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Short communication

Modification of adherence to plastic and to human buccal cells of *Candida albicans* and *Candida dubliniensis* by a subinhibitory concentration of itraconazole

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Exposure to subinhibitory concentrations of antifungal agents can influence the adherence of *Candida* spp. to the host cell. In this study the adherence of *Candida albicans* ATCC 10231 and *Candida dubliniensis* CECT 11455 to plastic and to human buccal epithelial cells was evaluated following pre-exposure to 0.5 × minimum inhibitory capacity (MIC) of itraconazole and compared with the corresponding cellular surface hydrophobicity. The yeasts were grown in Sabouraud broth or RPMI-1640 with itraconazole (0.5 × MIC) for 24–26 h at 37°C and the drug was then removed. The adhesion capacity to plastic was studied by turbidimetry in a polystyrene microtiter plate. The adhesion of the yeast to buccal epithelial cells was determined using microscopy techniques. The cellular surface hydrophobicity levels were determined by the microbial adhesion hydrocarbons test. Pre-exposure to itraconazole decreased plastic adherence and cellular surface hydrophobicity in both species when grown in RPMI. When *C. albicans* was grown in Sabouraud broth, it was nonhydrophobic and did not adhere and therefore no change was detected with the antibiotic. Itraconazole increased adherence to buccal epithelial cells in both species and media studied, as compared to controls without antifungal agents. To study the effects of these antifungal agents on pathogenicity mechanisms, it will be necessary to standardize the methodology for evaluation to determine their *in vivo* therapeutic efficacy.

Key words: *Candida* spp.; adhesion capacity; buccal cells; hydrophobicity; itraconazole

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Oral candidiasis constitutes one of the most important clinical manifestations of infection by *Candida* species. A major pathogenicity mechanism of *Candida* spp. is its adherence capacity to the host cell (8, 15). The mechanisms used by *Candida* spp. to adhere are multiple and have not been defined precisely (13, 16). Although there have been studies on the adhesion of *Candida albicans* versus that of *Candida dubliniensis,* it is difficult to compare the results of these studies due to differences in methods, culture conditions, cell types and surfaces (14). Cell surface hydrophobicity is thought to contribute during the initial steps of adherence to the attractive forces (9) and seems to be related to the adherence to plastic (2) and to play a role in the adhesion of yeast to cells (12). Several studies have examined the impact of azole on the cellular surface hydrophobicity (1, 6) and adherence capacity (3, 5, 7) of the yeasts, but have presented contradictory results depending on the conditions used.

The purpose of this work was to evaluate the adherence capacity of *C. albicans* and *C. dubliniensis* to plastic and to human buccal epithelial cells following pre-exposure for 24–26 h to a subinhibitory

concentration of itraconazole and to compare the capacity with the corresponding cellular surface hydrophobicity. We evaluated this effect using different growth media with an influence on the cellular surface hydrophobicity of the yeasts.

Material and methods

C. albicans ATCC 10231 (American Type Culture Collection, Manassas, VA), originally isolated from a case of human bronchomycosis, and *C. dubliniensis* CECT 11455 (Colección Epañola de Cultivos Tipo, Valencia, Spain), obtained from the oral cavity of an HIV-infected patient, were used. The strains were grown and subcultured in Sabouraud broth (Difco, Madrid, Spain) or RPMI-1640 medium with L-glutamine without sodium bicarbonate (Sigma, Madrid, Spain) buffered with 0.165 M 3-[N-morpholino]propanesulfonic acid (MOPS) and supplemented with 2% glucose. Cells were grown to stationary phase at 37°C.

Itraconazole powder (Janssen Pharmaceuticals, Titusville, NJ) was reconstituted in dimethylsulfoxide (Sigma) and further diluted to obtain the cultures with itraconazole (0.5 × minimum inhibitory capacity, MIC). MIC was determined by the microbroth dilution test performed as described in the NCCLS M27-A protocol (10). The two strains were incubated in the presence of a subinhibitory concentration of itraconazole (0.5 × MIC) for 24–26 h at 37°C in Sabouraud broth and in RPMI-1640 media. Two control tests were used, one with yeast grown in the culture media alone and another containing the solvent DMSO at the same concentration as that reached in the antifungal test. Controls and itraconazole-treated yeast cells were collected by centrifugation, washed, and resuspended in phosphate buffered saline (PBS) at pH 7.2. Following removal of the antifungal, a suspensions with an optical density (OD) at 490 nm of 0.4 for evaluation of adherence to plastic and cellular surface hydrophobicity, and of 0.2 for the evaluation of adherence to buccal epithelial cells corresponding to 1×10^7 yeast/ml, were made up. The prepared suspensions were sonicated in an ultrasound water bath for 5 min to avoid clumping.

The adhesion capacity of *C. albicans* and *C. dubliniensis* to polystyrene was assayed turbidimetrically (2) in microtiter plates (Greiner, Frickenhausen, Germany). The microplates containing 0.2 ml of the prepared suspensions of yeast were incubated for 24 h at 37°C and nonadhering cells were removed by aspiration. The wells were washed three times with cold PBS and the OD_{490} of the wells with attached cells read in a plate reader (ELx800, Bio-Tek, Winooski, VT). Values greater than 0.05 were considered adherent (this positive cut-off corresponds to the absorbance mean of all the visual negative results plus three times the SD). To verify the linearity of the results we quantified the adhered yeasts by a colorimetric assay using modified tetrazolium salt XTT (Sigma-Aldrich, Madrid, Spain). The viable cells convert XTT to a water-soluble formazan; this colorimetric substrate can be quantitated in a microplate reader at 570 nm. We related the OD of the attached cells and the color obtained with XTT, resulting in a linear regression $R^2 = 0.98$. Each assay was performed in quadruplicate and repeated two or more times.

The adhesion of the yeasts to buccal epithelial cells was assayed using the Kimura & Pearsall method as described by Ellepola & Samanarayake (5). The buccal epithelial cells were obtained by cotton-swabbing the mouths of two healthy individuals. The cells were washed three times by centrifugation in PBS, resuspended to a concentration of 2×10^5 buccal epithelial cells/ml, and used to study the adhesion of *Candida* spp. to buccal epithelial cells following exposure of yeast to itraconazole. Briefly, equal volumes of buccal epithelial cells (2×10^5 buccal epithelial cells/ml) and yeast cells (1×10^7 yeast/ml), treated as described above, were incubated in a rotor at 37°C for 1 h. Cells were filtered through a 10-μm pore size polycarbonate Isopore membrane filter (Millipore, Molshein, France) to remove nonadherent yeast. The filters were washed twice with 20 ml PBS and then placed on a glass slide, methanol-fixed, and stained with crystal violet for 1 min. The number of yeasts adhering to buccal epithelial cells was counted microscopically at a magnification of ×400. Counting was undertaken following the criteria described by Pizzo et al. (11) to standardize the counts. Each assay was carried out in triplicate.

Cellular surface hydrophobicity levels were determined by the microbial adhesion hydrocarbon test (MATH), using a modification of Rosenberg's method, as described by Blanco et al. (2). Briefly, we added 1 ml xylene to each 3 ml of the yeast suspension in PBS with an OD of 0.4, and mixed phases by vortexing for 1 min. The relative cellular surface hydrophobicity was expressed as the percentage reduction of initial turbidity of the aqueous suspension. Cellular surface hydrophobicity after pre-exposure of the two strains to 0.5 × MIC of itraconazole for 24–26 h was assessed in both media at 37°C.

Statistical analysis of variation due to itraconazole exposure in each of the experiments was carried out to compare the results of the drug-exposed test and the drug-free control using a paired *t*-test. Interspecies and intermedia variation was determined using an unpaired *t*-test.

Results

All the results are presented in Table 1. The results of the controls were not affected by the presence of DMSO at the concentrations used (data not shown). Figure 1 shows the adherence of *C. dubliniensis* to plastic (Fig. 1A) and adherence to buccal epithelial cells of *C. dubliniensis* and *C. albicans* (Fig. 1B), control and itraconazole (0.5 × MIC) treated yeast grown to stationary phase at 37°C.

The MIC of itraconazole was 0.125 μg/ml for *C. albicans and* 0.06 μg/ml for *C. dubliniensis.* The preincubation with 0.5 × MIC of itraconazole produced growth inhibition of *C. albicans* and *C. dubliniensis* which fluctuated from 39% to 47%. Cellular viability was quantified by vital methylene blue dye stain, and was in all cases greater than 95%.

C. albicans adhered to plastic in the microplates when grown in RPMI-1640 with an OD > 0.10, but not when it was grown in Sabouraud at 37°C. *C. dubliniensis* was adherent in both media, although in Sabouraud the adherence was low (OD = 0.07). The yeasts which had been treated previously itraconazole (0.5 × MIC) for 24–26 h at 37°C showed a relevant and significant decrease in their adherence to plastic, with an OD < 0.05 in most cases.

C. dubliniensis adhered to buccal epithelial cells in a similar way to *C. albicans* in RPMI 1640 (no significant variation) and better than *C. albicans* in Sabouraud ($P < 0.01$). Both strains were more adherent when grown in RPMI-1640 than in Sabouraud ($P < 0.01$). Pre-exposure to itraconazole (0.5 × MIC) of *C. dubliniensis* and *C. albicans* increased adherence to buccal epithelial cells 4–12-fold compared to controls without itraconazole.

C. albicans showed a variable cellular surface hydrophobicity depending on the culture medium. When grown in Sabouraud at 37°C *C. albicans* was always nonhydrophobic, whereas medium levels of cellular surface hydrophobicity were found when the yeast was grown in RPMI-1640 to stationary phase. In contrast, *C. dubliniensis* displayed high levels of cellular surface

Table 1. Effect of pre-exposure to 0.5 × MIC of itraconazole (ITZ) on growth, cell surface hydrophobicity, adhesion to plastic and to buccal epithelial cells of *Candida albicans* and *Candida dubliniensis* in RPMI-1640 and Sabouraud broth media

	C. albicans			*C. dubliniensis*		
	Control	ITZ	*P*-value	Control	ITZ	*P*-value
Growth (OD)						
RPMI-1640	1.4 ± 0.1[b]	0.9 ± 0.1	< 0.05	1.3 ± 0.1	0.8 ± 0.1	< 0.05
Sabouraud	1.6 ± 0.1	0.9 ± 0.1	< 0.05	1.4 ± 0.1	1.0 ± 0.2	< 0.05
Cellular surface hydrophobicity (%)						
RPMI-1640	43.3 ± 3.3	14.6 ± 2.9	< 0.01	95.8 ± 0.8	34.2 ± 8.1	< 0.01
Sabouraud	0	0	NS	65.1 ± 2.8	28.3 ± 6.0	< 0.01
Plastic adherence (OD)						
RPMI-1640	0.11 ± 0.01	0.05 ± 0.01	< 0.01	0.12 ± 0.01	0.07 ± 0.01	< 0.01
Sabouraud	0.04 ± 0.01	0.04 ± 0.01	NS	0.07 ± 0.01	0.05 ± 0.01	< 0.01
Buccal epithelial cell adherence[a]						
RPMI-1640	167 ± 60	790 ± 179	0.05	155 ± 23	563 ± 46	< 0.01
Sabouraud	13 ± 5	141 ± 57	0.05	41 ± 4	470 ± 31	< 0.05

[a]Buccal epithelial cells adherence expressed as no. of yeasts/100 buccal epithelial cells.
[b]Mean value of at least three different experiments ± SEM; NS: not statistically significant.

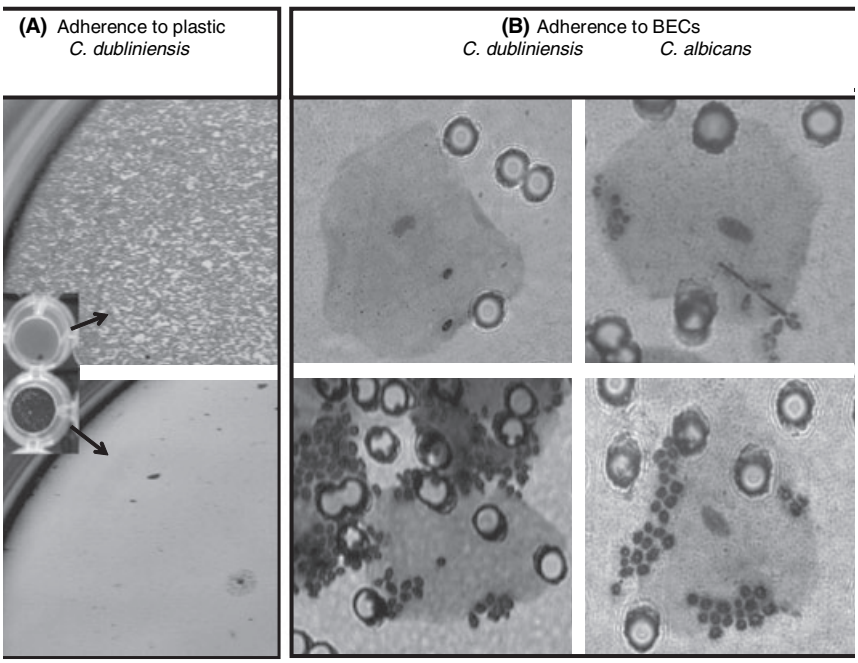

Fig. 1. Adherence of *C. dubliniensis* to plastic (A) and adherence to buccal epithelial cells (BEC)s of *C. dubliniensis* and *C. albicans* (B), control (upper row) and itraconazole (0.5 × MIC) (lower row) treated yeast grown to stationary phase at 37°C.

hydrophobicity at 37°C in both culture media. Treatment with itraconazole significantly reduced the cellular surface hydrophobicity of *C. dubliniensis* in both media. The nonhydrophobic character of the surface of *C. albicans* when grown in Sabouraud at 37°C did not permit the detection of change produced by itraconazole, but when grown in RPMI-1640, medium levels of cellular surface hydrophobicity were produced, which diminished after pre-exposure to itraconazole.

Discussion

Microbial adherence is an important factor in the pathogenicity of infections. Fungal adhesion is initially affected by a combination of hydrophobic and electrostatic interactions. Adherence capacity depends on several factors, such as the hydrophobic state of the fungal cell wall (2, 8), the characteristics of the substrate surface (7), and the conditions and methodology used (14). Our results confirm that the adhesion to plastic of both species of *Candida* is greater when the cellular surface hydrophobicity levels are high; this depends on several factors such as the culture media and strain used. The expression of cellular surface hydrophobicity of *C. albicans* is a dynamic process on which the culture conditions have a fundamental influence (2). *C. albicans* increased the hydrophobic properties of the surface in RPMI, probably due to the presence of hydrophobic germ tubes. *C. dubliniensis*, however, always exhibits high levels of cellular surface hydrophobicity when grown to stationary phase (8), and greater adherence to polystyrene than *C. albicans*, mainly in Sabouraud, where there is a clear difference in hydrophobicity between both strains.

In oral candidiasis, the therapeutic concentration of antifungal agents is reduced, especially during dosing intervals and also because of the diluent effect of saliva (1). The presence of subinhibitory concentrations of some antifungal agents can modulate adhesion of yeast (3–5) by altering the physical characteristics of the cell surface, inhibiting the formation of hyphae, or blocking the adhesins present on the fungal cell wall. In some studies it was noted that pre-exposure to sub-MIC concentrations of azole can affect cellular surface hydrophobicity and adherence. Ellepola & Samaranayake (5) noted a decrease in hydrophobicity, whereas Hazen et al. (6) found no differences between the cellular surface hydrophobicity levels of yeast treated with sub-MIC of fluconazole and the controls, although paradoxically they did detect greater susceptibility to phagocytosis. With our study conditions and methodology, a decrease in cellular surface hydrophobicity and adherence to plastic was detected in the presence of itraconazole. This effect was produced in *C. dubliniensis* regardless of the culture medium, but in *C. albicans* it was only observed in RPMI-1640 where medium levels of cellular surface hydrophobicity were reached. It is interesting to note the difference in the results according to the medium used.

Contradictory results have also been found concerning the relationship between adherence to buccal epithelial cells and

cellular surface hydrophobicity. Some authors state that hydrophobic cells, as compared to hydrophilic cells, are more adherent to buccal cells (8), whereas McCarron et al. (9) found an inverse relationship between cellular surface hydrophobicity and cell adherence. Samaranayake et al. (12) demonstrated a significant positive correlation in a single isotype, whereas no such correlation was observed when all tested *Candida* isolates were pooled and evaluated as a single, large group. Our results show a positive correlation between cellular surface hydrophobicity and adhesion to buccal epithelial cell in the controls: greater adherence of *C. dubliniensis* (hydrophobic) than *C. albicans* (less hydrophobic), and also greater adherence within the same species in RPMI-1640 (more hydrophobic) than in Sabouraud. However, when another modifying factor such as the presence of azole was introduced, we observed increased adherence to buccal epithelial cells as compared to controls without itraconazole, despite the decrease in cellular surface hydrophobicity produced, thus revealing the existence of different mechanisms involved in adherence. These results concur with the increase in adherence of *C. dubliniensis* to Vero cells found by Borg-von Zepelin et al. (3) in the presence of fluconazole, although they observed the inverse effect in *C. albicans*, which adhered better than *C. dubliniensis* and diminished in the presence of fluconazole.

Interpretation of the data obtained in this type of study is difficult as many factors are involved. Itraconazole acts directly by blocking the synthesis of ergosterol, although prolonged exposure to subtherapeutic concentrations of fluconazole can affect the cell wall and membrane structure of *C. albicans* isolates (6). In the literature, contradictory results were found, depending on the conditions used. We defined the study conditions *in vitro* to obtain reproducible results. Further studies are required with a greater number of isolates and a wider range of *in vivo* conditions, such as the presence of saliva in the oral cavity. San Millán et al. (13) have shown that saliva may stimulate or decrease adherence capacity, depending on the morphological phase of *C. albicans*. A similar explanation could be found for the apparent contradiction of the existing results on adherence capacity (17). However, though the action of azoles repressed hyphal development, adherence to buccal epithelial cells was stimulated, suggesting that other biological mechanisms such as accessibility of some adhesins may intervene.

In conclusion, this study demonstrates that *C. albicans* and *C. dubliniensis,* due to their polymorphism, have a high capacity to adapt to environmental conditions. This causes variations in the characteristics of the fungal cell surface and, consequently, modifications in adherence capacity. Sabouraud broth is not a valid medium to determine the influence of antifungal agents on the cellular surface hydrophobicity of *C. albicans* incubated at 37°C. Itraconazole altered adherence depending on the tested surface: adherence to plastic decreased in both species according to the diminution of cellular surface hydrophobicity; however, adherence to buccal epithelial cells increased in all cases regardless of cellular surface hydrophobicity. To study the effects of these antifungal agents on pathogenicity mechanisms, it will be necessary to standardize the methodology for evaluation in order to determine their *in vivo* therapeutic efficacy.

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