A novel murine model of pharyngeal candidiasis with local symptoms characteristic of pharyngeal thrush produced by using an inhaled corticosteroid

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We established a novel murine model of pharyngeal candidiasis maintaining stable yeast population and local symptoms characteristic of pharyngeal thrush. The persistent Candida-infection was prolonged by inhalation of beclomethasone dipropionate corticosteroid. The severity of infection lesions was evaluated by determining viable cell number of Candida albicans and scores representing symptomatic curd-like white patch on pharyngeal tissue. The utility of this model was shown by the disappearance of lesions and fungal cells after treatment with fluconazole (FLCZ). The model would be useful for evaluating new chemotherapeutic or immunotherapeutic approaches against pharyngeal candidiasis, as well as in pathological studies.

Keywords Steroid inhaler, antifungal, asthma, COPD, HIV, inflammation

Introduction

Inhaled corticosteroid (ICS) is the most effective and widely used form of anti-inflammatory therapeutics in bronchial asthma patients. Recently, ICS has also been used in chronic obstructive pulmonary disease (COPD) patients [1]. However, such treatment often predisposes the patients to Candida infections of the pharyngeal mucosa [2–9]. Moreover, oropharyngeal candidiasis has been reported to show incidence of 0–70% in ICS users [2,9–13].

On the other hand, oropharyngeal candidiasis is not only a side effect of inhaled corticosteroid but also the commonest fungal infection in HIV-infected patients [14–16]. To solve these problems and find available therapeutic treatments requires a suitable experimental animal model. There is no model of pharyngeal candidiasis that could be used to evaluate application of topical steroid ICS, although there are various oral candidiasis models that were induced by systemic steroids [16–20].

Therefore, in this study, we focused on the pharyngeal mucosa of mice and established a new murine model of pharyngeal candidiasis by using the inhaled corticosteroid beclomethasone dipropionate inhaler (BDI).

Materials and methods

Treating agents

The metered-dose inhalational drug, beclomethasone dipropionate (Becotide® 100 Inhaler), purchased from GlaxoSmithKline (Tokyo) was used in all experiments.

Candida albicans strain and growth condition

Candida albicans TIMM2640 was a clinical strain, isolated from a patient with cutaneous candidiasis (Teikyo University Institute of Medical Mycology, Tokyo) [21]. It was stored at −80°C in Sabouraud dextrose broth (Becton Dickinson, MD, USA) containing 0.5% yeast extract (Becton Dickinson) and 10% glycerol until the experiments were performed. The strain was grown on a Candida GS agar plate (Eiken...
Chemical Co., Ltd., Tokyo) at 37°C for 24 h. The yeast cells were harvested by a microspatula and suspended in RPMI1640 medium (Sigma Chemical Co., MO, USA) containing 2.5% fetal calf serum. The cell suspension was adjusted to a final concentration of $2 \times 10^8$ viable cells/ml. This *Candida* inoculum cell suspension gave reproducible results for pharyngeal *Candida* infection as found in cases of oral infection [20].

**Animal preparation and pharyngeal infection**

All animal experiments were done using female ICR mice (six-weeks-old, Charles River Japan, Inc., Yokohama Kanagawa, Japan), and were kept in cages which housed 4–6 animals and were given standard mouse food and water ad libitum. The environmental temperature was constantly maintained at 21°C, and the photoperiods were adjusted to 12 h of light and 12 h of darkness daily. Tests performed met the guidelines for the care and use of animals approved by Teikyo University.

The experiments were begun on the day before inoculation (day −1). Tetracycline hydrochloride (Takeda Shering Purau Animal Health Co., Osaka, Japan) was dissolved and given in drinking water at the concentration of 0.83 mg/ml. On day 0, the mice were anesthetized by intramuscular injection with 50 μl of 2 mg/ml chlorpromazine chloride (12 mg/kg; Wako Pure Chemical Industries, Ltd., Osaka) on each femur, and swabbed on the pharynx with a small cotton pad (baby cotton buds, Johnson & Johnson Co., Tokyo, Japan) which had been soaked with a suspension of *C. albicans* ($2 \times 10^8$ viable cells/ml). Eighteen hours post-inoculation (day 1), the mice were treated BDI (GlaxoSmithKline Inc., Tokyo) on their pharynxes using a spray device emitting one spray of 100 μg/day at 11 a.m. (once daily) or four sprays of 400 μg/day at 11 a.m. and 5 p.m. (twice daily) for three days. This device, shown in Fig. 1, was developed in our laboratory by modifying a spray device used with patients.

To evaluate the severity of lesions of the pharynxes, the mice were killed en masse by 30-sec exposure to CO$_2$ gas on day 4.

**Evaluation of development of infections**

The infections on the pharynxes of mice were examined by use of a stereoscopic microscope. The evaluation was expressed by scoring lesions from 0–4 on the basis of the extent of severity and relative thickness of whitish, curd-like patches on the surface of pharynx, as follows: score 0, normal; score 1, thin white patches occupying less than 30%; score 2: thin white patches occupying more than 30%; score 3: thick white patches occupying over less than 10%; score 4: thick white patches occupying more than 10%. Typical examples are shown in Fig. 2. This scoring was performed with recording photos of the lesions though it was not blinded.

![Fig. 1](image1.png)

**Fig. 1** The spray device for mice. 1. Bombe containing beclomethasone dipropionate (BD) with high pressure gas. 2. Spray device body: BD jets out when the body is pushed down to the direction indicated by the arrow. 3. Plastic corn prepared from 1–200 μl pipette tip. 4. Ejection pore for BD.

![Fig. 2](image2.png)

**Fig. 2** Macroscopic observations of typical lesions consisting of white patches on the pharynxes of pharyngeal candidiasis mice for each score (score 0–4). Scoring details are described in the text.
For microbiological evaluation, the pharyngeal mucosa tissues (size, 6 × 3 ml) were individually homogenized in 1 ml sterile saline. After 200-fold dilution, 50 μl of the homogenate suspension was incubated on a Candida GS agar (Eiken Chemical Co., Ltd, Tokyo) plate and incubated at 37°C for 24 h. The colony forming units (CFU) of C. albicans were calculated by counting the number of Candida colonies.

Histopathological study

For histopathological examination and fungal detection, tissue specimens of pharyngeal mucosa were taken from sacrificed animals, fixed in 10% formalin solution and embedded in paraffin. Five-μm sections were cut from the paraffin block and stained with periodic acid-Schiff (PAS) and hematoxylin eosin (HE) stain.

Statistical analysis

Scoring data were compared using the non-parametric Mann-Whitney u-test. The data of the log CFU of C. albicans isolated from the tongues and the pharynxes epithelium of the mice in the experimental groups were compared using the Student’s t-test. Multiple comparisons of both sets of data were made using one way analysis of variance, followed by Tukey test; P values of <0.05 and <0.01 were considered significant. All calculations were performed using a statistical software program (Stat View: Abacus Concepts, Berkeley, CA, USA). All mean values given in the text include the standard errors of the mean.

Results

Effects of BDI on pharyngeal candida infection in mice and the standard of evaluation against symptoms representing pharyngeal candidiasis

We first examined the effects of BDI-treatment on Candida infection in the mice which were given of 400 μg of BDI per day (twice daily). As expected, all the mice manifested C. albicans lesions consisting of the white patches or pseudomembranes on the pharynx on day 4 post-inoculation. To evaluate exactly the symptoms representing pharyngeal candidiasis, we introduced our scoring standard, described above, with the data shown in Fig. 2.

We then compared the effects of BDI-treatment on Candida infection between two groups of mice which were given of 400 μg of BDI per day (twice daily) or 100 μg of BDI per day (once daily). Fig. 3 shows that the colonization of C. albicans was increased depen-
candidiasis even in lower doses under our experimental condition.

Next, we kinetically checked the severity of this infection in the mice treated with or without 400 μg of BDI per day. As shown in Fig. 4, all mice on day 2 post-inoculation showed pharyngeal candidiasis with local symptoms mimicking those characteristic of human pharyngeal thrush. However, on day 4, the BDI-treated mice were carrying about $1 \times 10^5$ CFU of *C. albicans* and had developed the highest degree of the pharyngeal mucosa lesions that were given a score 4. In contrast, the control mice were recovering from pharyngeal candidiasis though they still carried some *C. albicans*. Moreover, only in the BDI-treated mice, did the *Candida* infection continue to day 8. These findings indicated that inhaled corticosteroid treatment was effective in causing persistent *Candida* infections of the pharyngeal mucosa and prolonging such infections.

**Characterization of new pharyngeal candidiasis model**

We checked the pharyngeal mucosa of control and BDI-treated mice on day 4. The BDI-treated mice showed thick whitish, curd-like patches (Fig. 5B) microscopically, and were given a score of 4. Microscopically and histopathologically, the PAS staining showed numerous hyphae of *C. albicans* organisms on the granular layer of squamous epithelium about 100 μm thick (Fig. 6B-PAS). In the HE stained sections, the inflammatory cells were found accumulating under the hyphal layer in the granular layer of squamous epithelium (Fig. 6B-HE). Comparatively, in the control mice (only received inoculation of *Candida*) showed no symptoms, and they were given a score of 0. Thus, the BDI-treated mice had the features of pharyngeal candidiasis lesions, but these were not found with the control mice.
strated the therapeutic activity of the antifungal agent FLCZ using this model. This suggests that the new model provides a useful tool for screening antifungal agents or evaluating new chemotherapeutic or immunotherapeutic approaches against pharyngeal candidiasis.

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**Table 1** Therapeutic efficacies of FLCZ against pharyngeal candidiasis mice on day 4 post-inoculation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log₁₀ CFU/mouse</th>
<th>Score of lesions</th>
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<tbody>
<tr>
<td>Control</td>
<td>5.29±0.59</td>
<td>3.25±1.5</td>
</tr>
<tr>
<td>FLCZ</td>
<td>&lt;1.40**</td>
<td>0.00±0.00**</td>
</tr>
</tbody>
</table>

All mice received BDI (400 µg/day) from day 1 to day 3 post-inoculation. FLCZ was administered in drinking water at 92 µg/ml (20mg/kg) from day 2 to day 4 post-inoculation and antifungal efficacy was assessed on day 4. **P <0.01 (versus Control). Data shown are the mean ± standard deviation for four mice.

**Estimation of therapeutic activity of anti-fungal agents in this pharyngeal candidiasis model**

To test the utility of this model, we examined the therapeutic activity of the antifungal agent FLCZ against pharyngeal candidiasis. As shown in Table 1, no C. albicans organisms were detected on the pharynxes of the FLCZ-treated mice, and their pharyngeal mucosa surfaces were observed to be normally glossy on day 4.

**Discussion**

In this study, we succeeded in developing a novel model of pharyngeal candidiasis that carried about 1×10^5.5 CFU of C. albicans and local symptoms characteristic of pharyngeal thrush by prolonging the persistence of the Candida-infection with the inhaled-corticosteroid, BDI. Microscopically, correlating with the fungal burden, white patches were observed which consisted of hyphae on the pharyngeal mucosa and germ tube formation was shown on the pharyngeal mucosa surface. When the white patches were mechanically removed, the pharyngeal mucosa on pharynx showed a reddish and irregular surface, perhaps indicating destruction of epithelial tissues (data not shown). However, in histopathological observation of the pharyngeal squamous epithelium, the inflammatory cells were accumulating under the hyphal layer of C. albicans in the granular layer of squamous epithelium (Fig. 6B-HE). These observations indicate that the model closely mimics the pathological situation seen in patients with oropharyngeal candidiasis. These indicate that the new model is a suitable experimental model for pathological studies of pharyngeal candidiasis.

Additionally, we also proposed an evaluation standard for the severity of pharyngeal mucosa lesions by scoring their characteristics. However, the scoring was not directly proportional to log₁₀ CFU of C. albicans in the lesion mucosa. Moreover, we successfully demon-

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**References**


