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Mannoprotein Adhesin of *Candida albicans* Germ Tubes

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Abstract: The production and detection of a mannoprotein adhesin (MPA) of the hyphal-form cells of *C. albicans* on plastic petri dishes was investigated. Using Concanavalin A-coated latex microspheres, the MPA was detected on the plastic surface on which *C. albicans* produced germ tubes. The adhesin was extracted using dithiothreitol and iodoacetamide. It did not inhibit the adhesion of the yeast-form *C. albicans* to buccal epithelial cells (BEC). This suggested that the MPA of the hyphal-form cells on the plastic was different from that on the yeast cells responsible for attachment to the BECs.

Key Words: *Candida albicans,* adhesin, germ tube, plastic petri dish, buccal epithelial cells.

Introduction

The pathogenic yeast *Candida albicans* has the ability to adhere to inert surfaces such as dentures, contact lenses, catheters and prostheses such as heart valves (1-3). Invasion of the human host by the fungus can therefore occur by adherence to such plastics followed by its dissemination through the vascular system (4). *C. albicans,* through its ability to bind to dentures, may cause denture stomatitis (1, 5-7). In the laboratory, small, transparent acrylic strips may be incubated with the yeast cells and, after removing the unattached organisms by rinsing, the adherent cells can be counted under a microscope (1). Similar methods have been applied to measure yeast adhesion to a variety of denture resin materials and plastics (8, 9).

The adhesion of *C. albicans* to plastic depends on the previous growth conditions of the organism. For example, *C. albicans* grown in 500 mM galactose was more adherent to acrylic strips than that grown in 50 mM glucose (1, 6). Mannoprotein which was isolated from the cell walls of *C. albicans* grown in a high concentration of galactose, reduced adherence to acrylic as it did to buccal epithelial cells (10). Hydrophobic interactions appeared to be of primary importance in the adhesion of *Candida* species to plastic surfaces *in vitro* (8, 9, 11). Adherence of *C. albicans* to acrylic was prevented by chitin derivatives (12).

Tronchin et al. observed that hyphal-form cells were more adherent than yeast forms to plastic petri dishes (13). The adhesin was extracted using dithiothreitol and iodoacetamide and, when analysed by SDS-PAGE, revealed that four specific proteins, 60, 68, 200 and >200 kDa were involved in *C. albicans* germ-tube adherence to the plastic. Using the same system to isolate hyphal-form cell wall extracts, two of these protein components (60, 68 kDa) interacted with host proteins such as laminin, fibrinogen or C3d (14, 15). Adherence of yeast and hyphal-form cells, which had been grown in yeast nitrogen base containing galactose and 199 Medium respectively, to whole saliva-coated acrylic was similar (16). Recently, Hawser and Douglas showed that the growth of *C. albicans* as biofilms on plastic catheters in a medium containing 500 mM galactose or 50 mM glucose reached a maximum after 48 h and then declined (17). However, the cell yield was lower with the cells grown in the glucose medium. The results also confirmed that *C. albicans,* in 500 mM galactose medium, synthesises a fibrillar mannoprotein which was responsible for the increased yeast adhesion to inert surfaces such as acrylic plastic *in vitro* (6, 17). According to Vasilas et al. yeast and hyphal-forms of a wild type strain did not differ substantially in their ability to adhere to saliva-coated acrylic *in vitro* but the germ tube-deficient mutant had a lower binding capacity (16). Hyphal and yeast-form cells were also observed as biofilms on the plastic catheters,

but the yeast form only was observed on an agar surface of the same medium (or in liquid culture). These results indicate that a contact-induced regulation of gene expression at the molecular level may be occuring during morphogenesis (17).

The aim of this study was to detect the production of mannoprotein adhesin of hyphal-form *C. albicans* on plastic by using Concanavalin A-coated latex microspheres. The adhesin was isolated from the plastic and then tested for its ability to inhibit the attachment of yeast cells of *C. albicans* to buccal epitheial cells.

Materials and Method

Organism

C. albicans strain GDH 2346, isolated at Glasgow Dental Hospital from a patient with denture stomatitis, was used in this study (1). It was deposited in the National Collection of Yeast Cultures, Food Research Institute, Norwich, England as strain NCYC 1467. The strain was supplied as freeze-dried samples from which further freeze-dried ampoules were prepared. The organism was maintained on the slope of Sabouraud dextrose agar (Difco) and subcultured monthly. Every two months, cultures were replaced by new ones freshly grown from freeze-dried stocks.

Germ tube formation on petri dishes

Germ tubes were produced by the modified method of Tronchin et al. (13). For the preparation of starved yeast-form cells, stock cultures were inoculated into Lee medium (18) containing the following: $(NH_4)_2SO_4$, 5.0 g; $MgSO_4.7H_2O$, 0.2 g; K_2HPO_4, 2.5 g; NaCl, 5.0 g; glucose, 10.0 g, biotin 0.04 g in 1.0 L distilled water (pH 6.8) and incubated at 25°C for 36 h with orbital shaking at 150 rpm. The cells were harvested by centrifugation (2300 g, 5 min, Megafuge 1.0 Heraeus, Sepatech) and washed twice with distilled water. The final pellet of starved yeast cells was resuspended in 2 ml distilled water. This suspension was added dropwise into 60 ml IX Medium 199 (Modified) with Hank's salts and 20 mM HEPES buffer, but without glutamine and sodium bicarbonate (Flow Laboratories, Irvine Scotland) pH 6.7 to adjust the concentration of the starved cells to $2x10^7 ml^{-1}$. This suspension in 15 ml aliquots in 199 Medium was poured into four petri dishes of 90 mm diameter made from biologically-neutral polystyrene (Philip Harris Scientific). Three of the petri dishes were incubated for 3 h at 37°C without shaking to produce germ tubes and the fourth at 22°C for yeast cells.

Germ tube mannoprotein adhesin on petri dishes

1. Con A-coated latex beads

Concanavalin A (Con A)-coated latex beads were used to detect *C. albicans* adhesins on the petri dishes prepared as described above. The detection procedure was based on the methods of Quash et al. and Tronchin et al. (13, 19, 20). Carboxylated polystyrene latex beads (0.9 µm, Sigma) were obtained as a 10% latex suspension in 0.14 M NaCl. The bead suspension (200 µl) in Eppendorf tubes was centrifuged with microcentrifuğe (1500 g for 10 min) and washed four times with 0.01 M borate HCl pH 8.1 (BBS). The washed beads, transferred to a universal container, were mixed with 2 ml of 1 mg ml^{-1} Con A (Sigma) and 1 mg 1-Ethyl-3-Dimethylamino propyl carbodiimide HCl, (EDC, Sigma) was added. The mixture was incubated for 2 h at 37°C with occasional shaking. After two washes with 20 ml BBS containing 1% (w/v) BSA (Sigma) (BBS-BSA), the latex beads coated with Con A were suspended in 20 ml BBS-BSA containing 0.1% (v/v) Tween 20.

2. Detection of adhesins

The yeast and hyphal-form cells were removed from the plastic surface of the petri dishes with a plastic scraper (Falcon, 3086-Becton Dickinson) designed for tissue culture work. The petri dishes were then washed three times with 10 ml distilled water and examined microscopically for completeness of removal of germ tubes. The dishes were then 'blocked' by incubation with 15 ml phosphate buffered saline (PBS) containing 0.5% (w/v) BSA (PBS-BSA) for 1 h at room temperature, to prevent nonspecific binding. The suspension of latex beads coated with Con A (5 ml) was poured into each petri dish and incubated for 30 min at room temperature to allow binding. Excess latex beads were withdrawn with a pipette and the dishes washed three times with 10 ml PBS-BSA prior to microscopical observation of the latex beads on the petri dishes. In each experiment, two control plates for the hyphal-form cells were prepared. One consisted of a scraped and blocked dish into which was poured a preincubated (for 30 min at room temperature) mixture of 0.2 M α-methyl-D-mannoside (Sigma) with Con A-coated latex beads. The other control was a dish which had not been treated with germ tubes but was subsequently incubated with Con A-coated latex beads.

Purification and characterization of germ tube adhesins

The hyphal-form cells were removed from the plastic surface with a scraper after incubation for 3 h. The petri dishes were washed three times with distilled water. Into

each washed petri dish was put 15 ml tris HCl-mannitol buffer at pH 7.5 and dithiothreitol (Sigma) (0.5 M) in tris HCl-mannitol buffer added to a final concentration of 12 mM. After incubation for 2 h at 37˚C with constant shaking, 0.5 M iodoacetamide in the same buffer was added to a final concentration of 17 mM. All samples were left for 1 h at room temperature for extraction. The extracts were collected and dialysed against four changes of deionized distilled water for 48 h at 4˚C in dialysis tubing with 12,000 Molecular weight (MW) exclusion and finally lyophilised. Carbohydrate was estimated by the method of Dubois et al. with mannose as a standard (21) and protein by the method of Lowry et al. with BSA as a standard (22).

Adhesion-inhibition assay

1. Preparation of yeast cells

In order to produce yeast-form cells, *C. albicans* was inoculated from stock cultures into yeast nitrogen base medium (Difco) supplemented with 500 mM galactose (YNBGal) as described previously (6). An additional filtration through a 0.45 µm membrane filter was carried out. This culture (5 ml) was used to inoculate a second flask of YNBGal medium (45 ml) and incubated at 37˚C for 24 h on an orbital shaker at 150 rpm. The yeast was harvested by centrifugation at 2300 g for 5 min in a bench centrifuge and the total number of yeasts was counted with an improved Neubauer haemocytometer and adjusted to 10^7 cells ml^{-1}.

2. Preparation of epithelial cells

Buccal epithelial cells (BEC) were collected from a single donor who was a 26-year-old healthy male nonsmoker who had had no recent antibiotic therapy, by gently swabbing the inside of the cheeks with a sterile swab. The swab was agitated in 6 ml PBS in a universal container. The BECs were harvested by centrifugation at 2300 g for 5 min in a bench centrifuge and washed twice in 5 ml PBS to remove loosely-bound microorganisms. After the second washing the BECs were counted and adjusted to 10^5 cells ml^{-1} using an improved Neubauer haemocytometer. The BECs were always collected at the same time of day to minimize variability.

3. Assay for inhibition of adherence

The method was based on that described by Duglas et al. (23). BEC suspensions (1 ml containing $1x10^5$ cells ml^{-1}) were centrifuged and the supernates discarded. Mannoproteins adhesin (1 ml) containing 1 mg ml^{-1} protein was added to the BEC pellets and mixed using a whirlimixer. The mixtures were incubated for 30 min at 37˚C on an orbital shaker. After this treatment, the BECs were recovered by centrifugation, resuspended in PBS (1 ml) and used for an adherence assay with *C. albicans.* The BECs (0.1 ml of 10^5 ml^{-1}) and the yeast were mixed in small bijoux bottles and incubated at 37˚C with gentle shaking for 45 min. Control mixtures were set up containing BECs and PBS. After incubation, 2 ml PBS was added to each bottle to stop any further attachment by dilution. The BECs were collected on polycarbonate filters (12 µm pore size; 25 mm diameter; Costar Nuclepore, UK), which retains them but allows free yeast cells to pass through. The filters were washed with 30 ml PBS to remove unattached yeasts. The washed BECs on the filters were placed on labelled slides and left for a few minutes until air-dry, followed by fixation with absolute methanol and staining by the Gram procedure. Finally, the whole filters were mounted under coverslips with DPX mountant (BDH Chemicals Ltd). The markers on the slides were covered and the slides mixed to avoid personal bias. The number of adherent yeasts on each of the 100 BECs was counted on each filter under 400x magnification. Three filters were prepared for each assay and all adherence assays were performed in triplicate.

Results

The production of mannoprotein by hyphal-form *C. albicans* on plastic petri dishes had previously been reported only by Tronchin et al. who used Con A-coated latex beads as a lectin-specific reagent for mannose (13). In this study, the same method was used to produce and detect the mannoprotein adhesin (MPA) on plastic petri dishes in which germ tubes had been produced so as to leave the cell wall fibrillar layer on the surface of the plastic. This material could subsequently be scraped off and concentrated. In four independent experiments, *C. albicans* in 199 Medium was allowed to produce germ tubes for 3 h at 37˚C in three petri dishes and, in parallel, yeast cells at 22˚C. The dishes were then washed three times in distilled water to remove yeast and hyphal-form cells, followed by a wash with PBS containing 0.5% (w/v) BSA, and incubation for 1 h at room temperature. The Con A-coated latex beads were added and, after 30 min, withdrawn, and the dish examined by bright-field microscopy at 400x. Uncoated latex beads and Con A-treated latex beads with α-methyl-D-mannopyranoside were used, both as negative controls.

The Con A-coated latex beads were seen to bind to the plastic of the petri dish which was allowed to produce hyphal-form cells, showing that mannoprotein of the cell wall of *C. albicans* had been produced during germ tube production and had remained during the subsequent

Table 1. Biochemical analysis of cell wall extract of germ tubes from petri dishes

Experiment	Protein (μg/ml)	Carbohydrate (μg/ml)	Ratio[a]
1	1.68	0.39	4.3
2	1.55	0.41	3.7
3	1.68	0.16	1.5
Mean±SEM	1.64±0.04	0.32±0.09	3.16±0.84

[a]protein / carbohydrate

Table 2. Effect of mannoprotein adhesin (MPA) of *C. albicans* at 1 mg ml^{-1} on the adhesion of the yeast to buccal epithelial cells.

Treatment of BEC	Mean of adherenta yeast cells/BEC±SEM	P[b] (%)
MPA + *C. albicans*	18.3+1.5	10
PBS + *C. albicans*	18.2+1.8	10
Control (BEC only)		

[a]Assay done in triplicate

[b]Probability values relative to adhesion of PBS+the yeast cells (control for MPA+the yeast cell)

scraping and washings. The beads were arranged in outlines that reflected the germ tube portion of the hyphal-form cells. Thereas no adhesion of either the uncoated latex beads or the Con-A treated beads with a-methyl-D-mannopyranoside (not shown.). The petri dish which had produced yeast cells contained very few Con-A coated latex beads.

In order to obtain soluble mannoprotein adhesin (MPA) for further experiments, *C. albicans* was grown in the hyphal-form on 70 plastic petri dishes in each of three separate batches. The cells were scraped off the surfaces and the dishes washed with distilled water as described above. Mannoprotein adhesin was extracted with dithiothreitol followed by iodoacetamide. The two extracts were pooled, dialysed, freeze-dried and reconstituted with PBS. Table 1 presents the analyses of protein and carbohydrate estimations in the three batches of soluble MPA, expressed as the yield from 10^{10} hyphal-form cells. The protein values averaged 1.64 mg, with little variation, and the carbohydrate averaged 0.32 mg, but with more variation than the protein values. The average ratio of protein to carbohydrate was approximately 3:1. It is clear from Table 2 that 1 mg ml^{-1} exhibited no detectable inhibitory activity towards the adhesion of *C. albicans* to BECs.

Discussion

Previous research has shown that both yeast cells and hyphal-form *C. albicans* can adhere to host surfaces. But it was suggested that the hyphal-form was more adherent than the yeast-form to epithelial cells (24). There was a strong correlation between germ-tube production and adherence to plastic of *C. albicans* as reported by Tronchin et al. (13). In this study, production of hyphal-form *C. albicans* in 199 Medium at pH 6.7 was carried out in polystyrene petri dishes. After removing the cells with a plastic scraper from the dishes, the mannoprotein adhesins were detected on the plastic surface by use of Con A-coated latex beads, the mannoprotein having a specific capacity to bind concanavalin A. This binding occurred on the plastic surface along the impressions left by the germ tubes which had previously been removed with the plastic scraper. This result indicated that germ-tube formation may cause chemical changes in the cell wall mannoprotein adhesins and enhance adherence to plastic. Confirmation of Con A-adhesins on the germ tube cell wall was obtained by labelling the cells with Con A-gold particles under electron microscopy by Tronchin et al. (13). Although these authors did not report the protein and carbohydrate content of mannoprotein, they purified 4 different proteins after dithiothreitol and iodoacetamide treatment of the washed plastic petri dishes. This study thus confirmed that the mannoprotein adhesins remained on the plastic, even after the organisms had been removed with a plastic scraper. Moreover, control petri dishes with uncoated-latex beads and Con-A treated latex beads, together with α-methyl-D-mannopyranoside, were free of beads. Control petri dishes in which only yeast cells were produced contained few Con A treated-latex beads, showing that yeast-form cells adhered only weakly to the plastic under the same conditions (except temperature) used with germ tubes.

Although mannoprotein adhesins (MPA) were observed on the plastic surface by Tronchin et al., (13) these authors did not test the possible inhibitory activity of hyphal-form MPA towards adhesion of yeast-form *C albicans* on human buccal epithelial cells (BEC). Other studies have isolated extracellular polymeric material (EP) of yeast-form *C. albicans* from culture supernates and

shown blockage of adherence by pretreating the epithelial cells before use in adherence assays (10, 25). In these experiments, EP (crude adhesin) was isolated from *C. albicans* 2346 grown in a defined medium containing a high concentration of galactose (25). Recently, Tosh and Doughas purified a fucose-binding protein fragment by affinity adsorption with the trisaccharide determinant of the H (type 2) blood group antigen which terminates in a residue of L-fucose (26). The purified adhesin inhibited yeast adhesion to BECs. Adhesion inhibiton was observed up to maximum of 80% at an adhesin concentration of 10 µg ml^{-1}. These observations thus implicated the fucose-binding protein as the major adhesin of the yeast cells of *C. albicans.*

In this study, mannoprotein adhesins of germ tubes of *C. albicans* from the petri dishes (1 mg ml^{-1} of protein) were used to pretreat BECs prior to their exposure in an adherence assay. These pretreated BECs were then mixed with yeast-form *C. albicans* to test for inhibitory activity of the mannoprotein adhesins, which had a protein to carbohydrate ratio of 3:1. The result was that the crude germ-tube mannoprotein adhesin, at the concentrations tested, were not able to block yeast adhesion to the BECs. Thus, the mannoprotein adhesin of the hyphal form cells was not identical to those from the yeast cells. Germ-tube mannoprotein adhesins have been used for the identification of laminin, fibrinogen and C3d receptors on SDS-PAGE followed by blotting on nitrocellulose membranes (14,15). The active components were molecules of 60 and 68 kDa and had multiple activities.

Critchley and Douglas reported that treatment of their crude extracellular material from yeast-cell supernates with dithiothreitol nullified its inhibitory effect on the adhesion of *C. albicans* to BECs (25). In this study, dithiothreitol was used to remove the mannoprotein adhesin from the petri dishes and the disulphide bonds thereby broken may have been involved in maintaining the integrity of the protein. Perhaps distraction of disulphide bridges caused the material to be inactive in the adhesion assay. Also, the adhesin molecules might be smaller than 12,000 Molecular weight (MW) and during dialysis, mihgt be lost.

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