Primary Interactions of Three Quaternary Ammonium Compounds with Blastospores of *Candida albicans* (MEN Strain)

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The absorption of three quaternary ammonium compounds (QAC), cetylpyridinium chloride, cetrimide and benzalkonium chloride, onto the surface of blastospores of *Candida albicans* (MEN strain) was examined at room temperature. Equilibrium uptake occurred in less than 30 seconds for cetylpyridinium chloride and cetrimide whereas 5 min contact time was required for benzalkonium chloride. The adsorption of all three agents may be mathematically described as Langmuirian and hence a concentration-dependent formation of drug-monolayer on the surface of the blastospore occurred. From this the number of molecules adsorbed onto the surface of a single blastospore was calculated to be 1.33×10^{12}, 3.17×10^{12} and 2.32×10^{12} for cetylpyridinium chloride, cetrimide and benzalkonium chloride, respectively. These dissimilarities are most likely due to differences in the orientations of both the cationic nitrogen atom and the accompanying lipophilic portions of each QAC at the blastospore surface. Relating these observations to the known anti-adherence effects of cetylpyridinium chloride and cetrimide, it can be concluded that monolayer coverage of the blastospore surface with QAC does not account for the observed reduced adherence. This suggests that the anti-adherence effects are due to either direct interaction with, or steric blockade of, adhesions on the blastospore surface.

KEY WORDS: adsorption isotherms; QAC; *candida albicans;* adherence.

INTRODUCTION

Candida albicans is an obligate associate of human beings and most warm blooded animals which is usually encountered as a harmless commensal of the gastrointestinal tract and vaginal tract. However, the incidence of both superficial and invasive candidosis has increased markedly due to iatrogenic factors, diabetes mellitus, immunological compromisation and trauma (1). There are several agents available for the treatment of candidosis, e.g. amphotericin B, nystatin, miconazole, ketoconazole, fluconazole, 5-fluorocytosine, however, the selection of the appropriate agent(s) is dependent on the site and severity of infection (2). Superficial candidosis, e.g. of the oral cavity, may be treated locally using mouthwashes and lozenges containing antifungal agents or non-antibiotic antimicrobial agents. Chlorhexidine is a member of the latter category which has been successfully used to treat oral candidosis (3).

In the pathogenesis of candidosis, it is accepted that the initial stage involves adherence of the pathogen to the host epithelium, and consequently, an interest has developed in the identification of agents which reduce or inhibit this process (4). Non-antibiotic, antimicrobial agents have been reported to reduce the adherence of micro-organisms to epithelial cells *in vitro*. Examples of these include: chlorhexidine gluconate, cetylpyridinium chloride, dequalinium chloride and cetrimide (2); taurolidine (5); polynoxylin (6), polyvinylpyrrolidone-iodine (7) and Brolene™ (8).

The primary interaction of any antimicrobial agent with a micro-organism is accepted to involve adsorption onto the cell surface. There are several patterns of adsorption, termed adsorption isotherms, and from such isotherms the rate and total amount of drug uptake may be calculated and an indication of adsorptive mechanism may be obtained (9). As adherence between micro-organism and host epithelia is a surface phenomenon and, in light of the ability of some quaternary ammonium compounds to reduce the adherence of *C. albicans* to epithelial cells (3), the purpose of this study was to examine whether saturation coverage of the surface of stationary phase blastospores of *C. albicans* with QAC accounts for their anti-adherence properties. Whilst, it has been reported that virulence potential of *Candida spp* may be correlated with the ability to adhere to epithelia (4), there is little experimental evidence to suggest that different growth phases of blastospores, either exponential or stationary phases, show differences in adherence to epithelial cells (10). Consequently, stationary phase blastospores of *C. albicans* (MEN strain), which were employed in a previous study, were again employed in this study.

MATERIALS AND METHODS

Chemicals

Cetylpyridinium chloride, Benzalkonium chloride and Cetrimide were purchased from Sigma Chemical Ltd., St. Louis, U.S.A.

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, cetrimide and benzalkonium chloride were obtained by dissolving the appropriate weight of pure substance in sterile deionised water.

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

Candida albicans Growth Conditions

Candida albicans (MEN strain, serotype B) (2,8) was used in this study and was maintained on Nutrient agar (Difco) slopes at 4°C. When required, stationary phase *C. albicans* blastospores were harvested by transferring two loopfuls into prewarmed sulphur-enriched glucose-salt-

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biotin (GSB) broth in which ammonium sulphate had been replaced with ammonium dihydrogen phosphate ($1 g l^{-1}$) and peptone had been replaced with bactopeptone (Difco, $1 g l^{-1}$) (11). This was incubated for 16 hours at 28°C in a shaking water bath (110 osc min^{-1}) following which blastospores were washed twice with and resuspended in sterile phosphate buffered saline (PBS, 0.03M, pH 7.4) to a total cell count of 1.0×10^9 cells ml^{-1}.

C. albicans dry weight was determined by adding aliquots of an aqueous suspension (1.0×10^9 cells ml^{-1}) into tarred oven-dried porcelain crucibles. These were dried at 120°C for 24 hours and the weights determined. This was performed in triplicate and an average dry weight ml^{-1} calculated.

Determination of Uptake of Cetylpyridinium Chloride, Cetrimide and Benzalkonium Chloride onto *C. albicans* Blastospores

All experiments were performed in triplicate at room temperature (*circa* 20°C). Stationary phase blastospores of *C. albicans* (1.3 mg dry weight equivalent to 1×10^8 cells) were suspended in solutions of cetylpyridinium chloride (0.147mMol), benzalkonium chloride (0.141mMol) or cetrimide (0.149mMol) for times ranging from 0.5 to 60 minutes. Each sample was then centrifuged (6,000g, 1 minute), the supernatant removed and the concentration of QAC assayed using the colorimetric ion-pair method previously reported by us (12). In brief, the supernatant samples were diluted and combined with excess eosin and Triton X-100 (0.025%v/v). The extinction of the QAC-eosin-Y ion-pair was determined at 534nm. The concentration of QAC absorbed per mg dry weight of blastospore cells was plotted against time of treatment. From this the time of treatment which corresponded to a plateau region of adsorption, i.e. equilibrium adsorption, was accepted as 15 minutes for each QAC.

In the determination of adsorption isotherms, blastospores of *C. albicans* (stationary phase, 1.3 mg dry weight equivalent to 1×10^8 cells) were treated for 15 minutes with various concentrations (0.141 - 0.358mMol) of cetylpyridinium chloride, cetrimide and benzalkonium chloride. Blastospores were centrifuged (6,000 x g, 1 minute) and the concentration of QAC in the supernatant determined colorimetrically, as described above. Adsorption isotherms were determined by plotting the supernatant concentration of QAC, after exposure to *C. albicans*, against the concentration of QAC adsorbed per dry weight of cells.

All experiments were performed in triplicate

Isolation of Cytoplasmic Materials from Blastospores of *C. albicans*

Isolation of cytoplasmic contents of blastospores were performed as described by Holmes *et al.* (13). A suspension of washed blastospores (2.0×10^9 cells ml^{-1}) was added to 1.5g of glass beads (400µm) in a 10 ml test-tube (cooled on ice). This mixture was vortexed twice (1 minute each), the extract decanted and the beads washed with 1.0ml sterile PBS. The pooled material was then centrifuged to remove cell debris (6,000 x g, 1 minute) and the supernatant containing the cytoplasmic material decanted.

RESULTS

The effects of time of treatment of stationary phase blastospores of *C. albicans* on the subsequent adsorption of cetylpyridinium chloride, cetrimide and benzalkonium chloride are shown in figure 1. Equilibrium adsorption of cetylpyridinium chloride and cetrimide occurred in less than 30s, whereas five minutes treatment was required for adsorption of benzalkonium chloride to reach equilibrium. Using a treatment time of 15 minutes to ensure equilibrium adsorption, adsorption isotherms for cetylpyridinium chloride, cetrimide and benzalkonium chloride were determined (Fig. 2, 3 and 4, respectively). All three isotherms may be described as Langmuirian (9). Mathematically transforming the data according to Florence and Attwood (14) allows the ratio of the equilibrium supernatant concentration of QAC to the concentration of QAC adsorbed per unit weight of adsorbate against the equilibrium concentration of QAC in the supernatant to be plotted. The resulting linear plot confirms Langmuirian adsorption. A summary of the slopes and intercepts of such plots is shown in table 1.

DISCUSSION

Cetylpyridinium chloride, cetrimide and benzalkonium chloride are frequently used as disinfectants, antiseptics and preservatives (15). The exact mode of antimicrobial action is unclear as there are thought to be several contributory factors; however, generally it is recognised that the cytoplasmic membrane is a major site of action resulting in increased cell permeability/leakage of cytoplasmic contents (16). In conducting adsorption studies it is important that the presence of cytoplasmic materials, released following contact with QAC, does not interfere with the analytical method for QAC. Therefore, a colorimetric ion-pairing method was used in this study (12) whose $\lambda_{max.}$ (534nm) is distant from the $\lambda_{max.}$ of cytoplasmic materials (260nm). To confirm this, analysis of cetylpyridinium chloride was performed in the presence and absence of ctyoplasmic materials. These were observed to be similar in both instances indicating that the presence of cytoplasmic contents did not interfere with the assay.

It is important in the determination of adsorption iso-

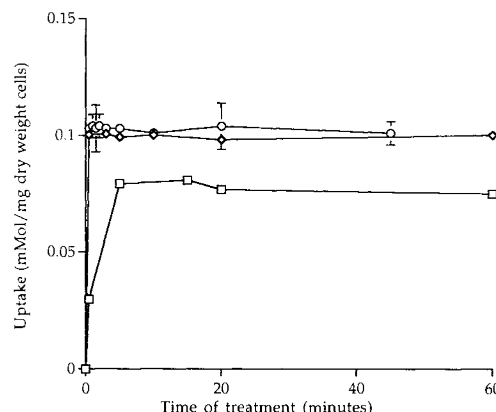

Figure 1. The effect of time of treatment on the uptake of cetylpyridinium chloride (0.147mMol, ○), Cetrimide (0.149mMol, ◇) and Benzalkonium chloride (0.141mMol, □) onto the surface of *Candida albicans* blastospores. Each point represents the mean ± standard deviation of three measurements.

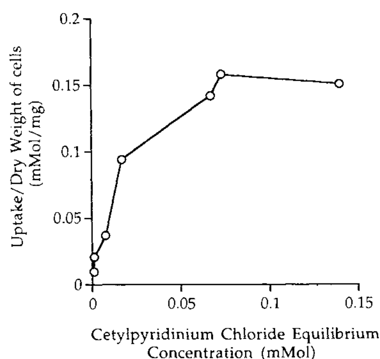

Figure 2. The adsorption isotherm of cetylpyridinium chloride onto blastospores of *Candida albicans*. Each point represents the mean ± standard deviation of three measurements.

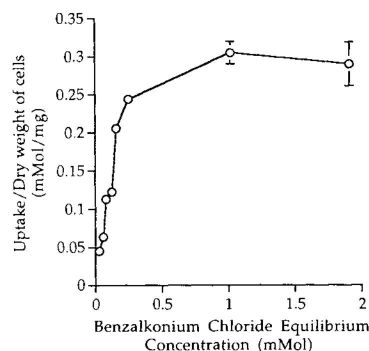

Figure 4. The adsorption isotherm of benzalkonium chloride onto blastospores of *Candida albicans*. Each point represents the mean ± standard deviation of three measurements.

therms to select a treatment time which ensures equilibrium uptake. Short equilibrium uptake times were observed in this study, however, such short times have been reported for the equilibrium uptake of other cationic agents, e.g. chlorhexidine diacetate, onto *E. coli* and *Ps. aeruginosa* (17) and onto *Saccharomyces cerevisiae* (18). This rapid uptake of chlorhexidine and QAC onto microbial cells is probably due to an electrostatic interaction between antimicrobial agent and microbial cell (19).

The adsorption patterns were observed to be Langmuirian. Therefore a concentration-dependent monolayer of QAC was adsorbed on the surface of the blastospore and is illustrated by the plateau region within the adsorption isotherm. From this, the amount of QAC covering the surface of each blastospore may be calculated (table 1). Statistical comparison of these values using a one-way Analysis of Variance ($p<0.05$ denoting significance) shows that the amount of QAC adsorbed as a monolayer is statistically dissimilar for each QAC. These dissimilarities are most likely due to differences in the orientations of both cationic nitrogen atom and the accompanying lipophilic portion at the blastospore surface.

We have previously reported that treatment of stationary phase blastospores of *C. albicans* (MEN strain, 1×10^8 cells) for 30 minutes with both supra and sub-minimum-inhibitory concentrations of cetrimide and cetylpyridinium chloride subsequently reduced their adherence to human

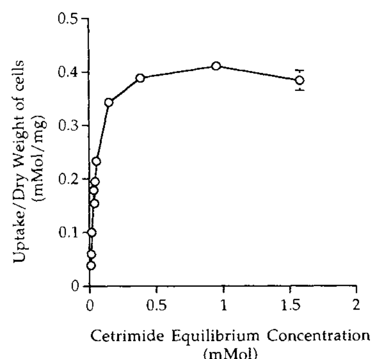

Figure 3. The adsorption isotherm of cetrimide onto blastospores of *Candida albicans*. Each point represents the mean ± standard deviation of three measurements.

buccal epithelial cells (2). Blastospore surface coverage with cetylpyridinium chloride and cetrimide following treatment with these "anti-adherence" concentrations are expressed in Table 2 in terms of drug monolayer presence, or absence. From this, it may be observed that the concentrations of cetylpyridinium chloride and cetrimide associated with the anti-adherence effects (2) were insufficient to produce QAC monolayer coverage of blastospores. In addition, marked differences in treatment concentrations of both cetylpyridinium chloride and cetrimide, reflecting differences in relative surface coverage of blastospores with QAC, produced statistically similar reductions in blastospore adherence. It can therefore be concluded that monolayer coverage of the blastospore surface with cetrimide or cetylpyridinium chloride is not required to illicit an anti-adherence effect. It is suggested that the anti-adherence effect observed in our ear-

Table 1. Data Derived from the Linear Plot of Langmuirian Isotherm

Drug	Intercept (mg)	Slope (mg mMol^{-1})	Regression	No. of molecules/ blastospore[a]
CPC	0.09 ± 0.02	5.83 ± 0.44	0.98	1.33×10^{12}
CT	0.12 ± 0.01	2.47 ± 0.17	0.99	3.17×10^{12}
BKC	0.41 ± 0.07	3.38 ± 0.31	0.98	2.32×10^{12}

1 Calculated as follows:

(a) Number of blastospores: $1 \times 10^8 = 1.3$ mg dry weight

(b) Mol of QAC required for complete monolayer formation over total cell population:

$$\frac{1}{[(Slope \times 10^3)]} \times 1.3$$

(c) Number of molecules of QAC required for complete monolayer formation over total cell population:

$$\frac{1}{[(Slope \times 10^3)]} \times 1.3 \times 6.02 \times 10^{23}$$

(d) Number of molecules required for complete monolayer formation over one blastospore:

$$\frac{\frac{1}{[(Slope \times 10^3)]} \times 1.3 \times 6.02 \times 10^{23}}{1 \times 10^8}.$$

Table 2. The Effect of Treatment of Blastospores of *Candida albicans* (MEN Strain) with Cetylpyridinium Chloride (CPC) and Cetrimide (Cet) on Their Subsequent Adherence to Buccal Epithelial Cells (BEC) *in Vitro** (From Fowler and Jones 1992, Reference 2)

Treatment[1]	Number of adherent *C. albicans* ± SE per BEC	Evidence of drug monolayer formation[2]
Water	1.40 ± 0.15	
CPC (1.47×10^{-1} mMol)	0.73 ± 0.01	No
CPC (1.47×10^{-3} mMol)	0.88 ± 0.10	No
Water	1.40 ± 0.15	
Cet (2.7×10^{-2} mMol)	0.65 ± 0.08	No
Cet (2.7×10^{-3} mMol)	0.63 ± 0.09	No

[1] Treatment of blastospores (1×10^8 cells) with either QAC or sterile water for 30 minutes.

[2] Concentrations ± SD of cetylpyridinium chloride and cetrimide required to produce monolayer coverage of blastospores were $2.23 \pm 0.02 \times 10^{-1}$ mMol and $5.26 \pm 0.36 \times 10^{-1}$ mMol, respectively, from table 1.

lier study (2) is due to specific interactions with, or stearic blockade of, adhesions on the blastospore surface.

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