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An ultrastructural and a cytochemical study of candidal invasion of reconstituted human oral epithelium

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BACKGROUND: Opportunistic yeast, *Candida albicans* causes superficial and systemic mycoses in compromised patients. Adhesion to host tissues, morphogenesis and extracellular phospholipases (PL) are thought to contribute to its virulence. The nature of numerous host–parasite interactions at the invasive phase of oral candidiasis is not fully understood. Hence in this study, we explore the ultrastructural features of oral candidiasis using a tissue culture model based on reconstituted human oral epithelium (RHOE).

METHODS: Reconstituted human oral epithelium (Skinethic Laboratory, Nice, France) was inoculated with *C. albicans* SC5314 and incubated up to 48 h. The infected tissue was harvested at 12, 24 and 48 h and examined using light, scanning (SEM) and transmission electron microscopy (TEM). Localized activity of PLs of *C. albicans* during tissue invasion was also examined using a cytochemical method.

RESULTS: Over a period of 48 h *C. albicans* invaded the RHOE, and histological examination revealed characteristic hallmarks of pathological tissue invasion. Hyphal penetration into the superficial epithelium, particularly at cell junctions, together with features of cellular internalization of yeasts was noted. Phospholipase activity was visible at the tips of hyphae and initial sites of bud formation. Further, SEM studies revealed cavitations on the surface epithelial cells particularly pronounced at the sites of hyphal invasion. Hyphal invasion was seen both at cell surfaces and intercellular cell junctions of the epithelium, the latter resembling thigmotropic behaviour.

CONCLUSIONS: Our findings confirm that multiple cellular interactions such as internalization, thigmotropism and extracellular PLs contribute to invasive candidiasis. The RHOE model, described here, appears to be a satisfactory model for the investigation of ultrastructural and histochemical features of invasive candidiasis in humans.

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Introduction

The yeast *Candida albicans* is a common, human commensal organism that inhabits the oral cavity. It is an important opportunistic pathogen, which causes superficial as well as potentially life-threatening systemic mycoses in compromised patients (1). Adhesion to host tissues, morphogenesis and extracellular enzyme production are some major virulent attributes of *Candida* species (2). During the initial stages of superficial mucosal infection *C. albicans* forms filamentous hyphae, which show thigmotropism (3, 4), a phenomenon also, known as contact guidance, in addition to releasing various hydrolytic enzymes (5). One of the more important hydrolytic enzymes produced by *C. albicans* is phospholipase (PL) that has now been classified into several subgroups.

Phospholipases, well-known for their cytolytic activity, are found in many bacterial toxins, arthropod poisons and snake venom (6). They are categorized as PL-A, -B, -C and -D, lysophospholipase and lysophospholipase-transacylase (7). Most of these are known to participate in the breakdown of phospholipids of host cell membranes during invasive candidal infections (8). Although there is data on PLs of *C. albicans* in *in vitro* culture media, only a few studies are available on their *in vivo* activity. In a pioneering study, Pugh and Cawson (9) using chick chorioallantoic membrane as a candidiasis model demonstrated the PL-A and lysophospholipase activity at the invading hyphal tips and initial budding. Recently, immunogold electron microscopic methods have also been used to demonstrate the distribution of *C. albicans* PL-B during tissue invasion in an experimental candidiasis model in mice (7, 10).

Oral epithelium serves as an invasive barrier against candidal infections and releases several immunomodulatory molecules. Although not phagocytic, like neutrophils and macrophages, cultured buccal and vaginal epithelial cells can exhibit a phenomenon called 'cellular

internalization' of organisms and behave as 'atypical' phagocytes (11). Moreover, it has been reported that *C. albicans* blastospores, irrespective of their viability are able to induce phagocytosis in cultured endothelial cells (12). This phenomenon together with receptor-mediated adhesion and hyphal invasion may modulate the process of invasive candidiasis. Therefore, ultrastructural investigations of the interphase of this opportunistic yeast and the oral epithelium may uncover important features, which may help to clarify the pathogenesis of superficial candidiasis.

Previously, ultrastructural studies of the invading front of mucosal candidiasis have been undertaken using biopsy specimens from humans (13–18) and animals (19, 20). Reconstituted human oral epithelium (RHOE), maintained as multilayer natural cell cultures, has been recently used as a satisfactory model for experimental candidiasis (21, 22), although the ultrastructural studies of this system have not been reported. Therefore in the present study, we evaluated cellular interactions of the yeast-epithelial interphase using the RHOE model. These ultrastructural studies with transmission (TEM) and scanning (SEM) electron microscopy focused on phenomena such as cellular internalization and thigmotropism, whilst in addition we evaluated, using cytochemical methods, the production of PL-A and lysophospholipase during candidal invasion.

Materials and methods

Culture media and growth conditions

A well-characterized wild-type strain of *C. albicans* SC5314 was used in all experiments. The identity of the organism was reconfirmed by the germ tube test, and fermentation reactions with commercially available API 20C auxonogram strips (Biomerieux, Marcy I'Etoile, France). In order to create artificial infection of the RHOE, inocula were prepared as previously described (21). Briefly, yeast cells were cultured for 24 h at 37°C on Sabouraud dextrose agar (Oxoid, Hampshire, England). The cell suspension obtained was then cultured in YPG medium (1% yeast extract, 2% peptone and 2% glucose; Difco, Detroit, MI, USA) for 16 h at 25°C with orbital shaking and the cells were harvested, washed in 0.9% NaCl and resuspended in fresh YPG medium and incubated in a shaker for 24 h at 37°C. Afterwards the cells were harvested by centrifuge and a cell suspension (4×10^7 yeast cells/ml) was prepared in phosphate-buffered saline (PBS, pH 7.2) for the experiments.

Model of oral candidiasis

Human oral epithelium (human keratinocytes derived from cutaneous carcinoma cell line TR146; Skinethic Laboratory, Nice, France) was reconstituted by incubating in serum-free, MCDB 153 defined medium (Clonetics, San Diego, CA, USA), containing 5 μg/ml insulin, 1.5 mM $CaCl_2$, 25 μg/ml gentamicin and 0.4 μg/ml hydrocortisone in 6-well tissue culture plates (Corning Inc., Corning, NY, USA) according to the manufacturer's instructions. The *in vitro* model and all culture media were prepared without antibiotics or antimycotics. After reconstitution, a 0.5 cm^2 segment of tissue was inoculated with 2×10^6 yeast cells in 50 μl of PBS (pH 7.2). Tissue cultures so inoculated were incubated at 37°C, at 5% CO_2 and saturated humidity for up to 48 h. They were harvested at 12, 24 and 48 h time intervals for ultrastructural and histochemical evaluation as described below. The medium was replenished once in every 24 h.

Cytochemical localization of extracellular phospholipases

Extracellular activity of PL-A and lysophospholipase was examined cytochemically as described by Pugh and Cawson (9). In brief, after 12, 24 and 48 h, part of the infected tissue was removed and fixed in formalin-calcium chloride solution for 2 h at 0–4°C. After washing in cold Ringer solution twice (5 min each), small blocks of tissue (1–2 mm diameter), dissected under the stereo-microscope were incubated with an incubation mixture of 0.2% lead nitrate and lecithin (Sigma Chemical Co., St Louis, MO, USA) as a substrate for PL-A and lysophospholipase, at 37°C for 20–30 min, pH 5 (lead precipitates due to interactions with the fatty acids derived by the action of PL-A and lysophospholipase were taken as evidence of the enzyme activity). In control experiments lecithin was not used as a substrate. After incubation, the tissue blocks were rinsed in distilled water and post-fixed in 1% osmium tetroxide for 4 h. Following serial dehydrations in ethanol the tissue blocks were embedded in LR White (Electron Microscopy Sciences, Hatfield, PA, USA) for ultrastructural studies.

Electron microscopy

Some blocks of the infected tissue were immersed overnight in 2.5% glutaraldyhyde and 2% formaldehyde in a 0.05 M cacodylate-buffered solution at pH 7.3 (23). Afterwards the tissues were washed briefly in phosphate buffer and post-fixed in 1% osmium tetroxide for 4 h. Then, the tissues were prepared for TEM and SEM examination separately.

TEM

Post-fixed tissues were washed in two changes of phosphate buffer for 30 min. Then they were dehydrated in graded concentrations of ethanol and were embedded in LR White (Electron Microscopy Sciences) and polymerized for 24 h at 60°C. Preliminary semithin sections were cut at 1 μm using ultramicrotome (Reichart Ultracut S, Leica, Nussloch, Germany) with glass knives and stained with toluidene blue for light microscopy. Some of the blocks thus selected were cut using the ultramicrotome at 60–90 nm with a diamond knife (Diatome, Bienne, Switzerland) and the resultant ultrathin sections were placed on cleaned uncoated 200 mesh copper grids, stained with 2% uranyl acetate and lead citrate before examination under a TEM (Philips EM208S: Philips, Eindhoven, USA).

SEM

Post-fixed tissues were washed in two changes of phosphate buffer. Then they were dehydrated in graded

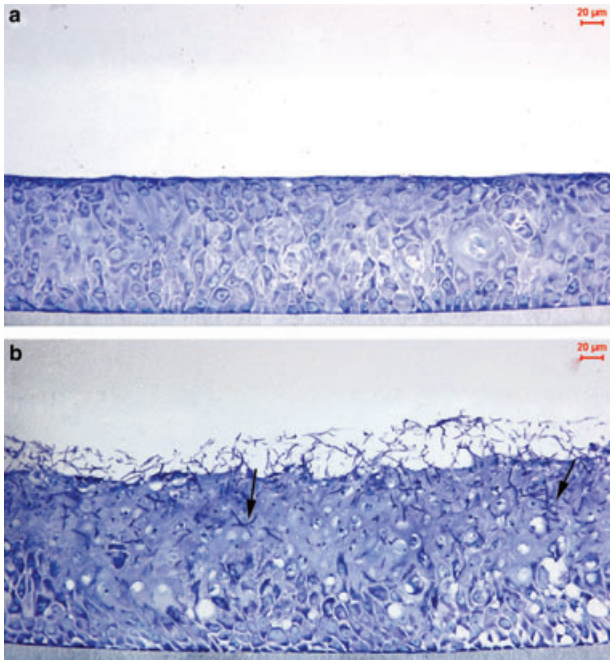

Figure 1 Light micrographs of reconstituted human oral epithelium (RHOE), after toluidene blue staining. (a) RHOE 12 h after culture (controls). Stratified keratinocytes with 10- to 12-cell layers are seen. (b) RHOE 12 h after inoculation with *Candida albicans*; adherence and invasion (arrowed) of *Candida* cells into the epithelium with severe oedema and disorganization of cell layers.

concentrations of ethanol and critical point-dried in 100% CO_2. Specimens were mounted on aluminium stubs, with copper tape, coated with gold in a low-pressure argon atmosphere with an ion sputter coater (Jeol JFC1100: Jeol, Tokyo, Japan). Tissues were viewed using a SEM (Philips XL 30CP) at an accelerating voltage of 10 kV.

Results

Light and electron microscopic features of normal and infected RHOE

Light microscopy

In general, the structural features of RHOE resembled the normal human oral epithelium except for some dyskeratotic cells seen rarely (Fig. 1a). After 12 h of yeast inoculation, clusters of yeast cells were detected on the superficial keratinocytes layer (Fig. 1b). At this stage some of the superficial cells displayed degeneration and intercellular oedema. Fungi in extra- and intra-cellular sites were then seen invading the basal cell layers especially at the latter stages of infection (24–48 h) causing significant structural damage to the epithelium.

Transmission electron microscopy

The tissue consisted of well-stratified keratinocytes, which were attached to each other by many zipper-like desmosomal cell junctions. Moreover, these keratinocytes were rich in irregularly arranged thick bundles of tonofibrils (Fig. 2).

Candida albicans produced both yeast and hyphal forms on RHOE. Hyphal elements were seen to penetrate the epithelial cells with no apparent disorganization of the cytoplasm of the host cells and, some cell membranes appeared to be intact covering the advancing hyphal tips (Fig. 2). Sometimes the hyphae were seen to invade through several epithelial cells changing their direction of penetration. Hyphal elements were also seen traversing intercellular gap junctions of the epithelium.

Yeast cells generally had a thick electron dense cell wall, and a dense floccular material external to the cell wall (Fig. 3). This material was particularly pronounced at the tips of the hyphae and formed a conical structure on occasions (Fig. 2). Furthermore, some hyphae demonstrated a 'bulge' at the tips, when they were penetrating the epithelial cells (Fig. 4).

Some of the superficial epithelial cells exhibited internalization of the yeast cells resembling phagocytosis. These epithelial cells produced cytoplasmic processes resembling pseudopodia embracing the yeast cells (Fig. 3). Yeast cells already within the host cells were seen lying in cytoplasmic vacuoles.

Scanning electron microscopy

Many *C. albicans* blastospores appeared randomly dispersed on the surface of the epithelium with hyphae extending from the parent cell during the first 24 h. Hyphal forms were predominantly seen at the late stage of infection (24–48 h). The surfaces of the blastospores and hyphae were generally smooth except for occasional bud scars and budding. Hyphal invasion of the epithelium was sometimes seen through the intercellular gaps (Fig. 5). However, some hyphae directly penetrated the epithelial cells and on doing so produced localized cavitations of the cell membrane at the site of penetration (Fig. 6).

Phospholipase activity

Cytochemical localization of combined activity of PL-A and lysophospholipase was mainly noted at the yeast cell peripheries. However, the enzyme activity appeared to vary depending on the yeast and hyphal phase of *C. albicans*. Whereas some yeast cells demonstrated generalized diffuse enzyme activity, in others the enzymes were markedly localized at initial bud sites and, tips of hyphae and occasionally associated with the extracellular floccular material (Fig. 7a,b).

In temporal terms localized enzyme activity was seen at all three-time points examined (i.e. 12, 24 and 48 h). However, activity was notable after 24 h of infection and was highly pronounced after 48 h, leading to heavy stain deposits.

Discussion

Ultrastructural features of superficial candidal infections have been studied using a number of different experimental models such as biopsy specimens (13–18), cultured cells (11, 12) and chick chorioallantoic membrane (24). These experimental models have been recently complemented by the cell culture-based RHOE. The advantages of this model are: (i) its

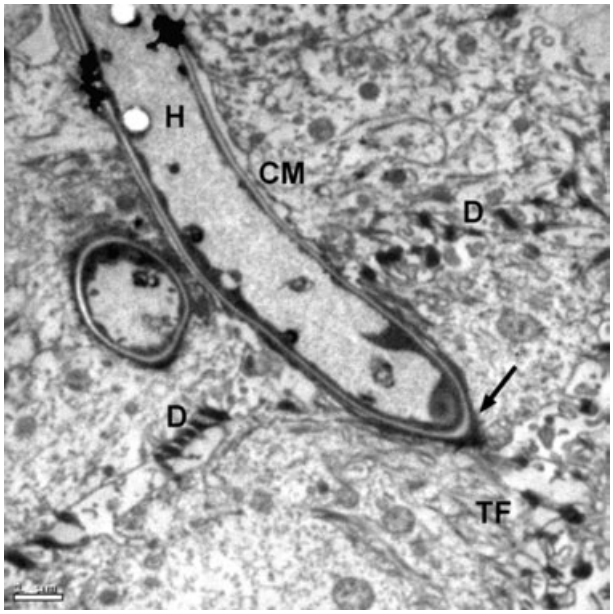

Figure 2 Longitudinal section of a *Candida albicans* hyphal element (H) within the reconstituted human oral epithelium (RHOE). Desmosomal cell junctions (D) of the host epithelium and dense tonofibrils (TF) are arranged irregularly in the host cytoplasm. Host cell membrane (CM) can be seen close to the invading hypha. Extracellular material can be seen as a conical structure (arrowed) at the tip of the hypha.

multilayer structure (up to 10- to 12-cell layers) closely resembling the oral epithelium, which contrasts with the single layer traditional tissue culture systems, (ii) the ability to artificially reproduce the internal milieu of the oral cavity within a pH range of 7.0–7.4 and (iii) its ready 'off-the-shelf' use for time-limited

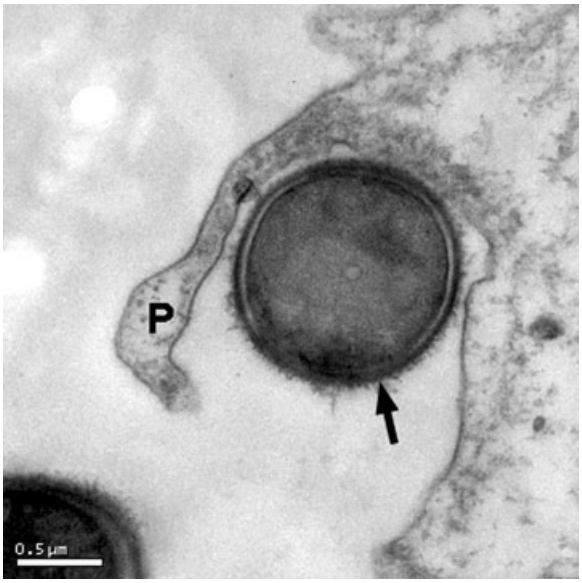

Figure 3 An electron micrograph showing the extension of the host cytoplasmic process (P) partially engulfing the adjacent yeast cell resembling pseudopodia formation during phagocytosis. The extracellular floccular material (arrowed) of the yeast is extensively seen here.

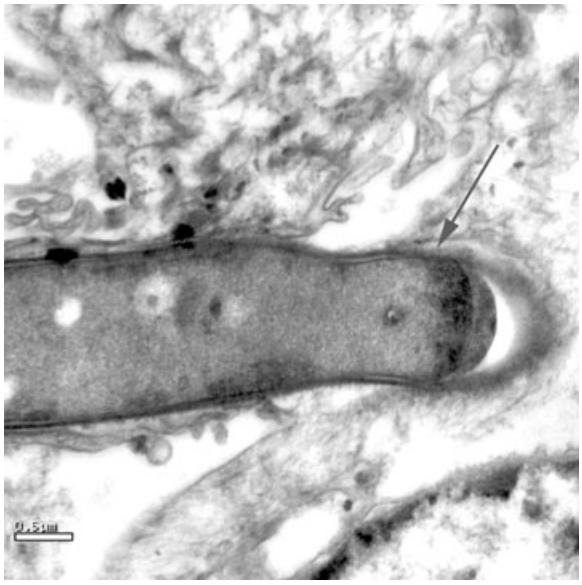

Figure 4 Characteristic apical bulging appearance of some hyphal tips (arrowed) resembling plant 'appressoria'.

experiments. Furthermore, previous work has shown that, histologically, RHOE resembles the normal human oral epithelium and the pathological changes that accompany candidal invasion are akin to human disease (21).

In the current study, the ultrastructural features of the RHOE, resembled closely the healthy human epithelium. Thus, numerous desmosomes arranged in a zipper-like manner between individual cells seem to provide tight attachment of subjacent cells leading to mechanical reinforcement. The cytoplasmic cytoskeleton of epithelial cells comprising thick bundles of tonofilaments appeared to provide additional internal support for the epithelium. Possibly because of this natural cellular architecture RHOE could withstand a 48-h period of candidal infection with relative convenience in experimental handling. Hence, we reconfirm the studies of Schaller et al. (21) that the RHOE could be used as a

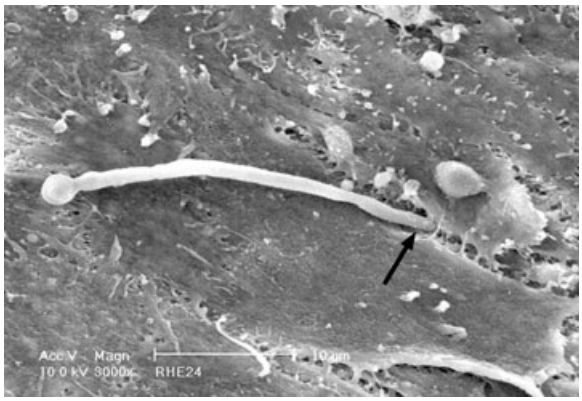

Figure 5 A single hyphal element originating from a *Candida albicans* blastospore following the intercellular channel-like topography and then penetrating the surface (arrowed) showing thigmotropic activity.

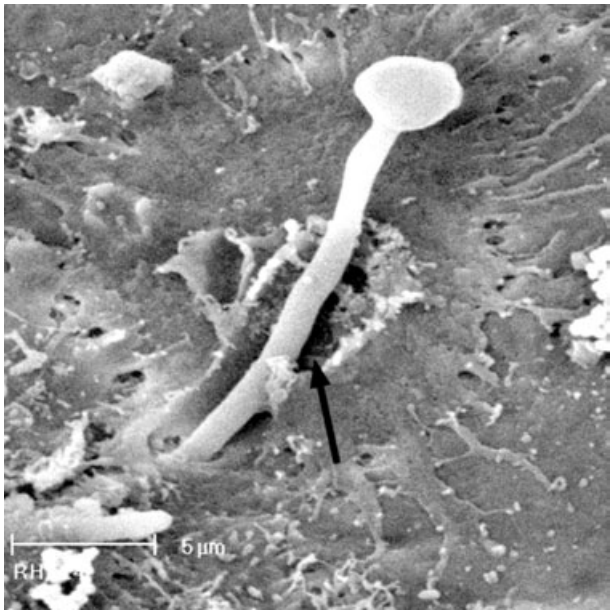

Figure 6 Hyphal element of *Candida albicans* invading the epithelium with marked cavitation (arrowed) on the cell surface possibly due to extracellular enzymic activity of the yeast.

flexible, simple model for the study of experimental candidiasis. However, ours is the first study to describe the ultrastructural features of this model system.

Incubation of stationary phase, *C. albicans* cells together with RHOE induced the classic growth stages of the mucosal candidiasis. *Candida* cells were able to invade the host tissue and the pathological changes were akin to those described in human biopsy specimens (13–18). For instance, the passage of hyphae through several epithelial cells, seen in our study confirms that *C. albicans* can grow intracellularly as observed previously in *in vivo* oral candidiasis (14). Hyphal elements were also seen traversing intercellular gap junctions of the epithelium. The proportion of hyphal elements directly penetrating the epithelial cell surfaces and their gap junctions were similar implying that the organisms do not preferentially utilize one mechanism over the other for tissue penetration. However, further quantitative studies are required to confirm or refute this observation.

Some of the plant pathogenic fungi such as *Magnaporthe grisea* are known to produce appressoria during invasion of plant tissues. These appressoria are modified hyphae specialized for invasion and are known to possess increased turgor pressure for facilitating the penetration process (25, 26). The occasional bulges of the invading hyphal tips noted in the present study resemble these plant 'appressoria', which have also been previously described in ultrastructural studies of invasive candidiasis (17, 27–29). The latter studies, which focused on the invasive phase of *C. albicans* in human oral and vaginal epithelia, described for the first time appressoria-like structures in *C. albicans*. We also noticed these structures in hyphal tips penetrating the RHOE (Fig. 4). Additionally, our cytochemical studies demonstrate that the PLs are particularly concentrated

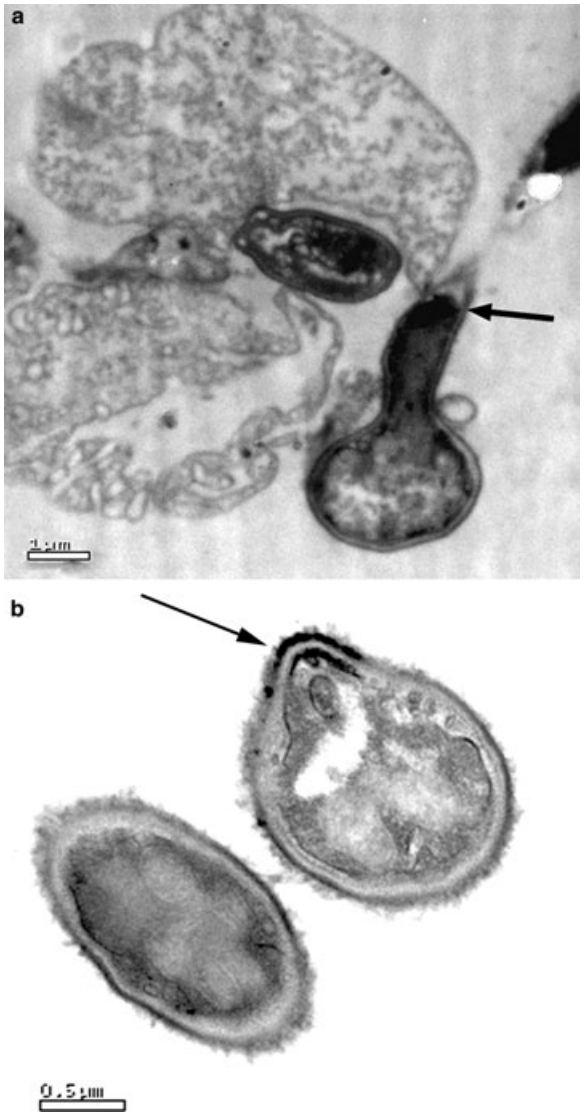

Figure 7 Phospholipase activity of *Candida albicans* invading the reconstituted human oral epithelium. (a) Blastospores forming germ tubes (initial hypha) with epithelial cells after 24 h of infection. The phospholipase activity can be seen at the tip of the hypha (arrowed). (b) Blastospores after 12 h of infection. The phospholipase activity is found at the initial site of bud formation (arrowed).

at the peripheries of the hyphal tips. This implies that appressoria, which are thought to exert turgor pressure, in tandem with extracellular PLs may synergistically act during successful hyphal invasion of the epithelium. Furthermore, the structural features of the bulging appressorium may facilitate and serve as a mechanical anchor for the yeasts to survive on the oral epithelium where the flushing action of saliva and other forces tend to dislodge the organism.

The mechanisms by which *C. albicans* gain entry into different host epithelia are unclear. Most of the intracellular pathogens enter cells by phagocytosis and it is evident that phagocytes such as reticuloendothelial cells engulf *C. albicans* (30, 31). It is also known that although epithelial cells or endothelial cells are not

phagocytic *per se*, they can engulf bacteria through a similar mechanism known as cellular internalization (32, 33). Interestingly, we noted under TEM, some superficial epithelial cells forming cytoplasmic processes encircling yeast blastospores (Fig. 3) resembling this phagocytic process. The present findings are consistent with the previous work of Filler et al. (12) who showed phagocytosis of *C. albicans* by cultured human vascular endothelial cells. Furthermore, in another ultrastructural study (11), where *C. albicans* was incubated with human buccal and vaginal cells and cultured HeLa cells, it was shown that all three cell types could internalize the yeast in a manner similar to phagocytosis. Although our observations support these findings, further work is needed to confirm this phenomenon.

Our observations on the PL activity of *C. albicans* during infection of RHOE were similar to that of Pugh and Cawson (9). Their findings on *C. albicans*-infected chick chorioallantoic membrane suggest that PLs could play an important role in the pathogenesis of invasive candidiasis. We also noted that *C. albicans* could exhibit PL activity during infection of RHOE. These enzymes were seen particularly localized at the tips of penetrating hyphae and initial sites of budding although few blastospores demonstrated generalized enzyme activity.

We also noted cavitations at sites of hyphal penetration of the host cell membrane. Ray and Payne (20) made similar observations in murine corneocytes, 8 h after inoculation of *C. albicans* blastospores and, they suggested that the cavitations were due to the action of *Candida* acid proteinases, which can degrade cellular keratin. Our ultrastructural and cytochemical studies confirm these and indicate that the cavitations may also be related to the activity of *C. albicans* PLs.

Developing hyphae could invade the host tissue either by penetrating the cell membranes or alternatively by traversing the intercellular spaces of the artificial epithelium where the surface integrity is possibly weak. The latter activity of *C. albicans* was first demonstrated elegantly by Gow et al. (4) *in vitro* where hyphal elements on nuclepore membrane filters entered the filter pores sensing the substrate topography and changes in the contour. Some hyphae in our study were noted to invade through intercellular gaps of the RHOE (Fig. 5), thus providing confirmatory evidence that *C. albicans* hyphae may indeed exhibit thigmotropic activity during mucosal candidiasis.

To conclude, our findings support the hypothesis that *C. albicans* invasion is a net result of the interaction of physical, mechanical and enzymatic processes of the pathogen. Furthermore, the multilayer RHOE, provides a convenient and satisfactory model for the investigation of ultrastructure of the invasive phase of candidiasis.

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