Effect of orally administered bovine lactoferrin on the immune response in the oral candidiasis murine model

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Therapeutic activity against oral candidiasis of orally administered bovine lactoferrin (LF), a multifunctional milk protein, was shown in a previous report using an immunosuppressed murine model. In the present study, the influence of orally administered LF on immune responses relevant to this therapeutic effect was examined. Because mice were immunosuppressed with prednisolone 1 day before and 3 days after the infection with Candida, the numbers of peripheral blood leukocytes (PBL) and cervical lymph node (CLN) cells were reduced. LF feeding prevented the reduction in the numbers of PBL on day 1 and CLN cells on days 1, 5 and 6 in the Candida-infected mice. The number of CLN cells of individual mice on days 5 and 6 was inversely correlated with the Candida c.f.u. in the oral cavity. Increased production of IFN-γ and TNF-α by CLN cells stimulated with heat-killed Candida albicans on day 6 was observed in LF-treated mice compared with non-treated mice. Concanavalin A (ConA)-stimulated CLN cells from LF-treated mice also showed a significant increase in the production of IFN-γ and IL-12 on day 5 and a tendency for increased production of IFN-γ and TNF-α on day 6. The levels of cytokine production by ConA-stimulated CLN cells on day 6 were inversely correlated with the Candida c.f.u. in the oral cavity. In conclusion, the alleviation of oral candidiasis by LF feeding in this model may correlate with the enhancement of the number of leukocytes and their cytokine responses in regional lymph nodes against Candida infection.

INTRODUCTION

Lactoferrin (LF) is an iron-binding glycoprotein that is present in milk, saliva and other exocrine secretions as well as in neutrophil granules. LF has a number of biological functions, including antimicrobial and immunomodulatory effects in vitro and in vivo (Brock, 1995; Tomita et al., 2002; Vorland, 1999). It has been reported that orally administered bovine LF reduces the number of pathogenic organisms not only in the gastrointestinal tract (Teraguchi et al., 1995; Wada et al., 1999) but also in tissues distant from the gastrointestinal tract in several animal models of infection (Abe et al., 2000; Bhimani et al., 1999; Haversen et al., 2000) and in humans with chronic hepatitis C and tinea pedis (Ishii et al., 2003; Iwasa et al., 2002; Yamauchi et al., 2000). It is known that LF and an LF-derived antimicrobial peptide, lactoferricin B (LFcin B), inhibit in vitro growth of fungi such as Candida albicans (Kuipers et al., 1999; Wakabayashi et al., 1996, 1998) and Trichophyton mentagrophytes (Wakabayashi et al., 2000); it has been shown that orally administered LF shows a host-protective effect against systemic C. albicans infection in mice (Abe et al., 2000) and cutaneous T. mentagrophytes infection in guinea pigs (Wakabayashi et al., 2000).

Oral candidiasis, caused by C. albicans, is most prevalent in infancy and old age and in individuals with immunosuppressive conditions. The clinical importance of oral candidiasis, which is not life-threatening but causes significant morbidity in patients, has increased recently (Hermann et al., 2001). Some drugs such as azole antifungal agents are used for chemotherapy of this fungal infection (Walsh et al., 2000), but long-term treatments sometimes lead to the appearance of drug-resistant Candida and side effects (Lopez-Ribot et al., 1999). Recently, we developed a new oral candidiasis model using immunosuppressed mice; the mice in this model have local symptoms characteristic of oral thrush (Takakura et al., 2003a). In an assessment of the potential of LF as a food component using this animal model, it was demonstrated...
that LF feeding improved oral candidiasis microbiologically and symptomatically (Takakura et al., 2003b). In that study, it was suggested that the effect of LF in this oral candidiasis model is not attributable simply to its direct antifungal activity. Therefore, the influence of orally administered LF on systemic or local immune responses relevant to its therapeutic effect in this model was examined in the present study.

**METHODS**

*C. albicans*. *C. albicans* strain TIM2640 (Teikyo University Institute of Medical Mycology, Tokyo, Japan), a clinical isolate from a patient with cutaneous candidiasis (Maebashi et al., 1994), was stored at −80 °C in Sabouraud dextrose broth (Becton Dickinson) containing 0.5 % (w/v) yeast extract (Becton Dickinson) and 10 % (v/v) glycerol until used for experiments. The strain was grown on Candida GS agar plates (Eiken Chemical Co.) at 37 °C for 24 h. Yeast cells were harvested using a microspatula and suspended in RPMI 1640 medium (Sigma) containing 2.5 % (v/v) heat-inactivated fetal calf serum (FCS). The cell density of the suspension was adjusted to 2.5 × 10^7 cells ml⁻¹ using a Celta haematocytometer (Nihon Kohden Co.) with a setting for counting *Candida* conidia.

**Experimental oral candidiasis of mice.** All animal experiments were performed according to the guidelines for the care and use of animals approved by Teikyo University. Six-week-old female ICR mice (Charles River Japan) were used for all animal experiments. The animals were randomized, assigned to groups of four to eleven individuals and given food and water *ad libitum*.

The experimental oral candidiasis model of mice was prepared as reported previously (Takakura et al., 2003a). Mice were immunosuppressed with two subcutaneous injections of prednisolone (PDS) (Mitaka Pharmaceutical Co.) at a dose of 100 mg (kg body weight)⁻¹ 1 day before and 3 days after the infection with *Candida*. Tetracycline hydrochloride in drinking water (0.83 mg ml⁻¹) was given to mice beginning 1 day before the infection. Animals were anaesthetized by an intramuscular injection of 50 μl of 2 mg chlorpromazine chloride ml⁻¹ in each femur. A small cotton pad was soaked in a *C. albicans* cell suspension (2.5 × 10^7 cells ml⁻¹) and used to swab the entire oral cavity of the anaesthetized mice to produce oral infections.

Bovine LF (Morinaga Milk Industry Co.) at a concentration of 0.3 % (w/v) in drinking water (equivalent to 0.5 g kg⁻¹ day⁻¹) was administered continuously from 1 day before the infection.

The end-point of infection evaluation was day 7 in this model. Mice were sacrificed under anaesthesia and subjected to daily evaluation of the severity of lesions of the tongue. Macroscopic evaluation of the infection was expressed by lesion score from 0 to 4 on the basis of the extent and severity of whitish, curd-like patches on the tongue surface as follows: 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 resembling pseudomembranes over more than 91 % of the tongue.

After scoring of tongue lesions, microbiological evaluation of progression of the infection was carried out as follows. The whole oral cavity was swabbed using a cotton pad. The end of the cotton pad was then cut off and placed in a tube containing sterile physiological saline. After mixing with a vortex mixer to release *Candida* cells from the swab into the saline, the undiluted cell suspension or dilutions of it were plated and incubated on Candida GS plates at 37 °C for 20 h. The number of c.f.u. of *Candida* cells was then counted. The detection limit was 50 c.f.u. per mouse.

**Determination of the number and subtyping of peripheral blood leukocytes (PBL).** Heparinized peripheral blood was collected from each sacrificed mouse on days 1, 3, 4, 5, 6 and 7 post-inoculation. PBL were counted using a haematocytometer. A drop of heparinized peripheral mouse blood was applied to a slide glass and a smear was made. After drying, cells in the preparations were stained with Wright–Giemsa stain and counted. The results are presented as the number of the following cell types: segments (neutrophils), eosinophils and lymphocytes.

**Determination of the number and cytokine production of cervical lymph node (CLN) cells.** Two CLNs were excised from each sacrificed mouse on days 1, 3, 4, 5, 6 and 7 post-infection. Single-cell suspensions of CLN cells were prepared by mechanical disruption through a nylon mesh. CLN cells were counted using a haematocytometer. *Candida* cells were grown and heat-killed as described previously (Akagawa et al., 1995). CLN cells obtained on day 5 or 6 were cultured at 10^5 cells ml⁻¹ in RPMI 1640 medium supplemented with 10 % FCS in the presence of 5 μg concanavalin A (ConA) ml⁻¹ or 10^8 heat-killed *C. albicans* cells in 96-well plates for 4 days. Culture supernatants were collected and then assayed for IFN-γ, IL12, TNF-α and IL2 by ELISA.

ELISA was performed using Maxisorp immunoplates (Nunc) and an OptiELA set for mouse IFN-γ, IL12 (p70), TNF-α and IL2 (BD Pharmingen). An Immuno Pure TMB substrate kit (Pierce) was used for detection of peroxidase activity in ELISA.

**Statistical analysis.** The log₁₀ c.f.u. of *C. albicans* isolated from the mouths of infected mice and numbers of PBL and CLN cells were analysed using Student’s *t* test for comparison of two groups. Lesion scores were analysed using the Mann–Whitney *U* test for comparison of two groups. Correlations were analysed by Pearson’s correlation test. *P* values < 0.05 were considered significant. All analyses were performed using a statistical software program (SAS version 8 and Stat View; SAS Institute Inc.). Data are expressed as means ± SEM.

**RESULTS AND DISCUSSION**

**Therapeutic effect of orally administered LF on oral candidiasis in mice**

The therapeutic effect of oral LF was examined over time in a murine model of oral candidiasis with local symptoms characteristic of oral thrush (Takakura et al., 2003a, b). Similar results were obtained repeatedly and are shown in Fig. 1(a, b). There were no differences in the severity of *Candida* infection between LF-treated and untreated mice until day 4. However, in LF-treated mice, the number of viable *Candida* cells in the oral cavity and the score of lesions on the tongue started to decrease compared with those in untreated mice from day 5. The differences in these parameters between the two groups became significant on days 6 and 7. In our previous study, we suggested that the therapeutic effect of LF may not be attributable simply to the direct antifungal activity of LF, since LF exerted this effect even under conditions in which LF did not come into direct contact with *Candida* cells in the oral cavity (Takakura et al., 2003b). Furthermore, LFcin B, which has a more potent antifungal effect than LF in *vitro*, failed to reduce the number of *C. albicans* cells in the oral cavity. Therefore, the influence
of orally administered LF on systemic or local immune responses relevant to its therapeutic effect in this model was examined.

**Kinetics of numbers of PBL and CLN cells**

We examined the time-courses of the numbers of PBL and CLN cells (Fig. 1c, d). The mean numbers of PBL and CLN cells were respectively about 8.4 and 8.1×10^6 cells per mouse before PDS treatment. In the oral candidiasis model, because mice were treated with PDS, a corticosterone, on days –1 and 3, the numbers of PBL on days 1 and 4 and CLN cells on days 1, 5 and 6 decreased. However, these reductions of cell numbers were partially prevented by LF feeding. We confirmed the reproducibility of these results at least three times.

The difference in the number of PBL between untreated and LF-treated mice was significant on day 1 (Fig. 1c). Analysis of PBL subtypes on day 1 revealed that the number of lymphocytes was reduced from 7.5±1.1×10^6 cells ml⁻¹ in normal mice to 2.6±0.4×10^6 cells ml⁻¹ in this model. However, LF feeding partially prevented the reduction in the number of lymphocytes in PBL on day 1 (4.0±0.8×10^6 cells ml⁻¹). On the other hand, there was no difference in the number of neutrophils in PBL between the two groups. This increase of lymphocytes in PBL by LF is in accord with the previous finding by Kuhara et al. (2000), that orally administered LF increased the number of cells in various lymphocyte subsets, including CD4⁺, CD8⁺ and asialoGM1⁺ cells, in the peripheral blood of tumour-bearing mice. CLNs are the most important lymph nodes in protection against oral infection (Elahi et al., 2000). The reduction in the number of CLN cells on day 1 was prevented by orally administered LF, as seen in PBL (Fig. 1d).

The enhancement by LF treatment of the number of PBL and CLN cells on day 1 was observed only in mice infected with Candida and also treated with PDS, and not in mice infected only, in mice treated with PDS only or in normal mice (Fig. 2a, b). Artym et al. (2003) demonstrated that the drop in the number of PBL and the strong reduction in the percentage of lymphocytes induced by cyclophosphamide, which is known to be a potent immunosuppressive drug, were prevented by orally administered LF in mice. In their study, LF decreased the number of PBL when administered to normal mice. Therefore, we can assume that oral LF may affect lymphocyte numbers in PBL and CLN cells in a manner dependent on the host’s immune status.
ings suggest that LF feeding may affect the number of lymphocytes systemically and/or locally, which may be relevant to its protective effects.

**Cytokine production by CLN cells stimulated with ConA or heat-killed *C. albicans***

After CLN cells were collected on day 5 or 6 and stimulated with ConA or heat-killed *C. albicans*, the levels of cytokines in the culture supernatants were measured. In this study, CLN cells were cultured for 4 days, because cytokine production was reduced by PDS treatment. On day 5, the level of IFN-γ and IL12 production by CLN cells stimulated with ConA was higher in LF-treated mice than in non-treated mice (Fig. 4). The level of production of IFN-γ by CLN cells stimulated with ConA was notably increased on day 6 compared with day 5, whereas that of IL12 was decreased. Augmentation of IFN-γ but not IL12 production by LF on day 6 was observed in CLN cells stimulated with *Candida* cells. Enhancement of TNF-α production by LF administration was also clearly seen on day 6 in CLN cells stimulated with *Candida*. IL2 production seemed somewhat higher in the LF group, but the difference between the two groups was not significant. Moreover, comparison of cytokine production by CLN cells and *Candida* c.f.u. in the oral cavity of individual mice on day 6 indicated that the levels of IFN-γ and TNF-α production after stimulation with ConA were inversely correlated with *Candida* c.f.u. (IFN-γ: $r = -0.75$, $P = 0.02$; TNF-α: $r = -0.83$, $P = 0.01$) and IL12 production by these cells was correlated with the number of *C. albicans* cells in the oral cavity ($r = 0.70$, $P = 0.06$).

IL12 production by CLN cells on day 5, it lowered IL12 production on day 6. Because a reduction in the number of *Candida* cells in the oral cavity was observed in mice that had lowered IL12 production on day 6, it is possible that lowered IL12 production indicates the early resolution of oral candidiasis. The earlier enhancement of IL12 secretion by LF feeding may have induced a rapid response, leading to early resolution of oral candidiasis.

It is well known that TNF-α and IFN-γ stimulate macrophages and neutrophils, which kill *C. albicans* directly (Tansho *et al.*, 1994). Farah *et al.* (2002) demonstrated that TNF-α in oral tissue was an important mediator in the recovery from oropharyngeal candidiasis in mice. Their findings suggest that T lymphocyte infiltration into the oral tissue activates resident or accumulated macrophages and neutrophils by releasing TNF-α and inducing a phagocytic response against invading hyphal elements. CLN cells activated by LF feeding may migrate to the circulation, with
some reaching the oral mucosa. Further examination is necessary of the influence of LF feeding on immunological events in the oral tissue using this model. In the past few years, several immunomodulatory effects of ingested LF in animals have been reported (Sekine et al., 1997; Wakabayashi et al., 2002). Very recently, we showed that LF feeding augmented macrophage activity at the local site where inactivated Candida was injected as a priming agent (Wakabayashi et al., 2003). These results support the possibility that LF feeding upregulates immune functions of effector cells and causes the early resolution of oral candidiasis in this model.

As shown by clinical observations and experimental data in animal models, impairment of cell-mediated immunity can enhance susceptibility to mucosal candidal infections (Fidel, 2002). Therefore, upregulation of cell-mediated immunity by LF feeding, as indicated by the enhancement of Th1-type cytokine production, may be beneficial for the treatment of oral candidiasis. Masco (2000) has already reported that a mouthwash containing LF and lysozyme was effective against oral candidiasis in an HIV patient. Our findings suggest that LF from cows’ milk can be used not only as a mouthwash but also as a dietary supplement with immunomodulatory action for antifungal treatment.

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REFERENCES


