Oral Lactoferrin Prevents Body Weight Loss and Increases Cytokine Responses during Herpes Simplex Virus Type 1 Infection of Mice

Hiroyuki WAKABAYASHI, Masahiko KUROKAWA, Kouichirou SHIN, Susumu TERAGUCHI, Yoshitaka TAMURA, and Kimiyasu SHIRAKI

Nutritional Science Laboratory, Morinaga Milk Industry Co., Ltd., Zama, Kanagawa 228-8583, Japan

Department of Virology, Toyama Medical and Pharmaceutical University, Toyama, Toyama 930-0194, Japan

Received August 8, 2003; Accepted December 12, 2003

Lactoferrin (LF), a multifunctional milk protein, is known to inhibit in vitro infection by viruses such as herpes simplex virus type 1 (HSV-1). We evaluated the influence of LF feeding on the HSV-1 cutaneous infection of mice. Bovine LF was administered to mice and, after 10 d, the mice were infected with HSV-1. LF administration did not affect the viral clearance in the skin, but inhibited the appearance of skin lesions. LF prevented body weight loss and the decrease of splenocyte number associated with HSV-1 infection. LF increased the serum interleukin (IL)-18 level and splenocyte production of interferon-γ and IL-12. These results suggest that LF feeding was not effective for eradication of the virus, but may contribute to the maintenance of homeostasis and the concomitant increases of cytokine responses during HSV-1 infection.

Key words: lactoferrin; body weight; herpes simplex virus type 1 (HSV-1); cytokine; homeostasis

Lactoferrin (LF) is a transferrin-family iron-binding glycoprotein present in milk and other exocrine secretions as well as in neutrophil granules. It is thought to play an important role as an innate-defense protein because its activities include antimicrobial effects and immunomodulatory effects, as shown by studies in vitro and in human LF-transgenic mice. LF displays in vitro antiviral activity against both DNA- and RNA-viruses, including rotavirus, respiratory syncytial virus, herpes viruses, human immunodeficiency virus, and hepatitis C virus, by preventing entry of the virus into the host cell.

It is increasingly reported that oral administration of LF or its fragment peptides exerts host-protective effects against infections, cancers, and inflammation in adults as well as in infants. Feeding of LF or its peptides improves resolution of the symptoms or the survival rate, and reduces pathogens in the body in animals or humans infected with bacteria, fungi, protozoa, and viruses. Immunomodulatory effects of LF rather than its direct antimicrobial activity may mediate these effects, because neither LF nor its fragment peptides could be detected in the blood of healthy adult rats after feeding. Some immunostimulatory features of LF feeding have been reported based on studies with normal, immunized, or tumor-bearing mice.

Herpes simplex virus type 1 (HSV-1) causes a number of human diseases, including cold sores, eye and genital infections, and encephalitis. LF inhibits the in vitro infection of cells by HSV-1 and acts at the stage of viral adsorption to the cells. Peptide fragments of bovine LF and lactoferricin B (LFcin B), an antimicrobial peptide derived from bovine LF, also inhibit in vitro infection with HSV-1, although their activity is weaker than that of the native LF. LF and LFcin B in combination with acyclovir synergistically inhibit in vitro infection with HSV-1. Topical administration of bovine LF prior to HSV-1 inoculation suppresses the corneal infection of mice. However, the effect of orally administered LF on HSV-1 infection in animals or humans has not been reported.

In this report, we evaluated the influence of oral administration of LF on the disease course and cytokine/chemokine responses in mice infected cutaneously with HSV-1 to explore the immunomodulatory effects of LF feeding.

Materials and Methods

Virus, cells, and compounds. Virus stock of the HSV-1 HF strain was prepared from infected Vero cells as reported previously. Vero cells were grown or maintained in Eagle’s minimum essential medium supplemented with 5 and 2% heat-inactivated fetal calf serum (FCS), respectively. Bovine LF (8.2% iron saturation) was produced from whey of cow’s milk by Morinaga Milk Industry.
Oral administration of LF and infection of mice. SPF female BALB/c CrSlc mice were obtained from Japan SLC and were used for animal experiments at 6 or 7 weeks of age. Mice were fed a commercial standard diet CE-2 (CLEA Japan). Water or a 1.5% solution of LF was administered orally ad libitum using an aseptic nursing AN pack and sterile SE nozzle (Musashi) until the end of the experiment. After 10 d of administration of the test solution, mice were infected cutaneously with HSV-1 at $10^6$ plaque forming unit (PFU)/mouse after scarification of the shaved right midflank with 27-gauge needles as described previously. The body weight and appearance of skin lesions were monitored through the examination period. The animal experimentation guidelines of Toyama Medical and Pharmaceutical University were followed in the animal studies.

Measurement of virus yields in the skin. After anaesthesia with chloroform, the skin in the area (2 × 2 cm) encompassing the inoculation site of the infected mice was removed and homogenized in 2 ml of phosphate buffered saline. The homogenate was centrifuged at 1000 g for 15 min and the virus yield in the supernatant was measured by a plaque assay on Vero cells.

Culture of splenocytes. The spleen was removed from mice, minced in Hanks’ balanced salt solution (HBSS), and passed through a Falcon cell strainer (Becton Dickinson Labware). After erythrocytes were removed by treating the cell suspensions with ACK lysing buffer, the splenocytes were washed with HBSS and suspended in RPMI1640 medium supplemented with 20 mm HEPES, 16 mm NaHCO3, 100 μg/ml penicillin, 100 μg/ml kanamycin and 10% heat-inactivated FCS. The number of splenocytes was found by the trypan blue exclusion test. Splenocytes at $5 \times 10^6$ cells/ml were incubated in the presence of UV-inactivated HSV-1 at $10^6$ PFU/ml at 37 °C under a humidified atmosphere of 5% CO2 and 95% air. After 2 d of incubation, the cell supernatant was collected for analysis as described below.

ELISA for cytokines and chemokines. Tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-10, IL-12(p70), IL-18, and MCP-1 were measured using Maxisorp immunoplates (Nunc) and OptEIA sets (BD Pharmingen) according to the manufacturer’s recommendations. The peroxidase activity in the ELISA was detected using an OPD peroxidase substrate tablet set (Sigma). MIP-1α, RANTES, and MIP-2 were measured using AN’ALYSA immunoassay systems (Genzyme Techne) according to the manufacturer’s instructions.

Statistical analysis. Data are expressed as mean±SE. The appearance of skin lesions and correlations were analyzed by the $\chi^2$ test and the Pearson correlation test, respectively, using the computer program StatView 5.0 (SAS Institute). Otherwise, statistical analysis was done by Student’s t test or ANOVA and Fisher’s PLSD post hoc test.

Results

Disease course of HSV-1 cutaneous infection. Mice were cutaneously infected with HSV-1. Oral administration of water or 1.5% LF in drinking water was started 10 d before the infection and continued until the end of the animal experiment. The amount of consumed drinking water (ml/mouse/d) was 4.9 ± 0.5 (d -10 to 0) and 3.4 ± 0.3 (d 0 to 10) in the water-administered control group, and 5.2 ± 0.5 (d -10 to 0) and 3.9 ± 0.4 (d 0 to 10) in the LF-administered group. Virus yields in the skin of HSV-1-infected animals were gradually decreased from d 2 until d 9 and no virus was detected at d 9 (Table 1). Virus yields were not significantly different in the control and LF groups. Virus was not detected in the brain throughout the experimental period. HSV-1 infection caused mild, transient lesions in the skin in 4 out of 15 water-administered control animals, while no lesions appeared in the LF group (Table 1).

Infection with HSV-1 impaired the body weight gain (Fig. 1A). LF administration prevented the body weight loss caused by HSV-1 infection at d 2 and 5. Infection with HSV-1 caused a decrease of the splenocyte number, especially immediately after the infection (d 2) (Fig. 1B). LF administration prevented the loss of splenocytes in the infected animals.

Responses of cytokines and chemokines. IFN-γ, IL-18, MCP-1, MIP-1α, RANTES, and MIP-2 levels in the serum of HSV-1 infected animals were measured by ELISA and are shown in Fig. 2 (except for the MCP-1 level, which was beneath the detection limit). All the cytokines and chemokines increased in response to HSV-1 infection, although the increase of RANTES was only moderate. In the infected animals, LF administration significantly increased the IL-18 level in the serum at d 9. The level of MIP-1α was somewhat higher in the LF group at d 5 and 9 and the level of MIP-2 was somewhat lower in the LF group at d 5, but these differences were not significant.

| Table 1. Virus Titer and Appearance of Lesions in the Skin of HSV-1 Infected Mice |
|-----------------|-----------------|-----------------|
| **Group** | **Virus titer (×10^6 PFU/skin)** | **Lesion-positive/total mice** |
| | **Day 2** | **Day 5** | **Day 9** |
| Water | 245 ± 108 | 12 ± 11 | <1 |
| LF | 348 ± 49 | 6 ± 6 | <1 |
| *** | 4/15 | 0/15 *** |

* The data are representative of two independent experiments with similar results.
** Virus titer was measured in 5 mice in each group.
*** P < 0.05 comparing the two groups.
The production of TNF-α, IFN-γ, IL-10, IL-12(p70), and IL-18 by inactivated HSV-1-stimulated splenocytes derived from HSV-1 infected animals was also measured (Fig. 3). All of the cytokines tested increased in response to HSV-1 infection. While the production of IFN-γ, IL-10, and IL-12 gradually increased and peaked at d 9, the production of IL-18 was highest at d 2. Increases of TNF-α, IL-12(p70), and IL-18 by HSV-1 infection was also observed in unstimulated splenocytes. In the infected animals, LF significantly increased the production of IFN-γ and IL-12 at d 5. The production of IFN-γ tended to be increased by LF administration in the infected mice throughout the experimental period (d 2 to 9), and a similar increase by LF was observed in splenocytes stimulated with concanavalin A or gB497-507 peptide, an epitope of an envelope glycoprotein of HSV-1 (data not shown). The level of production of TNF-α, IL-10, and IL-18 were not different between the control and LF groups.

LF administration significantly altered several parameters, including body weight, splenocyte number, and cytokines. We analysed possible correlations between these values by the Pearson correlation test. Figure 4 shows the parameters exhibiting significant correlations. The body weight change was significantly correlated with the splenocyte number at d 2 (r = 0.711, P = 0.0187) and d 5 (r = 0.657, P = 0.0371). The splenocyte production of IFN-γ and of IL-12 at d 5 was significantly correlated with the body weight change (r = 0.822, P = 0.0021) and splenocyte number (r = 0.729, P = 0.0142), respectively.

Discussion

In the mouse model of HSV-1 infection, viral clearance was not affected by oral administration of bovine LF, despite the in vitro anti-HSV-1 activity of LF. One possible reason for the failure of LF feeding to affect the viral clearance in vivo may be the very low level, if any, of LF absorbed in the body. Neither LF nor its fragment peptides could be detected in the blood of adult rats after feeding. A direct interaction of topically applied LF with the cornea decreased the virus titer in vivo. Another reason for the lack of effect of LF on viral clearance in our system may have been a faster and spontaneous clearance of the pathogen in this model of infection in contrast to a long-lived fungal infection model or patients with chronic hepatitis C virus infection, in which oral LF reduced the number of pathogens in the body.

On the other hand, beneficial effects of LF were observed in terms of the appearance of skin lesions, body weight, and splenocyte number in the infected mice. We also evaluated the effects of LF feeding on the responses of cytokines and chemokines in HSV-1 infected mice. In these tests, an increase of serum IL-18 and of the production of IFN-γ and IL-12 by splenocytes was observed as a result of LF administration. IFN-γ plays a pivotal role in the clearance of virus and in recovery in primary cutaneous infection with HSV-1. IL-12 protects hosts from HSV-1 infection by inducing IFN-γ production. IL-18 alone or in combination with IL-12 shows protective effects in mice infected with HSV-1 or HSV-2. However, in our experiments, increases of the levels of these cytokines by LF feeding did not result in accelerated clearance of HSV-1. Instead, increased IFN-γ and IL-12 production were correlated with body weight gain and maintenance of the splenocyte number, respectively.
Recently, Humphrey et al.\textsuperscript{36}) reported that chicks fed a diet containing human LF and lysozyme showed better feed efficiency (body weight gain/feed consumed) than chicks fed the control diet. They suggested that this effect of the proteins might be mediated by inhibition of the production of proinflammatory cytokines, because proinflammatory cytokines IL-1, IL-6 and TNF-\(\alpha\) cause decreases of the growth rate and feed efficiency during infections.\textsuperscript{37}) LF feeding did not affect the production of the proinflammatory cytokine TNF-\(\alpha\) by splenocytes in the HSV-1 infected mice.

One possible reason for the splenocyte loss observed
here is an emigration of splenocytes due to attraction by chemokines that were triggered by HSV-1 infection. LF administration prevented the splenocyte loss in HSV-1 infection and this may be related to the inhibition of the appearance of skin lesions caused by leukocyte infiltration. MIP-2, whose level was relatively low in LF-administered mice, may be involved in the inhibition of the splenocyte loss by LF. Alternatively, since murine gammaherpesvirus-68 infection in IFN-γ receptor knockout mice has been reported to result in the loss of...
of splenocytes, IFN-γ may play an important role in the maintenance of normal splenic architecture during herpes virus infection. The prevention of splenocyte loss by LF administration may be related to the increased production of IFN-γ induced by IL-12.

Here we showed that orally administered LF increases the splenocyte production of Th1 type cytokines (IFN-γ and IL-12) and also the serum level of IL-18 in response to HSV-1 infection. These results are in accord with previous findings that LF feeding increases the splenocyte production of IFN-γ in normal mice, the levels of IFN-γ and IL-12 in the peritoneal fluid of mice injected ip with inactivated Candida albicans, and the levels of IFN-γ and IL-18 in the intestinal mucosa of mice. These findings suggest that Th1 type cytokines may be key players in the host-protective effect of orally administered LF. A preliminary human trial of oral LF is now being conducted in recurrent genital herpes patients, and will further our understanding of the clinical effectiveness of oral LF in herpes virus infections.

Fig. 4. Correlations between Parameters That Were Significantly Affected by LF Administration in HSV-1-Infected Mice.

Parameters with significant correlation in mice administered water (n = 5, open circles) or LF (n = 5, closed circles) are shown. Values of body weight change and splenocyte number at d 2 (A) and 5 (B), body weight change and IFN-γ production of the stimulated splenocytes at d 5 (C), and splenocyte number and IL-12(p70) production of the stimulated splenocytes at d 5 (D) were significantly correlated.

References

33) Vollstedt, S., Franchini, M., Alber, G., Ackermann, M., and Suter, M., Interleukin-12- and gamma interferon-dependent innate immunity are essential and sufficient


35) Harandi, A. M., Svennerholm, B., Holmgren, J., and Eriksson, K., Interleukin-12 (IL-12) and IL-18 are important in innate defense against genital herpes simplex virus type 2 infection in mice but are not required for the development of acquired gamma interferon-mediated protective immunity. J. Virol., 75, 6705–6709 (2001).

