The Effects of Hexetidine (Oraldene™) on the Adherence of *Candida Albicans* to Human Buccal Epithelial Cells *In Vitro* and *Ex Vivo* and on *In Vitro* Morphogenesis

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Purpose. This study reports the effects of hexetidine (Oraldene™) on two virulence attributes of *Candida albicans*, namely, *in vitro* and *ex vivo* adherence of yeast cells to buccal epithelial cells (BEC) and *in vitro* morphogenesis.
Methods. The effects of hexetidine treatment of either yeast cells (stationary and exponential phases) or BEC on *Candidal* adherence, in terms of viable and non-viable adherent yeast cells, were evaluated using an acridine orange stain in conjunction with fluorescence microscopy. *Ex vivo* anti-adherence effects were determined by rinsing BEC *in vivo* with hexetidine (0.1%), removal of BEC after defined periods and inclusion in the adherence assay. The effects of hexetidine on morphogenesis were evaluated using light microscopy. Yeast cell viability following exposure to a range of concentration of hexetidine (0.005-0.1% v/v) for defined periods was determined following serial dilution and enumeration on solid media.
Results. Treatment of stationary and exponential phase yeast cells or BEC with hexetidine (0.1%) for a range of times (10–300 s) or, alternatively, with a range of concentrations of hexetidine (0.005–0.1%) for a fixed time (30s) significantly decreased the resultant *Candidal/* epithelial adhesion. No correlations were observed between reduced adherence and either time of treatment or hexetidine concentration. *In vivo* treatment of BEC with hexetidine (0.1%) for 30s resulted in prolonged and significant reductions in the *ex vivo* adherence of both viable and non-viable yeast cells for periods of up to (and including) four hours post-rinsing. Treatment of C. albicans blastospores with hexetidine (0.05, 0.1% v/v) for 10s and 30s totally inhibited *Candida* morphogenesis, whereas treatment with lower antiseptic concentrations significantly reduced the extent of Candida morphogenesis and the rate of hyphal development. The effects of hexetidine on yeast cell viability were both concentration and time-dependent.
Conclusions. The reduced adherence of *C. albicans* to BEC and the modification or inhibition of morphogenesis following exposure to hexetidine suggests a clinical role for hexetidine in the prophylaxis of both superficial candidosis and the systemic complications resulting from invasion of sub-epithelial tissue.

KEY WORDS: *candida albicans;* hexetidine; reduced adherence; *in vitro; ex vivo;* modified morphogenesis.

INTRODUCTION

Candida albicans is a normal resident of the oral cavity, gastrointestinal and female genital tracts of humans, that may under certain conditions e.g. trauma, diabetes mellitus, antibacterial chemotherapy and cancer chemotherapy, pathogenically invade a number of different sites including the oral cavity (1). The recent advent of the human immunodeficiency virus (HIV) and the acquired immunodeficiency syndrome (AIDS) has resulted in a marked increase in microbial opportunistic infections, one common example of which is oro-pharyngeal candidosis (2,3). Resistance of *C. albicans* to systemic and topical anti-fungal agents has been recognised in AIDS patients and consequently, in this patient group, complex and demanding treatment regimens are often required (2,3).

It is acknowledged that the initiation of candidosis involves adherence of the organism to host epithelial cells (4,5). This interaction ensures that the flushing actions of bodily secretions such as saliva are overcome, hence allowing retention of the organism on host epithelia (4). Microbial adherence to epithelial cells may be differentiated into two distinct stages. Initially, as the microorganism approaches the epithelial cell it is subjected to both attractive and repulsive forces. If the attractive forces exceed the repulsive forces, a reversible, non-specific adherence interaction occurs between microorganism and epithelium. From this location, adhesion molecules on the surface of the microorganism may then interact with receptors on the surface of the epithelia in a specific, irreversible, interaction (4,5).

Given the correlation between virulence potential of *Candida spp.* and adherence to epithelia (4) an interest has developed in the identification of agents which may interfere with this process. The ability of several non-antibiotic, antimicrobial agents to reduce the adherence of *C. albicans* to epithelial cells in vitro has been reported. Examples of these agents, all common constituents of mouthwash and lozenge preparations, include chlorhexidine gluconate, polyvinylpyrrolidone-iodine (1,6) cetylpyridinium chloride (1,5) and dequalinium chloride (1). Consequently, it has been suggested that these agents may be useful for the prophylaxis of superficial candidosis of the oropharynx (1).

In addition, *C. albicans* exhibits dimorphism and consequently may undergo morphogenesis (germination) from the yeast (blastospore) form to the hyphal forms (7). Whilst there is speculation as to the exact contributions of each form to the process of infection, the hyphal form *in vivo* is thought to be important in the initial process of tissue invasion, where it has been reported to be involved in the initial stages of epithelial penetration (8,9). Morphogenesis from the yeast form to the hyphal form is thus considered to be an expression of virulence (7) and, consequently, agents which can inhibit or reduce candidal morphogenesis may be of clinical benefit in preventing the systemic manifestations of candidosis.

Hexetidine (Oraldene™) is a cationic antimicrobial agent which has been reported to possess anti-plaque activity (10,11,12) and antifungal activity *in vivo* (12). However, there have been no reports of the use of this agent for the prophylaxis of infection. Therefore, the aims of this study were to investigate the effects of hexetidine on the *in vitro* and *ex vivo* adherence of *C. albicans* to human buccal epithelial cells, the requisitory step for colonisation and infection, using a recently reported assay to discern between viable (pathogenic) and non-viable (nonpathogenic) adherent yeast cells (13). Additionally, the

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effects of hexetidine on the *in vitro* morphogenesis of *C. albicans,* a primary virulence determinant, were examined.

MATERIALS AND METHODS

Chemicals

Oraldene™ (hexetidine 0.1%) was a gift from Warner Lambert Consumer Healthcare, (Dartford, U.K.). Appropriate concentrations of hexetidine were obtained by dilution of this stock solution using sterile water.

Acridine orange was purchased from Sigma Chemicals Ltd. (Poole, Dorset, UK).

All other chemicals were purchased from BDH Chemicals Ltd (Poole, Dorset, UK) and were of AnalaR, or equivalent, quality.

Candida albicans Strain and Growth Conditions

Candida albicans strain NCYC 1467 (from denture stomatitis) was employed in this study and was maintained on nutrient agar (Oxoid) slopes at 4°C. When required, two loopfuls were transferred into pre-warmed nutrient broth (Oxoid) and incubated at 25°C for 18 h to generate stationary phase yeast cells. Cells were removed by centrifugation (3000 × g for 15 minutes), the deposit washed twice in sterile phosphate buffered saline (PBS, pH 7.3, 0.01M) and resuspended to approximately 2×10^7 colony forming units (cfu) mL^{-1} in PBS. The absence of hyphal forms was confirmed by light microscopy (5,6).

Collection of Buccal Epithelial Cells

Buccal epithelial cells were collected from healthy male and female volunteers by scraping the buccal mucosa with sterile ampoule files as previously described (1,6,14). The ampoule files were then placed directly into sterile PBS and the adherent epithelia removed by vortexing. The cellular suspension was then passed through a 22 gauge needle to ensure cellular homogeneity and the cells were then washed twice and resuspended in sterile PBS to a defined cell density (2×10^5 cells mL^{-1}).

Cellular Pre-treatment Procedures Pertaining to Evaluation of *In Vitro* Antiadherence Effects

Standardised suspensions of either blastospores of *C. albicans* (2×10^7 cfu mL^{-1}) or buccal epithelial cells (2×10^5 cells mL^{-1}) were incubated with the required concentration of hexetidine or sterile water (as a control) for a defined period at 37°C in an orbital incubator (150 oscillations min^{-1}) (1,5). Following this period, hexetidine was removed by centrifugation (20,000 × g for 10 s) and decantation of the supernatant. The cellular deposit was then washed with, and resuspended in sterile PBS to the required cell density prior to inclusion in the adherence assay.

Determination of the Effects of Hexetidine on the *Ex Vivo* Adherence of C. albicans

The method used to determine the effect of hexetidine treatment on the *ex vivo* adherence of *C. albicans* to BEC was a modification of the method described by Tobgi *et al.* (15). In this, untreated (control) BEC were initially removed from the right cheek of twelve healthy, non-smoking male and female volunteers using ampoule files as described previously and standardised for inclusion in the adherence assay. Each volunteer then thoroughly rinsed their oral cavity with 15 mL of Oraldene™ (hexetidine 0.1%) for 30 s, in accordance with the manufacturer's directions. BEC (treated) were then collected from the left cheek of each volunteer at selected time intervals post-rinsing (30 min, 60 min, 120 min, 240 min and 24 h) with Oraldene™, standardised with respect to cell density for inclusion in the adherence assay, as described below. Only one sample of BEC was removed from the left and right mucosae from each patient in any particular day and there were no restrictions on food or fluid intake by patients between rinsing with Oraldene™ and collection of BEC.

Adherence Assay

The adherence assay employed in this study has been reported by us previously (12,13). In brief, yeast cells of *C. albicans* were initially suspended in a solution of acridine orange (0.025% w/v in citrate buffer at pH 6.8) for 5 min. Following this the cells were removed by centrifugation (3000 × g for 15 min.) and washed once with PBS. When required, the acridine orange-stained yeast cells were treated with hexetidine (0.005, 0.01, 0.05, 0.01%) or sterile water as described above. Equal volumes of suspensions containing either untreated or hexetidine treated, acridine orange-stained yeast cells of *C. albicans* (2×10^7 cfu mL^{-1}) and BEC were mixed (2×10^5 cells mL^{-1}) and incubated in a shaking water bath (110 oscillations min^{-1}) at 37°C for 2h. Following this, three loopfuls of the resulting mixed suspension were transferred onto a microscope slide, diluted with an equal volume of sterile PBS and allowed to air dry. Enumeration of candidal adherence (viable and non-viable yeast cells) to BEC was performed using fluorescence microscopy in which the number of adherent viable (orange fluorescing) and non-viable (green fluorescing) yeast cells to at least 150 epithelial cells was measured. The use of the acridine orange stain did not affect adherence of yeast cells to BEC (13). In addition, evaluation of the *in vitro* and *ex vivo* effects of hexetidine on candidal adherence to BEC was performed using a standard crystal violet staining technique as reported by us on several occasions (e.g. 1,6,14).

Hexetidine Effects on *In Vitro* Morphogenesis of C. albicans

The effects of hexetidine treatment on the morphogenesis of *C. albicans* were evaluated according to Jones (16). In brief, stationary phase yeast cells of *C. albicans* were treated with either hexetidine (0.005, 0.01, 0.05, 0.1%) or sterile PBS for 10 s or 30 s, as described above. The yeast cell suspensions were retained on a sterile 0.45 m filter by filtration, washed with PBS (15 mL) and the filter transferred into an Ehrlemeyer flask containing prewarmed nutrient broth at 37°C. The flask was vortexed to remove adherent yeast cells and then incubated at 37°C in an orbital incubator (150 oscillations min^{-1}). At hourly intervals, samples were removed, stained using crystal violet and the percentage of at least three hundred yeast cells that had undergone morphogenesis to hyphal forms and the hyphal lengths of at least 100 cells measured using light microscopy.

Hexetidine Effects on Yeast Cell Viability

To determine the effects of exposure time of yeast cells of *C. albicans* to hexetidine on viability, yeast cells (*circa* 1×10^7 cfu mL^{-1}) were suspended in known concentrations of hexetidine (0.005, 0.01, 0.05, 0.1%) at 37°C in a shaking water bath (150 osc min^{-1}). At predetermined periods, an aliquot was removed and serially diluted in Ringers solution. Dilution alone was sufficient to overcome the "carry-over" effect of hexetidine. The number of colony forming units was determined using the drop-plate method of Miles and Misra (17) onto nutrient agar plates which were incubated for a minimum of 48 h at 37°C.

Statistical Analysis of Results

Statistical analysis of the effects of hexetidine treatment (time of treatment and concentration effects) on the in vitro adherence of *C. albicans* yeast cells to BEC and yeast viability were performed using a one-way Analysis of Variance (ANOVA) (5). The effects of rinsing with Oraldene™ *in vivo* on the subsequent *ex vivo* anti-adherence properties at defined periods post-rinsing were analysed using a paired t-test. Hexetidine effects on the percentage morphogenesis of yeast cells and subsequent mean hyphal lengths at each sampling period were performed using Chi-squared analysis (χ^2) and Mann-Whitney U test, respectively (16). In all cases $P < 0.05$ denoted significance.

RESULTS

The effects of time of treatment of either *C. albicans* (stationary and exponential phases) or BEC on adherence are presented in Figure 1 wherein adherence is presented as total candidal adherence, i.e. viable and non-viable cells. Treatment of either cell population with hexetidine (0.1%) for 10, 30, 60 and 300 s significantly reduced adherence in comparison to their sterile-water treated controls. Exceptionally, the adherence of *C. albicans* to BEC that had been pretreated for 10 s with hexetidine was not significantly lower than adherence of untreated *C. albicans* to untreated BEC. No correlation was observed between hexetidine treatment time of either yeast cells or BEC and reduced adherence.

Figure 2 shows the effects of treatment of either *C. albicans* or BEC with a range of concentrations of hexetidine (0.005, 0.01, 0.05, 0.1%) for 30 s on the subsequent (total) adherence of *C. albicans* to BEC. In comparison to sterile water-treated treatments, significant reductions in adherence were observed following treatment with either cell type with hexetidine (0.005–0.1%). There was no correlation between increased treatment concentrations of hexetidine and reduced yeast/epithelial cell adherence. However, for all treatments, there was a greater reduction in adherence associated with the largest concentrations of hexetidine (0.1%) compared to the lowest concentration of hexetidine (0.005%) employed in this study.

Tables 1 or 2 show the effects of rinsing with Oraldene™ *in vivo* on the duration of the anti-adherence effect as determined using an *ex-vivo* adherence assay. For all subjects, a significant, prolonged anti-adherence effect was observed following *in vivo* treatment of BEC for all sampling periods up to and including four hours post-rinsing. In addition, at each of these post-rinsing sampling periods (0.5, 1, 2, 4 h), the adherences of both viable and nonviable yeast cells to treated BEC were significantly reduced in comparison to control samples. The adherence of both viable and non-viable yeast cells to BEC that had been removed from subjects twenty four hours post-rinsing were statistically similar to that of untreated (control) BEC and are therefore not included in Tables 1 or 2.

Tables 3 and 4 show the effects of treatment of *C. albicans* with a range of concentrations of Oraldene™ for 10 and 30 s,

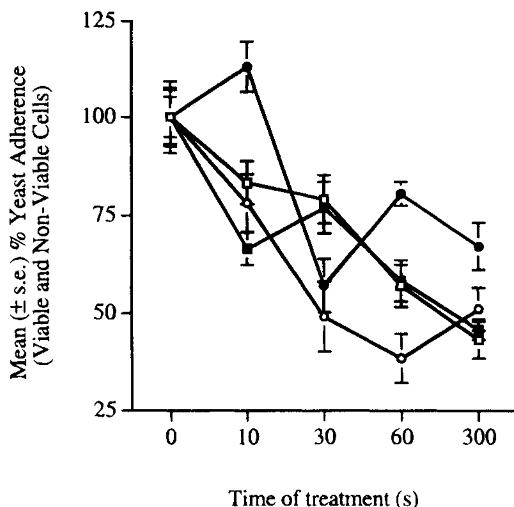

Fig. 1. The effects of time of treatment of either yeast cells of *Candida albicans* (stationary or exponential phases) or buccal epithelial cells (BEC) on their subsequent adherence *in vitro*. Key: Adherence of hexetidine-treated stationary phase yeast cells to BEC (open squares), Adherence of hexetidine-treated exponential phase yeast cells to BEC (closed squares), Adherence of stationary phase yeast cells to hexetidine-treated BEC (open circles), Adherence of exponential phase yeast cells to hexetidine-treated BEC (closed circles).

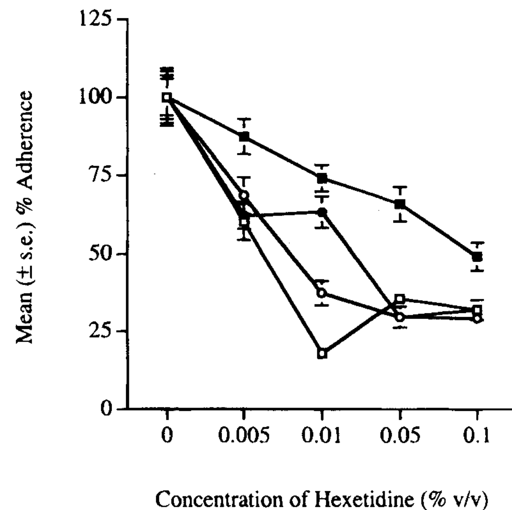

Fig. 2. The effects of concentration of treatment of hexetidine on the adherence of yeast cells of *Candida albicans* (stationary and exponential phases) to buccal epithelial cells *in vitro*. Key: Adherence of hexetidine-treated stationary phase yeast cells to BEC (open squares), Adherence of hexetidine-treated exponential phase yeast cells to BEC (closed squares), Adherence of stationary phase yeast cells to hexetidine-treated BEC (open circles), Adherence of exponential phase yeast cells to hexetidine-treated BEC (closed circles).

Table 1. The Effects of Rinsing with Oraldene™ *In Vivo* on the *Ex Vivo* Adherence of Yeast Cells of *Candida Albicans* to Human Buccal Epithelial Cells, Removed at 30 and 60 Minutes Post-Rinsing

Volumteer Number	Mean (± s.e.) % Adherence of *C. albicans* yeast cells (non-viable and viable) to untreated BEC and to Oraldene™-treated BEC							
	Control BEC		BEC removed after 30 min		Control BEC		BEC removed after 60 min	
	Non-Viable	Viable	Non-Viable	Viable	Non-Viable	Viable	Non-Viable	Viable
1	100.00 ± 8.50	100.00 ± 8.11	65.65 ± 5.78	52.66 ± 6.28	100.00 ± 8.01	100.0 ± 11.65	65.77 ± 5.87	21.98 ± 2.09
2	100.00 ± 7.95	100.00 ± 8.36	45.24 ± 3.75	45.25 ± 3.75	100.0 ± 11.85	100.0 ± 10.32	59.24 ± 11.85	7.14 ± 1.90
3	100.00 ± 6.95	100.00 ± 8.63	61.93 ± 5.14	61.93 ± 5.14	100.0 ± 10.53	100.00 ± 9.00	34.21 ± 5.92	16.33 ± 3.15
4	100.00 ± 6.95	100.00 ± 7.23	41.47 ± 3.56	41.47 ± 3.56	100.00 ± 9.41	100.0 ± 10.03	62.78 ± 4.76	24.45 ± 2.42
5	100.00 ± 6.65	100.00 ± 9.32	38.83 ± 4.17	38.83 ± 4.17	100.00 ± 7.41	100.0 ± 11.09	60.65 ± 12.50	10.33 ± 1.98
6	100.00 ± 6.40	100.00 ± 7.85	54.27 ± 4.57	54.27 ± 4.57	100.0 ± 10.82	100.00 ± 9.44	33.44 ± 5.90	26.64 ± 2.96
7	100.00 ± 7.71	100.0 ± 10.52	38.56 ± 3.46	38.56 ± 3.46	100.0 ± 10.75	100.0 ± 10.16	56.24 ± 7.49	31.42 ± 1.71
8	100.00 ± 7.07	100.00 ± 9.24	26.71 ± 2.51	26.71 ± 2.51	100.00 ± 9.88	100.00 ± 9.68	21.20 ± 4.03	19.00 ± 1.19
9	100.0 ± 11.52	100.00 ± 9.73	36.39 ± 3.40	36.39 ± 3.40	100.00 ± 9.42	100.0 ± 10.06	23.19 ± 5.31	23.87 ± 5.16
10	100.00 ± 8.51	100.00 ± 9.24	28.92 ± 2.43	28.92 ± 2.43	100.00 ± 9.04	100.0 ± 9.20	41.26 ± 5.38	27.81 ± 3.55
11	100.00 ± 7.36	100.0 ± 10.17	23.28 ± 2.15	23.28 ± 2.15	100.00 ± 7.53	100.0 ± 10.21	48.39 ± 7.96	24.39 ± 4.88
12	100.0 ± 10.86	100.00 ± 9.53	33.98 ± 2.79	33.89 ± 2.79	100.00 ± 9.44	100.0 ± 10.86	26.88 ± 4.64	43.83 ± 5.11

Table 2. The Effects of Rinsing with Oraldene™ *In Vivo* on the *Ex Vivo* Adherence of Yeast Cells of *Candida Albicans* to Human Buccal Epithelial Cells, Removed at 2 and 4 Hours Post-Rinsing

Volunteer No.	Mean (± s.e.) % Adherence of *C. albicans* yeast cells (non-viable and viable) to untreated BEC and Oraldene™-treated BEC							
	Control BEC		BEC removed after 2 hours		Control BEC		BEC removed after 4 hours	
	Non-Viable	Viable	Non-Viable	Viable	Non-Viable	Viable	Non-Viable	Viable
1	100.00 ± 9.39	100.0 ± 12.65	20.58 ± 2.17	20.63 ± 1.04	100.00 ± 8.41	100.0 ± 10.66	14.49 ± 3.27	7.94 ± 1.81
2	100.0 ± 11.84	100.0 ± 11.47	12.15 ± 2.08	5.59 ± 2.06	100.0 ± 9.35	100.0 ± 10.55	21.50 ± 4.21	7.14 ± 1.90
3	100.00 ± 6.70	100.0 ± 12.12	15.46 ± 2.06	9.34 ± 0.76	Not determined	Not determined	Not determined	Not determined
4	100.00 ± 9.12	100.0 ± 10.79	21.53 ± 3.65	15.18 ± 1.35	100.00 ± 7.23	100.0 ± 10.38	12.30 ± 0.90	13.77 ± 2.26
5	100.0 ± 12.07	100.0 ± 11.05	12.07 ± 1.38	12.50 ± 0.85	100.0 ± 13.17	100.0 ± 10.69	14.63 ± 4.34	11.95 ± 1.47
6	100.00 ± 6.47	100.00 ± 7.77	32.34 ± 3.98	16.29 ± 2.26	100.00 ± 9.52	100.0 ± 10.22	34.13 ± 7.14	26.52 ± 1.94
7	100.00 ± 8.58	100.0 ± 11.60	27.26 ± 2.78	32.71 ± 5.57	100.0 ± 13.23	100.00 ± 9.55	9.69 ± 1.42	25.63 ± 3.02
8	100.00 ± 8.28	100.0 ± 11.16	19.34 ± 1.87	22.53 ± 3.79	100.00 ± 9.19	100.00 ± 9.68	13.87 ± 0.53	62.37 ± 8.60
9	100.00 ± 8.92	100.00 ± 9.20	18.46 ± 1.69	42.94 ± 6.95	100.0 ± 10.09	100.0 ± 10.73	12.62 ± 1.56	28.71 ± 4.62
10	100.0 ± 12.98	100.00 ± 9.60	23.92 ± 4.10	23.65 ± 3.75	100.00 ± 8.63	100.0 ± 9.42	19.94 ± 1.79	17.17 ± 2.77
11	100.00 ± 9.83	100.00 ± 9.15	27.16 ± 3.66	17.25 ± 2.75	100.00 ± 7.67	100.0 ± 10.37	18.67 ± 1.67	14.10 ± 2.13
12	100.00 ± 9.32	100.00 ± 9.59	28.44 ± 3.26	9.81 ± 1.54	100.00 ± 9.56	100.0 ± 9.56	20.27 ± 1.82	21.50 ± 2.77

Table 3. The Effects of Treatment of Yeast Cells of *Candida Albicans* for 10 s with Hexetidine (Oraldene™) on the Subsequent Percentage Morphogenesis (Germination) and Hyphal Lengths of Germinated Yeast Cells

Time post-treatment	Mean (± s.e.) % germinated yeast cells following treatment with					Mean (±s.e.) hyphal lenghts (μm) of yeast cells following treatment with				
	Sterile water	Hexetidine 0.005%	Hexetidine 0.01%	Hexetidine 0.05%	Hexetidine 0.1%	Sterile water	Hexetidine 0.005%	Hexetidine 0.01%	Hexetidine 0.05%	Hexetidine 0.1%
1 h	6.3 ± 0.5	N.M.	N.M.	N.M.	N.M.	4.0 ± 0.1	N.M.	N.M.	N.M.	N.M.
2 h	9.7 ± 1.1	N.M.	N.M.	N.M.	N.M.	16.6 ± 0.3	N.M.	N.M.	N.M.	N.M.
3 h	13.0 ± 0.9	14.0 ± 0.7	3.3 ± 0.5	N.M.	N.M.	35.1 ± 0.5	9.9 ± 0.3	5.2 ± 0.1	N.M.	N.M.
4 h	20.7 ± 1.4	22.3 ± 1.2	9.0 ± 1.0	N.M.	N.M.	47.2 ± 1.5	35.0 ± 0.7	21.0 ± 0.6	N.M.	N.M.
5 h	22.7 ± 1.1	24.7 ± 2.0	12.3 ± 1.3	N.M.	N.M.	54.0 ± 2.0	45.4 ± 1.2	38.1 ± 0.9	N.M.	N.M.

N.M.: No morphogenesis observed in at least 300 yeast cells.

Table 4. The Effects of Treatment of Yeast Cells of *Candida Albicans* for 30 s with Hexetidine (Oraldene™) on the Subsequent Percentage Morphogenesis (Germination) and Hyphal Lengths of Germinated Yeast Cells

	Mean (± s.e.) % germinated yeast cells following treatment with					Mean (±s.e.) hyphal lengths (μm) of yeast cells following treatment with				
Time post-treatment	Sterile water	Hexetidine 0.005%	Hexetidine 0.01%	Hexetidine 0.05%	Hexetidine 0.1%	Sterile water	Hexetidine 0.005%	Hexetidine 0.01%	Hexetidine 0.05%	Hexetidine 0.1%
1 h	4.7 ± 0.7	N.M.	N.M.	N.M.	N.M.	6.1 ± 0.2	N.M.	N.M.	N.M.	N.M.
2 h	15.7 ± 1.2	N.M.	N.M.	N.M.	N.M.	12.0 ± 0.2	N.M.	N.M.	N.M.	N.M.
3 h	19.3 ± 1.1	13.0 ± 1.1	7.3 ± 0.6	N.M.	N.M.	21.5 ± 0.5	11.3 ± 0.7	5.3 ± 0.1	N.M.	N.M.
4 h	22.3 ± 1.6	20.3 ± 1.6	14.0 ± 0.4	N.M.	N.M.	42.8 ± 0.2	28.3 ± 1.1	10.9 ± 0.4	N.M.	N.M.
5 h	24.7 ± 1.7	22.3 ± 1.9	15.7 ± 1.8	N.M.	N.M.	50.1 ± 2.2	32.2 ± 1.2	23.2 ± 0.5	N.M.	N.M.

N.M.: No morphogenesis observed in at least 300 yeast cells.

respectively, on the percentage of germinated yeast cells and their mean hyphal length. Following incubation in nutrient media, untreated (control) yeast cells exhibited progressive morphogenesis, reaching a plateau at approximately 20–25%. Conversely, no morphogenesis was observed for up to 5 hours following treatment of yeast cells with hexetidine (0.05, 0.1%) for at least 10 s. Furthermore, significant reductions in both the percentage morphogenesis and mean hyphal lengths of germinated yeast cells were observed at each sampling period following treatment of yeast cells with lower concentrations of hexetidine (0.005 and 0.01%). However, the percentage germination (but not mean hyphal lengths) of yeast cells that had been treated with hexetidine (0.005%) for 10 and 30 s were statistically similar to those of untreated yeast cells at four and five hours, indicating cell recovery. The effects of hexetidine treatment of yeast cells on both their percentage morphogenesis and subsequent mean hyphal lengths was concentration-dependent.

The effects of time of exposure of yeast cells to a range of concentrations of hexetidine (0.005–0.1%) on their viabilities are presented in Figure 3. Maximal fungicidal effects were associated with the largest concentration of hexetidine examined (0.1%) and decreased with lower concentrations.

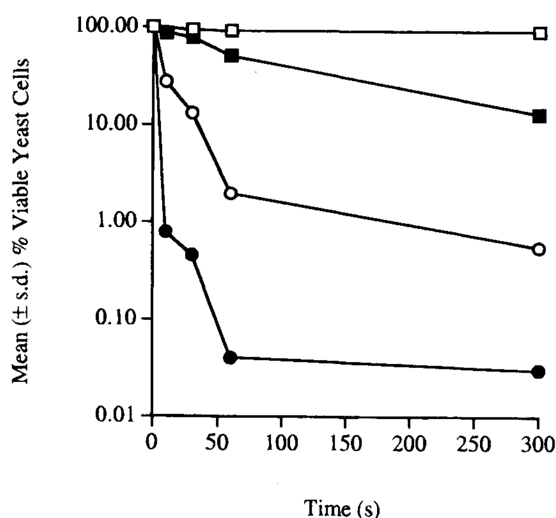

Fig. 3. The effects of time of treatment of yeast cells of *Candida albicans* with hexetidine (0.005 - 0.1% v/v) at 37°C on their resultant viability. Key: 0.005% v/v (open squares), 0.01% v/v (closed squares), 0.05% v/v (open circles), 0.1% v/v (closed circles).

DISCUSSION

Hexetidine (Oraldene®) is a non-antibiotic antimicrobial agent that has been reported to possess a broad spectrum of antimicrobial activity (10) and is consequently employed for the treatment of superficial infections of the oral cavity and for inhibition of plaque formation (11,12). However, little attention has been paid to the potential ability of hexetidine to significantly reduce or prevent the adherence of *C. albicans* to BEC, the requisitory step in the pathogenesis of superficial candidosis, and to its effects on candidal morphogenesis. Consequently, these deficiencies were adressed in this current study. In particular, as only viable adherent microorganims are capable of initiating infection, it is important to ensure that the anti-adherence effects of any antimicrobial agent include an effect on viable microorganisms. This has been overlooked in numerous studies due to the unavailability of an appropriate assay to measure adherent viable and non-viable microorganisms. Recently we described the use of the fluorescent dye, acridine orange, to discern between viable adherent and non-viable adherent yeast cells of *C. albicans*, and, to additionally examine the effects of non-antibiotic, antimicrobial agents on the adherence of these two yeast cell populations to BEC (13). Therefore, this assay was employed in the current study to fully quantify the effects of hexetidine on candidal adherence to BEC. Importantly, enumerations of the effects of hexetidine on the adherence (viable and non-viable) of *C. albicans* to BEC using the acridine orange staining method were statistically similar to those obtained using the conventional crystal violet stain (results not shown). This further confirmed that the effects of hexetidine on the adherence of *C. albicans* to BEC were not modified by the use of the acridine orange stain. Similar observations have been reported for chlorhexidine (13).

In this study, hexetidine treatment of either BEC or yeast cells with a wide range of concentrations of hexetidine for a range of times resulted in significant reductions in the yeast cell/epithelial cell adherence. Of particular importance were the anti-adherence properties of hexetidine associated with short treatment periods (*circa* 30 s) as these represent typical in use instillation periods. Hexetidine is a cationic antimicrobial agent which has been reported to possess a similar spectrum of antimicrobial activity as other cationic antimicrobial agents, e.g. chlor-

hexidine, cetylpyridiunium chloride (10). Interestingly, hexetidine mediated reductions in candidal adherence to BEC were broadly similar to those observed for other cationic antimicrobial agents in our previous studies, e.g. chlorhexidine, cetylpyridinium chloride, cetrimide and dequalinium chloride (1,5,6). However, it is important to remember that direct comparisons of the anti-adherence effects of these cationic agents cannot be accurately performed as the respective studies employed different isolates of *C. albicans*, different growth media and different BEC donors, factors which have been reported to affect microbial adherence (14). Previously, we have reported that the mechanism of anti-adherence effects of cationic, antimicrobial agents, e.g. cetylpyridinium chloride, may be attributed to a number of effects on the microbial cell, e.g. altered (decreased) cell surface hydrophobicity, altered microbial cell zeta potential, direct and/or indirect (stearic) blockade of microbial adhesins (5,18). In addition, it was also shown that the anti-adherence effects were not dependent on monolayer coverage of the microbial cell surface with antimicrobial agent (19). Given the cationic nature of hexetidine, it may be anticipated that the anti-adherence effects of this agent may be accredited to effects on the cell surface properties of *Candida albicans* and BECs, and also, due to the direct and indirect blockade of adhesins and/or receptors. In addition, due to the low aqueous solubility, hexetidine has been reported to partition into lipid domains, e.g. cell membranes (20). Consequently, the observed anti-adherence effects of hexetidine may also involve a direct alteration of the morphology of the outer cell walls of both yeast and epithelial cells, thereby potentially modifying the surface adhesive structures of *C. albicans* and the receptor domains on the surface of BECs. Interestingly, taurolidine, a poorly aqueous soluble non-antibiotic, antimicrobial agent that has been reported to reduce the adhesion of *C. albicans* to BEC (21), has also been shown to alter the surface adhesive structures of microorganisms (22).

Whilst *in vitro* studies of the anti-adherence properties of antimicrobial agents provide general information concerning the effects of concentration and treatment times on the microbial/epithelial cell adherence interaction, it is difficult to predict the potential *in vivo* consequences of such observations (1). Anti-adherence effects *in vivo* will be dependent on several factors including, the binding affinities of antimicrobial agents for both epithelial and yeast cells, and inactivation of the antimicrobial agent by salivary components, e.g. proteins. Importantly, following the period of instillation of antimicrobial agent within the oral cavity, there will be elimination of the antimicrobial agent, e.g. hexetidine, from the surface of both microbial and epithelial cells. However, *in vitro* studies do not consider the effects of this dynamic situation on the anti-adherence properties of anti-microbial agents present within mouthwash formulations. *Ex vivo* adherence studies attempt to reduce the extent of this problem by treating the BEC *in vivo* and allowing drug elimination to occur from the BEC for defined periods prior to their use in an adherence assay. Therefore, due to its greater relevance to the clinical situation, an *ex vivo* assay was employed to evaluate the potential consequences of the clinical use of hexetidine mouthwash (Oraldene™). Using this *ex vivo* assay, this study demonstrated that hexetidine, as Oraldene™, exerted a prolonged anti-adherence effect post-rinsing. In particular, the adherence of viable yeast cells to BEC was significantly decreased, thus reducing the propensity for infection on treated epithelia. The *ex vivo* assay does not however consider the effects of hexetidine treatment of, and elimination from the surface of the yeast cells on their subsequent adherence to BEC. However, as hexetidine treatment of yeast cells has been shown *in vitro* to significantly reduce adherence, it would be expected that the duration of anti-adherence effects observed *in vivo* would be equivalent, if not greater, than those observed in the *ex vivo* scenario. Thus, the anti-adherence effect associated with hexetidine would be predicted to be beneficial for the prophylaxis of superficial, oral candidosis in susceptible individuals.

The morphogenesis of *C. albicans* from the yeast cell (blastospore form) to the hyphal form has been suggested to play a number of significant roles in the pathogenesis of candidosis. Examples of these include the greater abilities of hyphal forms to adhere to epithelial cells *in vitro* in comparison to yeast cells (9) and also to directly breach epithelial barriers and hence facilitate entry of this organism into the systemic circulation (8). Treatment of yeast cells for short periods (*circa* 10 s) with a range of concentrations of hexetidine (Oraldene™) significantly reduced (or totally eliminated) the extent of morphogenesis and the subsequent rate of hyphal extension. As these treatment times were similar to those often employed by patients, it is likely that Oraldene™ would demonstrate similar activities *in vivo*, thus suggesting a potential clinical role for the prophylaxis of tissue invasion (and the resultant systemic complications). Hexetidine effects on morphogenesis and yeast cell viability were concentration dependent. Consequently, inhibitory effects of hexetidine on morphogenesis were observed following treatment of yeast cells with higher concentrations of this agent, concentrations which induced greater fungicidal effects, indicating a relationship between the two phenomena. The lower concentrations of hexetidine (0.005, 0.01%) were not significantly fungicidal following short exposure times of yeast cells and subsequently the effects on morphogenesis were significantly lower than for higher concentrations. These effects were most likely due to direct inhibitory effects on the metabolic processes associated with morphogenesis as recovery of some of these processes following treatment with 0.05% hexetidine was observed four hours post-treatment. This is consistent with previous observations concerning the effects of other cationic, non-antibiotic, antimicrobial agents, e.g. chlorhexidine gluconate, cetylpyridinium chloride, dequalinium chloride, on the morphogenesis of *C. albicans* (16).

In conclusion, this study has identified the ability of hexetidine to modify certain virulence attributes of *C. albicans* following short exposure times, namely, *in vitro* and *ex vivo* adherence to BEC, and, morphogenesis from the yeast cell form to hyphal forms. In light of the current clinical problems concerning both the emergence of azole-resistant *Candida spp* in AIDS patients and the implementation of effective disease treatment regimens (2,3), it would be beneficial in this patient group to introduce strategies to prevent candidosis. Given the clinical relevance of the *ex vivo* adherence assay, and, in addition, the effects of short exposure times of hexetidine on morphogenesis *in vitro*, it is suggested that the clinical use of hexetidine mouthwash represents a positive strategy for the prophylaxis of both superficial candidosis and epithelial invasion leading to systemic candidosis in such susceptible individuals.

REFERENCES

1. S. Fowler, and D. S. Jones. *Int J. Pharm.* **86**:193–199 (1992).
2. D. C. Coleman, D. E. Bennett, D. J. Sullivan, P. J., Gallagher, H. C. Henman, D. B. Shanley, and R. J. Russell. *Crit. Rev. Microbiol.* **19**:61–82 (1993).
3. D. W. Denning. *Lancet* **346**:454–455 (1995).
4. L. J. Douglas. *CRC Crit. Rev. Microbiol.* **45**:27–43 (1987).
5. D. S. Jones, L. J. Schep, and M. G. Shepherd. *Pharm. Res.* **12**(12):1896–1900 (1995).
6. S. P., Gorman, D. F. McCafferty, A. D. Woolfson, and D. S. Jones. *J. Clin. Pharm. Ther.* **12**:393–399 (1987).
7. J. E. Cutler. *Ann. Rev. Microbiol.* **45**:187–218 (1991).
8. J. A. Howlett, C. A. Squier. *Infect. Immun.* **29**:252–260 (1980).
9. M. L. Anderson and F. C. Odds. *Mykosen* **28**:531–540 (1985).
10. W. R. Roberts and M. Addy. *J. Clin. Periodontol.* **8**:295–310 (1981).
11. M. J. R. Williams, D. Adams, D. G. Hillam, and K. C. Ashley. *Br. Dent. J.* **163**(9):300–302 (1987).
12. D. B. Wile, J. R. M. Dinsdale, and D. H. M. Joynson. *Curr. Med. Res. Opin.* **10**(2):82–88 (1986).
13. S. P. Gorman, D. S. Jones, J. G. McGovern, and A. D. Woolfson, *J. Pharm. Pharmacol.* **48**(12):1315–1319 (1996).
14. L. J. Schep, D. S. Jones, G. Schep, and M. G. Shepherd, *Int. J. Pharm.* **105**:39–45 (1994).
15. R. S. Tobgi, L. P. Samaranayake, and T. W. MacFarlane, *J. Med. Vet. Mycol.* **25**:335–338 (1987).
16. D. S. Jones *Pharm. Res.* **12**(12):2057–2059 (1995).
17. A. A. Miles, and S. S. Misra. *J. Hyg.* **38**:732–749 (1938).
18. D. S. Jones, L. J. Schep, and M. G. Shepherd, 1995. *Pharm. Sci.* **1**(11):513–515 (1995).
19. L. J. Schep, D. S. Jones, and M. G. Shepherd. *Pharm. Res.* **12**(5):649–652 (1995).
20. V. Shakespeare, P. G. Shakespeare, and B. T. Evans. *Archs. Oral Biol.* **33**(12):881–885 (1988).
21. S. P. Gorman, D. F. McCafferty, A. D. Woolfson, and D. S. Jones. *J. Appl. Bact.*, **62**:315–320 (1987).
22. S. P. Gorman, D. F. McCafferty, A. D. Woolfson, and D. S. Jones. *Letts. Appl. Micro.* **4**:103–109 (1987).