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Pathological analysis of the *Candida albicans*-infected tongue tissues of a murine oral candidiasis model in the early infection stage

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ABSTRACT

Objective: The early pathological process of *Candida* infection and immunological responses in tongues of the mice with experimental oral candidiasis was analysed.
Methods: CD-1 mice, pretreated by prednisolone were orally inoculated with *Candida albicans*. Symptoms were monitored by measuring the area of white tongue coating and number of viable *Candida* cells in oral cavity. The histopathological analysis was carried by PAS-stain and immunofluorescent staining. IL-4, IL-12p70, IFN-γ, TNF-α in recovered from the homogenates of the tongues were measured by ELISA.
Results: The fungus invaded the tongue surface of the mice and white patches developed within 24 h after inoculation. Histopathological examination indicated the presence of local acute inflammation in superficial tissues of tongues covered by mycelium of *C. albicans*. Pathological exacerbation was observed from 24 to 48 h after the inoculation and from then the symptoms of oral candidiasis appeared to move into the recovery phase. Inflammatory cells mainly consisting of neutrophils was accumulated and located under the lesions covered by *Candida*-hyphae. An increase in IL-12p70 and IFN-γ in tongue homogenates was observed at 48 h after inoculation.
Conclusions: The worst condition in the pathological process in experimental oral candidiasis was found 48 h after *C. albicans* inoculation. When the surface of the *Candida*-inoculated tongues was covered with *Candida*-hyphae, a dense accumulation of neutrophils was observed under the lesions and homogenates of the tongues contained increased levels of IL-12p70 and IFN-γ. These suggested that local pathological condition of *Candida*-infected tongues may be affected by neutrophils accumulation and increased levels of some cytokines.

1. Introduction

Candidiasis of the oral cavity or oesophagus is a common disease that occurs in patients with underlying diseases such as malignant tumours, leukaemia, immunodeficiency syndrome related to human immunodeficiency virus, diabetes, etc., with long-term administration of adrenal cortex steroids or antibiotics, and in infants and elderly people whose host defense mechanisms are suppressed.[1,2] Antifungal agents

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Abbreviations: CFU, colony-forming unit; IFN, interferon; IL, interleukin; TLR, toll-like receptors; TNF, tumor necrosis factor.
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usually produce a beneficial response in treatment of these mycoses, but post-remission recurrence of the symptoms is not unusual.[3]

Several ex vivo research projects on host defense mechanisms against this condition have been conducted: host cells recognize *Candida albicans* cells via Toll-like receptors (TLR 2, 4 and 9) and produce several types of cytokines. In vitro experiments suggested that *C. albicans* can be eliminated from infected tissues by antifungal activities of effector cells, such as macrophages and neutrophils.[4] There are also reports of in vivo research using gene-knockout mice, indicating that the functions of Interleukin (IL)-12p40 and tumour necrosis factor (TNF)-α are important in host defense against oral candidiasis,[5] but we have only very limited information about kinetics of effector cells in local lesions in the oral cavity. Especially lacking is information about pathological changes and cytokine production in the early stage of oral candidiasis. We think that information about early pathological steps in oral candidiasis should give a clue to obtain rationales and strategies to develop a new prophylaxis against severe oral candidiasis.

Recently we reported that, in a murine oral candidiasis model developed by Takakura et al.,[6,7] penetration of *C. albicans* hyphae into the mucosal epithelium of tongues began 3 h after *Candida* infection.[8] On the basis of these studies, here we investigated the pathological process of oral candidiasis in this experimental model and examined kinetically the production of cytokines (IL-4, IL-12p70, interferon (IFN)-γ and TNF-α) in the *Candida*-infected tongues. Here we checked the level IL-4, IL-12p70, IFN-γ and TNF-α, since these cytokines can represent the humoral or cellular immunity and inflammatory responses.

2. Materials and methods

2.1. Organisms

This research used *C. albicans* TIMM1768 (serotype A, clinical isolate) stored at the Teikyo University Institute of Medical Mycology. The strain was stored at −80 °C in a YPD liquid culture medium containing 0.5% yeast extract (Becton Dickinson, Franklin Lakes, NJ), 2% glucose (Wako, Osaka, Japan), and 1% Bacto peptone (Becton Dickinson) to which 10% glycerol (Wako) had been added. The frozen yeast was thawed at room temperature and the fungal culture was spread over the surface of Sabouraud dextrose flat nutrient agar (Becton Dickinson) for 24 h at 37 °C. *Candida* cells were harvested from the culture and suspended in 2.5% foetal bovine serum in RPMI-1640 medium (Sigma Aldrich, St. Louis, MO); the number of cells was determined using a hemocytometer and adjusted to the designated concentration for inoculation.

2.2. Animals

All animal experiments were conducted in accordance with the policies for animal care, welfare, and use of the Teikyo University Institute of Medical Mycology. Six-week old female Crj:CD-1 (ICR) strain mice (Nihon Charles River, Kanagawa, Japan), weighing 25–30 g were used for animal experiments. During the experimental period, MR pellets (Nihon Nosan Kogyo, Kanagawa, Japan) and water were available ad libitum.

2.3. Oral candidiasis in mice

The murine oral candidiasis was induced by the method of Takakura et al.[6,7] During the experiment, in order to suppress commensal bacteria in the oral cavities, 1 mg/ml of Chlorin® solvent (chlortetracycline, hydrochloride, Serachem, Hiroshima, Japan) was added to the drinking water, which was available ad libitum, 24 h prior to oral inoculation. Prednisolone (100 mg/kg, Kawasaki Seiyaku, Kanagawa, Japan) was administered intraperitoneally 24 h prior to inoculation as an immunosuppressant.

For the *C. albicans* infection, 12 mg/kg of chlorpromazine hydrochloride (Wako) was administered intramuscularly into the thigh as a tranquilizer, and a cotton swab (Mentip, Japan Menbo, Tokyo, Japan) dipped into a cell suspension containing 2×10^8 cells/ml of *C. albicans* in RPMI-1640 medium with 2.5% foetal bovine serum was used to inoculate the entire oral cavity. The number of mice used for *Candida*-infection experiments was 5–8 mice/group, unless otherwise designated (total number 115 mice).

2.4. Quantitation of oral infection

The mice with oral candidiasis were euthanised by cervical dislocation and the entire tongue was removed from the radix linguae. Symptoms were observed by measuring the area of white patches. This area was evaluated by the method of Takakura et al.[6,7] with the visual observation converted to a 5-stage score: 0, normal; 1, white patches over less than 20%; 2, white patches over less than 90% but more than 21%; 3, white patches over more than 91%; 4, thick white patches like pseudomembranes over more than 91% of the tongue.

Viable cell counts were measured by applying tissue fluid, obtained by homogenising the sample in a homogenizer (POLYTRON PT1200, KINEMATICA AG, Central Kagaku Trading, Tokyo, Japan), placing the homogenate onto a GS flat medium plate (Eiken, Tokyo, Japan), cultivating it for 24 h at 37 °C, and counting the number of colonies that developed (defined as colony forming units [CFU]).

2.5. Histological examination

The sample was fixed by dipping it in 4% paraformaldehyde (Sigma Aldrich) in distilled water, pH7.4 at 4 °C for 48 h. The fixed sample was embedded in paraffin and prepared for staining by slicing it to a thickness of approximately 4 μm with a microtome (MICROM International GmbH, Germany) and periodic acid-Schiff staining of the slices by the prescribed method; following this, an inverted research microscope (IX71, Olympus, Tokyo, Japan) was used to observe the histopathology images.

Immunohistochemical staining was performed using the same samples. All fixed samples were embedded in OCT compound (Tissue-Tek, Sakura Finetek, USA), and kept at −80 °C until processing. Sample slices (8 μm) were cut using a cryostat, mounted on glass slides (SuperFrost, Matunami, Osaka, Japan), and air-dried for 10 min. The tissue was then