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Role of *Candida albicans* polymorphism in interactions with oral epithelial cells

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Background: *Candida albicans* is a polymorphic organism which undergoes morphologic transition between yeast, pseudohyphal and hyphal forms. The ability of *C. albicans* to change from yeast to filamentous types is a major virulence determinant of this organism. However, the exact role of hyphal transformation in establishing oral mucosal infection is still poorly understood.
Methods: In this study we used mutants with defects in filamentation, as well as oral strains, which differ in their capacity to form true hyphae, to examine the role of hyphal transformation in the interactions of *C. albicans* with oral epithelial cells *in vitro*. These interactions included the ability of these strains to adhere to and injure epithelial cells, as well as their ability to trigger a proinflammatory cytokine response.
Results: We found that strains SC5314 and ATCC28366 formed true hyphae on epithelial cells, whereas strain ATCC32077 and the *tup1/tup1* mutant formed only pseudohyphae. Double mutant *efg1/efg1cph1/cph1* grew exclusively as blastospores. We also found that yeast and pseudohyphal strains showed reduced adherence capacity to oral keratinocytes and caused minimal cell damage. Moreover, we showed that both yeast and pseudohyphal forms have a strongly attenuated proinflammatory phenotype, since they failed to induce significant interleukin (IL)-1α and IL-8 responses by oral epithelial cells.
Conclusions: Germination of *C. albicans* into true hyphae is particularly important in the interactions with oral epithelial cells *in vitro*.

Key words: *Candida albicans;* epithelial cell; yeast morphogenesis

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Candida albicans is an opportunistic pathogen, causing common superficial infections as well as life-threatening disseminated and deep-seated organ infections (33, 40). Fungal pathogens such as *C. albicans* are of increasing concern because of the rising incidence of immunosuppression induced by AIDS, diabetes, cancer, organ transplantation and other conditions, which predispose the host to oral opportunistic infection (40).

C. albicans is a polymorphic organism which undergoes morphologic transition between yeast, pseudohyphal, and hyphal forms (8). The ability of *C. albicans* to change from yeast to filamentous types is a major virulence determinant of this organism (for review see [4]). Mutants with defects in filamentation have reduced virulence in animal models of candidiasis (12). Although in animal models of disseminated infection it has been established that the ability to change from yeast form to hyphae is crucial for virulence (41), the exact role of hyphal transition during the development of oral candidiasis is still unclear. Germinated yeasts adhere better to oral epithelial and other cell types than blastospores (28, 44). Since the level of adhesion of *C. albicans* to host cells is positively related to the virulence of this organism (14), this can at least partly explain the great capacity of hyphal forms to establish infections.

Genetically manipulated *C. albicans* strains, with the ability to acquire exclusively hyphal, pseudohyphal or yeast forms, can differentially induce expression of leukocyte adhesion molecules by endothelial cells (37). Several lines of evidence also suggest that the morphogenetic status of *C. albicans* plays a significant role in

determining the secretory cytokine profiles of dendritic cells and macrophages in response to infection with this organism (5, 19).

All three morphogenetic forms of *C. albicans* are frequently encountered in the oral mucosa (11, 17). Oral epithelial cells constitute the first line of defense against oral *Candida* infection (24), and we therefore studied the interactions of these cells with the three different morphotypes of this pathogenic organism. More specifically, we compared the ability of yeast, pseudohyphal, and hyphal organisms to adhere to and lyse oral epithelial cells, as well as their ability to trigger a proinflammatory cytokine response. Production of proinflammatory cytokines by oral epithelial cells in response to fungal infection is expected to have a major impact in the initiation of the inflammatory response and possible activation of lysis of the invading microorganism by immune and nonimmune effector cells.

The central hypothesis of this work is that formation of true hyphae is necessary for the organisms to adhere to oral epithelial cells and trigger a proinflammatory cytokine response. To investigate this hypothesis, we compared *C. albicans* strains that differ in their capacity for hyphal transformation naturally, in response to environmental pressure, or by genetic manipulation, with respect to their ability to adhere to oral epithelial cells and trigger interleukin (IL)-1α and IL-8 responses. Furthermore, using this *in vitro* coculture system we investigated the ability of different *C. albicans* morphotypes to disrupt an oral epithelial cell layer.

Material and methods

Organisms

C. albicans strain SC5314, originally isolated from a patient with disseminated candidiasis (23), and its otherwise isogenic, germination-deficient mutants *efg1/efg1/cph1/cph1* (32) and *tup1/tup1* (7) were graciously provided by Aaron Mitchell (Columbia University, Department of Microbiology) and Alexander Johnson (University of California, San Francisco, Department of Microbiology and Immunology), respectively. Both mutant strains resulted from the homozygous disruption of their respective genes using the URA3 cassette (21), but express at least one functional copy of the URA3 gene, and have similar growth rates under our *in vitro* conditions (not shown). The *efg1/efg1/cph1/cph1* mutant grows in the yeast form under most *in vitro* conditions tested (32). The *tup1/tup1* mutant grows exclusively as pseudo-filaments (7). *C. albicans* strains 32077 and 28366 are human oral isolates and were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The organisms were routinely propagated in YPD agar (Difco Laboratories, Detroit, MI) at 25°C.

Oral epithelial cell cultures

SCC15 cells were obtained from Dr. D. Wong (UCLA), and have originated from a well differentiated squamous cell carcinoma of the ventral tongue (31). The cells were maintained in Keratinocyte Serum Free Media (KSFM, Invitrogen, Carlsbad, CA), supplemented with 0.4 mM $CaCl_2$, 0.1 ng/ml EGF, 50 μg/ml bovine pituitary extract (Invitrogen) and antibiotics (penicillin/streptomycin, 100 U/ml and 100 μg/ml, respectively).

Coculture of *Candida albicans* with oral epithelial cells

Epithelial cells were seeded at or near confluence in 12-well (2×10^5 cells/well) polystyrene plates (Corning Inc., Corning, NY) and were incubated overnight in complete KSFM at 37°C in 5% CO_2 until they became confluent. The following day the media were discarded and the cells were challenged with suspensions of stationary phase viable organisms at varying fungal cell to epithelial cell ratios for up to 20 h. Previous work in our laboratory showed that a 1 : 1 yeast to epithelial cell ratio triggers optimal cytokine responses in this *in vitro* coculture system (15, 16), therefore this infectivity ratio was used in most cytokine experiments.

Stationary phase organisms were prepared by growth for 18 h at room temperature in YPD agar broth (Difco Laboratories), supplemented with 2% (wt/vol) dextrose. The fungal cells were harvested by centrifugation and washed in phosphate-buffered saline (PBS). Subsequently, organisms were counted in a hemacytometer and adjusted to the final concentration in complete KSFM before adding to epithelial cells.

To inhibit hyphal transformation, in some experiments stationary phase wild type organisms (strain SC5314) were preincubated in KSFM supplemented with 25 or 50 mM aminotriazole (AT; 3-amino-1, 2, 4-triazole, Sigma, St. Louis, MO) on a shaker at 37°C for 24 h (47). After that, the organisms were adjusted to the final concentration in KSFM, supplemented with 25 or 50 mM AT before adding to epithelial cells. Negative controls for these experiments included uninfected cultures in KSFM with or without AT. At the end of the experimental period supernatants were collected and stored at −70°C until assayed.

Cytokine detection

Culture supernatants obtained from duplicate or triplicate experiments were analyzed for IL-1α and IL-8 content. The choice of cytokines was based on previously reported work from our laboratory indicating a strong IL-8 secretory response of oral epithelial cells to strain SC5314 under these coculture conditions, which induce true hyphal transformation (15). In each experiment supernatants from two replicate wells were pooled and assayed by duplicate sandwich enzyme-linked immunosorbent assays (ELISA) using commercially available antibody pairs (Endogen MiniKit, Pierce, Rockford, IL). Briefly, flat-bottomed 96-well microtiter plates (Fisher Scientific, Springfield, NJ) were coated with 1 μl/ml of antihuman IL-1α or IL-8 monoclonal antibodies in carbonate buffer, and blocked with 1% bovine serum albumin in PBS for 4 and 6 h, respectively. For IL-1α analyses, 50 μl of supernatant and 50 μl of rabbit antihuman IL-1α cytokine antibody (Endogen, Woburn, MA) at a dilution of 1 : 5000 were added at the same time in each well, and incubated for 2 h. For IL-8 analyses, 100 μl of supernatant was added in each well and incubated for 1 h, followed by the addition of 100 μl of rabbit antihuman IL-8 cytokine antibody at 1 : 5000. The reaction color was developed by the addition of HRP-conjugated streptavidin followed by addition of TMB Substrate Solution (1 Step™ Ultra TMB-ELISA, Pierce). The reaction was stopped with 2 M H_2SO_4 (100 μl per well). The absorbance values and corresponding cytokine concentrations were determined with an Opsys MR Microplate reader (Dynex Technologies Inc., Chantilly, VA) using the Revelation QuickLink® software (Thermo Labsystems, Chantilly, VA). The sensitivity of these assays was 16 and 8 pg/ml for IL-1α and IL-8, respectively.

Microscopic examination of *C. albicans* morphotypes

In order to examine the *C. albicans* morphotypes during coculture with oral epithelial cells we used the Calcofluor White fluorescent dye. Calcofluor White is a chitin-binding fluorescent agent that

stains the cell wall of *C. albicans* (9). Epithelial cells were seeded on glass slides (4×10^5 cells/slide) contained within 6-well polystyrene plates (Corning Inc.) and were incubated overnight in complete KSFM at 37°C in 5% CO_2. The following day the media were discarded and the cells were challenged with 1×10^5 stationary phase viable organisms in complete KSFM. After 5 h of incubation at 37°C in 5% CO_2 the coverslides were carefully removed from each well and were placed on frosted microscope slides. Each coverglass was stained with 100 µl of 0.05 mM Calcofluor White (Calcofluor White Fluorescent Brightener 28, Sigma), and the morphology of each strain was examined by fluorescence microscopy.

Adhesion assay

The adhesion assay was set up in 12-well polystyrene plates (Corning Inc.). Plates were seeded at confluence (2×10^5 cells/well) and epithelial cells were allowed to adhere overnight at 37°C in 5% CO_2. The following day the media were discarded and 2×10^2 stationary phase viable *C. albicans* suspended in 1 ml of KSFM were added. After 0, 0.5, 2, 4, and 6 h of incubation the culture plates were placed on a horizontal shaker for 2 min at 200 r.p.m. and the content of each well was aspirated and spread onto YPD agar plates. Subsequently, 1 ml of KSFM was added to each well and the epithelial cell monolayer was removed by scraping and spread onto YPD agar plates. The proportion of nonadherent and epithelial cell-associated *C. albicans* cells was determined according to the colony forming unit (CFU) counts in these plates after 48 h incubation at room temperature. The *C. albicans* CFU numbers were normalized by the total number of CFU retrieved at each time point, and results were expressed as percent of epithelial cell-associated *C. albicans* cells over the total number of cells retrieved from each well. The total number of the cells added did not vary significantly from the total number of the cells retrieved after 6 h of coculture ($P > 0.05$, not shown). Each mean value was based on at least three separate experiments, with experimental conditions set up in duplicate.

Assessment of the cytotoxicity of different *C. albicans* morphotypes

Oral epithelial cell lysis during coculture with *C. albicans* was assessed by the CytoTox-96 assay (Promega, Madison, WI), which measures release of lactate dehydrogenase (LDH) from dying cells. In these experiments SCC15 cells were cocultured with increasing doses of wild type, *efg1/efg1/cph1/cph1* and *tup1/tup1* strains for up to 20 h and the LDH released from this coculture system was quantified by spectrophotometry, according to the manufacturer's instructions. Uninfected cultures (control (1)) and *C. albicans* alone (control (2)) incubated under identical conditions, were included as negative controls. Total LDH release was estimated by treating control SCC15 cells with 9% Triton X-100 for 20 h. The LDH released in the presence of *C. albicans* was quantified according to the formula:

$$[\text{Experimental} - \text{control}(1) - \text{control}(2)/\text{Total-control}(1)] \times 100$$

and values were expressed as percent of the total LDH released.

Statistical analyses

The statistical significance of the differences in cytokine stimulation, adhesion and cytotoxicity among different morphotypes was determined by two-tailed *t*-test, assuming equal variances between groups. Differences were statistically significant at $P < 0.05$.

Results

Filamentation mutants and different oral strains exhibited variable morphogenetic patterns when cocultured with oral epithelial cells

Because hyphal transformation is strongly dependent on the growth conditions (35) and most germination-deficient mutant strains can still form true hyphae under certain experimental conditions (13), it was important to establish that the wild type and mutant strains retained their germination phenotype when infecting oral epithelial cells under these conditions. To examine the morphogenetic forms of *C. albicans* strains cocultured with oral epithelial cells, Calcofluor White was used to stain the fungal cell walls. Oral epithelial cells nonspecifically absorbed some of the Calcofluor White stain, in contrast to what has been published for kidney epithelial cells (6), and can be seen in the background (Fig. 1, 2). The wild-type SC5314 strain germinated within 2 h over the epithelial cell monolayer and produced long hyphae with parallel walls along the entire length, minimum branching and no evident constriction at the neck level.

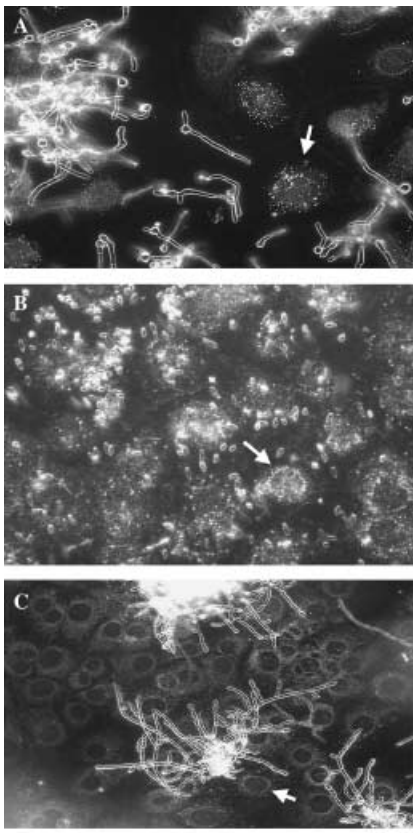

Fig. 1. Cellular morphology of strain SC5314 wild type (A), *efg1/efg1/cph1/cph1* mutant (B) and *tup1/tup1* mutant (C), cocultured with oral epithelial cells for 5 h. Epithelial cells are indicated by the white arrows. Cultures were stained with Calcofluor White and fluorescence micrographs are shown. Bar = 10 µm.

These hyphae have been classified as true hyphae (4) (Fig. 1A). Germination mutants, congenic to strain SC5314, were also examined microscopically to confirm differences in the germination pattern from the wild type strain when cocultured with oral epithelial cells under these experimental conditions. As expected, the *efg1/efg1/cph1/cph1* double mutant did not germinate and remained in the yeast form (4), observed as isolated ovoid cells, throughout the 24 h coculture period (Fig. 1B). As in previous reports using different growth conditions (7), strain *tup1/tup1* formed filamentous organisms, displaying constrictions at their septa between individual cell compartments, consistent with pseudohyphae (Fig. 1C).

In addition to strain SC5314 and its germination-deficient mutants, two oral strains were examined microscopically in order to elucidate their morphogenetic pattern in this coculture system. The choice of the two strains was based on our previous findings that strains ATCC28366 and

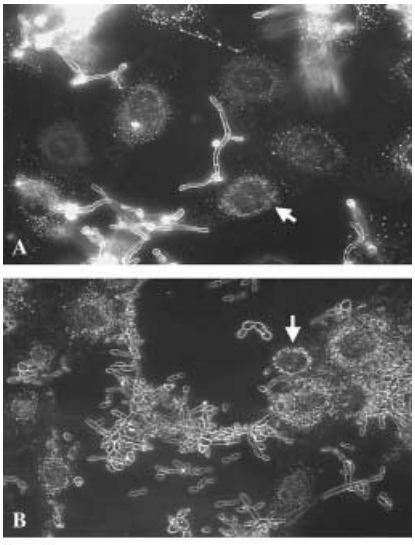

Fig. 2. Cellular morphology of oral strains ATCC28366 (A) and ATCC32077 (B), cocultured for 5 h with oral epithelial cells, indicated by the white arrows. Cultures were stained with Calcofluor White and fluorescence micrographs are shown. Bar = 10 μm.

ATCC32077 differ dramatically in their ability to trigger cytokine responses by oral epithelial cells, even though they exhibit filamentous growth and have similar growth rates in KSFM (15, 16). Calcofluor staining revealed distinct morphologic differences in the filaments formed by the two strains (Fig. 2). Specifically, strain ATCC28366 germinated within 2 h to produce hyphal structures with parallel walls, and some lateral branches along their length, showing a morphogenetic pattern that is consistent with true hyphal transformation (Fig. 2A). In contrast to strain ATCC28366, strain ATCC32077 formed pseudohyphae visualized as cells that remained attached to one another at the constricted septation site (Fig. 2B). This strain retained its pseudohyphal form even when cocultured with oral epithelial cells in the presence of 20% fetal bovine serum, a potent hyphal germination inducer (8, 10) (not shown). Morphogenesis under these growth conditions was not affected by the presence or absence of oral epithelial cells in any strain examined (not shown).

The ability of *C. albicans* to trigger proinflammatory cytokine responses by oral epithelial cells depended on its potential to form true hyphae

We have previously shown that strain ATCC28366 is capable of inducing a strong proinflammatory cytokine response by oral epithelial cells, whereas strain ATCC32077 is incapable of eliciting high cytokine responses (15, 16). Given the different morphologic phenotypes of these strains, we hypothesized that true hyphal transformation is important in triggering cytokine responses by oral epithelial cells. To test this hypothesis we compared wild type strain SC5314, which forms true hyphae, with its agerminative mutant *efg1/efg1/cph1/cph1* and pseudohyphal mutant *tup1/tup1.* These mutants had minimal effect on the IL-8 and IL-1α secretion by SCC15 cells after coculture for up to 18 h. In contrast, under these conditions, wild type strain SC5314, which forms true hyphae, stimulated high levels of IL-8 and IL-1α secretion by SCC15 cells (Fig. 3). These results were similar even when higher yeast inocula and longer incubation periods were tested (not shown).

True hyphal transformation of strain SC5314 in KSFM is inhibited by the presence of AT in a dose-dependent manner (Fig. 4). Therefore, in order to provide additional evidence that the proinflammatory cytokine responses of epithelial cells to this strain were dependent on its ability to form true hyphae, we performed oral keratinocyte experiments in the presence of increasing amounts of AT, using AT-induced mixed hyphal, pseudohyphal, and yeast forms of strain SC5314 as a stimulus. AT did not affect basal epithelial cell cytokine levels. AT-induced yeast, hyphal, and pseudohyphal forms of strain SC5314 triggered significantly reduced cytokine responses by oral epithelial cells as compared to true hyphal organisms, after 8 h of coculture ($P < 0.002$) (Fig. 5).

These results show that the ability of *C. albicans* to trigger proinflammatory cytokine responses by oral epithelial cells depends on its potential to form true hyphae.

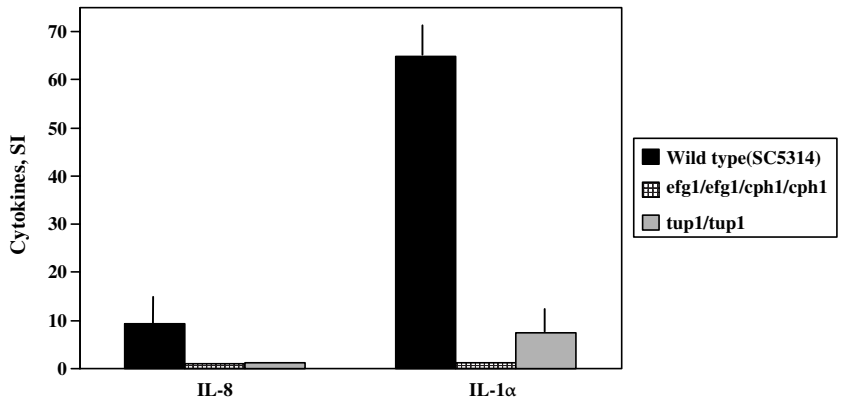

Fig. 3. Cytokine responses of SCC15 cells to different *C. albicans* morphotypes. Cells were exposed to viable *C. albicans* SC5314 (wild type) or its congenic germination-deficient mutants *efg1/efg1/cph1/cph1* and *tup1/tup1,* at 1 : 1 fungal cell to epithelial cell ratio, for 18 h. Results are expressed as the Stimulation Index (Stimulated/Basal, SI). Mean values were obtained by analysis of at least two separate experiments followed by duplicate ELISA of culture supernatants. The bars represent one SD of the mean.

Yeast and pseudohyphal strains showed reduced adherence to oral epithelial cells

In order to further investigate the mechanisms leading to differences in cytokine responses triggered by yeast, pseudohyphal, and hyphal forms of *C. albicans,* we compared the ability of strain SC5314 to adhere to oral epithelial cells with its germination-deficient mutants *efg1/efg1/cph1/cph1 and tup1/tup1.* In addition, strain ATCC32077, which germinates into pseudohyphae under our coculture conditions, was tested. Adherence of most strains to oral epithelial cells was time-dependent, with maximum levels obtained after 4 h of coculture. Strain SC5314 had the greatest ability to adhere to oral epithelial cells, with 81.82 ± 10.24% of fungal cells being associated with epithelial cells after 6 h of incubation (Fig. 6). In contrast, only 41.77 ± 15.51% of the pseudohyphal strains ATCC32077 and 31.27 ± 16.12% of the *tup1/tup1* mutant adhered to oral epithelial cells after 4 h of incubation, and longer incubation of these strains with epithelial cells did not result in additional adherence (Fig. 6). Finally, only 17.52 ± 12.11% of the yeast-forming mutant *efg1/efg1/cph1/cph1* adhered to oral epithelial cells during the experimental period (Fig. 6). These results suggest that the ability of *C. albicans* to adhere to oral epithelial cells is compromised when the organism is unable to form true hyphae.

Yeast and pseudohyphal forms of *C. albicans* did not cause injury to oral epithelial cells

Once in contact with epithelial cells, *C. albicans* is known to invade their

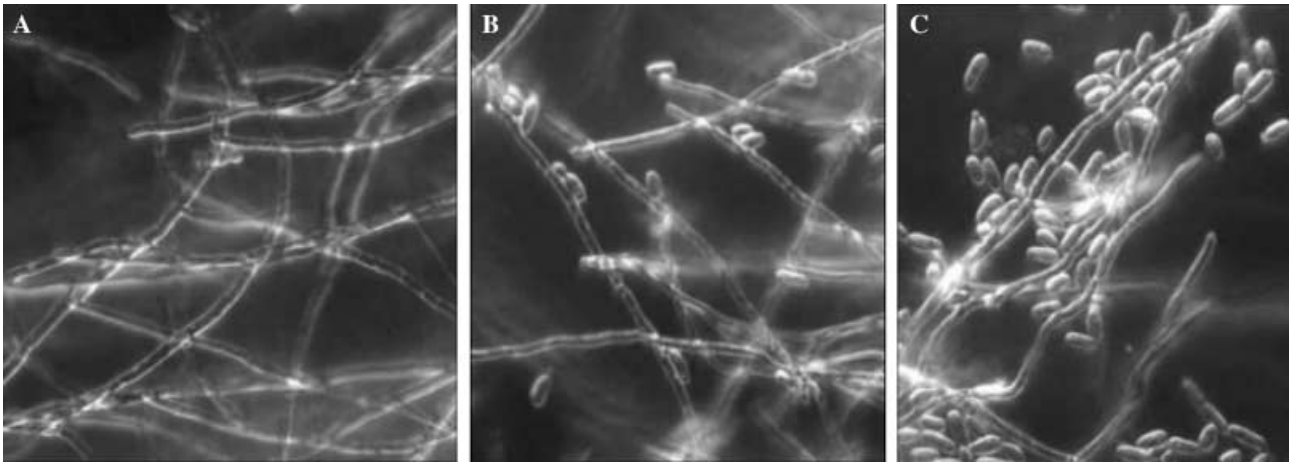

Fig. 4. Cellular morphology of strain SC5414 (wild type) grown in the absence (A) or presence of 25 mM AT (B) or 50 mM AT (C) for 24 h, at 37°C in KSFM. Cultures were stained with Calcofluor White and fluorescence micrographs are shown.

cytoplasm and trigger extensive cell damage (18). Since the ability of pseudohyphal and yeast forms of *C. albicans* to adhere to, or physically associate with, oral epithelial cells was compromised, we hypothesized that the ability of these morphotypes to inflict cell injury is also impaired. To test this hypothesis we compared the levels of LDH released when wild type strain SC5314 and its germination-deficient mutants were cocultured with SCC15 cells, at different infectivity ratios for 20 h.

When compared to its germination-deficient mutants, wild type strain SC5314 was the most powerful in promoting oral keratinocyte lysis, inducing the lysis of up to 96% of the oral epithelial cells at the end of the coculture period (Fig. 7). The germination impaired mutants *efg1/efg1/cph1/cph1* and *tup1/tup1* did not promote any considerable damage to keratinocytes in the range of infectious doses tested (Fig. 7). These results suggest that in contrast to true hyphal organisms, yeast and pseudohyphal forms of *C. albicans* cause minimal breakdown in the oral epithelial cell membrane integrity.

Discussion

This study has shown that *C. albicans* transformation into true hyphae promotes its interactions with oral epithelial cells. Specifically, we have shown for the first time that, under the conditions employed in this study, the ability to assume filamentous growth in the form of pseudohyphae is not sufficient to elicit a strong proinflammatory cytokine response by oral mucosal epithelial cells. Additionally, although differences in adhesion between yeast and hyphal forms of *C. albicans* have previously been reported (44), to our knowledge, this is the first study showing that pseudohyphal forms of this organism exhibit reduced capacity to adhere to oral epithelial cells, as compared to true hyphal forms.

There is a high degree of variability in the morphology of *C. albicans* clinical isolates. Some strains are characterized by vigorous hyphal growth, whereas others display deficiencies in true hyphal transformation (34). While the original thinking was that pseudohyphae represent a developmental stage between yeasts and true hyphae (34), more recent studies indicate that true hyphae have cell cycle characteristics that distinguish them from pseudohyphal and yeast cells (46). It has been hypothesized that the capacity of *Candida* strains to form true hyphae may be related to their potential for pathogenicity (41). In most oral infections, both yeast and filamentous organisms can be found in the infected tissues (36). However, clinicopathologic findings have correlated the presence of filamentous forms with localized tissue invasion in oral candidiasis (11, 39) and hyphal forms of *C. albicans* are in general thought to play an important role in the pathogenesis of invasive candidiasis (41).

We studied the role of hyphal transformation in the interactions with oral epithelial cells by examining two mutant strains of *C. albicans* SC5314 (*efg1/efg1/cph1/cph1* and *tup1/tup1*) that are not capable of true hyphal transformation. EFG1, CPH1, and TUP1 are transcriptional regulators that affect hyphal transition. The EFG1 and CPH1 transcription factors represent two distinct signaling

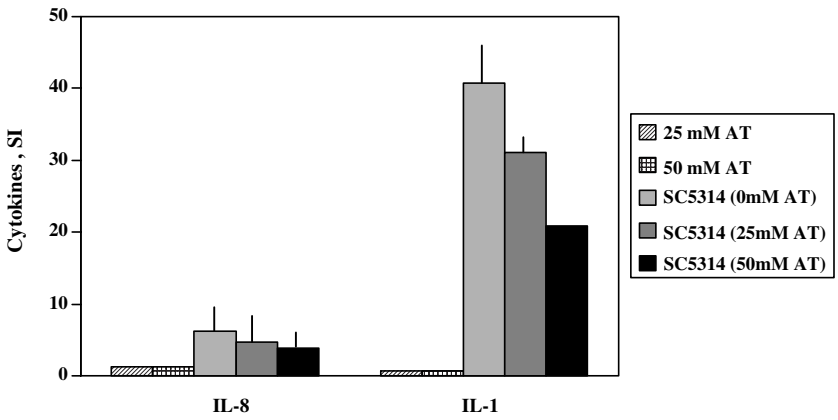

Fig. 5. Cytokine responses of SCC15 cells to *C. albicans* strain SC5314 in the presence or absence of AT. Epithelial cells were exposed to viable SC5314 organisms, which were preincubated with 0, 25, and 50 mM of AT for 24 h. The organisms were then added to epithelial cells at 1 : 1 fungal cell to epithelial cell ratio, for 8 h. Results are expressed as the Stimulation Index (Stimulated/Basal [absence of fungal stimulus and AT], SI). Mean values were obtained by analysis of at least three separate experiments followed by duplicate ELISA of culture supernatants. The bars represent one SD of the mean.

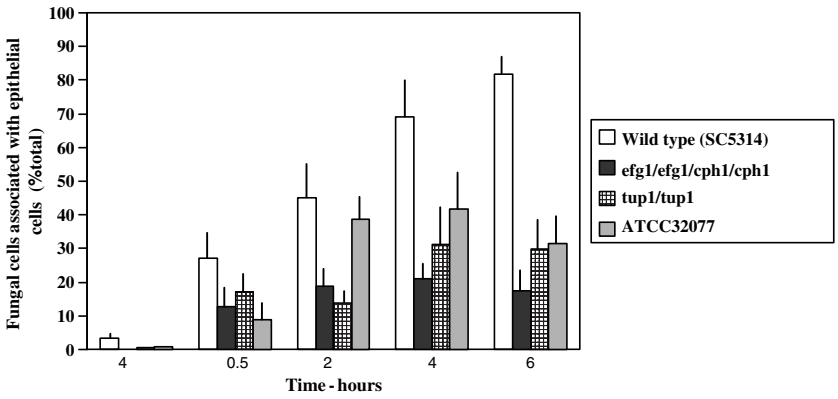

Fig. 6. Adherence of different *C. albicans* morphotypes to oral epithelial cells. Wild type and mutant organisms as well as pseudohyphal strain ATCC32077 were allowed to interact with oral epithelial cells for 0–6 h. Results are expressed as percentage of organisms physically associating with SCC15 cells at the end of each incubation period. Mean values were obtained by analysis of at least three separate experiments and the bars represent one SD of the mean.

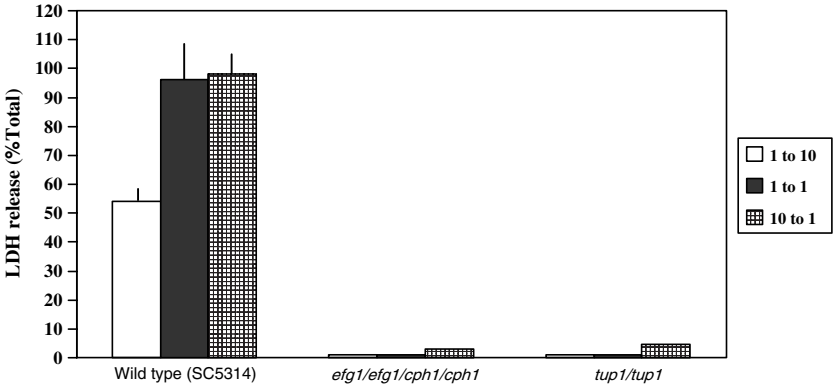

Fig. 7. Ability of different *C. albicans* morphotypes to injure oral epithelial cells. SCC15 cells were cocultured with *C. albicans* SC5314 or its congenic germination-deficient mutants *efg1/efg1/cph1/cph1* and *tup1/tup1* at increasing fungal cell to epithelial cell ratios, for 20 h, and LDH release was quantified in culture supernatants. Mean values were obtained by analysis of at least three separate experiments and the bars represent one SD of the mean.

pathways, promoting hyphal development (for review see [8]), whereas TUP1 represses the transcription of genes involved in hyphal growth (7). These transcription factors are essential for virulence of *C. albicans* in a mouse model of hematogenous infection (32). *Efg1/efg1/cph1/cph1* double mutants show reduced capacity to successfully colonize central venous catheters (30) as well as reduced ability to injure or invade epidermal, intestinal, and endothelial cells (14, 29, 37).

Adhesion of *C. albicans* to keratinocytes is thought to be a requirement for skin and mucosal infections, and mutants deficient in adherence show reduced virulence in animal models of candidiasis (1). The yeast mutant *efg1/efg1/cph1/cph1* has been shown to have diminished capacity to adhere to epidermal or intestinal mucosal systems *in vitro* (14), consistent with our results in oral epithelial cells. EFG1 is a major regulator of HWP1, a cell wall protein that contributes approximately 50% to the overall adhesion of *C. albicans* to buccal epithelial cells (45), and this may at least partly explain the inability of this mutant organism to adhere to SCC15 cells. It was previously shown that *C. albicans* germination leads to enhanced adherence to buccal epithelial cells (28, 44); however, this is the first study to show that the ability to assume elongated growth per se is not sufficient for optimal adherence to host cells, since pseudohyphal strains did not adhere to the same extent as hyphal strains.

In sharp contrast to strain SC5314, which formed true hyphae under these coculture conditions, germination-deficient mutants *efg1/efg1/cph1/cph1 and tup1/tup1* triggered essentially no proinflammatory cytokine responses in this coculture system. Although other cytokines were not tested, it is possible that the mutant strains trigger a different array of cytokines than the ones triggered by hyphal organisms. In support of this hypothesis, dendritic cells and macrophages are known to discriminate between yeasts and hyphae of *C. albicans* by generating a distinct pattern of cytokines to each form (5, 19). Our results with strain *efg1/efg1/cph1/cph1* are in agreement with recent reports concerning IL-1β and IL-8 responses in reconstituted oral epithelium (29). To our knowledge, this is the first report to describe failure of pseudohyphae to induce a proinflammatory cytokine response by host cells *in vitro*. It is likely that the inability of mutant strains *efg1/efg1/cph1/cph1* and *tup1/tup1* to trigger a proinflammatory cytokine response is due to multiple effects of these factors at different transcription levels.

Longer incubation (20 h) of hyphal strain SC5314 triggered damage of up to 96% of oral keratinocytes, whereas *efg1/efg1/cph1/cph1* and *tup1/tup1* mutants caused essentially no damage. These results were unrelated to differences in the number of viable organisms, since preliminary fungal growth experiments revealed similar numbers of microorganisms from each strain at the various time points of coculture studied ($P > 0.05$, not shown). This is supported by other reports in oral epithelial cell *in vitro* systems using the SC5314 congenic mutant *efg1/efg1/cph1/cph1,* which examined growth of these organisms in similar serum-free coculture media (29). Consistent with our findings, the *efg1/efg1/cph1/cph1* mutant was incapable of injuring endothelial cells *in vitro* (37). In addition, this mutant strain failed to damage or invade a reconstructed intestinal mucosal *in vitro* system, even after prolonged coincubation (14). Similar to our findings with the *tup1/tup1* pseudohyphal mutant, another pseudohyphal mutant (*fkh2/fkh2*) was recently shown to have significantly reduced capacity to injure an oral epithelial cell line (3).

Keratinocyte lysis by true hyphae may be mediated by secreted aspartyl proteinases (29), or phospholipases expressed at the hyphal tip (2, 22). A subset of *C. albicans* secreted aspartyl proteinases (SAP4-SAP6) comprises hypha-associated proteases, regulated by the EFG1 transcription pathway, which are required for tissue invasion (20). However, these proteases are only expressed in the presence of serum (26), and therefore are unlikely to be responsible for cell lysis in this *in vitro* system. Secreted aspartyl protease 2 contributes to endothelial cell injury by

C. albicans (27), and is important for epithelial cell damage in an *in vitro* model of oral candidiasis (43, 42). When cocultured with human oral reconstituted epithelium under serum-free conditions, wild type SC5314 expresses SAP2 whereas double mutant strain *efg1/efg1/cph1/cph1* does not (29). Therefore this protease is likely one of the lytic enzymes responsible for the oral epithelial cell damage inflicted by strain SC5314 in our system. *C. albicans* phospholipases may also play a role in cell lysis (2, 22). TUP1 mutants express high amounts of phospholipase B1 (25), an enzyme that probably does not contribute to epithelial cell damage in this coculture system, since the *tup1/tup1* mutant caused essentially no damage to oral keratinocytes. However, this does not preclude the possibility that other phospholipases expressed at the hyphal tip may play a role in cell lysis. Further experiments are needed, using soluble products from fungal cells or cell culture inserts separating fungal and host cells during coculture, to establish whether a secreted or surface-expressed *C. albicans* mediator is responsible for cell damage.

Taken together our data suggest that morphogenesis is an important determinant of the outcome from the interactions between oral epithelial cells and *C. albicans*. Based on our results we can speculate that yeast and pseudohyphal forms of this pathogen are unlikely to be responsible for the extensive epithelial damage, surface ulceration, and acute inflammatory response in the oral mucosa frequently associated with *C. albicans* infection (38). Future studies are needed to fully elucidate the specific recognition systems between true hyphae and oral epithelial cells that mediate adhesion, as well as stimulation of an inflammatory cytokine response, and the virulence factors expressed specifically by true hyphal organisms, which are responsible for oral epithelial cell damage.

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