

## Augmented inhibition of growth of *Candida albicans* by neutrophils in the presence of lactoferrin

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### Abstract

The combined inhibitory effects of neutrophils and lactoferrins on the growth of *Candida albicans* were examined. Murine or human neutrophils partially inhibited growth of *C. albicans* when cultured with *C. albicans* in vitro. The growth inhibition was augmented by a combination of neutrophils and more than 30 µg/ml of bovine lactoferrin or 1 µg/ml of human lactoferrin, concentrations less than 1/10–1/200 their inhibiting concentrations when used alone. The inhibition of *C. albicans* was also enhanced by combination of neutrophils and bovine apolactoferrin or iron-bound holo-lactoferrin, but not by transferrin. Combination effects of neutrophils and lactoferrin were also observed in a condition where there was no contact between neutrophils and *Candida* cells. These results suggest that neutrophils inhibit the growth of *C. albicans* regardless of whether there is direct contact between them and *Candida* cells: neutrophil growth inhibition effects were augmented in the presence of a physiological concentration of lactoferrin, perhaps through some action of lactoferrin other than chelation of ferric ion.

**Keywords:** *Candida albicans*; Neutrophil; Lactoferrin

### 1. Introduction

We are studying host defence mechanisms against *Candida* infection. An increase in immunocompromised hosts like AIDS patients have made mucosal candidosis clinically a more and more serious problem. Histological studies showing that mucosal lesions with *Candida* infection are associated with neutrophil accumulation suggest that neutrophils are one of the major participants in mucosal defence

mechanisms against *Candida* invasion [1]. It was reported that anti-*Candida* activity of neutrophils can be regulated by various immunomodulators; bacterial lipopolysaccharides and cytokines such as tumor necrosis factor and IL-8 augment the anti-*Candida* activity of neutrophils, while glucocorticoids and progesterone suppress it [2–4]. We speculated that anti-*Candida* activity of neutrophils could also be regulated by some other modulators existing in a histologically marginal area, the mucosa.

Lactoferrin, an antimicrobial glycoprotein with MW of 80 kDa, exists in exocrine secretions such as milk (2 mg/ml), tears (0.4–1.2 mg/ml), nasal secre-

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tions (0.1 mg/ml) and saliva (5–10 µg/ml) which cover various types of mucosae [5]. Lactoferrin is known to inhibit not only growth of bacteria but also *Candida* through deprivation of iron molecules from growth environment and direct interaction with the cell surface of target microorganisms [6–8]. Recently lactoferrin was reported to have activities to modulate phagocyte functions such as adhesiveness of neutrophils and phagocytosis of macrophages [9–12].

Here, we studied the interactions of neutrophils and lactoferrin in their growth inhibitory activity of *Candida albicans* in vitro.

## 2. Materials and methods

### 2.1. Mice

Seven- to eight-week-old female C3H/He N and C3H/He J mice were obtained from Japan SLC (Shizuoka, Japan) and Japan Clea (Tokyo, Japan), respectively.

### 2.2. Chemical reagents

Bovine lactoferrin, bovine apolactoferrin, holo-lactoferrin and human lactoferrin were generously provided by Morinaga Milk Industry Co. (Tokyo, Japan). Endotoxin content in the lactoferrin preparations was estimated to be below 1 ng/mg. Human transferrin and iron(III) chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

### 2.3. Culture of *C. albicans*

*C. albicans* TIMM1768, a clinically isolated serotype A strain, was passaged at 28°C by biweekly transfer onto fresh Sabouraud glucose agar slant. For experimental use a small colony was picked up from the agar slant by pipette and the yeast cells were washed with Dulbecco's phosphate-buffered saline (PBS) by centrifugation at 600×g for 5 min. The cells were suspended in RPMI 1640 medium with 2.5% heat-inactivated fetal calf serum (FCS) (complete medium).

### 2.4. Preparation of murine and human neutrophils

All animal experiments were performed according to the guideline for the care and use of animals approved by Teikyo University. Murine neutrophils were prepared from peritoneal exudate as described previously [4]. C3H/He N mice, unless otherwise designated, were injected intraperitoneally with 3 ml of 8% casein sodium (Tokyo Kasei, Tokyo, Japan) saline. Six hours later peritoneal cells were collected and contaminated erythrocytes were lysed by the addition of hypotonic PBS diluted to 1/3 by distilled water. After being washed with PBS, they were resuspended in 2 ml of complete medium and then layered on 10 ml of 90% Ficoll-Hypaque solution (Pharmacia Fine Chemicals, NJ, USA). After centrifugation at 300×g for 30 min at room temperature, the cells from the bottom phase were washed with PBS, and were shown to be more than 95% neutrophils by Giemsa staining. Human peripheral blood neutrophils were obtained as described previously [13]. Heparinized venous blood obtained from healthy male volunteers was mixed with dextran 70 and allowed to stand at room temperature for 30 min. The leukocyte-rich supernatant was collected and then centrifuged on a Ficoll-Hypaque density gradient. The neutrophil-rich layer at the bottom was washed with PBS, and contaminated erythrocytes were lysed by the addition of 0.83% NH<sub>4</sub>Cl in Tris-HCl buffer (pH 7.65). The mixture was suspended for 3 min and an equal volume of cold PBS was added. The residual leukocytes were washed and resuspended in complete medium. More than 95% of the suspension was neutrophils.

### 2.5. Assay for growth inhibition of *C. albicans* by neutrophils in the presence or absence of lactoferrin

To determine neutrophil-mediated inhibition of *C. albicans* growth, crystal violet staining assay was performed as described [2,3,14]. Fifty microliters of lactoferrin solution was put into a 96-well flatbottom microplate. Then, 50 µl of neutrophils and *C. albicans* ( $1 \times 10^4$  cells/ml) suspension were added. After the mixtures had been incubated at 37°C for 15 h, the medium in the wells was discarded by inverting the microplate. The *Candida* cells in the wells were

sterilized by immersion in 70% ethanol for 1 min and then adherent neutrophils were washed out with 100  $\mu$ l of 0.25% sodium dodecyl sulfate (SDS). The plates were washed twice by immersing them in distilled water, and mycelia attached to the wells were stained by 0.02% crystal violet in 100  $\mu$ l PBS for 15 min. After drying the microplate, 150  $\mu$ l of isopropanol containing 0.04 N HCl and 50  $\mu$ l of 0.25% SDS were added to the wells and mixed by a plate mixer for 30 s in order to extract crystal violet from the mycelia. The absorbance at 590 nm of triplicate samples was measured photometrically. The percent growth of *Candida* was calculated as follows: absorbance (*Candida* with neutrophils)/absorbance (*Candida* alone)  $\times$  100 (%).

In some cases, neutrophils and *Candida* cells were cultured separately in the upper and lower chamber, respectively, in order to keep them separated; the two chambers were connected by a filter with a pore size of 0.45  $\mu$ m (Falcon cell culture insert). *Candida* growth in the area of lower chamber corresponding to upper chamber was assessed by crystal violet staining assay described above.

## 2.6. Statistical analysis

Statistical analysis was carried out by Student's *t*-test.

## 3. Results

### 3.1. Effects of bovine lactoferrin on growth inhibition of *Candida* by neutrophils

We began our studies by examining the effects of bovine lactoferrin on anti-*Candida* activities of neutrophils, since various types of bovine lactoferrin preparations were available and this is an important product in the dairy industry. Murine peritoneal neutrophils and human neutrophils from peripheral blood inhibited the *Candida* growth depending on the effector to target ratio (E/T) as shown in Fig. 1. Although bovine lactoferrin was able to inhibit the *Candida* growth by itself, its effective dose to achieve 50% inhibition ( $ID_{50}$ ) was estimated to be as high as approx. 1000  $\mu$ g/ml, no complete inhibition was observed even at the concentration of 10 mg/ml (data

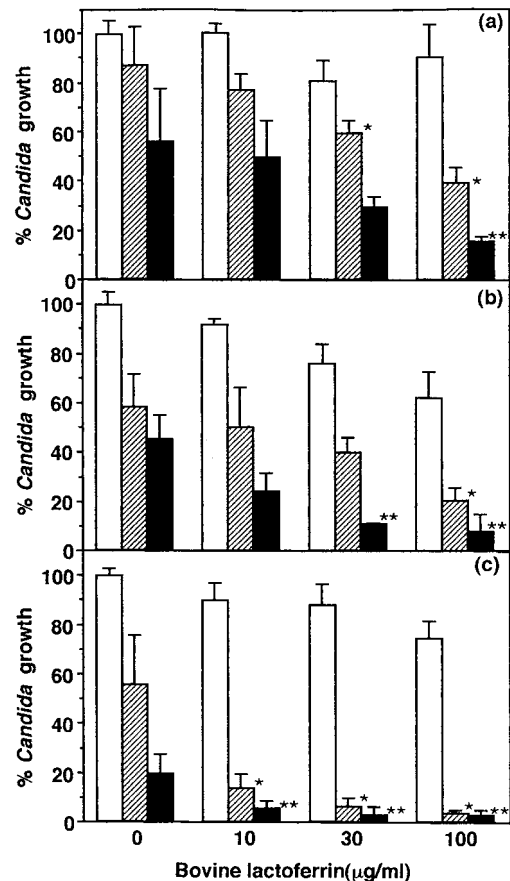


Fig. 1. Augmentation of anti-*Candida* activity of murine or human neutrophils by bovine lactoferrin. *C. albicans* was cultured with neutrophils of C3H/He N mouse (a) and C3H/He J mouse (b) at effector to target (E/T) ratios of 0 (open columns), 15 (hatched columns) or 30 (black columns) in the presence of various concentrations of bovine lactoferrin for 15 h. (c) *C. albicans* was cultured with human neutrophils at E/T ratios of 0 (open columns), 50 (hatched columns) or 100 (black columns) in the presence of various concentrations of bovine lactoferrin for 15 h. Percent *Candida* growth (%) was calculated as described in Section 2. The results represent the mean of three separate experiments  $\pm$  S.D. \*\*\*Statistically significant difference ( $P < 0.01$ ) from each control in the absence of bovine lactoferrin.

not shown). Fig. 1a shows that a relatively low concentration of lactoferrin in the presence of murine neutrophils at an E/T ratio of 15 and 30 clearly augmented the growth inhibition of *Candida*: concentrations causing 50% inhibition were 110  $\mu$ g/ml and 40  $\mu$ g/ml respectively, about 1/10 and 1/25 of the  $ID_{50}$  of lactoferrin alone (1000  $\mu$ g/ml). Fig. 1b shows that similar augmentation effects were also observed

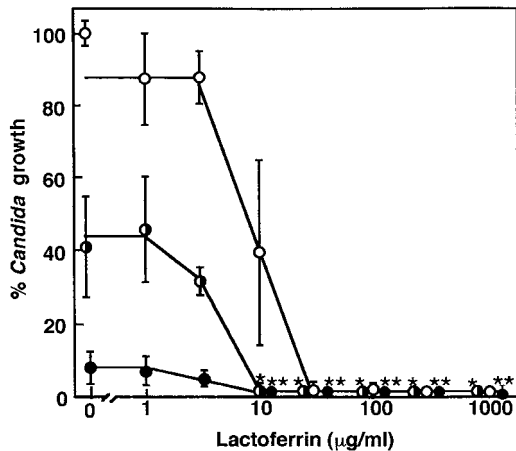


Fig. 2. Combination effect of human lactoferrin and neutrophils on growth of *C. albicans*. *C. albicans* was cultured with human neutrophils at E/T ratios of 0 (open circles), 50 (right-filled circles) or 100 (filled circles) in the presence of various concentrations of human lactoferrin for 15 h. The results represent the mean of three separate experiments  $\pm$  S.D. \*\*\*Statistically significant difference ( $P < 0.01$ ) from each control in the absence of human lactoferrin (\*E/T = 50, \*\*E/T = 100). For details, see legend to Fig. 1.

with the combination of bovine lactoferrin and neutrophils obtained from C3H/He J mice which are known to have low response to LPS.

As shown in Fig. 1c, enhanced anti-*Candida* activity by bovine lactoferrin was also seen in the co-culture of human neutrophils at an E/T ratio of 50

and 100; in this case the concentration of bovine lactoferrin to achieve ID<sub>50</sub> was 5 µg/ml, about 1/200 that of lactoferrin by itself (1000 µg/ml). In the presence of human neutrophils, complete inhibition of *Candida* growth was noted at the concentration of more than 30 µg/ml of bovine lactoferrin. These results indicate that bovine lactoferrin even at a low concentration effectively inhibits the growth of *Candida* when combined with murine or human neutrophils.

The antifungal activity of human lactoferrin was next examined. As shown in Fig. 2, human lactoferrin had a strong inhibitory effect on *Candida* growth when used alone: its ID<sub>50</sub> was 13 µg/ml and complete inhibition was observed at the concentration of 30 µg/ml. When human lactoferrin was added to the mixture of human neutrophils and *Candida* at the E/T ratio of 50, 50% growth inhibition was observed at the lactoferrin concentration of 4 µg/ml (Fig. 2). This shows that growth inhibition of *Candida* by human neutrophils was augmented in the presence of human lactoferrin at a very low concentration.

Thirdly, in order to clarify the role of iron ion, effects of bovine apolactoferrin or iron-bound holo-lactoferrin on anti-*Candida* activity of neutrophils were examined. As shown in Fig. 3a, the combination of neutrophils and apolactoferrin displayed potent anti-*Candida* activity when bovine apolactoferrin was added to the mixture of neutrophils and

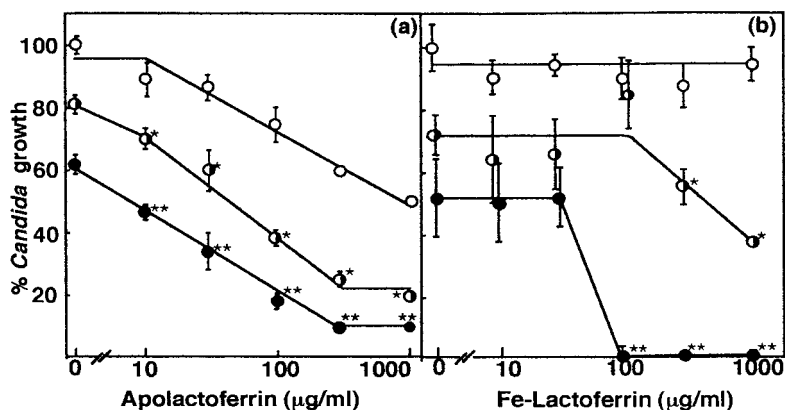


Fig. 3. Effects of apolactoferrin and iron-saturated lactoferrin on anti-*Candida* activity of murine neutrophils. *C. albicans* was cultured with murine neutrophils at E/T ratios of 0 (open circles), 15 (right-filled circles) or 30 (filled circles) in the presence of various concentrations of apolactoferrin (a) and iron-saturated lactoferrin (b). The data represent the mean of five determinations  $\pm$  S.D. \*\*\*Statistically significant difference ( $P < 0.01$ ) from each control in the absence of bovine lactoferrin (\*E/T = 15, \*\*E/T = 30). For details, see legend to Fig. 1.

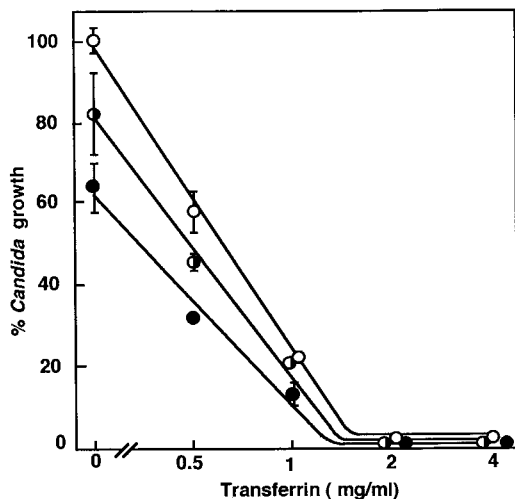


Fig. 4. Effect of transferrin on anti-*Candida* activity of murine neutrophils. *C. albicans* was cultured with murine neutrophils at E/T ratios of 0 (open circles), 15 (right-filled circles) or 30 (filled circles) in the presence of various concentrations of human transferrin. The data represent the mean of five determinations  $\pm$  S.D. For details, see legend to Fig. 1.

*Candida* at the E/T ratio of 0, 15 and 30;  $ID_{50}$  was 1000  $\mu$ g/ml, 90  $\mu$ g/ml and 40  $\mu$ g/ml, respectively. Fig. 3b indicates that iron-bound holo-lactoferrin at tested concentrations did not affect *Candida* growth in the absence of neutrophils but it augmented anti-*Candida* activity of murine neutrophils in a similar manner to the lactoferrin. Thus, lactoferrins, in spite of their ability of iron binding, appear to augment the growth inhibition of *C. albicans* in combination with neutrophils.

### 3.2. Effects of transferrin on anti-*Candida* activity of neutrophils

Effects of transferrin, an endogenous iron binding protein like lactoferrin, were examined on anti-*Candida* activity of neutrophils. As shown in Fig. 4, transferrin inhibited *Candida* growth in a dose-dependent manner at concentrations up to 500  $\mu$ g/ml, and at the concentration of about 600  $\mu$ g/ml transferrin caused 50% growth inhibition. The concentration of transferrin causing 50% inhibition of *Candida* growth in the presence of neutrophils, however, was estimated to be about 500  $\mu$ g/ml, similar to that in the absence of neutrophils. This suggests that trans-

ferrin does not augment the growth inhibition of *C. albicans* in combination with neutrophils.

### 3.3. Effective timing of addition of bovine lactoferrin for augmentation of anti-*Candida* activity of neutrophils

We checked whether or not *Candida* cells pretreated with bovine lactoferrin are more susceptible to anti-*Candida* activity of murine neutrophils and whether these neutrophils pretreated with bovine lactoferrin more effectively inhibit *Candida* growth. We found no significant effect of these pretreatments (data not shown). Therefore, we speculated that long-term interaction of bovine lactoferrin with neutrophils or *Candida* cells may be necessary for significant augmentation of neutrophil anti-*Candida* activity. The optimal timing of bovine lactoferrin addition for this augmentation activity was then examined.

Bovine lactoferrin was added at various times over the course of a 15 h culture period. As shown in Fig. 5, its addition within 3 h after the start of co-culture of neutrophils and *Candida* strongly augmented the anti-*Candida* activity of neutrophils. These augmentation effects of lactoferrin gradually decreased with delay in the timing of the addition ( $> 3$  h), suggesting that augmented anti-*Candida* activity depends on the long-term interaction of lactoferrin with neutrophils or *Candida* cells during the culture period, especially in the middle or late term.

### 3.4. Effects of lactoferrin on anti-*Candida* activity of neutrophils without contact between *Candida* cells and neutrophils

We speculated that neutrophils were able to inhibit *Candida* growth without contact between them [15]. We investigated the effect of lactoferrin on anti-*Candida* activity of neutrophils where there was no contact between neutrophils and *Candida* cells. Murine neutrophils were added in upper chamber and *Candida* cells in lower one; these were separated by a filter with pores allowing the diffusion of soluble factors. Fig. 6 shows that murine neutrophils inhibited the *Candida* growth even without contact with the target cells, and indicates that addition of lactoferrin to the lower chamber clearly augmented

the growth inhibition. These results demonstrated that lactoferrin could enhance the growth inhibition of *C. albicans* in combination with neutrophils regardless of whether or not there was contact between the neutrophils and *Candida* cells.

#### 4. Discussion

In this study we investigated the cooperative anti-*Candida* effect of neutrophils and lactoferrin which are major participants in the defence mechanism against mucosal candidiasis. Relatively low concentrations (30–100 µg/ml) of bovine lactoferrin strongly inhibited *Candida* growth in combination with neutrophils. The cooperative effects of neutrophils and lactoferrin on *Candida* growth inhibition were mainly observed in combination with bovine lactoferrin, and similar ones were also observed in human lactoferrin.

Lactoferrin is known to inhibit growth of microbials through deprivation of iron molecules by chelating effects [16]. The iron-chelating activity of hu-

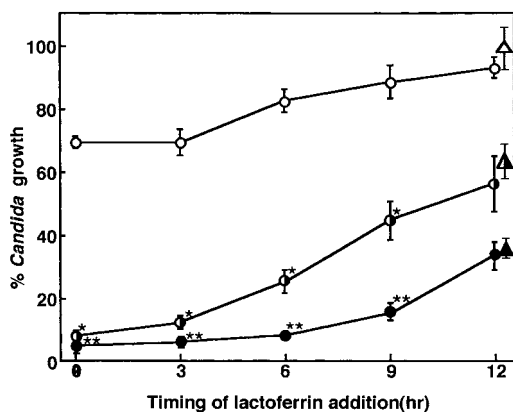


Fig. 5. Dependence of augmentation of anti-*Candida* activity of neutrophils on timing of addition of lactoferrin. *C. albicans* was cultured with murine neutrophils at E/T ratios of 0 (open circles and triangles), 15 (right-filled circles and triangles) or 30 (filled circles and triangles) in the presence (circles) or absence (triangles) of 1000 µg/ml of bovine lactoferrin. Bovine lactoferrin was added at various time points in the course of a 15 h culture period. The results of one of two separate experiments are shown and the data represent the mean of five determinations ± S.D. \*\*\*Statistically significant difference ( $P < 0.01$ ) from each control in the absence of bovine lactoferrin (\*E/T = 15, \*\*E/T = 30). For details, see legend to Fig. 1.

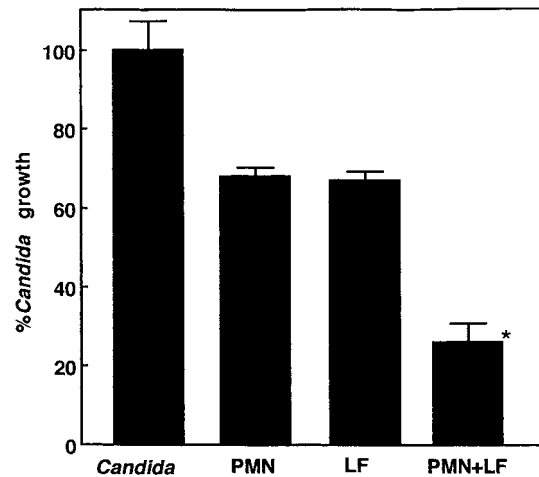


Fig. 6. Augmentation of anti-*Candida* activity of murine neutrophils by bovine lactoferrin under conditions of no contact between *Candida* cells and neutrophils. Murine neutrophils and *Candida* cells were cultured in a chamber divided by filter with a pore size of 0.45 µm for 15 h. Neutrophils were added in the upper chamber and *Candida* cells in the lower at the E/T ratio of 400 in the presence of 100 µg/ml bovine lactoferrin. Percent *Candida* growth (%) was calculated as described in Section 2. This shows one typical result obtained from three separate experiments and the data represent the mean of four determinations ± S.D. \*Statistically significant difference ( $P < 0.01$ ) from each control with bovine lactoferrin alone and in the absence of bovine lactoferrin.

man lactoferrin is reported to be stronger than that of murine lactoferrin [17]. Correspondingly, human lactoferrin used by itself showed stronger inhibitory effect than murine lactoferrin on *Candida* growth (Fig. 1). Therefore, anti-*Candida* activities of lactoferrins are assumed to depend on their iron-chelating activity. Anti-*Candida* effects of bovine and human lactoferrin, however, were equivalent when combined with human neutrophils (Fig. 1c, Fig. 2), suggesting that the iron-chelating effect of lactoferrin does not greatly contribute to the anti-*Candida* activity in a combination of lactoferrin and neutrophils. This assumption was supported by the following evidence. (1) No difference was observed in the combination effects of apolactoferrin, holo-lactoferrin and lactoferrin with neutrophils (Fig. 3). (2) No augmentation was observed in combined anti-*Candida* activity between neutrophils and transferrin which has iron-chelating activity as does lactoferrin (Fig. 4).

The mechanism(s) of inhibition of *Candida* growth

by the combination of lactoferrin and neutrophils remains to be clarified. Three hypotheses are offered: First, lactoferrins and some active substance produced from neutrophils may synergistically cooperate to inhibit *Candida* growth, since it is known that several intrinsic molecules produced from neutrophils participate in this inhibition [18–21]. This possibility is also supported by the observation that augmentation of anti-*Candida* activity is dependent on long-term interaction of lactoferrin with neutrophils or *Candida* cells during the culture period, especially late in that period (Fig. 5) and by our recent unpublished finding that lysozyme and lactoferrin synergistically induce *Candida* growth inhibition (data not shown). Secondly, anti-*Candida* activity of neutrophils may be augmented by lactoferrins. This is suggested by the finding of Oseas et al. that the adherence of human neutrophils to endothelial cells was promoted by 10 µg/ml of human lactoferrin [12]. Gahr et al. reported more recently that lactoferrin primed neutrophils to produce superoxide; however, a relatively high concentration (> 200 µg/ml) of human lactoferrin was required for the priming [9]. Thirdly, lactoferrin may enhance the interaction between neutrophils and *Candida*, allowing effective growth inhibition of the latter. In fact, it was reported that lactoferrin binds to LPS and aggregates gram-negative bacteria [22]. Further biochemical and morphological examination of the target organelles, when the growth of *Candida* cells is inhibited by the combination of lactoferrin and neutrophils, will provide a clue to details of the mechanisms.

The physiological role of the combined anti-*Candida* activity of lactoferrins and neutrophils in the mucosal defence against *Candida* infection can be speculated. *Candida* is one of the opportunistic fungi which colonize on mucosa and sometimes cause serious infection in immunocompromised hosts. Under physiologically conditions, lactoferrin is contained in secretions such as saliva, tears and nasal secretion, and the accumulation of neutrophils is observed in mucosal lesions caused by *Candida* infection [23]. These indicate that neutrophils and lactoferrin may coexist at the site of an inflammatory lesion with mucosal candidiasis. Therefore, it can be assumed that their combined effects play an important role in mucosal defence against *Candida* invasion. We plan to identify these cooperative effects of neutro-

phils and lactoferrin against mucosal candidiasis in *in vivo* experiments.

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