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Monoclonal antibodies directed against extracellular matrix proteins reduce the adherence of *Candida albicans* to HEp-2 cells

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Abstract

The presence of the extracellular matrix (ECM) proteins collagen types I and IV, laminin and fibronectin on the surface of HEp-2 cells was confirmed by flow cytometry using monoclonal antibodies. Monoclonal antibodies directed against these ECM proteins reduced the adherence of *C. albicans* ATCC 44990 to HEp-2 cells, the greatest reductions being evident in assays which incorporated anti-collagen type IV monoclonal antibody. The ability of sugaramines to inhibit the adherence of *C. albicans* to a variety of cell types has been demonstrated previously and the most significant reduction in *C. albicans* – HEp-2 adherence was in assays which incorporated 0.2M galactosamine. The combination of anti-collagen IV monoclonal antibody and galactosamine reduced the adherence of *C. albicans* to HEp-2 cells by approximately 70% ($p < 0.05$).

Key words: Adherence, *Candida*, Extracellular matrix, HEp-2

Abbreviations: ECM: Extracellular matrix, MAb(s): Monoclonal antibody (antibodies), PBS: Phosphate buffered saline

Introduction

The dimorphic yeast *Candida albicans* is an opportunistic fungal pathogen of humans and is capable of inducing a range of superficial and systemic diseases in the immunocompromised host [1]. A number of virulence factors enable the yeast to colonise the host and these include the ability to adhere to a range of tissues, to undergo phenotypic switching and to produce extracellular enzymes such as phospholipases and proteases [2]. Adherence of *C. albicans* to host tissue is considered to be crucial to the success of the pathogenic process and appears to be a prerequisite to colonisation and subsequent infection [3, 4]. Adherence is achieved by a combination of specific and non-specific mechanisms. Specific mechanisms involve the ability of the yeast to recognise a variety of host cell receptors using cell surface adhesins [5]. Non-specific mechanisms include electrostatic forces, aggregation and cell surface hydrophobicity [6, 7]. While it is clear that the latter forces are involved in adherence, their overall contribution appears to be less than that of the specific mechanisms [8].

A number of proteins associated with the extracellular matrix (ECM) of epithelial and endothelial cells have been implicated as possible targets for *C. albicans* adherence [9, 10]. These include fibronectin, located in the interstitium of the ECM, collagen types I and IV found in the interstitium and basement membrane of the ECM and laminin which is also located in the basement membrane [10]. Proteins on the surface of *C. albicans* mediate adherence to these molecules and are classified as integrin analogues due to their structural and functional homology to mammalian integrins [11–13].

Partial inhibition of adherence of *C. albicans* to buccal mucosal cells and corneocytes [15], an oesophageal cell line [16] and vaginal epithelial cells [17] has been achieved using sugaramines. This inhibition appears to result from a reversible interaction of the sugaramine with the fungal cell wall preventing direct contact between the fungal cell and the target surface [15].

The aim of the work presented here was to establish whether the ECM proteins laminin, fibronectin and collagen types I and IV were expressed on the surface of HEp-2 cells and to establish the ability of MAbs directed against these proteins to block the adherence of *C. albicans* to HEp-2 cells. The role of MAbs, directed against ECM proteins, in combination with sugaramines in altering the adherence of *C. albicans* to HEp-2 cells was also investigated.

Materials and Methods

Yeast strain and culture conditions

C. albicans ATCC 44990 was used throughout this study. Stationary phase cultures (5×10^8/ml) of *C. albicans* were obtained by growing cells in YEPD (2% (w/v) glucose (Sigma Chemical Co. Ltd., Dorset, England), 2% (w/v) Bactopeptone (Difco Laboratories, Michigan, USA), 1% (w/v) Yeast extract (Oxoid Ltd., Basingstoke, England) overnight at 30 °C and 200 rpm in an orbital incubator.

Cell culture.

The HEp-2 cell line (ATCC CCL23, derived from an epidermoid carcinoma of the larynx [18]) was obtained from the American Type Culture Collection (Maryland, USA) and was cultured in MEM (Sigma) supplemented with 5% (v/v) Foetal calf serum (GIBCO Laboratories, Paisley, Scotland) and 4mM L-glutamine (GIBCO). Cells were grown in tissue culture flasks (Nunc, Roskilde, Denmark) at 37 °C and 5% CO_2 in a humidified atmosphere and subcultured by trypsinisation every 3–4 days.

Monoclonal Antibodies

Commercially available MAbs were used to detect the presence of four ECM proteins. Monoclonal anti-fibronectin (Sigma) recognises an epitope located within the fifth type III repeat of human plasma fibronectin and is common to all fibronectin forms. Monoclonal anti-laminin (Sigma) has an affinity for the basal membranes of human, pig and cat cells but not those of rabbit, mouse or dog. Monoclonal anti-collagen type I antibody (Sigma) recognises the native form of collagen type I and shows no crossreactivity with other collagens such as collagen II, III, IV and V. Monoclonal anti-collagen type IV (Sigma) recognises epitopes located on the alpha 1 and 2 chains of human collagen type IV.

Binding of monoclonal antibodies to HEp-2 cells

Sub-confluent HEp-2 cells were harvested using a cell scraper (Nunc), washed and resuspended in PBS containing the MAbs directed against the ECM proteins. The anti-fibronectin MAb was used at a dilution of 1/100 in PBS while the other three MAbs were used at dilution of 1/200. Cells were incubated at 4 °C for 1 hr at 175 rpm in an orbital incubator to facilitate the binding of the MAbs to the ECM proteins [21]. Subsequently, cells were centrifuged at 1000 rpm for 5 mins, washed twice and resuspended in PBS.

Flow cytometry

Hep-2 cells, previously incubated in the presence of the anti-ECM protein MAbs, were resuspended in 1ml PBS containing anti-mouse IgG (Fab specific) FITC conjugate (Sigma) and incubated at 4 °C for 1 hour at 175 rpm. Cells were harvested by centrifugation, washed with PBS, resuspended in 1ml of FACSflow (Becton Dickinson, San Jose, California, USA) and analysed by flow cytometry on a FACScan (Becton Dickinson) using Lysis II software. In each case the fluorescence of 10,000 cells was analysed.

Adherence assay

Stationary phase cultures of *C. albicans* ATCC 44990 were harvested by centrifugation, washed and resuspended in PBS. Sub-confluent HEp-2 cells were harvested using a cell scraper (Nunc), centrifuged, washed and resuspended in PBS. Yeast and HEp-2 cells were mixed in a ratio of 50:1 (5×10^7: 1×10^5) in a final volume of 2 mls and incubated at 37 °C for 2 hrs. This mixture was subsequently smeared onto glass slides and allowed to air dry before being heat fixed and stained with a 1% (w/v) crystal violet solution. The number of yeast adhering to each of 200 HEp-2 cells was determined microscopically per treatment.

Inhibition assays

The effect of the four anti-ECM MAbs on the adherence of *C. albicans* to HEp-2 cells was determined by first binding the antibodies to the cells as described. Cells were then harvested, washed twice and resuspended in PBS. Yeast cells were added directly to this

cell suspension in a ratio of 50:1 and adherence was measured after 2 hours as described.

The inhibitory action of sugaramines was assessed by incubating yeasts and HEp-2 cells in PBS containing either 0.02 or 0.2 M galactosamine (Sigma) or mannosamine (Sigma). In the series of experiments which investigated the combination of anti-ECM protein MAb and sugaramine, cells were first treated with the MAb as described. Cells were subsequently harvested, washed and resuspended in PBS containing the stated concentration of sugaramine in the presence of *C. albicans*. Adherence was measured as described.

The percentage inhibition of adherence was calculated using the following formula:

$$\% \text{ Inhibition} = (1 - (A/B)) \times 100$$

Where A is the mean number of yeasts adhering to treated cells and B is the mean number of yeasts adhering to untreated cells.

Statistical analysis

The mean number of yeasts adhering to each of 200 HEp-2 cells was enumerated per treatment. Adherence assays were performed in duplicate on three separate occasions. Statistical analysis was performed using a Sigma Stat package (Sigma) with the Kruskial-Wallis Test and the Dunns Test being employed to establish the existence of a significant difference ($p < 0.05$) between the treatments and control.

Results

Assays were performed to determine whether the ECM proteins collagen types I and IV, laminin and fibronectin were expressed on the surface of HEp-2 cells with a view to establishing their relative importance in mediating yeast-HEp-2 adherence. Sub-confluent HEp-2 cells were exposed to each of the four MAbs as described. An anti-mouse IgG FITC conjugate was subsequently bound to the attached MAbs and the fluorescence of the cells was analysed by flow cytometry. FACS analysis (Figure 1) indicated the presence of fibronectin, collagen types I and IV and laminin on the surface of HEp-2 cells. The relative levels of fluorescence due to the anti-laminin, anti-fibronectin and anti-collagen I MAb-FITC complexes appeared similar while that due to the anti-collagen IV MAb was higher implying a greater abundance of collagen IV on the cell surface.

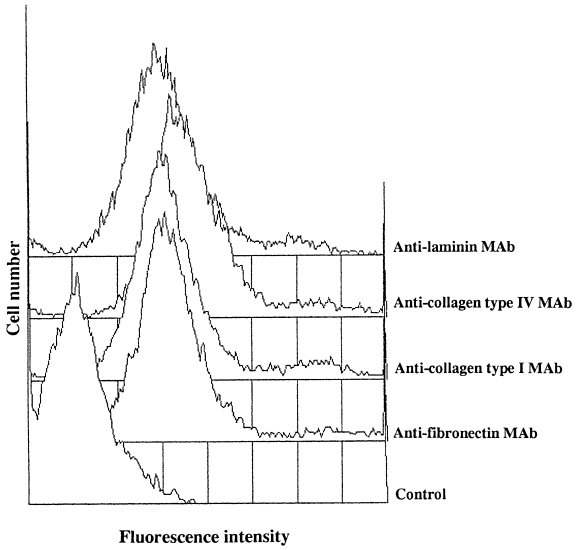

Figure 1. FITC fluorescence of HEp-2 cells treated with anti-ECM protein monoclonal antibodies.
HEp-2 cells were labelled with anti-ECM protein MAbs and exposed to the FITC conjugate. The fluorescence of ten thousand cells was analysed by flow cytometry using a Becton Dickinson FACScan with Lysis II software. The control represents the fluorescence due to the FITC conjugate binding to untreated HEp-2 cells.

Once the presence of the four ECM proteins on the surface of the HEp-2 cells was established, assays were performed to investigate whether MAbs directed against these could affect the adherence of *C. albicans*. The adherence of *C. albicans* to HEp-2 cells with bound MAbs was determined as described. When compared to the control, those HEp-2 cells with bound MAbs manifested reduced levels of yeast adherence (Figure 2) with reductions in adherence varying between 35 and 50% depending on the antibody. The greatest reduction in adherence was consistently observed when anti-collagen type IV MAb was used (typically in the order of 50% ($p < 0.05$)).

The inhibitory effect of sugaramines on the adherence of *C. albicans* to HEp-2 cells was determined. In all cases *C. albicans* and HEp-2 cells were incubated in 2 mls of PBS containing either 0.02 M or 0.2 M galactosamine or mannosamine [15]. The data obtained here (Figure 3) indicate that the inhibition of adherence was concentration dependent, with the greatest reductions being observed when the yeast-HEp-2 mixture was incubated in the presence of either 0.2 M galactosamine or mannosamine.

Significant reductions in the adherence of *C. albicans* to HEp-2 cells are achievable using either a MAb (particularly the anti-collagen type IV mono-

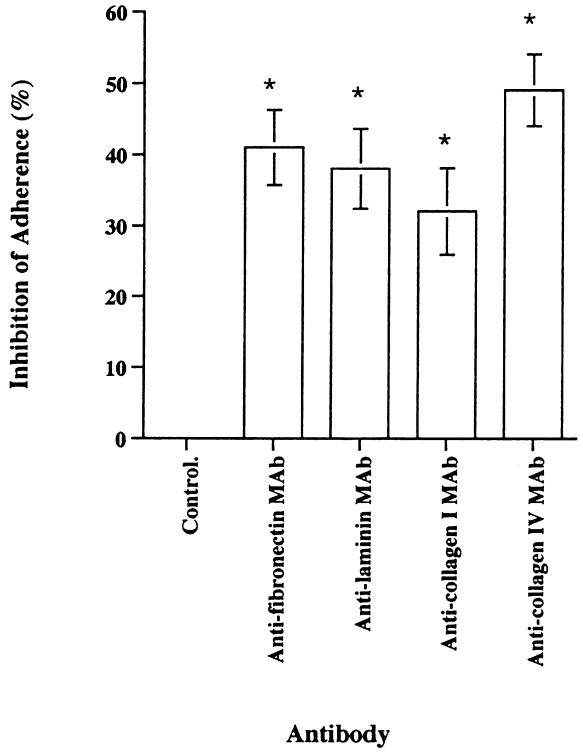

Figure 2. Inhibition of adherence of *Candida albicans* to HEp-2 cells which had been labelled with selected monoclonal antibodies. HEp-2 cells were labelled with selected MAbs and incubated with *C. albicans* ATCC 44990 in adherence assays as described. Values represent the mean±SE percentage inhibition of adherence (* indicates a significant difference ($p < 0.05$) compared to the control.)

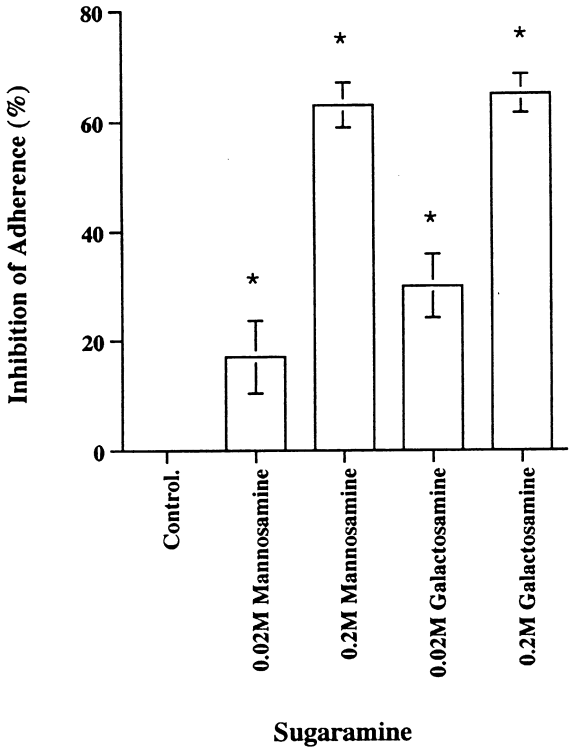

Figure 3. Adherence of *Candida albicans* to HEp-2 cells in the presence of sugaramines.
HEp-2 cells were harvested and incubated with cells of *C. albicans* ATCC 44990 in the presence of 0.02 or 0.2 M galactosamine or mannosamine. Values represent the mean percentage inhibition of adherence ± SE of the mean (* indicates a significant difference ($p < 0.05$) compared to the control.)

clonal antibody) directed against an ECM protein or a sugaramine. Assays to establish the potential of combining both of these strategies were performed. HEp-2 cells previously labelled with the MAb directed against anti-collagen type IV were mixed with yeast cells in PBS containing 0.2 M galactosamine and adherence was measure as described.

Reductions in *C. albicans* adherence due to the presence of anti-collagen type IV MAb on the surface of the HEp-2 cells were approximately 55% ($p < 0.05$) (Figure 4). The presence of galactosamine (0.2M) in adherence assays produced a similar sized reduction in adherence. The co-incubation of HEp-2 cells, pre-labelled with anti-collagen type IV MAb, and *C. albicans* in PBS containing 0.2 M galactosamine gave a reduction of approximately 70% ($p < 0.05$) in the overall adherence level.

Discussion

The adherence of *C. albicans* to host tissue is achieved by a combination of specific and non-specific interactions [21]. The specific adherence mechanisms include interactions with the proteins of the ECM such as collagen types I and IV, laminin and fibronectin [9, 10]. *C. albicans* has an affinity for fibronectin [20] and immobilised collagen type IV [10]. A fibronectin adhesin has been characterised for *C. albicans* [11] and laminin adhesins are present on the surface of *C. albicans* germ tubes [6, 21]. Results presented here suggest that all of these proteins, particularly collagen IV, are involved in facilitating the adherence of *C. albicans* to HEp-2 cells and their presence on the surface of HEp-2 cells was confirmed by flow cytometry. MAb labelled HEp-2 cells demonstrated reduced susceptibility to yeast adherence and the greatest inhibition of adherence was evident when anti-collagen

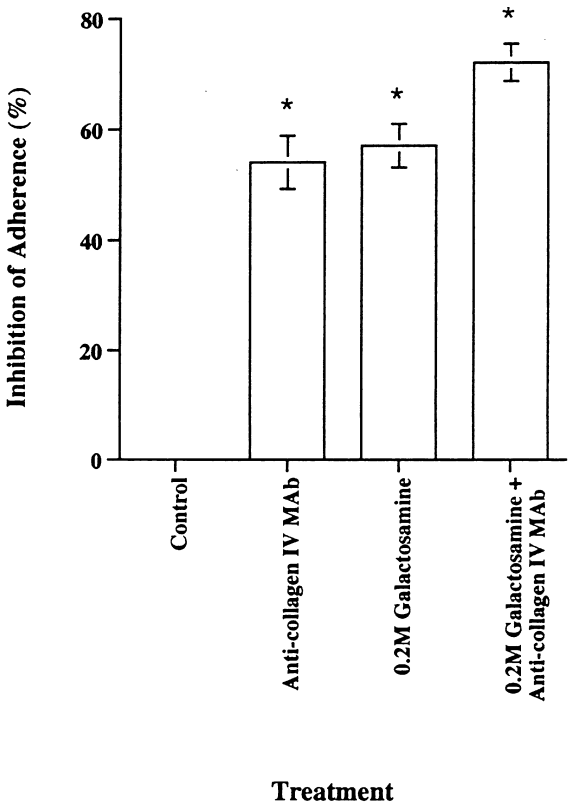

Figure 4. Adherence of *Candida albicans* to HEp-2 cells labelled with anti-collagen IV monoclonal antibody and in the presence of 0.2 M galactosamine.
Anti-collagen type IV MAb labelled HEp-2 cells were incubated with cells of *C. albicans* ATCC 44990 in the presence of 0.2 M galactosamine. Values represent the mean percentage inhibition of adherence ± SE of the mean. (* indicates a significant difference ($p < 0.05$) compared to the control.)

type IV MAb was used. This correlates with the presence of collagen type IV on the surface of HEp-2 cells as indicated by flow cytometry. Since the MAbs employed here were not directed specifically against the receptors for *C. albicans* the reductions in adherence may result from stearic hindrance rather the direct disruption of the receptor – adhesin interactions.

The inhibition of adherence of *C. albicans* to a variety of cell types in the presence of sugaramines has been demonstrated previously [15–17] and results from a reversible interaction of the sugaramine with the fungal cell wall preventing close contact occurring between the yeast cell and the epithelial cell surface [15]. A similar inhibition of adherence of *C. albicans* ATCC 44990 to HEp-2 cells was observed here with reductions occurring in a concentration dependent manner. Reductions in the order of 65% in *C. albicans* adherence to HEp-2 cells were observed in assays where the yeast and HEp-2 cells were incubated in the presence of either 0.2M galactosamine or mannosamine. Reductions of a similar magnitude in the adherence of *C. albicans* to corneocytes and buccal mucosa cells have previously been reported using sugaramines at the same concentrations as employed here [15].

The use of HEp-2 cells labelled with anti-collagen type IV MAb in assays incorporating 0.2M galactosamine reduced the adherence of *C. albicans* to HEp-2 cells by approximately 70% ($p < 0.05$) (Figure 4). This indicates that the reduction in adherence evident when a MAb directed against a target ECM protein can be augmented by performing the assay in the presence of a sugaramine. The anti-collagen IV MAb reduces adherence by indirectly disrupting the interaction of the yeast adhesin with one of the ECM proteins implicated as a target for yeast adherence while the sugaramine prevents close contact occurring between the cell types [15].

Previous work has demonstrated that HEp-2 cells constitute a good model for studying the interaction of *C. albicans* with cultured epithelial cells [14]. Cultured cells have been used extensively to study the adherence of *C. albicans* [18, 20, 21] and represent homogeneous populations of viable cells, the surfaces of which have not been subjected to bacterial or enzymatic modification as occurs with buccal and vaginal epithelial cells [1]. The work presented here indicates that a significant reduction in the adherence of *C. albicans* to HEp-2 cells is possible using agents which do not bind specifically to receptor sites but rather act by indirectly disrupting the yeast adhesin – cell receptor interaction or by preventing close contact occurring between the yeast and the epithelial cell.

While HEp-2 cells are a cultured cell line, an approach such as that described here may have potential in reducing the adherence of *C. albicans* to buccal or vaginal epithelial cells *in vitro*.

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