Antiviral Research 93 (2012) 340–345

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Lactoferricin but not lactoferrin inhibit herpes simplex virus type 2 infection in mice

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ARTICLE INFO

Article history:
Received 17 August 2011
Revised 4 January 2012
Accepted 9 January 2012
Available online 16 January 2012

Keywords:
HSV-2
Lactoferrin
Lactoferricin
Antiviral activity
Mouse

ABSTRACT

We have evaluated the potential of bovine lactoferrin and lactoferricin for their ability to prevent and/or treat genital HSV-2 infection in mice. We confirm previous data showing that both lactoferrin and lactoferricin have antiviral properties *in vitro* and can inhibit HSV-2 infection of GMK cells in a dose-dependent manner. When tested *in vivo*, lactoferricin but not lactoferrin was also a potent inhibitor of HSV-2 infection. When admixed with virus prior to inoculation, lactoferricin inhibited disease development and significantly reduced the viral load in a genital model of HSV-2 infection in mice. Lactoferrin and lactoferricin were also tested for their ability to stimulate the production of chemokines. Neither of the compounds induced the production of CCL3, CCL5, CXCL1 or CXCL2 by mouse splenocytes *in vitro*. However, when tested *in vivo*, both lactoferrin and lactoferricin were able to induce local vaginal production of CCL5. Lactoferrin also induced CXCL2 production. The prophylactic and/or therapeutic effects of lactoferrin or lactoferricin were also tested. But none of the compounds were efficient in blocking HSV-2 infection when given 24 h prior to HSV-2 infection. Lactoferricin however showed promising results as a therapeutic agent and delayed both disease onset by 3 days as well as reducing the viral load almost 15-fold when given as a single dose 24 h post-infection. These data show that lactoferricin can block genital herpes infection in mice, and perhaps also be used for post-infection treatment.

1. Introduction

Herpes simplex virus type 2 (HSV-2) is a sexually transmitted infection and one of the most common causes of genital ulcerative diseases worldwide (Halioua and Malkin, 1999). The infection is common in Sweden, with approximately 25% of the adult population being infected. The outcome of HSV-2 infection varies, with some individuals developing severe and recurrent episodes of genital herpes while others remain asymptomatic. The virus can, preferentially in neonates and immunosuppressed individuals, give rise to meningitis. During primary infection, HSV-2 replicates in the cervicovaginal epithelium. Thereafter, it enters axons of sensory neurons and dorsal root ganglia where it persists in neurons until reactivation. Virus is shed continuously in the genital tract irrespective of disease status, i.e. both during recurrent disease and during silent asymptomatic infection.

Standard treatment for HSV-2 is with acyclovir or penciclovir, or with their prodrugs valacyclovir or famciclovir, respectively. Both drugs have a similar mode of action: they inhibit the viral DNA-polymerase, and thus inhibit viral replication. Even though HSV-2 replication can be effectively blocked by acyclovir, these drugs do not eradicate the infection. Furthermore, development of drug resistant HSV strains is an escalating problem, especially in immunocompromised patients (Bacon et al., 2003; Duan et al., 2008; Frobert et al., 2008; Reyes et al., 2003). Many attempts have therefore been made to develop vaccines against HSV-2. Even though several vaccine candidates were highly efficient in animal models, they were ineffective against HSV-2 infections in humans (Kemble and Spaete, 2007).

Antimicrobial proteins are part of the first line of innate immune defense against infections and constitute a wide array of soluble proteins that are found in secretory fluids. Lactoferrin is one of the most abundant secretion proteins in man and is found in external secretions, especially milk, tears and saliva, but is also present in, e.g. seminal plasma and vaginal mucus (Caccavo et al., 2002). In the latter case, the levels of lactoferrin are related to the estrous hormone cycle. Lactoferrin is an iron-binding protein, but it has also strong antimicrobial properties and is efficient against bacterial, fungal and viral pathogens (Jenssen and Hancock, 2009). The antiviral activity occurs against many different viruses, but always involves interference with early events of the viral infectious cycle, either by a direct interaction with the viral particle or by inhibiting viral attachment/entry (Jenssen, 2005). Lactoferricin is a peptide generated by pepsin cleavage from lactoferrin (Tomita et al., 1991). The peptide shares many characteristics of lactoferrin, including the ability to interfere with several different viral infections (Jenssen, 2005).

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doi:10.1016/j.antiviral.2012.01.003

Both lactoferrin and lactoferricin show promise as anti-HSV-2 agents, at least *in vitro*. Bovine lactoferrin as well as both bovine and human lactoferricin inhibit *in vitro* replication of HSV-1 and HSV-2 (Andersen et al., 2003, 2004; Marchetti et al., 1998). Both compounds exert their anti-HSV effect by binding to heparan sulfate, and inhibit viral attachment and entry into the host cell (Andersen et al., 2004; Jenssen et al., 2004). However, lactoferricin but not lactoferrin is also able to interfere with HSV after the internalization stage, indicating a secondary antiviral effect (Andersen et al., 2004). However, the *in vivo* applicability of lactoferrin and lactoferricin has not yet been explored.

For that reason, we aimed at investigating the ability of lactoferrin and lactoferricin to block HSV-2 infection *in vivo* using a mouse model of genital HSV-2 infection. Both lactoferrin and lactoferricin were able to induce chemokine production *in vivo* in the genital tract implying that these proteins/peptides have immunomodulatory functions *in vivo*. However, only lactoferricin showed significant antiviral activity *in vivo*, and was even able to interfere with disease progression and viral load when given as a post-infection treatment. These findings were surprising as both lactoferrin and lactoferricin have demonstrated robust antiviral activity *in vitro*. Regardless, the data show that lactoferricin is a promising candidate for anti-HSV-2 treatments.

2. Material and methods

2.1. Antimicrobial reagents

Bovine lactoferrin was purchased from Sigma–Aldrich (Cat# L9507) and bovine lactoferricin from the Center for Food Technology (Hamilton, Queensland, Australia).

2.2. Virus preparation

HSV-2 strain 333 (Seth et al., 1974) was grown in African green monkey kidney cell (GMK-AH1) monolayers in Iscove's complete medium (Iscove's modified Dulbecco's medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% AB serum, 1% L-glutamine, 1% gentamicin sulfate and 1% mercaptoethanol). At 2–3 days post-infection the cells and supernatants were harvested and the virus retrieved by one cycle of freeze-thawing followed by centrifugation to remove cellular debris. Virus titers were determined by a plaque assay on GMK-AH1 cells monolayer.

2.3. Plaque reduction assay

GMK cells were cultured in 24-well plates until they reached 70–80% confluence. Synthetic and natural antimicrobials in different concentrations (0.1–1000 μg/ml) were admixed with HSV-2 and the mixture was then added to the cell monolayers. The infected cells were incubated for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2 whereafter 2% methylcellulose medium was added to block spreading of virus through the cell culture medium. Three days later plates were washed and stained with crystal violet for enumeration of plaques.

2.4. Mice

Female 6–10 weeks old C57/BL6 mice obtained from ScanBur, Sweden, were used for all experiments. The mice were kept under pathogen-free conditions in the animal facility at the Department of Rheumatology and Inflammation Research at the University of Gothenburg. The studies were approved by the ethical committee for animal experiments at the University of Gothenburg.

2.5. Genital HSV-2 infection

Mice were injected subcutaneously with 2 mg of Depo-Provera (Pharmacia) per mouse in 200 μl of PBS. Five days later the mice were anesthetized and inoculated with 20 μl of virus solution, containing 4×10^4 PFU ($LD_{50} = 4 \times 10^2$ PFU) of HSV-2 in 0.9% NaCl (McDermott et al., 1984; Parr et al., 1994). Lactoferrin and lactoferricin were diluted in 0.9% NaCl and were administered either together with the virus, or separately at different time-points prior to or after viral inoculation. Control mice were treated with 0.9% NaCl alone. When given simultaneously with HSV-2, virus and antimicrobials (200 μg) were mixed (total volume 40 μl) instantly before inoculation. When given prophylactically or therapeutically, the antimicrobials were given intravaginally (200 μg) in 40 μl. HSV-2-infected mice were examined daily for vaginal inflammation, neurological illness, and death. The severity of disease was graded from 0 to 5, i.e. healthy (0), genital erythema (1), moderate genital inflammation (2), genital lesion and/or generally bad condition (3), hind-limb paralysis (4), death or sacrifice due to paralysis (5) (Morrison et al., 1998). The mice were sacrificed at day 9 post-infection and their spinal cords were collected and frozen in 1 ml PBS.

2.6. Viral load

Viral load was analyzed in spinal cord at day 9 post-infection by quantitative PCR. For this analysis, the spinal cords were homogenized and DNA was extracted in a Magnapure LC robot (Roche Diagnostics, Mannheim, Germany) using the Magnapure DNA Isolation Kit according to the Manufacturer's instructions. Quantitative PCR was performed as earlier described (Namvar et al., 2005) and these analyses were performed by the clinical routine laboratory in Virology Department of Sahlgrenska University Hospital.

2.7. Chemokine induction in vitro

Spleens from naïve mice were passed through a 70-μm nylon mesh (BD Bioscience, Erembodegem, Belgium) into a Petri dish containing 10 ml phosphate-buffered saline (PBS). Cell suspension was centrifuged; the pellet resuspended in NH_4Cl solution (0.83%, pH 7.29) and kept on ice for 7 min to lyse erythrocytes, followed by two washing steps in cold PBS.

Cells were plated at a concentration of 1×10^5/ml in a volume of 100 μl per well in flat-bottom 96-well plates (Nunc, Denmark) in Iscove's complete medium in the presence or absence of 100 μg/ml of each antimicrobial. Cells were incubated for 48 h in a humidified atmosphere containing 5% CO_2 at 37 °C. Supernatants were further analyzed for content of CCL3 (MIP-1α), CCL5 (Rantes), CXCL1 (KC) and CXCL2 (MIP-2) using commercial ELISA kits from R&D Systems (Minneapolis, MN).

2.8. Chemokine induction in vivo

Antimicrobials (200 μg/mouse in 40 μl) or vehicle alone (0.9% NaCl) were inoculated intravaginally in progesterone-treated mice. The animals were sacrificed 8 and 24 h after inoculation and the vaginas were excised and weighed before storage at −70 °C in 300 μl of a PBS solution containing 2 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml of trypsin inhibitor from soybean (Sigma) and 0.05 M EDTA (Harandi et al., 2001). Vaginal samples were thawed and then permeabilized with saponin (Sigma) at a final concentration of 2% (w/v) in PBS at 4 °C overnight. Tissue samples were then centrifuged at 16,000*g* for 5 min, and supernatants were analyzed for chemokine content as described above.

2.9. Statistical analysis

Statistical differences between groups were verified with one-way ANOVA using a confidence interval of 95%, together with Bonferroni post-test. Weight loss differences at different time points were analyzed with two-way ANOVA using a confidence interval of 95%, together with Bonferroni post-test or Dunnett's Multiple Comparison Test (PRISM 5.0® GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Both lactoferrin and lactoferricin block HSV-2 infection in vitro

Lactoferrin and lactoferricin were tested for their ability to block HSV infection of GMK cells *in vitro*. As previously shown (Andersen et al., 2004; Jenssen et al., 2008) both lactoferrin and lactoferricin reduced the number of plaques by up to 60% and 100% respectively at the highest dose tested (1000 μg/ml) (Fig. 1). Lactoferricin maintained its antimicrobial activity (up to 60% plaque reduction) at the dose tested of 100 μg/ml.

3.2. In vivo antiviral activity of lactoferrin and lactoferricin

To study the *in vivo* effects of lactoferrin and lactoferricin we used a well-known mouse model of genital HSV-2 infection (Parr et al., 1994). Female mice were inoculated intravaginally with HSV-2 alone or with a mixture of HSV-2 and lactoferrin or lactoferricin. In this experimental setting, lactoferricin but not lactoferrin demonstrated a strong antiviral activity (Fig. 2). Mice treated with lactoferricin did not develop any signs of the disease (Fig. 2A) nor did they loose weight (Fig. 2B). The viral load in the spinal cords from lactoferricin-treated mice was more than 4000-fold (3 log 10) lower than in the untreated control group (Fig. 2C). Lactoferrin, however, was not effective in blocking HSV-2 infection (Fig. 2A–C).

3.3. Induction of chemokine production by lactoferrin and lactoferricin

We investigated the ability of lactoferrin and lactoferricin to induce the production of different chemokines. Neither lactoferrin nor lactoferricin induced any significant chemokine responses in spleen cell suspensions *in vitro* (not shown). However, when administered intravaginally, both lactoferrin and lactoferricin were able to induce a CCL5 response in the genital tract at 8 h post-administration (Fig. 3B). Lactoferricin also induced CXCL2 (Fig. 3D). These responses had all vanished by 24 h (data not shown). Neither lactoferrin nor lactoferricin induced any CCL3 or CXCL1 (Fig. 3).

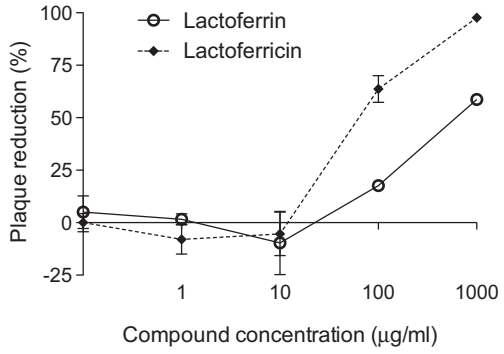

Fig. 1. Dose-dependent inhibition of HSV-2 infection *in vitro* using lactoferrin and lactoferricin. GMK cells were infected with HSV-2 in the presence or absence of different doses of lactoferrin or lactoferricin. Numbers of plaques were determined 3 days later. Data correspond to the relative change (in percentage) of the number of plaques in cultures of HSV-2 and lactoferrin or lactoferricin, compared to the number of plaques in cultures infected with HSV-2 alone.

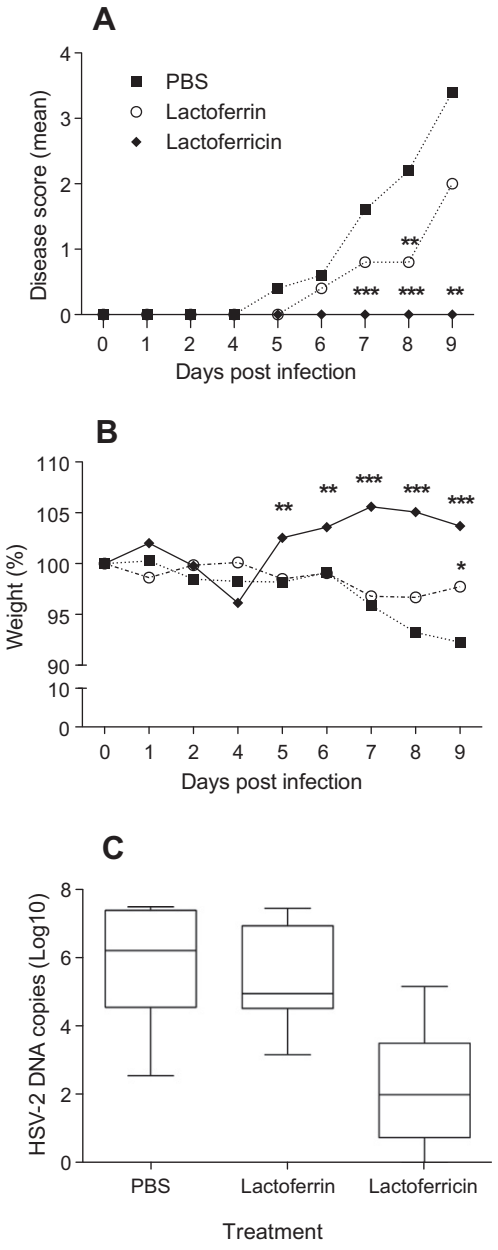

Fig. 2. Antiviral effect of the natural antimicrobials on HSV-2 infected mice. C57/BL6 mice were inoculated with HSV-2 alone or HSV-2 together with lactoferrin or lactoferricin, and disease development was followed daily over 9 days. In addition, viral load in the central nervous system was determined on days 2 and 9 after infection. (A) Disease development was scored between 0 and 5 as described earlier (Morrison et al., 1998) and is expressed as means. (B) Weight loss was determined daily and is expressed in percentage of weight loss comparing to day 0 of infection. (C) Viral load in the spinal cord, given as the number of HSV-2 DNA copies, were determined by quantitative PCR and is expressed as medians and the 25% and 75% percentile (the boxes) with the minimum and maximum responses for $n = 10$. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ using ANOVA with Bonferroni's post-test.

3.4. Lactoferricin show promise as a therapeutic treatment

Given the ability of lactoferricin to block HSV-2 infection as well as to induce chemokine responses *in vivo*, we wanted to assess if

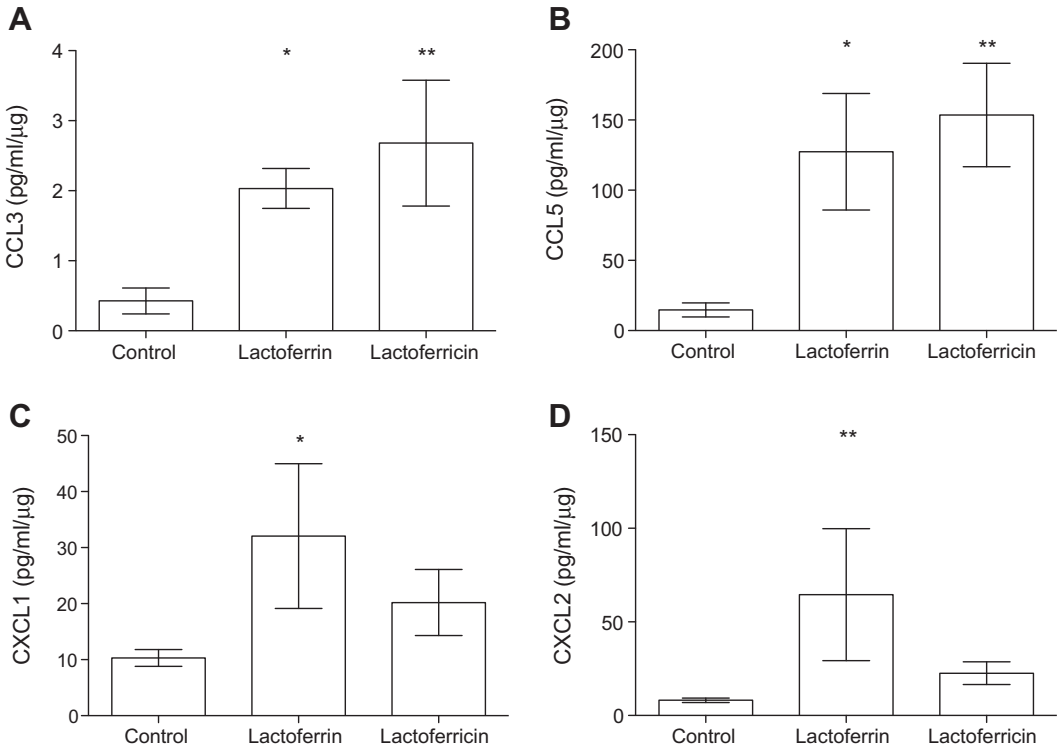

Fig. 3. Induction of chemokines by lactoferrin and lactoferricin in vivo. Hormone-treated naïve C57/BL6 mice were inoculated with lactoferrin or lactoferricin (200 µg/mouse) or left untreated as a control. Mice were sacrificed after 8 h. Levels of CCL3 (A), CCL 5 (B), CXCL1 (C), and CXCL2 (D) in the vaginal tissues were determined by ELISA. Data are expressed as mean values plus SEM ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ using ANOVA with Dunnett's Multiple Comparison Test.

lactoferricin could be used as prophylactic agent or as therapeutic treatment of genital HSV-2 infection. We therefore treated the mice intravaginally with lactoferricin either 24 h before or 24 h after HSV-2 inoculation. Lactoferrin was included as a control. We did not find any significant difference in viral load or disease scores in either the pre-treatment (Fig. 4A and C) or the post-treatment (Fig. 4B and D) groups when compared to PBS-treated animals. However, there was a strong trend that lactoferricin given 24 h post-infection reduced both the onset and the severity of the disease. Only 40% of animals treated with lactoferricin showed clinical signs of disease, and the disease onset was delayed by at least 2 days (Fig. 4B). This was associated with a 15-fold reduction in viral DNA content in the spinal cord (Fig. 4D).

4. Discussion

We have shown that lactoferricin but not lactoferrin could block HSV-2 infection *in vivo*, despite the fact that both substances could block infection *in vitro*. Lactoferricin completely prevented disease development when co-administered with HSV-2, and delayed both disease onset and disease severity when given 24 h post-viral inoculation. In addition, both lactoferrin and lactoferricin were immunomodulatory and induced the production of chemokines when deposited on the genital mucosa.

There are numerous studies showing that lactoferrin protects not only against HSV-2, but also against a panel of other viruses (van der Strate et al., 2001). Most of these studies have been carried out in tissue culture systems, but there are a few examples where lactoferrin has demonstrated a potent antiviral effect *in vivo*. For example, lactoferrin given intraperitoneally was proven to protect mice against enterovirus and murine cytomegalovirus (Shimizu et al., 1996; Weng et al., 2005). When deposited in the conjunctival sac it could protect mice against ocular HSV-1 infection (Fujihara and Hayashi, 1995). Consequently, the lack of effect in our genital *in vivo* HSV-2 infection model (despite a strong anti-HSV-2 activity *in vitro*) could simply be a dosage problem; high doses of lactoferrin (5–10 mg/mouse) were used to protect against enterovirus and cytomegalovirus, whereas, because of the limited volume that could be given intravaginally, we were able to administer only 200 µg. Alternatively, lactoferrin might be less efficient as a virucide when deposited at a mucosal surface compared to when it was given intraperitoneally or in the sterile environment of the eye. The latter is underscored by the lack of anti-HSV effects when lactoferrin was given orally to mice prior to infection. Even though the lactoferrin treatment had beneficial effects on body weight and Th1 cytokine responses, it did not affect susceptibility to or clearance of the virus (Wakabayashi et al., 2004). Another possibility is of course that lactoferrin is not effective against genital HSV-2 infections.

That lactoferricin but not the intact lactoferrin demonstrated antiviral activity *in vivo* could be related to their different modes of action. Even though both lactoferrin and lactoferricin can block viral entry *in vitro* (Andersen et al., 2004) and appears to co-localize with microtubules once internalized thus delaying trafficking of HSV particles to the nucleus (Marr et al., 2009), only lactoferricin appears to have a third antiviral mechanism (Andersen et al., 2004): lactoferricin but not lactoferrin maintains its antiviral activity even after it has disappeared from the cell surface (Andersen et al., 2004).

Lactoferrin as well as lactoferricin were previously shown to have heparan sulfate receptor tropism with high affinity (Jenssen et al., 2004), however this binding capacity cannot alone be used to predict antiviral peptide/protein activity, at least not *in vitro* (Jenssen et al., 2004). Nevertheless, we cannot rule out the possibility that the heparan sulfate binding capacity of lactoferrin and lactoferricin also is of importance in the antiviral defense, as it may serve as a docking site on the cell surface, thus aiding in the cellular uptake of both lactoferrin and lactoferricin.

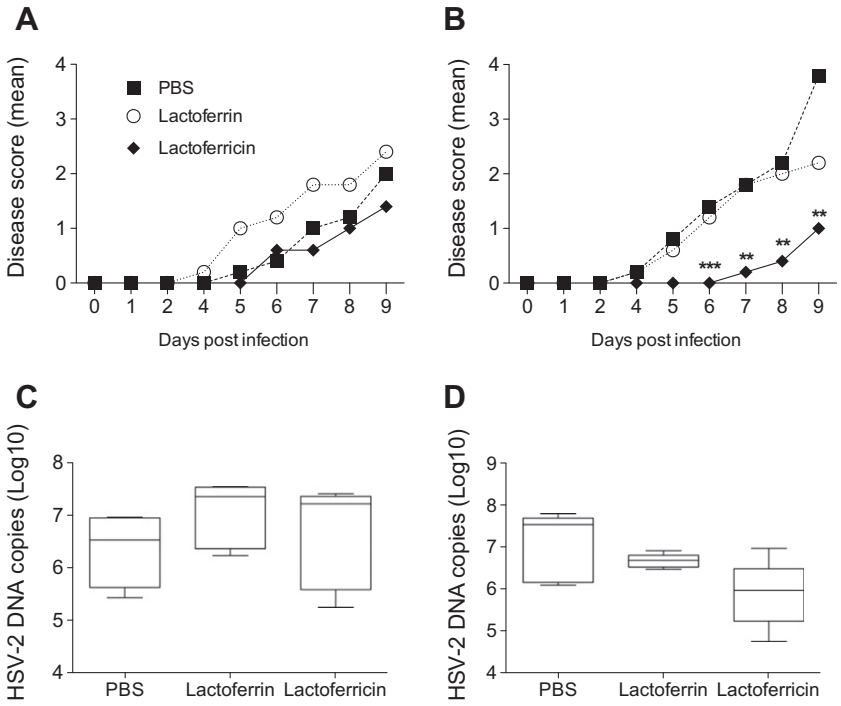

Fig. 4. Lactoferricin prevents disease development when given prophylactic. C57/BL6 mice were treated with lactoferrin, lactoferricin or PBS either 24 h before (A and C) or 24 h after (B and D) genital infection with HSV-2. Disease development was followed daily over 9 days and was scored between 0 and 5 as described earlier (Morrison et al., 1998). Data are expressed as the means (A and B). HSV-2 genome copies in the spinal cord were determined by quantitative PCR on day 9 (C and D). Data are expressed in Log scale as medians and the 25% and 75% percentile (the boxes) with the minimum and maximum responses ($n = 5$ per group). $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to PBS-treated mice using ANOVA with Bonferroni's post-test.

Both lactoferrin and lactoferricin were able to induce chemokine responses in the genital tract. Both compounds induced CCL5, one of the major T-cell and NK-cell attracting chemokines (Maghazachi, 2010; von Hundelshausen et al., 2007), and lactoferrin also induced the local genital production of CXCL2 (the murine analogue of human IL-8), which is chemotactic for neutrophils (Kobayashi, 2008). This implies that lactoferrin and lactoferricin are immunomodulatory. This is particularly interesting since CCL5, together with CCL3 and CCL4, has direct antiviral effects on HSV through its capacity to bind the envelope glycoprotein B and to generate pores in the HSV envelope (Nakayama et al., 2006). These chemokines were most likely produced by non-hematopoietic cells, given that neither lactoferrin nor lactoferricin were able to induce any chemokine production in spleen cells *in vitro*. The ability to induce chemokine responses in the genital tract, however, could not be correlated to the antiviral capacity of these compounds, as both lactoferrin and lactoferricin were immunomodulatory *in vivo* but only lactoferricin was able to block HSV-2 infection. Thus, the induction of CCL5 and CXCL2 alone does not lead to protection against HSV-2 infection *in vivo*. However, we cannot rule out the possibility that CCL5 contributed to the antiviral potential of lactoferricin.

Dosing and timing of drug administration are obviously vital parameters that need to be optimized. However, based on our initial experiments we would argue that lactoferricin showed considerable promise as a therapeutic agent. One single dose of lactoferricin given vaginally to mice 24 h after initial genital infection delayed the disease onset by at least 2 days and 60% of the animals were still symptom-free at day nine when the experiment was concluded. This was in sharp contrast with mock (PBS)-treated animals, where all mice showed signs of disease already by day 6. Furthermore, the levels of HSV-2 DNA in the central nervous system were 15-fold lower in the lactoferricin-treated group. Whether a continuous supply of lactoferricin given 24 h after viral exposure and onwards would have a more beneficial effect on disease development remains to be determined. To our knowledge, this is the first study showing that lactoferricin might have a therapeutic potential against genital HSV-2 infection in mice.

In conclusion, we show for the first time that lactoferricin but not lactoferrin is a potent antiviral agent *in vivo*. Genital co-administration of lactoferricin with HSV-2 completely blocked disease development whereas lactoferrin was ineffective. Even more importantly, lactoferricin given therapeutically delayed disease onset. These data suggest that lactoferricin is a promising microbicidal for *in vivo* use against genital herpes.

Acknowledgements

This work was supported by the Swedish Science Council VR (including a research position for Kristina Eriksson) and Västra Götalandsregionen through LUA/ALF.

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