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Adhesion of oral *Candida* species to human buccal epithelial cells following brief exposure to nystatin

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Opportunistic oral infections caused by *Candida albicans* and non-*albicans* *Candida* species are particularly common in compromised patients. Nystatin, which belongs to the polyene group of antimycotics, is frequently used as a topical agent in the treatment of oro-pharyngeal candidosis. It is recognized that due to the delivery mode of nystatin (i.e. topical, intermittent), as well as the cleansing effect of saliva within the oral environment, the yeasts undergo a relatively brief exposure to this drug during treatment. Nevertheless, there is a sparsity of data on the effect of such brief exposure to nystatin on the pathogenic attributes of *Candida* such as their adherence to host surfaces. The adhesion of microbes to host mucosal surfaces is a major determinant of successful colonization and infection. Thus the main aim of our investigation was to compare the *in vitro* adhesion of 30 oral isolates of *Candida* belonging to six different species (comprising *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida guilliermondii*, Candida krusei and *Candida parapsilosis*) to human buccal epithelial cells, following their brief exposure (1 h) to minimum inhibitory concentration of nystatin, and subsequent removal of the drug. The adhesion of these isolates to buccal epithelial cells was assessed by a previously described adhesion assay. Compared with the controls, there was a significant reduction in buccal epithelial cell adhesion of all six *Candida* species after drug exposure (54%–68%). However the adhesion of *C. albicans* isolates was the least affected by nystatin exposure, which was significantly different from that of the non-*albicans* species. These findings imply that sub-therapeutic levels of nystatin, which are likely to persist in the oral cavity during dosing intervals, may also be beneficial, as they inhibit candidal colonization. The significant difference in nystatin-induced suppression of adhesion between *C. albicans* and the non-*albicans* species investigated is a further testimonial for the pre-eminent virulence of the former species.

Key words: adhesion; *Candida*; nystatin

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Superficial and systemic fungal infections have alarmingly increased over the past two decades causing formidable morbidity and mortality among the immunocompromised, especially in HIV infection, and the yeast species *Candida* is the most frequently encountered opportunistic pathogen in these patients (21). In fact, oral candidosis is considered the commonest oral manifestation in such patients (2, 21, 23). Although *Candida albicans* is the most pervasive candidal pathogen, since it invades virtually all human tissues (21) non-*albicans* *Candida* species such as *Candida tropicalis*, *Candida glabrata*, *Candida guilliermondii*, *Candida krusei* and *Candida parapsilosis* are infre-

quently but consistently isolated from these patients (26, 32, 33). For instance, *Candida krusei*, which was considered a saprophyte, is currently regarded as an emerging pathogen because of the wide use of fluconazole, to which it is intrinsically resistant (26, 33).

The adhesion of microbes to host mucosal surfaces is a major determinant of successful microbial colonization and subsequent infection, and its critical role in the pathogenesis of many fungal infections is well recognized (11, 15, 29). Various *in vitro* studies (24, 25) and animal studies (3, 18) have provided evidence for a relationship between the proclivity of *Candida* species to adhere to mucosal surfaces and their presence in infections. Therefore candidal adherence to human buccal epithelial cells is considered as the critical initial step in the pathogenesis of oral candidosis, which may lead to eventual systemic infection, especially in compromised people (27).

Nystatin, which belongs to the polyene group of antimycotics is frequently used topically in the treatment of oropharyngeal candidosis. However, poor response to nystatin is not uncommon, as the therapeutic efficacy is compromised due to many factors. In addition to the intermittent topical administration, the diluent effect of saliva and the cleansing action of the oral musculature tend to reduce the availability of nystatin below that of the effective therapeutic concentration, especially during dosing intervals (17).

Several researchers have studied the effect of antifungal agents on candidal adhesion to buccal epithelial cells following i) incubation of the isolates with the agents (1, 16, 31), ii) pre-treatment of the buccal epithelial cells with the agent, both *in vivo* and *in vitro* (5), and iii) limited exposure (1 h) to sublethal concentrations of agents (7). On the contrary, there is no information characterizing the adhesion of a range of *Candida* species of oral origin to buccal epithelial cells, following brief exposure to nystatin and its subsequent removal. Hence the main aim of our investigation was to compare quantitatively the adhesion of 30 oral isolates of *Candida* belonging to six different species (five isolates each of *C. albicans, C. tropicalis, C. glabrata, C. guilliermondii, C. krusei* and *C. parapsilosis*) to human buccal epithelial cells, following their limited exposure (1 h) to minimum inhibitory concentration of nystatin.

Material and methods

Organisms

Five oral isolates each of *C. albicans, C. tropicalis, C. krusei, C. parapsilosis, C. glabrata* and *C. guilliermondii* were used. All isolates of *C. tropicalis, C. krusei, C. glabrata* and *C. guilliermondii* were derived from patients attending the Glasgow Dental Hospital, Glasgow, UK. *C. albicans* isolates were from patients attending the Prince Philip Dental Hospital in Hong Kong, and *C. parapsilosis* isolates were from both the Glasgow Dental Hospital and the Prince Philip Dental Hospital. *C. albicans* ATCC 90028 and *C. tropicalis* ATCC 13803 were used as the reference strains for determination of minimum inhibitory concentration. The organisms were identified using the germ tube test, and the commercially available API 20 (API System, Vercieu, France) *Candida* identification kits. Stock cultures were maintained at −20°C. After recovery these were maintained on Sabouraud's dextrose agar, stored at 4–6°C, during the experimental period.

Antifungal agents and media

Nystatin (Sigma Chemical Co., St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO) and absolute ethanol (3:2 ratio), respectively and was prepared initially as a 10,000 μg/ml solution and stored at −20°C before use. It was suspended/diluted in the following medium during the exposure period (1 h) of yeasts. RPMI 1640 medium buffered with 0.165 M MOPS (3-(*N*-morpholino) propanesulfonic acid) containing L-glutamine and lacking sodium bicarbonate (Sigma Chemical Co.), was dissolved in 1 liter of sterile distilled water and adjusted to a pH of 7.2 and filter sterilized. This liquid RPMI 1640 was stored at 2 to 8°C for 2–3 months.

Since nystatin was dissolved in DMSO and absolute ethanol, equivalent amounts of the latter chemicals were tested initially to ascertain whether they had an effect on the isolates tested. The minute volumes of the chemicals used did not have any effect on yeast survival/growth when compared with the controls.

Determination of minimal inhibitory concentration

The minimal inhibitory concentration values for all isolates with nystatin were determined by the broth dilution technique according to McGinnis & Rinaldi (20), by performing two-fold serial dilutions of the drug in microtiter plates using an inoculum of $1–5\times10^5$ colony-forming units/ml. The minimal inhibitory concentration, was determined visually and spectrophotometrically at 595 nm following 24 h of incubation at 37°C. The minimal inhibitory concentration was defined as the lowest concentration of the drug which inhibited growth of yeast cells, as indicated by the absence of turbidity (optically clear). *C. albicans* ATCC 90028 and *C. tropicalis* ATCC 13803 were used as the reference strains. All experiments were repeated on two separate occasions with duplicate determinations on each occasion.

Preparation of the cell suspension for the adhesion assay

Yeast cells, maintained on Sabouraud's dextrose agar, were inoculated onto fresh plates and incubated overnight for 24 h prior to use. The organisms were harvested and a cell suspension prepared in sterile phosphate-buffered saline at 520 nm to an optical density of 1.5. From this cell suspension, 1 ml was added to tubes containing 4 ml of RPMI broth (control) and 4 ml of RPMI/drug solution (test); the drug concentration used was minimal inhibitory concentration of nystatin. This gave a cell suspension of $10^6–10^7$ cells/ml in each assay tube.

The tubes were then incubated at 37°C for a period of 1 h in a rotary incubator. Following this limited exposure, the drug was removed by two cycles of dilution (with sterile phosphate-buffered saline) and centrifugation for 10 minutes at $3000\times g$ (4). Afterwards the supernatant was completely decanted and the pellets were resuspended in 3 ml of sterile phosphate-buffered saline. This procedure has proven to reduce the concentration of the drug as much as 10,000 fold (4), thereby minimizing any carry over effect of the drug following its removal. Prior to the adhesion assay, viable counts of the control and the test samples were performed after drug removal and control suspensions were reconstituted as needed to obtain a cell concentration comparable to the test.

Preparation of buccal epithelial cells

The method of Kimura & Pearsall (14), was used in preparation of buccal epi-

Table 1. The *in vitro* adhesion of *Candida* species to buccal epithelial cells (yeasts/50 buccal epithelial cells) following 1 h of exposure to minimal inhibitory concentration of nystatin and subsequent removal of the drug

Isolate	Control	Test	Percentage reduction in adhesion	*P*
Candida albicans				
A1	160.75 (4.75)[a]	65.35 (3.25)[a]	59.34	
A2	150.73 (5.00)	68.17 (3.95)	54.77	
A3	205.17 (8.13)	98.15 (7.95)	52.16	
A4	170.45 (6.55)	89.95 (4.52)	47.22	
A5	155.45 (6.13)	68.70 (3.55)	55.80	
Mean (SEM)	168.51 (9.73)	78.06 (6.67)	53.85 (2.02)	<0.01
Candida tropicalis				
T1	134.75 (6.25)	47.68 (3.72)	64.62	
T2	165.54 (3.75)	52.93 (4.95)	68.03	
T3	129.54 (7.23)	44.35 (4.93)	65.76	
T4	190.25 (3.55)	59.47 (5.85)	68.74	
T5	127.25 (1.75)	40.44 (7.20)	68.22	
Mean (SEM)	149.46 (12.30)	48.97 (3.32)	67.07 (0.79)	<0.01
Candida krusei				
K1	117.57 (3.87)	35.48 (9.45)	69.82	
K2	114.97 (6.20)	32.67 (2.35)	71.58	
K3	90.56 (2.61)	31.26 (3.57)	65.48	
K4	70.15 (4.92)	32.65 (4.35)	53.46	
K5	102.54 (9.25)	39.25 (3.75)	61.72	
Mean (SEM)	99.15 (8.70)	34.26 (1.42)	64.42 (3.23)	<0.01
Candida parapsilosis				
P1	155.34 (3.72)	54.93 (7.74)	64.63	
P2	175.45 (3.86)	60.25 (6.70)	65.66	
P3	112.24 (3.95)	41.45 (7.70)	63.07	
P4	109.54 (7.98)	37.74 (2.34)	65.55	
P5	101.45 (3.44)	39.02 (2.97)	61.54	
Mean (SEM)	130.80 (14.58)	46.67 (4.57)	64.09 (0.78)	<0.01
Candida glabrata				
GL1	162.33 (2.95)	51.35 (9.31)	68.37	
GL2	103.35 (7.75)	37.32 (2.95)	63.89	
GL3	95.75 (9.25)	31.56 (7.25)	67.04	
GL4	93.45 (2.97)	28.25 (6.33)	69.77	
GL5	142.35 (7.85)	43.25 (3.63)	69.62	
Mean (SEM)	119.44 (13.89)	38.34 (4.13)	67.74 (1.01)	<0.01
Candida guilliermondii				
GU1	101.39 (7.22)	36.54 (3.97)	63.96	
GU2	139.35 (2.97)	41.75 (5.37)	70.04	
GU3	131.25 (3.72)	47.76 (3.21)	63.61	
GU4	90.54 (7.21)	27.75 (1.33)	69.35	
GU5	83.95 (3.49)	31.59 (8.54)	62.37	
Mean (SEM)	109.29 (11.05)	37.07 (3.55)	65.86 (1.58)	<0.01

[a] Mean value of three different experiments with duplicate determinations on each occasion.

thelial cells for the adherence assay. Human buccal epithelial cells from three young healthy adults (two men and one woman) were collected each morning at the same time of the day, by gently rubbing the inner aspect of the right and left buccal mucosa with two sterile cotton swabs, and dispersed in 10 ml of sterile phosphate-buffered saline. The pooled buccal epithelial cells suspension was washed four times in phosphate-buffered saline to remove attached organisms, by centrifugation at $3500 \times g$ for 10 min. Washed epithelial cells usually had no attached yeasts before the adherence test. The buccal epithelial cells were then resuspended to a concentration of 1×10^5 cells/ml by hemocytometer counting (Neubauer's hemocytometer chamber, Superior, Germany), and were used immediately for the adhesion assay.

Adhesion assay

The adherence method of Kimura & Pearsall (14) was used with a few modifications. For the assay, 0.5 ml of the buccal epithelial cells and 0.5 ml of the yeast suspension were mixed gently in tubes and incubated on a rotary incubator with gentle agitation at 37°C for 1 h. The yeast/buccal epithelial cells suspension was thereafter diluted in 4 ml of sterile phosphate-buffered saline. The buccal epithelial cells were then harvested under negative pressure using a manifold filter (Sartorius, SM16547) onto 12-μm pore size polycarbonate filters (Millipore, UK), and washed twice with 50 ml of sterile phosphate-buffered saline to remove unattached fungi. Thereafter each filter was removed carefully with forceps and placed firmly on a glass slide with the buccal epithelial cells against the glass surface. After 10 s, the filter was removed gently, leaving the buccal epithelial cells adherent on to the glass slide. The preparations were air dried, fixed with methanol and stained with Gram's stain. The number of adherent yeast cells were quantified by light microscopy at ×400 magnification. Fifty buccal epithelial cells were observed for adherent yeast cells.

Clumped, folded or overlapping buccal epithelial cells were excluded. Each assay was performed on three separate occasions with duplicate determinations each time.

Statistical analysis

The statistical difference between the drug-free controls and drug exposed tests were analysed by Student's *t*-test. The inter-species variation of adhesion to buccal epithelial cells among the drug-free control groups and the isolates following limited exposure to nystatin was analysed by analysis of variance using Tukey-Kramer multiple comparison tests (INSTAT statistical analysis). A P value of <0.05 was considered as statistically significant (Tables 2, 3).

Results

The minimal inhibitory concentration (μg/ml) valueranges of nystatin for *C. albicans, C. tropicalis, C. krusei, C. parapsilosis*, C. glabrata and *C. guilliermondii* in RPMI broth were 0.78–1.56, 1.56–3.12, 3.12, 1.56–3.12, 0.78–1.56 and 0.39–0.78, respectively.

The *in vitro* adhesion of oral *Candida* isolates to buccal epithelial cells after 1 h of exposure to minimal inhibitory concentration of nystatin and sub-

Table 2. Statistical analysis of the inter-species variation in adhesion of the drug-free (control) *Candida* species to buccal epithelial cells (Tukey-Kramer multiple comparison post-hoc test)

	Candida albicans	*Candida tropicalis*	*Candida krusei*	*Candida parapsilosis*	*Candida glabrata*	*Candida guilliermondii*
Candida albicans	–	NS	$P<0.01$	NS	NS	$P<0.05$
Candida tropicalis	NS	–	NS	NS	NS	NS
Candida krusei	$P<0.01$	NS	–	NS	NS	NS
Candida parapsilosis	NS	NS	NS	–	NS	NS
Candida glabrata	NS	NS	NS	NS	–	NS
Candida guilliermondii	$P<0.05$	NS	NS	NS	NS	–

NS: statistically not significant.

Table 3. Statistical analysis of the inter-species variation in the mean percentage reduction on adhesion of *Candida* species to buccal epithelial cells following exposure to minimal inhibitory concentration of nystatin for 1 h (Turkey-Kramer multiple comparison *post-hoc* test)

	Candida albicans	*Candida tropicalis*	*Candida krusei*	*Candida parapsilosis*	*Candida glabrata*	*Candida guilliermondii*
Candida albicans	–	$P<0.001$	$P<0.01$	$P<0.01$	$P<0.001$	$P<0.01$
Candida tropicalis	$P<0.001$	–	NS	NS	NS	NS
Candida krusei	$P<0.01$	NS	–	NS	NS	NS
Candida parapsilosis	$P<0.01$	NS	NS	–	NS	NS
Candida glabrata	$P<0.001$	NS	NS	NS	–	NS
Candida guilliermondii	$P<0.01$	NS	NS	NS	NS	–

NS: statistically not significant.

sequent removal of the drug is shown in Table 1. Compared with the drug-free controls, a statistically significant ($P<0.01$) reduction in adhesion was seen in all 30 strains of oral *Candida*, with a mean percentage reduction in adhesion of 53.85%, 67.07%, 64.42%, 64.09%, 67.74% and 65.86% for *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. glabrata* and *C. guilliermondii*, respectively.

When the inter-species variation in adhesion of the drug free control yeasts to buccal epithelial cells was analysed, *C. albicans* isolates demonstrated the greatest adhesion to buccal epithelial cells (160.75 yeasts/50 buccal epithelial cells), which was significantly different to that of *C. guilliermondii* and *C. krusei* (109.29 yeasts/50 buccal epithelial cells; $P<0.05$ and 99.15 yeasts/50 buccal epithelial cells; $P<0.01$, respectively), However no such difference could be elicited between *C. albicans* and *C. tropicalis* or *C. parapsilosis* or *C. glabrata* (Tables 1, 2).

Interestingly, when the inter-species relationship for nystatin induced mean percentage reduction in adhesion to buccal epithelial cells was analyzed, a significant difference was noted between *C. albicans* isolates and all the non-*albicans* species tested (Table 3). Thus, nystatin induced suppression of adhesion was the least for *C. albicans* (53.85%) compared with the other five species (64.09%–67.74%). However such significant inter-species difference could not be elicited amongst the other five *Candida* species (Table 3).

Discussion

Compromised patients including those with human immunodeficiency virus (HIV) disease are prone to infection with an array of opportunistic pathogens, and oral candidosis caused by *Candida* species is widely recognized as the most frequently encountered oral manifestation in such cohorts (2). Although *C. albicans* is the commonest fungal pathogen in HIV disease and other compromised patient groups, oro-mucocutaneous infection due to non-*albicans* species including *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. glabrata*, and *C. guilliermondii* are not uncommon (21, 26, 32).

Among the plethora of antifungal agents nystatin is one of the most widely used topical agents in the management of oral candidosis (10, 11, 22). It belongs to the polyene group of antimycotics, and recent evidence implies that prophylactic oral application of nystatin can be of considerable benefit in preventing the systemic spread of oral candidal infection in the compromised (27). This antimycotic has also proven to be effective in modulating many pathogenic attributes of *Candida* both *in vivo* and *in vitro*. For instance, it has been shown that nystatin is capable of suppressing candidal adhesion to buccal epithelial cells (5, 7), and to denture acrylic surfaces (9, 19). Further, we have recently demonstrated its ability to perturb germ tube formation (6), and to modulate the relative cell surface hydrophobicity following brief exposure to the drug (8). Others have demonstrated the suppression of proteolytic activity of *Candida* following exposure to nystatin (34).

The exposure of yeasts to the concentration of nystatin used in the current study is known to elicit a post-antifungal effect in the organisms (10). The latter refers to the suppression of microbial growth that persists following brief exposure of an organism to antifungals and subsequent removal of the agent (4). In a previous study we have reported that nystatin induced a significant post-antifungal effect against the identical *Candida* isolates used in the current investigation i.e. 6.85 h, 12.73 h, 11.58 h, 15.17 h, 8.51 h and 8.68 h on *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, C. glabrata and *C. guilliermondii*, respectively. (10). Thus, the adhesion assays in this study were performed during the post-antifungal effect period of the yeasts, during which the cells were under the influence of the nystatin-induced metabolic shock.

The current results imply that brief exposure of all oral *Candida* isolates to nystatin significantly reduces their ability to adhere to buccal epithelial cells, compared with the unexposed controls. Thus, compared with the latter, a statistically significant ($P<0.01$) reduction in adhesion was seen in all 30 isolates of oral *Candida*, with a mean percentage reduction of 53.85%, 67.07%, 64.42%, 64.09%, 67.74% and 65.86% for *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. glabrata* and *C. guilliermondii*, respectively. Others have also shown that pretreatment of *C. albicans* with nystatin suppresses yeast adhesion to buccal epithelial cells (1, 16, 31). However, to our knowledge, the current study is the first to document the effect of limited exposure to nystatin on the adhesion of a battery of *Candida* species (30 isolates, belonging to six different species) to human buccal epithelial cells.

Interestingly, candidal adhesion to

denture acrylic is also reduced to a great extent following pre-treatment of acrylic strips and/or the yeasts with nystatin (9, 19). Further it has been recently documented that pre-treatment of buccal epithelial cells with nystatin resulted in reduction in candidal adhesion (5). These studies, together with ours, indicate that pre-exposure of either the target surface or the yeasts to nystatin results in reduced adhesion, which *in vivo* may act synergistically to potentiate the antimycotic effect of this commonly used topical agent.

The statistically significant suppression of yeast adhesion to buccal epithelial cells and the high post-antifungal effect elicited by limited exposure to nystatin may be related to the mechanism of action of the drug on the yeast cell membrane. Nystatin inhibits the biosynthesis of ergosterol in the fungal cell membrane, which contributes to a variety of cellular functions. Ergosterol is important for the fluidity and integrity of the membrane and for the function of many membrane-bound enzymes, including chitin synthetase, which is important for proper cell growth and division (33). The selectivity with which microbes attach to various epithelial cell surfaces suggests the existence of specific receptors on both microbial and epithelial cells. Hence, it is not surprising that nystatin-induced changes in the cell wall structure would contribute in suppressing candidal adhesion to human buccal epithelial cells (7, 8). However, further studies at a molecular level are necessary to evaluate the exact mechanisms by which adhesion is suppressed after antifungal exposure.

When the inter-species variation in adhesion to buccal epithelial cells was analyzed on the drug-free control groups, *C. albicans* isolates demonstrated the greatest adhesion to buccal epithelial cells, followed by *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. guilliermondii* and *C. krusei*, respectively. However statistically significant inter-species differences in adhesion was noted only amongst *C. albicans* and *C. krusei* ($P<0.01$) and *C. albicans* and *C. guilliermondii* species ($P<0.05$). A previous study by Tobgi (30) demonstrated a similar hierarchy of adherence to buccal epithelial cells amongst six species of *Candida*. Another study by Samaranayake & Samaranayake (26) has also demonstrated similar inter-species differences in *C. albicans* and *C. krusei* adhesion among 10 and 20 isolates, respectively.

When the inter-species relationship for the nystatin induced percentage reduction in adhesion to buccal epithelial cells was analyzed, a significant variation was noted between *C. albicans* and all five non-*albicans Candida* species tested. However there was no statistically significant nystatin-induced adhesion suppressive effect among the rest of the *Candida* species (64.09%–67.74%). Hence it seems that *C. albicans* is by far the most robust species in this genus, having the ability to sustain its cell wall characteristics conferring the potential to adhere, despite drug exposure. Further, in a previous investigation we have reported that nystatin also induced the least post-antifungal effect on *C. albicans* as compared with the rest of the non-*albicans* species used in the current study (10). This, together with the current findings, which have not been described previously, further substantiate the fact that *C. albicans* is the most virulent and pervasive of all the *Candida* species, and the reason for its pre-eminent position in the hierarchy of virulence among *Candida* species (21, 28).

Finally, in clinical terms our results unequivocally demonstrate that exposure to nystatin significantly reduces candidal adherence to buccal epithelial cells irrespective of the *Candida* species concerned. The sub-therapeutic levels of antimycotics likely to persist in the oral cavity during dosing intervals may therefore be beneficial in reducing candidal colonization though possibly ineffective in their total elimination.

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