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Inhibition of herpes simplex virus infection by lactoferrin is dependent on interference with the virus binding to glycosaminoglycans

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Abstract

Previous reports have indicated that lactoferrin inhibits herpes simplex virus (HSV) infection during the very early phases of the viral replicative cycle. In the present work we investigated the mechanism of the antiviral activity of lactoferrin in mutant glycosaminoglycan (GAG)-deficient cells. Bovine lactoferrin (BLf) was a strong inhibitor of HSV-1 infection in cells expressing either heparan sulfate (HS) or chondroitin sulfate (CS) or both, but was ineffective or less efficient in GAG-deficient cells or in cells treated with GAG-degrading enzymes. In contrast to wild-type HSV-1, virus mutants devoid of glycoprotein C (gC) were significantly less inhibited by lactoferrin in GAG-expressing cells, indicating that lactoferrin interfered with the binding of viral gC to cell surface HS and/or CS. Finally, we demonstrated that lactoferrin bound directly to both HS and CS isolated from surfaces of the studied cells, as well as to commercial preparations of GAG chains. The results support the hypothesis that the inhibition of HSV-1 infectivity by lactoferrin is dependent on its interaction with cell surface GAG chains of HS and CS.

Keywords: Lactoferrin; Antiviral activity; Heparan sulfate; Chondroitin sulfate; Herpes simplex virus

Introduction

Lactoferrin, an iron-binding glycoprotein composed of a single 80-kDa polypeptide chain that is folded into two homologous lobes (Moore et al., 1997), is a component of many different exocrine fluids such as milk, tears, saliva, bile, as well as bronchial, intestinal and vaginal secretions (Vorland, 1999). Lactoferrin plays a part in the innate host defense against pathogenic microorganisms and is a potent inhibitor of several viruses including herpesviruses (Marchetti and Superti, 2001; van der Strate et al., 2001; Vorland, 1999). The antimicrobial effects of lactoferrin may at least partly be explained by its ability to interact with cell surface molecules including glycosaminoglycans (GAGs) (van Berkel et al., 1997).

GAG chains are ubiquitous components of cell surfaces where most of them are covalently attached to core proteins to form proteoglycans. GAG chains are composed of repeating, specifically sulfated, disaccharide units of which two common classes are heparan sulfate (HS) and chondroitin sulfate (CS). In the former, the disaccharide unit is composed of uronic acid and glucosamine, whereas in the latter, the amino residue is galactosamine. During synthesis, these sugar residues are subjected to various modifications including sulfation, and the distribution of negatively charged sulfate groups along the chain is of prime importance for specific recognition of GAGs by different proteins including the viral attachment components. With regard to lactoferrin, GAG molecules have been reported to modulate various biological activities of this protein (Damiens et al., 1998), while lactoferrin in turn was found to regulate the GAG/proteoglycan activities in inflammation (Pejler, 1996) and during coagulation (Wu et al., 1995).

Herpes simplex virus (HSV) type 1 (HSV-1) initiates infection of cells by binding to cell surface GAG chains (WuDunn and Spear, 1989), and this interaction is a feature of both wild-type and laboratory strains (Terhune et al., 1998; Trybala et al., 2002). Binding of viral glycoproteins C (gC) and/or B (gB) to HS promotes virus

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adsorption to the cell surface (Herold et al., 1991, 1994) while the interaction of glycoprotein D with specifically 3-*O*-sulfated HS chains was shown to trigger the subsequent entry step of this virus (Shukla et al., 1999). In addition, HSV-1 and HSV-2, two related viruses that show preferential tropism for oral and genital tissue respectively, have been reported to differ in their interaction with HS (Gerber et al., 1995; Herold et al., 1996; Shukla et al., 1999; Trybala et al., 2000).

After Hasegawa et al. (1994) demonstrated inhibitory effects of lactoferrin against human cytomegalovirus and HSV, many other viruses have also been reported to be affected by this protein. In relation to the effect of lactoferrin on other viruses, herpesviruses were shown to be highly sensitive in various cell lines including those of human origin (Andersen et al., 2003; Hasegawa et al., 1994; Marchetti and Superti, 2001; Marchetti et al., 1996, 1998). Moreover, Fujihara and Hayashi (1995) demonstrated a protective effect of lactoferrin on murine herpetic keratitis. With regard to the mechanism of its antiviral action, we have previously demonstrated that the effect of lactoferrin occurs during the very early phases of the viral replicative cycle and seems unrelated to its iron content (Marchetti et al., 1996, 1998). It has been postulated that lactoferrin may compete with HSV for the attachment to cell surface HS since GAG binding sites has been demonstrated to be present on the N-terminal part of the protein (Ji and Mahley, 1994; Mann et al., 1994; Wu et al., 1995). In the present work we have addressed this issue by testing the effect of lactoferrin on HSV-1 infection of cells deficient or proficient in expression of HS and CS (Banfield et al., 1995a; Gruenheid et al., 1993; McCormick et al., 1998). Our study demonstrates that a direct binding of lactoferrin to cell surface GAG molecules constitute a hindrance for the gC-mediated attachment of HSV-1 to HS and/or CS, and that this feature may explain, at least in part, the anti-HSV activity of the protein.

Results

Antiviral activity of human and bovine lactoferrin in human fibroblasts

The inhibitory potency of human lactoferrin (HLf) and bovine lactoferrin (BLf) against different HSV-1 gC-positive and gC-negative strains was assayed in cultures of human embryonic fibroblasts. Both HLf and BLf were potent inhibitors of infection of cells by the gC-positive wild-type 2762 strain and laboratory KOS 321 strain (Figs. 1A and B). The IC_{50} (concentrations inhibiting plaque formation by 50%) values of HLf were 24 μg/ml for the 2762 strain and 52.5 μg/ml for KOS 321. The corresponding values for BLf were 75 and 78 μg/ml. Note that at relatively low concentrations, HLf was more active than BLf at inhibiting plaque formation by the wild-type

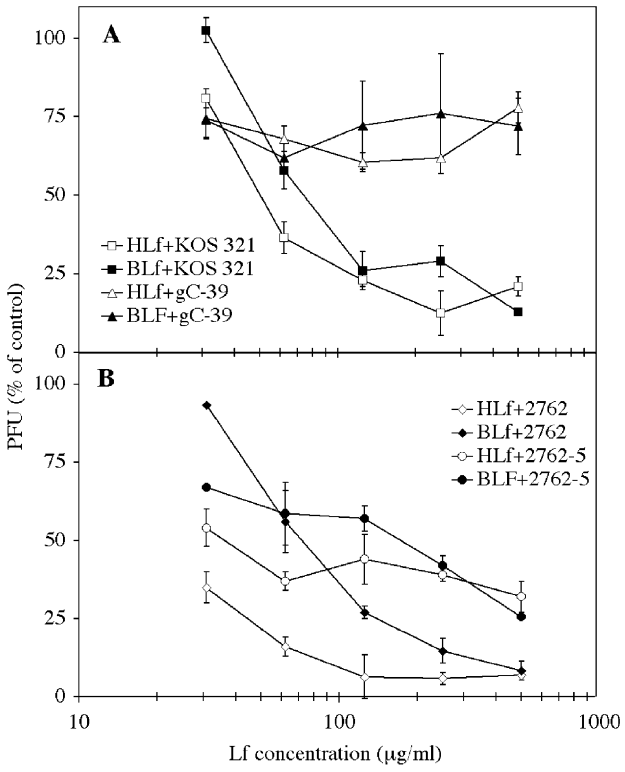

Fig. 1. Effect of HLf or BLf on HSV-1 KOS 321 and HSV-1 gC$^-$39 (A) and HSV-1 wild-type 2762 and its gC-negative derivate 2762-5 (B) strain infection in human embryonic fibroblasts. Lactoferrin and the virus were simultaneously added to cells and left on the cell monolayers during 1 h period of virus attachment to and infection of cells. The results are expressed as percentages of the number of plaques developed in untreated cultures. Data are means ± SD of duplicate samples of a representative experiment. Each experiment was repeated at least three times.

2762 strain (Figs. 1A and B). Both proteins were significantly less active at inhibiting the gC-negative mutant viruses gC$^-$39 and 2762-5 as compared to their parental strains KOS 321 and 2762, respectively. The difference between inhibition values found with KOS 321 and gC$^-$39 was more pronounced than that observed between 2762 and 2762-5.

Antiviral activity of human and bovine lactoferrin in GAG-deficient mouse L cells

The results obtained in human cells suggested that gC is important for an antiviral activity of lactoferrin. Since gC is one of the major components that mediate virus attachment to cells through interaction with HS chains, we sought to investigate an involvement of GAG chains in the antiviral activity of lactoferrin. To this end, we tested the effect of HLf and BLf on HSV-1 infectivity in mutant HS-deficient (gro2C) or HS- and CS-deficient (sog9) cells that were selected from murine L fibroblasts based on their partial resistance to HSV-1 infection. Gro2C cells were reported to be 90% resistant to HSV-1 infection compared with L cells, while sog9 cells showed approximately 95% resistance to

HSV-1 infection as related to gro2C cells (Banfield et al., 1995a). In addition sog9-EXT1, a cell line obtained by transfection of sog9 cells with the *EXT1* gene which restored expression of HS but of no other GAG molecules (McCormick et al., 1998), was used.

BLf and HSV-1 KOS 321 were simultaneously added to cells and incubated for 1 h at 37 °C. Under these conditions, BLf was inactive in sog9 cells even at the highest concentration tested, while in L and gro2C cells, BLf reduced the virus infection with IC_{50} values of 210 and 240 μg/ml, respectively. HSV-1 plaque formation in sog9-EXT1 cells was strongly inhibited by BLf with approximately 80% reduction noted at 250 μg/ml (Fig. 2A). These results indicated that the inhibitory effect of BLf was dependent on the expression of HS and/or CS at the cell surface.

Results presented in Fig. 2B show the modulation of HSV-1 infection by BLf (400 μg/ml), investigated under different experimental conditions. When BLf remained on cells during the adsorption and penetration step (1 h at 37 °C) or when it was present throughout the infection (1 + 72 h at 37 °C), the inhibition of plaque formation ranged from about 66% to 90% in L, gro2C and sog9-EXT1 cells. In contrast, when BLf was added after the adsorption and penetration step and incubated with cells for 72 h at 37 °C, no substantial inhibition in any of these cell lines was observed. In GAG-deficient sog9 cells, BLf showed only a weak effect on HSV infection. In these cells, the protein marginally inhibited plaque formation when added during the adsorption and penetration step, appeared to be completely ineffective when present throughout the infection, and enhanced infection by 30% when added after the adsorption and penetration step. Taken together, these results suggest that BLf interfered with the virus attachment to and/or entry into the cells through GAG interaction, but had little effect on the later stages of infection. To further investigate the effects of BLf on early events of HSV-1 infection, the cells were incubated with BLf for 1 h at 4 °C before the virus addition. The inhibitory effects were essentially similar to those observed upon addition of BLf during the adsorption and penetration step (data not shown), suggesting that the viral receptor molecules at the cell surface were the site of lactoferrin activity. However, the concentration of BLf required to obtain a similar degree of inhibition was higher at 4 °C than at 37 °C (2 mg/ml versus 400 μg/ml). We have no explanation for this phenomenon, but we can speculate that BLf-GAG binding may, in addition to electrostatic forces, rely on hydrophobic interactions that are known to be less efficient at low temperature.

The results presented in Fig. 2C show the modulation of HSV-1 infection by HLf at a concentration of 400 μg/ml, investigated under different experimental conditions, as already described for BLf (see Fig. 2B). The most striking difference between the two proteins was that HLf exhibited a clear inhibition of HSV-1 infection not only in L, gro2C and sog9-EXT1 cells, but also in sog9 cells. The observation that HLf showed antiviral activity during the initial phase of infection also in sog9 cells (Fig. 2C) suggested that this protein, in contrast to BLf, could inhibit the virus binding to cell surface molecules other than HS/CS.

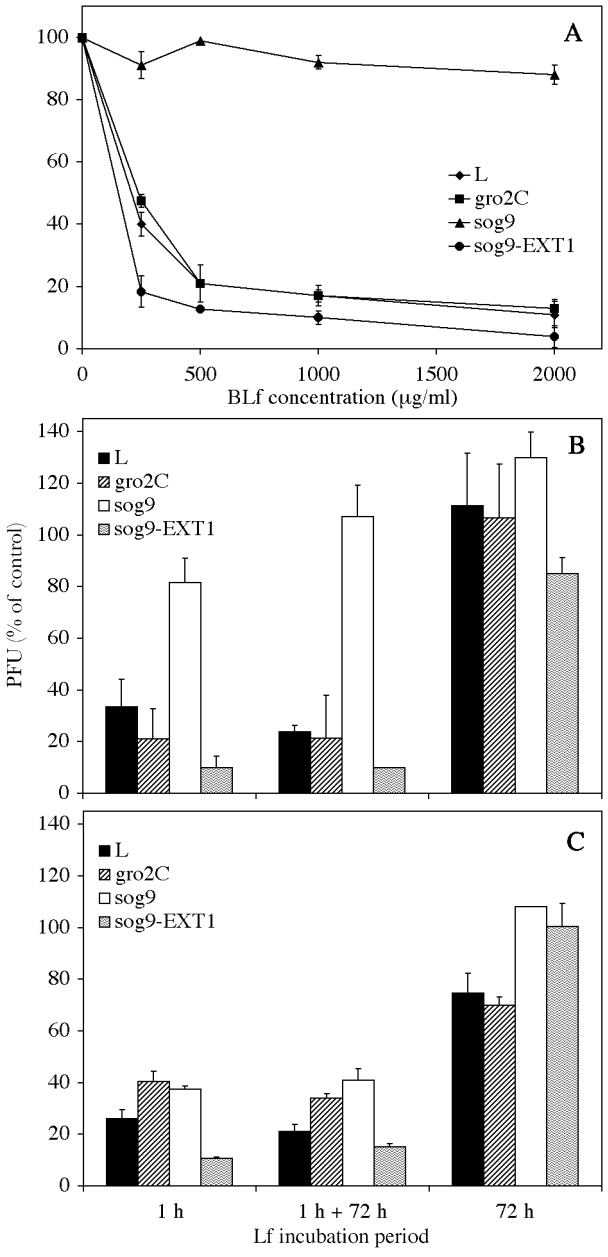

Fig. 2. Effect of BLf and HLf on HSV-1 KOS 321 infection in L, gro2C, sog9 and sog9-EXT1 cells. (A) Dose-dependent effect of BLf on HSV-1 infection in L, gro2C, sog9 and sog9-EXT1 cells. BLf and the virus inoculum were simultaneously added to and incubated with cells during 1 h period of virus attachment to and infection of cells. Effect of BLf (B) or HLf (C) on HSV-1 infection in L, gro2C, sog9 and sog9-EXT1 cells under different experimental conditions. Lactoferrin (400 μg/ml) was incubated with cells either during adsorption and penetration step (1 h at 37 °C), or throughout the infection (1 + 72 h at 37 °C), or during plaque formation only (72 h at 37 °C). The results are expressed as percentages of the number of plaques developed in untreated cultures. Data are means ± SD of duplicate samples of a representative experiment. Each experiment was repeated at least three times.

Binding of bovine and human lactoferrin to GAGs

The results presented in Fig. 2A suggested that GAGs are important for antiviral activity of lactoferrin. Consequently, BLf and HLf were tested for their ability to bind purified HS and CS chains. HS chains isolated from L cells or CS chains isolated from L or gro2C cells were mixed with lactoferrin thereafter the GAG–lactoferrin complexes formed were trapped on nitrocellulose filters. As shown in Fig. 3A, BLf bound to GAG molecules isolated from L and gro2C cells. At a concentration of 625 μg/ml, BLf bound a higher proportion of L cell-specific HS (90%) than CS from the same source (37%). Interestingly, at the same concentration, BLf bound efficiently to gro2C-specific CS (77%), a finding suggesting that the CS chains expressed on HS-deficient gro2C cells have been altered as compared with parental L cells. The binding of HLf to purified HS or CS chains is shown in Fig. 3B. HLf bound preferentially to HS and no significant difference in the binding capability could be noted between BLf and HLf at a concentration of 625 μg/ml. At a relatively low concentration (25 μg/ml), BLf bound 45% more HS than HLf. These experiments clearly demonstrated that BLf and HLf bound GAG molecules that are primarily responsible for attachment of HSV-1 to cells, that is, HS and to some lesser extent CS.

Table 1
Effect of GAG-degrading enzymes on BLf activity against HSV-1 infection

Enzyme	L cells	Gro2C cells
	% of BLf activity related to cells incubated without BLf	
Mock treatment	100	100
Heparinase	70	85
Chondroitinase	130	15

Cells were treated with heparinase III (4 U/ml) or Chondroitinase ABC (1 U/ml) or mock treated. The cells were then infected in the presence or absence of BLf (400 μg/ml).The activities of BLf were calculated according to the following formula:

$$\frac{1-(\text{No. of PFU with BLf and enzyme}/\text{No. of PFU with enzyme})}{1-(\text{No. of PFU with BLf}/\text{No. of PFU})}\times 100$$

Effect of enzymatic treatment of cells on the antiviral activity of bovine lactoferrin

Our data indicated that cell surface GAGs are important for antiviral activity of lactoferrin (Figs. 2 and 3). To further

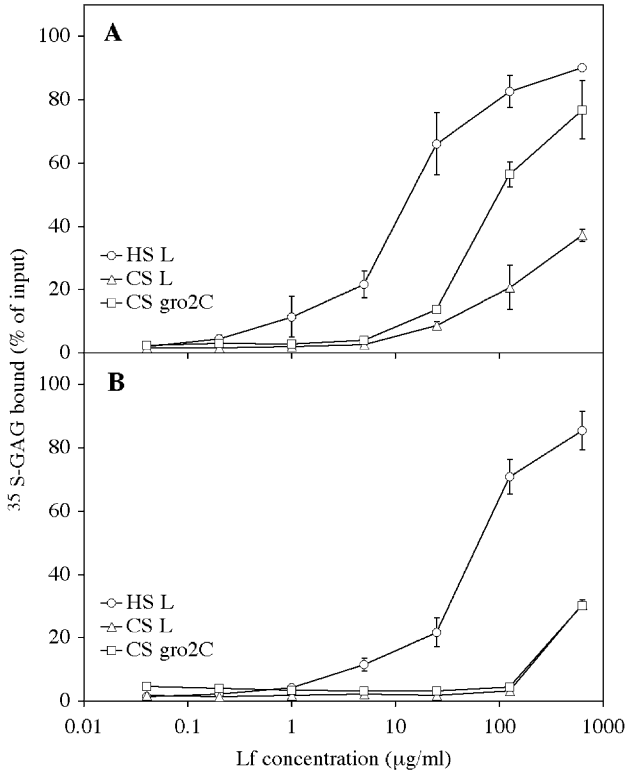

Fig. 3. Binding of BLf (A) or HLf (B) to HS and CS isolated from L and gro2C cells. ^{35}S-labeled HS/CS from L/gro2C cells were incubated with lactoferrin for 2 h at room temperature. The amounts of bound glycosaminoglycans were determined by the nitrocellulose membrane filtration method. Values shown are averages of two individual determinations from two separate experiments with two different glycosaminoglycan preparations.

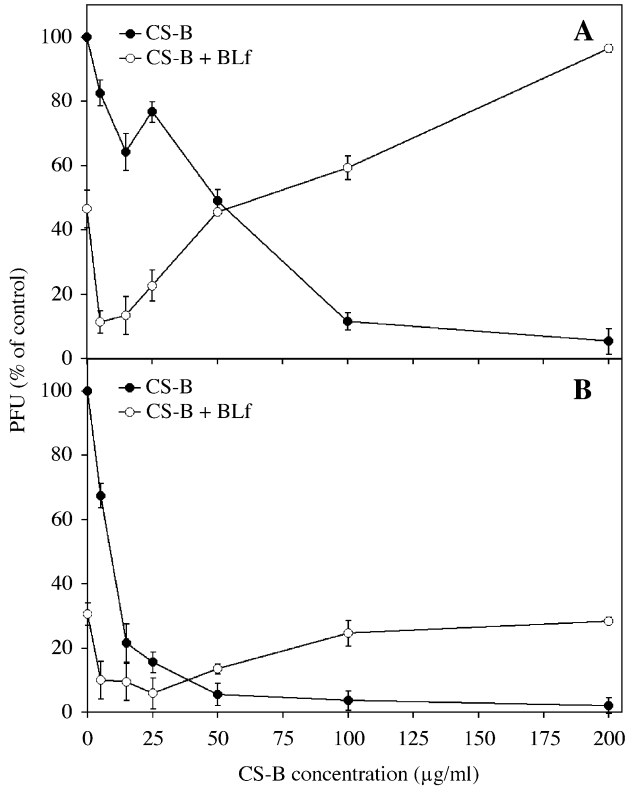

Fig. 4. Effect of CS-B and BLf on HSV-1 KOS 321 infection in L (A) and gro2C (B) cells. Serial dilutions of CS-B were incubated with BLf (400 μg/ml) at room temperature for 15 min before infection of cells for 1 h at 37 °C. The results are expressed as percentages of the number of plaques developed in untreated cultures. Data are means ± SD of duplicate samples of a representative experiment. Each experiment was repeated at least three times.

challenge this interpretation, the L or gro2C cells were treated with heparinase III and chondroitinase ABC before the addition of the virus–lactoferrin mixture or the virus alone (Table 1). As expected, treatment of L cells with heparinase and treatment of gro2C cells with chondroitinase reduced the number of viral plaques compared to mock-treated controls, while chondroitinase-treated L cells and heparinase-treated gro2C cells were readily infected by HSV-1 (data not shown). The antiviral effect of BLf in heparinase-treated cells was decreased as related to the effect of BLf in untreated controls. In contrast, in chondroitinase-treated L cells, BLf inhibited HSV-1 infection more efficiently than in mock-treated cells. Treatment of gro2C cells with heparinase did not significantly affect BLf antiviral activity while in chondroitinase-treated gro2C cells, BLf was almost unable to inhibit plaque formation. These results support our interpretation that the anti-HSV-1 effect of BLf is, at least partly, dependent on the blocking of the virus binding to cell surface HS and/or CS.

Effect of chondroitin sulfate preparations on antiviral activity of bovine lactoferrin

The inhibitory effect of CS-A, -B, -C or -D, alone or combined with BLf, on HSV-1 infection in L, gro2C, sog9 and sog9-EXT1 cells, was then assayed. In a first series of experiments, we have found that, of the CS preparations used, CS-B from pig skin was the most active inhibitor of HSV-1 infection (data not shown). Successively anti-HSV-1 effect of CS-B, alone or combined and preincubated with 400 μg/ml BLf before addition to L cells, was assayed (Fig. 4A). The modulation of BLf potency by CS-B was dependent on the concentration of this compound used, that is, at concentrations of <50 μg/ml CS-B enhanced, whereas above this threshold, diminished the anti-HSV-1 potency of BLf. These tendencies were markedly less pronounced in gro2C cells (Fig. 4B). Similarly to what was observed by Banfield et al. (1995b), HSV-1 infection of these HS-deficient cells was highly sensitive to inhibition with CS-B, and this feature might limit the discrimination of modulation of anti-HSV potency of lactoferrin by CS-B.

Effect of bovine lactoferrin on HSV-1 gC-null mutant infection of GAG-deficient mouse L cells

In addition to examining the contribution of GAGs to antiviral activity of lactoferrin (Figs. 2–4), we also investigated the involvement of viral gC in this process. Data shown in Fig. 1 demonstrated that HSV-1 gC-negative mutant viruses were significantly less sensitive to lactoferrin than parental strains. Given that, we assayed the activity of BLf against the HSV-1 gC-null mutant gC$^-$39 in GAG-deficient cells. BLf did not inhibit infection by gC$^-$39 in any of the examined cell line (Fig. 5). On the contrary, addition of lactoferrin during adsorption and penetration

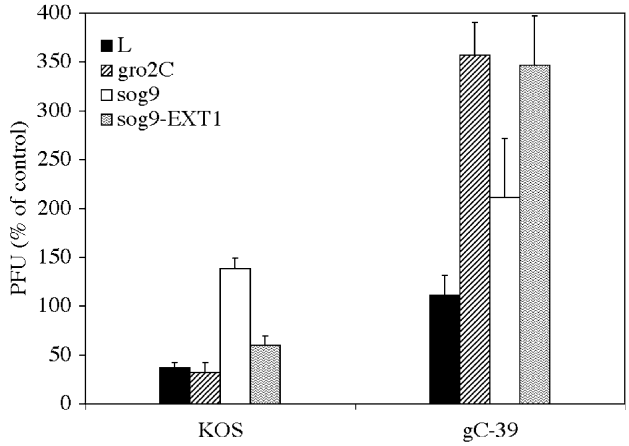

Fig. 5. Effect of BLf against infection with HSV-1 KOS 321 and its gC deletion mutant gC$^-$39 in L, gro2C, sog9 and sog9-EXT1 cells. BLf (400 μg/ml) and the virus inoculum were simultaneously added to and incubated with cells during 1 h period of virus attachment to and infection of cells. The results are expressed as percentages of the number of plaques developed in untreated cultures. Data are means ± SD of duplicate samples of a representative experiment. Each experiment was repeated at least three times.

step promoted the formation of plaques by this mutant virus, especially in gro2C, sog9 and sog9-EXT1 cells. These findings indicate that BLf functions by blocking the interaction of gC with cell surface HS and/or CS, and that this substance can enhance infectivity of the gC-negative variants.

Discussion

We attempted to elucidate molecular mechanisms behind the well-known anti-HSV activity of lactoferrin (Fujihara and Hayashi, 1995; Hasegawa et al., 1994; Marchetti et al., 1996, 1998). A proposal that lactoferrin blocks the virus adsorption to target cells was previously formulated (Fujihara and Hayashi, 1995; Marchetti et al., 1996). Here, by the use of cells with selectively modified expression of GAG molecules, we demonstrated that lactoferrin blocked HSV-1 infection by binding to HS and/or CS on cell surfaces, thereby occupying moieties functioning as initial receptors for this virus. Moreover, the antiviral activity of lactoferrin against HSV-1 depended on the presence of the attachment protein gC, earlier shown to mediate virus binding to HS (Herold et al., 1991) as well as to CS (Mårdberg et al., 2002).

As a common feature of protein–GAG interactions, the binding of lactoferrin to heparin, HS and CS have been attributed to electrostatic interactions between the negatively charged polysaccharides and clusters of basic amino acids that compose the positively charged domain at the N-terminal part of this protein (Mann et al., 1994; Shimazaki et al., 1998; Wu et al., 1995). Similarly, the domain of HSV-1 gC that is responsible for HS as well as for CS binding contains stretches of basic arginine residues enabling such

electrostatic interactions to occur (Mårdberg et al., 2002; Trybala et al., 1994). Therefore, a mechanism in which lactoferrin blocks HSV-1 infection by competing out gC-mediated binding to cell surface GAG molecules seems a plausible explanation of its antiviral activity. This assumption is supported both by the lack of antiviral activity of lactoferrin on GAG-deficient cells, and its impaired effect on gC-negative strains.

With regard to a comparison of HS and CS, respectively, as targets for the anti-HSV-1 activity of lactoferrin, this issue was investigated using BLf on mouse cells selectively expressing HS or CS. It should be noted that in mouse cells, HLf was a less effective antiviral compound (see below), and bound less amount of HS and CS as compared to its bovine homologue, why BLf was chosen for the comparison. BLf was a potent inhibitor of HSV-1 infection in L and sog9-EXT1 cells exposing HS on their surface. In the HS-deficient gro2C cells, in which HSV-1 utilizes CS as a substitute receptor for attachment (Banfield et al., 1995b), the function of BLf was dependent on binding to this GAG molecule since chondroitinase treatment strongly abrogated the antiviral activity of BLf. Taken together, these data strongly suggest that BLf requires the presence of at least one of the two GAGs HS/CS on host cell for antiviral activity. Furthermore, as judged by the results from selective enzymatic removal of HS or CS in L cells, it is likely that BLf preferably acts by binding to HS when both the GAGs are present (such as on L cells) and utilizes CS as a second choice when this molecule is the only alternative (i.e., as on gro2C cells).

Interestingly, heterogeneity of CS molecules present on L cells and its derivate HS-deficient CS-expressing gro2C cells was suggested by the present study. In particular, gro2C cell-specific CS appeared to be a better ligand for lactoferrin than L-cell-specific CS, suggestive of an adaptation of the synthesis and/or modification of CS in the absence of HS in the direction of higher affinity to a polycationic protein. Given the considerable structural diversity that GAG molecules from various sources may present (Shriver et al., 2002), the versatility of BLf in binding well to HS as well as to some CS populations might have bearings to the broad antimicrobial scope of the protein. When we analyzed different CS preparations, the CS-B was a strong blocker of HSV-1 infection in gro2C cells as shown earlier (Banfield et al., 1995b). Preincubation of CS-B with BLf showed an additive antiviral effect of the two substances at lower concentrations, and an abrogation of anti-HSV-1 activities at higher concentrations, suggesting a direct physical interaction between BLf and CS-B. It might be proposed that CS-B contains structural elements that can accommodate BLf as well as HSV-1 gC, but a more detailed analysis was beyond the scope of this article.

Since the viral envelope protein gC plays a key role in the adsorption of HSV-1 to cells by interacting with HS or CS, comparing the inhibitory activity of BLf toward gC-positive and gC-negative viruses could provide an independent challenge of the GAG dependency of the antiviral activity of lactoferrin. The finding that the infection by gC-negative mutants was significantly less inhibited or even enhanced by BLf (the latter probably by BLf-mediated bridging of viral and cellular surfaces), suggested that the anti-HSV-1 activity of the protein relied on interference with the binding of gC to cell surface GAG molecules. Attachment of gC-negative virions to cells was previously reported to be redundantly mediated by viral glycoprotein gB (Herold et al., 1994) and the two viral components gB and gC clearly differ in their interaction with HS with regard to contribution of electrostatic forces (Trybala et al., 2000). It may therefore be suggested that BLf affects viral adsorption by interacting with structural features of the HS/CS molecules that are involved in the binding to gC but not to other heparin-binding HSV glycoproteins, such as gB.

Finally, since BLf and HLf have been shown to differ in some structural features as well as in their binding to heparin-like molecules (Mann et al., 1994; Moore et al., 1997; Shimazaki et al., 1998; van Berkel et al., 1997; Wu et al., 1995), we studied the anti-HSV-1 activity of HLf in mutant L cells. In contrast to BLf, HLf inhibited viral infection in gro2C and sog9 cells to a similar extent. This finding, together with results showing that HLf bound much less gro2C-specific CS than BLf, suggests that HLf probably does not interact significantly with CS from gro2C cells. Therefore, it can be postulated that the presence of HS/CS on the surface of mouse L cells has a different relevance for the anti-HSV-1 activity of BLf as compared to that of HLf. Other mechanisms, unrelated to GAG binding, could have a preponderant role in HLf inhibition of viral infection in a situation where HS is not expressed on the surface of target cells. On the other hand, it should be noted that commercial preparation of HLf might contain variable amounts of molecules from which two or three N-terminal arginine residues have been removed by limited proteolysis (van Berkel et al., 1997), an alteration that could decisively affect lactoferrin–GAG interaction (Mann et al., 1994).

In conclusion, the results presented in this study could contribute to the explanation of the mechanisms involved in the anti-HSV activity of lactoferrin. Derivatives of this natural glycoprotein, blocking the early stages of the virus life cycle, might have therapeutic potential as an alternative to conventional drugs when the appearance of resistant virus strains or severe side effects compromise the effectiveness of the therapy. Furthermore, the implication of cell surface GAG binding as a mechanism for antiviral activity of lactoferrin may have some bearings on the wide spectrum of other viruses being inhibited by this protein.

Materials and methods

Cells and viruses

Human embryonic fibroblasts were grown in Eagle's minimal essential medium (MEM) supplemented with 2

mM L-glutamine, 4% fetal calf serum (FCS) and 4% newborn calf serum. Mouse L cells (clone 1D line of LMtk$^-$ murine fibroblasts) and the L-cell derivatives gro2C and sog9 were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/l) supplemented with 10% FCS in 5% CO_2 atmosphere. The gro2C cell line lacks expression of HS and the sog9 cell line lacks expression of both HS and CS (Banfield et al., 1995a; Gruenheid et al., 1993). Sog9-EXT1 cells were grown in DMEM high glucose supplemented with 10% FCS and 700 μg/ml G418 (Gibco BRL) in 5% CO_2 atmosphere. L, gro2C, sog9 and sog9-EXT1 cells were a generous gift of Dr. Frank Tufaro, University of British Columbia (Vancouver, Canada).

The HSV strains used were HSV-1 KOS 321, a plaque purified isolate of wild-type strain KOS (Holland et al., 1983), HSV-1 gC$^-$39, a gC-null derivative of KOS 321 (Holland et al., 1984), HSV-1 2762, a wild-type brain isolate (Bergström et al., 1990) and HSV-1 2762-5 a spontaneously occurring gC-negative variant of 2762. The latter virus strain contained a premature stop codon at position W13 of the gC gene, and its gC-negative phenotype was confirmed by the lack of reactivity to three different anti-gC MAbs (Bergström, unpublished).

Lactoferrin preparations and enzymes

Lactoferrin from bovine milk was supplied by Besnier Bridel (Laval, France), whereas human milk lactoferrin was purchased from Sigma Chemical Co. (St. Louis, MO). The stocks were prepared in phosphate-buffered saline (PBS, pH 7.2) at a concentration of 20 mg/ml and stored at − 20 °C. CS-A, -B, -C, and -D were purchased from ICN Biomedicals, Inc. (Irvine, CA). Heparinase III (heparitinase I) and chondroitinase ABC were purchased from Sigma. Concentrations of GAG lyases were expressed in Sigma units/ml (1 Sigma unit corresponds to approximately 0.0017 IU).

Cytotoxicity

Confluent monolayers of L, gro2C, sog9 or sog9 EXT1 cells grown in 96-well tissue culture plates were washed and exposed to serial 2-fold dilutions of CS-A, -B, -C, and -D or GAG lyases for 1 h at 37 °C, then washed and incubated with fresh medium. In the other experiments cells were incubated with serial 2-fold dilutions of lactoferrin. After 3/4 days at 37 °C, cell morphology was examined by light microscopy and cell viability was determined by neutral red uptake assay. Briefly, treated and untreated cells were stained for 3 h at 37 °C with neutral red (50 μg/ml), thereafter cells were washed with PBS and fixed with 4% formaldehyde, 10% $CaCl_2$. The uptaken dye was extracted by 1% acetic acid in 50% ethanol and the optical density was measured at 550 nm in an ELISA-reader. None of the drugs affected either morphology or viability of all cell lines tested.

Antiviral assay

Confluent monolayers of L, gro2C, sog9, sog9-EXT1 cells and human embryonic fibroblasts in six-well plates were washed twice with culture medium and incubated for 1 h at 37 °C with 900, 1300, 80 000, 5000 or 900 PFU/well, respectively. These inocula were chosen because they produced comparable numbers of plaques in each well (100–200). However, to exclude that the BLf inability of inhibiting HSV-1 infection in sog9 cells was due to the high viral inoculum, experiments were performed with serial 2-fold dilutions of HSV-1 inoculum starting from 1000 PFU/well. Lactoferrin was added in the following ways: before viral adsorption (1 h at 4 °C), during adsorption and penetration step (1 h at 37 °C), throughout the infection (1 + 72 h at 37 °C), or during plaque formation only (72 h at 37 °C). In experiments with CS, various dilutions of the compound were incubated with BLf at 400 μg/ml at room temperature for 15 min before infection. After viral adsorption and penetration step (1 h at 37 °C), cells were washed two times with medium, thereafter medium containing 1% methylcellulose, 2% FCS and antibiotics were added. Three days after infection, monolayers were stained with crystal violet and the plaques were counted.

Enzymatic digestion of cells with GAG lyases

Confluent monolayers of 3-day-old L and gro2C cells in six-well plates were washed twice with Hanks' medium and incubated with heparinase III or chondroitinase ABC for 1 h at 37 °C. The cells were washed twice with cold Hanks' medium and 900 or 1300 PFU (for L cells or gro2C cells, respectively) were added to each well. Virus adsorption was performed for 1 h at 4 °C with or without BLf in the medium.

Isolation of heparan and chondroitin sulfate chains

Subconfluent cell monolayers were grown for 48 h (L cells) or 65 h (gro2C cells) in the presence of $Na_2^{35}SO_4$ (50 μCi/ml; specific activity 1325 Ci/mmol; NEN Life Science Products, Boston, MA) in sulfate-free MEM supplemented with 10% FCS and antibiotics. Cell-associated HS and CS chains were purified by the method of Lyon et al. (1994). For the isolation of HS and CS chains from L cells, the recovered GAG chains, after dialysis, were digested with 2 Sigma unit/ml of chondroitinase ABC or 15 Sigma unit/ml of heparinase I, respectively, for 4 h at 37 °C.

Binding of lactoferrin to heparan and chondroitin sulfate

Lactoferrin was serially 5-fold diluted in 0.2 ml PBS supplemented with 0.05% bovine serum albumin. Each dilution was mixed with approximately 5000 cpm of ^{35}S-labeled HS from L cells or 3500 cpm of ^{35}S-labeled CS from L or gro2C cells. Following incubation for 2 h at room

temperature, the amounts of bound GAGs were determined by the nitrocellulose membrane (Aka Filter AB) filtration method (Maccarana and Lindahl, 1993).

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