Adherence of Candida albicans to epithelial cells: studies using fluorescently labelled yeasts and flow cytometry

Itzhack Polacheck, Adi Antman, Immanuel Barth, Ephraim Sagi and Haim Giloh

Candida albicans adherence to epithelial cells is the first step in the infectious process, but in spite of its importance, current methods for the quantitative measurement of adherence of C. albicans to epithelial cells in vitro have some serious limitations. They are based on filtration assays and either microscopic or radiometric analysis. The adherence reaction is usually carried out with a large excess of yeasts (100-fold) over epithelial cells in order to perform the microscopic analysis, which is slow, subjective and limited to 100–200 cells and thus lacks statistical power. The radiometric analysis fails to measure individual cells. A method for measuring yeast adherence that overcomes these problems has been developed. It is based on labelling the yeasts with the fluorogenic marker 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (BCECF) prior to the adherence reaction, and analysing 10⁴ epithelial cells by flow cytometry, while non-bound yeasts are excluded by gating. Two subpopulations of buccal epithelial cells (BECs) which differ in their mean fluorescence intensities per cell (MFI's) were observed: one with MFI which did not exceed nonspecific fluorescence, and the other with MFI as high or higher than the MFI of labelled yeasts. The two subpopulations represent yeast-free and yeast-binding epithelial cells, respectively, and the MFI increment of the BECs is a quantitative measure of the extent of yeast adherence. Control experiments confirming previously described basic features of adherence, such as enhanced adherence at increasing yeast excess, diminished adherence of trypsin-treated or heat-inactivated yeasts, and the differential adherence of various Candida species, supported the validity of the assay. The possibility of studying adherence reliably at low yeast: epithelial cell ratios, which better mimic adhesion as it occurs in vivo, is an important advantage of the assay. New findings, using this method, included the observation that exfoliated BECs from diabetic patients exhibited the same capacity for C. albicans adherence as cells from healthy controls, and that epithelial cells from early human ontogenic stages had a significantly lower adherence level than those from later stages.

Keywords: Candida albicans, adherence, epithelial cells, flow cytometry

INTRODUCTION

Candida albicans is known as a commensal microorganism on cutaneous and mucous membranes, particularly of the alimentary tract (Kwon-Chung & Bennett, 1992; Odds, 1988). It is the aetiologic agent of a wide spectrum of infections that vary from superficial mucosal...
lesions to life-threatening systemic or disseminated disease. The type of Candida infection mainly depends on the specific defects in host defence (Kwon-Chung & Bennett, 1992; Odds, 1988; Segal et al., 1984). Oral and vaginal infections are the most common forms of superficial candidiasis (Kwon-Chung & Bennett, 1992; Odds, 1988; Sobel et al., 1981).

Yeast adherence to host cells is the first step in the infectious process, preceding colonization and invasion (Calderone, 1993; Cutler, 1991; Kennedy, 1988; Kennedy et al., 1992; Odds, 1988). Adherence protects the microorganism from being removed by natural host defence and clearance systems such as peristalsis and the bathing actions of fluids over mucosal surfaces (Kennedy, 1988).

Yeast adherence has been studied mostly with C. albicans because of the increasing importance of this pathogen (Kennedy et al., 1992; Kwon-Chung & Bennett, 1992; Odds, 1988, 1994). Many factors influence yeast adhesion in vitro: they include host and fungus-related factors (Kennedy, 1990; Kennedy et al., 1992). Several reviews relating adherence of C. albicans to virulence have been published (Calderone & Braun, 1991; Cutler, 1991; Edwards et al., 1986; Hostetter, 1994; Kennedy, 1990; Kennedy et al., 1992; Segal et al., 1984).

The remarkable progress in revealing the existence of multiple mechanisms of adherence was made possible to a great extent by the development of an experimental system to reproduce and follow the process of adherence in vitro (Kimura & Pearsall, 1978; King et al., 1980). Adherence of C. albicans to a variety of surface cells that may represent attachment sites for Candida infections has been demonstrated in vitro: to exfoliated human buccal epithelial cells (BECs), vaginal, urogenital and corneal cells (Calderone & Braun, 1991; Kennedy, 1988; Segal et al., 1984). Candida interaction with various blood components has also been demonstrated (Edwards et al., 1986; Kennedy et al., 1992; Yeaman et al., 1994), and may be involved in complement-associated phagocytosis and haematoagogenous dissemination (Odds, 1994; Segal et al., 1984). In addition, Candida cells bind in vitro to several inert materials that are used to produce implantable medical and dental devices (Klotz et al., 1985; Rotrosen et al., 1986). In vitro adherence of C. albicans to BECs is influenced by environmental, yeast-related and epithelial-cell-related factors (Calderone, 1993; Kennedy, 1988, 1990).

Although extensive studies have been made on adherence in vitro, the assay system and its modifications have only occasionally been discussed (Kennedy, 1988). In vitro, host cells are exposed to untreated or radioactive-isotope-labelled yeasts and yeast adhesion is determined by the conventional filter assay (Kimura & Pearsall, 1978; King et al., 1980). The assay is based on the retention of the relatively large epithelial cells, including those with adherent yeasts, while nonadherent yeasts pass through the filter. It is then possible to determine the number of epithelial-cell-attached yeasts either microscopically (Kimura & Pearsall, 1978) or by a radiometric assay (King et al., 1980). Microscopy also allows the enumeration of host cells to which yeasts adhered. Microscopic analysis, however, has serious limitations, being rather slow, highly subjective (influenced by preparative and analytical differences of the individual researcher) and, most importantly, restricted to a small number of cells and therefore lacks statistical power. The radiometric assay, on the other hand, cannot estimate yeast adhesion to individual epithelial cells, which is important for the analysis of the phenomenon of adherence.

We describe a method for measuring adherence that overcomes these problems. Yeasts are labelled with a fluorescent marker prior to their adherence to epithelial cells, and adherence is measured by flow cytometry (Shapiro, 1988). This method enables the study of large populations of cells in a short time. The flow cytometer displays the histogram of individual cellular fluorescence intensities in the entire analysed cell population, and the characteristics of yeast adherence under varying experimental conditions are computed from the histogram data and can be compared directly. Using this method, previously described basic features of adherence, such as temperature and growth phase dependence, and the differential adherence of various Candida species, were confirmed. In addition, we studied the impact of a pathological state (diabetes mellitus) and the human ontogenic development on adherence.

**METHODS**

**Yeast strains, media and growth conditions.** The following yeast species were used in this study: Candida albicans CBS 562 (type strain), Candida parapsilosis, Candida tropicalis, Candida krusei and Torulopsis glabrata. Except for the type strain of C. albicans, all isolates were clinical, taken from our laboratory collection, including additional C. albicans strains. They were maintained on Sabouraud dextrose agar (SDA) at 4 °C and characterized by using the API 20C AUX (BioMérieux).

Yeasts cultured on SDA for 24-48 h at 30 °C were suspended in 20-40 ml yeast nitrogen base broth (YNB; Difco) containing 10 g glucose l⁻¹ and 10 mM phosphate buffer (pH 7), to give a concentration of 10⁶ cells ml⁻¹. This culture yielded 2 × 10⁸-4×10⁹ cells ml⁻¹ after 20 h at 30 °C (mid-late exponential phase). The cells were collected by centrifugation at 1800 g and 22 °C, washed three times with 10 mM PBS (0-145 M NaCl, 0.01 M sodium phosphate), pH 7-4, and counted in a haemocytometer.

**Preparation of fluorescent markers.** (i) A stock solution of 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF) (50 µg amperules; Molecular Probes) was prepared by adding 50 µl dried dimethylsulfoxide (DMSO; Fluka Chemie) to the original amouple. The stock solution was stored at −20 °C in small aliquots for no longer than 2 weeks. The stability of BCECF in solution was monitored by measuring the appearance of its fluorescent hydrolysis products by spectrofluorimetry. (ii) A stock solution of 5,6-carboxyfluorescein disodium (CFDA; Sigma) in DMSO (10 mg ml⁻¹) was prepared monthly and stored at 4 °C. (iii) 4,4'-bis-(4-Anilino-bis-diethylamino-5-triazin-2-ylamino)-2,2'-stilbene-disulfonic acid (Celuloflex stain; Polysciences) was diluted in PBS. (iv) FITC-ConA [concanavalin A type IV (fluorescein-isothiocyanate labelled; Sigma)] was diluted in PBS. (v) 4,6-Diamidino-2-phenylindole (DAPI; Sigma) was dissolved in ethanol (10 mg ml⁻¹) and diluted in PBS. (vi)
PKH26-GL (Zynaxis) was dissolved in the diluent supplied with the dye and finally mixed with the cell suspension at a 1:1 (v/v) ratio.

**Labelling of yeast cells.** Yeast cells (5 × 10^7 cells ml^-1) were incubated in YNB without amino acids and ammonium sulfate (Difco) and lacking the carbon source, on a shaker (100 oscillations min^-1) for 1 h at 30 °C in the presence of BCECF or one of the other markers that were tested. The final marker concentrations, after optimization of the labelling conditions, are detailed in Table 1. Subsequently, the cells were collected on a 0.45 μm pore-size cellulose nitrate filter of 25 mm diameter (Schleicher & Schuell) and washed five times by suction with a total of 150 ml PBS. Finally, the yeast cells were resuspended by vortexing the filter in a tube containing 1.5 ml PBS.

**Retention of the fluorescent label in yeasts.** Labelled, washed yeasts were incubated at 37 °C in fresh PBS for 1 h and then filtered as described above. The cells were resuspended in PBS and their fluorescence was determined by fluorescence microscopy or flow cytometry. Appearance of fluorescent compounds in the incubation medium was monitored by spectrofluorimetry.

**Exfoliated BECs.** BECs were collected by gentle scraping from ten healthy, nonsmoking, male and female adult donors, or from full-term or premature neonates. The cells were suspended in PBS, vortexed for 15 min to disrupt intercellular bonds, pooled, spun down by centrifugation at 400 g and 22 °C, washed twice and resuspended in 1 ml of the same buffer. The epithelial cells were centrifuged through a continuous density gradient of Percoll (Pharmacia LKB Biotechnology) to obtain a homogeneous single cell suspension minimally contaminated with cell debris and aggregates (Goldenbush et al., 1982). The cell suspension was layered over 7 ml 25% (v/v) Percoll in PBS and centrifuged at 20000 g and 22 °C for 30 min. The uppermost band from the gradient was collected and diluted fivefold with PBS. The cells were then washed twice in PBS and resuspended in the same buffer. Vortexing was avoided after the gradient centrifugation, since this had an adverse effect on epithelial cell integrity. Cell viability, checked by methylene blue exclusion (0.1% methylene blue in PBS), was greater than 90%.

**Foetal epithelial cells.** Amniotic fluid was obtained by amniocentesis from five or six healthy women in their 17th-22nd week of pregnancy. The cells were pelleted by centrifugation, washed twice in PBS similarly to the BECs and used without further treatment.

**Adherence of yeasts to epithelial cells.** In the standard adherence reaction, 1 × 10^6 epithelial cells were mixed with yeast cells in a final volume of 0.4 ml PBS at a varying yeast:epithelial cell ratio (1:1–100:1) as detailed for each experiment. The cell mixture and control tubes containing only yeasts or epithelial cells in PBS were incubated at 37 °C for 60 min with shaking (100 oscillations min^-1). At the end of the incubation period, the extent of yeast adherence was assayed by the conventional filter assay and microscopy, or by flow cytometry.

**Nonspecific fluorescent labelling of epithelial cells in the adherence reaction.** Evaluation of the extent of nonspecific BEC labelling, caused by efflux of fluorescent label from the yeasts during the adherence reaction, was performed in two steps. First, labelled yeast cells were incubated in the presence or absence of BECs in PBS under conditions mimicking those of the adherence reaction (60 min at 37 °C). Omission of BECs during this incubation did not affect the extent of efflux of fluorescent label from the yeasts. At the end of the incubation, the reaction mixture was passed through a 0.45 μm pore-size cellulose nitrate filter. In the second step, freshly prepared epithelial cells were incubated in the yeast-free filtrate for 60 min at 37 °C. Their fluorescence was analysed by flow cytometry and scanning fluorescence microscopy. Any increase in epithelial cell fluorescence during the second incubation was designated as nonspecific labelling. It increased as a function of incubation time and yeast: B EC (Y: BEC) ratio in the first incubation.

**The conventional filter assay for adherence.** At the end of the adherence reaction, the yeast/epithelial-cell mixture was filtered through a 25 mm diameter, 12 μm pore-size cellulose nitrate filter (Schleicher & Schuell). Nonadherent yeast cells were

---

**Table 1.** Fluorescent marker properties and optimal conditions for yeast labelling

<table>
<thead>
<tr>
<th>Fluorescent marker (concn)*</th>
<th>Labelling conditions</th>
<th>Cellular target</th>
<th>Yeast fluorescence intensity†</th>
<th>Yeast fluorescence uniformity</th>
<th>Marker leakage out of yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellufluor (1 μg ml^-1, 1.4 × 10^-4 M)</td>
<td>1 h, pH 4</td>
<td>Cell wall</td>
<td>++</td>
<td>Non-uniform</td>
<td>Significant</td>
</tr>
<tr>
<td>FITC-ConA (10 μg ml^-1, 1.8 × 10^-7 M)</td>
<td>1 h, pH 4</td>
<td>Cell wall</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DAPI (10 μg ml^-1, 2.9 × 10^-4 M)</td>
<td>1 h, pH 4</td>
<td>Nucleus</td>
<td>++</td>
<td>High</td>
<td>Significant</td>
</tr>
<tr>
<td>PKH26-GL (1 × 10^-9 M)</td>
<td>0.5 h, pH 7</td>
<td>Membrane</td>
<td>±</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CFDA (50 μg ml^-1, 9.4 × 10^-6 M)</td>
<td>1 h, pH 4</td>
<td>Vacuoles, cytoplasm</td>
<td>++</td>
<td>High</td>
<td>Significant</td>
</tr>
<tr>
<td>BCECF (2 μg ml^-1, 2.4 × 10^-6 M)</td>
<td>1 h, pH 7</td>
<td>Vacuoles, cytoplasm</td>
<td>++</td>
<td>High</td>
<td>Low‡</td>
</tr>
</tbody>
</table>

ND, Not determined.

* Markers were screened by labelling C. albicans cells with them under optimal conditions.

† Yeast fluorescence intensity was analysed by fluorescence microscopy and quantified by flow cytometry.

‡ Below the limit of detection by spectrofluorimetry of the incubation medium.
eliminated by washing the filter with PBS by suction. The epithelial cells, with or without adherent yeasts, remained on the filter and were subsequently fixed by inverting the filter onto a drop of ethanol on a microscope slide. The slides were air-dried. From each sample, 100 epithelial cells were examined with a Zeiss Standard microscope equipped with phase-shift optics. The percentage of cells to which yeasts adhered was determined, and the number of yeast cells that adhered to each of the epithelial cells was counted.

Flow cytometry. The FACScan flow cytometer (Becton Dickinson), calibrated routinely for immunofluorescence analysis, was used to determine the fluorescence intensities of yeasts and epithelial cells. Following yeast labelling or adherence of yeasts to epithelial cells, the entire reaction mixture was applied to the instrument without further treatment. To determine the extent of adherence, nonadherent yeasts were 'gated out' electronically as described in Results. Routinely, the fluorescence of $1 \times 10^4$ epithelial cells was analysed ($2-5 \times 10^3$ cells s$^{-1}$) in signal height mode. The excitation wavelength was 488 nm, and emitted light was collected via a 530/30 band pass filter. Data were processed and analysed in the FACScan by the Lysys II program.

Scanning fluorescence microscopy. Washed epithelial cells were resuspended in a small volume of PBS and analysed on the ACAS 570 (Meridian Instruments). Excitation and emission conditions were as described for flow cytometry. The fluorescence of 10–20 cells was scanned using the ×40 objective, and each cell was marked on its periphery by a polygon to quantitate the integrated fluorescence per unit cell area.

Trypsin and heat treatment of yeast cells. Prior to the adherence reaction, $5 \times 10^7$ BCECF-labelled C. albicans cells in 1 ml PBS were incubated either for 15 min at 56 °C, or at 37 °C with different concentrations of trypsin (20, 50 or 200 pg ml$^{-1}$; Sigma) or preheated trypsin (200 pg ml$^{-1}$, 100 °C for 5 min). Treatment was stopped by cooling to 4 °C and washing the cells with PBS. Neither yeast final fluorescence intensity nor the extent of label leakage from the yeasts was affected.

Miscellaneous fluorescence assays. Preliminary, qualitative fluorescence analysis of samples was performed using a CH-2 epifluorescence microscope (Olympus). The chemical stability of marker solutions and efflux of marker from labelled yeast cells into the incubation medium were measured with a MPF 44-A spectrofluorimeter (Perkin Elmer).

RESULTS

Screening of fluorescent markers for C. albicans

The development of the flow cytometric assay for adherence of C. albicans to epithelial cells required a fluorescent marker that labels C. albicans. Potential markers were screened for the following factors: (i) chemical stability under the experimental conditions; (ii) labelling of yeasts to high fluorescence intensity and with uniformity; (iii) relatively short labelling time; (iv) retention of the fluorescence marker within the labelled yeasts; (v) minimum effect on yeast adherence to epithelial cells. Table 1 summarizes the characteristics of yeast labelling with the tested fluorescent markers.

High yeast cell fluorescence was needed for reliable identification of the adhesion of a single yeast to a BEC. Marker retention, as monitored by reincubation of labelled washed yeasts and spectrofluorimetric examination of the fresh incubation medium, was, however, the most important criterion for marker selection. Marker efflux from the labelled yeasts resulted not only in the gradual decrease of yeast fluorescence, but in the subsequent nonspecific transfer of label from the medium to the epithelial cells during the adherence reaction.

Of all the markers tested, BCECF (Table 1), which was used for assaying yeast phagocytosis and killing and did not disturb cell growth and division (Martin & Bhakdi, 1991), led to the highest yeast fluorescence intensity and uniformity. Both CFDA (Table 1) and BCECF are fluorogenic esters of fluorescein that are hydrolysed to fluorescent products by intracellular esterases. BCECF was superior to CFDA. At equimolar concentrations, BCECF labelled yeasts more uniformly and five times more intensely. Most importantly, as determined by spectrofluorimetry, flow cytometry and fluorescence mi-
Adherence of *Candida* measured by flow cytometry

**C. albicans** labelling with BCECF

The effect of BCECF concentration on yeast labelling was studied by flow cytometry. Fig. 1(a) shows four FACScan histograms, each exhibiting the fluorescence intensity distribution of yeasts labelled with a different BCECF concentration. The quantitative expression that characterizes the fluorescence of an entire cell population is the mean fluorescence intensity per cell (MFI). The increase in yeast fluorescence due to labelling with increased BCECF concentrations is demonstrated by the shift of the histogram peaks towards the right (Fig. 1a) and the corresponding increase in MFI (Fig. 1b). A BCECF concentration of 2 μg ml⁻¹ was saturating (Fig. 1b), and accordingly was used routinely for labelling.

Additional conditions for labelling of *C. albicans* with BCECF were optimized by using the same approach of flow cytometric analysis. These included pH, temperature, incubation time, yeast concentration and composition of the incubation medium, and the yeast growth conditions prior to labelling.

Optimal labelling of yeasts with BCECF was achieved at a yeast concentration of 5 x 10⁷ cells ml⁻¹ and incubation in YNB medium lacking carbon source, ammonium sulphate and amino acids, at pH 7 and 30°C for 1 h. Labelling in this medium probably prevented amine-induced hydrolysis of the acetomethoxy esters of the fluorogenic dye (Haughland, 1992). Yeast cells harvested from the mid-late exponential phase (2–4 x 10⁷ yeasts ml⁻¹) were labelled more intensely and exhibited a smaller variance of fluorescence intensities than cells grown to stationary phase (1–2 x 10⁸ yeasts ml⁻¹).

Under optimal conditions, yeast MFI increased linearly with time and labelling was maximal after incubation of yeasts with BCECF for about 60 min (data not shown). In 21 different experiments the mean and standard error (SE) of yeast MFI was 2140 ± 162.

---

**The flow cytometric assay for adherence**

(i) **Discrimination between yeast and epithelial cells.** The flow cytometer (FACScan) analyses the light scatter and fluorescence intensities of every individual cell or particle that passes through. Both forward light scatter (parallel to the cytometer laser beam) and perpendicular light scatter are largely dependent on cell size and structure (Shapiro, 1988). Since yeast cells are substantially smaller than BECs (2–8 μm diameter vs 15–80 μm), the forward and perpendicular light scatter intensities of yeasts (Fig. 2a) and BECs (Fig. 2b) differed considerably, and thereby allowed discrimination between these two types of cells in the FACScan. By setting up appropriate windows of light scatter intensities and activating the electronic gating option of the flow cytometer, analysis of fluorescence was restricted to cells in region R1, which specifically included epithelial cells (compare Fig. 2a and b). Nonadherent fluorescently labelled yeasts were thus 'gated out' and the measured fluorescence of the cells in the restricted region (R1) reflected adherence of labelled yeasts to epithelial cells. No fluorescence data were displayed in the FACScan when a yeast suspension was run in the flow cytometer under these conditions, indicating the high accuracy of the gating procedure. The minor fraction of BECs, or particles present in the BEC suspension, which was characterized by lower light scatter intensities than those in the R1 region (Fig. 2b), was inversely dependent on the quality of cell purification in the Percoll gradient. It contained mainly small and round nonsquamous cells and cell debris.

(ii) **The effect of the Y:BEC ratio on adherence, measured by both flow cytometry and the filter assay.** One of the great advantages of using flow cytometry for measuring adherence is the possibility of studying adherence accurately at low Y:BEC ratios, which better mimic the adhesion as it occurs naturally.

A representative adherence experiment in which the effects of decreasing the Y:BEC ratio were studied, is shown in Fig. 3. Adherence reactions were performed at Y:BEC ratios of 3:1, 25:1 or 100:1. Two peaks which differ in their fluorescence intensities were clearly observed in the histograms obtained, and represent two subpopulations of epithelial cells, with and without
adherent yeasts. As described below, the one with adherent labelled yeasts exhibits high fluorescence intensity.

When the adherence reaction was performed at a low Y:BEC ratio, such as 3:1, the major peak arose from a low-fluorescence BEC subpopulation (Fig. 3) and its MFI was comparable with the autofluorescence intensity of control BECs (MFI values of 255 and 225, respectively; Table 2). Apparently, no fluorescently labelled yeasts adhered to BECs in this subpopulation. The second, minor peak of fluorescence in the same histogram reflected the presence of a highly fluorescent subpopulation of BECs comprising 12% of the $10^4$ BECs analysed. They had acquired an almost sevenfold stronger fluorescence intensity than the low-fluorescence cell majority, and their MFI was close to that of the labelled yeasts that were used in the experiment (MFI values of 1690 and 1725, respectively; Table 2). It therefore suggested that labelled yeasts had attached to these BECs.

On increasing the Y:BEC ratio to 25:1 and 100:1, two phenomena were observed: (i) the peak size of the highly fluorescent subpopulations, apparently yeast-carrying BECs, increased substantially to 35 and 75% of the total BEC population, while the proportion of the low-fluorescence subpopulations decreased correspondingly (Fig. 3, Table 2); (ii) the MFI of the highly fluorescent BECs rose to considerably higher values than the MFI found at the 3:1 Y:BEC ratio (MFI values of 1900 and 2650, respectively, compared to 1690; Table 2), suggesting that more yeasts adhered per epithelial cell with increasing yeast excess.

These results were in accordance with those of a similar experiment in which adherence was determined by enumerating the cells microscopically using the conventional filter assay. The data in Table 2 show for increasing Y:BEC ratios, a corresponding increase in both pairs of parameters: (i) the flow cytometrically measured percentage of highly fluorescent BECs to which, presumably, yeasts adhered, and the percentage of yeast-carrying BECs as counted microscopically; (ii) the MFI of the high-fluorescence BEC subpopulation as determined

---

**Table 2. Comparison of yeast adherence to BECs at various Y:BEC ratios using flow cytometry or microscopy**

Two standard adherence experiments were carried out at the specified Y:BEC ratios. One was analysed by the conventional filter assay and microscopy of $10^4$ BECs, the other by flow cytometry of $10^4$ BECs and is the same experiment described in Fig. 3. For statistical analyses the number of cells in each sample was calculated from their percentage. MFI ± SE of BEC autofluorescence was $225 ± 19^{f} \text{(n = 10^4)}$, and MFI ± SE of labelled yeasts in the experiment was $1725 ± 80^{f} \text{(n = 10^4)}$.

<table>
<thead>
<tr>
<th>Y:BEC ratio</th>
<th>Flow cytometry</th>
<th>Microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High fluorescently labelled BECs (%)</td>
<td>MFI – low-fluorescence BEC sub-population*</td>
</tr>
<tr>
<td>3:1</td>
<td>12</td>
<td>$255 ± 1.5$</td>
</tr>
<tr>
<td>25:1</td>
<td>35</td>
<td>$360 ± 1.7$</td>
</tr>
<tr>
<td>100:1</td>
<td>75</td>
<td>$760 ± 2.2$</td>
</tr>
</tbody>
</table>

* Non-specific fluorescence (see text) ± se.
† Fluorescence after yeast adherence ± se.
‡ MFI increment due to yeast adherence.
§ Mean number of yeasts per yeast-carrying BEC ± se.
by flow cytometry, and the mean number of yeasts adhering to a BEC as enumerated microscopically. These positive correlations between the two assays thus supported the notion that the flow cytometric assay indeed reflected adherence of yeasts to BECs in the reaction, the MFI of the highly fluorescent subpopulation reflecting the number of adherent yeasts in a nonlinear way. It should be noted that the number of cells analysed by flow cytometry was by two orders of magnitude larger than those counted by microscopy. Not surprisingly, therefore, the differences in MFI values were highly significant, as apparent from the small ss and relatively low significance of the differences between the numbers of adherent yeasts counted by microscopy (Table 2).

Each of the above experiments was repeated at least twice. In separate adherence experiments at Y:BEC ratios of 25:1 and 100:1, the mean values of MFI and their ss were 1867 ± 144 (n = 8) and 3995 ± 334 (n = 10), respectively. The difference between these values was significant at the level of a < 0.001, as determined by the Mann-Whitney U-test (Siegel & Castellan, 1988).

Analysis of the low-fluorescence peaks of BECs in the histograms at Y:BEC ratios of 25:1 or 100:1 (Fig. 3) showed that their MFI was somewhat higher than BEC autofluorescence MFI (Table 2). The increased fluorescence was however due to nonspecific fluorescent labelling of the BECs, as proved by a specifically designed negative control assay (see Methods; data not shown). It confirmed that the BEC subpopulation in the low-fluorescence peak represented yeast-free BECs, even at a large yeast excess.

In more recent experiments, adherence could be evaluated at a Y:BEC ratio as low as 1:1, and yeasts adhered to practically all BECs present in the reaction mixture at a 25:1–50:1 Y:BEC ratio; all other data were similar to those presented above.

Comparing the flow cytometric assay with the conventional filter assay suggested that BCECF-labelling did not affect yeast adherence to a significant extent. This was fully confirmed in an experiment using the filter assay and counting BECs to which either BCECF-labelled or nonlabelled yeasts were allowed to adhere (data not shown).

Negative control experiments supporting the accuracy of the novel adherence assay

In order to check the accuracy of the flow cytometric assay for adherence, the assay was applied under conditions known to decrease yeast adherence to epithelial cells. These conditions included: (i) treatment of BCECF-labelled yeasts with trypsin at different concentrations (20–200 µg ml⁻¹), which modifies the adhesins and changes the surface charge and hydrophobicity (Sobel, 1993); (ii) incubation of the labelled yeasts for 30 min at a high temperature (56 °C), which possibly inactivates the candidal adhesins (Kimura & Pearsall, 1978; Sobel, 1993); (iii) performing the adherence reaction at a low temperature (4 °C), where the kinetics are very slow. All treatments resulted in reduced adherence of the yeasts to BECs compared with the appropriate controls, as was demonstrated by the substantial increase in the proportion of the peak of the low-fluorescence subpopulation, which represented cells without adherent yeasts and which became the major one (data not shown).

Previously described basic features of adherence confirmed by the new method

Additional support for the validity of the flow cytometric assay came from the confirmation by flow cytometry of previously described basic features of adherence. These included the effect of the yeast growth phase on adherence capability (Kennedy, 1988, 1990; King et al., 1980), and the differential adherence of various Candida species (Kennedy, 1990; King et al., 1980; Odds, 1994; Segal et al., 1984).

Fig. 4(a) compares adherence to BECs of C. albicans harvested at the exponential or stationary growth phase.
Since stationary-phase yeasts were labelled considerably less than exponential-phase yeasts (compare the 'A' bars in Fig. 4a), the difference in adherence could not be assessed directly by comparing the respective MFI values of the yeast-binding BECs ('B' bars in Fig. 4a). Instead, the relative MFI increment (B/A) was calculated to reflect the number of BEC-adherent yeasts of the two growth phases ('C' bars in Fig. 4a). As shown in Fig. 4(a), the relative MFI increment of stationary-phase yeasts was about 60% higher than that of the exponential-phase yeasts. This result was confirmed by the conventional assay, and indicated that stationary-phase yeasts adhered to BECs to a greater extent, as previously noted, probably due to rearrangement and exposure of more adhesive molecules (Kennedy, 1988, 1990; King et al., 1980).

Another important feature confirmed by flow cytometry was the differential adherence capability of various Candida species. (Fig. 4b). Each Candida species was labelled to a different intensity with BCECF ('A' bars in Fig. 4b). Comparing the relative MFI increment of the BECs ('C' bars in Fig. 4b), as described above for Fig. 4(a), shows that C. albicans adherence to BECs exceeded greatly that of the other two Candida species. The order of differential adherence, where C. albicans presents the highest and C. krusei the lowest differential adherence, is in agreement with published results obtained by conventional procedures (King et al., 1980; Rotrosen et al., 1986) and correlates with the higher pathogenic potential of C. albicans (Kwon-Chung & Bennett, 1992; Odds, 1994; Segal et al., 1984). Clinical isolates of C. albicans taken from our laboratory collection exhibited similar adherence to the reference type strain (data not shown).

**A flow cytometric study of the impact of diabetes and ontogenic developmental stages on adherence**

Following the extensive validation of the flow cytometric assay of adherence, it was applied to study the adherence of C. albicans to epithelial cells from donors with a pathological condition and from different ontogenic human developmental stages.

Diabetic patients are more susceptible to developing oral, vaginal and cutaneous candidiasis (Lamey et al., 1988; Segal et al., 1984), therefore the relation of C. albicans adherence to this phenomenon was tested. When BECs obtained from 20 patients suffering from imbalanced type II diabetes (glucose levels between 300 and 400 mg per 100 ml and glucosylated proteins in the serum) were examined, no significant difference in C. albicans adherence was noted between these cells and BECs from healthy control donors in two separate experiments (MFI values of 2175 vs 2060 and 2300 vs 2400, at Y:BEC = 25:1).

The degree of yeast adherence, on the other hand, could be correlated with ontogenic developmental stages. Epithelial cells from four developmental stages, human foetuses (amniotic fluid from the 17th to 22nd week of pregnancy), premature neonates (born between the 25th and 36th week of pregnancy), mature babies (up to 7 d old, who were born after the 38th week of pregnancy) and adults (18 years or older), were tested by the flow cytometric assay for adherence of C. albicans (Fig. 5). Histogram 'A' in Fig. 5 demonstrates that the majority of cells from the amniotic fluid, which are mainly oral epithelial cells (Huisjes, 1973), did not bind yeasts. In the relatively minor proportion of amniotic cells that bound yeasts, the number of yeasts attached per epithelial cell was very heterogeneous, as exhibited by the broad distribution of fluorescence intensities. In premature neonates (histogram 'B', Fig. 5), high-fluorescence BECs which bound yeasts were more abundant (46% of the total BEC population) approaching the percentage (66%) in control adults' BECs (histogram 'C', Fig. 5). Moreover, the number of yeasts bound per epithelial cell from premature babies was close to that bound by cells from adults (the MFI was only 18% less). BECs from full-term newborns (mature babies) were indistinguishable in their yeast-binding capacity from those of adults (data not shown).

**DISCUSSION**

Adherence is the first and major step in the pathogenic mechanism of C. albicans, prior to the sequential process of colonization and invasion (Calderone & Braun, 1991; Cutler, 1991; Odds, 1988, 1994). The need for a simple and well-defined *in vitro* assay is still very apparent (Kennedy, 1990). It is therefore very important to have an accurate, sensitive and objective *in vitro* quantitative assay for its determination. A novel, flow cytometric method using fluorescently labelled yeasts was developed which not only meets the above requirements, but also overcomes some of the limitations of current conventional assays summarized in the Introduction.

The advantage of the flow cytometric assay is that it combines evaluation of a large number of cells, as in the
radiometric assay, with detailed single-cell information, as obtained by microscopy. Thus, flow cytometry yields direct data on the proportion of epithelial cells to which yeasts adhered, and provides an objective quantitative measure, the MFI, which is a function of the mean number of yeasts attached per yeast-binding epithelial cell. Under conditions of enhanced adherence, the MFI value of BECs was greater than four times that of single nonadherent yeast (Fig. 4).

Fluorescent labelling of the yeasts is a fundamental feature of the flow cytometric assay. Maximizing yeast labelling and minimizing leakage of fluorescent products from the yeasts is therefore very important and was achieved by selecting BCECF as the fluorescent marker (Table 1). The notable feature of flow cytometric analysis was the constant observation of two subpopulations of epithelial cells after coincubating the labelled yeasts and BECs in the adherence reaction (Figs 3 and 5). One subpopulation was yeast-free, as apparent from its low fluorescence intensity (not exceeding autofluorescence and nonspecific fluorescence); the other was characterized by high fluorescence intensities which could only be acquired by the adherence of one or more labelled yeasts. BEC autofluorescence and nonspecifically acquired fluorescence can be determined independently and subtracted. Nonspecific fluorescence increased with the Y:BEC ratio, apparently due to the increased concentration of fluorescent products in the adherence reaction. However, it did not interfere with evaluating yeast adherence up to a ratio of 100:1; the highest Y:BEC ratio tested (Table 2).

At a low Y:BEC ratio, the MFI of the highly fluorescent BEC subpopulation reflects adherence of a mean of one yeast per BEC as was also observed by microscopy (Table 2). At high Y:BEC ratios, when more than one yeast adheres to a BEC, the corrected MFI is still proportional to the number of bound yeasts, but the correlation between them is not a simple linear function. This is due to underestimation of the fluorescence increment as measured (in the standard ‘height’ mode of the FACScan) when a large epithelial cell carrying more than one yeast passes through the relatively narrow light beam of the flow cytometer (\(\sim 20\) μm diameter). Integrating the fluorescence signals over the entire cell (in ‘area’ mode of the instrument) could overcome this problem. Unfortunately, this was impractical because of the very large variance of BEC autofluorescence and nonspecific fluorescence, presumably due to the large heterogeneity of BEC sizes (unpublished results). Using a slit scan cytometer and time-of-flight analysis (Shapiro, 1988) should come closest to the expected linear correlation.

Conversion of MFI to the actual number of adhering yeasts is inapplicable since it must be based on the assumption that Candida-specific receptors are evenly and randomly distributed over the epithelial cell surface. However, no data supporting this view are available, and it is not valid for epithelial cells, which are polar in vivo. Nevertheless, since the flow cytometric assay readily analyses a large sample of epithelial cells, \(10^4\) or more, which is two orders of magnitude larger than in the conventional microscopic assay, even small differences in MFI values, exhibiting very low sse (Table 2), are statistically significant. Therefore the MFI represents a very valuable, objective, quantitative measure to compare the extent of yeast adherence per epithelial cell under a variety of conditions, and the effect on it of single factors, which is the main purpose of an in vitro assay. The flow cytometric assay thereby has a great advantage over the conventional one.

A direct quantitative statistical correlation between the conventional microscopic assay and the novel flow cytometric assay is impossible to perform since the two assays measure fundamentally different parameters in separate epithelial cells. The reliability and validation of the flow cytometric assay for measuring yeast adherence is therefore based on a number of independent semiquantitative correlations and is supported by the following findings: (i) the constant observation of the two distinct subpopulations of epithelial cells, either yeast-free or with one or more labelled yeasts adhering, as explained above (Figs 3 and 5); (ii) the consistent increase in the proportion of highly fluorescent cells, i.e. yeast-binding cells, as a function of increasing Y:BEC ratio (Kennedy, 1988; Kimura & Pearsall, 1978); (iii) comparable results with the conventional filter assay (Table 2), considering that different batches of yeasts and BECs were used in these experiments, and only 100 BECs were counted by microscopy; (iv) confirmation of reduced adherence after limited proteolysis or heat-inactivation of the yeasts; (v) differential adherence of various Candida species at different growth phases.

The greatest advantage of the flow cytometric method is the possibility of studying adherence at low yeast:epithelial cell ratios and still obtaining reliable quantitative results because of the large number of cells that are analysed. At decreasing Y:BEC ratios, yeast adherence decreases to very low levels. Evaluating the two parameters of adherence by counting 100–200 BECs, as usually performed by microscopy in the conventional method, becomes obviously less reliable. In fact, in experiments using the conventional methods for measuring adherence, the ratio of yeasts to exfoliated epithelial cells was usually 100:1 or greater (Kennedy, 1990). We wonder if these high ratios mimic adhesion of Candida to BECs as it occurs in vivo, as the initial step of an infection. Using these high Y:BEC ratios may lead to yeast coadhesion (Kennedy, 1988, 1990; King et al., 1980) and hence erroneous conclusions, due to considering the indirect binding of a yeast within an aggregate as equivalent to the direct adhesion of a single yeast to an epithelial cell. No coadhesion was observed at Y:BEC ratios of 25:1 or less under our experimental conditions (unpublished data).

Caution in using a large excess of yeasts is very important in the study of epithelial-cell-related factors. We therefore applied the new assay to studying the impact of diabetes on adherence. It was recently noted that epithelial cells from diabetic patients had a higher level of yeast adhesion than cells from nondiabetic controls (Darwazeh et al., 1990; Segal et al., 1984), suggesting a correlation between
the in vitro adhesion ability of the former cells and the known increased susceptibility of these patients to oral, vaginal and cutaneous candidiasis (Lamey et al., 1988; Segal et al., 1984; Segal, 1994; Srebnik & Segal, 1990). Contrary to this, our study showed no difference in adherence to BECs from diabetics and healthy subjects, questioning the existence of such a correlation. This discrepancy may be related to the lower Y: BEC ratio used in our study (25:1), which closer imitates the adherence to BECs from diabetics and healthy subjects, questioning the existence of such a correlation. This novel finding that expression of C. albicans-specific receptors develops in parallel with the development of the embryo in utero was highly interesting. While embryonic epithelial cells demonstrated very little expression of Candida receptors, high receptor expression was attained at 25–36 weeks of pregnancy (Fig. 5). During pregnancy, both the number of receptor-bearing cells and the number of receptors per cell appear to increase gradually. These findings are in agreement with the prevalence of oral candidiasis in premature newborns, which is as high as in full-term neonates or higher (Cox, 1986; Kozinn et al., 1958).

ACKNOWLEDGEMENTS

We thank Menachem Granat for generously providing the amniotic fluids and for helpful discussions. We also thank Itamar Raz for kindly providing the cells and data regarding the diabetic patients. Special thanks to Norman B. Grover for the assistance in the discussion of statistical analyses. This study was supported by grant no. 3033 from The Chief Scientist, Ministry of Health, Israel.

REFERENCES


Adherence of *Candida albicans* to human vaginal and buccal epithelial cells. *J Infect Dis* 143, 76–82.


Received 24 October 1994; revised 6 January 1995; accepted 6 April 1995.