Reversible Inhibition of Proliferation of Epithelial Cell Lines by *Agaricus bisporus* (Edible Mushroom) Lectin

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ABSTRACT

Galactosyl β-1,3-N-acetyl galactosamine (Gal β-1,3-GalNAc) (Thomsen Friedenreich antigen), the Class I core sequence in O-linked oligosaccharide chains, behaves as an oncofetal antigen showing increased expression in many epithelial malignancies. Previous work has shown that peanut agglutinin (PNA), a lectin that binds Gal β-1,3-GalNAc, stimulates proliferation in HT-29 (human colon cancer) cells and normal human colonic epithelium and this implies that cell surface glycoproteins which express Gal β-1,3-GalNAc may play a significant role in the regulation of epithelial cell proliferation. We have now studied the effect on epithelial cells of another dietary Gal β-1,3-GalNAc-binding lectin, the edible mushroom *Agaricus bisporus* lectin (ABL). This differs from PNA in its ability to bind also to sialylated Gal β-1,3-GalNAc.

In contrast to PNA, ABL (25 μg/ml) inhibited incorporation of \(^{3}H\)-thymidine into DNA of HT29 colon cancer cells by 87% (95% confidence limit, 85–89%), Caco-2 colon cancer cells by 16% (95% confidence limit, 12–20%), MCF-7 breast cancer cells by 50% (95% confidence limit, 47–52%), and Rama-27 rat mammary fibroblasts by 55% (95% confidence limit, 51–60%) when these cells were grown for 24 h in serum-free medium. When assessed by cell count, inhibition of proliferation of HT29 cells by ABL was found. In the presence of 2% fetal calf serum (which contains the ABL-binding glycoprotein fetuin), the inhibitory effect of ABL on cell proliferation was still demonstrable but at increased ABL concentration (60 μg/ml for 49% inhibition). Ten μg/ml ABL completely abolished the stimulatory effect on \(^{3}H\)-thymidine incorporation of epidermal growth factor (100 μg/ml) and PNA (25 μg/ml) and markedly inhibited the stimulatory effect of insulin (50 ng/ml).

ABL (0.2 mg/ml) caused no cytotoxicity to HT29, MCF-7, and Rama-27 cells as measured by trypan blue exclusion, and inhibition of proliferation in HT29 cells caused by 50 μg/ml ABL was reversible after removal of the lectin. Binding studies with \(^{125}\)I-labeled ABL suggested a single class of binding site with an apparent Kd value of (4.12 ± 0.29) \(\times 10^{-7}\) M with (3.6 ± 0.3) \(\times 10^{7}\) binding sites/ceil.

A. *bisporus* lectin is a reversible nontoxic inhibitor of epithelial cell proliferation which deserves study as a potential agent for cancer therapy.

INTRODUCTION

Colorectal cancer is the second most common cause of cancer-related death in the Western world and affects about 1 in 15 people at some time in their lives (1). In the colon, as in other epithelia, changes in surface carbohydrate expression are common in neoplasia and often represent neo-expression of oncofetal antigens (2, 3). These changes have often been identified in studies by ourselves (4) and others using enzyme-conjugated lectins as histochemical tools.

Until recently, very little has been known about the functional significance of these changes in carbohydrate expression. There is increasing evidence that changes in carbohydrate expression may play a key role in determining the metastatic behavior of tumor cells (5–9). Among the most commonly demonstrated abnormalities in malignant and hyperplastic epithelia has been the increased expression of the blood group TF-antigen which has the structure galactosyl β-1,3-N-acetyl-galactosamine β-1,3-GalNAc may play an important role in the regulation of epithelial cell proliferation. We have now studied the effect on epithelial cells of another dietary Gal β-1,3-GalNAc-binding lectin, the edible mushroom *Agaricus bisporus* lectin (ABL). This differs from PNA in its ability to bind also to sialylated Gal β-1,3-GalNAc.

MATTERIALS AND METHODS

Materials

All reagents were of analytical grade and purchased from BDH Chemicals Ltd. (Poole, United Kingdom) unless indicated otherwise. Culture medium, antibiotics, FCS, insulin, PBS, Nunc tissue-culture dishes and 24-multiwell plates were obtained from GHBCO Life Technologies Ltd. (Paisley, United Kingdom). [Methyl-\(^{3}\)H]thymidine and carrier-free \([^{125}\)I]-sodium iodide were obtained from Amersham International (Amersham, United Kingdom). EGF was purified from male mouse submaxillary glands (11). ABL, PNA, peroxidase conjugated PNA, bovine serum albumin, glucose, galactose, N-acetyl glucosamine, fucose, and N-acetyl galactosamine were obtained from Sigma Chemical Co. (Poole, United Kingdom). Galactosyl β-1,3-N-acetyl-galactosamine was from Biocarb Chemicals (Lund, Sweden).

Cell Lines

The HT29 cell line, established from an adenocarcinoma from a 44-year-old female Caucasian (12) and Caco-2 cell lines, isolated from a primary colon tumor in a 72-year-old Caucasian male (13), were obtained at unknown passage number from the European Cell Culture Collection at the Public Health Laboratory Service, Porton Down Wilts, United Kingdom. MCF-7 epithelial cell line, derived from a human malignant pleural effusion from a breast adenocarcinoma (14) and Rama-27 cell line, derived from the fast-sticking fraction of normal rat breast and defined as fibroblastic by its ability to differentiate toward the adipocyte phenotype (15), were from the Department of Biochemistry, University of Liverpool. Stock cultures in 9-cm diameter dishes were grown in monolayer and maintained at 37°C in a humidified atmosphere of 5% CO\(_2\)/95% air. The cell lines were maintained in DMEM supplemented with 5% FCS, 100 units/ml of penicillin, 100 μg/ml streptomycin, 4 mgl/ml glutamine, and 50 ng/ml of insulin. The cells were routinely passaged when they had become 80% confluent at a 1:6 subculture ratio and narrow passage cell lines (passaged fewer than 10 times) were used for all studies.

Cell Proliferation Assays

\[^{3}\text{H}]\)Thymidine Incorporation. Cells were seeded at a density of 1.2–2.0 × 10\(^{4}\)/well in 0.5 ml of DMEM containing 5% FCS in 24-well plates. After 48 h incubation at 37°C in a humidified atmosphere of 5% CO\(_2\)/95% air, each well was washed twice with 0.5 ml PBS and then 0.5 ml DMEM containing 250...
µg/ml bovine serum albumin was added to each well as described previously for short-term culture of rat mammary cell lines (16, 17). The cells were incubated at 37°C for a further 24 h in this medium. The lectin under test was then added to the cells for 24 h prior to a 1-h pulse with 0.5 µCi/well [methyl-3H]thymidine as described previously (10, 17). After 2 washes with PBS, the cells were precipitated by addition of 0.5 ml/well 5% trichloroacetic acid at 4°C. The precipitate was washed once with 5% trichloroacetic acid at 4°C, twice with 0.5 ml/well of 95% ethanol at 4°C followed by drying at room temperature, and then solubilized with 0.5 ml/well 0.2 M NaOH. 0.3 ml dissolved precipitate was added to 1 ml Optima Gold MV scintillation cocktail (Packard, Pangbourne, United Kingdom) and the cell-associated radioactivity was determined using a Packard scintillation counter.

These studies were performed in serum-free medium because of the possibility of interference by fetuin (which expresses sialyl-β-1, 3-GalNAc) in FCS. However, further studies were also carried out in the presence of 2% FCS. Studies were performed as described above except that the medium was replaced by 2% FCS in DMEM after the cells had been incubated for 24 h in DMEM containing bovine serum albumin.

Cell Counting. Cells were seeded and cultured as described above. At the end of the culture period, the cells were trypsinized with 0.5 ml 0.05% (w/v) trypsin in 1.6% (w/v) EDTA for 10 min and suspended after addition of 50 µl PBS, and 0.5 ml cell suspension was counted using a Coulter Counter (Luton, Beds, United Kingdom).

Reversibility of Lectin Effect and Viability of Lectin-treated Cells

Reversibility. Cells were seeded at 1.2–2.0 × 10^4/well in 5% FCS/DMEM in 24-well plates and after 48 h incubation at 37°C the medium was replaced with serum-free DMEM. The lectin was added after 24 h incubation in serum-free DMEM. Twenty-four h later the cells were washed twice with PBS containing 10 µm galactose and twice with PBS alone to remove ABL. Cells were then cultured in 5% FCS/DMEM for an additional 5 days and sample wells were counted daily.

Cell Viability. Cells were seeded and cultured as described above. After 24 h of incubation with lectin in 24-well plates, the medium was removed and the cells were washed twice with PBS and the cell viability was determined by Trypan blue dye exclusion (18).

Preparation of 125I-ABL and 125I-EGF

ABL and EGF were each radiolabeled with carrier-free 125I-sodium iodide (specific activity, 15.04 mCi 125I/µg) by a modification (17) of the Iodogen method (19). Briefly, 10 µl ABL, 1 mg/ml (5 µg of EGF), were dissolved in 40 µl 0.5 µM phosphate buffer, pH 7.4, and reacted for 10 min at room temperature with 5 µg Iodogen in the presence of 0.2 mCi Na[125I]. The reaction was terminated by the addition of 5 µl 100 mM sodium metabisulfite, 5 µl 100 mM potassium iodide, and 50 µl PBS containing 0.2% gelatin and 0.02% azide. The iodinated protein was separated from free 125I by chromatography on Sephadex G-25. The specific activity of the radiolabeled ABL was 8.8 µCi/µg (labeled EGF was diluted to 5 µg/ml; specific activity, 18.8 µCi/µg).

Binding Studies

Binding studies were performed as described previously (20). Briefly, confluent cells were released by trypsinization and seeded at 2.5 × 10⁴ cells/well in 24-well plates in DMEM containing 5% FCS. After 48 h, the cells were washed and the medium was changed to the serum-free medium followed by a further incubation for 24 h at 37°C. Cells were rinsed twice with 0.5 ml/well PBS and once with 0.2 ml binding medium (DMEM containing 10 µs Heps, pH 7.4, and 0.1% bovine serum albumin) at 4°C. New binding medium 0.2 ml/well containing 0.1 µg/ml 125I-ABL was added followed immediately by cold ABL at the concentrations indicated in Fig. 9. Cells were incubated at 4°C for 2 h. Preliminary studies of the time course of 125I-ABL binding had shown that a steady binding state was reached within 90 min (Fig. 9B). Unbound radioactivity was removed by washing twice at 4°C with 0.2 ml/well binding medium and then twice with 0.5 ml PBS. The washed cells were solubilized in 0.5 ml/well 0.2 M NaOH and the total cell-associated radioactivity was measured using a Cobra auto-gamma counter (Canberra Packard). The results were analyzed using the LIGAND program of Munson and Rodbard (21).

The effect of neuraminidase treatment on competition between PNA and ABL binding by HT29 cells was studied. After 24 h incubation in serum-free medium, 0.2 units/ml neuraminidase was added. Forty min later at 37°C, cells were washed twice with PBS followed by binding medium as before. Fresh binding medium, 0.2 ml, containing 0.1 µCi/ml of 125I-ABL was added into each well and PNA was added at a range of concentrations between 0 and 50 µg/ml.

Membrane Preparation and Lectin Blotting

Confluent HT29 cells (10⁶) were washed twice with PBS and scraped from the culture dishes and centrifuged at 1000 g. Two ml 20% Hepes-0.2 mM MgCl₂, pH 7.0, was added to the pellet and the cell suspension was sonicated twice for 30 s on ice using a MSE sonicator. EDTA was added to a concentration of 5 mM and the cells were sonicated twice more for 30 s followed by another centrifugation at 1000 g. After addition of 2% aprotinin (Sigma, St. Louis, MO) and 20 mg/ml of leupeptin (Sigma) the supernatant was centrifuged at 100,000 g for 60 min at 4°C. The precipitate was resuspended in 1 ml of 20 µg Hepes, pH 7.0, and stored at −80°C until use. The protein content was tested by modified Lowry’s method (22).

SDS-polyacrylamide gel electrophoresis was performed using a discontinuous buffer system (Tris-glycine, pH 8.0; 0.006% SDS) with 3% stacking and 5% running gels (23). The membrane preparation was mixed 1:1 with 0.125 M Tris buffer, pH 6.8, containing 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 0.01% bromophenol blue, and 10% glycerol and heated at 100°C for 5 min. A 25-µl sample (50 µg protein) was applied to each lane. After electrophoresis, the separated proteins were transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) in a Bio-Rad blotting apparatus with Tris-glycine-methanol buffer for 17 h with 70 mA current at room temperature. The nitrocellulose sheet was blocked in 50 µs Tris/HCl and 150 µs NaCl (pH 7.5) containing 1% bovine serum albumin and 0.5% Tween-20 for 2 h and then incubated with peroxidase-conjugated lectin with or without fetuin or galactose on a roller at room temperature for 1 h. After washes with Tris/HCl buffer, the peroxidase-conjugated lectin was visualized with 2-chloronaphthol (20 ml Tris/HCl buffer, 5 ml 3 mg/ml 2-chloronaphthol in methanol and 30 µl 30% H₂O₂).

RESULTS

Effect of ABL on Proliferation of HT29, MCF-7, Caco-2, and Rama-27 Cells. ABL produced marked inhibition of thymidine incorporation in a dose-dependent manner in human colon cancer cells HT29 and Caco-2, breast cancer cells MCF-7, and rat mammary fibroblasts Rama-27. Fifty % inhibition was observed at 0.3 µg/ml ABL for HT29 cells, 25 µg/ml for MCF-7, and 5 µg/ml for Rama-27 cells (Fig. 1). Twenty-five µg/ml ABL produced 87% (95% confidence, 85–89%; n = 6 separate experiments, each performed in triplicate) inhibition of [3H]thymidine incorporation by different cell lines. The ABL effect is expressed as percentage inhibition of the response obtained with medium alone. Values represent means ± SD of triplicate determinations from one of five experiments.

Fig. 1. Dose response relationship for ABL inhibition of incorporation of [3H]thymidine into DNA by various cell lines. The ABL effect is expressed as percentage inhibition of the response obtained with medium alone. Values represent means ± SD of triplicate determinations from one of five experiments.
inhibition of thymidine incorporation for HT29 cells, 16% inhibition (12–20%) for Caco-2 cells, 50% inhibition (47–52% n = 5) for MCF-7, and 55% inhibition (51–60% n = 5) for Rama-27. In the presence of 2% FCS, ABL caused 46% inhibition of thymidine incorporation by HT29 cells at 60 μg/ml (Fig. 2).

Parallel experiments were performed in which HT29 cells were released by trypsinisation and counted to confirm that changes in thymidine incorporation were true reflections of altered cell proliferation. After 24 h incubation with 10 μg/ml of ABL cell proliferation was almost totally inhibited (Fig. 3). A longer culture period was not possible using the serum-free medium. Further experiments showed that HT29 cell growth was inhibited 49% (45–53%; n = 3) by 60 μg/ml ABL after cells were incubated with ABL for 3 days in the presence of 2% FCS (Fig. 4).

**Inhibition of Proliferative Effect of EGF, PNA, and Insulin.** In the presence of 10 μg/ml ABL, the normal stimulatory effect of EGF (100 pg/ml) and PNA (25 μg/ml) was completely abolished (Fig. 5) and the stimulatory effect of insulin (50ng/ml) was markedly inhibited in a dose-dependent manner (Fig. 6). Further studies in the presence of EGF (100 pg/ml) and also in the presence of PNA (25 μg/ml) showed the inhibitory effect of ABL to have a similar dose-response (data not shown).

**Reversibility and Lack of Cytotoxicity.** HT29 cells incubated at 37°C for 24 h with ABL at 5, 10, and 50 μg/ml were able to proliferate again after removal of the lectin by 2 washes with 0.5 ml/well DMEM containing 10 mM galactose, and 2 washes with 0.5 ml/well of PBS (Fig. 7). Trypan blue dye exclusion analysis of cells incubated at 37°C for 24 h in the presence and absence of ABL at 5, 10, 50, 100, and 200 μg/ml showed >95% viability as did the control cells. MCF-7 and Rama-27 cells were also cultured for 24 h in the presence of ABL 200 μg/ml and showed >95% viability by trypan blue exclusion.

**Inhibition of ABL Effect by Gal β-1,3-GalNAc.** Gal β-1,3-GalNAc, 0.5 mM, blocked 90% (85–94%; n = 4) of the inhibitory effect of ABL (Fig. 8), showing that ABL inhibition was mediated through its sugar-specific binding site. α-L-fucose, α-galactose, N-acetyl-α-glucoosamine, N-acetyl-β-galactosamine, and α-lactose at 50 mM all failed to block ABL inhibition.

**Effect of Neuraminidase.** Since ABL can bind both Gal β-1,3-GalNAc (TF-antigen) and sialyl Gal β-1,3-GalNAc whereas PNA will not bind sialyl Gal β-1,3-GalNAc (24), the effect of prior neuraminidase treatment of HT29 cells on the inhibitory effect of ABL and the
stimulatory effect of PNA was investigated. Cells were incubated with 0.2 units/ml neuraminidase at 37°C for 40 min, which removes greater than 95% of the cell membrane-associated sialic acid while maintaining cell viability (25). This resulted in no significant difference to the inhibition of DNA synthesis caused by ABL or the stimulation caused by PNA as compared with control cells (Table 1).

**Cell Binding Studies.** 125I-ABL binding to HT29 cells was best fitted by a single binding site model with a $K_d$ of $(4.13 \pm 0.29) \times 10^{-7}$, corresponding to $(3.6 \pm 0.3) \times 10^7$ receptors/cell (Fig. 9A). The time course of 125I-ABL binding at 4°C is shown in Fig. 9B.

**PNA Interaction with 125I-ABL Binding.** PNA did not compete with ABL for binding sites on normal HT29 cells, but, if the cells were treated with neuraminidase first, PNA competed partially with ABL binding as shown in Fig. 10. This indicates that sialyl Gal β 1,3-GalNAc (cryptic TF-antigen) is expressed in addition to the TF antigen itself in HT29 cells.

**Lack of Effect of ABL on EGF Binding.** Competitive studies using 125I-EGF showed that under conditions in which ABL inhibited the proliferative effect of EGF, the binding of EGF was unaffected even when high concentrations of ABL were used (Fig. 11).

**Lectin Blotting.** SDS-polyacrylamide gel electrophoresis followed by lectin blotting demonstrated that the HT29 membrane preparation contained at least 12 glycoproteins which bind to ABL and PNA. No definite difference in the binding patterns of the two lectins could be observed (Fig. 12).

**DISCUSSION**

This study shows that *Agaricus bisporus* lectin causes dose-dependent inhibition of proliferation of HT29 human colorectal carcinoma cells, Caco-2 human colorectal cancer cells, human breast cancer MCF-7 cells, and rat mammary fibroblast Rama-27 cells. The effect on HT29 cells is reversible, is not associated with cytotoxicity, and seems to be both potent and nonspecific as shown by the inhibition of the growth of cells stimulated by diverse factors including peanut lectin, epidermal growth factor, and insulin.

Inhibition of proliferation of mitogen stimulated lymphocytes by ABL has been reported previously (26) but in contrast ABL has been shown to stimulate vascular smooth muscle and endothelial cell proliferation (27). However, both these studies were performed in the presence of serum, which contains glycoproteins which express sialyl
AGARICUS BISPORUS LECTIN INHIBITS EPITHELIAL CELL PROLIFERATION

Fig. 9. Binding of 125I-ABL to HT29 cells. a, effect of increasing lectin concentration; b, time course of binding (125I-ABL 0.1 μg/ml). The data presented in a represent the means ± SD of quadruplicate determinations from one of four similar experiments.

Fig. 10. Effects of neuraminidase treatment on the competition between PNA and ABL binding to HT29 cells. Values represent means ± SD of triplicate determinations from one of three experiments.

Fig. 11. Lack of effect of ABL on 125I-EGF binding; 0.2 ml per well of binding medium containing 13 ng/ml of 125I-EGF was used. Cells were incubated for 2 h at 4°C with 125I-EGF and ABL before cell-associated radioactivity was determined. The results represent the means ± SD of quadruplicate determinations from 1 of 2 similar experiments.

Fig. 12. Lectin blot of HT29 cell membrane preparation. a, blotted with 2.5 μg/ml peroxidase as control; b, 2.5 μg/ml peroxidase-ABL; c, 2.5 μg/ml peroxidase-ABL in the presence of 4 mg/ml fetuin; d, 2.5 μg/ml peroxidase-PNA; e, 2.5 μg/ml peroxidase-PNA in the presence of 0.4 M galactose.

Lectins such as phytohemagglutinin and wheat germ agglutinin are well known for their toxic effects on intestinal epithelial cells (29, 30) and Vicia faba lectin inhibits thymidine incorporation in Caco-2 cells (31) but there has been no previous demonstration of a lectin that has such a profound inhibitory effect on proliferation as ABL without apparent cytotoxicity. This lack of cytotoxicity suggests that it must be acting by a different mechanism than the irreversible mechanism by which ricin and abrin inhibit protein synthesis by inhibiting the ribosome-dependent GTPase activity (32).

We found that 50 mM L-fucose, β-galactose, N-acetyl-β-glucosamine, N-acetyl-β-galactose, and β-lactose all failed to block ABL

Galβ1,3-GalNAc, the binding site for ABL (e.g., fetuin in fetal calf serum; Ref. 28) and this makes interpretation difficult. Inhibition by ABL of HT29 cell proliferation was nevertheless observed in our study in the presence of 2% FCS but required an approximately 20-fold increase in lectin concentration to demonstrate an equivalent inhibitory effect. This is in keeping with the results of experiments (data not shown) in which 2% FCS could completely abolish the agglutinating effect of up to 12.5 μg/ml ABL on normal human red blood cells.

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inhibition. These results are consistent with those reported by Presant and Kornfeld (33) who found that whereas Gal-GalNAc-bearing glycoproteins could produce marked inhibition of red cell agglutination by ABL, single sugars produced only a slight effect but differ from those reported by Greene et al. (26) who found that 50 mM, β-galactose, N-acetyl-α-galactosamine, α-threonine, and α-lactose could block the inhibitory effect of ABL on lymphocytes.

It is intriguing that blotting studies showed no obvious difference between the binding profile of the two lectins to an HT29 membrane preparation although they have opposite effects on proliferation of the cell line. It is likely though that each lectin will be binding to a different range of oligosaccharide chains on the glycoproteins. Pretreatment of cells with neuraminidase produced no significant change in the effect of either lectin. This suggests that the binding of ABL but not PNA to sialyl Gal-1,3-GalNAc is not the main reason for their different effects.

ABL inhibits proliferation of a wide range of cells whether unstimulated or stimulated by a wide range of growth factors including FCS, EGF, insulin, and PNA, which suggests that ABL has a very general effect on cell growth, perhaps inhibiting a part of the intercellular signalling pathway or nutrient uptake. It has been shown that plastic cells may have a crucial role in determining their rate of proliferation effect to occur. Lectin-induced changes in metabolism in Caco-2 cell lines have been shown to be linked with internalization of lectin (29, 35), which lends indirect support for this idea.

These studies give further support to the hypothesis that lectins which interact with epithelial cell surface glycoproteins which express Gal-1,3-GalNAc (TF-antigen) may have very important effects on proliferation and that neo-expression of TF on malignant and hyperplastic cells may have a crucial role in determining their rate of proliferation. The potent and reversible inhibitory effect of the Agaricus bisporus lectin on proliferation in a range of cell lines suggests that it deserves study as a potential anticancer agent.

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