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Effect of antifungal agents on the adherence of *Candida albicans* to murine gastrointestinal mucosal surfaces

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The in-vitro adherence of *Candida albicans* to murine gastrointestinal mucosal surfaces was investigated in the presence of sub-inhibitory concentrations of amphotericin B, ketoconazole and itraconazole. Each antifungal drug showed a significant ability to reduce the adherence of *C. albicans* to gastric and jejunal mucosa. However, the outcome of an adherence assay was dependent on pH, drug concentration and *C. albicans* strain. The type of mucosal surface and its cellular arrangement also had an important role to play. Sub-inhibitory concentrations of amphotericin B, ketoconazole and itraconazole significantly reduced the adherence of an azole sensitive strain *C. albicans* 3436. These effects were influenced by the type of mucosal surface and the concentration of the antifungal drug. Sub-inhibitory concentrations of amphotericin B inhibited the adherence of an azole resistant strain, *C. albicans* 3310, to stomach and jejunal mucosal surfaces while sub-inhibitory concentrations of ketoconazole only significantly reduced this strain's adherence to gastric mucosal explants. Sub-inhibitory concentrations of itraconazole had little effect. Amphotericin B reduced the adherence of *C. albicans* 3436 to gastric mucosal explants when assays were performed at physiological pH levels, an effect dependent on concentration. This drug also reduced the adherence of strain 3436 to jejunal mucosal cells and explants at physiological pH levels, though results obtained from assays involving jejunal mucosal explants, incubated at pH 8·0, were concentration dependent.

Introduction

The adherence of *Candida albicans* to the mucosal surface of the gastrointestinal (GI) tract is considered to be the initiating event leading to the penetration of the mucosa, then haematogenous spread and disseminated infection in immunocompromised patients. The adherence of *C. albicans* blastospores to buccal and vaginal epithelial cells and plastic surfaces has been the subject of numerous in-vitro investigations (Kennedy, 1988). However, studies of the in-vitro adherence of *C. albicans* to GI mucosal surfaces are scarce (Segal & Savage, 1986; Kennedy *et al.*, 1987; Mehentee & Hay, 1989). Furthermore, there are no reports in the literature that describe the effects of antifungal drugs on this pathogen's adherence to GI mucosa. This paper describes the effects of sub-inhibitory concentrations of three antifungal drugs, amphotericin B, ketoconazole and itraconazole, on the in-vitro adherence of azole-sensitive and azole-resistant strains of *C. albicans* to murine GI mucosal cells and explants. The effect of pH level on adherence was also studied.

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Materials and methods

Strains and inoculum

C. albicans strains NCPF 3310 and NCPF 3436 were maintained on 'Sensitivity agar' at 4°C (Collins & Lyne, 1976). Strain NCPF 3310 is resistant to azole antifungals (Johnson, Richardson & Warnock, 1984). Blastospore suspensions were made by the method of Mehentee & Hay (1989). Briefly, 100 ml of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Paisley, UK) was added to 2 ml of HEPES pH 7·3 buffer (Gibco BRL) and 78 ml of sterile distilled water. This was inoculated with blastospores from 24 h old cultures and incubated in an orbital shaker at 37°C, 250 rpm for 18 h. Cells were harvested and washed three times in phosphate-buffered saline (PBS) and resuspended in supplemented DMEM (S-DMEM) (100 ml of DMEM supplemented with 2 ml heat inactivated fetal calf serum (Gibco BRL), 0·4 g $NaHCO_3$, 0·1 mg streptomycin sulphate (Gibco BRL) and 1×10^4 U sodium benzyl penicillin (Glaxo Pharmaceuticals, Greenford, UK)) to a concentration of 2×10^6 *C. albicans* blastospores/ml. Of this suspension 0·1 ml was plated out to test for viability, all showing a viability of 95% or greater.

Antifungal drugs

Ketoconazole was obtained from Janssen Pharmaceutical Ltd (Wantage, Oxfordshire, UK) and dissolved to a concentration of 1000 mg/l in a 1:1 (v/v) solution of sterile distilled water and acetone. Itraconazole (Janssen) was dissolved at a concentration of 1000 mg/l in dimethyl sulphoxide (DMSO). Amphotericin B (Squibb, Merseyside, UK) was dissolved at concentrations of 1000 mg/l in DMSO. All drug solutions were stored for up to six months at −20°C.

Susceptibility tests

Tests were performed in sterile, 96-well, round-bottomed Microtest plates (Sterilin Ltd, Teddington, Middlesex, UK) in S-DMEM. Serial two-fold dilutions of antifungal drug were made to give decreasing concentrations of 2–0·002 mg/l for ketoconazole and itraconazole and 1–0·001 mg/l for amphotericin B. An equal volume of *C. albicans* blastospores, at a concentration of 2×10^6 cells/ml, was added to each well. Plates were read after 18 h at 37°C. The lowest concentration of drug required to produce a visible reduction in growth compared with the control was recorded. This value was called the minimum reductive concentration (MRC).

Production of mucosal cell suspensions

Murine gastric and jejunal mucosal cells were prepared by the method of Mehentee & Hay (1989). Murine gastric and jejunal explants were removed, cleaned and then treated with a 0·05% trypsin solution (1 ml of 5% trypsin solution (Gibco BRL) dissolved in 99 ml of versene (0·1% ethylene diamine tetra-acetic acid (disodium salt) in PBS)). Tissues were initially trypsinized for 15 min to remove blood cells and adherent mesentery and then subjected to two 30 min trypsin washes to obtain mucosal cells. Suspensions were treated with 2% fetal calf serum to neutralize the trypsin, washed three times in PBS and resuspended in S-DMEM at a concentration of 2×10^5 mucosal cells/ml.

Mucosal cell suspension adherence assays

A suspension of 2 ml of mucosal cells was added to a sterile bijou bottle containing 2 ml of *C. albicans* blastospores at a concentration of 2×10^6 cells/ml. Then 0·1 ml of the mixture was removed and replaced with 0·1 ml of antifungal drug solution so as to give a final drug concentration that corresponded to a strain's MRC or 0·1 x MRC. Controls used were 4 ml yeast-free mucosal cell suspension and a drug-free mucosal-yeast cell mixture. Experiments using similar volumes of the drugs' solvents showed that they did not influence the strains' adherence to either type of mucosal cell suspension. Suspensions were shaken at 37°C, 250 rpm. Samples of 1 ml were taken after 2 h incubation and fixed in 1 ml of 6% glutaraldehyde (BDH).

The number of yeast cells adhering to individual mucosal cells was assessed by light microscopy (Mehentee & Hay, 1989) and frequency distributions of mucosal cells with 0, 1, 2, 3 and > 3 adherent *C. albicans* blastospores and germ tubes obtained. In addition, the number of mucosal cells in a sample with adherent *C. albicans* germ tubes was noted. The number of mucosal cells with adherent germ tubes was calculated as a proportion of the total number of mucosal cells studied.

Mucosal explant adherence assay

Blocks of gastric and jejunal explants were prepared as described by Mehentee & Hay (1989). Blocks were placed in a 3 ml solution of *C. albicans* cells (10^6 cells/ml) plus the antifungal drug at a concentration corresponding to the MRC and 0·1 x MRC of the yeast strain. Controls consisted of explants in an antifungal-free yeast cell suspension. Experiments using similar volumes of the drugs' solvents showed that they did not influence the strains' adherence to either type of mucosal explant. The cell suspensions were shaken at 37°C, 250 rpm for 2 h. Explants were washed in PBS to remove non-adherent *C. albicans* cells and stored overnight at −70°C in a 10% (v/v) glycerol-PBS solution.

The number of yeast cells adhering to explants was determined using a quantitative dilution plate counting technique (Mehentee & Hay, 1989). Explants were weighed, homogenized and the homogenate plated on to Sensitivity Agar containing 50 mg/l of chloramphenicol. Adherence was expressed as a number of colony forming units (cfu) adhering per 0·1 μg or 0·1 g wet explant.

Effect of pH on adherence to stomach and jejunal mucosal cells

HEPES buffer was omitted from S-DMEM and the appropriate pH obtained by the addition of either 5 M HCl, 0·1MHCl, 5 M NaOH or 0·1 M NaOH. The medium was buffered with either 2% (v/v) Sorensen's glycine buffer or 2% (v/v) Sorensen's phosphate buffer (Diem & Lentner, 1975). pH values selected for adherence assays involving gastric explant were 1·2, 2·3 and 3·4, while pH values used with jejunal explants were 6·5, 7·2 and 8·0. Assays were performed as above except that pH-adjusted S-DMEM was used.

Effect of pH on adherence to gastric and jejunal explants

Adherence assays were performed as described above and the same pH values were selected as in tests with mucosal cell suspensions.

Statistics

The chi-squared test was used to test for homogeneity between triplicate adherence frequency distributions so that the replicate experiments could be pooled. The median of the pooled frequency distribution was then calculated. The chi-squared test was used to test for differences between pooled adherence frequency distributions, in order to compare median values. The same test was used to compare the proportions of mucosal cells with and without adherent germ tubes of replicate experiments. The proportions obtained between the replicate experiments were found to be homogeneous, pooled and the percentage of mucosal cells with adherent germ tubes calculated from the pooled data. Variations between different percentage values were examined by performing chi-squared tests on the mucosal cell proportions. Student's *t*-test was used to test for statistical variance between cfu counts.

Results

Antifungal susceptibility tests

The minimum concentration of an antifungal required to reduce visible growth (MRC) was used since both ketoconazole and itraconazole gave a progressive diminution in growth with increasing drug concentration and a cut-off was not obtained. The MRCs of amphotericin B, ketoconazole and itraconazole obtained for strain 3436 were 0·5, 0·004 and 0·007 mg/l, respectively. The MRCs of each drug were monitored at frequent intervals throughout this work and were not found to vary by more than one two-fold dilution. Strain 3310 was found to be resistant to the maximum concentrations of 1 mg/l of amphotericin B and 2 mg/l of ketoconazole and itraconazole, used in the susceptibility tests. For comparative purposes these maximum values were used in experiments involving *C. albicans* 3310.

Effect of antifungal drugs on adherence of C. albicans *to stomach and jejunal mucosal surfaces*

The effect of amphotericin B, ketoconazole and itraconazole on the adherence of strains 3436 and 3310 to gastric and jejunal mucosal surfaces is summarised in Tables I to VI. Amphotericin B caused a significant reduction in the median number of *C. albicans* 3436 cells adhering to gastric mucosal cells (Table I). The proportion of gastric

Table I. The adherence of *C. albicans* strains 3436 and 3310 to murine gastric mucosal surfaces in the presence of amphotericin B

Strain	Drug concentration (mg/l)	Median number of *C. albicans* cells per mucosal cell	Percentage of mucosal cells with germinating *C. albicans*	Number of adherent *C. albicans* cells (cfu/0·1 μg wet explant)
3310	0	2·53	73	9·71 ± 5·60
	1·0	1·66	40[b]	0·19 ± 0·03[a]
3436	0	3·02	61	2·34 ± 1·26
	0·05	1·70[a]	49	2·44 ± 1·91
	0·5	1·31[b]	39[b]	1·83 ± 0·95

[a] $P < 0·05$; [b] $P < 0·01$.

Table II. The adherence of *C. albicans* strains 3436 and 3310 to murine jejunal mucosal surfaces in the presence of amphotericin B

Strain	Drug concentration (mg/l)	Median number of *C. albicans* cells per mucosal cell	Percentage of mucosal cells with germinating *C. albicans*	Number of adherent *C. albicans* cells (cfu/0·1 μg wet explant)
3310	0	0·94	40	0·19 ± 0·11
	1·0	0·83	20[b]	0·05 ± 0·001[a]
3436	0	1·52	36	1·00 ± 0·41
	0·05	1·17	17[b]	0·45 ± 0·22
	0·5	0·96	16[b]	0·32 ± 0·17[a]

[a] $P < 0·05$; [b] $P < 0·01$.

and jejunal mucosal cells to which germinating blastospores of both strains adhered was also reduced in the presence of amphotericin B (Tables I and II). This effect was dependent on the concentration of amphotericin B for strain 3436. Amphotericin B significantly reduced the adherence of strain 3310 to both gastric and jejunal mucosal explants and only significantly reduced the binding of strain 3436 to jejunal mucosal explants at the higher concentration used.

The median numbers of *C. albicans* 3436 cells adhering to gastric and jejunal mucosal cells were significantly reduced by ketoconazole (Tables III and IV). This effect was independent of the drug concentration in assays involving gastric mucosal cells, but was concentration dependent for assays involving jejunal mucosal cells. The higher concentrations of ketoconazole significantly reduced the percentage of gastric mucosal cells with adherent *C. albicans* 3436 germ tubes. Ketoconazole significantly reduced the binding of strain 3310 to gastric mucosal explants.

Itraconazole significantly reduced the median numbers of *C. albicans* 3436 cells adhering to both gastric and jejunal mucosal cells (Tables V and VI). This effect was dependent on the concentration of the drug. The median number of *C. albicans* 3310 cells adhering to jejunal mucosal cells was reduced by the drug. The percentage of jejunal mucosal cells with adherent *C. albicans* 3436 germ tubes was significantly reduced by itraconazole and this effect was dependent on the concentration of the drug. Independently of its concentration, itraconazole reduced the binding of *C. albicans* 3436 to gastric mucosal explants.

Table III. The adherence of *C. albicans* strains 3436 and 3310 to murine gastric mucosal surfaces in the presence of ketoconazole

Strain	Drug concentration (mg/l)	Median number of *C. albicans* cells per mucosal cell	Percentage of mucosal cells with germinating *C. albicans*	Number of adherent *C. albicans* cells (cfu/0·1 μg wet explant)
3310	0	2·15	61	7·54 ± 1·53
	2·0	2·00	52	1·71 ± 0·55[b]
3436	0	2 73	55	3·10 ± 0·79
	4×10^{-4}	1·43[b]	51	2·78 ± 0·20
	4×10^{-3}	1·45[b]	25[a]	2·60 ± 0·20

[a] $P < 0·05$; [b] $P < 0·01$.

Table IV. The adherence of *C. albicans* strains 3436 and 3310 to murine jejunal mucosal surfaces in the presence of ketoconazole

Strain	Drug concentration (mg/l)	Median number of *C. albicans* cells per mucosal cell	Percentage of mucosal cells with germinating *C. albicans*	Number of adherent *C. albicans* cells (cfu/0·1 μg wet explant)
3310	0	1·24	40	0·38 ± 0·16
	2·0	1·31	20	0·14 ± 0·12
3436	0	0·67	28	0·11 ± 0·11
	4×10^{-4}	0·61	35	0·14 ± 0·12
	4×10^{-3}	0·59*	31	0·17 ± 0·08

*$P < 0·01$.

Effect of pH on adherence of C. albicans *to gastric and jejunal and mucosal surfaces*

Tables VII and VIII summarize the effects of amphotericin B on the adherence of strain 3436 to gastric and jejunal mucosal surfaces at pH levels corresponding to physiological conditions *in vivo*. Amphotericin B significantly reduced the adherence of strain 3436 to gastric mucosal explants at pH 1·2 and 3·4; however, this effect was dependent on the concentration of the drug. When adherence assays involving jejunal mucosal cells were incubated at pH levels corresponding to conditions *in vivo*, amphotericin B significantly reduced germ tube adherence; this effect was independent of the drug's concentration. The drug also inhibited adherence to jejunal mucosal explants; however, this effect was dependent on the concentration of the drug when assays were incubated at pH 8·0.

Discussion

This study has shown that amphotericin B, ketoconazole and itraconazole can inhibit the adherence of *C. albicans* to both gastric and jejunal mucosal surfaces. However, the effect of an antifungal agent on adherence depended on its concentration, the susceptibility of the strain to the antifungal drug, and the source of the mucosal surface and its cellular arrangement.

Table V. The adherence of *C. albicans* strains 3436 and 3310 to murine gastric mucosal surfaces in the presence of itraconazole

Strain	Drug concentration (mg/l)	Median number of *C. albicans* cells per mucosal cell	Percentage of mucosal cells with germinating *C. albicans*	Number of adherent *C. albicans* cells (cfu/0·1 μg wet explant)
3310	0	2·73	66	2·31 ± 0·60
	2·0	2·30	69	2·44 ± 0·78
3436	0	3·33	69	3·68 ± 0·81
	7×10^{-4}	3·00*	69	0·60 ± 0·18*
	7×10^{-3}	3·12*	64	0·52 ± 0·40*

*$P < 0·01$.

Table VI. The adherence of *C. albicans* strains 3436 and 3310 to murine jejunal mucosal surfaces in the presence of itraconazole

Strain	Drug concentration (mg/l)	Median number of *C. albicans* cells per mucosal cell	Percentage of mucosal cells with germinating *C. albicans*	Number of adherent *C. albicans* cells (cfu/0·1 μg wet explant)
3310	0	0·80	31	0·78 ± 0·63
	2·0	0·68[a]	10	0·57 ± 0·37
3436	0	0·98	39	0·95 ± 0·28
	7×10^{-4}	0·73	27	0·96 ± 0·61
	7×10^{-3}	0·68[a]	19[a]	0·58 ± 0·19

[a] $P < 0·01$.

Table VII. The adherence of *C. albicans* strain 3436 to murine gastric mucosal surfaces in the presence of amphotericin B at different pH levels

pH	Drug concentration (mg/l)	Median number of *C. albicans* cells per mucosal cell	Percentage of mucosal cells with germinating *C. albicans*	Number of adherent *C. albicans* cells (cfu/0·1 g wet explant)
1·2	0	0·98	7	78 ± 45
	0·05	0·94	9	74 ± 77
	0·5	0·93	10	0[a]
2·3	0	0·65	6	491 ± 143
	0·05	0·69	7	404 ± 273
	0·5	0·73	8	15 ± 20
3·4	0	0·54	3	644 ± 599
	0·05	0·51	0	833 ± 85
	0·5	0·53	2	14 ± 2[a]

[a] $P < 0·05$.

Table VIII. The adherence of *C. albicans* strain 3436 to murine jejunal mucosal surfaces in the presence of amphotericin B at different pH levels

pH	Drug concentration (mg/l)	Median number of *C. albicans* cells per mucosal cell	Percentage of mucosal cells with germinating *C. albicans*	Number of adherent *C. albicans* cells (cfu/0·1 g wet explant)
6·5	0	0·58	13	1667 ± 228
	0·05	0·56	3[b]	592 ± 72[b]
	0·5	0·53	1[b]	9 ± 6[b]
7·2	0	0·56	10	1168 ± 38
	0·05	0·52	0[b]	645 ± 241[b]
	0·5	0·55	1[b]	9 ± 8[b]
8·0	0	0·65	19	309 ± 178
	0·05	0·54	1[b]	155 ± 129
	0·5	0·51	0[b]	16 ± 19[a]

[a] $P < 0·05$; [b] $P < 0·01$.

Amphotericin B reduced the adherence of both strains to gastric and jejunal mucosal surfaces. Sub-inhibitory concentrations of amphotericin B (0·5 mg/l) have been found to reduce the level of *C. albicans* cell wall mannans (Al-Bassam *et al.*, 1985). This could be the mechanism by which amphotericin B inhibits adherence since mannans are the carbohydrate component of mannoproteins which are believed to mediate the yeast's adherence to buccal and vaginal epithelial cells (Sandin, 1987).

Although amphotericin B failed to reduce the adherence of *C. albicans* 3436 to gastric mucosal explants in assays incubated at pH 7·3, it produced a significant reduction in adherence when similar assays were incubated at pH 1·2 and 3·4. One explanation for these findings is that *C. albicans* cultures grown at pH 3·0 have been found to be more susceptible to amphotericin B than those cultures grown at pH 5·0 and pH 7·0 (Johnson, White & Williamson, 1978). However, this does not explain why amphotericin B did not significantly reduce this strain's adherence to gastric mucosal cells. Clearly the cellular arrangement of the mucosal surface plays an important role in determining the effect of amphotericin B on adherence.

The inhibitory effects of ketoconazole and itraconazole on *C. albicans* were limited to the azole-sensitive strain, 3436. However, itraconazole did reduce the median numbers of cells of the azole-resistant strain *C. albicans* 3310 adhering to jejunal mucosal cells. Both ketoconazole and itraconazole indirectly increase the activity of chitin synthetase (Vanden Bossche, 1985) by inhibiting ergosterol synthesis (Chiew, Sullivan & Shepherd, 1982). The subsequent increase in cell wall chitin may affect the cell's adherence properties since chitin (Segal, Soroka & Lehrer, 1984) and its constituent N-acetyl D-glucosamine (Segal, Lehrer & Ofek, 1982; Collins-Lech *et al.*, 1984) and chitin soluble extracts (Segal & Savage, 1986) have been shown to inhibit the adherence of *C. albicans*.

Our work has shown that there is a good correlation between a reduction in germ tube adherence and a corresponding reduction in either the median number of *C. albicans* blastospores adhering to mucosal cell, mucosal explant, or in some cases, both. Neither ketoconazole nor itraconazole reduced the adherence of the azole-resistant strain 3310 while both drugs reduced the germ tube adherence of strain 3436. This suggests that an antifungal drug's influence on the development and elongation of *C. albicans* germ tubes may also affect its adherence properties as ketoconazole (Johnson, Richardson & Warnock, 1984) and itraconazole (E. M. Johnson, personal communication) inhibit the elongation of azole-sensitive *C. albicans* strains but not azole-resistant strains, including strain 3310.

The reason why strains 3436 and 3310 should display different binding characteristics in the presence of antifungal drugs is not clear. While the cell wall of *C. albicans* is considered to be the surface that is most likely to be involved in its adherence (Douglas, 1987), there are no reports that suggest that either biochemical or structural differences occur between the cell walls of azole-sensitive and azole-resistant strains.

In conclusion, this investigation has shown that at least three antifungal drugs can inhibit the adherence of *C. albicans* to murine GI mucosal surfaces. Further work should include a larger cross-section of *C. albicans* strains and other *Candida* spp. However, this work does suggest that the outcome of oral antifungal prophylaxis of patients at risk from disseminated candidosis depends on a number of factors which may include the susceptibility of the yeast to the drug, its ability to form germ tubes, the type of mucosal surface to which the yeast is adhering and the concentration of the drug.

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