

## Innate Defense Mechanisms in Oral Candidiasis

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## 1. Introduction

*Candida* species can be isolated from the oral cavity of up to 80% of healthy individuals as a commensal organism (Odds, 1988; Wilkieson et al., 1991). Despite these extremely high reported carriage rates, very few healthy carriers will develop clinical signs of the oral infection. This commensal relationship however can change in response to a change in the local oral microenvironment. Breakdown of mucosal integrity, qualitative or quantitative shifts in oral microbial flora, or an inadequate innate host response can lead to increased fungal burden, which can cause a chronic oral mucosal inflammatory response to the organism, also known as *Candida* stomatitis (reviewed in Scully et al., 1994). Perhaps the most important host defense strategy against *Candida albicans* is the activation of the innate arm of the immune system, principally represented by the polymorphonuclear leukocyte (PMN) or neutrophil. This is evidenced by the fact that neutropenic patients are highly susceptible to the fungus, and that the disappearance of yeast cells from tissues parallels the appearance of neutrophils (reviewed in Ashman and Papadimitriou, 1995). Although PMN are considered important in the resistance to and eradication of fungi, it appears that these functions in vivo require stimulation with an activating agent. CD4<sup>+</sup> T cells maintain a central role in the defense against *Candida* since they provide such activating signals to PMN through the release of specific cytokines (Ashman and Papadimitriou, 1995). The importance of the CD4<sup>+</sup> T helper

cell in the eradication or control of *Candida* growth in vivo is evidenced by the increased incidence of oral mucosal candidiasis in patients affected by HIV disease or immunosuppressive treatment, which primarily targets the CD4<sup>+</sup> T cell subset (Coleman et al., 1997; O'Daniels et al., 2000). Thus it appears that oral candidiasis emerges at later stages of HIV infection or during chronic pharmacological immunosuppression, when *Candida*-specific CD4<sup>+</sup> T cell clones are materially or functionally depleted, as innate immunoeffectors are faced with a remarkably increased burden of commensal microorganisms, and with essentially minimal activating help from circulating T helper cells. Interestingly, even though PMN activation may be compromised in these individuals, cases of oral invasive candidiasis or disseminating candidiasis through the oral mucosa are rare among these patients and seem to be associated only with additional risk factors such as severe neutropenia or high doses of corticosteroid treatment (Imam et al., 1990). Thus it is possible that either (a) unique innate defense mechanisms operate in the oral mucosa, which control invasive infection and are independent of T cell or neutrophil function; or (b) a certain degree of PMN activation still takes place at the infection site, which may be due to cytokines released from structural oral mucosal cells. This chapter will focus on the innate immune and nonimmune defense mechanisms, which may play a role in limiting oral *Candida* infections. Finally a critical evaluation of the inherent defense mechanisms unique to the oral mucosa that may prevent

invasive infection in the immunocompromised host will be presented.

clearance of adherent bacteria and fungi (Samaranayake and Samaranayake, 2001).

## 2. Unique Structural and Ecological Features of the Oral Mucosa

### 2.1. Structure of the Oral Mucosa

The oral mucosa is a highly permeable tissue, with regional variations, as the type and keratinization status of the epithelial cell layer varies in different locations of the oral cavity. The oral cavity is lined by at least four different types of mucosa (Squier and Finkelstein, 2003). While nonkeratinized epithelium lines the majority of the oral cavity, covering the hard palate and gingiva is the masticatory mucosa, which receives the most severe mechanical forces and has stratified keratinized epithelium. The dorsal surface of the tongue is covered by specialized mucosa and is also lined by keratinized epithelium, which specializes in taste and other neurophysiologic functions. Junctional epithelium is a relatively undifferentiated and nonkeratinized epithelium, which forms the junction between the oral mucosa and the teeth. Another unique structural feature of the oral cavity is the localized presence of submucosa containing adipose tissue and minor salivary glands (Squier and Finkelstein, 2003).

The most common form of *Candida* stomatitis affects the palatal and dorsal tongue mucosa (Samaranayake and MacFarlane, 1990), and therefore this infection mostly relates to oral stratified keratinized squamous epithelium. In general, stratified squamous epithelia are thought to protect the underlying tissues by the process of keratinization, which decreases the mucosal permeability, and also by the process of desquamation of keratinized cells, which is thought to play an important role in the

### 2.2. Oral Microbial Ecology

The oral mucosa is colonized by over 200 microbial species. Thus, the potential for bacterial and fungal infections is high, with a need for innate defense mechanisms. The oral cavity is comprised of at least four microbial ecological niches with a certain degree of variability in the composition of their indigenous flora: the saliva, the tongue, and the tooth-associated supragingival and subgingival plaques (Slots, 1992). The most predominant indigenous bacterial flora in saliva, tongue and supragingival plaque are members of the *Streptococcus* species. These commensal bacteria may modulate yeast colonization by competing for nutrients and adhesion sites. Evidence for a protective role of the oral bacterial flora against fungal infection is derived from the fact that use of broad-spectrum antibiotics in humans and animals promotes oral *Candida* infection (Samaranayake et al., 1994; Deslauriers et al., 1995). In fact, many animal and in vitro studies have shown that oral *Candida* colonization can be inhibited by oral *Streptococci* (Liljemark and Gibbons, 1973; Samaranayake et al., 1994). Coaggregation of *C. albicans* or *C. dubliniensis* with the oral bacterium *Fusobacterium nucleatum* has been described in subgingival plaque, and may play a role in the pathogenesis of periodontal infections. The extent of coaggregation varied between the two *Candida* species, was inhibitable by mannose or  $\alpha$ -methyl mannoside in both cases, and appeared to be due to the presence of a heat-stable *Candida* receptor (Jabra-Rizk et al., 1999).

*Candida* species are also part of the commensal flora in the oral cavity, with oral asymptomatic *Candida* carriage rates varying among different age groups. The highest asymptomatic *Candida* carriage rates (65–80%) were reported for healthy children

(Odds, 1988), the elderly (Wilkieson et al., 1991), and HIV<sup>+</sup> patients (Campisi et al., 2002; Myers et al., 2003). *C. albicans* colonizes the oral mucosa at higher rates than many other mucosal sites in humans, but very few healthy carriers develop clinical signs of the oral infection (Odds, 1988). This is in contrast to other mucosal sites such as the vagina, where the carriage rate by healthy individuals is approximately 25% and clinical infection can be observed in otherwise healthy women (Sobel, 1988). *C. albicans* is frequently isolated from the saliva, tongue, and subgingival plaque (Redding et al., 1988, 2002; Phelan et al., 1997), and it is a change in the oral host environment that determines whether colonization will progress to infection.

### 3. Epidemiology, Clinical, and Histopathologic Characteristics of Oral Candidiasis

#### 3.1. Epidemiology

General risk factors for oropharyngeal candidiasis (OPC) are the two extremes of age (Odds, 1988), trauma (O'Grady and Reade, 1993), salivary gland hypofunction (Scully et al., 1994), dental prostheses (Odds, 1988), broad-spectrum antibiotic therapy, and topical use of corticosteroids (Deslauriers et al., 1995), as well as nutritional factors (Rennie et al., 1983; Samaranayake, 1986). Among the systemic conditions that predispose patients to OPC are diabetes mellitus, HIV infection, immunosuppressive therapy, Sjogren's syndrome, and radiation therapy for head and neck cancer (reviewed in Scully et al., 1994). In most types of high-risk patients, *C. albicans* is still the main etiologic agent of oral candidiasis (Scully et al., 1994). However, over the last 15 years new *Candida* species have emerged as the infectious agent respon-

sible for some of these infections in special patient categories, such as HIV<sup>+</sup> patients and patients receiving radiation treatment of head and neck tumors (Redding et al., 1999; O'Daniels et al., 2000). The most commonly reported non-*albicans Candida* species involved are *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. tropicalis* (O'Daniels et al., 2000; Redding, 2001).

Interestingly, up to 90% of HIV<sup>+</sup> patients have had at least one episode of OPC, and their susceptibility to oral candidiasis is not paralleled by susceptibility to vaginal or disseminated infection (Scully et al., 1994). Although HIV-associated OPC is predominantly caused by *C. albicans* (Vargas and Joly, 2002; Myers et al., 2003), non-*albicans Candida* species have emerged as etiologic agents of oral candidiasis in certain HIV<sup>+</sup> patients (O'Daniels et al., 2000; Redding, 2001). *C. dubliniensis* was isolated from as many as 32% of HIV<sup>+</sup> patients with clinical signs of this infection (Coleman et al., 1997) and it can apparently be the sole causative agent detectable in some of these patients (O'Daniels et al., 2000; Vargas and Joly, 2002). More recent epidemiologic evidence suggests that the prevalence of *C. dubliniensis* infection in this patient population is much lower than originally proposed and ranges between 5% and 10% (Giammanco et al., 2002; Vargas and Joly, 2002). Because typically *C. dubliniensis* shows the same pattern of antifungal susceptibility as *C. albicans*, in the vast majority of cases distinction between the two species is not required for successful treatment (Redding, 2001).

OPC is also a common infection in patients receiving radiation treatment of head and neck tumors (Redding et al., 1999). This infection is thought to be due to destruction of salivary gland tissue and hyposalivation (Fotos and Hellstein, 1992). Recently, an increase in oral candidiasis has been reported in head and neck cancer patients receiving radiation therapy, which is due to infection with one or more non-*albicans Candida* species (Redding, 2001).

In fact, fungi other than *C. albicans* were detected in 59% of the head and neck cancer patients with positive cultures, whereas 27% of the culture-positive patients harbored *C. albicans* in combination with other species (Redding et al., 1999, 2001). One of the most frequently isolated *Candida* species from these patients is *C. glabrata* (Redding et al., 1999). In recent years *C. glabrata* has emerged as an important pathogen in humans, being the second or third leading agent of candidiasis at all sites (reviewed in Fidel et al., 1999). Because *C. glabrata* is most often co-isolated with *C. albicans*, its role as a causative agent in OPC has been controversial. Also, its pathogenicity has been difficult to demonstrate experimentally due to its much lower virulence in animal models of infection (reviewed in Fidel et al., 1999). However, oral infection with mixed *C. albicans* and *C. glabrata* may be clinically more severe (Redding et al., 2002) and reports of *C. glabrata* as the only detectable species from oral lesions have been rising steadily (Redding et al., 1999, 2001, 2002). This is particularly important since unlike *C. dubliniensis*, *C. glabrata* isolated from oral lesions is much more resistant to standard antifungal treatment than *C. albicans* (Redding et al., 1999, 2001). As a result, *C. glabrata* oral infection is suspected in most cases when the patient does not respond to routine doses of fluconazole (Redding et al., 2002). Interestingly, *C. glabrata* is also associated with increased oral carriage rates among the elderly, especially the ones wearing oral prostheses (Lockhart et al., 1999). In the elderly, denture stomatitis and angular cheilitis are the most common denture-related infections (Espinoza et al., 2003), and the most frequently isolated species from these lesions is *C. albicans* (Leigh et al., 2002; Dar-Odeh and Shehabi, 2003).

### 3.2. Clinical Features of Oral Candidiasis

All *Candida* species form the same type of oral lesions clinically (Redding, 2001).

However, recent evidence suggests that mixed infections with more than one species may be associated with more severe symptoms and are more difficult to treat (Redding et al., 2002). There are three main clinical variants of oral candidiasis: the pseudomembranous (also known as thrush), the hyperplastic, and the erythematous (Axell et al., 1997). The hyperplastic form is accompanied by extensive epithelial hyperplasia and hyperkeratosis, also termed candidal leukoplakia. The erythematous form has been the predominant clinical form in HIV<sup>+</sup> patients with CD4<sup>+</sup> lymphocytes >400, whereas as the lymphocyte counts drop, the lesions appear to become more of the pseudomembranous type (Weinert et al., 1996). Frequently all three forms coexist and the term “multifocal candidiasis” is used to describe the lesions. *Candida* is also frequently responsible for inflammatory lesions found between the lips (angular cheilitis), under dentures (denture stomatitis), and on the dorsal surface of the tongue (median rhomboid glossitis). Symptoms associated with this infection are pain, burning mouth, and dysphagia, which can lead to poor nutrition and significant patient morbidity (Fotos and Hellstein, 1992).

### 3.3. Histopathologic Characteristics

Chronic hyperplastic or pseudomembranous candidiasis is a form of infection with distinct clinical and histopathological characteristics. The histologic features of this infection include a hyperplastic and parakeratotic response of the surface epithelium, which is invaded by hyphal organisms. The inflammatory infiltrate consists primarily of PMN, which form microabscesses within the epithelium, whereas very few PMN are found within the lamina propria in association with blood vessels (Eversole et al., 1997). A chronic inflammatory infiltrate is also present in the superficial lamina propria

close to the epithelial border, with a characteristic high presence of IgA-expressing lymphocytes (Williams et al., 1997).

In HIV<sup>+</sup> patients, neutrophils appear to be a rare finding in oral candidiasis lesions and are only encountered in a limited number of erythematous forms. The inflammatory cell infiltrate is primarily mononuclear in both pseudomembranous and erythematous cases of HIV-associated infection (Romagnoli et al., 1997). Few *Candida* hyphae are associated with the atrophic epithelium in erythematous candidiasis, whereas numerous organisms are found invading into the prickle cell layer of oral epithelium in pseudomembranous candidiasis. In HIV<sup>+</sup> patients the inflammatory infiltrate is heavier in erythematous candidiasis and consists of CD8<sup>+</sup> lymphocytes and CD1a<sup>+</sup> Langerhans cells (Romagnoli et al., 1997). In fact, in this study CD1a<sup>+</sup> dendritic cells were the only cell type to be significantly increased in HIV<sup>+</sup> oral candidiasis as compared to HIV<sup>+</sup> or HIV<sup>-</sup> controls. These cells were almost exclusively restricted to the basal layer of the oral epithelium. Overall a change in localization of inflammatory cells such as macrophages and dendritic cells from the lamina propria into the basal epithelial cell layer was observed, as opposed to an increase in cell number (Romagnoli et al., 1997). A more recent immunohistochemical analysis of the T cell populations in HIV-associated oral candidiasis showed an intriguing accumulation of high numbers of CD8<sup>+</sup> T cells at the lamina propria–epithelium interface of the infected sites as compared to uninfected controls, and a positive correlation between the numbers of CD8<sup>+</sup> T cells and oral fungal burden in HIV<sup>+</sup>OPC<sup>-</sup> individuals (Myers et al., 2003). Interestingly, CD4<sup>+</sup> cells were also found in these lesions. These cells did not colocalize with CD3<sup>+</sup> cells and were highly irregular in shape, suggesting that the majority were not T cells but macrophages or dendritic cells (Myers et al., 2003).

## 4. Role of Oral Fluids in the Control of *Candida* Infection

### 4.1. Saliva

Whole saliva is comprised of a mixture of molecules and cells derived from the major and minor salivary glands, with mucosal epithelium and the serous exudate originating in the gingival crevices (gingival crevicular fluid (GCF)). Collectively, these components form a strong innate defense barrier to infection. Abnormal salivary function, caused by reduced salivary flow or altered composition, leads to increased levels of *C. albicans* in the oral cavity, often culminating in overt OPC (Fotos and Hellstein, 1992). This is largely due to the fact that *C. albicans* species colonizing the oral cavity are constantly bathed in saliva and interact with salivary constituents, which can significantly modulate their growth, metabolic activity, and adhesion to oral mucosa.

In general, both unstimulated and stimulated salivary flow rates are decreased in patients with oral candidiasis (Ueta et al., 2000) and reduced salivary flow rates, associated with senescence, have been reported to be a risk factor for oral candidiasis (Tanida et al., 2001), believed mainly to be due to compromised mechanical clearance. Xerostomia due to pathologic changes in salivary glands from disease (e.g. Sjogren's syndrome) or treatment (e.g., head and neck radiation therapy) promotes chronic *Candida* colonization and predisposes patients to oral infection (MacFarlane, 1975), an effect that has been confirmed in sialoadenectomized animals (Jorge et al., 1993). However, history of recurrent oral candidiasis was not found to be associated with reduced salivary flow rates, but rather with a significantly more acidic saliva in a small group of patients as compared to healthy controls (Bercier et al., 1999). In general, saliva inhibits adhesion of *C. albicans*

to oral epithelial cells (Ueta et al., 2000), at least partly due to the inhibitory activity of salivary mucins (de Repentigny et al., 2000) and in oral candidiasis patients this inhibitory activity appears to be somewhat suppressed (Ueta et al., 2000). Several other innate effector molecules with direct antifungal activity are contained in human saliva such as histatins, calprotectin, defensins, secretory leukocyte protease inhibitor (SLPI) and others, which are listed in Table 2.1, and will be discussed in more detail later in this chapter.

## 4.2. Gingival Crevicular Fluid

GCF is an inflammatory exudate originating from the leaky venules next to the oral sulcular and junctional epithelia. An increase in the flow rate of GCF has been associated with inflammatory changes in the gingival tissues, secondary to bacterial infection (reviewed in Tonetti et al., 1998). In addition to salivary PMN and macrophages that exude through the oral junctional and sulcular epithelia into the GCF, the GCF also contains relatively high levels of

**Table 2.1.** Oral Innate Effector Molecules with Anti-*Candida* Function

| Effector                                    | Source  | Mechanism of action   | Reference   |
|---|---|---|---|
| Salivary mucins and proteolytic derivatives | Mucous salivary gland cells                                       | Modulate adhesion, candidacidal activity via electrostatic interactions with yeast membrane | de Repentigny et al. (2000), Gururaja et al. (1999) |
| Histatins                                   | Salivary gland epithelium   | Efflux of <i>Candida</i> ATP, deprivation of energy stores                                  | Koshlukova et al. (1999)                            |
| $\beta$ -Defensins                          | Oral mucosal and salivary gland epithelia                         | Pore-forming cationic peptides  | Lehrer and Ganz (1996)                              |
| Calprotectin                                | Neutrophils, monocytes, and oral mucosal epithelium               | Zinc deprivation  | Sohnle et al. (2000)                                |
| Peroxidase/MPO                              | Salivary gland acinar cells, neutrophils, and monocytes           | Oxidative damage  | Lehrer and Cline (1969)                             |
| Lysozyme                                    | Salivary gland epithelium, neutrophils, and monocytes             | Insertion of cationic regions into yeast membrane   | During et al. (1999)                                |
| Lactoferrin                                 | Acinar cells in major salivary glands, neutrophils, and monocytes | Possibly by iron deprivation  | Wakabayashi et al. (1996)                           |
| Secretory leukoprotease inhibitor           | Oral mucosal epithelium   | Candidacidal mechanism unknown  | Tomee et al. (1997)                                 |



serum-derived complement components and the antimicrobial enzymes lactoferrin and peroxidase, presumably derived from these leukocytes (Miyachi et al., 1998; Tonetti et al., 1998).

In conjunction with PMN, the complement system may play an important role in the innate immune protection of the oral mucosa. Complement activation takes place in the gingival crevice via the classical and alternative pathways (Cutler et al., 1991). Although formation of membrane attack complex (MAC) on the surface of *C. albicans* has been demonstrated (Lukasser-Vogl et al., 2000), direct killing of pathogenic fungi through this mechanism has not been conclusively proven (Kozel, 1996). In addition, although C5-deficient mice were extremely susceptible to systemic challenge with *C. albicans*, they cleared the oral infection at the same rate as controls (Ashman et al., 2003). Therefore it appears that complement is less crucial in the clearance of oral infection than it is for disseminated disease, thus affirming the localized nature of protective host responses in *Candida* infections.

## **5. Cellular Components of Innate Defense Mechanisms against Oral Candidiasis**

### **5.1. Oral Epithelial Cells**

#### **5.1.1. Immune Regulatory Function**

In oral mucosal infections, *C. albicans* organisms colonize the uppermost layers of epithelium, rarely invading past the spinous cell layer (Reichart et al., 1995; Eversole et al., 1997). As a result, the oral mucosa is chronically inflamed with intense intraepithelial and subepithelial infiltration by leukocytes (Reichart et al., 1995; Eversole et al., 1997; Myers et al., 2003). Although

the role of epithelial cells as an infection barrier against *Candida* is well recognized (Hahn and Sohnle, 1988), new information is emerging about the role of these cells in orchestrating the oral mucosal inflammatory response to this pathogen by synthesizing immunoregulatory cytokines.

Oral epithelial cells respond to infection with the release of a number of proinflammatory cytokines (Bickel et al., 1996), which can initiate and perpetuate mucosal inflammation. The response of oral epithelial cells to *C. albicans* infection in vitro includes an array of proinflammatory cytokines, namely interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-8, IL-18, tumor necrosis factor alpha (TNF- $\alpha$ ), and granulocyte-macrophage colony-stimulating factor (GM-CSF), which have been detected at the protein and/or mRNA level (Rouabhia et al., 2002; Schaller et al., 2002; Steele and Fidel, 2002; Dongari-Bagtzoglou and Kashleva 2003a,b; Dongari-Bagtzoglou et al., 2004). These cytokine responses of oral epithelial cells to *C. albicans* infection are strain-specific, require direct epithelial cell-fungal cell contact, and are optimal when viable yeast, germinating into hyphae, are used in cell interactions (Schaller et al., 2002; Dongari-Bagtzoglou and Kashleva, 2003a,b). Strong evidence also supports the fact that IL-1 $\alpha$ , resulting from the interactions of oral epithelial cells with *C. albicans*, autoregulates other cytokines secreted in response to this pathogen (Dongari-Bagtzoglou and Kashleva, 2003a).

IL-1 $\alpha$  is a major constitutive and inducible proinflammatory product of epithelial cells, which can act as a key cytokine to amplify the inflammatory response by neighboring mucosal cells, or activate local leukocyte antifungal activities (reviewed in Dinarello, 1997). Studies screening cell supernatants or lysates of *C. albicans*-infected epithelia for various proinflammatory cytokines have identified IL-1 $\alpha$  as one of the major cytokines upregulated at both the mRNA and protein levels (Schaller et al., 2002; Dongari-Bagtzoglou et al., 2004). Epithelial cell IL-1 $\alpha$  has also



been found to be present in human oral mucosal candidiasis lesions (Eversole et al., 1997). As our laboratory has shown, *Candida*-infected oral epithelial cells release this proinflammatory cytokine in its mature protein form in their microenvironment upon cell lysis. We have hypothesized that most of the IL-1 $\alpha$  processing in the epithelial cell-*C. albicans* coculture system takes place at the plasma membrane where the cytolytic actions of *C. albicans* phospholipases and proteases trigger a release of membrane phospholipids. Membrane phospholipids may in turn activate the IL-1 convertase, which cleaves membrane-associated pro-IL-1 $\alpha$  and triggers the release of the mature protein in culture supernatants (Kobayashi et al., 1990). Similarly, the ability of *C. albicans* to induce cleavage of the inactive IL-18 pro-peptide and trigger release of the active mature IL-18 protein has been demonstrated, an event temporally associated with the presence of the active form of the IL-1 convertase in these cells (Rouabhia et al., 2002).

IL-1 $\alpha$  released by injured epithelial cells increases the proinflammatory cytokine production (IL-8, GM-CSF) by neighboring uninfected mucosal and stromal cells (Dongari-Bagtzoglou et al., 2004). Such a mechanism could serve to amplify and extend the local inflammatory response, even in the absence of direct fungal invasion of the deeper mucosal and submucosal tissues. The local release of cytokines such as IL-1 $\alpha$ , IL-8, and GM-CSF by oral epithelial cells and fibroblasts could explain the histopathologic finding of neutrophilic microabscesses in these lesions (Eversole et al., 1997), since these cytokines are potent chemoattractants and/or activators of PMNs (Baggiolini et al., 1989; Blanchard et al., 1991). Recently, we established that the activation of PMN antifungal activity can take place in response to cytokines from *C. albicans*-infected oral epithelial cells in vitro. Hyphal growth inhibition experiments with human PMN from multiple donors revealed that the antifungal activity

of PMN can be enhanced by two- to three-fold over basal levels by *C. albicans*-infected oral epithelial cell supernatants, an effect largely dependent on the presence of bioactive IL-1 $\alpha$  in these supernatants (Dongari-Bagtzoglou and Kashleva, in press).

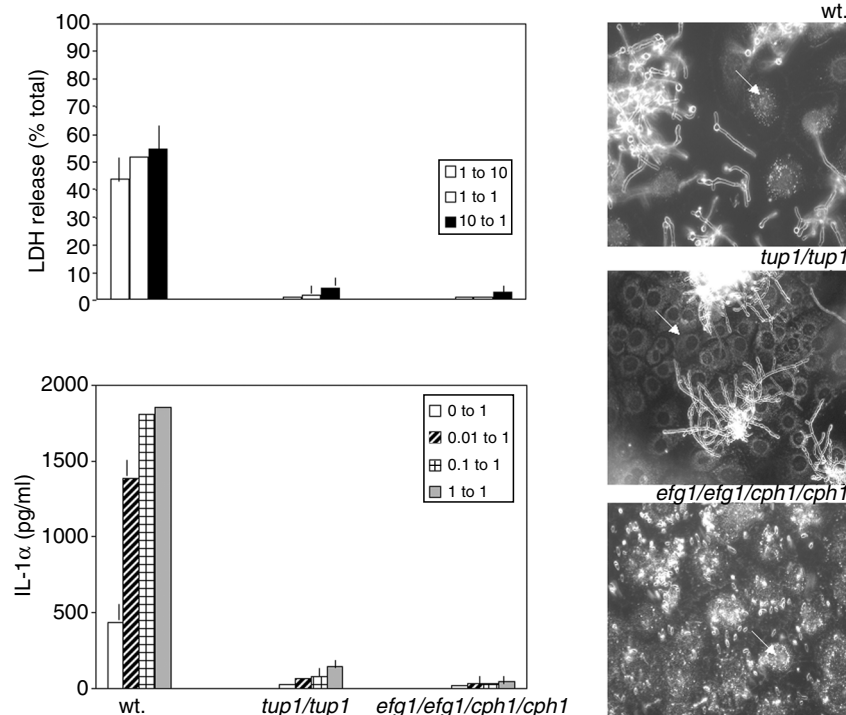
We and other researchers have shown that only live, germinating organisms are capable of stimulating proinflammatory cytokine responses by oral epithelial cells, consistent with reports in endothelial cells (Orozco et al., 2000; Schaller et al., 2002; Dongari-Bagtzoglou and Kashleva, 2003a,b). *C. albicans* is a polymorphic organism which undergoes morphological transition between yeast, pseudohyphal, and hyphal forms. All three morphogenetic forms of *C. albicans* are frequently encountered in the oral mucosa (Cox et al., 1996) and in most oral infections both yeast and filamentous organisms can be found in the infected tissues (Olsen and Birkeland, 1977). 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 (Saville et al., 2003), the exact role of hyphal transition during the development of oral candidiasis is still unclear. However, clinicopathologic findings have correlated the presence of filamentous forms with localized tissue invasion in oral candidiasis (Reinhart et al., 1995; Cox et al., 1996). Recently we studied the interactions of oral epithelial 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 (IL-8, IL-1 $\alpha$ ) response. By using mutant strains with defects in hyphal transformation or by applying environmental pressure, which affected filamentation in wild-type organisms, we found that morphogenesis is an important determinant of the outcome from the interactions between oral epithelial cells and *C. albicans*. When germination-deficient *C. albicans* mutants that form exclusively yeast (*efg1/efg11*

*cph1/cph1* mutant, Lo et al., 1997) or pseudohyphae (*tup1/tup1* mutant, Braun and Johnson, 1997) were cocultured with oral epithelial cells, they exhibited a significantly reduced capacity to adhere to oral epithelial cells and disrupt their cell membrane (Fig. 2.1). Also, in sharp contrast to strains, which formed true hyphae under these coculture conditions, germination mutants and oral strains naturally deficient in germination, triggered essentially no proinflammatory cytokine responses by these cells (Villar et al., 2004). In addition to morphogenesis, invasion of oral epithelial cells and tissues is a critical determinant of the oral mucosal inflammatory

response to infection. Highly invasive *C. albicans* strains trigger a wider array and overall greater levels of proinflammatory cytokines in oral epithelial cells compared to invasion-deficient organisms (Villar et al., in press).

### 5.1.2. Innate Immune Effector Function

Oral epithelial cells are constantly exposed to microbial challenge and therefore play an important role as the first line of defense against infection. In addition to secretion of natural antibiotic peptides (i.e., calprotectin and defensins) with antifungal



**Figure 2.1.** Yeast and pseudohyphal forms of *C. albicans* do not injure oral epithelial cells or trigger a proinflammatory cytokine response. SCC15 oral epithelial cells were cocultured with *C. albicans* SC5314 (wt.), or its congenic yeast (*efg1/efg1/cph1/cph1*) and pseudohyphal mutants (*tup1/tup1*), at increasing fungal cell to epithelial cell ratios, for up to 20 h. IL-1 $\alpha$  and lactate dehydrogenase (LDH) release were quantified in culture supernatants using colorimetric assays (left panel). The cellular morphology of these strains, cocultured with oral epithelial cells for 5 h, is shown on the right panel. Cultures were stained with Calcofluor White and epithelial cells are indicated by the white arrows.

activity, recently, a contact-dependent oral epithelial cell anti-*Candida* activity was described, which was significantly greater than that of vaginal epithelial cells (Steele et al., 2000). More specifically, primary human oral epithelial cells inhibited the growth of 40–85% of *C. albicans* at ratios ranging between 0.6 and 1, and 5:1 effector to target. This antimicrobial activity extended to other *Candida* species, including *C. glabrata*, *C. dubliniensis*, and *C. krusei*. Saliva appeared to have no effect on growth inhibition and cells isolated from HIV<sup>+</sup> patients with OPC had reduced antifungal activity as compared to HIV<sup>+</sup>OPC<sup>-</sup> controls (Steele et al., 2000). Mechanistic studies also confirmed a growth inhibitory rather than a fungicidal effect (Nomanbhoy et al., 2002), and further demonstrated that an acid labile molecule was involved in the growth-inhibiting interactions (Steele et al., 2001; Yano et al., in press), although the specific molecule was not identified.

## 5.2. Neutrophils

Neutropenia has long been recognized as the primary risk factor for invasive candidiasis, the mortality rate of which rises up to 90%, even with maximal antifungal treatment (Rodriguez-Adrian et al., 1998). Recently it has become evident that apart from neutropenia, a decrease in function of circulating neutrophils may also be responsible for reduced resistance to infection, thus cytokine treatment combined with white blood cell transfusions have been successfully tested in neutropenic patients with refractory-invasive fungal infections (Rodriguez-Adrian et al., 1998). Like G-CSF, GM-CSF is a cytokine, which also augments neutrophil antifungal activities in vitro and may have a protective function in oral candidiasis in vivo. It has been reported that administration of rhGM-CSF, as adjunctive treatment of fluconazole-refractory OPC in AIDS patients, exerts a

significant beneficial effect on the oral mycoflora and may help to clear the infection in these patients (Vazquez et al., 2000).

PMN and macrophages are constantly entering the oral cavity by transepithelial migration through the oral mucosal epithelium, as well as through the oral sulcular and junctional epithelia in the gingival crevice (Tonetti et al., 1998). Salivary PMN (sPMN) is the most abundant salivary immune cell type and can be easily isolated from human saliva (Ueta et al., 1993). Although there is no information on the functional status of the macrophages in saliva, a number of investigations have examined the functional role of sPMN. It has been shown that the activation potential of sPMN is inferior to that of peripheral blood PMN, and more specifically sPMN produce reactive oxygen metabolites and possess candidacidal activity equivalent to 65% of the peripheral blood PMN (Ueta et al., 1993). Even greater sPMN hypofunction (reduced superoxide generation and candidacidal activity) has been described in patients with oral candidiasis (Ueta et al., 2000) and in high-risk individuals such as cancer patients receiving chemoradiotherapy (Ueta et al., 1993), or the elderly (Tanida et al., 2001), and thus sPMN hypofunction can be considered a possible risk factor for oral candidiasis.

## 5.3. Other Cell Types

Quantification of immunolabeled cells in lesions from HIV<sup>+</sup> persons with OPC showed that the inflammatory infiltrate consisted mainly of CD1a<sup>+</sup> Langerhans cells and macrophages, both of which moved from the lamina propria into the basal epithelial cell layer (Romagnoli et al., 1997). In this study, CD1a<sup>+</sup> cells were the only cell type, which increased considerably in numbers, as compared to healthy controls (Romagnoli et al., 1997). A significant increase in the number of CD8<sup>+</sup> cells was observed at the lamina propria-epithelial

cell interface in *C. albicans*-infected oral tissues of HIV<sup>+</sup> individuals in a more recent study (Myers et al., 2003). The functional role of these cells in oral candidiasis is currently unknown, however, it is tempting to speculate that they may be directly involved in the clearance of the microorganism acting as innate effectors in an major histocompatibility complex (MHC)-unrestricted manner, similar to the IL-2-activated CD8<sup>+</sup> cells in mice (Beno et al., 1995).

CD4<sup>+</sup> T cells and NK cells maintain a central role in the defense against *Candida* in vivo since they provide activating signals to PMN through the release of specific cytokines (Ashman and Papadimitriou, 1995). Although NK cells are unable to kill *C. albicans* directly (Djeu and Blanchard, 1987; Zunino and Hudig, 1988; Arancia et al., 1995), in a mouse model of OPC, these cells could substitute for T cells in phagocytic cell activation and protect the animals from lethal oral infection (Balish et al., 2001). Invasive oral infection in otherwise immunocompetent mice was demonstrated using a combined neutrophil and macrophage depletion approach, implying a synergistic role of these phagocytic cell types in clearing the oral infection (Farah et al., 2001).

## 6. Effector Molecules with Anti-*Candida* Function in the Oral Mucosa

### 6.1. Salivary Mucins

These are high molecular weight glycoproteins, synthesized by mucous cells in submandibular, sublingual, and minor salivary glands. Mucins are natural lubricants of the oral mucosa, which form a protective barrier and may act as potent agglutinators of potentially pathogenic microbes. Among the salivary constituents that have been shown to serve as binding ligands for *C. albicans* in vitro, salivary mucins appear to have a

particularly high binding affinity to *C. albicans* (Hoffman and Haidaris, 1993). After binding, *C. albicans* enzymatically digests mucins by the action of secreted aspartyl proteinases (de Repentigny et al., 2000). Enzymatic degradation of mucins may facilitate adhesion to buccal epithelium since intact mucins normally block the adherence of the microorganism to these cells (de Repentigny et al., 2000). Microbial proteolysis of the low molecular weight salivary mucin MUC7 in the oral cavity may also result in the release of smaller, bioactive candidacidal peptides, which share sequence and functional similarities with histatins (Gururaja et al., 1999). Based on extensive biochemical analyses of these molecules, it has been hypothesized that the candidacidal activity of cationic MUC7 histatin-like peptides probably involves electrostatic adsorption to the negatively charged phospholipid polar groups in the yeast cell membrane, followed by membrane instability, and abnormal distribution of the cytoplasm (Gururaja et al., 1999).

### 6.2. Histatins

These are histidine-rich proteins, which originate in the salivary glands of humans and higher primates (Oppenheim et al., 1988). Histatins 1 and 3 are synthesized in the parotid and submandibular glands, and together with histatin 5, which is a proteolytic product of histatin 3, represent about 80% of the total histatin content in human saliva (Edgerton and Koshlukova, 2000). Histatin 5 is the most effective candidacidal peptide within the histatin family, killing both yeast and hyphal organisms in vitro when used within a physiologic range of concentrations (Raj et al., 1990; Xu et al., 1991). Unlike other cationic antimicrobial peptides, histatin 5 does not kill target cells by insertion into fungal cell membranes and pore formation (Raj et al., 1998). Edgerton and coworkers (2000) have demonstrated

that *C. albicans* expresses a 67-kDa histatin-binding protein receptor, detectable in the cytoplasm and cell membrane, but not the cell wall (Edgerton et al., 2000), which may provide the basis of selectivity in the killing of fungal but not mammalian cells. Biochemical and pharmacological studies of this group further demonstrated that binding of histatin 5 to its receptor triggers massive efflux of intracellular ATP without loss of membrane integrity (Koshlukova et al., 1999). Based on these findings it was further hypothesized that binding of the newly released ATP to yeast membrane ATP receptors would initiate a cascade of cytotoxic events, analogous to ATP-triggered apoptosis in mammalian cells.

The relationship between salivary levels of histatins and protection from oral *Candida* infection in vivo is unclear. Quite unexpectedly, the concentration of total histatins was elevated in a small group of clinically healthy patients with recurrent oral candidiasis as compared to controls with no history of infection (Bercier et al., 1999), a finding which suggests that repeated infection may trigger a rise in histatin synthesis by salivary gland cells. Investigators in this study explained the absence of protection despite high salivary histatin concentrations to a significantly more acidic pH of the saliva of these patients, as histatin antifungal activities are pH-dependent (Santarpia et al., 1990).

### 6.3. Defensins

Defensins are pore-forming cationic peptides with broad antimicrobial activity against bacteria and fungi (reviewed in Lehrer and Ganz, 1996; Diamond and Bevens, 1998). There are 28 types of human beta defensins, but only four have so far been fully characterized. Defensins are divided into  $\alpha$  and  $\beta$  classes. The  $\alpha$  defensins are expressed in neutrophils and intestinal Paneth cells, whereas the  $\beta$  defensins are expressed by epithelial cells of multiple

organs, including but not limited to the lung, pancreas, kidney, salivary glands, skin, and oral mucosa (Diamond and Bevens, 1998).

Oral mucosal and salivary gland epithelia synthesize beta defensin 1 (hBD-1), which is constitutively expressed and is effective in killing Gram(+) and Gram(-) bacteria (Bals et al., 1998; Krisanaprakornkit et al., 1998). This defensin is believed to be involved in homeostatic interactions between the oral mucosa and the commensal flora. Another type of hBD, hBD-2, which is transcriptionally upregulated in oral epithelia in response to infectious and inflammatory stimuli (Krisanaprakornkit et al., 2003), is very effective against Gram(-) bacteria and fungi. hBD-2 Protein expression is absent in poorly differentiated oral epithelia, such as the junctional epithelium, or nonkeratinized oral epithelia, such as the buccal epithelium and the epithelium lining the floor of the mouth (Abiko et al., 2001, 2002; Dale et al., 2001). Human alpha defensins colocalized with elastase in the oral mucosa, indicating that they are expressed by neutrophils (Dale et al., 2001).

An immunohistochemical study of human oral candidiasis biopsies showed that hBD-2 is expressed in infected as well as adjacent uninfected epithelium, although the staining intensity was much more pronounced in the presence of *Candida* (Sawaki et al., 2002). Neutrophils and oral epithelial cells present in infected tissues only expressed alpha defensins (human neutrophil peptide (HNP)-1, HNP-2, and HNP-3). However, interpretation of the findings of this study are limited by the fact that it did not include control biopsies from healthy subjects (Sawaki et al., 2002).

Expression levels of hBD-1 vary from person to person. A single-nucleotide polymorphism (SNP) at site 668 of the hBD-1 gene exhibited a significant association with oral *Candida* carriage levels. Individuals carrying one or two copies of this SNP are predisposed to a lower *Candida* carriage in the oral mucosa (Jurevic et al., 2003). However, the biological relevance of this finding is uncertain since this

SNP does not confer a change in the amino acid composition of the peptide as it lies within the 5' untranslated region of the gene and not within the coding region.

#### 6.4. Calprotectin

Calprotectin is a protein complex consisting of two noncovalently linked peptide chains that are abundantly synthesized by neutrophils, monocytes, certain subpopulations of macrophages, and squamous epithelia, such as oral epithelium and activated epidermal keratinocytes (reviewed in Brandtzaeg et al., 1995). In situ hybridization studies revealed that calprotectin is synthesized in the upper and middle spinous cell layers in the oral mucosa in oral candidiasis as well as normal tissues, with no clear quantitative differences between infected and uninfected tissues (Eversole et al., 1997). This protein complex is also found in many human body fluids, including saliva (Kleinegger et al., 2001).

Calprotectin has candidastatic activity and inhibits yeast to hyphal transformation (Murthy et al., 1993). The mechanism of the antifungal function of calprotectin is unknown, however it has been suggested that it is based on zinc binding via histidine-containing sequences, which deprives the organism from this essential metal (Sohnle et al., 1991, 2000). In fact, zinc deficiency has a negative effect on *C. albicans* growth and germination in vitro (Yamaguchi, 1975). The individual peptide calprotectin chains do not exhibit antifungal activity even though they both have zinc-binding capabilities (Sohnle et al., 2000). To explain this finding it was suggested that formation of a stable heterodimer is necessary for maximum zinc affinity (Sohnle et al., 2000). Absence of a requirement for direct contact between calprotectin and the microorganism and reversibility of the antimicrobial activity in the presence of zinc also support zinc scavenging from a distance as a likely mechanism of action (Sohnle et al., 1991).

Although variability in study design and methods make it difficult to compare clinical studies, results comparing salivary calprotectin levels in health and oral candidiasis appear to be in conflict with one another. It has been reported that patients with HIV who develop oral candidiasis have significantly lower levels of calprotectin in saliva than those who do not (Muller et al., 1993). On the contrary, two other studies reported higher salivary calprotectin levels associated with higher *Candida* counts, or presence of oral infection (Kleinegger et al., 2001; Sweet et al., 2001). Inadequate control for confounding factors such as concurrent presence of active periodontal infection, and oral hygiene practices, as well as limited number of subjects examined, may have been the source of these discrepant results. Recently clinical evidence has emerged supporting the notion that salivary calprotectin levels are nonspecifically increased secondary to mucosal inflammation and that HIV infection may negatively affect calprotectin synthesis (Sweet et al., 2001).

#### 6.5. Peroxidase and Myeloperoxidase

Peroxidase originates from two main sources in the oral cavity. Salivary peroxidase is synthesized by acinar cells in major salivary glands and myeloperoxidase (MPO) is derived from the neutrophil primary granules. Monocytes also contain MPO in their primary granules, whereas macrophages are known to lack this enzyme (Marodi et al., 1991). These enzymes combine with  $H_2O_2$  and thiocyanate or iodide ions to produce hypothiocyanate, or hypoiodite, which are powerful oxidizing agents. In the presence of chloride ion, MPO also converts  $H_2O_2$  into hypochlorous acid and monochloramine, the two potent microbiocidal agents. MPO plays a critical role in the fungicidal activity of PMN in vitro, and phagocytes genetically deficient in MPO fail to kill *C. albicans* (Lehrer and Cline, 1969). Exogenously supplied MPO also elevates the ability of human



macrophages to kill *C. albicans* (Weber et al., 1987) and activates cytokine secretion and the respiratory burst of these cells (Lefkowitz et al., 1996; Marodi et al., 1998).

Salivary peroxidase has potent fungicidal activity in vitro (Majerus and Courtois, 1992; Bosch et al., 2000). However the role of salivary peroxidase in oral *Candida* clearance in vivo is unclear since the presence of phosphate at concentrations equivalent to those found in saliva suppresses its fungicidal activity (Lenander-Lumikari, 1992). Human MPO deficiency is the most frequently encountered neutrophilic lysosomal enzyme deficiency. The importance of MPO in clearing *Candida* infections in vivo has been suggested by case reports, which have demonstrated that patients with this deficiency may develop rapidly disseminated cutaneous *C. albicans* infection (Nguyen and Katner, 1997). Similarly, patients with hereditary MPO deficiency have an increased susceptibility to oral thrush and invasive oral candidiasis (Okuda et al., 1991), therefore it appears that MPO activity may also play a role in limiting oral infection in vivo.

## 6.6. Lysozyme and Lactoferrin

Salivary lysozyme is a product of the ductal epithelium of major and minor salivary glands, in addition to being potentially synthesized by sPMN. The muramidase activity of lysozyme causing degradation of the murein in bacterial cell walls is mainly responsible for its potent bactericidal activity, which has mostly been characterized for oral streptococci (Laible and Germaine, 1985). Recently, small cationic amphipathic regions were identified in the peptide sequence of lysozyme that exhibited fungicidal activity by inserting into and damaging the fungal cell membrane (During et al., 1999). Exposure of *C. albicans* oral isolates from HIV-infected patients to physiological concentrations of lysozyme triggered a rapid loss of viability to a variable extent among isolates (Samaranayake et al., 2001).

On a limited number of genotypically defined strains, there was a significant negative correlation between lysozyme resistance and the duration of HIV disease, potentially implying that certain strains of *C. albicans* may develop progressive resistance over time to innate antifungal defenses such as lysozyme (Samaranayake et al., 2001).

The major sources of lactoferrin in the oral mucosa are the serous cells in salivary glands and the secondary granules of PMN. Although several antimicrobial mechanisms have been identified for lactoferrin, the classical mechanism involves high affinity for iron, which causes inhibition of microbial iron-dependent metabolism (Bellamy et al., 1992). While the iron-binding domain of this molecule is located at the carboxyterminus, the aminoterminal contains a microbicidal peptide sequence, known as lactoferricin (Bellamy et al., 1992). This peptide may be released by enzymatic degradation, which takes place in the gastrointestinal (GI) tract, and is active against fungi like *C. albicans* (Wakabayashi et al., 1996), although the exact mechanism of its fungicidal activity is still unknown. The fungicidal role of lactoferrin in saliva has been questioned by certain investigators since phosphate and bicarbonate ions at physiological salivary concentrations completely blocked its antifungal activity in vitro (Soukka et al., 1992). However, other studies dispute these findings by showing that addition of whole saliva in this in vitro system does not reduce its antifungal activities (Kuipers et al., 2002). As with salivary lysozyme, clinical or animal data supporting a role of lactoferrin in oral candidiasis are lacking.

## 6.7. Secretory Leukoprotease Inhibitor

SLPI is a natural anti-inflammatory and antimicrobial peptide found in mucous secretions of the oral, respiratory, and genital mucosa, and is secreted by epithelial cells lining these mucosal surfaces (reviewed in Tomee



et al., 1998). SLPI is a relatively small cationic peptide (12 kDa), which acts primarily as an endogenous inhibitor of neutrophil elastase, thus limiting tissue injury and inflammation (Bingle and Tetley, 1996). In addition, SLPI has antiretroviral activity and can kill bacterial and fungal targets, through mechanisms that have not yet been defined (reviewed in Tomee et al., 1998). A pronounced fungicidal and fungistatic activity against metabolically active *C. albicans* yeast organisms has been demonstrated at physiologic concentrations of this peptide, which was localized primarily in the NH<sub>2</sub>-terminal domain. On a molar basis this activity was similar with that of defensins and lysozyme (Tomee et al., 1997).

Although the first report on the in vitro antimicrobial activity of SLPI was in saliva (McNeely et al., 1995), to date only one study has addressed the functional role of salivary SLPI during oral infection in vivo (Chattopadhyay et al., 2004). This report on HIV-associated oral candidiasis found significantly higher levels of SLPI among participants with a history of OPC as compared to those with no history of this oral infection, but failed to show significantly higher levels in individuals with current oral infection as compared to uninfected controls. In an attempt to explain these findings the authors suggested that elevated levels of SLPI in response to recurrent infection is an attempt of the host to limit oral infection, a response that may persist long after the infection is resolved (Chattopadhyay et al., 2004).

## 7. Summary and Future Directions

Oral candidiasis is characterized by a recurrent, persistent, acute inflammatory reaction to *Candida* infection, which is limited to the uppermost epithelial layers of the oral mucosa. The inflammatory response to this pathogen elicits chronic pain and discomfort upon mastication, but it may also be responsible for activation of immunoeffector cells and the prevention of invasive infection.

Although this chapter has concentrated on the innate immune and nonimmune mechanisms of the oral mucosal defense against *Candida*, it is well recognized that an intact arm of the adaptive immunity, represented mainly by Th1 cells, plays an instrumental role in regulating the clearance of this infection by innate immunoeffectors. The mechanisms that trigger the acute inflammatory response in the oral mucosa are currently unknown. However, dissection of this process is critical to the understanding of the pathogenesis of this fungal infection and may be important for the development of strategies to prevent invasive infection in immunocompromised hosts. Evidence is accumulating that demonstrates that the acute host response to oral infection with *Candida* is initiated and perpetuated by oral epithelial cells, the first and principal targets of infection. A number of studies support the hypothesis that oral epithelial cells, just like epithelial cells from other mucosal sites that normally harbor a great number of commensal organisms (e.g., colon, vagina), require contact with the microorganism and active invasion of the host cell cytoplasm, possibly via destruction of the plasma membrane, for a proinflammatory response. Further studies are needed to demonstrate that induction of specific cytokines in oral epithelial cells in vivo may promote the ability of PMN, monocyte, and/or keratinocyte antifungal activities. Increased production of proinflammatory cytokines by oral epithelial cells combined with the cytolytic activity of the inflammation-inducing hyphal forms of *C. albicans*, are also likely responsible for the clinical findings of redness and surface ulceration of the oral mucosa during this superficial oral infection. Further studies are also necessary to explore the contribution of other *Candida* species such as *C. glabrata* or *C. krusei* to the oral mucosal inflammatory response and epithelial cell damage in a monoinfection as well coinfection model system with *C. albi-*

*cans*. Future animal studies are also needed to determine whether mitigation of these proinflammatory cytokine responses and the ensuing acute inflammation would ameliorate the clinical symptoms of oral candidiasis, and/or promote invasion into the submucosal tissues. Limited knowledge is available about the contribution of salivary and oral epithelial cell antifungal peptides in the innate defense mechanisms in oral candidiasis in vivo. Knockout animal model systems will be instrumental in fully elucidating the role of such antifungal peptides (histatins, defensins, calprotectin) in the innate immune protection of the oral mucosa from this microorganism in vivo.

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