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Variation in adhesion and cell surface hydrophobicity in *Candida albicans* white and opaque phenotypes

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Abstract

A previous study had established that a select group of pathogenic isolates of *Candida albicans* was capable of switching heritably, reversibly and at a high frequency (10^{-2} to 10^{-3}) between two phenotypes ('white' or 'opaque') readily distinguishable by the size, shape, and color of colonies formed on agar at 25 °C. This paper describes experiments designed to determine the ability of these two phenotypes to attach to buccal epithelial cells (BECs) and plastic, and to compare the cell surface hydrophobicities of white and opaque phenotypes from three clinical isolates. 'White cells' were found to be significantly more adhesive to BECs, and a strong correlation was also found between phenotype adhesiveness and the percentage of BECs to which *C. albicans* had attached. The percentage of BECs with one or more attached *C. albicans* was approximately 90% for the white phenotype and approximately 50% for the opaque phenotype. 'Opaque cells', in contrast, were twice as hydrophobic as white cells, and the percentage of opaque cells bound to BECs by coadhesion was also double that of white cells. The differences in adhesion to plastic between the two phenotypes were not statistically significant and there was no distinct trend to suggest which phenotype might be more adhesive to plastic. These results indicate that several factors are involved in the adhesion of *C. albicans* to plastic, and confirm the hypothesis that cell surface hydrophobicity is of minor importance in direct adhesion to epithelial cells but that it may contribute to indirect attachment to epithelial cells by promoting yeast coadhesion. Moreover, the data presented in this paper also revealed that under identical growth conditions, adhesion of *C. albicans* was significantly altered depending on the phenotypic state of the organism tested. Therefore, because *C. albicans* can switch at a high frequency to various phenotypes *in vitro*, it may be that in future adhesion studies involving *Candida* the phenotypic state of the organism at the time of testing will have to be determined. Otherwise, the results, even within the same laboratory, may be difficult to interpret.

Introduction

Recently, it was reported that a select group of pathogenic isolates of *Candida albicans* was capable of switching heritably, reversibly and at a high frequency (10^{-2} to 10^{-3}) between two general phenotypes ('white' or 'opaque') readily distinguishable by the size, shape, and color of colonies formed on agar at 25 °C [38]. The differences noted in colony characteristics apparently were due to the dramatic difference in cell size, shape, and budding pattern. 'White cells' are round to ellipsoidal and exhibit a budding pattern similar to most strains of *C. albi-*

cans; in contrast, 'opaque cells' are elongate, or bean shaped, and exhibit a different budding pattern. While white cells undergo the bud to hypha transition under standard laboratory conditions, opaque cells do not [38]. In addition, opaque cells exhibit an unusual pimpled pattern on the cell surface and express an opaque-specific antigen (J. M. Anderson and D. R. Soll, manuscript submitted for publication and unpublished observations). Because the white-opaque transition has now been observed in a number of pathogenic isolates obtained from several body sites [41], it may be that the ability of *C. albicans* to switch between various phenotypes [37] *in vivo* may play a role in pathogenicity [38]. However, it is unclear what role the opaque phenotype might play since opaque cells are differentially sensitive to the normal temperature of the human body, at least under laboratory conditions [38]. It is possible that the opaque phenotype plays only a transient role *in vivo*, for instance, if it were more resistant to host defense mechanisms. Alternatively, it may be that the opaque phenotype plays a role in another habitat such as water or in the hospital environment. Such environments would foster a temperature range that would allow for maximal growth of opaque cells. Consequently, we examined cells in the white and opaque phenotypes of several independently isolated strains of *C. albicans* for adhesion to both buccal epithelial cells and plastic surfaces. Significant differences in adhesion characteristics as well as cell surface properties were observed between these two phenotypes. This report describes these differences, considers them in relation to recently observed differences in cell surface architecture (J. M. Anderson and D. R. Soll, manuscript submitted for publication and unpublished observations), and discusses the putative role white and opaque phenotypes may play in pathogenesis or alternative habitats.

Materials and methods

Yeast, culture conditions, and cell preparation

Three isolates of *C. albicans* exhibiting a white-opaque transition were recovered from patients with systemic or vaginal candidiosis. These isolates were used throughout the study and were passed less than six times from the original isolation to minimize changes in adhesive properties. For all assays, *C. albicans* cells were inoculated onto a modification [37] of the medium of Lee *et al.* [21], designated here MLBC agar, and grown aerobically at 24 °C for four to five days. A colony of cells was transferred to 100 ml of MLBC broth, which was then incubated aerobically with shaking (180 rpm) at 24 °C. Cells were grown to stationary phase, and were selected for study because they have been shown to adhere more readily than logarithmic phase cells [17, 18, 35]. One portion of the culture was then processed for adhesion to buccal epithelial cells (BECs). Yeast cells were collected by centrifugation at 3000 rpm for 20 min at 4 °C, washed three times in buffered KCl, and resuspended to 2×10^7 cells/ml to give a final yeast:buccal cell ratio of 100:1 for use in the epithelial cell adhesion assay. Another portion of the culture was collected by centrifugation, washed three times in PUM buffer, which consisted of 22.2 g $K_2HPO_4 \cdot 3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$ and distilled water to 1000 ml [28], and resuspended to 5×10^6 cells/ml for use in an assay to measure adhesion to plastic. The remainder of the culture was then harvested by centrifugation, washed three times in PUM buffer, and used for hydrophobicity testing at a concentration of 1×10^8 cells/ml [16]. All cultures were examined by light microscopy prior to testing to confirm the phenotypic state of the organism.

Mucosal cells

Buccal epithelial cells were collected by gently rubbing the inside of the cheeks of ten healthy adult volunteers with sterile cotton swabs and suspending the cells in 0.05 M KCl containing 1 mM phosphate 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ ('buffered KCl' [4] at pH 7.2. This assay medium was chosen because it mimics the ionic composition of saliva [10] The donors were not suffering from signs or symptoms of oral thrush and had not taken antibiotics for at least 12 months prior to the present study Cells were washed three times in buffered KCl and resuspended to concentrations of 2×10^5 cells/m

of assay medium, as determined by hemacytometer count. This single, large batch of cells was used throughout the study to assure that differences observed in adhesion could be attributed to variables of yeast phenotypic parameters and not to changes or differences in the BECs [32]. When examined by light microscopy prior to experimentation, none of the cell samples collected from the different donors contained mucosal cells already colonized by yeasts.

Adhesion assays

The adhesion of *C. albicans* white and opaque phenotypes to BECs was studied using a previously described assay [32]. Briefly, 0.2 ml samples of BECs and yeast cells were placed into small test tubes (12 × 75 mm) and incubated on a rotary shaker (180 rpm) for 1 h at 37 °C. Three tubes were used for each experiment. After the incubation period BECs were collected and washed on polycarbonate filters (12 μm pore size) (Nucleopore Corporation, Pleasanton, California). Cells were washed with approximately 100 ml of PBS under continual (gentle) agitation. The filters were then stained with Gram crystal violet and the number of *C. albicans* adhering to 200 BECs was determined by light microscopy at 430×. Double-blind conditions were used.

The adhesion of *C. albicans* cells to plastic was studied using polystyrene microtiter trays containing 24, 16 mm-diameter wells (Costar, Cambridge, MA) as follows. A 0.5-ml sample of a suspension of white or opaque *Candida* cells was placed in each well, the tray incubated for 1.5 h at 37 °C without shaking, and the wells then washed three times with 1.0 ml of assay medium. The trays were inverted and allowed to dry overnight at room temperature. Adherent *Candida* cells were counted by light microscopy at 100×. Five to ten 1 mm^2 fields were counted per well, and the assay was performed in quadruplicate. Only the center area of each well was included in the counting procedure because several nonadherent *Candida* cells were deposited at the outer edge of the wells during the drying process. This procedure provided an efficient and reproducible method of quantitating the adhesion of *C. albicans* to plastic surfaces (M. J. Kennedy, A. L. Rogers, R. V. Thomas, P. A. Volz and R. J. Yancey, Jr., manuscript submitted for publication).

Phase-partition test for cell surface hydrophobicity

A modification [16] of the phase-partition method of Rosenberg *et al.* [28], with hexadecane as the hydrocarbon phase, was employed to test for *Candida* cell surface hydrophobicity. Briefly, yeast were grown as described, washed twice in PUM buffer, and resuspended to a final concentration of 1×10^8 yeast/ml. To round-bottom test tubes (12 mm-diameter), containing 2.5 ml of washed cells in PUM buffer, 0.5 ml of hexadecane (Sigma Chemical Co., St. Louis, MO) was added. The suspension was preincubated at 37 °C for 10 min, then mixed on a vortex mixer for 2 min. After separation of the aqueous and hydrocarbon phases, the aqueous phase was measured at 400 nm, using a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, NY). Control cells were put through the identical procedure without the addition of hexadecane to the system. The relative cell surface hydrophobicity was then determined by measuring the change in absorbance between test and control cells, and determining the percentage of cells that entered the hydrocarbon phase. This method was selected because of its ease, and because it has previously been shown to give results similar to all other current methods of testing cell surface hydrophobicity [19, 25].

Results

Adhesion of C. albicans *phenotypes to BECs*

To determine the effect the phenotypic state of *C. albicans* had on adhesion to BECs, three individual isolates of *C. albicans* (WO-1, BL6, and 6a) were grown under identical growth conditions in either the white or opaque phenotype [38] and tested by the *in vitro* assay described in *Materials and methods*. To assure that differences were due to phenotype induced changes and not to differences in epithelial

Table 1. Adhesion of *Candida albicans* white and opaque phenotypes to buccal epithelial cells (BECs).

Strain	Phenotype	Mean no. yeasts/ BEC (±SD)	% of yeast co-attached	% of BECs with yeast attached
WO-1	White	14.9±5.8	6.1	92.0
	Opaque	2.1±3.3	12.2	50.0
BL6	White	13.5±3.4	12.8	88.7
	Opaque	1.9±4.7	27.5	31.2
6a	White	18.2±2.4	13.8	96.0
	Opaque	3.0±5.3	23.2	44.0

cells [7, 13, 18, 33, 34], a constant and standardized pool of BECs was used throughout the study [32]. The ability of *C. albicans* to attach to BECs was found to be highly dependent on the phenotype used. These results are summarized in Table 1. For all three isolates, the white phenotype was significantly ($p < 0.001$) more adhesive to BECs than was the opaque phenotype. A correlation was also found between phenotype adhesiveness and the percentage of BECs to which *Candida* cells had attached (Table 1). The percentage of BECs with one or more attached *Candida* cells was approximately 90% for the white phenotype and approximately 50% for the opaque phenotype. In general, as the mean number of attached *Candida* cells increased, so did the percentage of BECs with attached *Candida* cells. Nevertheless, the white phenotype attached to significantly more BECs than did the opaque phenotype.

The ability of *Candida* cells to associate with BECs indirectly, i.e. by coadhesion to adherent *Candida* cells, was also studied. It was found that yeast-to-yeast coadhesion contributed to an increase in the total number of *Candida* cells that attached to BECs for both phenotypes (Table 1). However, although the percentage of the total coadherent yeast population was low for both phenotypes, opaque phenotypes had approximately twice as many cells attaching to BECs in this manner.

Adhesion of C. albicans *phenotypes to plastic*

The adhesion of white and opaque *Candida* cells to plastic was studied using a modification of a microtiter tray assay described by Klotz *et al.* [19]. In preliminary studies (M. J. Kennedy, A. L. Rogers, R. V. Thomas, P. A. Volz, and R. J. Yancey, Jr., manuscript submitted for publication) it was found that two types of adhesion occurred in this system. The first was the direct adhesion of *Candida* cells to plastic, and the second was adhesion to the liquid-plastic interface. Consequently, as plates dry, cells floating freely in a thin layer of fluid are deposited at the outer edge of the wells. Therefore, to accurately determine the relative number of *Candida* cells adherent to the plastic surface, only the center area of the wells were counted. Using this procedure, the adhesion of *C. albicans* phenotypes to plastic was determined. The results are summarized in Table 2. The differences in adhesion to plastic between the two phenotypes were not statistically significant for any of the isolates tested, and there was no distinct trend to suggest which phenotype was more adhesive to plastic. For instance, for the WO-1-isolate, the white phenotype was more adhesive to plastic than the opaque. In contrast, this trend was reversed for isolate BL6. Furthermore, for isolate 6a the cells of both the white and opaque phenotype attached in similar numbers. This suggests that several factors may be involved in adhesion of *C. albicans* to plastic surfaces.

Table 2. Adhesion of *Candida albicans* white and opaque phenotypes to plastic.

Strain	Phenotype	Mean no. yeasts/ mm^2 (± SD)
WO-1	White	154.2±31.8
	Opaque	94.8±41.3
BL6	White	76.2±12.4
	Opaque	110.8±26.0
6a	White	62.7±30.1
	Opaque	76.1±33.6

Effect of the phenotypic state on C. albicans *cell surface hydrophobicity*

To test the hypothesis that cell surface hydrophobicity may influence the adhesion of *C. albicans* to BECs

Table 3. Hydrophobic properties of *Candida albicans* white and opaque phenotypes.

Strain	Phenotype	% Δ in absorbance (± SD)
WO-1	White	45.2 ± 1.1
	Opaque	82.7 ± 3.8
BL6	White	53.2 ± 1.3
	Opaque	80.0 ± 0.5
6a	White	40.3 ± 1.6
	Opaque	96.8 ± 0.5

or plastic [11, 19], the relative hydrophobic affinity of *C. albicans* phenotypes was determined. As is shown in Table 3, both the white and opaque phenotypes of the three isolates of *C. albicans* used in this study proved to be relatively hydrophobic after growth in MLBC at 24 °C since about 50% of the cells entered the hydrocarbon phase. Furthermore, it was also noted that the opaque cells were significantly more hydrophobic than the white cells, with at least twice as many cells adhering to the hydrocarbon phase.

Discussion

It was recently demonstrated that most strains of *C. albicans* switch heritably and reversibly at high frequency between a number of general phenotypes distinguishable by colony morphology [37–39, 41]. There are at least three switching systems which are strain specific [40]. The 'white-opaque transition' is perhaps the most interesting system since it appears to represent a phase transition and involves a dramatic effect on cell shape, cell size, gene expression, growth dynamics, budding dynamics, actin localization and cell wall morphology [38, 39] (J. M. Anderson and D. R. Soll, manuscript submitted for publication and unpublished observations). Strains which possess a white-opaque transition system are capable of switching back and forth indefinitely between two general phenotypes at frequencies of 10^{-2} to 10^{-3} [39].

The results presented in this report demonstrate that switching between the white and opaque phenotype dramatically influences the adhesive and cell surface properties of individual cells. White cells of three independently isolated strains of *C. albicans* were significantly more adhesive to BECs than their opaque cells counterparts. In addition, there was a higher percentage of BECs with attached *Candida* cells of the white phenotype. This was true for all the isolates tested, and was similar to previous reports that showed a strong correlation to exist between *C. albicans* adhesiveness and the percentage of BECs to which *C. albicans* had attached [32, 36]. In contrast, the opaque cells of each *C. albicans* isolate tested were far more hydrophobic than their white cell counterparts. The present data, therefore, clearly indicates that under identical growth conditions, adhesiveness, and other cell surface properties, can be significantly different depending on the phenotypic state of the organism tested.

One factor which may be involved in the apparently superior adhesion of white cells to BECs is the differences in size and shape of the phenotypes. White cells are relatively round, whereas opaque cells are elongate, or bean shaped. The mean volume of a population of *C. albicans* WO-1 white cells was shown to be 33 μm^3, whereas the mean volume of opaque cells was 114 μm^3 [38]. Because curved bodies require less kinetic energy to overcome repulsive interactions during adhesion [12, 14], such as between *C. albicans* and epithelial cells [14, 29], the smaller, rounder white cells may more effectively reduce the yeast-epithelial gap to allow binding to take place [14]. Indeed, it was previously shown that small blastospores of *C. albicans* were significantly more adhesive to BECs than larger yeast cells taken from the same culture [32]. However, because *C. albicans* is known to bind essentially irreversibly to BECs [14, 15, 29], and nonspecific binding is not strong enough to account for this type of adhesion [12], it is likely that specific adhesion-receptor binding was also involved. Although both factors are important in adhesion to epithelial cells, the latter is probably more important since even mild fluid shear can prevent adhesion by dislodging *Candida* cells that are 'attached' only by nonspecific mechanisms [14].

While the data presented here do not allow definitive conclusions to be drawn regarding the increased adhesiveness of white cells to BECs compared to opaque cells, there are, nevertheless, at least two possible mechanisms which could account for this

difference [1]. There may be major changes in the organization of the cell wall of these phenotypes [8, 14] that allows cells of the white phenotype to produce more or different adhesins that increase their adhesiveness. There is some evidence to suggest that *C. albicans* does produce more than one type of adhesion [16, 23, 31], but it is not clear if these types are phenotypically regulated [24] or whether a cell can produce two distinct types of adhesion simultaneously. Although there is no evidence indicating that differences in the composition of the wall exist between white and opaque *Candida* cells, both scanning and transmission electron microscopy have revealed dramatic differences in wall morphology (J. M. Anderson and D. R. Soll, manuscript submitted for publication and unpublished observations). The function of 'pimples' on opaque cells is still unknown, but such dramatic differences in wall morphology may indeed be related to the difference in adhesion delineated by the BEC adhesion experiments and the hydrophobicity measurements [2]. Because ultrastructural and surface characteristics of white and opaque cells are different, other surface features (e.g., charge) might also be different. Such differences may allow white cells to favor adhesion to BECs by further reducing repulsive interactions that occur during adhesion between cells [14, 29]. Moreover, such changes may also allow surface molecules adjacent to adhesions to bind nonspecifically to BECs and thereby facilitate and strengthen the adhesion [14].

Factors involved in the adhesion of *C. albicans* to plastic surfaces were previously investigated by Klotz *et al.* [19], who found that hydrophobic properties predominately governed *Candida*-plastic interactions. However, kinetic analyses also revealed negative cooperativity due to electrostatic repulsion [19]. Thus, if cell surface hydrophobicity was the only factor involved in the adhesion of *Candida* to plastic, opaque cells should have attached in higher numbers. The present study, therefore, corroborates the view that other factors are involved in the adhesion of *C. albicans* to plastic. Factors other than cell surface hydrophobicity that may have influenced the attachment of *C. albicans* phenotypes to plastic include cell size and surface charge [14, 19].

Previous studies have also shown that *C. tropicalis* was more hydrophobic and attached in greater numbers to these surfaces than did *C. albicans* [19, 25–27, 30]. In those studies SDB (at 37 °C) was used for cultivation of *Candida* cells, whereas in this study MLBC (at 24 °C) was used. We have found that growth of *C. albicans* under the latter conditions produced cells that were significantly more hydrophobic [32]. The observations of the present study, therefore, were also consistent with previous findings that *C. albicans* is not very hydrophobic after growth in SDB [16, 19, 25–27, 32], and confirms the suggestion that growth in the medium of Lee *et al.* [21] at 25 °C produces cells that would attach to plastic in the greater numbers [32]. Furthermore, the differences in adhesion and coadhesion to BECs noted here for white and opaque phenotypes, corroborates the hypothesis that cell surface hydrophobicity plays only a small role in direct adhesion to BECs but that this property may be important in promoting yeast coadhesion. Opaque cells were generally twice as hydrophobic as white cells, and the percentage of opaque cells bound by coadhesion was also double that of white cells. In contrast, white cells were significantly more adhesive to BECs.

Finally, it is worth considering whether the white-opaque transition, the resulting differences in adhesion, or both, play a role in colonization and pathogenesis by *C. albicans*. To begin with, it should be noted that strains exhibiting a white-opaque transition have been isolated from systemic infections, vaginal infections and oral lesions [39]. The transition between white and opaque occurs frequently and spontaneously, and appears to involve the activation of opaque specific genes (J. M. Anderson and D. R. Soll, manuscript submitted for publication and unpublished observations). The transition also involves very refined changes in nearly every aspect of growth and cell architecture [38, 39], suggesting that the transition is a highly evolved form of heritatable, high frequency variability. It seems likely, therefore, that this transition does play some role in the pathogenic success of this organism. However, because white cells are significantly more adhesive to epithelial cells than opaque cells, and because white cells have selective growth advantage at ~37 °C [38], it seems likely that of the two phenotypes the white phenotype would predominate *in*

vivo. Nevertheless, it is possible that the opaque phenotype may play a transient role during the initial phases of colonization, or that the opaque phenotype may be more resistant to irradication by antifungal chemotherapy (B. Slutsky, Ph.D. thesis, University of Iowa; D. R. Soll, M. Staebell, S. Eisely, and B. Slutsky, manuscript in preparation). It is equally plausible that the dramatic alteration of cell surface properties may offer the opaque phenotype an increased resistance to host defense mechanisms similar to that of encapsulated forms of *Cryptococcus neoformans* [2, 20]. Alternatively, the opaque phenotype may predominate in some other environment. Indeed, *C. albicans* has been isolated from IV lines, hospital linens, soil, water, and toothbrushes [3, 5, 9]. Further studies will be necessary, therefore, to characterize the specific role(s) of the white-opaque transition in pathogenesis, and to determine which phenotypes are presenting the host during colonization, invasion, and infection of various body sites. Differences in adhesive properties, nevertheless, may be a major factor in the alternative roles of white and opaque switch phenotypes.

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