In vitro Adherence of Candida albicans Strains to Murine Gastrointestinal Mucosal Cells and Explants and the Role of Environmental pH

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Two in vitro adherence assays involving isolated mucosal cells or mucosal explants were used to study the adherence of five Candida albicans strains to murine gastrointestinal mucosal surfaces. Adherence was found to be dependent on the strain used, and on the cellular arrangement, as well as the site of origin of the mucosal surface. Adherence of strains NCPF 3436 and 3310 to stomach and jejunal surfaces was affected by the pH of the medium. Binding between the C. albicans strains and stomach mucosal cells fluctuated as the pH was raised from pH 1.2 to pH 3.4. However, adherence increased with a rise in pH when the strains were incubated with stomach mucosal explants. Optimal adherence by both strains to jejunal mucosal surfaces occurred at neutral pH.

INTRODUCTION

An important facet of a micro-organism's pathogenicity is its ability to adhere to mucosal surfaces of a prospective host (Smith, 1985). The adherence by Candida albicans to the mucosal surface of the gut is considered to be the initiating event leading to penetration of the mucosa and haematogenous spread in the immunocompromised host, the patient with reduced indigenous microflora, or both (Myerowitz, 1981).

Methods used to study adherence by C. albicans have employed surfaces that include buccal cells (Kimura & Pearsall, 1978), vaginal epithelial cells (Segal et al., 1982) and plastic surfaces (Rotrosen et al., 1986). The influence of bacterial populations (Centeno et al., 1983) and antifungal antibiotics (Anderson & Odds, 1985) upon adherence have also been examined.

There are no published studies of comparisons, under similar conditions, between the adherence of yeasts to single epithelial cells or to intact mucosal surfaces. Such a comparison might indicate whether the adherence by different isolates of Candida is influenced by the type of mucosal cell used or by an arrangement of cells in a more complex and contoured surface which closely resembles conditions in vivo.

This report describes the adherence of C. albicans to gastrointestinal (GI) mucosa using either mucosal cell suspensions or intact explants. The effect of pH upon adherence by C. albicans is also described.

METHODS

Fungi. Five clinical isolates of C. albicans were supplied by the Mycological Reference Laboratory, Central Public Health Laboratory, Colindale, London. Strains NCPF 3310 (Johnson et al., 1984) and NCPF 3363 (Smith et al., 1986) were isolated from patients with chronic mucocutaneous candidosis and have been reported to be cross-resistant to azole antifungal antibiotics. C. albicans strain NCPF 3328 was isolated from a renal transplant patient, while strains NCPF 3435 and NCPF 3436 were laboratory strains originally isolated from clinical material.

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Abbreviations: (S-)DMEM, (supplemented) Dulbecco's modified Eagle's medium; FCS, foetal calf serum; GI, gastrointestinal.
Each strain was maintained on Sensitivity Agar (Collins & Lyne, 1976) at 4 °C with a purity plate, of the same medium, made 24 h prior to use. Cultures were grown in 100 ml of Dulbecco's Modified Eagle's Medium (DMEM) [20 ml of a x 5 DMEM solution (Gibco BRL) was added to 2 ml of HEPES pH 7.3 buffer (Gibco BRL) and 78 ml of sterile distilled water] in an orbital incubator (New Brunswick Scientific UK) at 37 °C, 250 r.p.m., 18 h before setting up an experiment. Cultures were still in the exponential phase of growth. Cells were harvested by centrifugation, washed three times in phosphate buffered saline (PBS) [Oxoid recipe A] and resuspended in supplemented DMEM (S-DMEM) [100 ml DMEM supplemented with 2 ml heat inactivated foetal calf serum (FCS; Gibco BRL), 0.4% NaHCO₃, 0.1 mg streptomycin sulphate (Glaxo) and 1 x 10⁵ U sodium benzylpenicillin (Glaxo)].

The blastospores were counted with an improved Neubauer counting chamber and adjusted to the required concentration. To test for viability 0.1 ml of the suspension was plated out separately onto Sensitivity Agar.

Production of a GI mucosal cell suspension. BALB/c mice (National Institute of Medical Research, Mill Hill, UK) were killed by cervical dislocation and their GI tracts removed aseptically. Tissues removed included the lower third of the oesophagus, the stomach, duodenum, jejunum, ileum, and colon. Each explant was slit along its longitudinal axis to expose the mucosal surface and washed three times in PBS to remove the luminal contents.

With the exception of the oesophageal explants, which were stored prior to use in PBS at 4 °C, tissues were placed in a 0.05% trypsin solution [1 ml 5% trypsin solution (Gibco BRL) dissolved in 99 ml verseone (0.1% Na₂, EDTA in PBS)] and shaken at 37 °C, 250 r.p.m. for 15 min to remove blood cells and adherent mesentery. The supernatant was discarded, a further 10 ml trypsin solution added to each explant, including the oesophageal tissue, and the preparation was shaken for 30 min at 37 °C, 250 r.p.m. in an orbital incubator. The supernatant from each tissue type was collected and the trypsin neutralized by the addition of an equal volume of 2% heat inactivated FCS. A second 30 min trypsinization and neutralization step was performed. The initial trypsinization of oesophageal tissue was omitted since an optimal yield of mucosal cells was obtained in a single step.

The cell suspensions were passed through sterile 100 μm mesh gauze to remove large pieces of debris and then centrifuged at 2000 r.p.m. for 10 min. After discarding the supernatant, pellets were washed and resuspended in 2 ml S-DMEM, counted with a counting chamber and adjusted to a concentration of 2 x 10⁶ cells ml⁻¹. Cell suspensions were used immediately after preparation.

Mucosal cell suspension adherence assay. Volumes (2 ml) of suspension containing 2 x 10⁵ mucosal cells ml⁻¹ were added to sterile bijoux bottles containing 2 ml suspension of C. albicans at a concentration of 2 x 10⁶ C. albicans cells ml⁻¹ and shaken at 37 °C, 250 r.p.m. Controls consisted of a 4 ml yeast-free mucosal cell suspension. Samples (1 ml) were taken after 2 h incubation and fixed directly in 1 ml of 6% (v/v) glutaraldehyde (BDH).

The accepted method for separating host cells with bound C. albicans from unbound C. albicans would be to pass the cells through a filter. However, to avoid changes due to cell packing, cells were fixed and not differentially filtered.

Adherence was assessed by mounting 50 μl of a sample directly on to a microscope slide and examining 30 randomly chosen mucosal cells at X400 magnification, using a Leitz Laborlux K light microscope.

The numbers of C. albicans adhering to a single mucosal cell, usually within a range between zero to three or more bound fungal particles per cell, was recorded and a frequency distribution of the sample obtained. The median of the frequency distribution, the median adherence, was used to describe the numbers of C. albicans blastospores and germ tubes adhering per mucosal cell, since distributions were skewed. The median of the frequency distribution was found using linear interpolation (Armitage, 1973). All experiments were repeated three times.

Effect of the trypsinization procedure on adherence. Human buccal cells were preincubated in either a 0.05% trypsin solution or trypsin-free verseone solution for 75 min, the maximum period a mucosal cell would be subjected to the enzyme. An adherence assay was then performed using strain 3436.

Mucosal explant adherence assay. After removal and preparation of the GI tissues, as described above, 5 x 5 mm blocks were cut from the mid-regions of each explant (stomach explants were obtained from longitudinal strips of the greater curve). Individual blocks were placed in bijoux bottles containing a 3 ml suspension of 10⁶ C. albicans (ml S-DMEM)⁻¹. Controls consisted of explants in yeast-free S-DMEM. The bijoux were shaken at 37 °C, 250 r.p.m. for 4 h. After 2 h tissue samples were aseptically removed, washed three times in PBS to remove any non-adhering yeast and stored overnight at -70 °C in 10% (v/v) glycerol/PBS solution (BDH), which prevented ice crystallization.

The numbers of C. albicans adhering to explants were determined by a quantitative dilution plate counting technique. Each explant was placed in a preweighed universal bottle containing 5 ml of sensitivity broth, weighed, and homogenized using an Ultra-Turrax homogenizer (Orme Scientific Ltd) and dilutions of the homogenate plated out in triplicate onto sensitivity agar plates containing 50 μg chloramphenicol ml⁻¹. Plates were incubated at 37 °C for 24 h or until colonies could be seen. Adherence was expressed as the number of colony forming units (c.f.u.) adhering per 0.1 g wet tissue. All experiments were repeated three times.

Assessment of C. albicans-serosal attachment. Binding by C. albicans 3328 to the serosal surface of GI explants was assessed visually after staining with a 0.05% (w/v) aqueous solution of calcofluor white (Polysciences). The
adherence of strain 3328 was studied because this strain showed a greater binding affinity to murine GI serosa when compared with the other C. albicans strains.

Adherence by C. albicans 3436 and 3310 to stomach and jejunal mucosal cells under varying pH conditions. Adherence assays were carried out as previously described; however, HEPES buffer was omitted from the S-DMEM and the required physiological pH obtained by the addition of an appropriate volume of either 5 M-HCl, 0.1 M-HCl, 5 M-NaOH or 0.1 M-NaOH. The medium was then buffered with either 2% (v/v) Sorensen’s glycine or 2% (v/v) Sorensen’s phosphate buffer (Diem & Lentner, 1975).

Physiological pH ranges at different sites in the GI tract have been shown to be 1.2, 1.8, 2-3, 2.8 and 3.4 for the stomach, while jejunal pH levels included pHs of 6.5, 6.9, 7-3, 7-6, and 8-0 (Guyton, 1981). These values were used in different assays. All experiments were repeated three times.

Adherence by C. albicans 3436 and 3310 to stomach and jejunal explants under varying pH conditions. Adherence assays were performed under similar conditions as those described above. All experiments were repeated three times.

Statistics. The chi-squared test was used to test for homogeneity between the triplicate adherence frequency distributions in order that the data from the replicate experiments could be pooled. The median of the pooled distribution was then calculated. The chi-squared test was also used to compare pooled adherence frequency distributions, in order to statistically compare median values. The Student t-test and one-way analysis of variance were used to test for statistical variance between c.f.u. counts.

RESULTS

Effect of the trypsinization procedure on adherence

The median number of C. albicans 3436 adhering to a buccal mucosal cell after 75 min incubation with 0.05% trypsin was 2.0. This value was not significantly different to the control’s median value of 2.8 C. albicans cells per mucosal cell (P < 0.05). Therefore, the trypsinization procedure was considered to have no effect on adherence.

Assessment of C. albicans-serosal attachment

The adherence of C. albicans 3328 to mucosal and serosal surfaces of GI explants is shown in Table 1. Measurements were limited to oesophageal, gastric and colonic explants because the small intestinal explants produced secretions that took up the fluorochrome and obscured their mucosal surface detail. The adherence of strain 3328 to GI mucosa was significantly greater than its involvement with the respective serosa. Since the adherence of this strain to serosal surfaces was proportionately lower than its attachment to mucosal surfaces, serosal involvement was considered to be insignificant.

Median adherence of five C. albicans strains to separated GI tract mucosal cells

The median adherence values after 2 h incubation are shown in Fig. 1. The adherence between C. albicans and substrate statistically varied with the yeast strain and type of mucosal cell used in the assay (all tests gave confidence limits of 95% or greater).

Table 1. Comparison of adherence by C. albicans 3328 to the mucosal and serosal surfaces of murine GI explants

<table>
<thead>
<tr>
<th>Explant</th>
<th>Surface</th>
<th>No. of C. albicans adhering mm⁻² (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oesophagus</td>
<td>Mucosal</td>
<td>673.3 ± 62.3</td>
</tr>
<tr>
<td></td>
<td>Serosal</td>
<td>32.3 ± 11.3</td>
</tr>
<tr>
<td>Stomach</td>
<td>Mucosal</td>
<td>332.9 ± 180.2</td>
</tr>
<tr>
<td></td>
<td>Serosal</td>
<td>19.6 ± 24.5</td>
</tr>
<tr>
<td>Colon</td>
<td>Mucosal</td>
<td>149.1 ± 43.0</td>
</tr>
<tr>
<td></td>
<td>Serosal</td>
<td>8.0 ± 6.7</td>
</tr>
</tbody>
</table>
Adherence of five *C. albicans* strains to mucosal cells isolated from six sites along the murine GI tract. Assays were incubated at 37 °C for 2 h and the results are the medians of the pooled adherence frequency distributions of three experiments. □, *C. albicans* 3435; □, *C. albicans* 3436; ●, *C. albicans* 3328; △, *C. albicans* 3310; △, *C. albicans* 3363; O, oesophagus; S, stomach; D, duodenum; J, jejunum; I, ileum; C, colon.

When assays were incubated for a further 2 h little or no difference in median adherence values for a single isolate was found between the two incubation periods (results not shown). There were exceptions which depended on the strain and mucosal cell type under study. For instance, the median adherence of *C. albicans* 3436 to jejunal mucosal cells was higher after 4 h incubation, while that of strain 3310 at 4 h was lower than its median adherence seen after 2 h.

**Adherence by *C. albicans* to murine GI tract mucosal explants**

Adherence to explant tissues after 2 h incubation is shown in Fig. 2. With the exception of strain 3435, all *C. albicans* strains adhered in greater numbers to oesophageal explants than any other type.

The adherence by strains 3328, 3310, and 3363 varied significantly with each type of mucosal explant (all tests gave confidence limits of 95% or greater) while no statistical differences were
found between the c.f.u. counts of *C. albicans* 3435 and *C. albicans* 3436. Variations in adherence between the strains occurred with duodenal mucosal explants (*P* < 0.005), jejunal mucosal explants (*P* < 0.05), and ileal mucosal explants (*P* < 0.005). When assays were incubated for a further 2 h the adherence by each strain to mucosal explants was not generally affected by the length of incubation. Exceptions included an increase in attachment of *C. albicans* 3328 to oesophageal (*P* < 0.04) and colonic explants (*P* < 0.05), and an increase in c.f.u. counts by strain 3363 to duodenal mucosal explants (*P* < 0.01) and jejunal mucosal explants (*P* < 0.05) at the longer period of incubation (results not shown).

**Median adherence by *C. albicans* 3436 and 3310 to stomach and jejunal mucosal cells at physiological pH values**

This varied significantly at each pH value (all tests gave confidence limits of 95% or greater) (Figs 3 and 4).
Optimal adherence by both strains to jejunal mucosal cells occurred at neutral pH. But interestingly the adherence of the strains to stomach mucosal cells at pH 1.2 was not less than that seen at pH 3.4. Where statistical differences occurred strain 3436 adhered to stomach mucosal cells in greater numbers than strain 3310. *C. albicans* 3310 adhered to jejunal mucosal cells in greater numbers than strain 3436 where statistical differences arose.

No significant difference occurred in the median adherence of *C. albicans* 3436 (*P* > 0.05 for all tests) at neutral (7.3) versus acidic pH values. However, the median adherence by *C. albicans* 3310 at pH 7.3 was greater than that obtained under acidic conditions (all tests gave confidence limits of 95% or greater).

After a further 2 h incubation the adherence to stomach mucosal cells by strain 3436 at pH 3.4 increased (*P* < 0.03) and the adherence of strain 3310 at pH 2.8 increased (*P* < 0.001) (results not shown). The binding between strain 3436 and jejunal mucosal cells increased after a further 2 h incubation at pH 8.0 (*P* < 0.001) while the adherence by strain 3310 increased after a further 2 h at pH 6.5 (*P* < 0.001), pH 6.9 (*P* < 0.001) and pH 8.0 (*P* < 0.03).

**Adherence by strains 3436 and 3310 to stomach and jejunal mucosal explants at physiological pH values**

The attachment by *C. albicans* 3436 and 3310 to stomach mucosal explants was not influenced by pH (see Fig. 3; *P* > 0.05 for all tests). Differences between the adherence of the strains to stomach mucosal cells and explants at similar pH levels can be seen. While the adherence to stomach mucosal cells appeared to fluctuate with pH, the adherence to stomach mucosal explants increased with increasing pH. Where statistical differences occurred strain 3436 adhered to stomach mucosal explants in greater quantities than strain 3310.

Both strains showed optimum adherence to stomach explants at pH 7.3 compared to acid pH ranges (all tests gave confidence limits of 95% or greater).

The adherence by strain 3436 to jejunal mucosal explants varied significantly with pH while strain 3310 was not affected (Fig. 4). Where statistical differences occurred strain 3310 showed greater adherence.

Once again after a further 2 h incubation both strains showed isolated decreases in adherence at acid pH ranges (e.g. strain 3436 at pH 1.2; *P* < 0.001) and increases, by the same strain, at the neutral pH range (pH 6.5–7.6) (results not shown).

**DISCUSSION**

In this report the development of two *in vitro* assays for the study of adherence by *C. albicans* to murine GI mucosal surfaces has been described. With the single cell adherence assay, the median number of adherent *C. albicans* cells was used to examine changes with mucosal cell type, *C. albicans* strain or both. Adherence assays based on GI mucosal explants were used to study the adherence of *C. albicans* to a more physiological but complex surface with an intact mucus layer.

When the adherence values of the five *C. albicans* strains for the six types of GI mucosal surfaces were compared, the extent of binding between *C. albicans* and a particular substrate was dependent on three main variables: strain, the source of the mucosal cells and their arrangement as either separated single cells or explants. An epithelial cell's source, outside the GI tract (Bibel et al., 1987), the age of the donor (Cox, 1986), cell size (Sandin et al., 1987b) and in some cases the time of its collection (Sandin et al., 1987a) have also been shown to influence the interaction with *C. albicans*.

Most of the five *C. albicans* strains adhered most strongly to oesophageal explants, even though these were obtained from the lower oesophagus, which is non-keratinized. In a study of *Candida* infection of the human GI tract, Eras et al. (1972) concluded that *Candida* infection was most common in the oesophagus and stomach. Oral and oesophageal candidosis occurs frequently in immunocompromised hosts (Mathieson & Dutta, 1983) and is particularly common in patients with acquired immunodeficiency syndrome (Tavitian et al., 1986). Rats treated with a combination of cortisone and antibiotics developed superficial focal invasion of
the oesophagus while those treated with azathioprine and antibiotics developed severe lesions of both the oesophagus and stomach (DeMaria et al., 1976).

The adherence by C. albicans to vaginal mucosal cells can also be affected by pH (Persi et al., 1985; King et al., 1980; Sobel et al., 1981). These studies gave an optimum pH for adherence of between pH 6–8, which is within the range for extracellular phospholipase activity (Barrett-Bee et al., 1985). This complements our findings where the incubation of strain 3310 with either stomach mucosal cells or explants and strain 3436 with stomach mucosal explants at physiological pH (i.e. acidic pH between 1·2 and 3·4), gave adherence values that were less than those obtained when similar assays were performed at pH 7·3.

However, when C. albicans was incubated with buccal epithelial cells at pH values ranging from 3 to 8, peak adherence by C. albicans was reported at pH 3 (Samaranayake & MacFarlane, 1982). This suggests that the effect of pH on C. albicans adherence varies with the source of the mucosal cell. Since the binding affinities of strains 3436 and 3310 varied as the pH levels changed the effect of pH on adherence also appears to be strain dependent.

Other ambient conditions such as temperature may also affect adherence. Assays incubated at 37 °C have shown adherence yields greater than those obtained at 25 °C (Kimura & Pearsall, 1978). Interestingly, blastospores from cultures grown at 25 °C have been shown to adhere to vaginal epithelial cells in higher numbers than those blastospores isolated from cultures containing a high proportion of pseudomycelia grown at 37 °C (Lee & King, 1983). These differences, the authors suggest, were due to temperature induced variations in the C. albicans cell wall.

Our results have shown that it is important to select the strain of C. albicans, the type of mucosal surface used, and the ambient pH when designing an adherence assay for examining the interaction between yeasts and the GI tract, especially if antifungal antibiotics or inhibitors of adherence are to be tested.

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