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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

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Abstract

This study describes the use of radiolabelled ($^{35}SO_4$) blastospores to examine the adherence of *Candida albicans* (MEN strain serotype B, isolated from an eye infection) to human buccal epithelial cells in vitro. The isotope showed good retention (> 85%) within the blastospore over the time of the adherence assay. Separation of the adherent from non-adherent blastospores was successfully achieved using a Percoll™ gradient and two successive centrifugation steps using a vertical rotor centrifuge. The results obtained from this method were compared to those obtained using a modified light microscopic assay which employs periodic acid Schiff reagent to selectively stain the blastospore and buccal epithelial cell population. This staining technique allowed visualisation, and hence estimation of the number, of adherent *C. albicans* on the underside of the epithelial cell. The results showed that there was a good correlation between the two methods ($r = 0.946$, $p > 0.4$ paired one tailed t-test); however, the radiometric method was found to be less tedious to perform and less prone to subjective variation. Therefore, it is suggested that this method may be of use in future assessments of the in vitro adherence of *C. albicans* to epithelial cells.

Key words: Candida albicans; Adherence assay; Radiolabelled blastospore; Microscopy; Percoll™

1. Introduction

Candida albicans is a commensal of humans which is commonly found in the oral cavity, gastrointestinal and female genital tracts. However, under certain circumstances in which there is debilitation of the host, e.g., tissue trauma, dietary deficiency, physiological disorders or compromisation following cancer chemotherapy or excessive antibiotic usage, this organism may become pathogenic (Odds, 1979).

It is accepted that the adherence of microorganisms, e.g., *C. albicans*, to epithelial cells is the initial (requisitory) step in the process of infection as it enables the pathogen to overcome the normal flushing mechanisms of body secretions, e.g., saliva (Beachey, 1981; Kennedy, 1988). The adherence phenomenon has been suggested to be composed of two distinct phases, termed reversible and irreversible. The initial phase is

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thought to be governed by the Deryagin, Landau, Verwey and Overbeek theory and therefore as the pathogen approaches the host epithelium it will be subjected to both attractive (due to hydrophobic bonding between hydrophobic molecules on the surface of the yeast and epithelial cells) and repulsive forces, the latter due to the similarity of charge on the surface of the two interacting cells. If the attractive forces predominate, then the yeast may approach and interact with the epithelial cell at short distances, referred to as the primary minimum. At this stage the attachment is enhanced via the interaction between adhesin molecules on the surface of the yeast cell and the corresponding receptors on the surface of the epithelial cell (Douglas, 1987). The identity of the candidal adhesin(s) is still under debate, with the majority of experimental evidence suggesting a role for mannoproteins (Douglas, 1987). However, some authors have reported that chitin and chitin-soluble extracts (Segal et al., 1982) and lipids (Ghannoum et al., 1986) may function as the candidal adhesin(s).

Whilst the majority of studies concerning microbial adherence to epithelial cells have involved bacteria (Jones and Isaacson, 1983; Savage and Fletcher, 1985), there are a number of reports concerning the adherence of *C. albicans* to human epithelial cells in vitro. These include: buccal epithelial cells (Kimura and Pearsall, 1978; King et al., 1980; Segal et al., 1982; Calderone et al., 1984; Jones et al., 1986), human vaginal epithelial cells (King et al., 1980; Sobel et al., 1981), gastrointestinal epithelial cells of mice (Pope and Cole, 1981), uroepithelial cells (Botta, 1981; Centeno et al., 1983) and epidermal cornecytes (Botta, 1981). Most commonly, due to the ease of collection and the incidence of candidosis of the mouth and vagina, the adherence of *C. albicans* to buccal and vaginal epithelial cells has been investigated (Douglas, 1987).

The assessment of candidal adherence in vitro is usually performed using light microscopy. In this method equal volumes of standardised yeast and epithelial cells are mixed and agitated at 37°C for a specified time interval. After this, the epithelial cells (±adherent yeast cells) are collected, stained and the number of adherent yeasts per epithelial cell counted using light microscopy (Sobel et al., 1981; Epstein et al., 1982; Segal et al., 1982). However, the light microscopic method, despite its widespread usage, is plagued by inherent faults including the tedium associated with counting of adherent yeasts and the small population size of epithelial cells counted. Therefore, the aim of this study was to develop a rapid, reproducible, efficient assay for the measurement of adherence of *C. albicans* to human buccal epithelial cells in vitro. This method employs radiolabelled yeast cells and a Percoll™ density gradient to separate the non-adherent from adherent yeast cells. In addition, the results from this method have been compared to those obtained using a modified light microscopic method.

2. Materials and methods

2.1. Chemicals

$^{35}SO_4$ and Aqueous Scintillation Cocktail (ACS) were obtained from Amersham (U.K.).

Percoll™ was obtained from Pharmacia (Auckland, New Zealand).

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

2.2. Epithelial cells

Buccal epithelial cells (BEC) were collected by scraping the buccal mucosa of healthy male and female volunteers using sterile ampoule files as previously described (Jones et al., 1986; Gorman et al., 1987a,b). The volunteers were non-smokers and were not taking medication. Following scraping, the cells were placed in sterile phosphate-buffered saline (PBS, 0.01 M, pH 7.3), shaken and washed. The cells were then passed through a 22 gauge needle to disperse and remove sheets of epithelial cells and resuspended in sterile PBS to approx. 4.0×10^5 cells ml^{-1}. Epithelial cell counts were performed using an electronic particle counter (Coulter ZM). Epithelial cells were collected daily for use on the same day.

2.3. Yeasts and culture conditions

C. albicans (MEN serotype B, isolated from an eye infection) was maintained on yeast extract

agar slopes at 4°C. Blastospores were radiolabelled with $^{35}SO_4$ using a modification of a method previously reported (Farley et al., 1986). Two loopfuls of *C. albicans* were inoculated into sulphur minimum glucose salts biotin (GSB) medium (pH 5.2), in which $(NH_4)_2SO_4$ had been replaced with $(NH_4)H_2PO_4$ (1 g l^{-1}) and spiked with approx. 7.0 μCi $^{35}SO_4$ and this was incubated at 28°C in a shaking water bath for 12 h at 150 oscillations/min. The cells were then centrifuged (1000 × *g* for 10 min) and resuspended in GSB medium containing $(NH_4)_2SO_4$ (1 g l^{-1}). In both GSB media, peptone had been replaced with bactopeptone (Difco, 1 g l^{-1}). The blastospores were then grown for a further 6 h at 28°C, washed in PBS and resuspended to the required cell count. The absence of germinated cells was confirmed using light microscopy.

2.4. Adherence assay

Equal volumes of *C. albicans* (4×10^7 cfu ml^{-1}) and BEC (4×10^5 ml^{-1}) were mixed and incubated at 37°C for 30 min using a shaking water bath (120 cycles/min). Examination of the candidal/BEC adherence was then performed using two methods, the radiometric method and light microscopy. In the radiometric method, a sample of the incubated cells (600 μl) was removed, mixed with PBS (0.1 M, 150 μl) and Percoll™ (750 μl) and centrifuged at 10 000 × *g* for 30 min (Biofuge B-vertical rotor). This separated the cell population into two layers, the upper layer containing BEC ± adherent yeast cells and the lower layer containing non-adherent yeast cells. The top layer (600 μl) was removed, mixed with further aliquots of PBS (150 μl, 0.1 M) and Percoll™ (750 μl) and centrifuged as before. The epithelial cell (upper) layer (600 μl) was then removed, an aliquot mixed with saline (100 μl) and the number of BEC assayed using a Coulter counter (orifice size 200 μm). Two centrifugation steps were required to fully separate the cell populations. The value obtained from this assay was used in the final calculation of the number of *C. albicans* adherent per BEC. Epithelial cell integrity, following the centrifugation steps and the adherence assay, was determined by comparing size distribution profiles with the stock solution of epithelial cells. To a further sample of the epithelial cell layer (400 μl), which had been removed from the Percoll™ gradient, was added 5 ml of aqueous scintillation cocktail and the counts per min (cpm) associated with the sample assayed using a liquid scintillation counter (Beckman model 6000).

As a control, the extent of adherence which occurred during the centrifugation step was investigated by adding 600 μl of the yeast/BEC mixture (before incubation) to PBS (0.1 M, 150 μl) and Percoll™ (750 μl) and centrifuging twice as before. The cpm associated with the epithelial cell layer was determined as outlined previously. The cpm per yeast cell of the stock suspension was determined following shaking a sample of known cell density (approx. 2×10^7 cfu ml^{-1}) in a shaking water bath at 37°C and centrifugation as outlined previously. Therefore, the cpm associated with the epithelial cell layer may be converted into a number of adherent *C. albicans*. Furthermore, as the number of epithelial cells present in this sample is known (estimated using the Coulter counter), the number of adherent *C. albicans* per BEC may be calculated. The number of adherent yeast cells per BEC which was a result of the centrifugation steps must be deducted from this value to obtain a final value of the number of adherent yeast cells per BEC.

All radiometric evaluations of adherence were performed in quadruplicate.

In the light microscopic method, two loopfuls of the *C. albicans*/BEC incubation mixture were placed onto a microscope slide and stained using the periodic acid Schiff reaction (Hotchkiss, 1948; McManus, 1948). This reagent stained the blastospores magenta and the epithelial cells green. Following staining the number of adherent *C. albicans* to at least 200 BEC was counted (Jones et al., 1986; Gorman et al., 1987a,b).

3. Results and discussion

Table 1 shows the mean (± standard deviation) of adherent blastospores per BEC as evaluated by both the light microscopic and the radiometric methods. Within the fourteen experiments, the

Table 1
The number of adherent *Candida albicans* [a] ± standard deviation (S.D.) per buccal epithelial cell as determined by light microscopy and a radiometric assay using $^{35}SO_4$ blastospores

Assay no.	Radiometric assay	Light microscopy
1	12.30 ± 1.60	12.90 ± 10.60
2	15.20 ± 2.10	13.60 ± 13.00
3	12.80 ± 1.50	12.10 ± 13.60
4	11.00 ± 1.80	12.00 ± 13.10
5	11.50 ± 0.23	11.90 ± 13.80
6	8.30 ± 1.40	9.00 ± 10.90
7	5.00 ± 0.32	6.30 ± 6.50
8	9.30 ± 1.00	11.50 ± 15.00
9	10.70 ± 1.20	10.90 ± 11.00
10	7.80 ± 1.90	8.40 ± 9.60
11	9.90 ± 0.80	10.00 ± 9.80
12	11.30 ± 1.90	11.60 ± 10.50
13	11.00 ± 2.00	10.90 ± 11.50
14	12.60 ± 1.20	12.60 ± 13.70

[a] MEN serotype B, isolated from an eye infection. Differences in mean values analysed by a one-tailed paired *t*-test ($p > 0.4$).

range of the number of adherent blastospores per BEC was 5.0–15.2 for the radiometric method and 6.3–13.6 for the light microscopic method. These upper and lower variations in adherence were obtained from the same experiment and hence from the same pool of *C. albicans* and BEC, in each case.

The correlation between the results obtained from the two methods was assessed by linear regression ($r = 0.946$). This implies that there is a good correlation between the results obtained from the two methods.

Since the early reports of candidal adherence (Liljemark and Gibbons, 1973; Kimura and Pearsall, 1978), numerous workers have attempted to identify the nature of the interaction between *C. albicans* and epithelial cells and the environmental factors which affect this interaction (King et al., 1980; Douglas et al., 1981; Segal et al., 1982; McCourtie and Douglas, 1984, 1985). In addition, several investigators have considered methods by which this interaction may be reduced or inhibited, including the use of isolated adhesin or adhesin analogs (Lee and King, 1983; Collins-Lech et al., 1984), lectins (Sandin et al., 1982; Critchley and Douglas, 1987), sub-lethal concentrations of antibiotics (Douglas and McCourtie, 1983) and non-antibiotic antimicrobial agents (Jones et al., 1986; Gorman et al., 1987a,b). In the majority of these investigations, the assay employed to quantify the adherence interaction has involved light microscopy which, while uncomplicated in approach, is laborious, may be subjective and has associated inherent problems. One such problem is the choice of staining technique to allow adequate visualization, and thus quantification, of the *C. albicans*/epithelial cell interaction. Previously, authors have employed crystal violet (Kimura and Pearsall, 1978; Jones et al., 1986; Gorman et al., 1987a,b; Kennedy and Sandin, 1988) whilst others (King et al., 1980) have employed wet mounts which were examined by dark field microscopy. Crystal violet stain is non-specific and consequently, the epithelial cells are stained a similar colour to the microbial cell. Therefore, whenever this stain is employed to visualise adherence interactions, *C. albicans* adherence is frequently examined from a two dimensional perspective, i.e., only the *C. albicans* adherent to the top side of the epithelial cell are counted. This leads to difficulty whenever a correlation between results obtained using light microscopy and radiometric methods is required as the latter method accounts for adherence in three dimensions. An obvious approach to overcome this problem would be to use a stain which colorimetrically differentiates between yeast and epithelial cells. In the present study, this criterion was achieved by the use of a periodic acid Schiff reaction (Hotchkiss, 1948; McManus, 1948). This stains the cell wall of fungi magenta and epithelial cells a pale green, thus allowing visualization of adherent blastospores on the underside of the epithelial cell.

Despite the widespread use of the light microscopic method to monitor candidal adherence, it is accepted that the method is tedious (Douglas, 1987). In general, most investigators count the number of *C. albicans* adherent to at least 100 epithelial cells, a process which requires several hours. In addition, considering that initially approx. 1×10^5 epithelial cells are included in the assay, a population size of 100–200 cells would seem an inadequate number to statistically de-

scribe and hence interpret the results from an adherence assay. Subsequently, other authors have explored alternative approaches including the use of the Coulter counter (Gorman et al., 1986) and radiolabelling methods. For example, King et al. (1980) examined the adherence of ^{14}C-labelled *C. albicans* to mucosal epithelial cells. In the present study, *C. albicans* was labelled with $^{35}SO_4$ as previously described (Farley et al., 1986). Whilst it has been recognised that one of the problems concerning the use of isotopes is leaching of the isotope from the microbial cell (Douglas, 1987), in this study the retention of $^{35}SO_4$ by *C. albicans* was always greater than 85% for the duration of the adherence assay and greater than that for ^{14}C (which ranged from 12 to 37% retention) and ^{3}H (which was approx. 11%), (results not shown). To our knowledge, $^{35}SO_4$ has been employed in only one other adherence study, in which the adsorption of radiolabelled bacterial (*Proteus mirabilis*) outer membrane material to uroepithelial cells was examined (Wray et al., 1986). However, these authors used light microscopy to quantify the adherence of *Pr. mirabilis* (whole cells) to uroepithelial cells.

One important factor in the development of a reliable radiometric assay is the complete separation of adherent from non-adherent *C. albicans*. Typically, after the incubation period, the standardised suspension of *C. albicans* and epithelial cells has been filtered through 10 μm pore size polypropylene filters. Theoretically, this filter retains the epithelial cells, with or without adherent *C. albicans*. These filters are usually digested with a solubiliser, scintillation cocktail is added and the associated disintegrations per min are monitored using liquid scintillation (King et al., 1980). The use of filters has also frequently been used as part of the light microscopic method (Kimura and Pearsall, 1978; Sandin et al., 1987). However, in some reports, this separation step has been omitted and the yeast/epithelial cell suspension has been directly placed on a microscope slide and stained before counting the number of adherent yeasts/epithelial cell (Jones et al., 1986; Gorman et al., 1987a,b).

Initially, in this current study the separation of adherent from non-adherent *C. albicans* was attempted using 10 μm polypropylene filters. However this method produced unreproducible results (not shown), which were probably a consequence of filter blockade by epithelial cells. Therefore, as a alternative, the use of Percoll™, a density gradient centrifugation medium commonly used to separate cell populations, was investigated. Percoll™ has been previously used in a bacterial/epithelial cell adherence assay to separate adherent from non-adherent *Streptococcus mitis* C5, *Actinomyces naeslundii* 12104 and *Bacteroides gingivalis* 381 (Childs and Gibbons, 1988). These workers reported that a Percoll™ density gradient separated the cell population into two layers, one containing the epithelial cells ± adherent bacteria and the other containing the non-adherent bacteria. In the present study, the epithelial cells (± adherent yeast cells) were retained at the top of the density gradient whereas the unattached yeast cells collected as a pellet at the bottom of the centrifuge tube following centrifugation.

One advantage of the method developed in the current study over that reported by Childs and Gibbons (1988) is the use of a Coulter counter to exactly count the number of epithelial cells recovered from the Percoll™ density gradient. This improves the accuracy of the final calculation of the number of adherent yeast cells per epithelial cell. Childs and Gibbons reported that the recovery of epithelial cells from the Percoll™ gradient was greater than 95%, although in our present study we rarely achieved greater than 85% recovery, hence the importance of an accurate technique to exactly count the number of BEC.

Another important factor in the experimental design was the use of a vertical rotor in the centrifuge. Originally, an angle rotor was employed, however, this failed to separate the cell population into two distinct bands. Instead, the candidal layer was smeared along the length of the centrifuge tube. The use of a vertical rotor was observed to effect an adequate separation of the two cell populations.

One final comment comcerning the radiometric assay method is the necessity of a control in the calculations to account for the adhesion which occurs during the centrifugation steps, if correla-

tion with the light microscope method is required. This consistently exhibited values ranging from 0.8 to 1.4 *C. albicans*/BEC.

There is a good correlation ($r = 0.946$) between the number of yeast cells/BEC as evaluated by the radiometric and light microscopic methods (Table 1). Application of a one tailed paired *t*-test to this data confirms that any differences between the values occurs only by chance ($p > 0.4$), i.e., any difference is insignificant. Upon first observation, one striking difference between the two pools of data displayed in Table 1 is the disparity between the standard deviation (S.D.) values associated with the two methods. The S.D. values associated with the light microscopic method represent the variation in the number of adherent yeasts/BEC about the mean in a population size of 200 BEC, i.e., it is an indication of intraassay variation. Such large values of S.D. associated with microbial/epithelial cell adherence assessed using light microscopy have been previously reported (Gorman et al., 1986; Woolfson et al., 1987) and are accredited to the skewed nature of adherence data. The S.D. values reported for the radiometric assay represent the variation about the mean of four replicate experiments, i.e., an indication of interassay variation. The observed low values of S.D. associated with the radiometric assay would indicate that this method exhibits good reproducibility.

In conclusion, the radiometric assay reported offers a quick, convenient (non-tedious), reproducible method by which the adherence of *C. albicans* to buccal epithelial cells may be examined. The results from this assay are expressed as a mean number of adherent organisms per epithelial cell and therefore may be useful whenever the effect of a certain parameter on candidal adherence (either environmental or chemical) is under examination. However, unlike the light microscopic method, this radiometric method cannot be used to characterise the number of adherent yeast cells on each individual BEC and, in addition, is reliant upon the availability of an accurate system (a Coulter counter or cell counting chamber) to count the number of epithelial cells after separation from non-adherent yeasts using Percoll™. It is also suggested that, whenever light microscopy is used to investigate candidal adherence, the periodic acid Schiff reagent should be employed to stain the yeast and BEC. This staining system allows adequate visualisation of *C. albicans* adherence to the underside of the epithelial cell.

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