The Effects of Sub-Inhibitory Concentrations of Cationic, Non-Antibiotic, Antimicrobial Agents on the Morphogenesis of *Candida albicans in Vitro*

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INTRODUCTION

Candida albicans is an obligate commensal associate of the digestive and vaginal tracts of humans and most warm blooded animals which, under certain conditions, may become pathogenic (1). There have been reports of candidal infections of most tissues in the human body however, the most frequently encountered sites of infection are the oral cavity and vaginal tract (2).

In the treatment and prophylaxis of oral candidosis, mouthwashes and lozenges containing non-antibiotic, antimicrobial agents, e.g. chlorhexidine may be prescribed (3, 4). More recently, non-antibiotic, antimicrobial agents have received attention due to their ability to reduce the adherence of micro-organisms to epithelial cells *in vitro*. As the adherence of micro-organisms, e.g. *C. albicans*, to host epithelia is thought to be the primary (requisitory) step in the process of infection, and, given the correlation between virulence potential of *Candida spp.* and adherence to epithelia (5), it has been suggested that these agents may also be of use in the prophylaxis of superficial candidosis (1, 6). Examples of non-antibiotic, antimicrobial agents which have reduced adherence of *C. albicans* to epithelial cells *in vitro* include chlorhexidine gluconate, cetylpyridinium chloride, cetrimide and dequalinium chloride, all common constituents of mouthwash and lozenge preparations (1).

C. albicans is a dimorphic microorganism which may undergo morphogenesis from the yeast form to the hyphal forms (7, 8). The hyphal form *in vivo* is thought to be of importance in the initial processes of tissue invasion, where it has been reported to adhere better than yeast cells to epithelia (9, 10) and also has been shown to be involved in the initial stages of epithelial penetration (9, 11).

In a previously study it was reported that sub-inhibitory concentrations of chlorhexidine gluconate, cetrimide, cetylpyridinium chloride and dequalinium chloride decreased the adherence of *C. albicans* to buccal epithelial cells *in vitro* (1). This study therefore investigates the effects of these concentrations on other potential virulence factors, namely, the dimorphic transition from yeast to hyphae and the rate of hyphal extension, collectively referred to as morphogenesis.

MATERIALS AND METHODS

Chemicals

Chlorhexidine gluconate was obtained from Imperial Chemical Industry plc (ICI) Macclesfield, Cheshire, England.

Cetylpyridinium chloride and cetrimide were purchased from Sigma Chemicals Ltd., St. Louis, Missouri, USA.

Dequalinium chloride, as Dequadin™ throat lozenges was purchased from Crookes Healthcare, Nottingham, England.

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

The appropriate concentrations of cetrimide and cetylpyridinium chloride were obtained following addition of the required weight of pure substance to sterile deionised water. For chlorhexidine gluconate, the appropriate dilutions of the stock solution (20%v/v) were performed using sterile deionised water.

Dequalinium chloride was extracted from Dequadin™ by dissolving one lozenge in sterile water. This was then diluted to the required concentration again using sterile deionised water.

All solutions were prepared daily for use on the same day.

Candida albicans Isolates and Growth Conditions

One strain, MEN and one isolate of *C. albicans* (from a diagnosed oral infection) were employed in this study. These have been used in previous investigations (1, 6) and were stored on the surface of Nutrient agar (Difco) slopes at 4°C. When required, stationary phase blastospores were harvested by transferring two loopfuls into prewarmed Nutrient broth (Difco) at 25°C for 18 h in an orbital incubator (150 oscillations min^{-1}). Cultures were centrifuged (2,000g, 15 min.) and the deposit washed once with and resuspended in Phosphate-Buffered Saline (PBS, pH 7.3, 0.1M) to the required cell density.

Determination of the Effects of Sub-Inhibitory Concentrations of Chlorhexidine Gluconate, Cetylpyridinium Chloride, Cetrimide and Dequalinium Chloride on Morphogenesis of *Candida albicans*

The effects of these agents on the morphogenesis of *C. albicans* were investigated as previously reported (6, 12). Minimal inhibitory concentrations were determined by a doubling dilution method as previously described (13). Stationary phase blastospores of *C. albicans* (*circa* 1×10^8 colony forming units) were suspended in either sterile water, chlorhexidine gluconate (5.0×10^{-5}%v/v), cetrimide (1.0×10^{-4} %w/v, cetylpyridinium chloride (5.0×10^{-5}%w/v) or dequalinium chloride (1.9×10^{-5}%w/v) and incubated at

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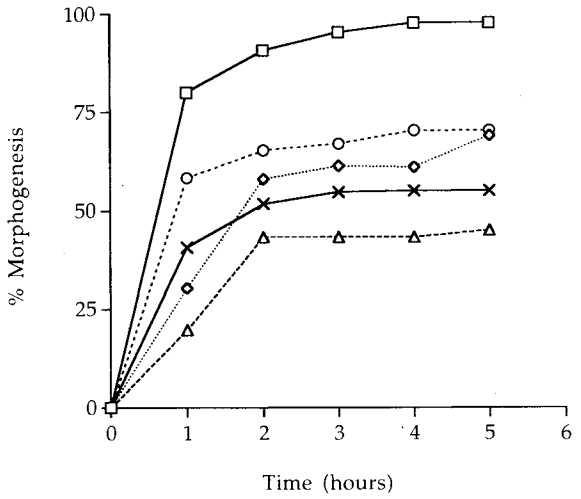

Fig. 1. The effect of treatment of yeast cells of *Candida albicans* (oral isolate) with sterile water (□), Cetylpyridinium chloride (5.0×10^{-5}%w/v, ◇), Chlorhexidine gluconate (5.0×10^{-5}%v/v, ○), Cetrimide (1.0×10^{-4}%w/v, △) or Dequalinium chloride (1.9×10^{-5}%w/v, *) for 30 minutes at 37°C on subsequent morphogenesis. In all cases, the percentage coefficient of variation did not exceed 15%.

37°C for 30 minutes in an orbital incubator (150 oscillations min^{-1}). The concentrations were selected due to their previously reported abilities to decrease the adherence of this strain and isolate of *C. albicans* to human buccal epithelial cells *in vitro,* and never exceeded 1/4 of the minimal inhibitory concentrations. The samples were then filtered through a 0.45μm pore-sized filters (Millipore), the filters washed with PBS and then transferred into Ehrlenmeyer flasks containing pre-warmed Nutrient broth (50mL). The flasks containing the broth were then vortexed to displace attached blastospores and then transferred to an orbital incubator and incubated at 37°C (150 oscillations min^{-1}). At hourly intervals samples were removed, stained using crystal violet and the percentage germinated of at least 300 blastospores and the mean hyphal length of at least 100 germinated blastospores determined using light microscopy.

Statistical Analysis

Statistical analysis of the effects of these non-antibiotic antimicrobial agents on the percentage morphogenesis of yeast cells and on subsequent mean hyphal lengths were performed using Chi-squared analysis (χ^2) and Mann-Whitney U test, respectively, as previously reported (6). In all cases, $p < 0.05$ was accepted to denote significance.

RESULTS

The percentage morphogenesis of the MEN strain and the oral isolate of *C. albicans* following treatment with sterile water were greater than 80% after one hour and was observed to reach a plateau, after approximately three hours, at *circa* 95%. Figure 1 illustrates the effects of treatment with the non-antibiotic antimicrobial agents on the % morphogenesis of the oral isolate with respect to time post-treatment. The profile for the MEN strain was similar and is consequently not shown. In all cases, the difference between the MEN strain and the oral isolate with respect to time was nonsignificant. Conversely, the percentage morphogeneses of chlorhexidine gluconate-treated, cetylpyridinium chloride-treated, cetrimide-treated and dequalinium chloride-treated blastospores, at each time point, were significantly lower than their water-treated counterparts ($p < 0.05$).

The effects of treatment of yeast cells with sub-inhibitory concentrations of cationic antimicrobial agents on the mean hyphal lengths of *C. albicans* are depicted in Table 1. There were statistically significant differences observed between water-treated and antimicrobial agent-treated *C. albicans* at all sampling periods. These observations indicated differences in the rate of hyphal development between water-treated and drug-treated yeast cells. Complete inhibition of morphogenesis was not observed in this study, regardless of treatment type.

Table I. The Effects of Treatment of Yeast Form of *C. albicans* (MEN Strain and an Oral Isolate) with Cetylpyridinium Chloride (CPC, 5.0×10^{-5}%w/v), Chlorhexidine Gluconate (CHG, 5.0×10^{-5}%v/v), Cetrimide (Cet, 1.0×10^{-4}%w/v) or Dequalinium Chloride (DQC, 1.9×10^{-5}%w/v) on the Subsequent Mean Hyphal Length with Respect to Time

	Mean hyphal length (±S.E.) of *Candida albicans* following treatment with									
	Sterile water		CPC		CHG		Cet		DQC	
Time (h) posttreatment	MEN strain	Oral isolate	MEN strain	Oral isolate	MEN strain	Oral isolate	MEN strain	Oral isolate	MEN strain	Oral isolate
1	2.62 ± 0.10	2.24 ± 0.99	2.22 ± 0.11	3.09 ± 0.14	1.57 ± 0.07	2.41 ± 0.12	1.50 ± 0.01	3.29 ± 0.26	1.89 ± 0.08	2.33 ± 0.11
2	6.80 ± 0.31	8.07 ± 0.37	5.45 ± 0.27	6.61 ± 0.35	3.93 ± 0.18	5.24 ± 0.26	2.28 ± 0.12	7.96 ± 0.43	4.63 ± 0.33	4.12 ± 0.23
3	12.34 ± 0.46	12.63 ± 0.06	8.51 ± 0.46	8.20 ± 0.55	8.26 ± 50.49	9.53 ± 0.61	6.37 ± 0.30	9.44 ± 0.72	6.32 ± 0.32	5.11 ± 0.33
4	17.71 ± 0.73	19.05 ± 0.86	9.04 ± 0.56	13.10 ± 0.77	12.05 ± 0.63	15.09 ± 0.81	10.15 ± 0.5	13.10 ± 0.75	8.43 ± 0.37	7.77 ± 0.52
5	18.56 ± 0.75	24.00 ± 2.46	13.22 ± 0.64	15.82 ± 0.98	14.89 ± 1.07	19.05 ± 1.01	13.23 ± 0.76	19.46 ± 0.79	9.84 ± 0.48	9.15 ± 0.55

DISCUSSION

The exact role of morphogenesis of *C. albicans* from the blastospore form to the hyphal form is at present unclear, however, one primary role for the hyphal form would appear to be in the initial processes of tissue invasion (7). In this hyphal forms have been reported to exhibit superior adherence to buccal and epithelial cells *in vitro* than their yeast cell counterparts (9, 10). Secondly, hyphal forms have been shown to be involved in the penetration of epithelia (9, 11), hence allowing access of *C. albicans* to the systemic circulation from an originally superficial infection. Therefore, the partial inhibitory effects of chlorhexidine gluconate, cetrimide, cetylpyridinium chloride and dequalinium chloride on the extent of germination and hyphal development of blastospores of *C. albicans* may be of clinical interest as both the incidence of tissue invasion and the spread of, and severity of candidosis may be reduced.

Previously we reported that treatments (30 min at 37°C) of *C. albicans* blastospores (MEN strain and oral isolate) or human buccal epithelial cells with these sub-inhibitory concentrations of chlorhexidine gluconate, cetrimide, dequalinium chloride and cetylpyridinium chloride reduced the subsequent adherence of the two cell types (1). Therefore, the clinical usefulness of these agents may be considered to be firstly prophylactic, in that the adherence of the potential pathogen is modified, dimorphic transition and the rate of hyphal development are impeded thus limiting epithelial invasion and systemic spread, and secondly, therapeutic, as these agents may provide direct fungicidal or fungistatic activity at concentrations usually found in mouthwashes and lozenges. Chlorhexidine, cetylpyridinium chloride and hexadecyltrimethylammonium bromide (a component of cetrimide) have been reported to be present in saliva at sublethal concentrations several hours after rinsing twice daily with these agents (14). Therefore blastospores of *C. albicans* will be bathed with subinhibitory concentrations of these antimicrobial agents which have been shown in this *in vitro* study to decrease both the dimorphic transition and subsequent rate of hyphal extension and also, in a previous study, to decrease the adherence of this organism to epithelial cells *in vitro*.

In conclusion this study has demonstrated that treatment of *C. albicans* blastospores (MEN strain and an oral isolate) with sub-minimal inhibitory concentrations of chlorhexidine gluconate, cetylpyridinium chloride, cetrimide or dequalinium chloride significantly reduced both the extent of morphogenesis and the subsequent rate of hyphal development. In the light of these current observations, the reported anti-adherent properties of these agents (1), the good retention of these agents within the oral cavity following rinsing (14) and also the current problems of emergent azole-resistant *Candida spp.* associated with the prophylactic use of azole anti-fungal agents, it is suggested that these agents may be clinically useful, as mouthwash or lozenge preparations, in the prophylaxis of both oral candidosis and the resultant tissue invasion by *C. albicans*. Consequently, it is recommended that suitable comparator clinical trials should be performed to determine the *in vivo* potential of these antimicrobial agents as first-line prophylaxis agents.

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