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Internalisation of the Bowman–Birk Protease Inhibitor by Intestinal Epithelial Cells

Paul C. Billings, David L. Brandon and Joan M. Habres

Protease inhibitors have been shown to be effective suppressors of carcinogenesis *in vitro* and *in vivo*. For example, the soybean-derived Bowman–Birk inhibitor (BBI) suppresses dimethylhydrazine-induced colon carcinogenesis in mice. Relatively little is known about the effects of protease inhibitors on intestinal epithelial cells. In the present study, we have investigated the interaction of the anticarcinogenic BBI with intestinal epithelial cells. At the concentrations examined, BBI was non-toxic and had no effect on the doubling time, saturation density or rate of DNA synthesis by these cells. This compound was taken up by these cells in a time dependent manner and was present in the cells for 12 h following a 2 h incubation with BBI. Subcellular fractionation experiments demonstrated that the bulk of the internalised inhibitor was present in the cytosol. Analysis of BBI from treated cells on a chymotrypsin affinity column revealed that active inhibitor was present in the cells. Our results indicate that the BBI is internalised by colonic epithelial cells which would allow BBI to inhibit critical intracellular proteases and thus suppress malignant transformation.
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INTRODUCTION

COLON CANCER is the second leading cause of cancer death in adults in the USA [1]. The incidence and mortality rates of colon cancer have remained unchanged for the past 50 years [1, 2]. Since colon cancer has been difficult to combat with contemporary treatment regimens [2], an alternative approach towards controlling this disease is through prevention. Dietary manipulation may well be the most cost-effective approach to control this disease. Protease inhibitors have been shown to be effective suppressors of carcinogenesis *in vitro* and *in vivo* [3–11]. For example, the Bowman–Birk inhibitor (BBI) has been shown to suppress colon carcinogenesis in mice [3, 10]. In addition, human populations consuming high levels of protease inhibitor-rich legumes in their diet have a lower incidence of breast, colon, pancreatic and prostate cancer [12–14]. Consequently, we believe that protease inhibitors will be potentially useful for reducing the incidence of cancer, particularly colon cancer, in the US population.

A major interest of our laboratory is to identify and study compounds which will inhibit or retard tumour cell development. We have extensively studied the anticarcinogenic activity of the soybean-derived Bowman–Birk protease inhibitor. BBI is an 8000 dalton protein which inhibits both trypsin and chymotrypsin and contains 7 disulphide bonds, making it an extremely stable molecule [15]. BBI has been shown to suppress dimethylhydrazine-induced colon carcinogenesis in mice [3, 10] as well as chemical carcinogen-induced cheek pouch carcinogenesis in hamsters [7] and liver carcinogenesis in mice [8]. Further, BBI reduces chemical and radiation induced transformation of mouse embryo fibroblast cells *in vitro* [6, 11]. BBI has also been found to down regulate c-*myc* oncogene expression in colonic epithelium [16]. Although the precise mechanisms by which protease inhibitors suppress carcinogenesis are not known, it is believed that these compounds exert their anticarcinogenic effects by inhibiting one or more enzymatic activities involved in the induction and/or expression of the transformed phenotype [17, 18]. Relatively little information is available about the effect of protease inhibitors such as BBI on colonic epithelium. In the present report we have examined the interactions of BBI with the intestinal epithelial cell (IEC) line IEC-17.

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MATERIALS AND METHODS

Chemicals

Tetramethyl-rhodamine-isothiocyanate (TRITC) was obtained from Molecular Probes, Eugene, Oregon. BBI was purified by DEAE ion exchange chromatography [8]. Insulin was obtained from Sigma.

Cells

The normal rat intestinal epithelial cell line IEC-17 was grown in Dulbecco's modified Eagle's medium containing 5% heat-inactivated fetal calf serum and insulin (1 μg/ml) in a humidified atmosphere of 5% CO_2 [19].

DNA synthesis studies

The rate of DNA synthesis in these cells was determined by pulsing logarithmically growing IEC cells with ^{14}C-thymidine (37 kBq/ml) for 3 h in 60 mm dishes. The cells were washed three times with phosphate-buffered saline (PBS) scraped from the dishes and pelleted by centrifugation at 1000 *g* for 10 min. The cell pellets were resuspended in 2 ml PBS and precipitated with ice-cold 10% trichloroacetic acid onto Whatman GF/C glass fibre filters; acid precipitable counts were measured in a Beckman LS 6800 liquid scintillation counter.

Fluorescent labeling of BBI

Pure BBI was labeled with the fluorescent label TRITC using a modified procedure [5]. Briefly, 5 mg BBI was dissolved in 0.5 mol/l sodium bicarbonate buffer (pH 9.2) to a final concentration of 1 mg/ml. 65 μl TRITC (dissolved in acetone

at a concentration of 25 μg/ml) was mixed with 5 ml inhibitor solution and incubated for 12 h at 20°C on a rocker platform. After incubation, the sample was passed over a Bio-Gel P-6PD column (1.5 × 20 cm) in PBS to remove free TRITC, dialysed overnight against water at 4°C and lyophilised to dryness. Rhodamine labeling had no effect on the ability of the BBI to inhibit chymotrypsin activity. Insulin was labeled with TRITC in a similar manner. For the fluorescent microscopic studies, 10^5 IEC cells were seeded into slide chambers (Lab-Tek tissue culture chamber/slide) and grown for 24–48 h prior to use. Cells growing in logarithmic phase were treated at 37°C with TRITC-BBI (10 μg/ml) in 2 ml medium containing 5% fetal calf serum. After the desired incubation period, the medium was removed, the cell monolayers were washed five times with PBS and the cells were fixed with buffered formalin and covered with glass coverslips. The coverslips were sealed onto the slides with nail polish. The slides were examined with a Nikon Microphot-FX fluorescence microscope; representative fields were photographed with Kodak Tmax 3200 film.

Kinetic uptake studies

10^5 IEC cells were seeded into 60 mm dishes and grown for 48–72 h. Log phase cells were treated with TRITC-BBI (10 μg/ml) for 5 min to 4 h, washed five times with PBS, scraped from the dishes with a rubber policeman and pelleted by low-speed centrifugation. The cells were resuspended in 2 ml PBS, disrupted by sonication and the fluorescence was determined in a Perkin Elmer LS-5 spectrofluorometer at excitation and emission wavelengths of 578 and 604 nm, respectively.

Subcellular fractionation

Logarithmically growing IEC cells were treated with TRITC-BBI (10 μg/ml) for 16 h, washed twice with PBS, trypsinised from the dishes and pelleted by low-speed centrifugation. The cells were resuspended in ice-cold isotonic sucrose buffer (10 mmol/l Tris (pH 7.0), 250 mmol/l sucrose, 1 mmol/l $MgCl_2$) and homogenised on ice in a Dounce homogeniser (100 strokes). The homogenate was centrifuged at 1000 *g* for 10 min, 10 000 *g* for 10 min and 100 000 *g* for 60 min; all centrifugation steps were carried out at 4°C. Each subcellular fraction was assayed for TRITC-BBI and marker enzyme activity.

Marker enzymes

The lysosomal enzyme, β-Glu, was assayed in 0.1 mol/l sodium acetate buffer (pH 4.5) containing 0.5 mmol/l phenolphthalein glucuronidate as substrate [17]. The samples were incubated at 37°C for 12 h and the release of phenolphthalein was determined spectrophotometrically at 540 nm. LDH, a cytosolic marker enzyme, was assayed in 0.2 mol/l Tris (pH 7.3) containing 1 mmol/l pyruvate and 0.22 mmol/l NADH as substrate. The disappearance of NADH was determined spectrophotometrically at 340 nm [17]. The protein present in each sample was determined by the method of Bradford [20] by using bovine serum albumin as standard.

Affinity chromatography

Chymotrypsin was covalently attached to a 1 ml N-hydroxysuccinimide affinity-filter (Affinity Technology, Leonia, New Jersey) according to the manufacturer's instructions. The 100 000 *g* supernatant (cytosolic) fraction, obtained from the subcellular fractionation studies, was applied to the column in 100 mmol/l Tris (pH 8), 10 mmol/l $CaCl_2$ (loading buffer). The column was washed with 10 ml of loading buffer; bound BBI was eluted with 0.1 mol/l HCl. The amount of labeled BBI binding to the column was determined in a spectrofluorometer at excitation and emission wavelengths of 578 and 604 nm, respectively.

Table 1. Effect of BBI on IEC-17 cell growth parameters

Treatment (μg/ml BBI)	Survival fraction*	DNA synthesis†	Saturation density‡
Untreated	1.00	7.5 (0.2)	11.9 (0.3)
1	1.00	6.2 (2.6)	11.2 (0.8)
5	0.98	ND	ND
10	1.00	7.1 (0.8)	12.5 (0.5)
25	1.00	ND	12.2 (0.4)

* The absolute plating efficiency of control cultures was approximately 70% and the doubling time for cells in all treatment groups ranged from 20–23 h.
† Counts (S.E.) per minute of ^{14}C-thymidine incorporated (× 10^{-4}).
‡ Number (S.E.) of IEC cells/60 mm dish (× 10^{-5}).
ND = not determined.

RESULTS

Although the BBI has been shown to be an effective suppressor of colon carcinogenesis in mice [3, 10], relatively little is known about the effect of this inhibitor on intestinal epithelial cells. In the first series of experiments, we determined the effect of the BBI on several growth parameters of IEC cells. The BBI was not toxic to these cells up to a concentration of 25 μg/ml. In addition, the BBI had no effect on the rate of DNA synthesis, doubling time or saturation density of these cells (Table 1).

An unresolved question about the mechanism of action of the anticarcinogenic protease inhibitors is their site of action (i.e. whether these compounds act extracellularly or intracellularly). If the BBI suppresses colon carcinogenesis by inhibiting intracellular proteases involved in oncogenic transformation, then one must assume that these compounds are internalised by the colonic epithelium. In the present study, the fate of TRITC-BBI in IEC cells was determined. TRITC-labeled BBI was administered to the cells in complete medium and after incubation times ranging from 5 min to 4 h, the cells were fixed in buffered formalin and subsequently examined under a fluorescence microscope. No autofluorescence was observed in untreated cells. After 15 min of incubation with TRITC-labeled inhibitor, traces of fluorescence were visible in the cells (data not shown). At 1 and 4 h of incubation, labeled inhibitor could be seen accumulating in the cytoplasm surrounding the nucleus (Fig. 1). These results indicate that the BBI is internalised by these cells. In contrast, when the cells were incubated on ice, no uptake of BBI was observed.

In the next series of experiments, the kinetics of uptake of the BBI by IEC cells was examined. In these studies, cells were grown in the presence of TRITC-BBI for varying lengths of time, the cells were washed extensively with PBS, scraped from the dishes and pelleted. The cell pellet was resuspended in 2 ml PBS and sonicated and the amount of BBI taken up by the cells was determined spectrofluorometrically. We observed rapid uptake of the BBI for the first 30 min with a slower rate of uptake at later time points. The results from a representative experiment are presented in Fig. 2. The uptake of the BBI, as measured using this procedure, was qualitatively similar to that observed by fluorescence microscopy (Fig. 1).

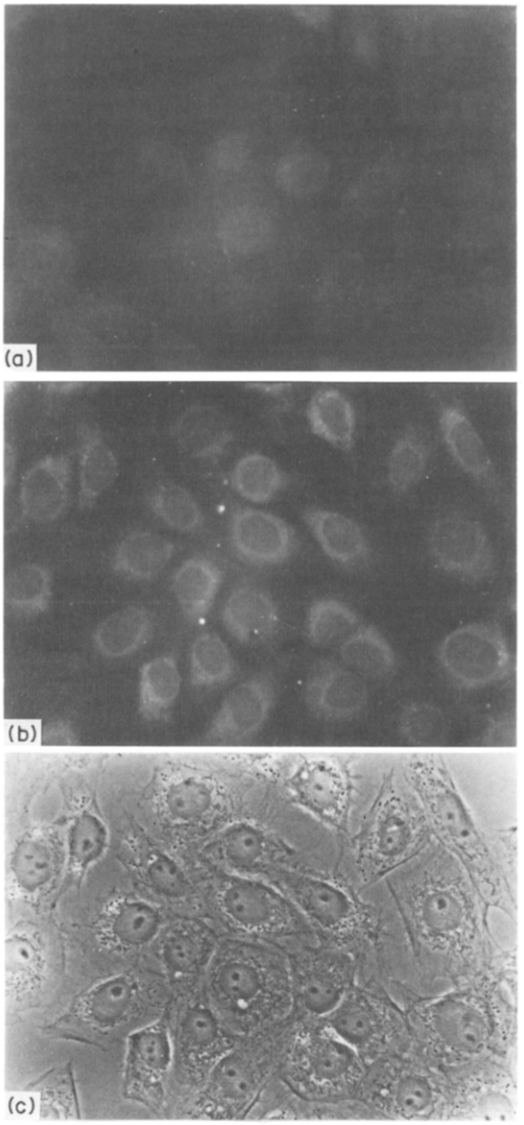

Fig. 1. Uptake of TRITC-labeled BBI by logarithmically growing IEC-17 cells. (a) Cells incubated with TRITC-BBI for 1 h; (b) cells incubated with TRITC-BBI for 4 h; (c) phase contrast micrograph of (b) (× 630).

To examine further the uptake of this inhibitor, the cells were treated with 10 μg/ml TRITC-BBI for 2 h, washed twice with complete medium and incubated for increasing periods of time in complete medium lacking protease inhibitor. After the desired incubation period, the cells were analysed for the presence of labeled inhibitor by fluorescence microscopy. Labeled inhibitor could be observed in the cells 12 hr after removal of the BBI from the medium (Fig. 3). These results indicate that once taken up, BBI remains in the cells.

To verify that labeled inhibitor was present in the cells, rather than on the cell surface, IEC cells were grown in the presence of the labeled inhibitor for 2 h, the cells were washed extensively with PBS, trypsinised and replated. After the cells attached to the substratum, they were washed, fixed and examined by fluorescence microscopy. TRITC-BBI was observed in reseeded cells grown for 4 h or 16 h (Fig. 4). This is strong evidence that the BBI is present inside the cells. If BBI were sticking to the cell surface, trypsinisation should remove any surface-bound material. To determine whether the TRITC label could be

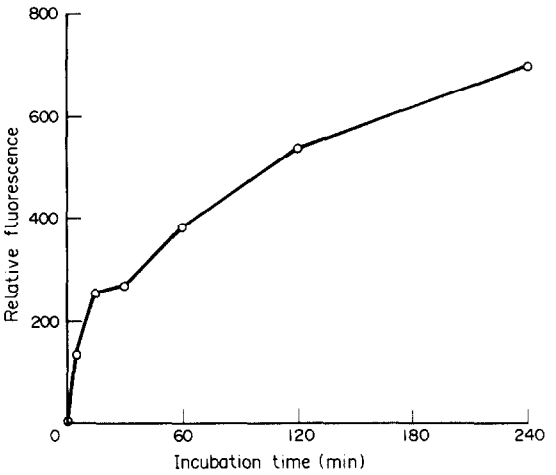

Fig. 2. Time course of uptake of TRITC-BBI by logarithmically growing IEC-17 cells. Cells were treated with labeled inhibitor in complete medium for 0–4 h. Fluorescence was determined in a spectrofluorometer at excitation and emission wavelengths of 578 and 604 nm, respectively.

removed from the BBI by serum proteases, we examined the labeled BBI, by gel filtration chromatography, after it had been stored in complete medium for 12 h. All of the TRITC label remained protein-bound (data not shown). Therefore, these results indicate that IEC-17 cells are taking up TRITC-BBI rather than free TRITC.

To determine which intracellular compartment the BBI was associated with, subcellular fractionation studies were performed. Cells were grown in TRITC-BBI, trypsinised from the dishes and disrupted by Dounce homogenisation; subcellular fractions were obtained by differential centrifugation. Each subcellular fraction was assayed for marker enzyme activity (Table 2) and TRITC-BBI (Table 3). The bulk of the labelled BBI was present in the 100 000 *g* supernatant (cytosolic) fraction. To investigate whether the internalised BBI is still active (i.e. still possesses protease inhibitor activity) we determined the ability of BBI, present in the cytosolic fraction, to bind to chymotrypsin immobilised on a solid support (the BBI contains a chymotrypsin inhibitory domain [15]). We found that the 100 000 *g* supernatant contained inhibitor which bound to the chymotrypsin-affinity resin (Fig. 5). Hence, active protease inhibitor is present in the cells.

In a control experiment, we examined the uptake of TRITC-labeled insulin by the IEC cell line. For these studies, IEC cells were treated with TRITC-insulin for 2 h, washed extensively with PBS, fixed and examined by fluorescence microscopy (Fig. 6). These cells internalised insulin, as expected [21].

DISCUSSION

In the present report we have examined the effect of BBI on the normal rat intestinal epithelial cell line, IEC-17. At the concentrations tested (0–25 μg/ml), BBI had no effect on any of the growth parameters measured including toxicity, DNA synthesis, doubling time or saturation density. These results are in agreement with previous studies which have shown that the BBI, as well as several other anticarcinogenic protease inhibitors, are non-toxic and have no effect on the growth (doubling time, rates of DNA and RNA synthesis) of C3H/10T1/2 mouse embryo fibroblast cells in culture [4, 22]. Further, the levels of dietary protease inhibitors required to suppress carcinogenesis in ani-

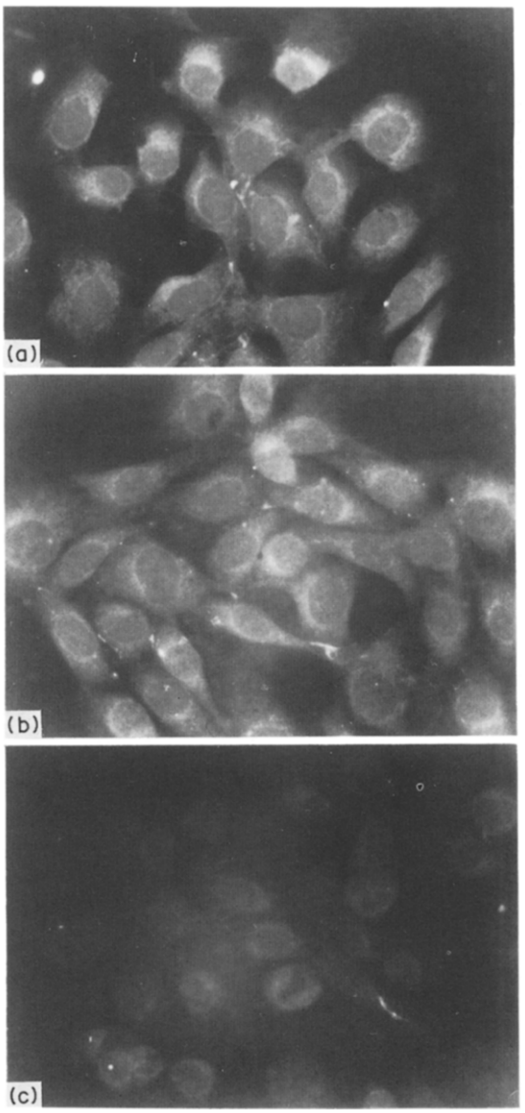

Fig. 3. Decrease of TRITC-labeled BBI in IEC cells. (a) Cells grown for 0 h, (b) cells grown for 2 h, (c) cells grown for 12 h (× 630).

mals have been found to have no adverse effects on the general health of the animals including toxicity or reduced weight gain [4, 7, 8, 10].

We have also demonstrated that BBI was taken up by IEC cells in a time-dependent manner. TRITC-BBI could be visualised in the cells within 15 min of incubation and the amount present in the cells increased with longer incubation times. Further, we found that labeled BBI could be observed in the cells for 12 h after removal of the inhibitor from the tissue culture medium. Preliminary studies using a monoclonal antibody to BBI have also confirmed the uptake of BBI by IEC-17 cells [23]. In prior studies, C3H/10T1/2 cells were shown to internalise several anticarcinogenic protease inhibitors such as BBI, as well as insulin [5, 24]. The mechanism of uptake of the BBI, or other anticarcinogenic protease inhibitors, by mammalian cells is not known. We have not detected surface receptors, capable of reversibly binding anticarcinogenic protease inhibitors, on the surface of C3H/10T1/cells [4]; these results suggest that the uptake of these compounds is not mediated by a specific receptor on the cell surface. The uptake of the BBI by IEC cells was qualitatively similar to the uptake of BBI and other vegetable-

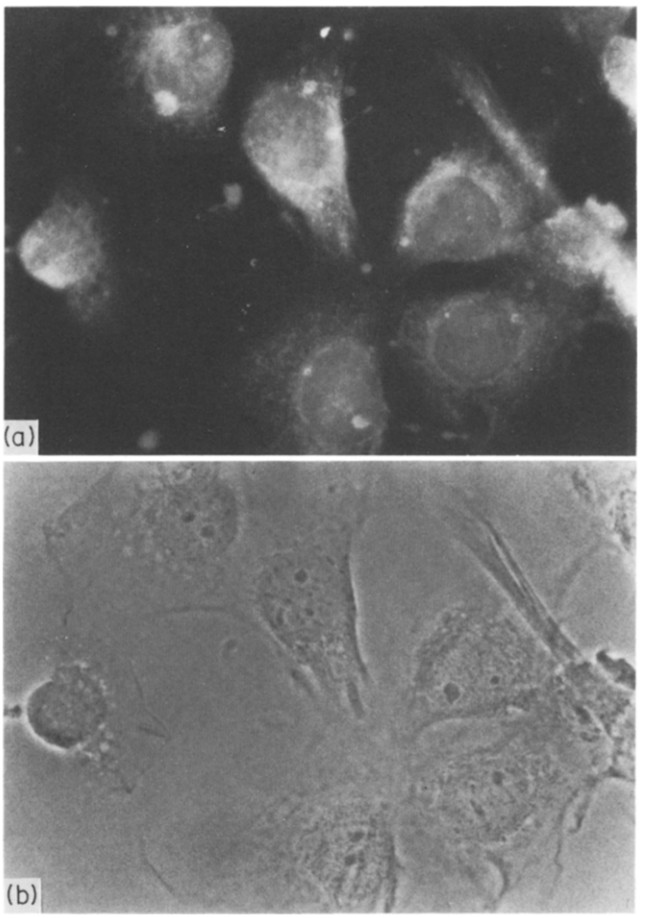

Fig. 4. TRITC-BBI in reseeded cells. (a) Fluorescent micrograph, (b) phase contrast micrograph of (a) (× 1000).

derived anticarcinogenic protease inhibitors by mouse fibroblasts [4, 24, 25]. Further, the uptake of BBI by IEC cells and mouse fibroblasts is inhibited when the cells were incubated on ice [24] suggesting that protease inhibitor uptake takes place by a similar mechanism in both cell lines.

Although the mechanisms by which protease inhibitors suppress carcinogenesis are not fully understood, we have hypothesised that these compounds exert their anticarcinogenic effects

Table 2. Marker enzyme activity in IEC subcellular fractions

Subcellular fraction (1000 *g*)	Enzyme activity	
	β-Glu	LDH
1 pellet	0.26 (0.01)	2.01 (0.05)
10 pellet	1.95 (0.10)	1.41 (0.04)
100 pellet	0.69 (0.04)	0.97 (0.02)
100 supernatant	0.60 (0.03)	8.07 (0.20)

β-Glu = β-Glucuronidase activity (lysosomal marker enzyme) expressed as $\triangle OD_{540\ nm}$/12 h per mg protein.
LDH = Lactate dehydrogenase (LDH) activity (cytosolic marker enzyme) is expressed as $\triangle OD_{340\ nm}$/min/mg protein.
Numbers in parentheses indicate the total amount of enzyme activity present in the sample expressed as the specific activity × total protein present in the sample ($\triangle OD_{540\ nm}$/12 h for β-Glu, $\triangle OD_{340\ nm}$/min for LDH).

Table 3. TRITC-BBI in IEC subcellular fractions

Subcellular fraction (1000 *g*)	Total fluorescence*	Specific activity†
1 pellet	658	3.29
10 pellet	66	6.44
100 pellet	34	1.31
100 supernatant	1000	99.50

* Total amount of fluorescent BBI present expressed as relative fluorescent units (RFU).
† RFU/mg protein ($\times 10^{-3}$).

by inhibiting cellular enzymes involved in the induction and/or expression of the transformed phenotype [17, 18]. We have found that BBI is a highly effective suppressor of colon carcinogenesis [3, 10] and can downregulate c-*myc* gene expression in the mouse colon [16]. We believe that both of these effects are directly related to the ability of BBI to be internalised by the stem cells at the base of the colonic crypts. In the present investigation, we have shown that BBI is internalised by intestinal epithelial cells. The majority of the internalised BBI is present in the cytosol. Further, we were able to recover fluorescent-labeled material capable of binding to a chymotrypsin affinity column, indicating that active protease inhibitor is present inside the cells. Once inside the cells, the BBI would be free to interact with the critical "target enzymes" in these cells to suppress carcinogenesis.

In conclusion, protease inhibitors have been shown to suppress carcinogenesis in several animal model systems including dimethylhydrazine-induced colon carcinogenesis in mice [3, 10]. Since this is considered to be a representative model for the human disease [26], we feel that these results have direct relevance to the human disease. Further, human populations consuming high levels of legumes in the diet have a lower incidence of colon, breast, pancreatic and prostate cancer [12–14]. Consequently, we believe that protease inhibitors will be potentially useful for reducing cancer incidence, and in particular colon cancer rates in the US population. The present report demonstrates that BBI is internalised by normal intestinal epithelial cells, is non-toxic and has no effect on the growth of these cells.

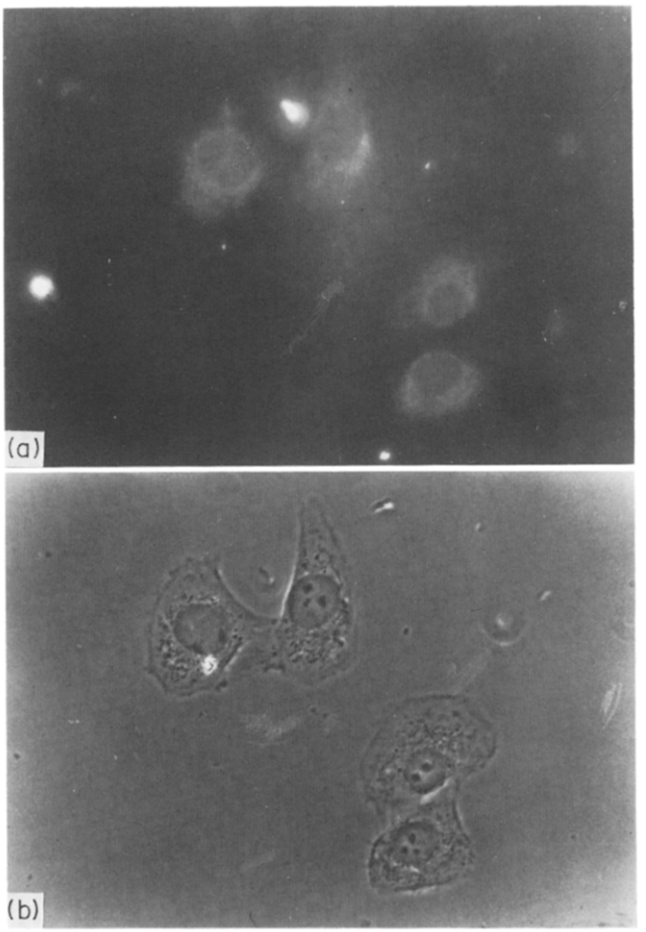

Fig. 6. Uptake of TRITC-labeled insulin by logarithmically growing IEC-17 cells. (a) Fluorescent micrograph, (b) phase contrast micrograph of (a) (× 1000).

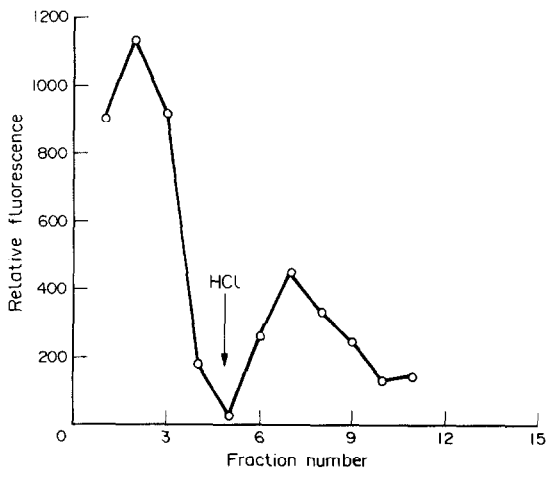

Fig. 5. Affinity chromatography analysis of cells incubated with BBI. Bound BBI was eluted with 0.1 mol/l HCl (arrow). The amount of labeled-BBI binding to the column was determined in a spectrofluorometer at excitation and emission wavelengths of 578 and 604 nm, respectively.

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Hormone Sensitivity in Breast Cancer: Influence of Heterogeneity of Oestrogen Receptor Expression and Cell Proliferation

R.I. Nicholson, N. Bouzubar, K.J. Walker, R. McClelland, A.R. Dixon, J.F.R. Robertson, I.O. Ellis and R.W. Blamey

The percentage of oestrogen receptor (ER) positive cells in a series of 118 breast cancers has been examined by immunohistochemistry in relation to patients' response to endocrine therapy. Positive and negative predictive values have been used to calculate appropriate cut-off points. The rate of response to treatment was significantly higher in women with receptor positive tumours, especially where the tumours contained more than 70% positive cells. Tumours that were apparently negative for ER expression rarely responded to endocrine therapy. The hormone sensitivity of ER positive breast cancer was also influenced by the rate of tumour cell proliferation, with tumours expressing high levels of Ki67 immunostaining rarely responding to therapy.
Eur J Cancer, Vol. 27, No. 7, pp. 908–913, 1991

INTRODUCTION

THE PREDICTION of hormone sensitivity in breast cancer has occupied clinical and biochemical laboratories for many years. This preoccupation is based on the relatively complication free and lengthy tumour remissions that may be achieved in advanced breast cancer patients with endocrine therapy and the need to preselect patients with primary breast cancer for adjuvant therapies. Some success has already been achieved in this area using the hormone receptor status of either the primary or secondary disease [1, 2], especially when quantitative aspects of the assays are taken into account [3] or when more than one hormone receptor is measured [4]. Unfortunately, the steroid binding assays [1–4], and more recently the enzyme immunoassays [5] that have been employed in these studies have required the solubilisation of the receptor prior to its measurement and have necessitated access to appreciable quantities of tissue. Thus, information on the heterogeneity of hormone receptor expression is lost and tumour tissue is often not available for other assays. These technical difficulties have, however, now been overcome through the use of monoclonal antibodies to