Mo, USA. Meyer's haematoxylin solution was prepared in the routine histopathology laboratory.

METHODS

The slides were placed in haematoxylin for 1 min, drained and washed for 2 min in isotonic saline. The haematoxylin stained sections were treated with 10% formaldehyde in isotonic saline for 30 min, followed by washing in isotonic saline for 2 min and equilibrating in isotonic saline containing 10^{-3}M $NaHCO_3$, pH 8.4 for 5 min. The slides were then treated with 9-AA (10^{-3}M) in isotonic saline at pH 8.4 for 5 min, followed by 2 min in a similar 9-AA solution containing 10^{-4}M propidium iodide.

Finally the slide was washed in isotonic saline and examined by conventional light and fluorescent microscopy. We employed a Leitz Diaplan fluorescent microscope, with filter cube [N] for fluorescent studies and filter cube [G] in the conventional light path, for nuclear location. Kodak ASA 400 colour film in an Olympus camera, fitted with automatic exposure, was used for photographing the cells.

RESULTS AND DISCUSSION

Staining the smears with propidium iodide alone resulted in nuclear fluorescence of the leukaemia cells, as would be expected with a fluorescent marker for double stranded DNA (Figure 1). Leukaemia cells consist of a large nucleus with a rim of cytoplasm surrounded by the cell surface. It would obviously be advantageous if we could mask the nucleus with a quenching non-fluorescent stain, so that fluorescent staining of the cell surface could be visualised against a darkened nucleus. This approach would have the added advantage that the cells' nuclear to cytoplasmic ratios

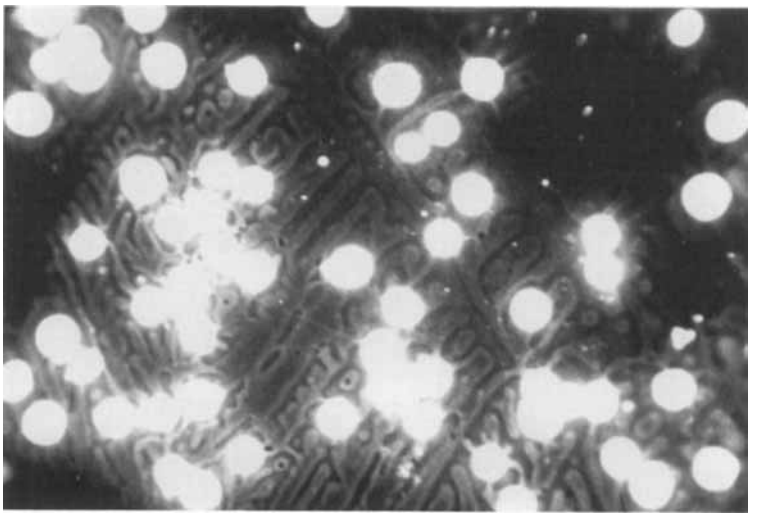

FIGURE 1 Rat leukaekmia cells stained with propidium iodide only. The nuclei bind propidium iodide and fluoresce red; the nucleus occupies most of the leukaemia cell's volume. Magnification × 500. (See colour plate at back of issue).

could be studied by conventional light microscopy, as is usual in cytological examination of cervical smears. We therefore incorporated haematoxylin staining as the first step in our double staining technique.

Haematoxylin is an acidic stain and these conditions are detrimental to the activity of GB on the surface of cells. However we observed that latent GB is protected by the

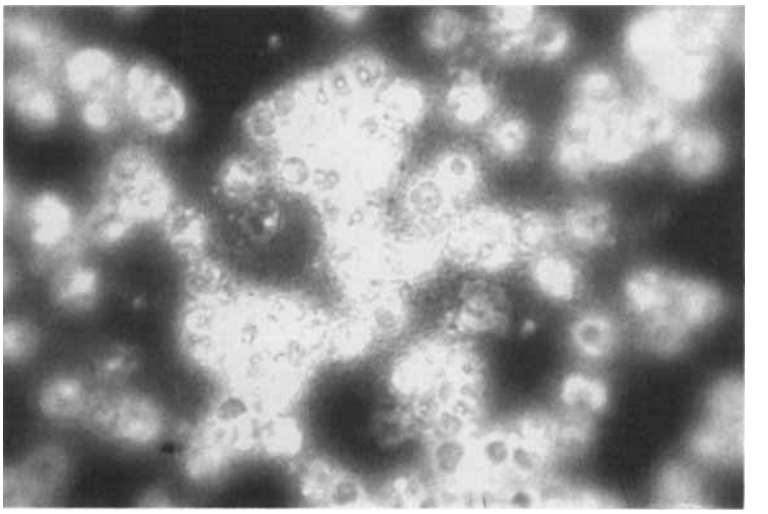

FIGURE 2 Rat leukaemia cells fixed with formaldehyde prior to ring staining of guanidinobenzoatase on cell surface. The nuclei were first blocked with haematoxylin and then the latent enzyme was activated by formaldehyde fixation, followed by location of guanidinobenzoatase with 9-amino acridine and propidium iodide as red fluorescence. Magnification × 500. (See colour plate at back of issue).

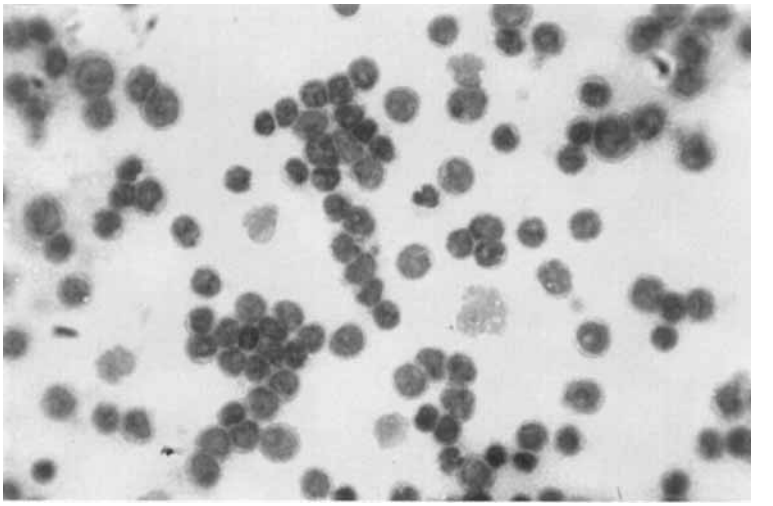

FIGURE 3 Rat leukaemia cells showing nuclear staining with haematoxylin. These are the same cells as shown in Figure 2, but seen by conventional light microscopy. The nuclei appear black. Magnification × 500. (See colour plate at back of issue).

acid-stable enzyme-inhibitor complex.[7] This observation has been taken advantage of in order to achieve good double labelling of leukaemia cells.

The need to displace the inhibitor from the GB in haematoxylin treated leukaemic smears was achieved with formaldehyde.[7] The reactivated GB on the leukaemia cells was then stacked[3] with 9-AA followed by co-stacking with propidium iodide[8] to enhance the fluorescent contrast of the labelled cell surface GB.

Typical examples of the fluorescent ring staining of the surface of GB on leukaemia

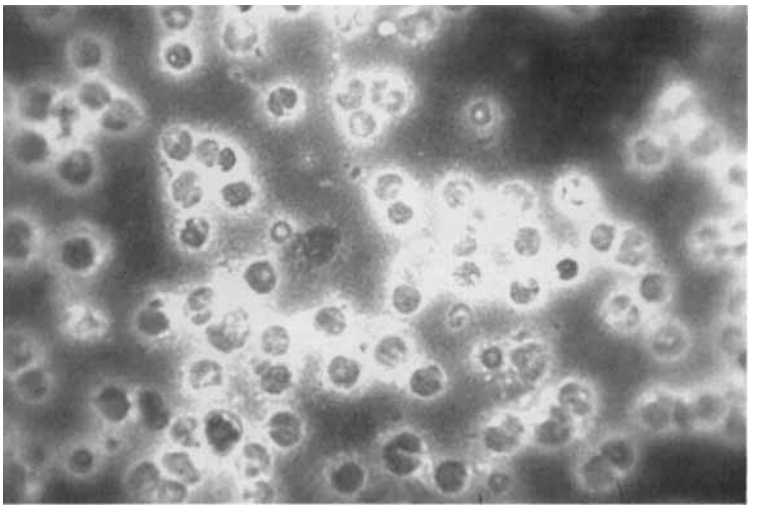

FIGURE 4 Human leukaemia cells fixed with formaldehyde prior to ring staining of guanidinobenzoatase on cell surface. Method as described for Figure 2. Note the extra-nuclear red fluorescence indicating the presence of guanidinobenzoatase. Magnification × 500. (See colour plate at back of issue).

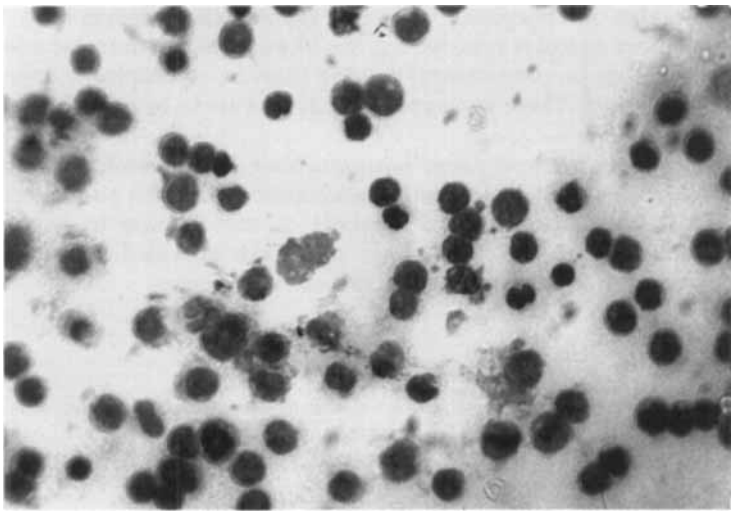

FIGURE 5 Human leukaemia cells showing nuclear staining with haematoxylin. These are the same cells as shown in Figure 4, but are seen by conventional light microscopy. The nuclei appear black. Magnification × 500. (See colour plate at back of issue).

cells, together with the nuclear location of the same cells by conventional microscopy is presented in Figures 2–5. In this study propidium iodide was used for fluorescent enhancement of GB for the following reasons. Propidium iodide binds to both DNA (Figure 1) and stacked 9-AA (Figures 2 and 4); in the present technique the propidium iodide does not cause red nuclear fluorescence but surface ring staining (Figure 2). The cells which show clearly the fluorescent surface ring staining with propidium iodide also show large well stained nuclei (Figure 3), typical of leukaemia cells.

These haematoxylin stained nuclei are particularly obvious even within thick layers of cells in which the fainter red cell surface fluorescence may be out of the focal plane when photographs were taken. As a consequence, not all cells with obvious large dark nuclei show red fluorescent ring staining in Figure 2. Similar results were obtained with human leukaemic blood smears (Figures 4 and 5).

Without the formaldehyde treatment (to displace the inhibitor from the cell surface GB) the leukaemia cells failed to bind 9-AA and propidium iodide. Under these conditions the leukaemia cells did not fluoresce. These experiments acted as controls for the fluorescent techniques and for the claim that the 9-AA and propidium iodide only locate the leukaemia cells when the inhibitor has been displaced from the latent GB enzyme-inhibitor complex.

In this study two inhibitors of cell surface GB were used. Firstly, the natural protein inhibitor already on the GB of the leukaemia cells was employed for protection of the active centre of GB during the haematoxylin staining of the cells' nuclei. Secondly, the protein inhibitor was displaced and the fluorescent competitive inhibitor of GB, 9-AA used to stack on the active centre of the re-activated GB. This stacked 9-AA has then allowed the co-stacking of propidium iodide and the fluorescent location of cells possessing active GB, e.g. leukaemia cells. The haematoxylin in the nucleus masks any nuclear staining with propidium iodide, thus the cell surface binding of propidium iodide can be visualised.

The use of this leukaemia cell line as a simple model system for designing double

labelling techniques for neoplastic cells has enabled these concepts to be applied successfully to more complex systems, e.g. the location of pleomorphic adenoma cells of the salivary glands [in preparation] and the location of neoplastic cells in cervical smears [in preparation]. These more complex systems are to be published in medically oriented journals.

The purpose of the present paper is to establish the rationale of employing the enzyme-inhibitor complex as a protective mechanism which can possibly be made use of in a clinical setting where large numbers of cells require to be screened for neoplastic characteristics. The leukaemia cells have provided an excellent model system to demonstrate the advantage of this protective step.

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