The Effect of Cetylpyridinium Chloride (CPC) on the Cell Surface Hydrophobicity and Adherence of *Candida albicans* to Human Buccal Epithelial Cells *in Vitro*

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Received May 8, 1995; accepted July 19, 1995

Purpose. This study examined the effects of cetylpyridinium chloride (CPC) on cell surface hydrophobicity (CSH) and adherence of blastospores of *Candida albicans* (MEN strain) to human buccal epithelial cells (BEC) *in vitro*.

Methods. The effect of CPC treatment of either *C. albicans* blastospores or BEC on their subsequent adherence was determined using $^{35}SO_4$ labelled blastospores in association with a Percoll™ gradient. The effects of CPC treatment of blastospores on their CSH was determined using Hydrophobic Interaction Chromatography.

Results. Treatment of exponential and stationary phase blastospores with CPC (50 μg mL^{-1}) for 0.5–30 minutes, or with CPC (0.5–50 μg mL^{-1}) for 15 minutes resulted in significant reductions in both blastospore CSH and adherence to BEC *in vitro*. No correlation was apparent ($r < 0.8$) between reduced CSH and reduced blastospore adherence following treatment with CPC (0.5–50 μg mL^{-1}). Significantly reduced adherence of *C. albicans* (stationary or exponential growth phases) to human BEC was also observed following treatment of BEC with CPC (50 μg mL^{-1}) for 0.5–30 minutes or with CPC (0.5–50 μg mL^{-1}) for 15 minutes. Antiadherence effects were observed at both sub and super-minimum inhibitory concentrations of CPC.

Conclusions. It is suggested that, whilst the ability of CPC to reduce the CSH of *C. albicans* may contribute to its reduced adherence to human BEC *in vitro*, reduced CSH is only one of several possible factors that contribute to the observed antiadherence effects.

KEY WORDS: *Candida albicans;* cetylpyridinium chloride; reduced adherence; reduced cell surface hydrophobicity.

INTRODUCTION

Candida albicans is an obligate associate of humans which under certain conditions, e.g. immunological compromisation, diabetes mellitus, acquired immune deficiency syndrome and antimicrobial chemotherapy may become pathogenic (1). In the pathogenesis of candidiasis the primary step involves adherence of *C. albicans* to the host epithelium (2,3). As the organism approaches the host epithelia, it is subjected to both attractive and repulsive forces. If the attractive forces exceed the repulsive forces, the organism may approach the epithelial cell at close distances and irreversibly adhere to receptor molecules on the surface of the epithelial cell using adhesin molecules on the surface of the yeast cell in a "lock and key" type interaction (2).

Several approaches to reduce or inhibit candidal adherence have been investigated *in vitro,* including, the use of isolated adhesin or adhesin analogues, sub-lethal concentrations of antibiotics (2) and lectins (4). However, more recently, it has been reported that some non-antibiotic, antimicrobial agents significantly reduced that adherence of *C. albicans* to human buccal epithelial cells *in vitro*. Examples include; chlorhexidine, cetrimide, dequalinium chloride (3), taurolidine and polyvinylpyrrolidone-iodine (5).

Microbial cell surface hydrophobicity (CSH), which contributes to hydrophobic interactions between cells and between cells and surfaces, is thought to be of importance in the adherence of *C. albicans* to inert surfaces (6). However, the role of CSH in candidal adherence to epithelial cells is still under debate with some reports proposing, and others rejecting a correlation between CSH and adherence (7–9). More recently, it was suggested that the CSH of *C. albicans* contributes to adherence by both promoting the initial docking process of adhesin on receptor and also by maintaining the fidelity of adhesin-receptor bonds (10).

Therefore, the aims of this study were to investigate the effects of the non-antibiotic antimicrobial agent, cetylpyridinium chloride, a common constituent of mouthwashes and lozenges, on the adherence of *C. albicans* to human buccal epithelial cells *in vitro* using a radiometric method developed by us (11), and also on blastospore CSH. This potentially allows an evaluation of the contribution of altered CSH on the antiadherence effects, if present. The strain of *C. albicans* employed in this study has been the subject of our previous adherence studies (3,11,12).

MATERIALS AND METHODS

Chemicals

$^{35}SO_4$ and aqueous scintillation cocktail (ACS) were obtained from Amersham (U.K.) and Percoll™ from Pharmacia (Auckland, New Zealand).

Cetylpyridinium chloride was purchased from Sigma Chemicals Ltd., St. Louis, U.S.A, Bactopeptone from Difco (Detroit, USA) and Octyl-Sepharose CL4B from Pharmacia (Uppsala, Sweden).

All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. and were of AnalaR or equivalent quality.

The appropriate concentrations of cetylpyridinium chloride were obtained by adding the appropriate weights of pure substance to sterile deionised water. All solutions were prepared daily for use on the same day.

Yeast and Culture Conditions

C. albicans (MEN strain) was maintained on Nutrient Agar (Difco) slopes at 4°C. For adherence experiments blastospores were radiolabelled with $^{35}SO_4$ as previously described (11). In brief, *C. albicans* was inoculated into (sul-

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0724-8741/95/1200-1896$07.50/0

phur minimum) GSB broth containing approximately 7.0μCi $^{35}SO_4$ and incubated at 28°C in a shaking water bath for 12 hours. The radiolabelled yeast cells were then centrifuged, resuspended in GSB medium containing ammonium sulphate (1 g L^{-1}) and incubated at 28°C in a shaking water bath for a further 4 hours (to produce exponential phase blastospores) or 12 hours (to produce stationary phase blastospores). The absence of germinated cells was confirmed using light microscopy.

Buccal Epithelial Cells (BEC)

BEC were collected daily for use on the same day as previously described by us (3,5,11) by gently scraping the buccal mucosa of healthy male and female volunteers with sterile ampoule files. The ampoule files containing adherent BEC were placed in sterile phosphate buffered saline (PBS, 0.01M, pH 7.3), thoroughly shaken and the detached BEC washed twice and resuspended in sterile PBS to approximately 4.0×10^5 cells mL^{-1}.

Pretreatment Procedures

Cell treatments were performed by incubating suspensions of blastospores or BEC with either sterile water or CPC, initially 50.0 μg mL^{-1} for 0.5–30 minutes, or a range of concentrations of CPC (0.5–50 μg mL^{-1}) or sterile water for a standard treatment time of 15 minutes. Following these, the cell suspensions were centrifuged (1000g × 15 minutes) and the supernatant removed by decantation. The remaining pellet of either *C. albicans* blastospores or BEC was washed with, and resuspended in PBS to the required cell count before inclusion in the respective assays (3,5,13).

Adherence Assay

The adherence assay employed in this study has been previously reported by us (11). In brief, equal volumes of $^{35}SO_4$ labelled blastospores (5.0×10^7 cells mL^{-1}) and BEC (2.5×10^5 cells mL^{-1}) were mixed and incubated for 30 minutes at 37°C in a shaking water bath (100 oscillations/minute). Aliquots of this cell suspension were then twice centrifuged (10000 × g, 30 min) in a Percoll™ gradient and the upper cell layer containing BEC with adherent *C. albicans* blastospores removed. The numbers of BEC and blastospores in this layer were determined using a Coulter Counter (orifice size 200 μm) and a Liquid Scintillation Counter (Beckman model 6000), respectively, and from this the uncorrected number of adherent blastospores per BEC was calculated. The extent of adherence which occurred during the centrifugation step was evaluated as described previously (11) and was deducted from the uncorrected number of adherence blastospores per BEC to produce a final value of the number of adherent blastospores per BEC. All experiments were performed in triplicate.

Determination of Cell Surface Hydrophobicity (CSH)

Blastospore CSH was determined using Hydrophobic Interaction Chromatography (HIC), as previously reported (13). In this, an aliquot of blastospores (100 μL, 6.0×10^8 cells mL^{-1}), either treated with CPC or sterile water, was added to an Octyl-Sepharose column (3 cm length) and washed through with 5 × 1.0 mL aliquots of PBS. The eluent was collected and its absorbance determined at 540 nm. All absorbances were measured 15 min. after the final addition of the buffer and calculated as a percentage of the applied cell suspension absorbance. All experiments were performed in triplicate.

Statistical Analysis of Results

Statistical evaluations of the effects of CPC treatment on the CSH and adherence of *C. albicans* blastospores to BEC *in vitro* was performed using a one way ANOVA (Stat View™, $p < 0.05$ indicating significance). Fischer PLSD test was employed to compare differences between the mean values of water-treated and cetylpyridinium chloride-treated groups ($p < 0.05$ denoting significance).

Minimum Inhibitory Concentration (MIC) of Cetylpyridinium Chloride

The MIC of CPC was determined using a macrodilution method with doubling dilutions of CPC (3). Washed stationary phase *C. albicans* blastospores (1×10^6 colony forming units mL^{-1}, 0.1 mL) was added to each tube containing broth and antimicrobial agent (9.9 mL). These were incubated for 24 hours at 28°C in a shaking water bath. The MIC was read as the first concentration where no growth occurred.

RESULTS

The effects of time of treatment of *C. albicans* blastospores (stationary and exponential growth phases) and BEC with CPC (50 μg mL^{-1}) on the subsequent adherence of these two cell populations are shown in Figures 1 and 2, respectively. At all treatment times examined there was a significant reduction in the adherence of *C. albicans* to BEC following treatment of blastospores (stationary or exponential growth phases) or BEC with CPC.

Therefore, selecting a cell treatment time of 15 minutes (representing a standard treatment time associated with an anti-adherent effect), the effect of CPC concentration on the

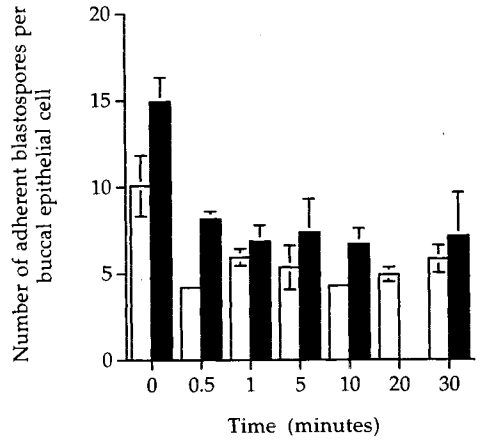

Fig. 1. The effects (±S.D.) of time of treatment with cetylpyridinium chloride (50 μg mL^{-1}) of exponential (■) or stationary (□) growth phases of *C. albicans* (MEN strain) blastospores on their subsequent adherence to buccal epithelial cells.

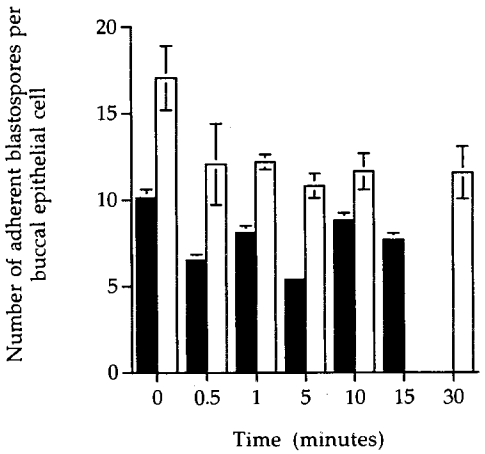

Fig. 2. The effects (±S.D.) of time of treatment of human buccal epithelial cells with cetylpyridinium chloride (50 μg mL^{-1}) on the subsequent adherence of *C. albicans* (MEN strain) blastospores, exponential (■) or stationary growth phases (□).

adherence of *C. albicans* (exponential and stationary growth phase) to BEC was evaluated (Tables 1 and 2). In general, significant reductions in adherence of candidal blastospores to epithelial cells were observed over the entire concentration range of CPC examined (0.5–50 μg mL^{-1}), following treatment of either cell type. However, on one occasion, namely the treatment of BEC with CPC (0.5 μg mL^{-1}), a significant reduction in blastospore adherence to BEC was not observed. For the most part, treatment of blastospores or BEC with increasing CPC concentrations (0.5–50 μg mL^{-1}) did not result in a significant concentration-dependent reduction of blastospore adherence to BEC. Consequently, it is proposed that the concentrations of CPC examined exhibited statistically similar effects on the adherence process.

Typical reductions in adherence (compared to the water treated control) following CPC treatments of BEC and blastospores ranged from 13.23–54.97% and 21.01–63.60%, respectively.

The MIC of CPC against *C. albicans* (MEN strain) blastospores was 5.0 μg mL^{-1} and hence significant reductions in the mean number of adherent *C. albicans*, following treatment of either blastospores or BEC, were observed at both sub and super-MIC of this non-antibiotic antimicrobial agent.

Tables 1 and 3, show the effects CPC treatment concentration and time of treatment with CPC on blastospore CSH, respectively. All treatment times and concentrations were associated with a significant reduction in CSH. Typical CSH of water and CPC treated blastospores ranged from 69.5–79.8% and 60.6–68.7%, respectively.

DISCUSSION

Cetylpyridinium chloride has commonly been used as an oral and pharyngeal antiseptic where it is commonly administered as mouthwash or lozenge preparations (3). Given the correlation between *C. albicans* adherence to epithelial cells and colonisation and infection (2), the antiadherence effects of CPC reported in this current study may also indicate a role for this agent in the prophylaxis of superficial candidosis of, e.g., the oral cavity, and further illustrates the anti-adherence properties of non-antibiotic antimicrobial agents.

Cetylpyridinium chloride has been reported to be well retained within the oral cavity and, in addition, when used two to four times daily, to exhibit a plaque-inhibiting effect (14). The mechanism for this retention is thought to involve an electrostatic interaction between the positively charged antimicrobial agent and the net negative charge on the epithelial cells in the oral cavity. In addition, CPC has previously been reported to interact strongly with other negatively charged surfaces, e.g. blastospores (12). The ability to interact well with blastospores and the epithelia of the oral cavity is an interesting property of this agent, especially as microbial/epithelial cell adherence is a surface interaction and therefore the antiadherence effect of CPC would typically involve an interaction with the surfaces of both cell types. Of potential clinical interest are the significant antiadherence effects following treatments of blastospores or BEC for short periods (*circa* 0.5–1.0 min) as such times represent the typical in-use instillation times of mouthwash preparations and also represent the times required for max-

Table 1. The Effect of Treatment of *Candida albicans* Blastospores (MEN Strain) with Cetylpyridinium Chloride (CPC, 15 min. Treatment Time) on the Resultant Cell Surface Hydrophobicity (CSH) and Subsequent Adherence to Buccal Epithelial Cells (BEC) *in Vitro*

CPC conc[n] (μg/mL)	Mean number[a] of adherent *Candida albicans*/BEC (±SE)		Mean percentage reduction in adherence[b]		Mean[a] (±S.E.) relative cell surface hydrophobicity[c]	
	Exponential	Stationary	Exponential	Stationary	Exponential	Stationary
0 (water control)	17.09 ± 0.88	26.76 ± 1.10	0	0	71.0 ± 0.73	70.5 ± 1.91
0.5	N.D.[d]	18.83 ± 1.50	N.D.[d]	29.63	64.0 ± 2.13	48.0 ± 0.83
1.0	13.50 ± 0.55	18.23 ± 0.43	21.01	31.88	65.0 ± 0.56	49.3 ± 0.68
5.0	11.41 ± 1.04	14.51 ± 1.62	33.24	45.78	65.6 ± 1.33	53.3 ± 0.34
10.0	6.22 ± 0.91	12.36 ± 0.45	63.60	53.81	68.0 ± 1.49	51.7 ± 1.28
50.0	10.70 ± 0.48	N.D.[d]	37.39	N.D.[d]	64.7 ± 2.75	55.3 ± 0.87

[a] Mean ± S.E. calculated from three replicates.
[b] Calculated using [(control) − (sample)/control] × 100.
[c] Calculated using [(extinction of original inoculum) − (extinction of eluent)/(extinction of original inoculum)] × 100.
[d] N.D. = not determined.

Table 2. The Effect of Treatment of Human Buccal Epithelial Cells with Cetylpyridinium Chloride (CPC, 15 min. Treatment Time) on the Subsequent Adherence of *Candida albicans* Blastospores (Clinical Isolate) *in Vitro*

CPC Conc[n] (μg/mL)	Number[a] of adherent *C. albicans*/BEC (±SE)		Mean percentage reduction in adherence[b]	
	Exponential	Stationary	Exponential	Stationary
0 (water control)	28.78 ± 1.51	21.16 ± 1.93	0	0
0.5	12.96 ± 0.63	18.36 ± 0.60[c]	54.97	13.23[c]
1.0	17.50 ± 2.92	N.D.[d]	39.19	N.D.[d]
5.0	16.58 ± 0.08	12.94 ± 0.43	42.39	38.85
10.0	N.D.[d]	11.30 ± 1.07	N.D.[d]	46.60
25.0	18.73 ± 2.78	16.20 ± 0.53	34.92	23.44
50.0	14.80 ± 0.99	14.59 ± 1.88	48.58	31.05

[a] Mean ± S.E. calculated from three replicates.
[b] Calculated using [(control) − (sample)/control] × 100.
[c] Difference not significant ($p > 0.05$).
[d] N.D. = not determined.

imal uptake of CPC onto the surface of *C. albicans* blastospores (MEN strain) (12).

In this study, the antiadherence effects of CPC were observed at sub-MIC. Clinically, this may infer that several rinses with saliva may be required to reduce the concentration of CPC on the oral mucosa to a level not associated with an anti-adherence effect and hence the duration of anti-adherent effect may markedly exceed that of the direct effects on candidal viability. It has been reported that, several hours after rinsing, CPC was present in saliva in sub-lethal concentrations (14), and therefore, as saliva bathes the epithelial cells and the potentially pathogenic *C. albicans* blastospores, this may also influence the anti-adherence properties of CPC *in vivo*.

It is difficult to predict accurately the implications of these *in vitro* anti-adherence observations to the *in vivo* situation. Tobgi *et al.* (15) administered mouthwashes containing chlorhexidine to volunteers for differing time periods, subsequently removed the treated BEC and monitored the adherence of *C. albicans* to the exfoliated BEC. These authors reported that such treatments with chlorhexidine were sufficient to significantly reduce the adherence of *C. albicans* to BEC. Given that CPC interacts both strongly and swiftly with oral surfaces and, in light of the observed *in vitro* anti-adherence properties, it may be suggested that this agent will act similarly to chlorhexidine with respect to anti-adherence properties under similar experimental conditions.

Table 3. The Effect of Time of Treatment of Blastospores of *Candida albicans* (MEN Strain, Exponential and Stationary Growth Phases) with Cetylpyridinium Chloride (CPC, 50 μg mL^{-1}) on Their Surface Hydrophobicity as Determined by Hydrophobic Interaction Chromatography (HIC)

Time of treatment with CPC (minutes)	Mean % of initial eluent (±S.E.)[a] retained on HIC column (% relative cell surface hydrophobicity)	
	Exponential phase	Stationary phase
Water treatment (control)	79.8 ± 2.37	69.5 ± 1.11
0.5	66.0 ± 2.33	61.7 ± 1.61
1.0	68.7 ± 2.19	62.4 ± 2.32
5.0	65.9 ± 1.74	62.4 ± 1.16
10.0	65.6 ± 0.19	63.7 ± 2.46
15.0	64.4 ± 1.27	62.9 ± 0.85
20.0	64.2 ± 1.08	64.0 ± 2.31
30.0	60.6 ± 2.31	63.5 ± 0.92

[a] Mean ± S.E. calculated from three replicates.

Whilst it has been shown in this study that CPC treatment of blastospores of *C. albicans* significantly reduces both CSH and adherence to BEC, there was no direct correlation between these two parameters. Therefore, this would infer that reduced blastospore adherence, as a result of pretreatment with CPC, may not be fully explained in terms of the concurrent reduction in CSH. Statistically maximum reductions in blastospore surface hydrophobicity were observed following short treatment times (*circa* 30s), once again highlighting the need for maximum adsorption of CPC onto the blastospore surface (12) to facilitate maximum reduction in surface hydrophobicity. The limited contribution of reduced CSH to reduced adherence following treatment with CPC would infer that other factors may contribute to the antiadherence effects, e.g., direct, or stearic blockade of adhesins and receptors and morphological alteration of the topological arrangement of the fibrillar layer on candidal blastospores by CPC.

In conclusion, this study has shown that CPC significantly reduced the adherence of *C. albicans* to human BEC *in vitro*. Such effects were exhibited following short cell treatment times (≤30 s) and also over a large (100-fold) range of concentrations, both super and sub-MIC. Such treatment times and treatment concentrations also reduced *C. albicans* CSH however, this is suggested as being only a contributory factor in the anti-adherence properties of CPC. As adherence to the host epithelia is accepted to be the initial step in the infection process, these antiadherence effects may be of clinical use in the prophylaxis of candidosis of e.g. the oral cavity or the vaginal tract.

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