**Invasion process of Candida albicans to tongue surface in early stages of experimental murine oral candidiasis**

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We analyzed the morphologic and microbiologic aspects of the process of adhesion and invasion in the early stages of *Candida albicans* oral infection in a murine system. ICR mice were anesthetized by intramuscular injection with chlorpromazine chloride and then orally inoculated by swabbing with the *C. albicans* yeast cells. Their tongues were resected 1–3 h after inoculation, washed sequentially with a physiological saline and 0.25% trypsin-solution and then homogenized. The number of viable *C. albicans* cells on the tongue surface was counted and found to increase from 1–3 h after inoculation. Most of the *Candida* cells attached to the tongue surface were present in clusters, mainly located in the gaps between lingual papillae and were covered with a mucoidal substance. By 3 h after inoculation, these clusters frequently formed mycelia and could not be easily detached from the tongue surface by trypsin treatment. Observation of SEM and histological sections stained by Fungiflora Y revealed that the *Candida* hyphae at 3 h stretched out of the cluster and entered the tongues through the surface. These results indicate that *Candida* hyphae begin to invade the tongue surface within 3 h after inoculation and suggest that the mucus-like substance covering these cells may have an important early role in the interaction between the *Candida* cells and the tongue mucosal epithelium.

**Keywords** mucosal infection, colonization, mucoidal substance, hypha, scanning electron microscope

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**Introduction**

*Candida albicans* is an endogenous microbe in the oral cavity and small intestine, as well as being a major cause of oral and esophageal infections in immunocompromised patients with hyposalivation, diabetes mellitus, and prolonged use of antibiotics or immuno-suppressive drugs [1,2]. Mucosal candidiasis is one of the most common opportunistic infections in HIV-infected patients [3–5].

In order to rationally develop a new therapeutic strategy against oral candidiasis, it is essential to investigate the conditions involved in pathogenesis. In oral *C. albicans* infections, it is presumed that the ragged construction of the dorsal tongue surface contributes to the invasion process, including involvement in adhesion, proliferation, and hyphal formation of *Candida* cells. Many authors reported that hyphal invasion of the tongue surface and pathological change of the dorsal tongue occurred more than 12 h after initiation of experimental oral candidiasis [6,7]. These events were then followed by the host-parasite interaction between *Candida* cells and host defense mechanisms including inflammatory responses in the oral mucosa. We speculate that the early interaction between *Candida* cells and the tongue epithelial surface plays an important role in the infection process.
There are many animal models available for experimental investigation of the early interaction events in candidiasis which have included the use of rats [8–10] and mice [11–15]. Our murine oral candidiasis model, in which the mice were anesthetized for about 3 h by the administration of chlorpromazine (CPZ) after the initial oral inoculation of \textit{C. albicans} [16,17], made it possible to study very early contact between \textit{Candida} cells and the tongue surface. Using this model, we analyzed the morphologic and microbiologic aspects of the early stages of the invasion process in \textit{Candida} infection.

**Materials and methods**

**Organism**

Two \textit{C. albicans} strains (TIMM 1768 which was isolated from the blood of a candidiasis patient, and TIMM 2640 recovered from a patient with cutaneous candidiasis), were maintained in our laboratory and stored at \( -80 \)°C in Sabouraud dextrose broth (Becton Dickinson, MD, USA) containing 0.5% yeast extract (Becton Dickinson) and 10% glycerol until used in the studies [16]. The isolates were grown on \textit{Candida} GS agar plate (Eiken Chemical Co., Ltd. Tokyo, Japan) at \( 37 \)°C for 24 h and then harvested and suspended in RPMI 1640 medium containing 2.5% fetal calf serum (FCS) for oral inoculation.

**Animals**

All animal experiments were performed according to the guidelines for the care and use of animals approved by Teikyo University. A total of 131, six-week-old, female ICR mice (Charles River Japan, Inc., Yokohama, Kanagawa) were employed in the investigations. The photoperiods were adjusted to 12 h of light and 12 h darkness daily, and the environmental temperature was maintained at a constant 21°C. The mice were kept in cages, each housing 5–6 animals and were given \textit{ad libitum} access to food and water.

**Oral candidiasis in mice**

The experimental procedure for the oral candidiasis model has been described previously [16]. Briefly, immunosuppression was induced by subcutaneous treatment of the mice with a dose of 100 mg/kg of prednisolone (Mitaka Pharmaceutical Co., Japan) 1 day prior to oral infection. Tetracycline hydrochloride (Takeda Shering Purau Animal Health Co., Japan) in drinking water at a dose of 0.08% was also given to the mice beginning 1 day before infection. The animals were anesthetized by intramuscular injection in each foot with 50 μl of 0.2% chlorpromazine chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan). They were orally infected through the use of cotton swabs (baby cotton buds; Johnson & Johnson Co., Tokyo) with \( 2.0 \times 10^8/\text{ml} \) viable cells of \textit{C. albicans} TIMM 2640 in RPMI 1640 containing 2.5% FCS.

**Quantitation of oral infection**

The \textit{Candida}-inoculated mice (\( n = 5 \) per group) were sacrificed 1 h and 3 h after the introduction of the yeast, and their tongues were resected, washed sequentially with a sterile physiological saline and a trypsin solution, and then homogenized as follows: shaken by hand in saline for 5 sec, then after washing were again shaken by hand in 0.25% trypsin solution for 10 sec, sequentially incubated for 20 sec at 37°C, and finally disrupted by vortex mixing for 10 sec. The residual tongue samples were homogenized in saline by homogenizer (Polytron PT 1200, Kinematica AG, Switzerland). The number of viable yeast cells of \textit{C. albicans} (CFU) recovered in the saline-washing, trypsin-washing and tongue homogenates was determined by colony counts on 20 h old \textit{Candida} GS plates incubated at 37°C and the totals per mouse were calculated.

**Scanning Electron Microscope (SEM)**

One, three and six h after inoculation, mice were anesthetized with pentobarbital sodium (Nembutal, Abbott Laboratories, IL, USA) and perfused through the left ventricle, first for exsanguination with phosphate buffer (pH 7.4) containing heparin, and then with fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB (pH 7.4) at 4°C. After fixation, tongues were resected and immersed in the same fixative overnight at 4°C. The samples were post-fixed with 1% osmium tetroxide in 0.1 M PB (pH 7.4) for 2 h at 4°C, dehydrated in ethanol, then desiccated in a critical point drying apparatus using liquid CO₂ (Hitachi HCP-2, Japan). The tissue samples were affixed on aluminum stubs by silver conductive paint and sputter-coated with gold-palladium using a Quick Cool Coater sputter-coating apparatus (SC-701MC, Sanyu Denshi Co., LTD., Tokyo). They were then examined in a SEM (JSM-5400LV, JEOL Co., Tokyo) operated at 20 kV, and images were prepared at a magnification of 2.0–5.0 K.

**Light Microscopy (LM)**

To obtain specimens for light microscopy, the mice were anesthetized and exsanguinated as above, and
then perfused with fixative containing 4% paraformaldehyde in 0.1 M PB (pH 7.4) at 4°C. For preparation of sections, tongues were excised after fixation by perfusion and immersed in 0.1 M PB containing 30% sucrose for 12 h at 4°C. Specimens were sectioned in a cryostat at 8 μm, postfixed in 0.1 M PB containing 4% paraformaldehyde. Subsequently, the preparations were stained by Fungiflora Y, which specifically binds to beta-linked polysaccharides of the fungal cell wall, such as glucan and chitin (Fungiflora Y, Biomate, Ltd., Osaka) [18], and mounted in glycerin PB (1:1). In these histological processes, the tongues were carefully treated in order to avoid detachment of mucoidal materials from the tongue surface.

Statistical analysis
Data were expressed as mean ± SD and evaluated by Kruskal Wallis H-test, Mann-Whitney U-test with Bonferroni correction and Mann-Whitney U-test. A p-value of less than 0.05 was considered to be statistically significant.

Results
Distribution of Candida cell population on infected-tongues
To understand the early invasion process in the pathogenesis of murine oral candidiasis, we focused on early phenomena in the interaction between C. albicans and the tongue surface after the inoculation of experimental animals. As shown in Fig. 1, a mucoidal substance, containing yeast-like cells (Fig. 2C, 2D) appeared and increased on the tongue surface from 1–3 h after inoculation (Fig. 2A, 2B). In contrast, no attachment was observed in the control group.

To assess kinetic changes associated with the adherence of Candida cells to the tongue surface, the tongues covered with the substance were washed with saline and trypsin solutions 1 h and 3 h after inoculation. The number of viable Candida cells washed off the tongues into solution and the residual cells adhering to the surface 1 h after inoculation could not be easily detached by saline washing. The changes on the tongue surface after trypsin treatment were morphologically analyzed. The surfaces of Candida-infected tongues were cleaned with trypsin solution 1 h after inoculation (Fig. 4A), but not at 3 h after inoculation (Fig. 4B), suggesting that this coating substance containing yeast cells was resistant to washing by trypsin solution 3 h after inoculation.

Histological sections of tongues removed from the experimental animals 1 h after Candida inoculation were stained by Fungiflora Y. Candida cells adhering to the surface of lingual papillae were present even after saline washing, but few were observed after trypsin treatment (Fig. 5A, 5C), suggesting that these yeast cells on the surface 1 h after inoculation could be detached by the trypsin treatment. Three hours after inoculation, the Candida cells on the tongue surface remained mainly in the gaps between lingual papillae, even after trypsin treatment (Fig. 5B, 5D).

Hyphal formation of Candida cells and invasion of hyphae into the tongue surface
We next analyzed the morphology of the hyphal formations, clusters and invasion of the tongues. Yeast cells were always observed on the surface of Candida-infected tongues, but not on that of uninfected tongues (Fig. 6A). One hour after inoculation, very few yeast cells appeared on the tongue surface (Fig. 6B); however, their numbers increased for up to 3 h and some of them formed hyphae (Fig. 6C). The hyphae seemed to elongate along the tongue surface from 3–6 h (Fig. 6D).

High magnification SEM photographs of Candida cells on the tongues surfaces 3 h after inoculation revealed that the hyphae were frequently found in the clusters of yeast cells (Fig. 7A). These hyphae were also obviously and strongly stained by Fungiflora Y (Fig. 7B). Finally, we found that the hyphae seemed to stretch out of the clusters and to enter the tongue through the surface at 3 h after inoculation (Fig. 7C). Observation of the sections stained by Fungiflora Y also confirmed that the hyphae elongated from the Candida clusters and entered the non-nucleated epithelial cells through the surface (Fig. 7D). The invasions of TIMM1768 hyphae were also observed by both SEM and LM in a tongue surface 3 h after inoculation. Their hyphae had invaded a widespread area of the tongue surface more frequently than those of TIMM2640 (Fig. 7E, 7F).
In this study, we analyzed the early process of Candida infection in a murine oral candidiasis model. The experimental animals showed typical symptoms, such as a white patch within three days after inoculation [16,17]. The most important morphological observation was that Candida hyphae began to invade the surface of lingual papillae 3 or 6 h after inoculation.

**Discussion**

In this study, we analyzed the early process of Candida infection in a murine oral candidiasis model. The experimental animals showed typical symptoms, such as a white patch within three days after inoculation [16,17]. The most important morphological observation was that Candida hyphae began to invade the surface of lingual papillae 3 or 6 h after inoculation.
(Fig. 7), at least into the cornified layer. This is, to the authors’ knowledge, the first report indicating that *Candida* hyphae begin to enter the tongue’s surface within 6 h of infection. This does not contradict the previous findings that in a rabbit model, *C. albicans* invasion into tissue was observed 12 h after inoculation [6], or that *C. albicans* cells invaded, *in vitro*, a reconstituted human oral epithelium within 12–24 h after inoculation [19,20].

Furthermore, we made the following findings with respect to the *Candida* invasion process: (i) Most *Candida* cells attached to the tongue surface were present in cluster form, and the clusters were covered with a mucoidal substance; (ii) most of the clusters were located in the gaps between lingual papillae; And (iii) 3 h after inoculation, these clusters frequently formed hyphae and the yeast cells were not easily detached from the tongue surface by trypsin treatment.

The mucosal invasion process of *C. albicans* cells in oral candidiasis is usually recognized to consist of three steps; (i) *C. albicans* adhesion to mucosa, (ii) proliferation and hyphal formation, and (iii) invasion into the epithelium [2]. In the *C. albicans*-adhesion step, our results clearly indicated that most of the *Candida* cells adhered to the tongue surface in an aggregated form covered with a mucoidal substance. This suggests the possibility that the adhesion is dependent on not only interaction between individual cells and epithelium but also between *Candida* clusters in the mucoidal substance and epithelium. It is notable that the period of 3 h after inoculation, when adhesion of the mucoidal clusters to tongues developed resistance to trypsin treatment, corresponded to the beginning of the invasion of *Candida* cells into the epithelium.

In this oral candidiasis model, *Candida* cells formed clusters covered with a mucoidal substance soon after inoculation. Thus cluster formation with the substance may be one step in the pathologic process of oral candidiasis. Though the mechanism of *Candida* cluster formation within a mucoid substance remains to be clarified, we can speculate on the following two possible processes. First, host-derived substances, such as components of saliva may contribute to the appearance of the mucoidal substance, because mucin in saliva could be condensed by oral dryness of the mice treated with CPZ. Since the *Candida* cells suspended in this infection model contain FCS, the mucoidal substance could be composed of serum factors which may have oozed from the tongue surface damaged by swabbing at the time of inoculation. Serum contains an agglutinin like mannan-binding protein and a cell clumping factor, and these may affect the cluster or germ tube formation of *C. albicans* [21].

Second, *Candida* clusters may be formed by a matrix of extracellular polymeric material produced by *Candida* cells. Biofilms of *C. albicans* were reported to form on disks of a catheter and consisted of a thin basal yeast layer and a thick hyphal layer, and the biofilm cells were surrounded with a matrix of extracellular polymeric material, primarily polysaccharides [22–25].

![Fig. 3](https://academic.oup.com/mmy/article-abstract/46/7/697/970680)
In our mice experiments, the yeast cells were covered with a mucoidal substance. This finding may support the possibility that part of the mucoidal substance is a Candida-derived polysaccharide. Additionally, we can speculate that these serum components including fibronectin may have an important role in the cluster formation of C. albicans, because these factors are known to enhance Candida biofilm formation [26].

We observed that clusters of Candida cells and the mucoidal substance were mainly located in the crypts between lingual papillae and could not be easily removed by trypsin treatment 3 h after inoculation.

**Fig. 5** Fluorescence micrographs with sagittal section of the tongue after saline- and trypsin treatments. The tongues which had been washed by saline and trypsin were stained by Fungi flora Y, which stains the polysaccharides in cell walls of Candida (arrow). Candida cells remained on the surface of lingual papillae even following saline washing 1 h after inoculation, but they were almost completely removed by trypsin treatment (A and C). However, 3 h after inoculation, Candida cells on the tongue surface were not detached by trypsin washing (B and D), and the residual Candida cells were distributed characteristically in gaps between lingual papillae. Bar = 20 μm.

**Fig. 6** SEM photographs of yeast cells and hyphae adhering to the surface of lingual papillae. (A) Control group; (B) 1 h after inoculation; (C) 3 h after inoculation; (D) 6 h after inoculation. An arrow shows yeast cells (B). Arrowheads are hyphae (C and D). There were many more yeast cells 3 and 6 h than at 1 h, and hyphae were first confirmed on the surface of papillae 3 h after inoculation. Six hours after being inoculated, they were more clearly distinguishable. Bar = 10 μm.
These crypts may provide a good environment for the adhesion of clusters of Candida. This information may provide a clue to the understanding of how the lesional distribution of Candida infection in the oral cavity can be determined clinically.

Finally, we wish to note that our basic findings about the early steps of C. albicans invasion in the oral cavity may suggest that frequent oral care treatment, which removes C. albicans cells from this cavity, is very important for prophylaxis and avoidance of oral candidiasis.

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