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Frequency Distribution of *Candida albicans* Blastospores Adhered to Mucosal Epithelial Cells In-vitro

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Abstract

Although several methods are available for examination of microbial adherence to epithelial cells, these do not distinguish between adherence of viable and non-viable micro-organisms. This study reports the use of acridine orange-stained blastospores of *Candida albicans* in conjunction with direct epifluorescence microscopy to determine viable (orange-fluorescing) and non-viable (green-fluorescing) blastospore adherence to buccal epithelial cells. The method was also employed to examine the effects of chlorhexidine treatment at sub-minimum inhibitory concentrations on the adherence of viable and non-viable blastospores.

There was good correlation in the assessment of blastospore viability between the direct epifluorescence microscopy technique and the standard serial dilution and plating method for viable counting, confirming the reliability of direct epifluorescence microscopy. Chlorhexidine treatment before acridine orange staining did not alter this assessment of viability. Blastospore adherence to buccal epithelial cells resulted in a similarly skewed distribution whether examined using a crystal violet stain in conjunction with light microscopy or using direct epifluorescence microscopy, therefore validating the direct epifluorescence microscopy technique for the enumeration of blastospore adherence. Chlorhexidine treatment (0.0005% v/v, 30 min) of either blastospores or buccal epithelial cells altered the distribution of adherent blastospores per epithelial cell by increasing the number of epithelial cells having no adherent blastospores. No differences in adherence were, however, observed between blastospore or epithelial cells after treatment with this agent. Examination of the adherence of viable and non-viable blastospores to buccal epithelial cells using direct epifluorescence microscopy revealed a greater adherence capacity of non-viable than viable blastospores for buccal epithelial cells. Treatment of blastospores with chlorhexidine altered the frequency distributions of viable and non-viable blastospores with lower numbers of blastospores adherent per epithelial cell. The larger reduction in adherent viable blastospores in comparison with their non-viable counterparts is, however, an important observation which might have clinical relevance.

Microbial cells adhere to epithelial cells resulting in a skewed distribution; study of this distribution gives useful information about the adherence process. Viable and non-viable components of a microbial population have different adherence capabilities and treatment of such populations with an antimicrobial agent exerting anti-adherent activity at sub-minimum inhibitory concentrations reduces the amount of adherence of these viable/non-viable components to different extents.

The yeast *Candida albicans* is a commensal of the oral cavity, gastrointestinal and female genital tracts in man (Odds 1988). In certain circumstances, however, e.g. immunocompromization, diabetes mellitus or antimicrobial chemotherapy, this organism might become pathogenic, resulting in superficial or systemic infections, or both (Odds 1988). Adherence of *C. albicans* to epithelial cells represents the initial step in the pathogenesis of infection as this enables the organism to overcome the normal flushing mechanisms of body secretions (Douglas 1987; Fowler & Jones 1992). Indeed, amongst *Candida* species a correlation has been reported between the relative virulence and ability to adhere to epithelial cells in vitro (King et al 1980; Douglas 1987). Given the importance of the adherence process to infection, a potential role for agents which reduce or inhibit the adherence of *C. albicans* to epithelial cells in the prophylaxis of candidosis has been proposed (Fowler & Jones 1992). We have recently reported that treatment of either blastospores of *C. albicans* or buccal epithelial cells with non-antibiotic, antimicrobial agents significantly reduced subsequent adherence (Fowler & Jones 1992).

The most frequently employed method of assessing microbial adherence to epithelial cells involves light-microscopy in conjunction with an appropriate stain, e.g. crystal violet (Gorman et al 1987; Sandin et al 1987; Fowler & Jones 1992), periodic acid Schiff reagent (Schep et al 1994) or alternatively dark-field microscopy (King et al 1980). These methods, however, provide no information about the number of viable and non-viable adherent blastospores per epithelial cell, considerations which will affect the potential virulence in-vivo. The direct epifluorescence filter technique was developed for the enumeration of viable bacteria in milk and food samples (Pettipher et al 1980) and utilizes the ability of acridine orange stain, in conjunction with fluorescence microscopy, to distinguish between viable micro-organisms, which are observed as orange-fluorescing cells, and green-fluorescing cells which are thought to be non-viable, or at least inactive, micro-organisms. This paper reports the use of an acridine orange stain and direct epifluorescence microscopy to enumerate the adherence of both viable and non-viable blastospores of *C. albicans* to buccal epithelial cells in-vitro. In addition, chlorhexidine gluconate, a non-antibiotic antimicrobial agent which is a common constituent of topical oral medications, was examined for its effect on both the frequency distribution of blastospores to buccal epithelial cells and the ratio of viable to non-viable adherent blastospores.

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Materials and Methods

Chemicals

Chlorhexidine gluconate (hibitane) was purchased from Zeneca, Wilmslow, Cheshire, UK. Acridine orange and all other chemicals were obtained from Sigma (Poole, Dorset, UK) and were of analytical-reagent quality. The appropriate concentration of chlorhexidine gluconate was obtained by dilution of the received stock solution with sterile deionized water. All solutions were prepared daily for use on the same day.

Candida albicans isolate and growth conditions

Candida albicans, an oral isolate NCYC 1467 from denture stomatitis, was maintained on Nutrient Agar (Oxoid) at 4°C and transferred to Nutrient Broth (Oxoid) at 37°C for growth, in blastospore form, to late-exponential phase. Cultures were washed twice by centrifugation at 2000 rev min^{-1} for 15 min in sterile phosphate-buffered saline (PBS; pH 7·3, 0·01 M) and resuspended to approximately 5×10^7 cfu mL^{-1} in PBS. The absence of hyphal forms in the cultured candida was confirmed by use of light microscopy.

Collection of epithelial cells

Buccal epithelial cells, collected from healthy male and female volunteers by scraping the buccal mucosa with the rounded ends of sterile ampoule files, were placed in sterile PBS, vortex mixed to remove the cells and the cellular suspension passed through a 22-gauge needle to ensure cellular homogeneity, as previously described (Jones et al 1995). The cells were washed and resuspended in sterile PBS. Volunteers were not in receipt of antibiotics or topical oral medication. Standardization of buccal epithelial cells density (cells mL^{-1}) was performed using optical density measurements (400 nm) that had previously been calibrated against buccal epithelial cell density enumeration using a haemocytometer ($r > 0·9$).

Minimum inhibitory concentration

The minimum inhibitory concentration of chlorhexidine gluconate for the isolate of *C. albicans* employed in this study was determined using a doubling dilution method described elsewhere (Gorman et al 1987; Jones et al 1995). In brief, a suspension of washed blastospores (0·1 mL, 1×10^6 colony forming units mL^{-1}) was added to nutrient broth (9·9 mL) containing antimicrobial agent and incubated at 25°C for 24 h. The minimum inhibitory concentration was taken as the first concentration where no growth was observed.

Pre-treatment procedures

Standardized suspensions of *C. albicans* blastospores or buccal epithelial cells were incubated with sub-minimum inhibitory concentrations of chlorhexidine gluconate (0·0005% v/v) or sterile water (control) for 30 min at 37°C in an orbital incubator (150 oscillations min^{-1}) (Gorman et al 1987; Fowler & Jones 1992). The samples were then centrifuged (3000 *g*, 15 min) and the supernatant fluid was removed by decanting. The deposit of blastospores of epithelial cells was then washed in sterile PBS.

Adherence assay

The adherence assay employed in this study was a modification of a method reported elsewhere (Gorman et al 1987). In this, equal volumes of *C. albicans* (1×10^7 cfu mL^{-1}) and buccal epithelial cells (1×10^5 cells mL^{-1}) were mixed and incubated at 37°C for 2h in a shaking water-bath (110 oscillations min^{-1}). Three loops-full of the suspension were then placed on to a microscope slide and diluted with an equal volume of sterile PBS and left to dry in air. To obtain total adherent blastospore counts, samples were stained with crystal violet (2·0% w/w for 15 s) directly on the microscope slide and examined by light microscopy. The fluorescent dye acridine orange (0·025% in citrate buffer at pH 6·8) was used to differentiate between viable and non-viable adherent *C. albicans*. *C. albicans* blastospores were suspended in the fluorescent dye for 5 min, precipitated by centrifugation (2000 *g*, 15 min) and the cells resuspended in PBS for inclusion in the adherence assay. Fluorescence microscopy (Vickers M17) was used to view acridine orange-stained specimens and thus differentiate between viable (orange fluorescing) and non-viable (green fluorescing) blastospores. In this system a filter was used which blocked UV light yet transmitted fluorescence equivalent to that of fluorescein isothiocyanate (absorbance λ_{max} 495 nm; fluorescence λ_{max} 520 nm). A minimum of 150 epithelial cells was examined for adherent blastospores. The reliability of detection of viable and non-viable blastospores, treated with either sterile water or chlorhexidine (0·0005% v/v) by fluorescence, was shown from the total blastospore count obtained in a haemocytometer and from viable blastospore counts on nutrient agar after serial dilution in sterile quarter-strength Ringer's solution (Miles & Misra 1938).

The absence of adverse effects of acridine-orange staining on cell adherence characteristics and viability was demonstrated by the colony-forming ability and adherence of stained blastospores in relation to crystal-violet-stained blastospores.

Statistical analysis of the effects of chlorhexidine gluconate treatment on the mean total, mean viable and mean non-viable number of adherent blastospores per buccal epithelial cell was performed using a two-tailed unpaired *t*-test, $P < 0·05$ denoting significance, as described elsewhere (Gorman et al 1987; Fowler & Jones 1992). The effect of treatment with chlorhexidine gluconate on the frequency distribution of adherent blastospores per buccal epithelial cells was also determined by comparison of their skewness coefficients (Woolfson 1993).

Results

Fig. 1 illustrates the determination of the viability of blastospore suspensions using both the acridine orange/direct epifluorescence microscopic technique and the serial dilution method (Miles & Misra 1938). Good correlation ($r = 0·91$) was generally observed between the two methods confirming the reliability of the direct epifluorescence technique for determination of blastospore viability. Pre-treatment of blastospores with sub-minimum inhibitory concentrations of chlorhexidine did not, furthermore, interfere with the determination of blastospore viability using the acridine orange staining procedure (results not shown).

The pattern of adherence of *C. albicans* blastospores to epithelial cells is demonstrated by the frequency distribution curves in Fig. 2. No significant difference ($0·375 < P < 0·4$) was obtained between adherence observed after staining with either crystal violet or acridine orange. Mean number (± s.e.)

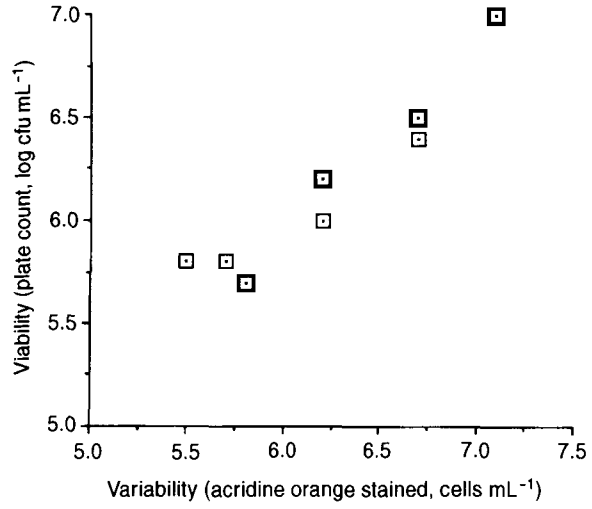

FIG. 1. Determination of the viability of blastospores of *Candida albicans* using direct epifluorescence microscopy (x axis) and the serial dilution method (Miles & Misra 1938; y axis). The observed correlation (r) between the two methods was 0·91.

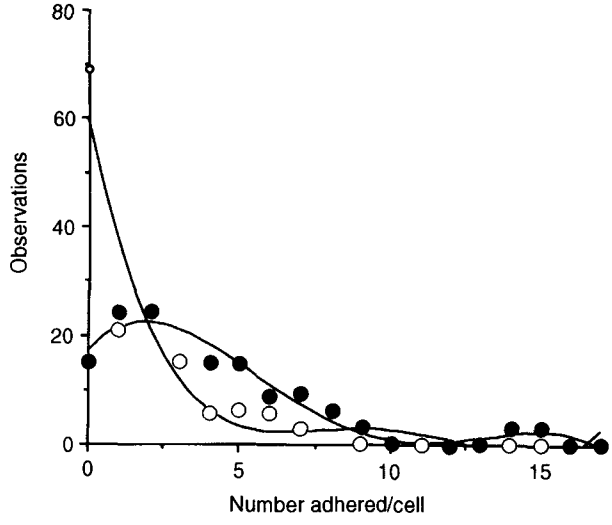

FIG. 3. Frequency distribution plot showing adherence of *Candida albicans* blastospores to human epithelial cells after treatment of either blastospores (○) or epithelial cells (●) with a sub-minimum inhibitory concentration of chlorhexidine gluconate (0·0005% w/v, 30 min). Acridine orange-stained.

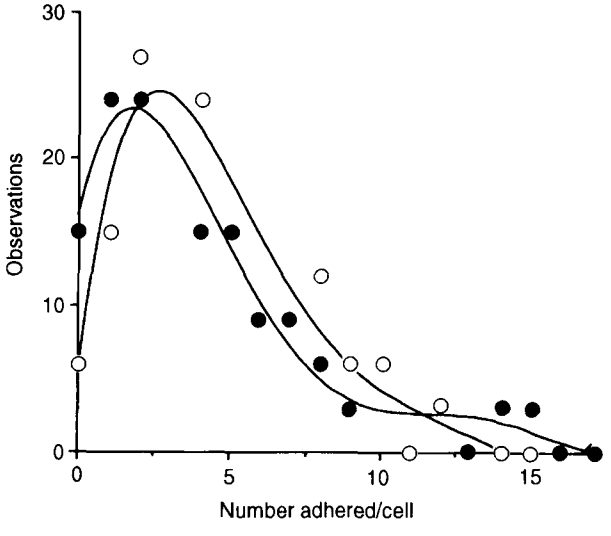

FIG. 2. Frequency distribution plot showing adherence of *Candida albicans* blastospores to human epithelial cells. ○ Crystal violet-stained blastospores, ● acridine orange-stained blastospores.

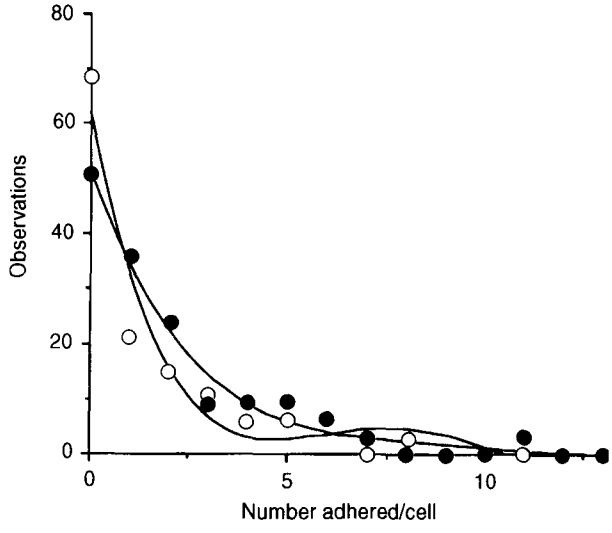

FIG. 4. Frequency distribution plot showing adherence of chlorhexidine-treated (sub-minimum inhibitory concentration 0.0005% w/v, 30 min) *Candida albicans* blastospores to human epithelial cells (○ treated, ● untreated). Acridine orange-stained.

of adherent blastospores (epithelial cell)$^{-1}$ was 4·32 ± 0·41 and 4·52 ± 0·40, respectively. The effect on this distribution of chlorhexidine-treatment of blastospores of *C. albicans* at the sub-minimum inhibitory concentration of 0·0005% is shown in Fig. 3. The resulting distribution is highly skewed and the number of epithelial cells having no adherent blastospores is greatly increased (52·6% treated in comparison with 10% untreated). No significant difference was observed in the adherence patterns resulting from treating either blastospores or buccal epithelial cells with chlorhexidine ($0·1 < P < 0·375$, Fig. 4). The mean (± s.e.) adherence values were, respectively, 1·52 ± 0·15 and 1·88 ± 0·15 per epithelial cell and both populations exhibited similar skewness coefficients (1·60 and 1·65, respectively). This contrasts with the levels exhibited with no chlorhexidine treatment as shown in Fig. 2, and the difference is highly significant ($P < 0·0005$).

The application of epifluorescence microscopy to investigation of microbial adherence to epithelial cells enables differentiation of viable and non-viable microbial cells. Fig. 5 shows a typical frequency distribution plot obtained for adherence of *C. albicans* to epithelial cells. Different mean numbers of adherent blastospores per epithelial cell and different distributions for viable and non-viable *C. albicans* are observed within the population employed in the adherence assay. Within this normal, untreated *C. albicans* population, the number of non-viable blastospores observed to adhere per

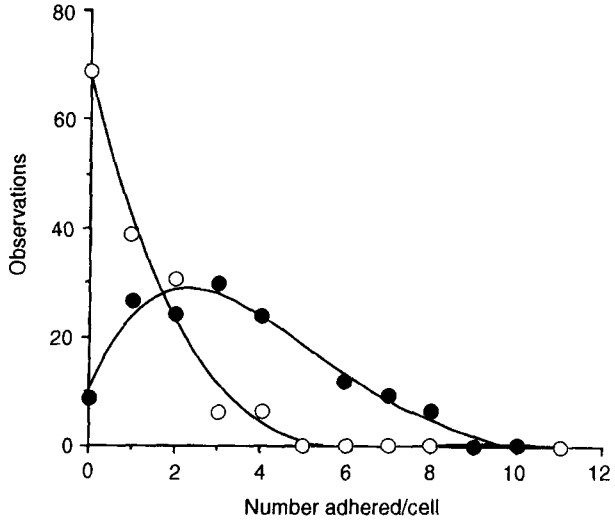

FIG. 5. Frequency distribution plot showing adherence to human epithelial cells of viable and non-viable blastospores in a population of *Candida albicans* as determined by epifluorescence microscopy after acridine orange staining (○ viable, ● non-viable blastospores).

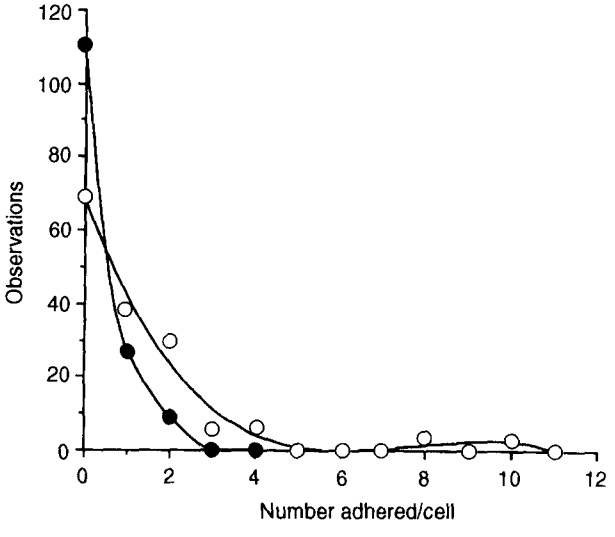

FIG. 6. Frequency distribution plot showing adherence to human epithelial cells of viable blastospores in a population of *Candida albicans* as determined by epifluorescence microscopy before (○) and after (●) treatment with a sub-minimum inhibitory concentration of chlorhexidine (0.0005% w/v, 30 min).

epithelial cell was significantly greater than the number of viable blastospores (3·10 ± 0·18 and 0·94 ± 0·09, respectively). The percentage of epithelial cells (46%) having no adherent blastospores was significantly higher when viable blastospores are considered in contrast with non-viable blastospores (6%). Treatment with sub-minimum inhibitory concentrations of chlorhexidine significantly reduced adherence but had the further effect of altering, to different extents, the distributions of viable and non-viable *C. albicans* adhered to epithelial cells. The larger decrease in the number of viable compared with non-viable blastospores adhered after treatment with chlorhexidine is an important observation (Figs 6 & 7).

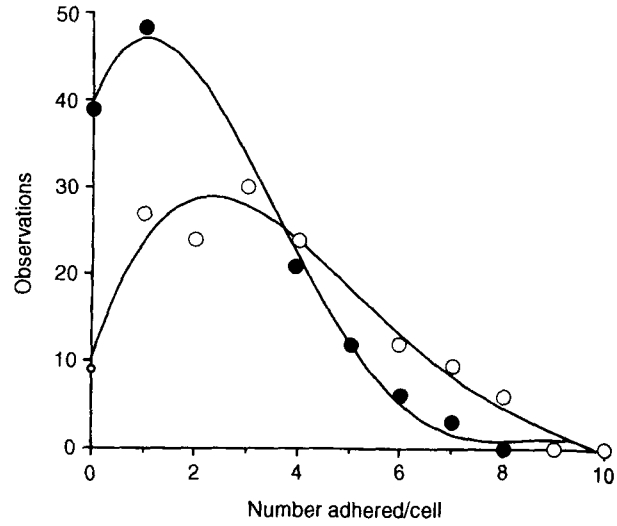

FIG. 7. Frequency distribution plot showing adherence to human epithelial cells of non-viable blastospores in a population of *Candida albicans* as determined by epifluorescence microscopy before (○) and after (●) treatment with a sub-minimum inhibitory concentration of chlorhexidine (0.0005% w/v, 30 min).

Discussion

Despite the availability of several anti-fungal drugs for the treatment of oral candidosis, there are reports of incidences of strains of *C. albicans* that are resistant to anti-fungal chemotherapy (Coleman et al 1993; Denning 1995). Given the correlation between the adherence potential of Candida species and virulence (King et al 1980), reduction or inhibition of adherence might be a method of providing prophylaxis from oral candidosis (Tobgi et al 1987; Fowler & Jones 1992; Jones et al 1995). Several workers have reported reduced adherence of blastospores of *C. albicans* to buccal epithelial cells after treatment with oral topical antimicrobial agents, e.g. chlorhexidine (Gorman et al 1987; Fowler & Jones 1992); cetylpyridinium chloride, cetrimide and dequalinium chloride (Fowler & Jones 1992); and octenidine and pirtenidine (Ghannoum et al 1990). It has, as a consequence, been suggested that these agents might offer the required prophylaxis against this condition (Fowler & Jones 1992). In these studies, adherence is typically reported as a mean value of adherent blastospores per epithelial cell. This, however, provides no information about the variation in adhesion from cell to cell nor the relative contributions of viable and non-viable blastospores adhering per epithelial cell (Woolfson 1993). As only viable blastospores are pathogenic and, as the presence of adherent non-viable blastospores on epithelial cells might block potential receptor sites for the adherence of viable blastospores, the development of an adherence assay which can discern between viable and non-viable adherent blastospores on the surface of epithelial cells would be beneficial. In this study we have described the use of acridine orange stain in conjunction with epifluorescence microscopy to illustrate the different adherence of viable and non-viable adherent blastospores. Previously, this stain has been used for reliable enumeration of the viability both of bacteria and of yeasts (Rodrigues & Kroll 1985, 1986) and, similarly, in this study good correlation was observed between the serial dilution method and the acridine orange-epifluorescence microscopic method for viability assessment of blastospores.

Typically, the distribution of data from adherence assays tends to be skewed, usually positively, rather than normally distributed (Rosenstein et al 1985). To calculate the extent to which the distribution of adherence data deviates from a normal distribution, a skewness coefficient might be calculated for each set of adherence data (Woolfson 1993), values exceeding unity indicating significant deviations from normality. The frequency distributions observed in this study were, in general, significantly positively skewed and were independent of the type of stain employed for sample visualization. The similar frequency distributions obtained using either the widely employed crystal violet stain or the acridine orange stain would imply that the acridine orange stain might be reliably used to characterize total blastospore adherence. The acridine orange stain enables further characterization of the frequency distribution in terms of viable and non-viable blastospore adherence. Interestingly, the apparent differences in frequency distribution and mean ($\pm$ s.e.) adherence of viable blastospores between viable and non-viable blastospores might suggest that non-viable blastospores possess a greater adherence capacity.

Treatment of blastospores of *C. albicans* with sub-minimum inhibitory concentrations of antimicrobial agents has previously been reported to reduce significantly adherence to buccal epithelial cells (Gorman et al 1987; Fowler & Jones 1992). Similarly, in this study chlorhexidine (0·0005% v/v, 30 min) treatment of blastospores significantly reduced their adherence to epithelial cells from $3{\cdot}86 \pm 0{\cdot}31$ to $1{\cdot}50 \pm 0{\cdot}15$. This treatment also altered the distribution of adherent blastospores by reducing the number of epithelial cells with larger numbers of adherent blastospores, and also increasing the number of buccal epithelial cells with either zero or few adherent organisms. The use of the acridine orange stain illustrated that whereas chlorhexidine significantly reduced the mean number of both viable and non-viable blastospores adhering to buccal epithelial cells, a statistically greater anti-adherence effect was observed for viable blastospores. This might suggest the existence of a preferential anti-adherence ability of this compound for viable cells.

In conclusion, microbial cells adhere to epithelial cells resulting in a skewed distribution. Importantly, study of such distributions gives more information about the adherence process than the mean adherence values normally generated from adherence studies. The data presented indicate that viable and non-viable components of a microbial population have different adherence capabilities and that treatment of such populations with an antimicrobial agent exerting anti-adherent activity at sub-minimum inhibitory concentrations reduces the amount of adherence of these viable/non-viable components to different extents. Extensive reductions in the number of viable cells adhering are observed, in sharp contrast with the marginal reductions observed with non-viable cells.

References

Coleman, D. C., Bennett, D. E., Sullivan, D. J., Gallagher, P. J., Henman, M. C., Shanley, D. B., Russell, R. J. (1993) Oral *Candida* in HIV–infection and AIDS–new perspectives, new approaches. Crit. Rev. Microbiol. 19: 61–82

Denning, D. W. (1995) Can we prevent azole resistance in fungi? Lancet 346: 454–455

Douglas, L. J. (1987) Adhesion of *Candida* species to epithelial surfaces. CRC Crit. Rev. Microbiol. 15: 27–43

Fowler, S., Jones, D. S. (1992) Modified adherence of *Candida albicans* to human buccal epithelial cells in vitro following treatment with cationic, non-antibiotic antimicrobial agents. Int. J. Pharm. 86: 193–199

Ghannoum, M. A., Abu Elteen, K., Stretton, R. J., Whittaker, P. A. (1990) Effects of octenidine and pirtenidine on adhesion of *Candida* species to human buccal epithelial cells in-vitro. Arch. Oral Biol. 35: 249–253

Gorman, S. P., McCafferty, D. F., Woolfson, A. D., Jones, D. S. (1987) A comparative study of the microbial anti–adherence capacities of three antimicrobial agents. J. Clin. Pharm. Ther. 12: 393–399

Jones, D. S., Schep, L. J., Shepherd, M. G. (1995) The effect of cetylpyridinium chloride (CPC) on the cell surface hydrophobicity and adherence of *Candida albicans* to human buccal epithelial cells in vitro. Pharm. Res. 12: 1896–1900

King, R. D., Lee, J. C., Morris, A. L. (1980) Adherence of *Candida albicans* and other *Candida* species to mucosal epithelial cells. Infect. Immunol. 27: 667–674

Miles, A. A., Misra, S. S. (1938) The estimation of the bactericidal power of blood. J. Hyg. 38: 732–749

Odds, F. C. (1988) *Candida* and Candidosis, a Review and Bibliography, Baliere Tindall, London, pp 115–230

Pettipher, G. L., Mansell, R., McKinnon, C. H., Cousins, C. M. (1980) Rapid membrane filtration-epifluorescent microscopy technique for the direct enumeration of bacteria in raw milk. Appl. Environ. Microbiol. 39: 423–429

Rodrigues, U. B., Kroll, R. G. (1985) The direct epifluorescent filter technique (DEFT): increased selectivity, sensitivity and rapidity. J. Appl. Bacteriol. 59: 493–499

Rodrigues, U. B., Kroll, R. G. (1986) Use of epifluorescent filter technique for the enumeration of yeasts. J. Appl. Bacteriol. 61: 139–144

Rosenstein, I. J., Grady, D., Hamilton-Miller, J. M. T., Brumfitt, W. (1985) Relationship between adhesion of *Escherichia coli* to uroepithelial cells and the pathogenesis of urinary infection: problems in methodology and analysis. J. Med. Microbiol. 20: 335–344

Sandin, R. L., Rogers, A. L., Fernandez, M. I., Beneke, E. S. (1987) Variations in affinity to *Candida albicans* in vitro among human buccal epithelial cells. J. Med. Microbiol. 24: 151–155

Schep, L. J., Jones, D. S., Schep, G., Shepherd, M. G. (1994) A method to study the adherence of *Candida albicans* to human buccal epithelial cells in vitro using $^{35}SO_4$ radiolabelled blastospores and a Percoll gradient. Int. J. Pharm. 105: 39–45

Tobgi, R. S., Samaranayake, L. P., MacFarlane, T. W. (1987) Adhesion of *Candida albicans* to buccal epithelial cells exposed to chlorhexidine gluconate. J. Med. Vet. Mycol. 25: 335–338

Woolfson, A. D. (1993) The statistical evaluation of adherence assays. In: Denyer, S. P., Gorman, S. P., Sussman, M. (eds) Microbial biofilms: formation and control, Society for Applied Bacteriology, Technical Series Number 30, Blackwell Scientific Publications, Oxford, pp 315–325