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Lactoferrin inhibits herpes simplex virus type 1 adsorption to Vero cells

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Abstract

This paper describes the ability of human and bovine lactoferrins (HLf; BLf), iron-binding proteins belonging to the non-immune defense system, to interfere with herpes simplex virus type 1 (HSV-1) infection. Since lactoferrins are known to bind to heparan sulphate proteoglycans and to low density lipoprotein receptor, which in turn act as binding sites for the initial interaction of HSV-1 with host cells, we tested the effect of these proteins on HSV-1 multiplication in Vero cells. Both HLf and BLf are found to be potent inhibitors of HSV-1 infection, the concentrations required to inhibit the viral cytopathic effect in Vero cells by 50% being 1.41 μM and 0.12 μM, respectively. HLf and BLf exerted their activity through the inhibition of adsorption of virions to the cells independently of their iron withholding property showing similar activity in the apo- and iron-saturated form. The binding of [^{35}S]methionine-labelled HSV-1 particles to Vero cells was strongly inhibited when BLf was added during the attachment step. BLf interacts with both Vero cell surfaces and HSV-1 particles, suggesting that the hindrance of cellular receptors and/or of viral attachment proteins may be involved in its antiviral mechanism.

Keywords: Lactoferrin; Herpes simplex virus type 1 (HSV-1); Antiviral agents; Viral adsorption

1. Introduction

Herpes simplex virus type 1 (HSV-1) infects a wide range of cells and causes disease in a variety of different tissues. Electron microscopy studies suggested that this virus enters host cells by means of either endocytosis or fusion between the membranes of the virus and the cell (Rosenthal et al., 1984). The envelope of the HSV-1 virion contains at least ten different viral glycoproteins, several of which project as distinct spikes from the membrane surface and are likely to interact sequentially or simultaneously with different binding sites on the cell surface (Fuller and Lee, 1992; Herold et al., 1994).

The initial attachment of virions to cells is shown to be mediated independently by interactions of either glycoprotein C (gC) or glycoprotein B (gB) with heparan sulphate moieties of cell

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surface proteoglycans (Campadelli-Fiume et al., 1990; Spear et al., 1992; Shieh et al., 1992; Gruenheid et al., 1993; Herold et al., 1994; Trybala et al., 1994). Heparin, an anionic related glycosaminoglycan, has been demonstrated to block HSV-1 adsorption to cells (WuDunn and Spear, 1989). There is also evidence that glycoprotein D (gD) may interact with its own cell receptor (Johnson et al., 1990) and oligomers of glycoprotein H (gH) and glycoprotein L (gL) are also known to be required for HSV-1 penetration (Fuller and Lee, 1992; Forrester et al., 1992; Roop et al., 1993). Moreover, it has been recently suggested that the low-density lipoprotein receptor present in coated pits may interact with domains in gB, gC or gD allowing the virions to penetrate by an endocytosis process (Becker et al., 1994).

Heparan sulphate, the primary cell surface receptor for HSV-1, is an ubiquitous and multifunctional constituent of most mammalian cell plasma membranes and of extracellular matrices and has been recently identified as a binding site for human and bovine lactoferrin (Ji and Mahley, 1994; Mann et al., 1994; Wu et al., 1995). Lactoferrin is a monomeric glycoprotein with a molecular mass of about 80 kDa, constituted by two lobes each possessing one iron-binding site (Metz-Boutigue et al., 1984; Pierce et al., 1991); it is present, at relatively high concentrations (up to 12 mg/ml) in various biological fluids and in specific granules of polymorphonuclear leukocytes (Brock, 1980; Reiter, 1983; Chasteen and Woodworth, 1990). Its concentration in the plasma increases 2- to 3-fold in certain pathological circumstances, such as severe infections or autoimmune diseases (Nuijens et al., 1992; Wu et al., 1995). Lactoferrin possesses a broad-spectrum antimicrobial property and it is considered an important component of the non-immune defense system active at mucosal surfaces and in secretions, such as colostrum, milk, saliva and tears; its antibacterial and antimycotic activity have been commonly attributed to the ability of the molecule to bind and sequester environmental iron (Bullen et al., 1978; Chasteen and Woodworth, 1990). However, evidence from a number of studies indicates that the antimicrobial mechanism of lactoferrin is more complex than simple iron deprivation (Arnold et al., 1980; Bellamy et al., 1992; Longhi et al., 1993). A bactericidal activity responsible for the release of lipopolysaccharide in Gram-negative bacteria has been correlated to the binding of lactoferrin to bacterial surfaces (Ellison et al., 1988; Visca et al., 1990). The bactericidal domain of lactoferrin has been localized near the N-terminus of the molecule in a region distinct from its iron-binding sites (Bellamy et al., 1992). Recently, the existence in lactoferrins of a glycosaminoglycan-binding site in the N-terminus as well as of a binding site for the low density lipoprotein receptor has been reported (Ji and Mahley, 1994; Mann et al., 1994; Wu et al., 1995).

The evidence that heparan sulphate proteoglycans and the low density lipoprotein receptor-related protein are capable of binding to lactoferrin and to act as receptors for initial cell-HSV-1 interactions, suggested the idea that lactoferrin could interfere with early events of viral infection. Here, we investigated the effect of human and bovine lactoferrin on different steps of HSV-1 multiplication in Vero cells. Both these glycoproteins were able to strongly inhibit viral attachment to cell monolayers, bovine lactoferrin being the most effective. In an initial attempt to elucidate its antiviral mechanism, we also examined the binding properties of bovine lactoferrin to Vero cells and viral particles.

2. Materials and methods

2.1. Cells and virus

Vero (African green monkey kidney) cells were cultured at 37°C in MEM (Eagle's Minimal Essential Medium) containing 1.2 g/l $NaHCO_3$ and supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. For cell maintenance the serum concentration was lowered to 2%.

Herpes simplex virus type 1 (HSV-1), strain F, was propagated in Vero cells and the virus concentration was estimated by plaque assay.

2.2. Chemicals

The antiviral activity of the following iron-binding proteins (MW 80 000) was tested: lactoferrin from human milk (HLf), lactoferrin from bovine milk (BLf), ovotransferrin from chicken egg white (Otf), were 70–75% iron-saturated. Apo-ovotransferrin (apo-Otf) was purchased from Sigma; apo-lactoferrin from human milk (apo-HLf) and apo-lactoferrin from bovine milk (apo-BLf) were purchased from Fluka. The purity of compounds was tested by SDS electrophoresis and their iron-saturation was controlled by titration with iron citrate, followed by optical absorbance at 468 nm. The glycoproteins were dissolved in phosphate-buffered saline (PBS) at a concentration of 20 mg/ml and stored at −20°C.

2.3. Cytotoxicity

Confluent Vero cell monolayers grown in 96-well tissue culture plates (Falcon) were washed and exposed to 2-fold serial dilutions of the proteins for 1 h at 4°C and then for 47 h at 37°C. At this time the following parameters were evaluated: cell morphology and viability (determined by neutral red staining) were examined by light microscopy; cell proliferation was evaluated quantitatively by microscopic counts after dispersion into individual cells with trypsin. Results were expressed as complete cytotoxicity (++) when at least one of the parameters was affected in 100% of cells, or partial cytotoxicity (+) when one parameter was affected in 50% of cells, or absence of cytotoxicity (−) when none of the parameters was affected.

2.4. Cytopathic effect assay

Cytopathic effect (CPE) assays were performed in 96-well tissue culture microplates. Confluent cell monolayers were infected with 100 cell culture infectious doses 50% ($CCID_{50}$)/well and incubated for 1 h at 4°C. After removal of viral inoculum, cells were washed and the temperature was shifted to 37°C for 47 h. Proteins were present through the infection. The cytopathic effect induced by HSV-1 was measured by the neutral red uptake assay. Briefly, at 48 h post infection treated and untreated infected cells were stained for 3 h with neutral red (50 μg/ml, 200 μl/well, 37°C, 5% CO_2); then the cells were washed with Hank's salt solution and fixed with 4% formaldehyde, 10% $CaCl_2$ (200 μl/well). The uptaken dye was extracted by 1% acetic acid in 50% ethanol (200 μl/well) and the disruption of the cells by the virus or the possible protection by the compounds were measured at 550 nm in an ELISA-reader. Results were expressed as percentage of cytopathic effect inhibition by comparison with untreated control cultures.

2.5. Virus yield assay

Proteins were incubated with cell monolayers before (1 h at 4°C) or during (1 h at 4°C) viral adsorption or throughout the viral multiplication cycle (1 h at 4°C and 23 h at 37°C). At 24 h post infection supernatants from drug-treated and untreated cells were collected and virus yield was determined by plaque forming unit (PFU) assay. Then confluent Vero cells, grown in 24-well tissue culture plates, were infected with different dilutions of the supernatants for 1 h at 37°C. After removal of viral inoculum, monolayers were washed and overlaid with 0.5% agarose. After 3 days incubation at 37°C, cells were fixed with 40% formaldehyde, stained with 1% crystal violet in methanol, and the number of plaques was counted.

2.6. Particle agglutination assay (PAA)

Latex particle suspensions (bead diameter 0.8 μm, Difco Lab.) were coated with BLf or Otf according to a minor modification of the procedure of Naidu et al. (1988). Briefly, HSV-1 was pelleted (1 h; 42 000 × *g*) and resuspended in 0.004 M potassium phosphate buffer (pH 6.8) at a concentration of 10^6 PFU/ml. For PAA, equal volumes of viral suspension and latex reagent were placed on a glass slide. The reactions were scored from positive (++) to weakly positive (+) and negative (−). All tests included controls performed using ovalbumin-coated beads to detect non-specific agglutination reactions.

2.7. Binding of BLf to Vero cells detected by immunofluorescence assay

Semi-confluent Vero cell monolayers, grown in microtissue chamber slides, were washed and then incubated with different concentrations of BLf for 1 h at 4°C. Drug-treated and control cells were washed three times with PBS, incubated for 1 h at 37°C with anti-bovine lactoferrin from rabbit, washed again and finally stained with FITC-labelled anti-rabbit gamma globulins plus Evans blue for 1 h at 37°C. Binding of BLf to Vero cell surfaces was examined by a fluorescence microscope.

2.8. Binding of BLf to Vero cells detected by ELISA assay

The binding of HSV-1 to plastic-adsorbed proteins was examined by an ELISA method. Different concentrations of BLf and Otf were attached to the inner bottom surface of a Linbro microtitration plate (Flow Laboratories). For the assay, 100 μl of each protein dilution in PBS were applied to the wells in quadruplicate. After incubation at 4°C overnight, the plate was rinsed three times with PBS containing 0.5% bovine serum albumin (PBS-BSA). Unoccupied binding sites were blocked by incubation for 30 min at 37°C with PBS-BSA. The plate was rinsed with the same buffer and then incubated for 90 min at 37°C with 2×10^6 PFU of HSV-1 suspended in PBS-BSA (100 μl/well). After washing three times with PBS-BSA, virus binding was evaluated by means of an anti-HSV rabbit serum (1 h at 37°C) and peroxidase-linked donkey-anti-rabbit antibody (1 h at 37°C). The substrate (2,2′-azinobis[3-ethylbenzthlazoline-6-sulphonic acid]) reaction was stopped after 15 min at room temperature with 2 N H_2SO_4 and read in a Titertek Multiscan (Flow Laboratories) at 405 nm. Thorough washing with PBS between assays was included.

2.9. Assay for entry of radiolabelled virus particles

Monolayers of Vero cells cultured in 75-cm^2 flasks were infected at a multiplicity of 0.1 PFU per cell. After 90 min of adsorption at 37°C, maintenance medium was added. All incubations were carried out at 37°C. After 5 h the medium was replaced with MEM without serum. Infected cells were labelled with [^{35}S]methionine (50 μCi/flask) from 6 to 48 h after infection. At 48 h post infection, cells were frozen, thawed twice and then extracellular virions were harvested from the media. Cell debris were removed by low-speed centrifugation. Virions were pelleted by ultracentrifugation and suspended in TNE buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA). The virus suspension was then layered onto a 20–60% (w/v) continuous sucrose gradient and centrifuged for 20 h at 50 000 $\times$ g. Upon completion of centrifugation, the sucrose gradient was fractionated. The recovered virus suspension was then pelleted, diluted in MEM and stored at -80°C. The infectivity of purified virus was determined by titration in Vero cell monolayers by plaque assay.

For binding assays confluent Vero cells, grown in 24-well tissue culture plates, were infected with radiolabeled purified virus. BLf (2 mg/ml) was added to the monolayers together with virus inoculum and incubated at 4°C. After 90 min, supernatants were collected and cells were washed once in MEM to remove unadsorbed virions. The washing media, added to the supernatants, were counted in a liquid scintillation counter. Cell-associated radioactivity was recovered by treating cell monolayers with a lysis buffer (10 mM Tris–HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 1 mM phenylmethylsulphonylfluoride). The amount of radiolabeled virions adsorbed to cell cultures was measured by counting an aliquot of the acetone-precipitated proteins.

2.10. Assays for rate of virus penetration

The rate of virus penetration was measured by inactivation of extracellular virus with a low-pH citrate buffer according to the procedure of Herold et al. (1991). Confluent Vero cell monolay-

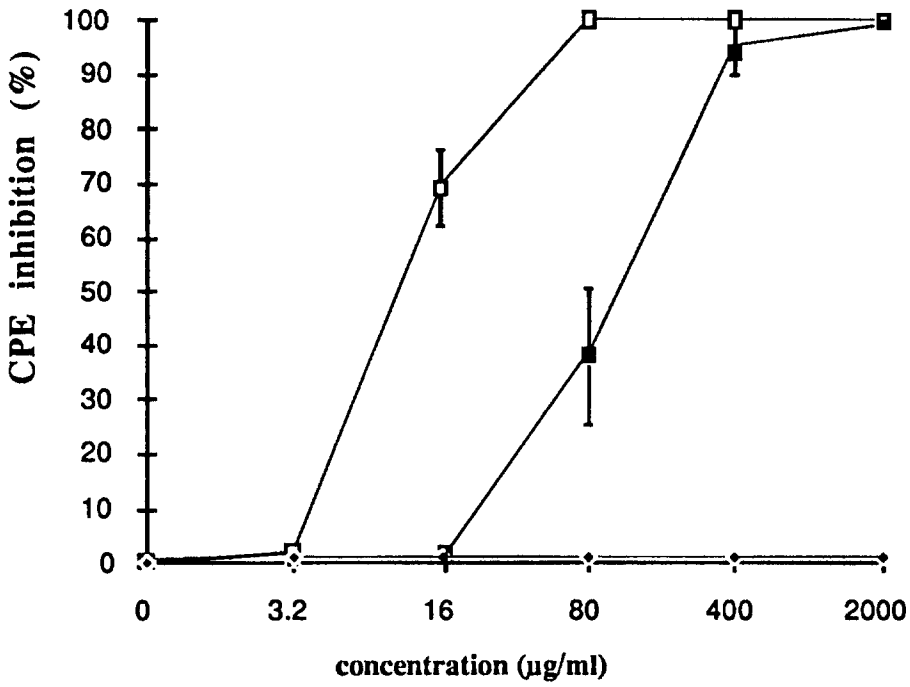

Fig. 1. Dose-response curves of Otf, HLf and BLf towards HSV-1 cytopathic effect in Vero cells. Data are expressed as mean ± S.D. ($n = 3$). ◆, Otf; ■, Hlf; □, Blf. Proteins were present throughout the infection (1 h at 4°C plus 47 h at 37°C).

ers in 24-well tissue culture plates were overlaid with 100 µl/well (0.01 PFU/cell) of virus inoculum with or without BLf (800 µg/ml). The plates were then incubated at 4°C for 1 h, washed twice with MEM, overlaid with 0.5 ml of MEM 2% fetal calf serum, 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 overlaid with agarose and after 3 days infection was titrated by plaque assay. The fraction of intracellular virus at a given time was the percent of viral PFU surviving citrate treatment, where 100% was the number of plaques formed on a monolayer not treated with citrate buffer.

3. Results

3.1. Effect of lactoferrins on cytopathic effect (CPE) by HSV-1 in Vero cells

HLf and BLf were tested for their ability to alter cell morphology, viability and yield after incubation for 1 h at 4°C and 47 h at 37°C of confluent cell monolayers with serial drug concentrations from 20 mg/ml. Under these conditions, up to the highest doses, none of the cytotoxicity parameters was affected. Then, the ability of 5-fold dilutions of the lactoferrins, starting from 2 mg/ml, to inhibit HSV-1 induced cytopathic effect in Vero cells was tested and compared with that of ovotransferrin, an iron-binding protein present in white egg. The compounds were present thor-

ough the infection. The concentration-response curves of these glycoproteins towards HSV-1 infection are presented in Fig. 1. Otf was ineffective whereas HLf and BLf showed a concentration-dependent effect and they completely prevented HSV-1 cytopathic effect in Vero cells at 2 mg/ml and 80 μg/ml, respectively.

3.2. Selectivity indexes of human and bovine lactoferrin towards HSV-1 infection

In order to determine the selectivity indexes (SI) of HLf and BLf, the ratio between the 50% drug cytotoxicity concentration (CC_{50}) and the concentration required to inhibit CPE by 50% (EC_{50}) was calculated. HLf was capable to reduce CPE by 50% at 1.41 μM and BLf at 0.12 μM. Since none of the parameters of cytotoxicity was affected at concentrations up to 20 mg/ml, as reported above, the selectivity indexes of HLf and BLf were higher than 177 and 2083, respectively (Table 1).

3.3. Effect of lactoferrins in apo- and iron-saturated form on HSV-1 yield

To evaluate whether the antiviral activity was a function of the iron binding property of the proteins or not, the ability of lactoferrins and ovotransferrin in the apo- and iron-saturated form to inhibit HSV-1 infection was tested following different experimental procedures: (i) Vero cells were pretreated for 1 h at 4°C with the drugs

Table 1
In vitro antiviral activity of HLf and BLf against HSV-1 monitored by the cytopathic effect assay (CPE)

Lactoferrins	CC^a_{50} (μM)	EC^b_{50} (μM)	SI[c]
HLf	>250	1.41 + 0.33	>177
BLf	>250	0.12 + 0.01	>2083

[a]CC_{50}, cytotoxic concentration, 50%.
[b]EC_{50}, effective concentration, 50%. The EC_{50} values are in μM and represent the concentration required to inhibit CPE by 50%. Proteins were present thorough the infection (1 h at 4°C plus 47 h at 37°C).
[c]SI, selectivity index (SI = CC_{50}/EC_{50}).
Data are expressed as mean ± S.D. (n = 3).

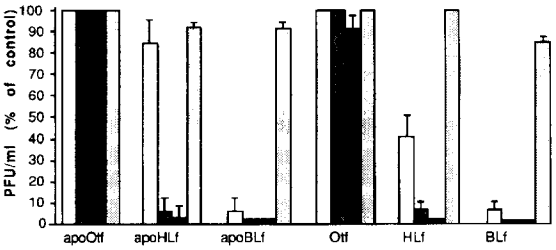

Fig. 2. Effect of 800 μg/ml lactoferrins and ovotransferrin in the apo- and iron-saturated form on HSV-1 yield in Vero cells. Data are expressed as mean ± S.D. (n = 3). □, proteins were incubated with cell monolayers for 1 h at 4°C before the infection. ▧, proteins were present during viral adsorption step (1 h at 4°C). ■, proteins were present for the entire period of viral multiplication (1 h at 4°C + 23 h at 37°C). ▨, proteins were present after the adsorption step (23 h at 37°C).

before the viral infection; (ii) the drugs were added together with virus inoculum during the adsorption step for 1 h at 4°C, and then washed away, fresh medium was added to the cell cultures and the virus infection was allowed to progress by raising the temperature to 37°C in the absence of the compounds; (iii) the drugs were present during the adsorption step for 1 h at 4°C and after the temperature shift to 37°C for 23 h; (iv) the drugs were present after the adsorption step for 23 h at 37°C.

Fig. 2 presents the data on the virus yield in the supernatants expressed as PFU/ml percentage of untreated infected monolayers. No antiviral effect could be noted with iron-saturated Otf and apo-Otf, whereas high inhibition values were reached with lactoferrins. No consistent differences in the inhibiting activity could be noted between the apo- and the iron-saturated lactoferrins, thus demonstrating that their antiviral effect was independent of their iron-binding ability. The block of HSV-1 infection by apo-BLf and BLf was observed in all the experimental conditions tested. Similar results were obtained for apo-HLf and HLf in all experimental conditions with the exception of the pretreatment of cell monolayers which was less effective in preventing HSV-1 infection.

3.4. Effect of bovine lactoferrin on the early steps of HSV-1 infection

Since BLf showed the highest inhibition towards HSV-1 infection, further approaches were carried out to ascertain the effect of this drug at the level of virus attachment to cell surfaces. The adsorption of [^{35}S]-labelled HSV-1 to Vero cells and the rate of viral penetration were measured when viral attachment was performed at 4°C in the presence of BLf.

3.4.1. Adsorption of [^{35}S]-labelled HSV-1 to Vero cells

HSV-1 virions were labelled with [^{35}S]methionine and binding to the cells was measured, in the presence or in the absence of BLf, by counting radiolabeled bound virions. Vero cells were incubated at 4°C with [^{35}S]-labelled HSV-1 at a m.o.i. of 0.1 PFU/cell. At different time intervals the bound radioactivity was compared with that obtained in cells preincubated or in cells infected in presence of 2 mg/ml BLf. The adsorption of virus after 1 h, given as cpm of radiolabeled bound virions, was 4540 as compared with 50 000 of untreated control cultures, thus demonstrating a noticeable inhibition of viral binding when viral adsorption was performed in the presence of BLf.

3.4.2. Rate of penetration of HSV-1 in Vero cells

The entry of adsorbed virions in the presence of BLf, was evaluated by measuring the rate of HSV-1 penetration in Vero cells in the presence or in the absence of 800 μg/ml BLf. Virions (0.01 PFU/cell) were allowed to bind at 4°C for 1 h, then the cell monolayers were incubated at 37°C for various times and exposed to a low pH citrate buffer to inactivate any bound virus that had not yet penetrated the cells. The results presented in Fig. 3 show that after the temperature shift at 37°C no viral particles were internalized when the attachment step was performed in the presence of BLf.

3.5. Binding of bovine lactoferrin to Vero cells

The interaction of BLf with Vero cells was checked by indirect immunofluorescence. Different concentrations of BLf were incubated for 1 h at 4°C with semi-confluent Vero cell monolayers. Microscopic observations showed BLf associated to Vero cell surfaces. The intensity of fluorescence exhibited a concentration-dependent relationship.

3.6. Binding of bovine lactoferrin to HSV-1

In order to test whether bovine lactoferrin could directly interact with HSV-1 virions, trials of virus particle binding to latex beads coated with BLf or to plastic-adsorbed BLf were performed. Control experiments were also carried out with Otf which was deprived of antiviral effect.

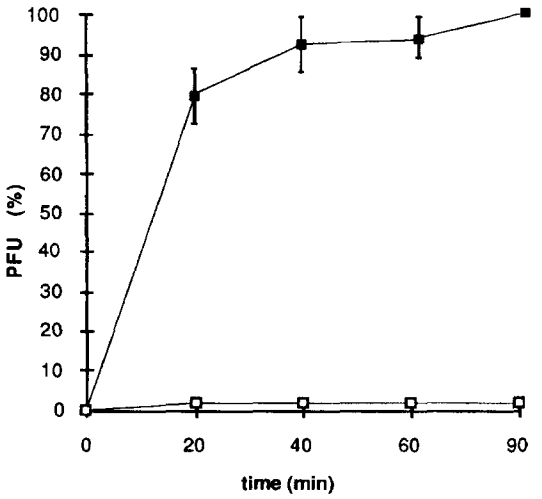

Fig. 3. Rate of penetration of HSV-1 in Vero cells. ■, HSV-1. □, HSV-1 + BLf. Cells were infected with HSV-1 (0.01 PFU/cell) in the presence or in the absence of 800 μg/ml BLf for 1 h at 4°C. Then cultures were shifted to 37°C and washed with citric acid buffer (pH 3). The times shown started after shift to 37°C. Virus plaques were counted after 3 days at 37°C. The number of plaques in the control cultures treated with PBS was essentially the same at all time points and the average value 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 expressed as mean ± S.D. ($n = 3$).

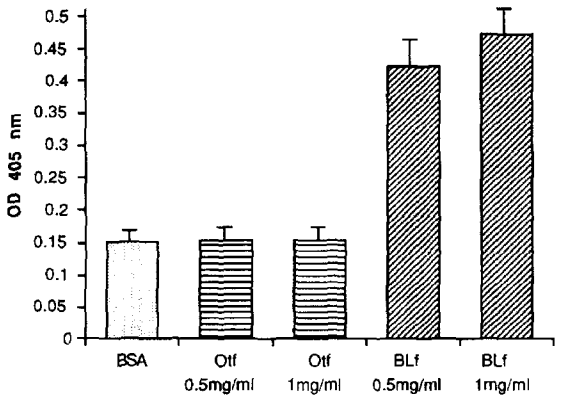

Fig. 4. Amount of HSV-1 binding to BSA-treated plastic surfaces (BSA) or to plastic-adsorbed BLf and Otf (0.5–1 mg/ml) quantified by means of ELISA. The absorbance at 405 nm depicted along the ordinate was obtained by subtracting from the original values the background values which were obtained by incubating the plastic adsorbed proteins with buffer instead of HSV-1. Data are expressed as mean ± S.D. ($n = 3$).

3.6.1. HSV-1 binding to latex beads coated with BLf or Otf

The putative binding of BLf and Otf to HSV-1 was evaluated with a rapid PAA by coating latex beads with the proteins. Viral suspensions (1×10^6 PFU/ml) in 0.004 M potassium phosphate buffer (pH 8) caused the clumping of latex beads coated with BLf. Strongly positive (+ +) BLf-PAA reactions were readily visible within 1 min. Otf-PAA showed negative reactions. Appropriate controls, performed with uncoated latex beads or ovalbumin-coated latex beads in order to check non-specific agglutination reactions, always gave negative agglutination.

3.6.2. HSV-1 binding to plastic-adsorbed BLf or Otf

The binding of HSV-1 to BLf and Otf was investigated by an ELISA procedure. In this test, plastic-adsorbed proteins were incubated with either virus or PBS-BSA (Fig. 4). Control experiments in which HSV-1 was incubated in the absence of proteins were also carried out. Binding of HSV-1 to BLf (0.5–1.0 mg/ml) was clearly stronger than to BSA-treated plastic surfaces, whereas no significant differences in the binding to plastic-adsorbed Otf and controls could be observed.

4. Discussion

Lactoferrin is an iron binding glycoprotein secreted by polymorphonuclear leukocytes as well as by exocrine glands, which is found in milk and at the mucosal surfaces (Brock, 1980; Chasteen and Woodworth, 1990). Its role in the human defense against bacterial and mycotic infections has been recognized (Bullen et al., 1978; Brock, 1980; Bellamy et al., 1992). Since iron is essential for microbial growth, the activity of lactoferrin has been commonly attributed to its ability to sequester environmental iron (Bullen et al., 1978; Chasteen and Woodworth, 1990). However, many lines of evidence demonstrated a more complex antimicrobial mechanism of this protein, independent from its iron withholding ability, consisting in a direct damage of the Gram-negative bacterial outer membrane (Ellison et al., 1988). Lactoferrin has been also shown to modulate bacterial invasion and phagocytosis (Longhi et al., 1993; Donnarumma et al., 1995).

Results reported here provide evidence for the existence of a further biological role of lactoferrin as we demonstrated that human and bovine lactoferrin potently inhibit infection of Vero cells by HSV-1. It could be hypothesized that the antiviral effect of lactoferrins was linked to their iron-binding property, similar to other iron-chelating substances known as inhibitors of herpesvirus ribonucleotide reductases (Spector et al., 1989, 1991). In contrast, their effect towards HSV-1 infection does not appear related to the iron-withholding from the environment since no significant differences in the inhibition could be found between lactoferrins in apo- and iron-saturated form.

Our findings suggest that the block of infection occurs during the very early phases of the viral multiplication cycle, since the highest inhibitory effect took place when lactoferrins were added during the attachment step. It must be emphasized that in this experimental condition, both viral binding of [^{35}S]methionine-labelled HSV-1

particles and the rate of viral penetration were almost completely inhibited, clearly demonstrating that BLf acts on viral adsorption to Vero cells.

To formulate an hypothesis on the mechanism of the antiviral action of lactoferrins, the events involved in the adsorption and entry of HSV-1 virions into cells must be considered. The virion-associated glycoproteins B and C possess aminoacidic domains interacting with at least two receptors on the cell membrane. Heparan sulphate, a proteoglycan present on cell membranes acts as a receptor for a polycationic arginine-rich segment of HSV-1 gC and for HSV-1 gB (Herold et al., 1994; Trybala et al., 1994), and the LDL receptor, present in coated pits of the cell membranes, seems to bind a minority of virions, probably interacting with HSV-1 gB, gC or gD polypeptides (Becker et al., 1994).

The antiviral effect of lactoferrins correlates well with their affinity for the virus receptor binding sites. In fact, polyanionic glycosaminoglycan chains of heparan sulphate and apo-lipoprotein-E receptor have been shown to interact with lactoferrins which are well known for their highly cationic features (Pierce et al., 1991). Consequently, it can be assumed that the capability of lactoferrins to inhibit HSV-1 infection at the level of viral attachment may rely to a large extent on their competitive interaction with cell receptors for HSV-1 which can hinder the binding of the virus attachment proteins. The negative electric charge of ovotransferrin should be taken into account to explain its lack of antiviral activity.

BLf was a significantly better inhibitor than HLf, its selectivity index being over 10-fold higher. A 25-fold lower concentration of BLf was needed to exert a complete inhibition of viral infectivity. Thus, as the effectiveness of BLf was markedly higher than that of HLf, we focused our interest on the deeper understanding of its mechanism of inhibition. The effect of BLf on HSV-1 infection probably involves more than a simple mechanism of interference at the level of cell receptors. This hypothesis has been supported by our findings that virus binds efficiently to BLf immobilized on a solid-phase surface, as revealed by an ELISA method, and causes the rapid agglutination of BLf coated latex beads. These experiments allowed demonstration of a direct interaction of bovine lactoferrin with virus particles which could play a pivotal role in the antiviral mechanism. Nevertheless, taking into account that many outer envelope proteins of HSV-1 are involved in virus-cell early interactions, further studies are needed to evaluate which one of these viral proteins can act as a binding site for BLf.

It can be put forward that the lower activity of HLf against HSV-1, as compared with that of BLf, is linked to differences in the molecular structure. Bovine lactoferrin is 69% identical to human lactoferrin, but, in spite of this high degree of similarity, their comparison shows that the glycan chains of the molecules and the number of disulphide bridges vary (Metz-Boutigue et al., 1984; Pierce et al., 1991). These variations are likely to contribute to differences in the functional domains responsible for the binding properties of the lactoferrins to host cells and viral particles.

Thus, although a large number of antiviral drugs with different targets has been recognized to control infections caused by viruses belonging to the *Herpesviridae* family (De Clercq, 1993; De Clercq et al., 1980), the present study can represent a useful tool in the discovery of new natural anti-herpetic drugs. In fact, among non-antibody inhibitors, we identified the lactoferrins which, besides the well known role in the non-immune defence system, exert also a significant effect toward the early steps of HSV-1 infection.

Acknowledgments

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