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Bovine lactoferrin prevents the entry and intercellular spread of herpes simplex virus type 1 in Green Monkey Kidney cells

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Abstract

Lactoferrin (Lf) is a multifunctional glycoprotein that plays an important role in immune regulation and defence mechanisms against bacteria, fungi and viruses. Bovine lactoferrin (bLf) has been recognized as a potent inhibitor of human herpetic viruses, such as cytomegalovirus and herpes simplex virus type 1 and 2. BLf has been found to prevent viral infection by binding to heparan sulphate containing proteoglycans that also act as cell receptors for herpetic viruses.

In this study we further investigated the inhibiting activity of bLf against herpes simplex virus type 1 (HSV-1) in Green Monkey Kidney (GMK) cells and found that, in addition to the viral adsorption step, bLf also targets the HSV-1 entry process and cell-to-cell viral spread. Our study showed that the inhibition of HSV-1 infectivity by bLf is dependent on its interaction with specific structural viral proteins. Apart from the prevention of early phases of viral infection, cell-to-cell spread inhibition activity of HSV-1 by bLf confirmed that this protein is an outstanding candidate for the treatment of herpetic infections since it would offer the advantage to prevent also viral infections caused by cell-associated virus.
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Keywords: Lactoferrin; HSV-1; GMK cells; Viral entry; Viral spreading

1. Introduction

Herpes simplex virus (HSV) causes a wide range of infections, which are usually self-limiting in the immunocompetent host. HSV-1 is generally associated with primary and recurrent mucocutaneous facial, ophthalmic or genital lesions. Infection with HSV-1 normally results in limited benign lesions; but, in some instances, this virus is capable of causing serious, life-threatening disease or blindness (Spruance et al., 1977; Whitley et al., 1981; Whitley and Roizman, 2001; Remeijer et al., 2004; Kolokotronis and Doumas, 2006). The virus establishes a latent ganglionic infection in the neurons that innervate the area of primary infection. This location then can serve as a site for re-occurring infections, which, in turn, may become a source of infection for an unwitting host. Many therapeutic agents have been developed and used for treatment of HSV disease, most of these are nucleoside analogs with selective antiviral activity, acyclovir (ACV) being the most common. Resistance to nucleoside analogs has been reported (Coen, 1994). Therefore, the development of different antiviral compounds with alternative mechanisms of action is important. Among natural compounds exhibiting anti-microbial activity, lactoferrin (Lf) showed peculiar features that make it suitable for use as anti-herpetic agent alone or combined with other drugs (Andersen et al., 2003).

Human Lf is an 80 kDa multifunctional, monomeric glycoprotein (Metz-Boutigue et al., 1984) present in external secretions, especially milk, tears and saliva possessing a variety of biological functions, such as promotion of iron absorption, immunomodulation and inhibiting activity towards different pathogens (Levay and Viljoen, 1995; Valenti et al., 1998; Vorland, 1999; van der Strate et al., 2001; Marchetti and Superti, 2001). Bovine Lf (bLf) has been recognized as a potent inhibitor of different enveloped viruses such as human cytomegalovirus (HCMV) (Harmsen et al., 1995; Beljaars et al., 2004), HSV-1 and HSV-2 (Hasegawa et al., 1994; Fujihara and Hayashi, 1995; Marchetti et al., 1996, 1998), human immunodeficiency virus (HIV) (Harmsen et al., 1995; Swart et al., 1996; Puddu et al., 1998; Berkhout et al., 2002, 2004), human hepatitis C virus (HCV) (Ikeda et al., 1998, 2000), hantavirus (Murphy et al., 2000), hepatitis B virus (Hara et al., 2002) and respiratory syncytial virus (Sano et al., 2003). The antiviral effect of bLf

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against some non-enveloped viruses has been also demonstrated (Superti et al., 1997, 2001; Marchetti et al., 1999; Arnold et al., 2002; Lin et al., 2002; McCann et al., 2003; Di Biase et al., 2003; Pietrantoni et al., 2003, 2006; Drobni et al., 2004; Seganti et al., 2004; Tinari et al., 2005; Ammendolia et al., 2007).

In most cases studied, it appears that Lf exhibits its antiviral activity at an early phase in the infection process (Valenti and Antonini, 2005; Jenssen, 2005). It is known that Lf binds to cell surface heparan sulphate (HS) containing proteoglycans (Ji and Mahley, 1994) which in turn act as binding sites for different viruses (WuDunn and Spear, 1989; Giroglou et al., 2001; Dechecchi et al., 2001) and its inhibiting activity has been ascribed to a competition for cell receptors leading to prevention of virus adsorption to host cells (Di Biase et al., 2003; Marchetti et al., 2004; Drobni et al., 2004).

With regard to HSV, viral adsorption is effectively blocked by Lf (Andersen et al., 2004), most likely as a result of its interaction with cell surface HS (Hasegawa et al., 1994; Marchetti et al., 1996, 2004). Anti-HSV activity of Lf has been investigated with several cell lines, deficient in, or expressing, different glycosaminoglycan (GAG) molecules at the cell surface. The results have shown that HS at the cell surface is important for Lf to exert its antiviral activity (Andersen et al., 2001, 2004; Marchetti et al., 2004). In particular, Andersen et al. (2001) showed that the antiviral activity of Lf was not improved by pre-incubation of drug with HSV-1 or HSV-2 prior to infection, indicating that the effect of Lf must be exerted through its interaction with a cellular target, rather than with the viral particle itself (Andersen et al., 2004). Conversely, Marchetti et al. (1996, 1998) suggested that Lf prevents HSV entry in part by binding to the virus particles.

In the present study we have further investigated the interaction between bLf and HSV-1 in order to better characterize the mechanism of the antiviral activity of this protein. Results obtained showed that, in addition to the viral adsorption step, bLf also targets HSV-1 entry process and cell-to-cell viral spread. In particular, our findings demonstrated that the viral inhibition by Lf is dependent on its direct interaction with specific structural viral proteins.

2. Materials and methods

2.1. Cells and virus

Green Monkey Kidney (GMK) cells were grown at 37 °C in a humidified atmosphere with 5% CO_2 in minimal essential medium (MEM; Gibco, Paisley, UK) containing 1.2 g/l $NaHCO_3$, and supplemented with 10% inactivated foetal calf serum (FCS; Flow Laboratories), 2 mM glutamine, non-essential amino acids, penicillin (100 IU/ml) and streptomycin (100 μg/ml).

Virus strain used was HSV-1 KOS 321, a plaque purified isolate of wild-type strain KOS (Holland et al., 1984). Virus was inoculated onto confluent GMK cell monolayers at a multiplicity of infection (MOI) of 0.1 plaque forming unit (PFU)/cell. After 60 min at 37 °C, the inoculum was removed, and the monolayers were washed in MEM and then incubated at 37 °C in MEM containing 1.2 g/l $NaHCO_3$, and supplemented with 2% inactivated FCS, 2 mM glutamine, non-essential amino acids, penicillin (100 IU/ml) and streptomycin (100 μg/ml). When an extensive cytopathic effect (c.p.e.) was observed, infected cultures were frozen and thawed three times, centrifuged (3000 × *g*; 10 min), and supernatants were stored at −70 °C. Stock virus titer was determined by plaque assay on GMK cells.

For co-localization experiments, the K26GFP viral strain (kindly provided by Dr. S. Person) was utilized (Desai and Person, 1998).

For some experiments purified virus was used. Virus purification was carried out from infectious culture media of GMK cells as described by Trybala et al. (2000) and virion purity checked by SDS-PAGE under reducing conditions.

2.2. Lf preparation

Lf from bovine milk (bLf), purchased from Morinaga Milk Industries (Zama City, Japan), was dissolved as stock solution (10 mg/ml) in pyrogen-free PBS. Protein purity was checked by SDS-PAGE stained with silver nitrate and was judged to be greater than 95%. Protein concentration was determined by UV spectroscopy on the basis of the extinction coefficient of 15.1 (280 nm, 1% solution) (Groves, 1960).

2.3. Cytotoxicity

The maximal non-cytotoxic dose of protein was calculated by evaluating cell morphology and viability (determined by neutral red staining) and cell enumeration (by microscopic counts after dispersion into individual cells with trypsin) (Ammendolia et al., 2007).

2.4. Dose-dependent effect of bLf on HSV-1 infection

GMK cell monolayers in six-well plates (Costar, Cambridge, MA, USA) were washed twice with culture medium and incubated for 1 h at 37 °C with a MOI of 0.01 PFU/cell in presence of different concentrations of bLf. After the adsorption step, monolayers were washed twice with medium and layered with MEM containing 1% methylcellulose, 2% FCS and antibiotics. Three days after infection, monolayers were stained with crystal violet and the plaques were counted.

2.5. Time of addition assay

To establish the step of the viral cycle affected by bLf in our cell system, a time of addition assay was performed. For these experiments, cell monolayers were infected with HSV-1 at a MOI of 0.01 and bLf (0.4 mg/ml) 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), during plaque formation only (72 h at 37 °C) or 1 h after adsorption step (71 h at 37 °C). Viral infection was monitored by the plaque assay as described above.

2.6. BLf activity on HSV-1 antigen synthesis

GMK cells were grown in tissue culture chamber slides (Lab-Tek; Nunc, Naperville, IL, USA) for 24 h at 37 °C in 5% CO_2. Viral attachment was synchronized by infecting the cells for 1 h at 4 °C. Monolayers were incubated with a solution of bLf (1 mg/ml) before (1 h at 4 °C), during, after virus attachment step, or throughout the whole experiment. As viral inoculum was utilized HSV-1 at a MOI of 0.1 PFU/cell. After 16 h at 37 °C in 5% CO_2 the cells were washed in PBS, fixed in acetone at −20 °C for 5 min, incubated with rabbit anti-HSV-1 antibodies (Dako Cytomation, Glostrup, Denmark) for 45 min at 37 °C and washed in PBS. Viral antigen synthesis was estimated as previously described by Marchetti et al. (1996) by utilizing (FITC)-conjugated anti-rabbit gamma-globulin antibodies (Sigma Chemical Company, St. Louis, MO, USA) and an UV Leitz microscope.

2.7. BLf activity on HSV-1 antigen synthesis at different time intervals after the adsorption step

BLf activity on HSV-1 antigen synthesis was monitored at different time intervals after viral adsorption. For these experiment, after the viral attachment step (MOI 1 for 2 h at 4 °C), cells were washed twice with MEM and incubated at 37 °C. The inhibitory activity of 1 mg/ml bLf was assessed by two different experimental procedures: (i) protein was added to the cells after the temperature shift and incubated for different lengths of times and (ii) protein was added to the cells at different times after the viral attachment step until the end of the infectious cycle. Virus antigen synthesis was monitored at 8 h after infection by indirect immunofluorescence.

2.8. Neutralization assay

Neutralization of virus binding to GMK cells was carried out by incubating 1 mg/ml bLf with HSV-1 (10^5 PFU/ml) for 1 h at 37 °C. After incubation, two-fold serial dilutions of virus-bLf suspensions were added to GMK cells grown in 24-well tissue culture plates (Flow Laboratories, Costa Mesa, CA, USA) for 24 h at 37 °C in 5% CO_2. After 1 h adsorption at 37 °C, cells were rinsed thoroughly and incubated at 37 °C for plaque assay as above described.

2.9. Penetration assay

GMK cells seeded on 24-well tissue culture plates were overlaid with 100 μl/well (0.01 PFU/cell) of virus inoculum and incubated for 2 h at 4 °C. After washing (two times), infected cells were overlaid with MEM with or without bLf (0.4 mg/ml) and shifted to 37 °C to allow virus penetration to proceed. At various times after temperature shift, each well was treated with 0.4 ml of citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) for 1 min. Then monolayers were extensively washed and layered with MEM containing 1% methylcellulose, 2% FCS and antibiotics in the presence or absence of bLf. After 3 days, virus infection was titrated by plaque assay. The fraction of intracellular virus at a given time was expressed as the percent of viral PFU surviving citrate treatment, where 100% was the number of plaques formed on infected monolayers not treated with citrate buffer.

2.10. BLf localization in GMK cells

Monolayers of GMK cells were exposed to bLf (1 mg/ml) for 1 h at 4 °C. After washing, the incubation temperature was shifted to 37 °C and cells were incubated for 8 h. BLf localization was monitored at different time intervals (every hour until 8 h) by indirect immunofluorescence assay. Briefly, cells were fixed in acetone at −20 °C for 5 min and incubated with a rabbit serum raised against Lf (Dako Cytomation) for 45 min at 37 °C. After washing in PBS, bLf localization was visualized by utilizing (FITC)-conjugated anti-rabbit antibodies (Sigma Chemical Company, St. Louis, MO, USA) and an UV Leitz microscope.

2.11. SDS-PAGE, Western and Far-Western blot assays

Purified HSV-1 proteins were resolved by polyacrylamide-SDS gel electrophoresis (SDS-PAGE), as described by Laemmli (1970) under denaturing or non-denaturing conditions on 10% acrylamide gel. Separated proteins were then transferred from gel to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), blocked with 5% skim-milk solution and washed with PBS/0.05% Tween-20 (washing solution).

For Far-Western blot, strips were incubated for 90 min with washing solution containing 1 mg/ml bLf and immuno-stained as previously described (Ammendolia et al., 2007).

For Western blot, strips were incubated with either mouse monoclonal anti-ICP-5 (Abcam plc, Cambridge, UK) or goat polyclonal antibodies anti-VP-16 (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. After washing with PBS–0.5% Tween-20, anti-mouse (Bio-Rad) or anti-goat (Santa Cruz Biotechnology Inc.) HRP-conjugated antibodies were added and incubated for 1 h at room temperature. Following extensive washing, staining was achieved by using TMB substrate kit for peroxidase (Vector Laboratories Inc., Burlingame, CA, USA) according to manufacturer's instructions.

2.12. Immunoprecipitation

Immunoprecipitation of ICP-5 and VP-16 proteins was performed on crude infected cell lysates. Infected monolayers were lysed with RIPA buffer (140 mM NaCl, 20 mM Tris–HCl, pH 7.8, 1% NP-40, 0.5% Na-deoxycholate, 1 mg/ml BSA, 0.5 mM PMSF). Monoclonal anti-ICP-5 or goat polyclonal anti-VP-16 antibodies were added to one hundred microliters of lysate and incubated overnight at 4 °C on a rotating wheel. After incubation, 20 μl of Protein G-Sepharose beads (Santa Cruz Biotechnology Inc.) were added to each and samples were returned to 4 °C for 2 h. The precipitates were washed three times with RIPA buffer and resuspended in SDS sample buffer. After SDS-PAGE, immunoprecipitated proteins were electroblotted onto nitrocellulose paper and processed for Western blot with

anti-ICP-5 and VP-16 antibodies or probed with bLf by Far-Western blot assay as above described.

2.13. Co-localization of bLf and HSV-1 at early stage of infection

To evaluate the interaction between HSV-1 and bLf at very early stages of infection, co-localization tests were carried out. As ICP-5 is strictly associated to VP26 in the exon regions of the capsid, the following experiments were carried out by utilizing the K26GFP strain, a green fluorescent protein (GFP)-tagged HSV-1 generated by introducing the VP26-GFP marker into the HSV-1 (KOS) genome (Desai and Person, 1998). GMK cells were infected with a MOI of 10 at 4 °C. After 2 h of infection, bLf (2 mg/ml) was added and temperature was shifted to 37 °C to permit the internalization of attached virions. At various times after temperature shift, each well was treated with 0.4 ml of citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) for 1 min to inactivate extracellular virions and infected cells were treated with bLf containing medium. Thirty and 60 min temperature shift, infected cells were washed with PBS, fixed with paraformaldehyde (3.7%) for 10 min and processed for indirect immunofluorescence by using anti-Lf rabbit serum (Dako Cytomation) and Texas-Red-conjugated anti-rabbit antibodies (Molecular Probes, Eugene, OR, USA). Cells were then examined in an UV Leitz microscope and collected images (Hamamatsu 3CCD Camera Controller) were processed and annotated with Adobe Photoshop software.

2.14. VP-16 transport to the nucleus

To assess the effect of bLf on VP-16 translocation, cells were infected with HSV-1 (MOI 10). After a 2 h adsorption step, bLf (2 mg/ml) was added and cells were incubated at 37 °C for 3 h. Then, treated and untreated infected cells were scraped into PBS containing protease inhibitors, pelleted, resuspended in the same buffer containing 0.4% Nonidet P-40 (NP-40) and pelleted again. The pellet was washed once in PBS containing protease inhibitors plus 0.1% NP-40, pelleted and resuspended in PBS containing protease inhibitors and 0.4% NP-40. This fraction was considered the nuclear fraction (Pomeranz and Blaho, 1999). The protein concentrations of all extracts were determined by a modified Bradford assay (Bio-Rad) as specified by the manufacturer. Equal amounts of nuclei extracts were separated in SDS-PAGE 10%, electrically transferred to nitrocellulose and probed with anti-VP-16 polyclonal antibodies (Santa Cruz Biotechnology) as described above.

2.15. Cell-to-cell spreading

GMK cell monolayers, seeded onto chamber slides (Lab-Tek), were infected with HSV-1 at a MOI of 0.01. At 8 h post-infection, cells were incubated with or without 0.4 mg/ml bLf and a 1:100 dilution of pooled human gamma globulins. At 8 or 16 h post-infection, the infected monolayers were washed with PBS, fixed, permeabilized and processed by indirect immunofluorescence using a polyclonal anti-HSV-1 serum (Dako Cytomation) and FITC-anti-rabbit antibodies (Sigma Chemical Company, St. Louis, MI, USA).

3. Results

3.1. Dose-dependent anti-HSV-1 activity of bLf

BLf was first tested for the ability to alter cell morphology and viability after incubation of cell monolayers for 24, 48 and 72 h at 37 °C with serial drug concentrations from 2 mg/ml. Under these conditions, no cytotoxicity was observed. Then, the ability of two-fold serial dilutions of bLf, starting from 2 mg/ml, to inhibit viral cytopathic effect in GMK cells was tested. The drug was present throughout the infection. Under these experimental conditions, bLf showed a dose-dependent inhibitory activity (Fig. 1).

3.2. Effect of time of bLf addition on HSV-1 production

To establish the step of viral cycle affected by bLf, a time of addition assay was performed. Results showed that bLf was able to inhibit plaque formation of HSV-1 when added to the GMK cells at different time-points before and after virus infection (Fig. 2). Pre-incubation of the GMK cells with bLf before infection or bLf treatment for the whole experiment resulted in inhibitory effect similar to that obtained by adding bLf and virus simultaneously (about 90%). A certain inhibition was also detected after the binding of the virus to the cells (about 48%) and 1 h after the adsorption step (about 40%). Moreover, a characteristic pattern of small plaques was observed in bLf-treated cells, suggesting that the protein inhibited viral dissemination to neighbouring cells.

The effect of bLf on HSV-1 infectivity was also assayed by incubating the virus with the protein for 1 h before addition to the GMK cells. Results obtained demonstrated no inhibitory effect with all dilutions of virus-bLf mixture tested (data not shown).

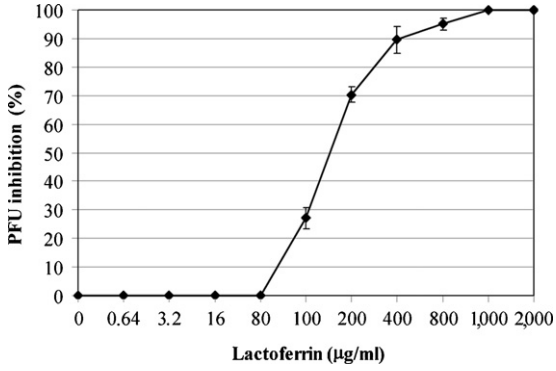

Fig. 1. Dose-dependent effect of bLf against HSV-1 infection. BLf was added together with the virus inoculum during the adsorption step (1 h at 37 °C). Then monolayers were washed and incubated with fresh medium containing methylcellulose for 72 h at 37 °C. Data are means ± standard deviation of triplicate samples of a representative experiment. Each experiment was repeated at least three times.

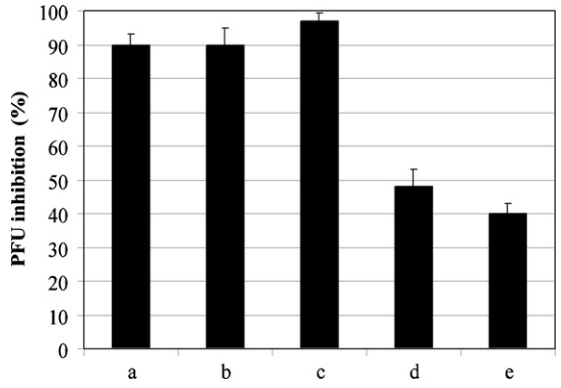

Fig. 2. Time of addition assay. Virus adsorption was performed 1 h at 37 °C and bLf was added in the following ways: before viral adsorption (1 h at 4 °C) (a), during adsorption and penetration step (1 h at 37 °C) (b), throughout the infection (1 + 72 h at 37 °C) (c), during plaque formation only (72 h at 37 °C) (d) or 1 h after viral adsorption and penetration step (71 h at 37 °C) (e). Cells were washed two times with culture medium and incubated for plaque assay as described above. Data are means ± standard deviation of triplicate samples of a representative experiment. Each experiment was repeated at least three times.

3.3. Effect of bLf on expression of antigen synthesis

To determine the effect of time-of-addition of bLf on viral antigen synthesis, GMK cells were infected with HSV-1, treated with bLf before (a) or during (b) the adsorption step, for the whole experiment (c) or after the attachment step (d), and processed for immunofluorescence assay at 16 h post-infection using polyclonal antiserum against all HSV-1 proteins. As shown in Fig. 3, maximum reduction of the number of fluorescent cells was detected when infected monolayers were treated with bLf for the whole experiment (100%) or during the virus adsorption step (about 99%). This effect was only slightly reduced when the infected cells were pre-treated with bLf before viral adsorption step (about 95% reduction of the number of fluorescent cells). Interestingly, bLf inhibited HSV-1 antigen expression also after the initial binding of the virus to the cells, albeit to a lower extent (about 58%).

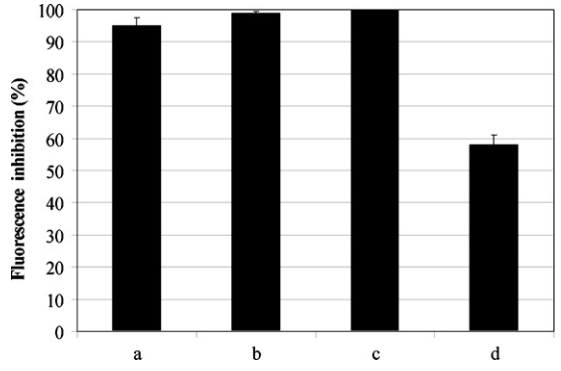

Fig. 3. BLf activity on HSV-1 antigen synthesis. Virus adsorption was performed 1 h at 4 °C, after that cells were washed and incubated at 37 °C. (a) BLf was pre-incubated 1 h at 4 °C prior viral adsorption; (b) bLf was incubated together with virus inoculum; (c) bLf was present throughout the infection; (d) bLf was added after the viral adsorption step. Viral antigen synthesis was evaluated 16 h after adsorption by indirect immunofluorescence. Data are means ± standard deviation of duplicate samples of a representative experiment. Each experiment was repeated at least three times.

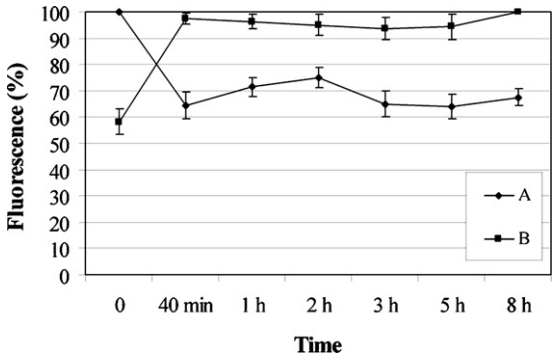

Fig. 4. BLf activity on HSV-1 antigen synthesis at different time intervals post-adsorption step. Virus adsorption was allowed for 2 h at 4 °C, thereafter cells were washed and incubated at 37 °C. (A) BLf was added soon after viral adsorption and removed at different time intervals. (B) BLf was added either soon or at different time intervals after viral adsorption. Viral antigen synthesis was evaluated at 8 h after adsorption by indirect immunofluorescence. A 100% refers to the number of fluorescent cells in untreated cultures. Data are means ± standard deviation of duplicate samples of a representative experiment. Each experiment was repeated at least three times.

3.4. Effect of bLf on viral antigen synthesis during post-adsorption step

The effect of bLf on viral post-adsorption step was further investigated and experiments were carried out in which cells were incubated with the virus for 2 h at 4 °C and then protein was added at different times or for various lengths of time after the viral attachment step. The data in Fig. 4 revealed that the earlier bLf was added to the cells after infection with HSV-1, the better was its effect. In fact, when it was added immediately after viral adsorption step, about 40% of virus production was inhibited (Fig. 4B). If the drug was added 40 min after infection, there was no inhibitory effect of bLf on viral replication (Fig. 4B). These results were confirmed by experiments in which GMK infected cells were treated with bLf for different time intervals after the adsorption step. Viral antigen synthesis was reduced to about 38% when GMK infected cells were treated with bLf during the first 40 min after virus attachment compared to untreated GMK infected cells (Fig. 4A). The same inhibitory effect was revealed when bLf was allowed to interact with infected cells for longer time intervals (Fig. 4A).

3.5. Effect of bLf on early stages of viral infection

As previously suggested (Andersen et al., 2001; Marchetti et al., 1996, 2004) the inhibitory effect observed when bLf was added during the adsorption step is probably due to competition mechanisms between protein and virus for heparan sulphate receptors on the cell membrane. As our results showed that bLf activity was exerted also after HSV-1 adsorption to the cell surface, a penetration assay was performed to further explore this mechanism. After virus binding to the cells at 4 °C, incubation temperature was shifted to 37 °C to allow viral penetration in the absence (Fig. 5A) or presence (Fig. 5B and C) of bLf. At various times cells were washed with citrate buffer to inactivate extracellular virus and fresh medium, with (Fig. 5C) or without

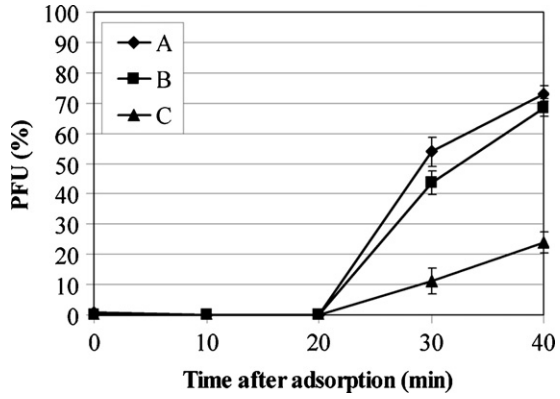

Fig. 5. Penetration assay. Virus suspension was adsorbed on cells for 2 h at 4 °C, then cultures were shifted to 37 °C and washed with citric acid buffer (pH 3) at indicated time points. (A) Untreated virus control. (B) BLf added soon after viral adsorption (and removed before low pH treatment). (C) BLf added soon after adsorption and newly added after low pH treatment. Virus plaques were counted after 3 days at 37 °C. The average of the number of plaques in the control cultures treated with PBS was taken as 100%. The results are presented as the PFU surviving to low pH treatment at each time, expressed as percentage of the PBS control value. Data are means ± standard deviation of triplicate samples of a representative experiment. Each experiment was repeated at least three times.

(Fig. 5B) bLf, was added. Results, reported in Fig. 5, indicate that bLf was not able to inhibit the very early stage of HSV-1 penetration into GMK cells, but it appeared to affect a late step of viral entry, since 40 min post-binding, when most virions were penetrated into the cells, bLf yet exerted about 65% of viral inhibition (Fig. 5C). Moreover, to exert its antiviral activity, bLf had to be located at the cell membranes or interact with cells since its removal by citrate treatment abolished viral inhibition (Fig. 5B).

3.6. BLf localization

Since bLf exerts its inhibitory activity also after the initial binding of the virus to the cells and it has been reported that it must be located at the cell surface to exert antiviral activity against HSV (Andersen et al., 2004), we performed a time-course by immunofluorescence assay to establish whether bLf was able to persist on GMK surface membranes. After 1 h incubation at 4 °C, the protein appeared localized at the cell surface, whereas 1 h after temperature shift to 37 °C, bLf appeared to be localized either at the plasma membrane or into the cytoplasm of GMK cells. From 2 to 8 h post-binding, bLf was localized mainly into the cell cytoplasm but was also detected in large amount at the cell surface (data not shown).

3.7. BLf binding to viral proteins

BLf binding to viral proteins was detected by both Far-Western and Western blots. As shown in Fig. 6A, a lot of bands were detected by bLf binding to mock-infected GMK cells and virus sample in denaturing conditions (Fig. 6A, lanes 1 and 2, respectively). Similar results were obtained when non-denaturing conditions for electrophoresis separation were used (data not shown).

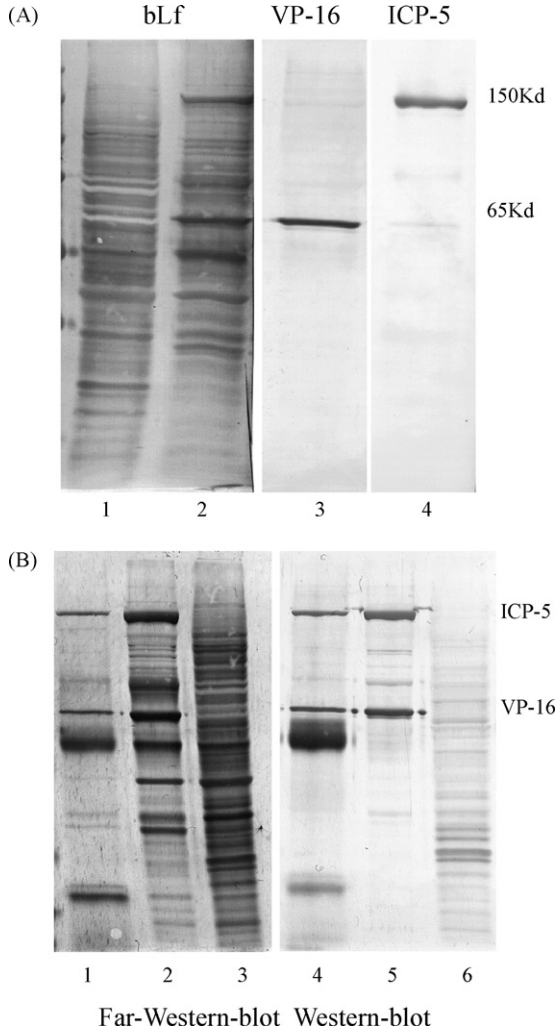

Fig. 6. SDS-PAGE, Western and Far-Western blot assays. (A) Purified HSV-1 (lanes 2, 3 and 4) and mock-infected GMK cells (lane 1) were electrophoresed on 10% polyacrylamide gel, blotted on nitrocellulose and processed for Far-Western blot or Western blot. BLf (4 mg/ml) was overlayed onto viral proteins (lane 2) and mock infected cells (lane 1) and bLf binding was detected by polyclonal anti-bLf and anti-rabbit HRP-conjugated antibodies. Blots with purified HSV-1 proteins were also stained by antibodies against VP-16 (lane 3) and ICP-5 (lane 4). Molecular weights (in kDa) are shown on the right. (B) Far-Western blot and Western blot of immunoprecipitated VP-16 and ICP-5 proteins. VP-16 and ICP-5 proteins precipitated by specific antibodies were separated by SDS-PAGE and blotted on nitrocellulose paper. Blots containing immunoprecipitated proteins (lanes 1 and 4), purified virions (lanes 2 and 5) and mock-infected cells (lanes 3 and 6) were overlayed with bLf for Far-Western blot and with anti-VP-16 and ICP-5 antibodies for Western blot.

Only two bands in virus sample of apparent molecular masses of 150 and 65 kDa, that did not appear in mock-infected GMK cells (Fig. 6A, lane 1), were revealed by bLf binding (Fig. 6A, lane 2). Based on molecular weight, protein bands could correspond to the viral protein ICP-5 (major capsid protein) and VP-16 (viral tegument protein), respectively.

To verify this hypothesis, three kinds of experiments were carried out: (i) Western blot of viral proteins performed with anti-ICP-5 or anti-VP-16 antibodies; (ii) ICP-5 and VP-16 immunoprecipitation followed by electrophoresis separation and Far-Western blot assay with bLf as a probe and (iii) West-

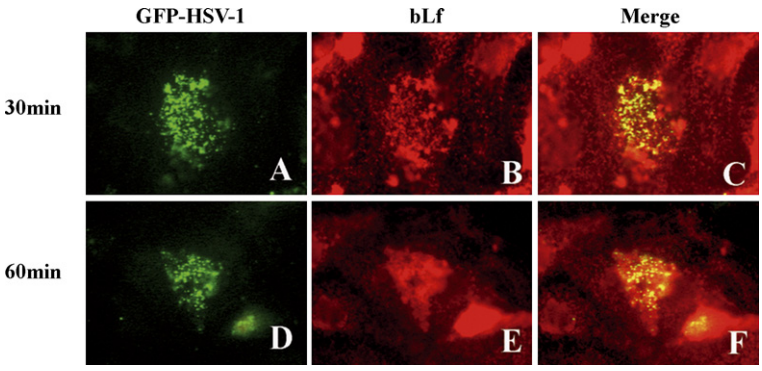

Fig. 7. Co-localization of bLf and HSV-1 at early stage of infection. The interaction between HSV-1 and bLf during early phases of viral infection was analyzed after viral adsorption step. BLf was added to infected cells and temperature shifted to 37 °C to permit virus internalization. Thirty (panels A–C) and 60 (panels D–F) minutes after adsorption, bLf (panels B and E) and GFP-HSV-1 (panels A and D) were detected by immunofluorescence. Panels (C and F) represent merged images.

ern blot of immunoprecipitated ICP-5 and VP-16 proteins with specific antibodies.

Results of Western blot performed on purified virus sample (Fig. 6A, lanes 3 and 4) showed that VP-16 and ICP-5 detected by specific antibodies correspond to the protein bands detected by bLf (Fig. 6A, lane 2). These results were confirmed by Western blot performed on immunoprecipitated ICP-5 and VP-16 proteins (Fig. 6B, lane 4).

Far-Western blot assay performed on immunoprecipitated viral proteins using bLf as a probe, showed that the drug was able to bind to these viral proteins, reinforcing the results obtained by Western blot analysis (Fig. 6B, lane 1). To reveal non-specific binding to viral or cellular proteins, nitrocellulose strips were incubated with anti-bLf and peroxidase-conjugated-anti-rabbit antibodies. No significant staining was detected (data not shown).

3.8. Co-localisation of HSV-1 and bLf during early step of viral infection

To assess the interaction between HSV-1 and bLf during early phases of viral infection, co-localization experiments were carried out by using a GFP-tagged HSV-1 strain. After adsorption step at 4 °C, bLf was added and the temperature shifted to 37 °C to permit virus penetration. At 30 and 60 min after infection, cells were fixed and processed by indirect immunofluorescence to detect bLf. HSV-1 localization was monitored by the intrinsic fluorescence of GFP-HSV. Images obtained showed that, either 30 min (Fig. 7, panels A–C) or 60 min (Fig. 7, panels D–F) after temperature shift, bLf appeared in part to co-localize (yellow signal) with HSV-1 in cytosolic patches located at cell surface membrane (Fig. 7, panels C and F).

3.9. VP-16 transport to the nucleus

Since our results suggested that an early event after virus penetration was being affected by bLf, we tested for VP-16, a viral tegument protein that, after virus entry, is translocated to the nucleus to activate viral immediate genes (IE). Inhibition of VP-16 translocation to the nucleus can severely impede viral replication (Weinheimer et al., 1992). Western immunoblots were utilized to examine infected nuclei extracts for the occurrence of VP-16 in the presence and absence of bLf. It can be seen in Fig. 8 that, when cells were examined for VP-16 3 h after infection with HSV-1, the amount detected in bLf-treated cultures was considerably lower than in cultures devoid of bLf.

3.10. Cell-to-cell spreading

Since bLf was shown to inhibit viral replication and to reduce the area of viral plaques, we wondered whether it was able to influence cell-to-cell spread of HSV-1 in GMK cells. Cell monolayers were infected at a low MOI in the absence of the drug. At 8 h post-infection, pooled human sera and bLf were added to the infected cells. In the presence and absence of neutralizing antibody, HSV-1 was able to spread to adjacent cells, as shown by the distribution of the immunofluorescence signal from single cells at 8 h post-infection to surrounding cells at 16 h post-infection (Fig. 9, compare panels A and B). If bLf was added at 8 h post-infection, we were unable to demonstrate the development of multicellular foci at 16 h post-infection (Fig. 9, panel C). These results indicate that bLf added at 8 h post-infection inhibited the spread of virus to adjacent cells.

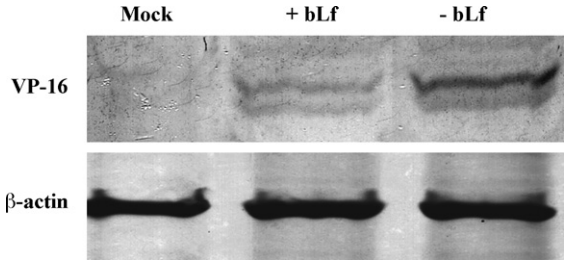

Fig. 8. VP-16 transport to the nucleus. To assess the effect of bLf on VP-16 translocation, cells were infected with HSV-1. After 2 h adsorption step, bLf was added and incubated at 37 °C for 3 h. Extracted nuclei of mock-, bLf-treated- and untreated-infected cells were electrophoresed and blotted onto nitrocellulose paper. VP-16 was detected by goat polyclonal antibodies followed by anti-goat HRP-conjugated antibodies. As loading control anti-beta-actin monoclonal antibodies were used.

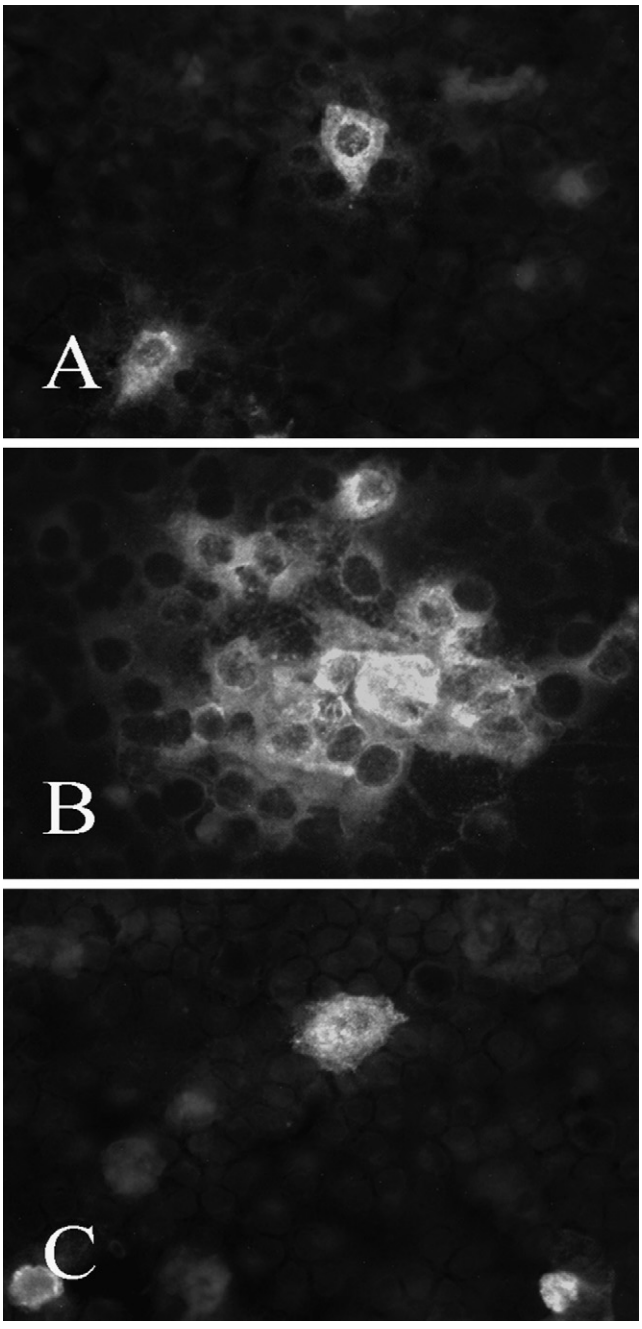

Fig. 9. Cell-to-cell spreading. GMK cell monolayers were infected for 8 h (A) or 16 h (B and C). At 8 h p.i., the medium was replaced with medium supplemented with a 1:100 dilution of pooled human sera with (C) or without (B) bLf. Infected monolayers were fixed at 8 h p.i. (A) or 16 h p.i. (B and C) and stained for HSV-1 with a rabbit polyclonal antibody and goat anti-rabbit IgG conjugated to fluorescein.

4. Discussion

Results presented in this study confirmed that bLf exerts its effect during the early phases of HSV-1 infection in GMK cells. As expected, the highest viral inhibition was detected when bLf was added before or during viral attachment suggesting that bLf competes also in GMK cell system with HSV-1 for heparan sulphate receptor on cell surface (Andersen et al., 2004; Marchetti et al., 2004; Jenssen, 2005). The initial step in HSV infection involves glycosaminoglycans on the cell surface and glycoproteins in the viral envelope (WuDunn and Spear, 1989). The major type of GAG chains found at the cell surface is heparan sulphate (Lindahl et al., 1994). Lf also has the ability to interact with HS (Ji and Mahley, 1994; Wu et al., 1995; Jenssen et al., 2004; Jenssen, 2005) as a consequence of its high net charge and the presence of two GAG binding domains in its N-terminal portion (Mann et al., 1994; Shimazaki et al., 1998; Wu et al., 1995). HSV-1 entry is effectively blocked by Lf (Andersen et al., 2004), as a result of its interaction with cell surface HS (Hasegawa et al., 1994; Lin et al., 2002; Marchetti et al., 2004).

However, anti-herpetic activity of bLf could be due to other mechanisms in addition to competition with virus for HS binding on cell surface, as suggested by Marchetti et al. (1996, 1998). Consistent with previous reports (Marchetti et al., 1996, 1998), we found that bLf was able to reduce HSV infectivity also when added immediately after viral binding, as showed by reduced plaque formation. The observation that the addition of bLf after the initial binding of the virus resulted in an inhibition of plaque formation could be due either to bLf blocking secondary infections of neighbouring cells or to bLf blocking cell-to-cell spreading. To verify these hypotheses, experiments of antigen expression were performed in which bLf was added at different times post-adsorption step during one round of viral replication. Results obtained indicate that bLf has to be present within the first 40 min after attachment to exert its antiviral activity. If added after this time, bLf failed to inhibit viral replication. Since the infection was allowed to proceed for only 8 h, there was no time for HSV-1 to produce secondary infections; then, reduced plaque number observed when bLf was added after viral attachment, was probably caused by bLf inhibition of the early stages of infection rather than by the prevention of viral dissemination to neighbouring cells.

Attempting to investigate the post-binding stage affected by bLf treatment, we performed a penetration assay. HSV-1 was allowed to attach to the cells at 4 °C; after shifting of temperature to 37 °C to permit virus internalization, the addition of bLf was observed to reduce viral infection. One possibility to explain a post-binding effect of bLf could be that it removes bound virus. We assessed this by washing the cells after bLf treatment with buffer citrate to remove virus and drug. Such washing completely abolished the antiviral activity of bLf, suggesting that removal of bound virus did not account for the post-binding antiviral activity. In contrast, when the protein was added to the medium after citrate treatment and incubated for the entire experiment, bLf appeared to prevent viral penetration of about 65%. Therefore, the observed reduction in virus infection after the attachment step did not appear to be caused by the direct inactivation of HSV-1 bound to cell membrane nor did it appear that the drug prevented the virus from penetrating the cells. In fact, bLf still exerted significant viral inhibition 40 min post-binding, when most virions had penetrated into the cells. Furthermore, tests performed to evaluate the virucidal effect of bLf, demonstrated that the drug had no distinct effect directed against the extracellular virus, since allowing bLf to interact directly with the virus the inhibitory effect was not observed, as previously reported also by Andersen et al. (2004). These observations strongly sug-

gested that an event downstream of entry was the target for the drug.

We therefore assayed the ability of bLf to bind virions immediately after envelope fusion with cell membrane to determine if the drug was able to block HSV-1 before transport to the nucleus. Immunofluorescence experiments indicate that GFP-tagged virions in part co-localized with bLf when HSV-1 infected cells were treated with drug after virus attachment. Thirty minutes after starting of virus entry, when most of the virus particles had penetrated into the cells, bLf was visualized at the plasma membrane or into the cytoplasm partly overlapping GFP-virions. Since we demonstrated that bLf was able to persist on cell membrane and/or penetrate GMK cells, it is likely that it could target HSV-1 particles after the membrane fusion step, preventing capsid transportation to the nucleus.

One of the earlier detectable measures of viral entry is the translocation to the nucleus of VP-16, a viral tegument protein. To assess capsid translocation to the nucleus, we extracted nuclei 3 h after infection from HSV-1 infected cells treated or not treated with bLf. Western blot assay revealed that bLf was able to prevent VP-16 from being translocated to the nucleus, suggesting that it affected a post-entry step of viral infection.

Moreover, since co-localization studies suggested that bLf could bind de-enveloped viral capsids after attachment step, a series of Western and Far-Western blot assays, by using purified HSV-1 virions and bLf as a probe, were carried out to investigate bLf-viral protein binding. Results obtained indicate that bLf was able to recognize two marked bands in HSV-1 sample that were not present in mock-infected cells. Based on molecular weights, viral proteins seemed to be the major capsid protein ICP-5 and the tegument protein VP-16. Immunoprecipitation studies and subsequent Far-Western blots with bLf as a probe confirmed that the proteins targeted by bLf really were ICP-5 and VP-16.

The inhibition of VP-16 translocation to the nucleus by bLf could explain reduced viral protein expression and virus replication in GMK cells. In fact, when viral antigen synthesis was analyzed by Western blot, we observed a reduced virus protein accumulation in infected cells treated with bLf compared to non-treated infected cells, starting with the immediate early ICP-4 protein (data not shown).

The same mechanism could be hypothesized to explain bLf activity on viral spreading. In GMK cells, bLf appeared to exert antiviral activity not only by competing for cell receptors with viral particles or blocking IE gene expression but also preventing cell-to-cell spreading. The observation that the addition of bLf after virus infection resulted in reduced plaque size suggested that bLf might also prevent the spread of virus from infected cells to neighbouring uninfected cells as hypothesized by Andersen et al. (2004).

To gain further insight into the mode of action of bLf, we examined whether the virus was able to spread to adjacent cells in the presence of bLf. In the presence of neutralizing antibody, HSV-1 was able to spread to adjacent cells, as demonstrated by the diffusion of the immunofluorescence signal from single cells at 8 h p.i. to surrounding cells at 16 h p.i. Addition of bLf to cells previously infected with HSV-1 at very low MOI rendered them unable to form multicellular foci.

Although bLf interference with one or more viral glycoproteins involved in fusion events between adjacent cells cannot be ruled out, we hypothesized that bLf was able to prevent cell-to-cell spreading by the same mechanism involved in virus entry inhibition. Late in infection, when nascent virions with surrounding tegument proteins were produced, bLf located at the cell surface or penetrated into the cell, might interact with VP-16 and/or ICP-5 interfering with viral assembly or virion translocation during cell-to-cell spreading. On the other hand, VP-16, akin to other tegument proteins such as VP-22, plays an important role during viral assembly (Weinheimer et al., 1992; Elliott et al., 1995); it was observed that the efficient assembly of virions requires large amounts of VP-16 (Weinheimer et al., 1992). VP-16 deletion mutants of HSV-1 proved defective in their replication in cell culture with reduced virus yield and plaque size in some cell cultures (Weinheimer et al., 1992). Our findings suggested that the interaction of bLf with VP-16 resulted in the alteration or sequestration of this viral protein, so that it was unable to serve as a virion structural component.

Since a rapid cell-to-cell spread of HSV-1 infecting oral and genital mucosa is of vital importance in avoiding the developing immune response and thus in establishing productive primary or recurrent infections in humans, cell-to-cell spread inhibition of HSV by bLf could offer the advantage to prevent viral infections caused by cell-associated virus.

Taken together, our results provide further insights in the antiherpetic activity of bLf and confirm bLf as an excellent candidate for the treatment of herpetic infections, as it is able not only to hinder the viral adsorption to the cells but also to prevent the dissemination of infection to adjacent cells.

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