Condition for effective inhibition of *Candida albicans* growth by lactoferricin B and its therapeutic activity with fluconazole against oral candidiasis in mice

Takashi Tanaka\(^{1,2}\), Takafumi Okutomi\(^1\), Hiroyuki Wakabayashi\(^2\), Hiroko Ishibashi\(^1\), Shigeru Tansho\(^2\), Kentaro Ninomiya\(^1\), Hideyo Yamaguchi\(^1\), Yasuo Ono\(^2\) and Shigeru Abe\(^{1,2,4}\)

\(^1\) Teikyo University Institute of Medical Mycology
\(^2\) Department of Microbiology and Immunology, Teikyo University School of Medicine
\(^3\) Food Science & Technology Institute, Morinaga Milk Industry Co., Ltd.,
\(^4\) Faculty of Medical Technology, Teikyo University

**Abstract**

Effects of various medium conditions on anti-*Candida* activity of an antimicrobial peptide, lactoferricin B (LFcin B), were examined. Presence of sodium chrolide and \(\alpha\)-macroglobulin lowered the anti-*Candida* activity. This suggests that LFcin B is not suitable to therapeutic use against *Candida* infection in blood containing physiological salts. The therapeutic effect was examined in a murine model of oral candidiasis. The infected animals were orally administered LFcin B and/or fluconazole in the drinking water. The oral administration of LFcin B resulted in a significant decrease in number of viable *Candida* cells in oral cavity but did not improve topical lesions. The combination of LFcin B and fluconazole showed a significant therapeutic effect. LFcin B is potentially useful for management of oral candidiasis when used alone or in combination with fluconazole.

Key words: oral candidiasis, lactoferricin B, mice

**Introduction**

Lactoferricin (LFcin) is an antimicrobial peptide derived from lactoferrin (LF), an innate defense protein present in exocrine secretions and neutrophils, by pepsin digestion\(^{1,2}\). LFcin B has 25 amino acid residues and can be produced by proteolysis of bovine LF\(^3\), chemical synthesis\(^5\), and recombinant expression\(^\text{1}\). It has antifungal activities against various fungal pathogens\(^6,8\) and inhibits hyphal growth, an invasive form, of azole-resistant *C. albicans* synergistically withazole antifungal agents\(^7\). Truncated derivatives of LFcin B have been shown to be efficacious for treatment of *C. albicans* systemic infections by its intravenous injection in alone or in combination with antifungal agents\(^6,8,10\). N-terminal 11-mer peptide of LFcin H, a human homolog, was effective in disseminated *C. albicans* infection of mice\(^10\). A Dutch company announced that they completed phase I clinical trials with this peptide and have suggested that they will develop it as a systemic antifungal agent\(^11\). Therefore, LFcin-related peptides would be one of candidates for future antifungal drugs.

In recent years, owing to an increase in the number of compromised hosts, the incidence of oral infections caused by *Candida* species, in particular *Candida albicans*, has increased\(^12\).

We have established oral candidiasis model of mice having characteristics of human oral candidiasis\(^13\). Using this model, we have evaluated efficacy of antifungal drugs\(^14\) and natural defense
factors\textsuperscript{15}). Previously we reported that oral administration of bovine LF improves symptoms and reduces the number of \textit{Candida} cells in this oral candidiasis model\textsuperscript{15}. However, LFcin B was unexpectedly not effective in this study.

To further evaluate whether LFcin B is effective for oral candidiasis or not, we performed \textit{in vitro} and \textit{in vivo} studies. First, we investigated influences of physiological factors including medium strength, salt strength, osmotic pressure, and an anti-inflammatory plasma protein, \(\alpha_2\)-macroglobulin, on the activity of LFcin B against \textit{in vitro} hyphal growth of \textit{C. albicans} to predict its anti-\textit{Candida} activity in the oral cavity. It is known that antimicrobial activity of LFcin B is substantially influenced by the pH\textsuperscript{16} and the peptide shows excellent anti-\textit{Candida} activity at pH 5.5\textsuperscript{17}. Therefore, as the second study, we tested the effect of LFcin B with or without fluconazole, a currently used antifungal agent, in the oral candidiasis model by the drinking water (pH 5.5) containing LFcin B.

**Materials and Methods**

**Reagents**

Intravenous solution of fluconazole (Diflucan) was purchased from Pfizer (Tokyo, Japan). \(\alpha_2\)-Macroglobulin was purchased from Life Institute (Yamagata, Japan). LFcin B was purified from bovine LF pepsin hydrolyzate as described previously\textsuperscript{3}).

\textit{C. albicans} strains

\textit{C. albicans} TIMM1768 and TIMM2640, clinically isolated strains, were maintained at the Research Institute of Medical Mycology of Teikyo University. These strains were stored at \(-80^\circ\text{C}\) in Sabouraud dextrose broth (Becton Dickinson, MD, USA) containing 0.5% yeast extract (Becton Dickinson, MD, USA) and 10% glycerol in our laboratory until the experiment was performed. \textit{C. albicans} was grown on \textit{Candida} GS agar plate (Eiken Chemical Co., Ltd. Tokyo, Japan) for 24 h at 37°C and then the cells were harvested, suspended in RPMI1640 medium containing 2.5% fetal calf serum (FCS) (RP medium) for \textit{in vitro} test and oral inoculation.

**Measurement of \textit{in vitro} activities of LFcin B against \textit{Candida} growth**

\textit{C. albicans} TIMM1768 at \(1 \times 10^3\) cells/well were incubated in RPMI1640 or 1/3-diluted RPMI1640 + 2.5% FCS containing test compounds in 96-well microplate in 5% CO\textsubscript{2} atmosphere at 37°C for 16 h. To determine the extent of mycelial growth of \textit{C. albicans}, the crystal violet (CV) staining assay was performed as described previously\textsuperscript{18}. Briefly, the medium in the wells was discarded and the adhesive \textit{Candida} mycelia were sterilized by treatment with 70% ethanol. The mycelia were stained with 0.01% CV and washed with water. After the microplate was dried, 150 \(\mu\text{L}\) of isopropanol containing 0.04 N HCl and 50 \(\mu\text{L}\) of 0.25% sodium dodecyl sulfate were added to the wells and mixed. The absorbances at 620 nm of the wells were measured spectrophotometrically.

**Animals**

All animal experiments were performed according to the guideline for the care and use of animals approved by Teikyo University. Six week-old female ICR mice (Charles River Japan, Inc., Yokohama, Japan) were used for all animal experiments. The photoperiods were adjusted to 12 h of light and 12 h darkness daily, and the environmental temperature was constantly maintained at 21°C. The mice were kept in cages housing 5-6 animals and were given \textit{ad libitum} to food and water.

**Oral candidiasis in mice**

Experimental procedure of oral candidiasis model was described previously\textsuperscript{13}). Briefly, immuno-suppression of mice was induced by subcutaneous treatment with a dose of 100 mg/kg of prednisolone (Mitaka Pharmaceutical Co., Japan) 1 day prior to oral infection. The drinking water containing 0.08% of tetracycline hydrochloride (Takeda Shering Purau Animal Health Co., Japan) was adjusted its pH to 5.5 by addition of sodium dicarbonate and was given to the mice, beginning 1 day before infection. Mice were anesthetized by intramuscular injection with 100 \(\mu\text{L}\) of 0.2% chlorpromazine chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in foot. They were orally infected with about \(2 \times 10^6\) cells/ml viable cells of \textit{C. albicans} TIMM2640 in RP medium. Oral infection was performed by means of a cotton swab (baby cotton buds; Johnson & Johnson, Co., Tokyo, Japan) rolled over all parts of the mouth. The cell number of \textit{Candida} inoculated in oral cavity was
calculated to be about $1 \times 10^6$ cells/mouse by the difference in viable cell number associated to cotton swabs before and after oral inoculation. LFcin B at a concentration of 0.04% and/or fluconazole (1.2µg/ml) in the drinking water described above was consecutively administered just after the infection. Three days after infection, all mice were sacrificed, and tongue symptom and viable Candida cell number in the oral cavity were evaluated as described below.

**Scoring of tongue’s lesions and histological analysis**

Groups of mice were sacrificed, and each tongue’s fur and squamous disorder was scored as follows: 0, normal; 1, fur in less than 20%; 2, fur in less than 90% more than 21%; 3, fur in more than 91% and squamous layer; 4, thick fur in more than 91% and squamous layer. Macroscopic and microscopic histological observations were done as described previously.

**Measurement of the number of viable Candida cells in oral cavity of mice**

The oral cavity (i.e. cheek, tongue, and soft palate) was swabbed using a cotton swab. After swabbing, the cotton end was cut off and placed in a tube containing 5 ml sterile saline. The fungal cells were resuspended by mixing on a vortex mixer. Then the suspension in 100-fold dilution was incubated on Candida GS plate for 20 h at 37°C and the CFU (colony forming unit) was counted.

**Statistical analysis**

The data of scores were compared using the non-parametric Mann-Whitney U test. The data of the log$_{10}$ CFU of C. albicans isolated from mice were compared using a Student’s t test. Multiple comparisons of both data were performed using one-way analysis of variance, followed by the Tukey test. P values of <0.05 were considered significant. All calculations were performed using a statistical software program (Stat View: Abacus Concepts, Berkeley, Calif.). All mean values in the text were given with the standard deviation of the mean.

**Results**

**Condition of culture medium necessary for anti-Candida activity of LFcin B**

The anti-Candida activity of LFcin B was assessed by its *in vitro* growth-inhibitory activity against a typical C. albicans strain under several different assay conditions using RPMI1640 medium or 1/3-diluted RPMI1640 medium.

Fig. 1 shows that in RPMI1640 medium, LFcin B was only partly effective even at the concentration of 100 µg/ml, giving 80% relative Candida growth. On the other hand, in 1/3-diluted RPMI1640 medium, LFcin B at concentration range between 12.5 and 100 µg/ml strongly inhibited the growth of Candida in a dose-dependent manner. This means that 1/3-diluted RPMI1640 medium was suitable for anti-Candida activity of LFcin B and that conventional RPMI1640 medium may contain excessive components suppressing anti-Candida activity of LFcin B. We adopted the diluted medium for the following experiments to detect the anti-Candida activity of LFcin B as described below.

RPMI1640 medium contains 103 mM of sodium chloride as a main salt component. To examine the effects of salt concentration, sodium chloride was added to the culture medium. As shown in Fig. 2A, addition of sodium chloride at more than 51 mM to the diluted medium suppressed the anti-Candida activity of LFcin B dose-dependently. Fig. 2 also shows that D-sorbitol did not affect the activity even at 240 mM giving a high osmotic pressure. This result suggested that inhibition of the anti-Candida activity of LFcin B by sodium chloride was not caused by high osmotic pressure.

To learn whether LFcin B can be active in the blood and tissue environment of human hosts, effects of α₂-macroglobulin were examined. Fig. 3 shows that 0.67 mg/ml of α₂-macroglobulin blocked the anti-Candida activity of LFcin B.

**Anti-Candida activity of LFcin B combined with fluconazole**

As shown in Fig.4, more than 0.17 µg/ml of fluconazole inhibited the growth of C. albicans dose-dependently. LFcin B enhanced the anti-Candida activity of fluconazole.
Protective activity of LFcin B preparation in experimental oral candidiasis

In this study, the therapeutic effect of LFcin B was examined in a murine model of oral candidiasis. The infected animals were orally administered LFcin B in the drinking water for three successive days and the efficacy of LFcin B was evaluated on the basis of fungal burden in the oral cavity. Macroscopic lesions of the tongue surface and the histopathology of lingual tissues at 3 days after infection were examined.

Preliminary experiments showed that the number of Candida CFU in the oral cavity of the mice given 0.01% or 0.04% LFcin B in drinking water was significantly less than that of control, but these treatments did not improve tongue’s lesion.

Since in vitro anti-Candida activity of LFcin B was enhanced by the presence of low concentration of fluconazole, therapeutic activity of administration of this combination was examined. Table 1 shows that administration of either 0.04% LFcin B or 1.2 µg/ml fluconazole (equivalent to 0.2 mg/kg/day) reduced Candida CFU in the oral cavity but did not significantly improve scores of tongue’s lesions. The combination gave significantly lower average of the lesion score. Fig. 5 shows that tongues of the mice treated with the combination appeared to be normal macroscopically. By histological studies, PAS-positive fungi could be observed in the lesions near oral epithelium of dorsal tongues of control mice. Fig. 5 also shows that there was only few PAS-positive fungal hypha in the tongue of Candida-
infected mice treated with the combination.

Discussion

In the former part of this study, we evaluated influence of several physiological factors on anti-
*Candida* activity of LFcin B. We showed that LFcin B clearly displays its anti-*Candida* activity in 1/3-diluted RPMI1640 medium but not in the conventional RPMI1640 medium. This effectiveness of LFcin B in the diluted medium was at least partially attributed to low ionic milieu of the medium, because the anti-*Candida* activity was clearly suppressed by the addition of sodium chloride to the medium. This suppression of LFcin B activity did not appear curious, because various antimicrobial peptides are known to be salt-sensitive\(^9\). \(\alpha\)-Macroglobulin at 0.67 mg/ml, which is lower than physiological range in the human plasma (1.8-3.6 mg/ml), suppressed anti-*Candida* activity of LFcin B. \(\alpha\)-Macroglobulin is known to bind proteases and antimicrobial peptides such as human defensin HNP-1, and inhibits their activities\(^{20}\). These results suggest that the direct anti-*Candida* activity of LFcin B may be limited to some extent in the blood or perhaps in the tissue fluids. The reported effects of LFcin B derivative peptide in the systemic *C. albicans* infections might be depend on its activity to upregulate functions of neutrophils and macrophages rather than its direct antimicrobial activity\(^9,10\). Instead, we expected that LFcin B would
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Fig. 3 Suppression of anti-\textit{Candida} activity of LFcin B by the addition of \(\alpha_2\)-macroglobulin.\textsuperscript{23)} \textit{C. albicans} cells were cultured in 1/3-diluted medium with LFcin B and various concentrations of \(\alpha_2\)-macroglobulin for 16 h. Concentration of LFcin B: 0 µg/ml (○), 50 µg/ml (●).

Fig. 4 Inhibition of mycelial growth of \textit{C. albicans} by fluconazole in the presence of LFcin B\textsuperscript{23}. \textit{C. albicans} cells were cultured in 1/3-diluted medium with LFcin B and various concentrations of fluconazole for 16 h. LFcin B at 0µg/ml (○), 12.5µg/ml (▲), 25µg/ml (●), 50µg/ml (■) and 100µg/ml (■) *p<0.05, ** <p 0.01

TABLE1 Protection of murine oral candidiasis by lactoferricin B and/or fluconazole

<table>
<thead>
<tr>
<th>Group</th>
<th>Viable \textit{Candida} cells</th>
<th>Symptomatic observation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>CFU(X10\textsuperscript{5})</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>8.28±1.47</td>
</tr>
<tr>
<td>LFcinB 0.04%</td>
<td>4</td>
<td>3.23±1.38\textsuperscript{a}</td>
</tr>
<tr>
<td>FLCZ 1.2µg/ml</td>
<td>4</td>
<td>2.68±1.00\textsuperscript{a}</td>
</tr>
<tr>
<td>LFcinB 0.04%+FLCZ 1.2µg/ml</td>
<td>4</td>
<td>4.20±0.31\textsuperscript{a}</td>
</tr>
</tbody>
</table>

The mice were sacrificed at 3 days after infection. \textit{Candida} cell number in oral cavity and tongue score were determined as described in “Materials and Methods.” *p<0.05
be potentially useful for management of oral candidiasis, because it develops on the oral epithelium surrounded by saliva with low concentrations of ions and proteinous compounds.

We reported an experimental oral candidiasis model of mice, which made it possible to estimate therapeutic efficacy of antifungal compounds by two parameters, CFU of *C. albicans* in the oral cavity and score of clinical manifestation of the infected tongues^{13}. In our previous study, LFcin B was not effective in this model, where pH of the drinking water containing the test compound had not been adjusted^{15}. We noted that the anti-*Candida* activity of the peptide is influenced by pH and it is most effective around pH 5.0-6.0^{6,17}. Therefore, we reexamined the efficacy of LFcin B by dissolving it in the drinking water adjusted to pH 5.5 in the oral candidiasis model. In this condition, administration of LFcin B reduced the number of *Candida* CFU in the oral cavity of mice. The *Candida*-suppressive effect of LFcin B was seen rapidly (at day 3 after the infection) in this test, whereas the anti-*Candida* effect

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Fig. 5 Macroscopic observation of typical lesions on the tongues of oral candidiasis mice (A, B) and microscopic observation of typical lesions (C, D)\(^{23}\). Mice were orally infected with *C. albicans* 3 days before. Control mice (A, C) were given water and the tested mice (B, D) were administered water containing LFcin B(0.04%) and fluconazole (1.2µg/ml). The tongue sections (C, D) were stained with PAS stain. There are numerous hyphae of *Candida* in the epithelium surface layer on the dorsum of tongue of control mouse (C).
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of bovine LF was observed more slowly (since 5 days after the infection and significant at day 7) in the previous study and the immunomodulatory effect was implicated in this effect\textsuperscript{15,21}. The exact mechanism underlying the protective action of LFcin B against oral candidiasis remains to be clarified. However, it is possibly supposed that the antimicrobial activity of LFcin B mainly contributed to the observed reduction of Candida cells in the oral cavity.

Notably the combination of LFcin B and fluconazole displayed significant therapeutic efficacy in macroscopic lesional pathogenesis of tongues estimated by scoring. This effectiveness of the combination would be explained by their \textit{in vitro} anti-Candida activity. Our previous studies showed that the combination of azole antifungal agents and LFcin B synergistically inhibits the growth of azole-resistant or non-resistant strains of \textit{C. albicans}\textsuperscript{7,22}. In addition, we also confirmed the anti-\textit{Candida} effect of LFcin B in combination with fluconazole in this study. Therefore, we can suspect that LFcin B would be an effective agent as single use, but it could be developed as a supporting agent for clinical therapy by existing azole antifungal agents against oral candidiasis caused by \textit{C. albicans} including azole-resistant strains.

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\textbf{References}


