HOST RESPONSE TO INFECTION

Effect of lactoferrin feeding on the host antifungal response in guinea-pigs infected or immunised with *Trichophyton mentagrophytes*

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Earlier studies revealed that oral administration of lactoferrin (LF), a multi-functional milk protein, facilitated curing of dermatophytosis in guinea-pigs and man by an unknown mechanism. The present study aimed to assess the effect of feeding bovine LF on the host antifungal defence systems in guinea-pigs infected or immunised with *Trichophyton mentagrophytes*, a dermatophytosis-causing fungus. The unbound iron-binding capacity (UIBC) of the plasma of individual animals varied, and plasma with higher UIBC inhibited growth of *T. mentagrophytes* *in vitro*. However, LF administration did not enhance plasma UIBC or the anti-*T. mentagrophytes* activity of plasma in infected or uninfected animals. Phagocytic activity and reactive oxygen (RO) production of blood neutrophil polymorphonuclear leucocytes (PMNLs) were estimated by flow cytometry. LF administration caused no significant effects on phagocytic activity or RO production of neutrophil PMNLs in infected or uninfected animals. The functions of mononuclear cells (MNC) from the spleen were investigated in guinea-pigs immunised with heat-killed *T. mentagrophytes* conidia. The MNC were cultured with concanavalin A or inactivated *T. mentagrophytes*. In the bromo-deoxyuridine incorporation assay, the stimulation index was higher for MNC derived from LF-treated animals than for those from control animals. The culture supernates of MNC enhanced the ability of macrophages to kill *T. mentagrophytes* conidia. Furthermore, stronger augmentation was observed with the culture supernate from LF-treated animals than with that from control animals. In conclusion, LF feeding may potentiate the host antifungal defence systems by modulating MNC function rather than plasma antifungal activity or peripheral blood neutrophil PMNL activity.

Introduction

Lactoferrin (LF) is a transferrin-family iron-binding glycoprotein present in milk and other exocrine secretions as well as in neutrophil polymorphonuclear leucocyte (PMNL) granules. It is thought to play an important role in host defence because it exhibits a variety of biological activities, including antimicrobial activity and immunomodulatory effects, as shown by *in-vitro* studies [1, 2]. It is increasingly appreciated that oral administration of LF or its fragment peptides

improves the survival rate and enhances elimination of pathogens from the body of animals or human patients infected with bacteria, fungi, protozoa or viruses [3–7]. Recent studies showed that feeding bovine LF enhances the elimination of pathogens and the rate of symptomatic cure in guinea-pigs and man with dermatophytosis, a very common fungal infection on the skin [8, 9]. LF exhibits *in-vitro* growth-inhibitory activity against the dermatophytes *Trichophyton mentagrophytes* and *T. rubrum*, causative fungi of dermatophytosis [8]. However, the mechanism by which LF provides protection against dermatophytosis *in vivo* remains unknown.

The host antifungal response against dermatophytosis is considered to involve the following mechanisms. First,

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it has been demonstrated that the anti-dermatophyte activity of serum is due to iron-unsaturated transferrin, which is expressed as serum unbound iron-binding capacity (UIBC) [10, 11]. Likewise, iron-unsaturated LF may have a role in the inhibition of dermatophytes [8]. Second, phagocytes (including neutrophil PMNLs and macrophages) may be involved in the eradication of dermatophytes, because dense infiltration of neutrophil PMNLs is observed in infected areas of the skin of man and animals with dermatophytosis [12, 13], and neutrophil PMNLs and macrophages exhibit killing activity against *Trichophyton* spp. *in vitro* [14]. Third, the development of cell-mediated immunity correlated with delayed hypersensitivity and an inflammatory response is associated with clinical cure, whereas the lack of, or a defective, cell-mediated immunity predisposes the host to chronic or recurrent dermatophyte infection [15, 16]. In response to stimulation by *Trichophyton* antigens, interferon (IFN)- γ , interleukin (IL)-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are produced by peripheral blood MNC obtained from patients with acute dermatophytosis, whereas markedly lower levels of IFN- γ production are found in the case of chronically infected patients [17–20]. These cytokines released from MNC may participate in the activation of phagocytic cells in the infected site.

To evaluate the effect of feeding bovine LF on the host antifungal response, the following activities were tested in guinea-pigs infected or immunised with *T. mentagrophytes*. In infected or uninfected animals, plasma UIBC, plasma anti-*T. mentagrophytes* activity and blood neutrophil activities – phagocytic activity and reactive oxygen (RO) production – were compared in the LF-treatment group and non-treatment group. In immunised or non-immunised animals, the proliferative response and the effector activity of splenic MNC were examined. Culture supernates of MNC were assessed for their capacity to augment the ability of macrophages to kill *T. mentagrophytes* conidia.

Materials and methods

T. mentagrophytes

T. mentagrophytes TIMM2789 was obtained from the culture collection of Teikyo University Institute of Medical Mycology (Tokyo, Japan) and maintained on Sabouraud glucose agar (peptone 1%, glucose 2%, agar 1.5%) slants. A conidial suspension was prepared in physiological saline containing Tween 80 0.05% from cultures grown on modified Sabouraud glucose agar (peptone 0.2%, glucose 0.1%, KH_2PO_4 0.1%, MgSO_4 0.1%, agar 2%) slants at 27°C for 2 weeks. The suspension was filtered through sterilised gauze to remove hyphal fragments and agar debris, and adjusted to a cell concentration of 2×10^7 conidia/ml for inoculation of animals or 10^7 conidia/ml for tests of *T. mentagrophytes*-killing by macrophages or 10^4

conidia/ml for plasma antifungal tests. For use in immunisation, the suspension was resuspended in distilled water at 10^7 conidia/ml and autoclaved at 121°C for 15 min.

Guinea-pigs and oral administration of LF

Female Hartley SPF guinea-pigs (7–9-week-old) (Japan SLC, Shizuoka, Japan) were used for all animal experiments. Animals were given bovine LF (Morinaga Milk Industry Co., Tokyo, Japan) solution (250 mg/ml), orally, twice a day at a daily LF dose of 2.5 g/kg body weight by gavage. The untreated control group received the same volume of water or did not receive any test solution. The animal study protocol was approved by the Morinaga Milk Industry Animal Research Committee, and the guinea-pigs were maintained according to the guidelines for the care and use of laboratory animals of Morinaga Milk Industry.

Dermatophytosis model

Experiments with dermatophytosis on the back (tinea corporis) of guinea-pigs were performed as described previously [8]. Briefly, each animal's back was inoculated with 50 μl of *T. mentagrophytes* conidial suspension. Three weeks after infection, animals were killed and heparinised blood was collected by cardiac puncture. LF was administered from 3 days after infection to 1 day before the sampling day (for 2.5 weeks) or from 7 days before infection to 1 day before the sampling day (for 4 weeks).

Immunisation and isolation of MNC

Equal volumes of the heat-killed *T. mentagrophytes* cell preparation and complete Freund's adjuvant were mixed and emulsified, and 200 μl of the solution were injected subcutaneously into a footpad of each animal. Thereafter, LF was administered for 7 days. At 7 days after immunisation, the spleen was removed, minced in Hanks's Balanced Salts Solution (HBSS), and passed through a Falcon cell strainer (Becton Dickinson Labware, Franklin Lakes, USA). After erythrocytes were removed by treating the cell suspensions with ACK lysing buffer [21], the cell suspensions were layered on Ficoll-Paque Plus (Pharmacia Biotech, Tokyo, Japan) and centrifuged at 550 g for 30 min at room temperature. The MNC layer was collected, washed with HBSS and suspended in RPMI 1640 medium supplemented with 20 mM HEPES, 16 mM NaHCO_3 , penicillin 100 mg/L, kanamycin 100 mg/L and heat-inactivated FCS 5% (complete medium).

*UIBC and anti-*T. mentagrophytes* activity*

Plasma UIBC of animals was estimated by the UIBC-Test Wako (Wako Junyaku Kogyo, Osaka, Japan); 50 μl of filter-sterilised plasma, 20 μl of *T. mentagrophytes* conidial suspension, and 130 μl of RPMI 1640

supplemented with 0.165 M MOPS, a medium recommended by the NCCLS for susceptibility testing of filamentous fungi [22], were added to each well of a 96-well flat-bottomed microtitration plate. The inoculated plates were incubated at 27°C for 7 days. The fungal growth was measured spectrophotometrically at OD₆₃₀.

Phagocytic activity and RO production of neutrophils

To measure the phagocytic activity, 100 µl of heparinised blood and 100 µl of FITC-labelled beads (Polysciences, Warrington, USA) at 5×10^7 /ml were mixed and incubated for 15 min at 37°C. To measure the RO production, heparinised blood was incubated at room temperature for 30 min after mixing with an equal volume of dextran (Nakarai Tesque, Kyoto, Japan) 5% in phosphate-buffered saline (PBS), and the buffy coat was resuspended in PBS. Then 20 µl of the separated blood and 130 µl of 5 µM 2',7'-dichlorofluorescein diacetate (Kodak, Rochester, USA) in PBS were mixed and incubated for 15 min at 37°C; 30 µl of 20 mM EDTA and 20 µl of phorbol myristate acetate (PMA) 25 µg/ml were added to each tube and incubated for 15 min at 37°C. Finally, red blood cells were lysed with Lysing Solution (Becton Dickinson, San Jose, USA). The cells were resuspended in PBS and the fluorescence in the neutrophil PMNLs was analysed by flow cytometry (EPICS, Coulter, Hialeah, USA).

Proliferation of MNC

MNC at 5×10^5 cells/ml were incubated in complete medium at 37°C for 3 days in an humidified atmosphere of CO₂ 5% in air 95% with stimulation by concanavalin A (ConA; Sigma) 10 µg/ml or a 10% suspension of heat-killed *T. mentagrophytes* cells (final concentration 10⁶ conidia/ml). The cells were pulse-labelled with bromo-deoxyuridine (BrdU) solution (Cell Proliferation ELISA System, Amersham Life Science, Tokyo, Japan) during the last 2 h of incubation. Then BrdU incorporated into the cells was quantified with an anti-BrdU monoclonal antibody (MAb) according to the method recommended by the manufacturer. The stimulation index was calculated by dividing the BrdU incorporation of cells with stimulation by the BrdU incorporation of cells without stimulation.

T. mentagrophytes-killing activity of macrophages

MNC at 5×10^6 cells/ml were stimulated by incubation with ConA 10 µg/ml or a 10% suspension of heat-killed *T. mentagrophytes* cells. After incubation for 2 days, the culture supernate was collected. The macrophage killing activity was assayed according to the method of Calderon and Hay [14]. Peritoneal cells containing resident macrophages were collected after

injection of PBS. Various numbers of the cells suspended in complete medium were added to the wells of a 96-well flat-bottomed plate and incubated for 2 h. After removal of non-adhesive cells, *T. mentagrophytes* conidia at an E/T ratio of 0/1 to 25/1 and defined amounts of MNC culture supernate or recombinant rat IFN-γ (Genzyme, Cambridge, USA) were added to the wells and incubated in complete medium at 37°C for 19 h. After vigorous shaking of the plates, 30 µl of the cell suspension from each well were transferred to a petri dish and mixed with 20 ml of Sabouraud glucose agar warmed to 45°C. The agar plates were incubated at 27°C for 2–3 days and the number of fungal colonies formed was counted. The percentage *T. mentagrophytes*-killing was calculated as follows: killing (%) = 100 – (test cfu/control cfu) × 100.

Results

UIBC and antifungal activity of plasma

Normal guinea-pigs were administered water for 2 weeks or LF for 1 or 2 weeks. One day after the administration period, blood plasma was collected for measurement of the UIBC and anti-*T. mentagrophytes* activity (Fig. 1a). The individual plasma samples had UIBC ranging from 0.6 to 1.5 µg/ml. Whereas the plasma showing higher UIBC inhibited growth of *T. mentagrophytes*, the plasma showing lower UIBC promoted the growth compared with medium only. However, no difference of plasma UIBC or the effect on *T. mentagrophytes* growth was observed between control animals and LF-treated animals. Guinea-pigs were infected with *T. mentagrophytes* and administered nothing or LF for 2.5 or 4 weeks. Three weeks after infection, plasma UIBC and anti-*T. mentagrophytes* activity were measured (Fig. 1b). Plasma collected from infected animals exhibited UIBC ranging from 0.8 to 2.0 µg/ml. No obvious difference of plasma UIBC and the effect on *Trichophyton* growth was seen between control animals and LF-treated animals. The plasma from two LF-treated animals promoted rather than inhibited growth of *T. mentagrophytes*, whereas none of the plasma from control animals showed such an effect.

Neutrophil PMNL activities

The phagocytic activity and RO production of neutrophil PMNLs was measured in control and LF-treated animals. The phagocytic activity of neutrophil PMNLs from control animals was lower in infected animals than in uninfected animals (Fig. 2a). LF administration tended to enhance the phagocytic activity in infected animals, but the effect was not significant ($p = 0.128$ for LF2.5w and $p = 0.061$ for LF4w). RO production of neutrophil PMNLs was higher in infected animals than in uninfected animals (Fig. 2b). However, LF

Fig. 1. Plasma UIBC and the effect on growth of *T. mentagrophytes*. Normal guinea-pigs were given water ($n = 6$) or LF ($n = 12$). *T. mentagrophytes*-infected guinea-pigs were given nothing ($n = 6$) or LF ($n = 8$). One day after the administration period, the plasma of each individual guinea-pig was diluted to 25% concentration in RPMI 1640 medium and incubated with *T. mentagrophytes* conidia for 7 days, and the fungal growth was measured spectrophotometrically. UIBC and the effect on *T. mentagrophytes* growth of the plasma from each individual normal animal (a) and infected animal (b) were plotted for the control (○) and LF-treatment (●) groups. △, results obtained with medium alone.

administration did not significantly change the RO production of neutrophil PMNLs.

Proliferation of MNC

Guinea-pigs were immunised with heat-killed *T. mentagrophytes* cells or were non-immunised, and LF was administered for 1 week. One week after immunisation, the proliferative response of splenic MNC to stimulation by ConA or heat-killed *T. mentagrophytes* during a 3-day incubation period was examined. The stimulation index determined by BrdU incorporation is shown in Fig. 3. In both non-immunised and immunised animals, LF administration significantly enhanced the stimulation index of MNC stimulated with ConA or heat-killed *T. mentagrophytes*. The stimulation index of MNC from LF-treated

Fig. 2. Phagocytic activity and RO production of neutrophils. Normal guinea-pigs were given water for 2 weeks or LF for 1 or 2 weeks. *T. mentagrophytes*-infected guinea-pigs were given nothing or LF for 2.5 or 4 weeks. One day after the administration period, phagocytic activity (a) and PMA-stimulated RO production (b) of neutrophil PMNLs were estimated by flow cytometry. Data represent mean and SEM ($n = 5-7$).

animals appeared to be higher in immunised animals than in non-immunised animals.

Effect of MNC culture supernate on *T. mentagrophytes*-killing activity of macrophages

The effector activity of MNC for modulation of the *T. mentagrophytes*-killing activity of macrophages was examined in guinea-pigs that had been immunised or were non-immunised. One week after immunisation of the guinea-pigs, the MNC were isolated and cultured with stimulation by ConA or heat-killed *T. mentagrophytes* cells, and the culture supernate was then collected and added to the *T. mentagrophytes*-killing assay system at 10% concentration. The resident peritoneal macrophages alone killed 26% of *T. mentagrophytes* conidia (Fig. 4). The killing activity

Fig. 3. Proliferative response of MNC. Non-immunised guinea-pigs and guinea-pigs immunised with inactivated *T. mentagrophytes* were given nothing or LF for 7 days. One day after the administration period, splenic MNC were incubated with ConA or inactivated *T. mentagrophytes* for 3 days and BrdU incorporation was assayed in triplicate. Data represent mean and SEM ($n = 3$) of the stimulation index relative to basal BrdU incorporation for the control (□) and LF-treatment (■) groups. * $p < 0.05$ versus control by two-tailed t test.

Fig. 4. Effect of MNC supernate on the *T. mentagrophytes*-killing activity of macrophages. Non-immunised guinea-pigs and guinea-pigs immunised with inactivated *T. mentagrophytes* were given nothing or LF for 7 days. One day after the administration period, splenic MNC were incubated with ConA or inactivated *T. mentagrophytes* for 2 days and the culture supernate was collected. The supernate was added at 10% concentration to naive peritoneal macrophage cultures, and the *T. mentagrophytes*-killing activity of the macrophages (E/T ratio = 5/1) was determined in triplicate. Data represent mean and SEM ($n = 3$) of *T. mentagrophytes*-killing activity for macrophages alone, macrophages with supernates from the control (□) and LF-treatment (■) groups. * $p < 0.05$ versus control by two-tailed t test.

of the macrophages was increased to 60% as a result of the addition of the MNC supernate. Comparison of control and LF-treated animals showed that *T. mentagrophytes*-killing appeared to be higher when macro-

phages were treated with MNC supernate derived from LF-treated animals and the enhancement by LF-treatment was significant in the case of supernate from *T. mentagrophytes*-stimulated MNC of immunised animals.

Supernate was collected from *T. mentagrophytes*-stimulated MNC of immunised and LF-administered animals. The effect of the supernate on the *T. mentagrophytes*-killing of macrophages was observed dose-dependently over the concentration range of 2–20% (Fig. 5). However, the supernate alone without macrophages did not show any killing activity. Similarly, recombinant rat IFN- γ enhanced the *T. mentagrophytes*-killing activity of guinea-pig macrophages at the relatively high concentration of 1 $\mu\text{g/ml}$ (3200 U/ml) (Fig. 6).

Discussion

This study investigated the effects of oral administration of LF on the host antifungal response in guinea-pigs infected or immunised with *T. mentagrophytes*. The plasma UIBC value was correlated with the effect on growth of *T. mentagrophytes*. This indicates that the iron-binding capacity of plasma, which is considered to reflect the transferrin content, plays a role in limiting growth of *T. mentagrophytes*, as demonstrated previously [10, 11]. The UIBC of infected animals seemed to be somewhat higher than that of uninfected animals. The infection or the state of inflammation may have influenced the plasma UIBC value. However, LF

Fig. 5. Dose-response of MNC supernate on the *T. mentagrophytes*-killing activity of macrophages. Guinea-pigs were immunised and given LF, and supernate was collected from MNC isolated from these guinea-pigs and cultured with inactivated *T. mentagrophytes*. Various concentrations of the supernate (0, 2, 5, 10, 20%; left to right columns) were assayed for *T. mentagrophytes*-killing activity without macrophages (E/T = 0/1) or with macrophages (E/T = 5/1 or 25/1) in triplicate. Data represent mean and SEM ($n = 3$). * $p < 0.05$ versus 0% of the supernate by two-tailed t test.

Fig. 6. Effect of recombinant rat IFN- γ on the *T. mentagrophytes*-killing activity of macrophages. Various concentrations of IFN- γ (0, 1, 10, 100, 1000 ng/ml; left to right columns) were assayed for *T. mentagrophytes*-killing activity without macrophages (E/T = 0/1) or with macrophages (E/T = 5/1 or 25/1) in triplicate. Data represent mean and SEM (n = 3). *p < 0.05 versus 0 ng/ml of IFN- γ by two-tailed *t* test.

administration did not enhance UIBC or the anti-*T. mentagrophytes* activity of the plasma in uninfected or infected animals. Indeed, plasma from two LF-treated, infected animals showed the lowest UIBC and promoted growth of *T. mentagrophytes*. This may have been due to accelerated resolution of the infection in the LF-administered animals [8]. In fact, these two animals showed a low fungal burden of the skin (score of 1, data not shown).

LF administration did not significantly alter the blood neutrophil PMNL activities in uninfected or infected animals, although it augmented to some extent the phagocytic activity in infected animals. It is known that LF or its peptide fragment(s) stimulate phagocytic activity [23] and superoxide production [24] of neutrophil PMNLs *in vitro*. Recently, it was reported that intravenous injection of LF peptide upregulates neutrophil PMNL functions, including superoxide generation, in *Candida albicans*-infected mice [25]. Such effects of LF may not be exerted by orally administered LF, because substantial amounts of LF or LF fragments cannot be absorbed from the intestinal lumen, as shown previously [26].

In guinea-pigs immunised with heat-killed *T. mentagrophytes* cells, the study examined the functions of splenic MNC. First, enhancement of the proliferative response of MNC upon stimulation with ConA or inactivated *T. mentagrophytes* was observed as a result of LF administration. Second, an *in-vitro* system was constructed for measuring *T. mentagrophytes*-killing by macrophages and the modulating activity of MNC supernate was tested in this system. When guinea-pigs

were immunised and the MNC collected from these guinea-pigs were stimulated with inactivated *T. mentagrophytes*, the culture supernates of MNC derived from LF-administered animals induced significantly higher *T. mentagrophytes*-killing by macrophages than supernates from animals not given LF. These results indicate that, upon ingestion, LF acts as a second signal that augments the function of MNC when animals have received foreign antigens. Augmentation of cell-mediated immunity by oral administration of LF is suggested by the present results and previous findings. A previous study in guinea-pig models of dermatophytosis found that LF feeding did not prevent inflammation related to cell-mediated immunity but promoted the clinical curing of skin lesions after the development of symptoms [8]. Another group showed that oral administration of LF at the time of immunisation with several antigens increased the DTH responses [27]. Facilitated elimination of fungi by LF administration in the dermatophytosis models [8] may be mediated at least partly by enhanced cell-mediated immunity.

It has been reported that the ability of ConA-stimulated murine splenic cell supernate to induce antimicrobial effects of macrophages is attributable to IFN- γ [28]. Although it is known that guinea-pigs produce IFNs [29], the identification of guinea-pig IFN- γ has not been reported yet. Accordingly, the present study could not identify an active component released from MNC as IFN- γ . Instead, it showed that a relatively high concentration of recombinant rat IFN- γ augmented the *T. mentagrophytes*-killing activity of guinea-pig macrophages. A previous report indicated that IFN- γ production by CD4⁺ splenocytes in mice is increased by oral administration of LF [30]. LF feeding also increased the number of IFN- γ ⁺ cells in the small intestinal mucosa of mice [31]. These findings suggest the possibility that the macrophage-activating component released from the guinea-pig MNC is an IFN- γ -like cytokine.

This study could not detect any obvious activation of peripheral blood neutrophils by LF feeding. Phagocytic cells, including neutrophil PMNLs and macrophages, may be activated at the infected locus with the help of MNC. Further investigations are under way to clarify the immunological and molecular events induced by the oral administration of LF.

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