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A Comparative Study of Candidal Invasion in Rabbit Tongue Mucosal Explants and Reconstituted Human Oral Epithelium

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Abstract The purpose of this study is to compare the light and scanning electron microscopic (SEM) features of tissue invasion by three *Candida* species (*C. albicans*, *C. tropicalis*, and *C. dubliniensis*) in two different tissue culture models: rabbit tongue mucosal explants (RTME) and reconstituted human oral epithelium (RHOE). Tongue mucosal biopsies of healthy New Zealand rabbits were maintained in explant culture using a transwell system. RHOE was obtained from Skinethic Laboratory (Nice, France). RTME and RHOE were inoculated with *C. albicans*, *C. tropicalis*, and *C. dubliniensis* separately and incubated at 37°C, 5% CO_2, and 100% humidity up to 48 h. Light microscopic and SEM examinations of uninfected (controls) and infected tissues were performed at 24 and 48 h. *C. albicans* produced characteristic hallmarks of pathological tissue invasion in both tissue models over a period of 48 h. Hyphae penetrated through epithelial cells and intercellular gaps latter resembling thigmotropism. SEM showed cavitations on the epithelial cell surfaces particularly pronounced at sites of hyphal invasion. Some hyphae on RTME showed several clusters of blastospores attached in regular arrangements resembling "appareil sporifere". *C. tropicalis* and *C. dubliniensis* produced few hyphae mainly on RTME but they did not penetrate either model. Our findings indicate that multiple host–fungal interactions such as cavitations, thigmotropism, and morphogenesis take place during candidal tissue invasion. RTME described here appears to be useful in investigations of such pathogenic processes of *Candida* active at the epithelial front.

Keywords *Candida* · Mucosal explants · Reconstituted human oral epithelium · Thigmotropism

Abbreviations

DMEM	Dulbecco's modified eagle medium
PAS	Periodic acid Schiff
PBS	Phosphate-buffered saline
RHOE	Reconstituted human oral epithelium
RTME	Rabbit tongue mucosal explants
SEM	Scanning electron microscopy

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Introduction

Oral candidiasis is a common opportunistic infection in compromised patients. While *C. albicans* is the main etiology for oral candidiasis, other *Candida* species such as *C. tropicalis* and *C. dubliniensis* have emerged as pathogens. Increasing incidence of oral

candidiasis due to HIV outbreak and wide use of antibiotics and immunosuppressives in the recent years have aroused much interest in investigation of *Candida* colonization and invasion of the oral mucosa.

Oral epithelium constitutes an effective front line barrier against numerous oral commensals including *Candida* species. Important host–pathogen interactions at the oral epithelium have been extensively explored using biopsy specimens from humans [1–4] as well as animals [5]. In addition, different tissue culture models have been used to reproduce mucosal candidiasis in vitro. In a pioneering study, Partridge et al. [6] used chick-chorioallantoic membrane to assess relative pathogenicity of different *Candida* species. Furthermore, monolayer cell cultures derived from a variety of human epithelial cells have also been used for investigation of *Candida* host–pathogen interactions [7, 8]. Recently, many researchers have used reconstituted human oral epithelium (RHOE) which is available commercially as multilayer cell cultures [9–12] for experimental candidiasis.

Apart from these cell cultures, mucosal biopsies maintained as explant cultures have elicited interesting features of the host–fungal interactions in oral candidiasis [13, 14, 15]. Although these tissue models possess superior tissue topography close to their natural counterpart, invasion of fungal elements at the exposed explant peripheries [14, 15] may lead to spurious results. However, in an experiment to investigate HIV transmission through the human vaginal epithelium Collins et al. [16] suggested that an agar layer around the explant tissue could successfully overcome pathogens seeping through the periphery. Using this novel technique, we developed a modified rabbit tongue mucosal explant (RTME) model for experimental oral candidiasis. We explored using light and scanning electron microscopy, the features of candidal invasion in novel RTME and RHOE and, various host–fungal interactions observed are discussed here.

Materials and Methods

Candida Growth Conditions and Preparation of Inoculum

Candida isolates used in this study were *C. albicans* SC5314 (gracious gift of Professor NAR Gow, The University of Aberdeen, UK), *C. tropicalis* HK192552, and *C. dubliniensis* HK186434 (isolated from nasopharyngeal carcinoma patients attending the Prince Philip Dental Hospital, The University of Hong Kong, Hong Kong). They were subcultured from thawed suspensions of pure isolates at the Oral Biosciences laboratory (The Prince Philip Dental Hospital, The University of Hong Kong, Hong Kong) and their identities were reconfirmed by the standard germ tube test, and fermentation reactions in commercially available API 20C auxonogram strips (Biomerieux, Marcy I'Etoile, France). The yeast cells were cultured for 24 h at 37°C on Sabouraud dextrose agar (Difco, Hampshire, England), washed thrice in 0.9% NaCl and an inoculum of approximately 2×10^5 cells were suspended in 10 ml of YPG medium (1% yeast extract, 2% peptone and 2% glucose; Difco). The suspension was shake-cultured for 16 h at 37°C. Afterwards, the resultant cells were harvested by centrifuging and the inocula containing 4×10^7 cells per ml were prepared in phosphate-buffered saline (PBS, pH 7.2) using hemocytometer quantification.

Rabbit Tongue Mucosal Explant (RTME)

For the establishment of a novel explant culture system, we used a method described by Collins et al. [16] with some modifications. The glossal epithelium was harvested from healthy adult New Zealand white rabbits within 30 min of sacrifice. Harvested tissues were transported in Dulbecco's modified eagle medium (DMEM) (Gibco BRL, Gaithersburg, Maryland, USA) supplemented with 10,000 U/ml penicillin and streptomycin. On arrival at the laboratory, tissues were washed in sterile PBS and transferred to fresh DMEM. The mucosa was trimmed into 3–4 mm thickness and cut approximately into 8×8 mm^2 under a stereomicroscope. Mucosal squares oriented on top were then placed on the upper compartment of a transwell (24 mm diameter with 0.4 μm pore size polycarbonate membrane) (Corning Inc., Corning, NY) (Fig. 1). A 3% solution of agarose was then added to the area surrounding the tissue in the top well, which upon solidification created a tight seal around the explant periphery. Subsequently, 1.5 ml of DMEM was added to the bottom chamber to keep the transwell membrane in contact with the medium. Afterwards,

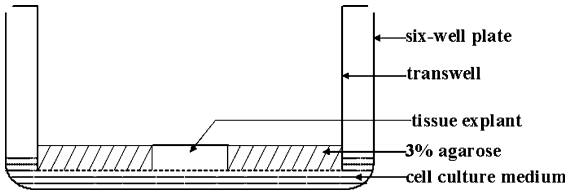

Fig. 1 A schematic representation of setting up of the rabbit tongue mucosal explant (RTME) model

0.5 ml of DMEM was added to the top chamber to keep the tissue moistened and the model was incubated at 37°C, 5% CO_2, and 100% humidity for 1 h. After this incubation, DMEM in the upper compartment was aspirated and an inoculum of yeast (2×10^6 cells in 50 µl of PBS) was placed on the center of the mucosal explants. Controls were inoculated with 50 µl of PBS (pH 7.2).

Reconstituted Human Oral Epithelium (RHOE)

Commercially available 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), containing 5 µg/ml insulin, 1.5 mM $CaCl_2$, and 25 µg/ml gentamicin in six-well tissue culture plates (Corning Inc., Corning, NY) for 24 h according to manufacturer's instructions. The in vitro model and all culture media were prepared without antimycotics. After reconstitution, a 0.5-cm^2 segment of tissue was inoculated with 2×10^6 yeast cells in 50 µl of phosphate-buffered saline (PBS, pH 7.2). Control samples were inoculated with 50 µl PBS.

Uninfected (controls) and infected tissues of RTME and RHOE were incubated at 37°C, 5% CO_2, and saturated humidity up to 48 h. The respective culture media were replenished in every 24 h. Tissues were harvested at 24 and 48 h time points, washed gently with PBS, and processed for light and scanning electron microscopic investigations as described below.

Light Microscopy

Tissue samples were fixed in 10% formalin in PBS, for at least 2 h at room temperature. After series of dehydrations, they were embedded in paraffin and 3–4 µm thick sections were cut using the Leica RM 2155 rotary microtome (Nussloch, Germany). The sections were stained with periodic acid Schiff (PAS) and counterstained with Myers Hematoxylin (Dako, Carpinteria, CA, USA).

Scanning Electron Microscopy

Parts of each specimen were immersed overnight in 2.5% glutaraldehyde and 2% formaldehyde in a 0.05 M cacodylate-buffered solution (pH 7.3). Afterwards, the tissues were washed briefly in phosphate buffer and post-fixed in 1% osmium tetroxide for 4 h. Post-fixed tissues were washed in two changes of phosphate buffer. Then, they were dehydrated in graded 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). Tissues were viewed using a scanning electron microscope (Philips XL 30CP) at an accelerating voltage of 10 kV.

Results

Light Microscopic Features of RTME

The mucosa of the dorsal surface of rabbit tongue consisted of a stratified squamous, keratinizing epithelium that was organized into discrete filiform papillae (Fig. 2). These papillae were supported by a cellular connective tissue core beneath which areas of muscle fibers could be seen. Generally, this structure was well maintained during the study period up to 48 h, although later they showed signs of structural disorganization.

All *Candida* inoculated explants showed evidence of infection, but the only invasive species was *C. albicans*. In *C. albicans* infected tissues, blastospores and hyphal forms could be seen invading the superficial epithelial cell layers at 24 h. The invasion intensified with more hyphae penetrating the deeper layers of the epithelium toward the latter part of the incubation period at 48 h (Fig. 3). Extensive invasion of hyphae disrupted the ordered epithelial structure with inter- and intracellular edema. Interestingly, the fungal elements invading through the superficial epithelium did not penetrate into the connective tissue layer (i.e. lamina propria) during the study period.

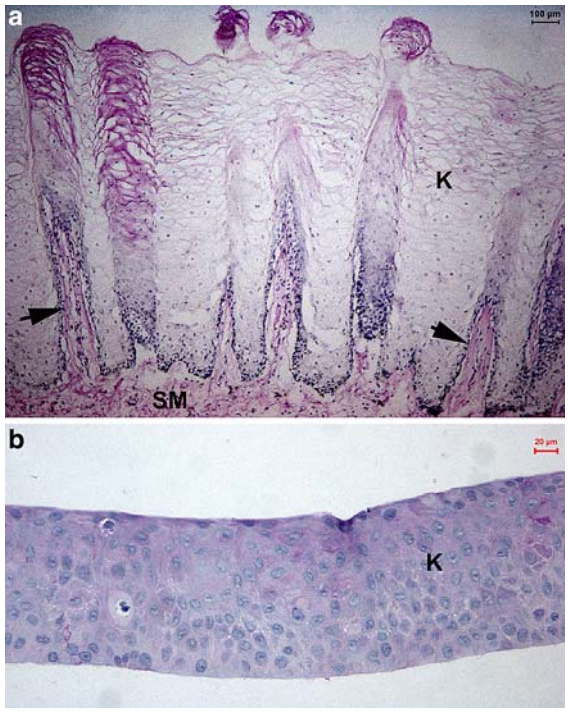

Fig. 2 Light micrographs of uninfected (control) (**a**) rabbit tongue mucosal explant (RTME) and (**b**) reconstituted human oral epithelium (RHOE) after 24-h culture (periodic acid-Schiff stain). Stratified keratinocytes (K), submucosa (SM), connective tissue cores (arrowed)

Macroscopic observations revealed that both *C. tropicalis* and *C. dubliniensis* grew well over RTME under the culture conditions used. However, these yeasts did not invade the tissue and were especially present in the blastospore phase throughout 48-h incubation period. Most of fungal growth on RTME surface was removed in PBS during fixation, leaving a few organisms attached to the epithelium. Nonetheless, they produced variable degree of pathologic tissue changes such as intercellular edema and disorganization during 48 h, in common with *C. albicans*.

Light Microscopic Features of RHOE

RHOE consisted of stratified keratinocytes without stratum corneum. In general, the structural features of the uninfected (control) RHOE resembled normal human oral epithelium except for some dyskeratotic cells, seen rarely (Fig. 2).

Three *Candida* species (*C. albicans*, *C. tropicalis*, and *C. dubliniensis*) showed differential host–fungal interactions with RHOE. *C. albicans* formed hyphae and penetrated RHOE while *C. tropicalis* and *C. dubliniensis* were non-invasive. After 24 h of yeast infection, clusters of *C. albicans* yeast cells were detected on the superficial keratinocyte layer. Fungi in extra- and intracellular sites were then seen invading the basal cell layers especially at the latter stage of infection causing significant structural damage to the epithelium (Fig. 3). After 48 h, there was exfoliation of superficial cell layers of RHOE with severe intercellular edema and structural disorganization. These exfoliated epithelial cells together with candidal hyphae formed a delicate pseudomembrane over RHOE.

In contrast, *C. tropicalis* and *C. dubliniensis* remained mainly in the blastospore phase and they were easily washed off in PBS during the fixation procedure. Nonetheless, these non-*albicans Candida* species produced structural disorganization of RHOE at late stages of incubation (48 h) akin to *C. albicans* cultures.

Scanning Electron Microscopic (SEM) Features of RTME

On SEM observation, RTME showed numerous filiform papillae, lined with epithelial cells having surface microridges (Fig. 4). SEM observations also showed that all three *Candida* species colonized RTME. *C. albicans* formed hyphae profusely and penetrated the epithelium successfully as opposed to *C. tropicalis* and *C. dubliniensis*. Occasionally, surface cavitations were observed on epithelial cells associated with candidal blastospores and hyphae (Fig. 4). Some hyphal elements of *C. albicans* on RTME showed maturation with subclusters of blastospores regularly arranged on their hyphal surface (Fig. 5) toward the latter stage of infection (48 h).

Scanning Electron Microscopic Features of RHOE

SEM examination showed that RHOE is composed of squamous epithelial cells with regular surface. Cytoplasmic processes extending from the cell peripheries were seen intermingled with adjacent cells giving structural stability.

On examination of infected RHOE, many *C. albicans* blastospores appeared randomly dispersed

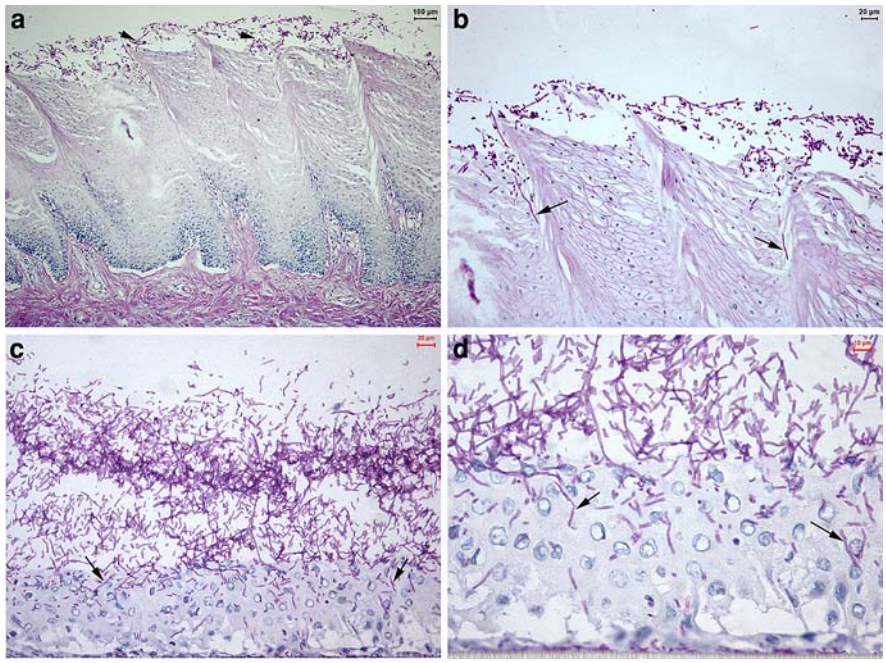

Fig. 3 Light micrographs of *C. albicans* infected tissues after 48-h culture (periodic acid-Schiff stain). Low (**a**) and high (**b**) magnification of rabbit tongue mucosal explant (RTME). Low (**c**) and high (**d**) magnification of reconstituted human oral epithelium (RHOE). Adherence and invasion of yeast and hyphal forms (arrowed) through the superficial cell layers are seen. Extensive tissue invasion has caused disorganization of the tissues with intercellular edema, exfoliation of the superficial cell layers and detachment of the basal cell layers

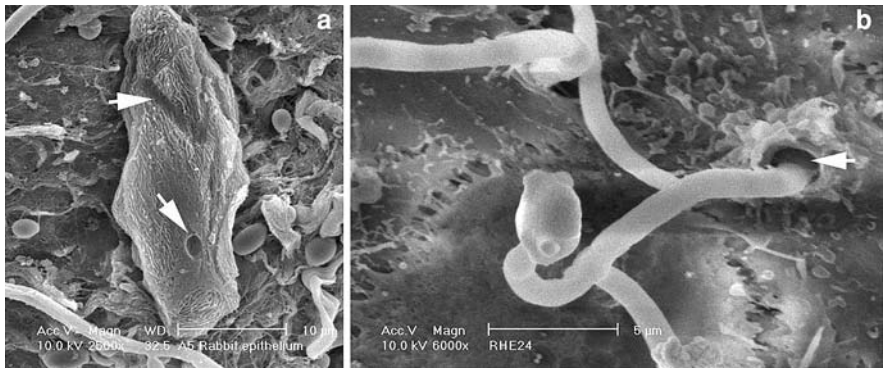

Fig. 4 Cavitations (arrowed) on the cell surface possibly due to extracellular enzymic activity of fungal elements (**a**) rabbit tongue mucosal explant (RTME); (**b**) reconstituted human oral epithelium (RHOE) after 48-h incubation. Micro-ridges are seen on the RTME cells whereas RHOE surface is smooth

on the surface of the epithelium with a few hyphae during first 24 h whereas hyphal forms were predominant at the late stage of infection (48 h). Blastospores and hyphal surfaces were generally smooth except for a few buds and bud scars. As hyphae matured, they started to invade the epithelium either passing through the cells intracytoplasmically or between the cell junctions (Fig. 6). Hyphal penetration created localized cavitations on the epithelial cells occasionally (Fig. 4). On the contrary, neither *C. tropicalis* nor *C. dubliniensis* fungal elements were identified penetrating RHOE by scanning electron microscopy.

Discussion

Candida–host interactions at the mucosal level have been explored using various in vitro models based on tissue culture techniques. Recently, these investigations have been extended up to artificial epithelial models such as reconstituted [9–12] and engineered [17, 18] human epithelia. However, the absence of normal immune mechanisms (humoral or cell mediated) is an important delimitation of these tissue models when they are used to investigate host–pathogen interactions [9]. On the other hand, the epithelial integrity, surface architecture, and the

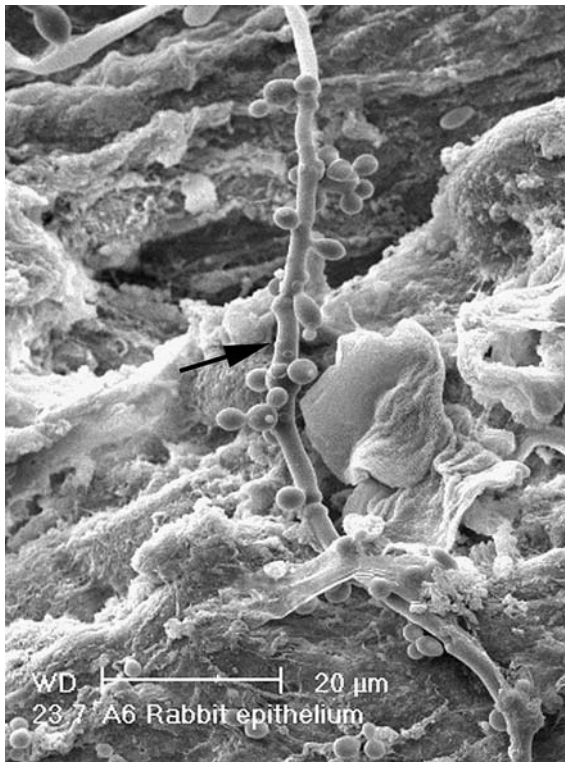

Fig. 5 RTME infected with *C. albicans* showing characteristic arrangements of newly formed clusters of blastospores on the surface of long hyphae (verticillia)—"spore outfits" arising from long hyphae known as "appareil sporiferes" (arrowed) after 48-h incubation

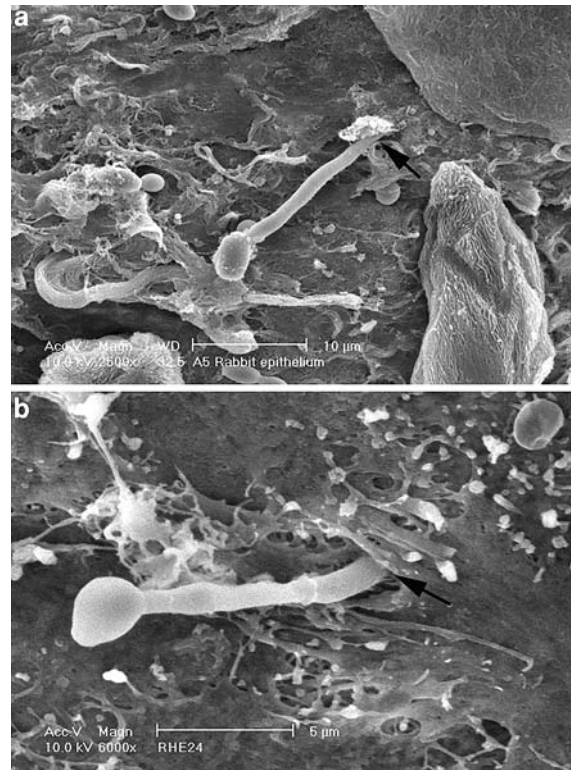

Fig. 6 Hyphae originating from *C. albicans* blastospores penetrating through the intercellular gaps (arrowed) showing thigmotropic activity at 24 h of incubation (**a**) rabbit tongue mucosal explant (RTME), (**b**) reconstituted human oral epithelium (RHOE)

tissue topography of these reconstituted tissues differ from the natural counterpart.

Mucosal biopsies on the other hand, maintained as explant cultures seem to help overcome such drawbacks with preserved normal immune responses and physiological tissue architecture. Pemberton and Turner [13], in a pioneering study demonstrated *C. albicans* invasion of human gingival epithelium using explant cultures. Howlett [14, 15] used explants obtained from different sites of the oral mucosa of neonatal rats and rabbits to examine the differential invasion of five species of *Candida*: *C. albicans*, *C. tropicalis*, *C krusei*, *C. parapsilosis*, and *C. guilliermondii* in vitro. These experiments have demonstrated that oral mucosa maintained in explant cultures retain most of the in vivo conditions. Nonetheless, the invasion of fungal elements into the subepithelial tissues through the exposed explant peripheries was a major drawback of latter models. Our RTME model developed using a method described by Collins et al. [16] seems to minimize the latter problems. We found that the use of a 3% agarose layer around the explant considerably limits fungal penetration at the tissue peripheries by building a tight seal around the explant tissue. This allowed direct interaction of fungi with the surface epithelium by preventing them entering into the submucosa through the explant peripheries. Moreover, existence of culture media in a separate chamber minimized the contamination of culture system with fungal elements. Therefore, present RTME model seems to overcome most of the limitations of previous explant cultures used in experimental oral candidiasis [5].

Two epithelial models used in this study, rabbit tongue mucosal explants (RTME) and reconstituted human oral epithelium (RHOE), were successfully colonized by *C. albicans* in comparison with *C. tropicalis* and *C. dubliniensis*. This reconfirms the fact that *C. albicans* possesses superior virulence

mechanisms particularly for adhesion and colonization of mucosal surfaces compared with non-*albicans Candida* species. The success of *Candida* as a pathogen in the oral cavity depends on yeast being prevented from dislodgement by saliva, water, food, and forces of masticatory musculature. This study shows that *C. albicans* gain a sound foothold on the mucosa by forming hyphae unlike the latter non-*albicans Candida* species. Furthermore, light and scanning electron microscopy (SEM) confirmed that *C. albicans* hyphae invade epithelia in both RTME and RHOE inter- and intracellularly. These features are similar to previous reports [1, 2] where *C. albicans* hyphae were seen in inter- and intracellular sites of epithelial plaques collected from different oral candidiasis lesions. Therefore, it is likely that the conversion from blastospores to hyphal phase is an initial factor for *C. albicans* to be a successful opportunistic pathogen, leading to superficial candidiasis.

It was striking to note that the invading fungal elements penetrated RHOE up to the deep layers after 48-h incubation whereas they could not do so in RTME indicating that the superficial keratinocyte layer of the glossal epithelium may help resist candidal invasion. This could also be due to the various immuno-inflammatory mediators such as cytokines and growth factors released by intact submucosa of the RTME. The integrity of an active submucosal defense system has shown to potentiate the resistance of various reconstituted epithelia against candidal challenge [17, 18] and these investigators have suggested that the submucosal support is critical for conditioning the immune response. Therefore, the natural submucosa and the tissue architecture of the modified RTME validate its usefulness in better assessment of host response toward candidal challenge.

Scanning electron microscopy demonstrated *C. albicans* hyphae penetrate cell layers by traversing intercellular gaps of RTME and RHOE, showing thigmotropic behavior. This concurs with the previous reports on candidal hyphae growing through polycarbonate neuclepore membranes and on various surface topographies following contact guidance [19]. Comparable observations have been made by Wilborn and Montes [4] who described that candidal hyphae penetrate through the "holes" present on keratinocytes in biopsy specimens obtained from chronic oral candidiasis patients. This thigmotropic behavior of candidal hyphae could be an important predisposing factor in triggering *Candida* superinfections on traumatized mucosa, for instance chronic irritation due to ill-fitting dentures, appliances, and septic teeth.

Odds [20] argued that *C. albicans* is pleomorphic rather than dimorphic fungus due to its transforming morphologies (e.g. blastospores, pseudohyphae, hyphae, and chlamydospores) in different growth environments. Interestingly, SEM observations on RTME infected with *C. albicans* showed for the first time, characteristic arrangements of newly formed clusters of blastospores on the surface of long hyphae (Fig. 5). These "spore outfits" arising from long hyphae (verticillia) have been described as "appareil sporiferes"—one of the stages of vegetative reproduction of *C. albicans* [21]. However, further investigations on candidal morphogenesis during fungal reproduction are necessary to confirm this phenomenon.

There were occasional cavitations on the epithelial cells in either model that were in close contact with invading fungal elements (Fig. 4). Hoshika and Mine [22] and Hoshika et al. [23] have demonstrated cavitations and depressions caused by *C. albicans* on rabbit esophageal epithelium and, they suggested that cavitations help candidal adherence to the mucosa. Moreover, in an early study, Ray and Payne [24] found cavitations of murine corneocytes after 48 h of *C. albicans* inoculation and the cavitations were attributed to the activity of secretory aspartyl proteinases (SAP) of the invading fungi. Secretion of lytic enzymes such as SAPs and phospholipases by *Candida* species into their immediate environment has also been well demonstrated [10, 12, 25]. In particular, our recent investigations using ultrastructural and cytochemical methods on the invasive interface of *C. albicans* and RHOE demonstrated phospholipase activity localized at the invading hyphal tips and initial sites of bud formation [12]. Therefore, it is conceivable that these cavitations in relation to invading fungal elements are caused by fungal SAPs and phospholipases.

Both *C. tropicalis* and *C. dubliniensis* were unable to invade either RTME or RHOE and were seen predominantly in blastospore phase. However, it was evident that these non-*albicans Candida* species were more adhesive on RTME than RHOE. This could be due to the irregular topography of the filiform papillae and micro-ridges of the epithelial cells of the rabbit

tongue. Yet, SEM gave evidence of spares hyphae formed by *C. tropicalis* whereas *C. dubliniensis* remained predominantly in blastospore phase. However, despite being non-invasive, these non-*albicans Candida* species produced other pathogenic effects such as intercellular edema and exfoliation of the superficial cell layers in infected tissues in common with *C. albicans*. This could be attributed to virulence factors such as SAPs and phospholipases of these non-*albicans Candida* species released into the tissues.

To conclude, this study compares invasion of *C. albicans*, *C. tropicalis*, and *C. dubliniensis* on two oral candidiasis models based on RTME and RHOE. *C. albicans* invades both culture models in yeast and hyphal forms and the invading hyphae show thigmotropism. Fungal elements produce cavitations on the epithelial cells possibly due to extracellular enzyme activity. Furthermore, *C. albicans* forms characteristic morphologic structures known as "appareil sporifere" or "spore outfit" during infection of RTME. RTME offers potential utility for the study of host response toward infection by *Candida* species.

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