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Bovine lactoferrin peptidic fragments involved in inhibition of Echovirus 6 in vitro infection

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Abstract

Bovine lactoferrin is a multifunctional glycoprotein folded in two symmetric globular lobes (N- and C-lobes), each being able to bind one ferric ion. We have previously demonstrated that this protein is able to prevent echovirus-induced apoptosis. In the present study, we have investigated both the role of tryptic fragments of bovine lactoferrin and the mechanism of lactoferrin effect on echovirus infection. Results obtained showed that bovine lactoferrin inhibits echovirus-induced cytopathic effect and antigen synthesis in a dose-dependent manner and that this protein is able to prevent viral replication when added not only during the entire cycle of infection but also before, during or after the viral adsorption step. The N-terminal cationic peptide was sufficient to prevent viral binding. Our data suggest that lactoferrin inhibition of echovirus attachment to cell receptors could be mediated by the cluster of positive charges at its N-terminus (lactoferricin).

Keywords: Lactoferrin; Echovirus; N-lobe; Lactoferricin; Antiviral activity

1. Introduction

Echoviruses are small, naked, single-stranded RNA viruses which are members of the enterovirus genus. Enteroviruses, belonging to the Picornaviridae family, represent 68 human serotypes that have been classified into five species (A–D and the polioviruses). In particular, human enteroviruses are enterically transmitted viruses responsible for various illnesses among infants and children, with a seasonal peak of infection in the summer and autumn. Aseptic meningitis, which affects mainly very young children, is the most commonly encountered illness associated with enterovirus infections, and members of the human enterovirus B species, especially echovirus and coxsackievirus B, have been recognized as the most common viruses responsible for aseptic meningitis worldwide (Hyypiä et al., 1997; Zeichard and Grunert, 2000). Echoviruses may also be the cause of devastating disease and death in neonates (John and Walker, 1999; Modlin, 2000; Ventura et al., 2001). In adults and immunocompetent children fatal echovirus infections are rare, while neonates are more receptive to echovirus infection, probably because of a relative immunodeficiency and of a lack of transplacentally acquired specific neutralizing antibodies. It has also been demonstrated that breast-feeding is strictly associated with protection from enterovirus infection (Jenista et al., 1984).

It is well known that milk, in addition to secretory IgA and IgM, also contains numerous non-antibody components with known antiviral activity, including lactoferrin (Laegreid et al., 1986; Levay and Viljoen, 1995; Peterson et al., 1998; Portelli et al., 1998). Bovine lactoferrin (bLf) is a glycoprotein consisting of a single polypeptide chain of 689 amino acidic residues, with a molecular mass of about 80 kDa, which binds two iron atoms with very high affinity (Legrand et al., 1990). Bovine lactoferrin, like lactoferrin of other mammalian species, is folded in two symmetric globular lobes. Each one is itself folded into two domains (N-lobe: N1 and N2; C-lobe: C1 and C2) each containing a Fe^{3+} binding site (Norris et al., 1986; Moore et al., 1997). Moreover, bLf has an alkaline isoelectric point (about p*I* 9) and its cationic nature could have a major role in the ability to bind cells and many anionic molecules, such as glycosaminoglycans. In particular, at physiological pH, bLf possesses noticeable

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clusters of positively charged amino acids at the N-terminus that have been shown to be important for glycosaminoglycan and lipopolysaccharide binding (Shimazaki et al., 2000). BLf is present in various biological fluids and in specific granules of polymorphonuclear leukocytes (Gennaro et al., 1983), and possesses 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). BLf has been recognized as potent inhibitors of the replication of different enveloped viruses such as human cytomegalovirus (HCMV) (Hasegawa et al., 1994; Harmsen et al., 1995; Andersen et al., 2001), herpes simplex virus (HSV) 1 and 2 (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), human hepatitis C virus (HCV) (Ikeda et al., 1998, 2000), hantavirus (Murphy et al., 2000), and hepatitis B virus (Hara et al., 2002). The antiviral effect of bLf 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; Drobni et al., 2004; Seganti et al., 2004; Tinari et al., 2005). Although for almost all viruses investigated to date bLf exerts its inhibiting activity in the early phases of infection, its antiviral effect seems to be exercised in different ways among different viral species. A direct bLf binding to HIV-1 (Swart et al., 1996), hepatitis C virus envelope proteins (Yi et al., 1997), rotavirus (Superti et al., 1997), poliovirus (Marchetti et al., 1999), adenovirus (Pietrantoni et al., 2003), and enterovirus 71 (Weng et al., 2005) has been suggested. Moreover, as bLf is known to bind cell surface heparan sulphate containing proteoglycans (Ji and Mahley, 1994), which in turn act as binding sites for different viruses such as HSV-1, human papillomavirus, and adenovirus (WuDunn and Spear, 1989; Giroglou et al., 2001; Dechecchi et al., 2001), its inhibiting activity on these viruses has been ascribed to a competition for cell receptors (Di Biase et al., 2003; Marchetti et al., 2004; Drobni et al., 2004). Interestingly, a further effect on a later intracellular step of virus infection has been described for rotavirus (Superti et al., 1997) and, more recently, we demonstrated that bLf treatment can prevent echovirus-induced programmed cell death (Tinari et al., 2005). Twenty-five amino acid residues of the N-terminal part of bLf correspond to a peptide called lactoferricin (bLfcin), which is generated upon gastric cleavage of the protein (Tomita et al., 1991). Even if an activity of bLfcin against bacteria, fungi, and protozoa (Bellamy et al., 1992, 1993; Isamida et al., 1998; Turchany et al., 1995), is well known, an antiviral activity of this peptide has only recently been reported for herpesviruses (Hammer et al., 2000; Andersen et al., 2001, 2003, 2004), feline calicivirus (McCann et al., 2003), and adenovirus (Di Biase et al., 2003). It has been also demonstrated that bLfcin is able to bind glycosaminoglycans and, in particular, heparin (Shimazaki et al., 1998).

In the present study, we have attempted to clarify the mechanism of the anti-echovirus 6 effect of bLf and the role of its tryptic fragments (the N- and C-lobes and the N-terminal peptide bLfcin) in the antiviral activity. Results obtained demonstrated that the anti-echovirus activity of bLf is due to an interaction with a very early phase of the viral infection. In particular, the activity of the N-lobe was comparable to that of the entire protein, in that it was able to prevent both viral binding to cell receptors and the step immediately after virus attachment. Moreover, we demonstrated that the fragment containing 25 amino acid residues (17–41) of the N-terminal part of bLf, corresponding to bLfcin, sufficed to prevent echovirus 6 attachment to target cells.

2. Materials and methods

2.1. Cells

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).

2.2. Virus

Echovirus type 6 (kindly provided by Prof. Lucilla Seganti, University “La Sapienza”, Rome) was grown in GMK cells. Virus was inoculated onto confluent monolayers grown in roller bottles at a multiplicity of infection (m.o.i.) of 1 plaque forming unit (p.f.u.)/cell. After 90 min at 37 °C, the inoculum was removed, and the monolayers were washed once in phosphate-buffered saline (PBS, pH 7.4) 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 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. This stock virus was titered by plaque assay on GMK cells.

2.3. Lactoferrin

Lactoferrin from bovine milk (bLf), kindly supplied by Dicofarm (Rome, Italy), 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).

2.4. Endotoxin removal from bLf

BLf was solubilized in NaCl 0.5 M (pyrogen-free) at a concentration of 10 mg/ml. The removal of lipopolysaccharide (LPS; Endotoxin), putatively conjugated to bLf, was obtained by Detoxy-Gel (Pierce Chemical Company, Rockford, IL, USA). The Detoxy-Gel support consisted of polimyxin B, immobilized on agarose, which binds and removes pyrogen from solutions.

Briefly, 1 ml of the gel, packed in a column, was washed with five column volumes of 1% sodium deoxycholate to strip the putatively bound endotoxin from the gel. Then, five washings of pyrogen-free water were performed to remove the detergent and the gel was equilibrated with NaCl 0.5 M (pyrogen-free). Successively 10 mg/ml of bLf were applied and 300 ml of the sample were eluted at time 0. After 1 h incubation, 200 ml of NaCl 0.5 M (pyrogen-free) were added and the detoxified bLf was collected.

2.5. Enzymatic hydrolysis of bLf and HPLC separation, purification and characterization of N- and C-lobes

BLf (4 mg/ml) was dissolved in 50 mM ammonium bicarbonate, pH 8.5, and trypsin (from bovine pancreas TPCK treated, Sigma Chemical Company, St. Louis, MO) digestion was performed at 37 °C overnight using an enzyme to substrate ratio of 1:50 (w/w). The N- and C-lobes obtained by enzymatic hydrolysis were purified by reverse phase HPLC on a Vydac C18 column (250 mm × 10 mm, 5 μm) using a Waters HPLC System (Datasystem Millenium, HPLC pumps Waters 510, Detector Waters 486). Eluents were 0.1% trifluoroacetic acid (solvent A) and 0.07% trifluoroacetic acid in 95% acetonitrile (solvent B). The elution was performed by means of a short linear gradient from 35 to 55% solvent B over 20 min at a flow rate of 3.5 ml/min and monitored at 220 nm. The trypsin-containing fraction was discharged since it did not co-elute with fractions containing bLf lobes. In addition, control experiments did not show residual tryptic activity in the digested fractions of bLf since the enzyme underwent auto-hydrolysis and inactivation due to denaturing conditions of HPLC. Electrophoretic analysis (SDS-PAGE) of the HPLC fractions was carried out using 12.5% gels stained by Comassie Blue R250. Purification and characterization of bLf N- and C-lobes were carried out as previously described (Siciliano et al., 1999; Superti et al., 2001). Fractions containing bLf lobes were collected, dried in a Speed-Vac centrifuge (Savant), lyophilized twice and stored at −20 °C.

2.6. Lactoferricin

BLfcin was isolated according to Recio and Visser (1999), dissolved in minimal essential medium (Gibco, Paisley, UK), pH 7.2, at a concentration of 4 mg/ml, sterilized by filtration with 0.45 μM Millipore filters (Millipore SA, Molsheim, France) and stored at −20 °C in 1 ml aliquots.

2.7. Cytotoxicity assay

To establish the maximal non-cytotoxic dose of proteins, two-fold serial dilutions of each substance in MEM were incubated at 37 °C with confluent GMK cells grown in 96-well tissue culture microplates (Flow Laboratories). After 24, 48, and 72 h, the following parameters were evaluated: cell morphology and viability (determined by neutral red staining) were examined by light microscopy, cell enumeration 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. Protein dilutions that did not affect any of these parameters were considered as non-cytotoxic concentrations and utilized for antiviral assays.

2.8. Action of bLf on echovirus antigen synthesis and viral cytopathic effect

GMK cells grown in tissue culture chamber slides (Lab-Tek, Nunc Laboratories) or 96-well tissue culture microplates for 24 h at 37 °C in 5% CO_2, were incubated with different concentrations of bLf during the virus attachment step (1 h, 4 °C). As viral inoculum was utilized echovirus at a m.o.i. of 1 p.f.u./cell. Then, cells were rinsed thoroughly and incubated with the same concentrations of bLf for 5 or 24 h at 37 °C in 5% CO_2. Viral antigen synthesis was monitored at 5 h after infection by indirect immunofluorescence. The cytopathic effect induced by echovirus was measured at 24 h after infection by the neutral red uptake assay as previously described (Marchetti et al., 1996). Results are expressed as percentage of fluorescence or cytopathic effect inhibition by comparison with untreated infected control cultures.

2.9. Effect of bLf, N- and C-lobes and bLfcin on different steps of echovirus infection

GMK cells, grown in tissue culture chamber slides (Lab-Tek, Nalge Nunc International, Rochester, NY) for 24 h at 37 °C in 5% CO_2, were infected with echovirus (1 p.f.u./cell) for 1 h at 4 °C, then viral inoculum was removed, cell monolayers were washed three times with MEM and incubated at 37 °C in 5% CO_2. Virus antigen synthesis was monitored at 5 h after infection by indirect immunofluorescence. To ascertain whether the antiviral effect of proteins took place on viral adsorption or on a different step of viral replication, the inhibiting activity of 12.5 μM bLf, N- or C-lobes, and bLfcin was assessed by the following experimental procedures: (i) the cells were incubated with proteins (30 min at 37 °C), washed three times with medium and than infected (1 h at 4 °C), (ii) proteins were added together with the virus inoculum during the adsorption step (1 h at 4 °C), (iii) proteins were incubated with the cells after the viral adsorption step (5 h at 37 °C), and (iv) proteins were present during the whole experiment.

2.10. Time of addition assay

BLf, N-lobe, and bLfcin (12.5 μM) were also tested in a time of addition assay. For these experiments, after the viral attachment step (1 h at 4 °C) cells were washed twice with MEM and incubated at 37 °C. The inhibiting activity of 12.5 μM bLf, N-lobe, and bLfcin was assessed by two different experimental procedures: (i) proteins were added to the cells after the temperature shift and incubated for different lengths of times and (ii) proteins were 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 5 h after infection by indirect immunofluorescence.

2.11. Immunofluorescence

GMK infected cells were washed in PBS, fixed in acetone at −20 °C for 5 min, incubated with monoclonal anti-echovirus 6 antibodies (Immunological Sciences, Rome, Italy) for 45 min at 37 °C. After washing in PBS, viral antigen synthesis was estimated by utilizing (FITC)-conjugated anti-mouse gamma-globulin antibodies (Sigma Chemical Company) and an UV Leitz microscope.

2.12. Transmission electron microscopy

For transmission electron microscopy (TEM), infection was synchronized (1 h at 4 °C) and proteins were incubated with the cells after the viral adsorption step. Mock-infected cells, virus-infected cells, and infected cells (m.o.i. 1) treated with bLf, N-, C-lobe or lactoferricin were harvested at 24 h post infection, washed in PBS, and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 20 min. Cells were then post-fixed in 1% osmium tetroxide in cacodylate buffer, dehydrated through a graded series of ethanol solutions and, finally, embedded in Agar 100 epoxy resin. Thin sections were stained with lead citrate and uranyl acetate and examined with a Philips 208s electron microscope.

2.13. Statistical analysis

Statistical analysis was performed using the Student's *t*-test for unpaired data. Data were expressed as the mean ± S.D. and *p*-values of <0.05 were considered significant.

3. Results

3.1. Cytotoxicity

A preliminary set of experiments was carried out in order to determine the maximal non-cytotoxic concentration of bLf, bLf-derived N- and C-lobe, and bLfcin. For this purpose, two-fold serial dilutions of proteins up to 25 μM in MEM were incubated with GMK cells for 24, 48, and 72 h at 37 °C. Under these conditions, all compounds tested did not affect any of the cytotoxicity parameters up to the highest concentration used.

3.2. Action of bLf on echovirus infection

To establish whether bLf could inhibit echovirus antigen synthesis and cytopathic effect in GMK cells, two-fold serial dilutions of protein, starting from the highest non-cytotoxic concentration, were incubated with the cells throughout the infection (5 and 24 h at 37 °C). Under both experimental conditions, bLf showed a concentration-dependent inhibitory activity, being able to completely prevent infection at the concentration of 12.5 μM (Fig. 1).

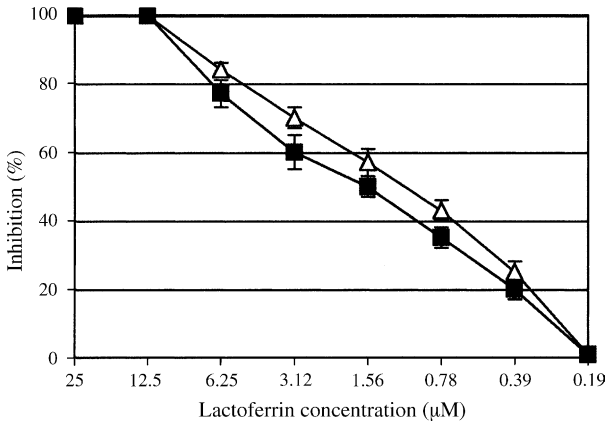

Fig. 1. Concentration-response curves of bLf towards echovirus cytopathic effect (c.p.e.) and antigen synthesis [determined by immunofluorescence (IF)] in GMK cells. BLf was present throughout the infection (1 h at 4 °C during virus adsorption, plus 24 or 5 h at 37 °C). Data showed represent the mean of at least quadruplicate samples. S.D. < 5% are not shown. (△) c.p.e. and (■) IF.

3.3. Effect of bLf on different steps of viral infection

To ascertain whether the antiviral effect of bLf took place on viral adsorption or on a different step of viral replication, the inhibiting activity of protein (12.5 μM) was assessed by the following experimental procedures: (i) the cells were incubated with bLf (30 min at 37 °C), washed three times with medium and than infected (1 h at 4 °C), (ii) bLf was added together with the virus inoculum during the adsorption step (1 h at 4 °C), (iii) bLf was incubated with the cells after the viral adsorption step (5 h at 37 °C), and (iv) bLf was present during the whole experiment. Echovirus antigen synthesis was measured by immunofluorescence. Results reported in Fig. 2 show that a high inhibition was obtained under the conditions used in all procedures (i)–(iv).

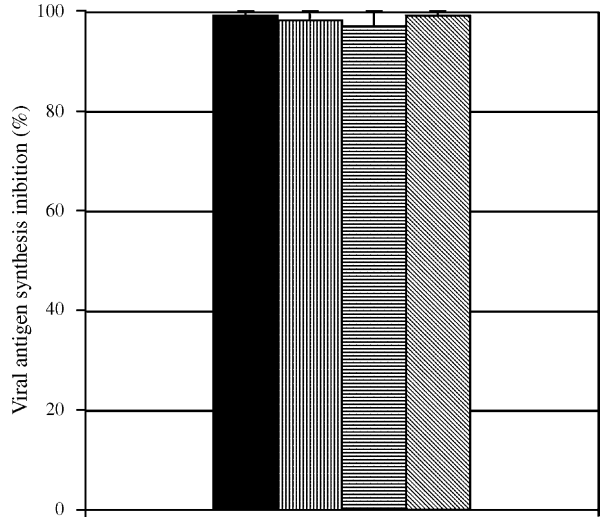

Fig. 2. Effect of 12.5 μM bLf on echovirus antigen synthesis. BLf was incubated with the cells before (30 min, 37 °C) (■), during (1 h, 4 °C) (▥), after (5 h, 37 °C) the viral adsorption step (▤) or during the whole experiment (▨). Virus antigen synthesis was monitored at 5 h after infection by immunofluorescence. Data shown represent the mean of at least quadruplicate samples. For each sample, 600 cells were examined.

3.4. Effect of bLf peptidic fragments on different steps of viral infection

As bLf was able to inhibit different steps of echovirus infection, other experiments were carried out to analyze the role of N- and C-lobes of bLf and of its N-terminal peptide bLfcin in the prevention of viral adsorption or other steps of viral infection. In these experiments, the inhibiting activity of bLf peptidic fragments was assessed as previously described for bLf. Briefly, 12.5 μM N- or C-lobes or bLfcin were added to the cells before (30 min at 37 °C) or during (1 h at 4 °C) or after (5 h at 37 °C) the viral adsorption step or were present during the whole experiment. Echovirus antigen synthesis was measured by immunofluorescence and results obtained were compared to that achieved with the entire protein.

Results obtained are reported in Fig. 3. Under all experimental conditions, a high inhibition of viral infection was obtained with N-lobe, whereas C-lobe showed a very slight inhibitory activity only when present during the post-adsorption step. BLfcin was able to prevent infection when present during the virus attachment step (1 h at 4 °C) and it retained some of its inhibiting effect also when added to the cells after the initial binding of the virus (5 h at 37 °C). The inhibitory effect of bLfcin was highly reduced when the peptide was pre-incubated with the cells for 30 min at 37 °C before infection.

3.5. Addition time point assay

The effect of bLf, N-lobe, and bLfcin on viral post-adsorption step was further investigated and experiments were carried out in which cells were incubated with the virus for 1 h at 4 °C and then proteins (12.5 μM) were added for various lengths of time (Fig. 4A) or at different times (Fig. 4B) after the viral attachment step. It was found that bLf totally prevented viral antigen synthesis when present for only 10 min after infection (Fig. 4A), whereas when it was added at 30 min after infection, the inhibition was 40% and it was ineffective when added 2 h after viral internalization (Fig. 4B). A similar behaviour was observed with the N-lobe, whereas bLfcin was less inhibitory as it was completely deprived of activity when added 30 min after viral adsorption (Fig. 4B).

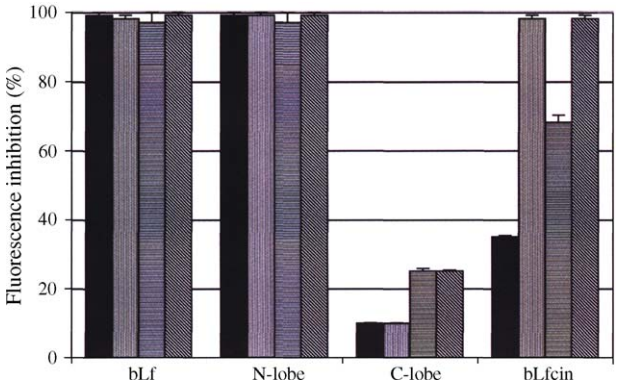

Fig. 3. Effect of bLf and its tryptic fragments (each at 12.5 μM) on echovirus antigen synthesis. BLf, N-, C-lobe, and bLfcin were incubated with the cells before (30 min, 37 °C) (■), during (1 h, 4 °C) (▥), after (5 h, 37 °C) the viral adsorption step (▤), or during the whole experiment (▧). Virus antigen synthesis was monitored at 5 h after infection by immunofluorescence. Data shown represent the mean of at least quadruplicate samples. For each sample, 600 cells were examined.

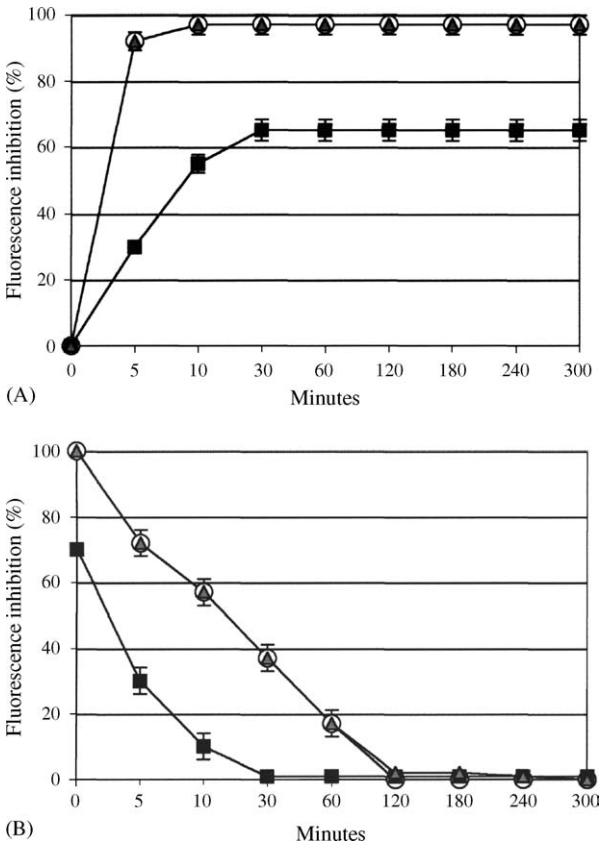

Fig. 4. Addition time effect of 12.5 μM bLf, N-lobe, and bLfcin on echovirus infection. Proteins were added for various lengths of time immediately after the 1 h virus adsorption period (A) or at different times (B) after the viral attachment step. Viral antigen synthesis was measured by immunofluorescence 5 h after infection. Data shown represent the means of at least quadruplicate samples. S.D. < 5% are not shown. (◯) bLf, (■) bLfcin, and (▲) N-lobe.

As bLf and N-lobe showed a similar anti-echovirus activity, experiments were carried out in which two-fold serial dilution of N-lobe, starting from the highest non-cytotoxic concentration, were incubated with the cells throughout the infection (5 h at 37 °C). The ratio between the 50% N-lobe cytotoxicity concentration (CC_{50}: >25 μM) and the concentration required to inhibit the viral antigen synthesis by 50% (EC_{50}: 1.56 μM) was calculated in order to determine the selectivity index (SI) of the N-lobe that was >16.

3.6. Transmission electron microscopy

The effect of bLf and bLf derived peptidic fragments (12.5 μM) on viral infection was also analyzed at ultrastructural level. For these experiments mock-infected cells, virus-infected cells (m.o.i. 1 and 24 h), and infected cells treated with bLf, N-, C-lobe, or bLfcin were processed for transmission electron microscopy (Fig. 5). As expected, both bLf-treated (Fig. 5C) and N-lobe-treated (Fig. 5D) infected cells appeared similar to mock-infected cells (Fig. 5A), did not displaying pathological mor-

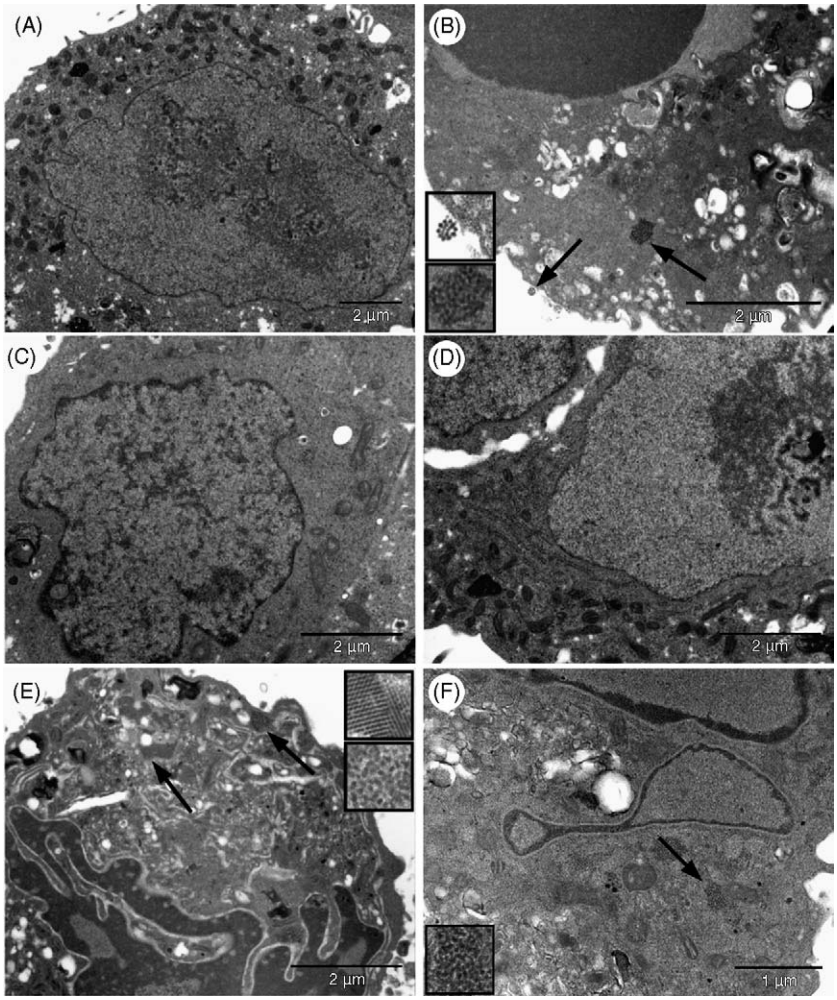

Fig. 5. Electron micrographs showing the effect of bLf peptidic fragments on echovirus infection. (A) Mock-infected cell. (B) Echovirus-infected cells without any treatment display viral particles at different stages of their morphogenesis: insets show clusters of both cytoplasmic particles and extracellular mature virions. (C) BLf-treated infected cells and (D) N-lobe-treated infected cells do not exhibit any presence of viral morphogenesis and appear similar, in their ultrastructure, to mock-infected ones. (E) In C-lobe-treated infected cells, various stages of viral morphogenesis are detectable: insets show a typical paracrystalline inclusion of viruses under formation and a cluster of viral particles inside the cytoplasm. (F) In bLfcin-treated infected cells, viral particles are present in the cell cytoplasm only, as shown in inset. Arrows indicate viral paracrystalline inclusions or clusters of virions shown in the insets.

phological changes or presence of viral particles. Only infected cells in which viral morphogenesis took place (Fig. 5B, E, and F) displayed typical morphological markers of programmed cell death, such as chromatin condensation and marginalization and cytoplasm derangement. Interestingly, in bLfcin-treated infected cells (Fig. 5F) apoptosis seems to be at an earlier stage, displaying only initial chromatin marginalization in the absence of cytoplasm injury. Moreover, echovirus-infected cells (Fig. 5B) and C-lobe-treated infected cells (Fig. 5E) showed the presence of numerous viral particles. In these two last experimental conditions, typical viral paracrystalline inclusion and cluster of virions both inside and outside the cytoplasm were detectable. Finally, in bLfcin-treated cells (Fig. 5F) infection seems to be delayed, as viral particles were never observed outside of the cytoplasm.

4. Discussion

This is the first study showing that the attachment of echovirus to susceptible cells and the first steps of echoviral infection are prevented by bLf. Several studies analyzed the effect of lactoferrin on picornavirus infection with different results. In a previous study (Marchetti et al., 1999), we demonstrated that bLf and human lactoferrin (hLf) inhibited the early phases of poliovirus infection, whereas they were ineffective when added after the viral adsorption step. Successively, it was reported that hLf did not affect rhinovirus growth (Clarke and May, 2000), whereas bLf exerted an inhibitory effect on enterovirus 71 attachment to target cells (Lin et al., 2002) and was able to bind enterovirus 71 VP1 protein (Weng et al., 2005). As we have recently demonstrated that bLf was able to prevent echovirus-mediated programmed cell death (Tinari et al., 2005), in the present research we performed further experiments to better characterize the anti-echovirus activity of bLf and to identify the bLf structural domains responsible for this effect.

For this purpose, experiments were carried out to determine which step of echovirus infection was susceptible to bLf treatment. Results obtained indicate that bLf exerts its effect not only on virus adsorption or receptor-mediated binding to target cells,

as already reported for several virus–cell systems (Valenti et al., 1998; Marchetti and Superti, 2001; van der Strate et al., 2001; Arnold et al., 2002; Seganti et al., 2004), but, more interestingly, once the virus has bound to the receptor it was still able to prevent infection. To find out the role played by different bLf moieties in such double antiviral effect, bLf was enzymatic digested to obtain the N- and C-lobes and the cationic peptide bLfcin (Shimazaki et al., 1998; Andersen et al., 2001).

Our results on the anti-echovirus effect of N-, C-lobe, and bLfcin, demonstrate that only N-lobe and bLfcin are able to inhibit viral infection, as already reported for adenovirus (Di Biase et al., 2003). Conversely, it has to be underlined that the antiviral activity of bLf and N-lobe on a post-adsorption step was not observed for adenovirus (Arnold et al., 2002).

Moreover, in our virus–cell system, N-lobe displays the same anti-echoviral activity as the entire protein and bLfcin alone is able to prevent viral attachment to target cells. It must be pointed out that, in our experimental conditions, we utilized the cyclic form of bLfcin and this could explain the strong anti-echovirus activity because, as previously reported for cytomegalovirus, herpes simplex virus, and adenovirus (Andersen et al., 2001, 2003; Di Biase et al., 2003), bLfcin must be in its cyclic form to exert antiviral activity. BLfcin completely prevents viral infection when added during the attachment step, whereas when pre-incubated with the cells before infection, a very slight inhibition of viral antigen synthesis was observed, probably because this peptide enters cell membranes. Moreover, bLfcin was able to inhibit viral antigen synthesis by about 70% when added to the cells after the viral attachment step. Our results are in agreement with data previously reported by Andersen et al. (2004), which demonstrated that bLfcin exerts an anti-herpes simplex virus activity also after the initial binding of the virus to the host cells. The same authors reported that bLf had no effect against herpes simplex virus after viral entry. In regard of HIV-1, it has been demonstrated that bLf targets the entry process, showing a considerable inhibitory activity, whereas modest inhibition was obtained with blfcin, indicating that other domains within the native bLf protein may also be required for inhibition (Berkhout et al., 2002).

It is well known that bLf is a potent antiviral agent acting on early phases of infection and it has been demonstrated that this glycoprotein is able to bind heparan sulphate (HS)-glycosaminoglycans present on cell surfaces (Ji and Mahley, 1994) and that some positively charged amino acid residues involved in this binding are located on N-lobe (Shimazaki et al., 2000). It has also been demonstrated that bLfcin is able to bind glycosaminoglycans and, in particular, heparin (Shimazaki et al., 1998). As cell surface HS-glycosaminoglycans are co-receptors for different pathogens (Rostand and Esko, 1997), it has been suggested that bLf binding to HS blocks cell receptors for several viruses such as adenovirus (Di Biase et al., 2003), herpesvirus (Marchetti et al., 2004; Andersen et al., 2004), papillomavirus (Drobni et al., 2004), and enterovirus 71 (Lin et al., 2002). With regard to echoviruses, it has been reported that some of these, in particular echovirus 6, are able to bind HS (Goodfellow et al., 2001). These published studies demonstrated that: (i) HS binding phenotype is widespread within the human enterovirus B species; (ii) HS binding by echovirus may be of in vivo relevance. So it can be speculated that the effect of bLf, its N-lobe, and bLfcin on echovirus binding could be due to a direct competition for a common glycosaminoglycan receptor.

To further investigate the step of viral infection sensitive to the action of bLf, N-lobe, and bLfcin, experiments were carried out in which these molecules were incubated with the cells for different periods of time or were added to the infected monolayers at different times after viral binding. Results from time of addition experiments showed that bLfcin acts on an earlier step of viral infection, probably by a competition with viral particles for common cell receptors, whereas both bLf and N-lobe also exert some inhibitory effects also on internalization.

The effect of bLf and its derived tryptic fragments on the viral post-adsorption step was also investigated at the ultrastructural level. Transmission electron microscopy studies revealed that in bLfcin-treated infected cells, when positive for the presence of echovirus particles, cytopathological changes and viral replication cycle seems to be delayed, compared to untreated infected cells or C-lobe-treated infected cells. These results suggest that bLfcin, competing for common glycosaminoglycan cell receptor, could delay echovirus entry. This observation confirmed results of time of addition studies showing that bLfcin is deprived of action when added to the cells at 30 min after viral internalization.

In conclusion, our findings provide further evidences that bLfcin plays an important role in the modulation of infection by viruses. The higher antiviral activity of bLf or its N-lobe fragment at a post-adsorption step, compared to bLfcin, suggests that other domains, in addition to those involved in glycosaminoglycan binding, are important for the anti-echovirus activity of bLf. Therefore, we can conclude that the inhibitory effect of bLf on echovirus infection is mediated by the N-lobe and can be attributed to its interference with viral binding to glycosaminoglycans (mediated by bLfcin) and with an early event of infection such as viral internalization. Taken together results of our study suggest bLf as an excellent candidate for the prevention or treatment of enteroviral infections, as it is able not only to hinder viral adsorption to the cells but also to prevent early events of infection.

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References

Andersen, J.H., Osbakk, S.A., Vorland, L.H., Traavik, T., Gutteberg, T.J., 2001. Lactoferrin and cyclic lactoferricin inhibit the entry of human cytomegalovirus into human fibroblasts. Antiviral Res. 51, 141–149.

Andersen, J.H., Jenssen, H., Gutteberg, T.J., 2003. Lactoferrin and lactoferricin inhibit herpes simplex 1 and 2 infection and exhibit synergy when combined with acyclovir. Antiviral Res. 58, 209–215.

Andersen, J.H., Jenssen, H., Sandvik, K., Gutteberg, T.J., 2004. Anti-HSV activity of lactoferrin and lactoferricin is dependent on the presence of heparan sulphate at the cell surface. J. Med. Virol. 74, 262–271.

Arnold, D., Di Biase, A.M., Marchetti, M., Pietrantoni, A., Valenti, P., Seganti, L., Superti, F., 2002. Antiadenovirus activity of milk proteins: lactoferrin prevents viral infection. Antiviral Res. 53, 153–158.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., Tomita, M., 1992. Identification of the bactericidal domain of lactoferrin. Biochim. Biophys. Acta 1121, 130–136.

Bellamy, W., Wakabayashi, H., Takase, M., Kawase, K., Shimamura, S., Tomita, M., 1993. Killing of *Candida albicans* by lactoferricin B, a potent antimicrobial peptide derived from the N-terminal region of bovine lactoferrin. Med. Microbiol. Immunol. 182, 97–105.

Berkhout, B., van Wamel, J.L., Beljaars, L., Meijer, D.K., Visser, S., Floris, R., 2002. Characterization of the anti-HIV effects of native lactoferrin and other milk proteins and protein-derived peptides. Antiviral Res. 55, 341–355.

Clarke, N.M., May, J.T., 2000. Effect of antimicrobial factors in human milk on rhinoviruses and milk-borne cytomegalovirus in vitro. J. Med. Microbiol. 49, 719–723.

Dechecchi, M.C., Melotti, P., Bonizzato, A., Santacatterina, M., Chilosi, M., Cabrini, G., 2001. Heparan sulfate glycosaminoglycans are receptors sufficient to mediate the initial binding of adenovirus types 2 and 5. J. Virol. 75, 8772–8780.

Di Biase, A.M., Pietrantoni, A., Tinari, A., Siciliano, R., Valenti, P., Antonini, G., Seganti, L., Superti, F., 2003. Heparin-interacting sites of bovine lactoferrin are involved in anti-adenovirus activity. J. Med. Virol. 69, 495–502.

Drobni, P., Naslund, J., Evander, M., 2004. Lactoferrin inhibits human papillomavirus binding and uptake in vitro. Antiviral Res. 64, 63–68.

Fujihara, T., Hayashi, K., 1995. Lactoferrin inhibits herpes simplex virus type-1 (HSV-1) infection in mouse cornea. Arch. Virol. 140, 1469–1472.

Gennaro, R., Dewald, B., Horisberger, U., Gubler, H.U., Baggiolini, M., 1983. A novel type of cytoplasmic granule in bovine neutrophils. J. Cell. Biol. 96, 1651–1661.

Giroglou, T., Florin, L., Schafer, F., Streeck, R.E., Sapp, M., 2001. Human papillomavirus infection requires cell surface heparan sulfate. J. Virol. 75, 1565–1570.

Goodfellow, I.G., Sioofy, A.B., Powell, R.M., Evans, D.J., 2001. Echoviruses bind heparan sulfate at the cell surface. J. Virol. 75, 4918–4921.

Groves, M.L., 1960. The isolation of a red protein from milk. J. Am. Chem. Soc. 82, 3345–3350.

Hammer, J., Haaheim, H., Gutteberg, T.J., 2000. Bovine lactoferrin is more efficient that bovine lactoferricin in inhibiting HSV-I/-II replication in vitro. In: Shimazaki, K., Tsuda, H., Tomita, M., Kuwata, T., Perraudin, J.P. (Eds.), Lactoferrin: Structure, Function and Applications. Elsevier Science B.V., Amsterdam, pp. 217–224.

Hara, K., Ikeda, M., Saito, S., Matsumoto, S., Numata, K., Kato, N., Tanaka, K., Sekihara, H., 2002. Lactoferrin inhibits hepatitis B virus infection in cultured human hepatocytes. Hepatol. Res. 24, 228–235.

Harmsen, M.C., Swart, P.J., de Béthune, M.P., Pawels, R., De Clercq, E., Thé, T.H., Meijer, D.K.F., 1995. Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication in vitro. J. Infect. Dis. 172, 380–388.

Hasegawa, K., Motsuchi, W., Tanaka, S., Dosako, S., 1994. Inhibition with lactoferrin of in vitro infection with human herpes virus. Jpn. J. Med. Sci. Biol. 47, 73–85.

Hyypiä, T., Hovi, T., Knowles, N.J., Stanway, G., 1997. Classification of enteroviruses based on molecular and biological properties. J. Gen. Virol. 78, 1–11.

Ikeda, M., Sugiyama, K., Tanaka, T., Tanaka, K., Sekihara, H., Shimotohno, K., Kato, N., 1998. Lactoferrin markedly inhibits hepatitis C virus infection in cultured human hepatocytes. Biochem. Biophys. Res. Commun. 245, 549–553.

Ikeda, M., Nozaki, A., Sugiyama, K., Tanaka, T., Naganuma, A., Tanaka, K., Sekihara, H., Shimotohno, K., Saito, M., Kato, N., 2000. Characterization of antiviral activity of lactoferrin against hepatitis C virus infection in human cultured cells. Virus Res. 66, 51–63.

Isamida, T., Tanaka, T., Omata, Y., Yamauchi, K., Shimazaki, K., Saito, A., 1998. Protective effect of lactoferricin against *Toxoplasma gondii* infection in mice. J. Vet. Med. Sci. 60, 241–244.

Jenista, J.A., Powell, K.R., Menegus, M.A., 1984. Epidemiology of neonatal enterovirus infection. J. Pediatr. 104, 685–690.

Ji, Z.S., Mahley, R.W., 1994. Lactoferrin binding to heparan sulfate proteoglycans and the LDL receptor-related protein. Further evidence supporting the importance of direct binding of remnant lipoproteins to HSPG. Arterioscler. Thromb. 14, 2025–2031.

John, T.J., Walker, D.H., 1999. Enterovirus infections including poliomyelitis. In: Guerrant, R.I., Walker, D.H., Weller, P.F. (Eds.), Tropical Infectious Diseases: Principles, Pathogens, and Practice. Churchill Livingston, Philadelphia, pp. 1123–1132.

Laegreid, A., Kolsto Otnaess, A.B., Orstavik, I., Carlsen, K.H., 1986. Neutralizing activity in human milk fractions against respiratory syncytial virus. Acta Paediatr. Scand. 75, 696–701.

Legrand, D., Mazurier, J., Colavizza, D., Montreuil, J., Spik, G., 1990. Properties of the iron-binding site of the N-terminal lobe of human and bovine lactotransferrins. Importance of the glycan moiety and of the non-covalent interactions between the N- and C-terminal lobes in the stability of the iron-binding site. Biochem. J. 266, 575–581.

Levay, P.F., Viljoen, M., 1995. Lactoferrin: a general review. Haematologica 80, 252–267.

Lin, T.Y., Chu, C., Chiu, C.H., 2002. Lactoferrin inhibits enterovirus 71 infection of human embryonal rhabdomyosarcoma cells in vitro. J. Infect. Dis. 186, 1161–1164.

Marchetti, M., Longhi, C., Conte, M.P., Pisani, S., Valenti, P., Seganti, L., 1996. Lactoferrin inhibits herpes simplex virus type 1 adsorption to Vero cells. Antiviral Res. 29, 221–231.

Marchetti, M., Pisani, S., Antonini, G., Valenti, P., Seganti, L., Orsi, N., 1998. Metal complexes of bovine lactoferrin inhibit in vitro replication of herpes simplex virus type 1 and 2. Biometals 11, 89–94.

Marchetti, M., Superti, F., Ammendolia, M.G., Rossi, P., Valenti, P., Seganti, L., 1999. Inhibition of poliovirus type 1 infection by iron-, manganese- and zinc-saturated lactoferrin. Med. Microbiol. Immunol. 187, 199–204.

Marchetti, M., Superti, F., 2001. Antiviral activity of lactoferrin. In: Pandalai, S.G. (Ed.), Recent Developments in Antiviral Research. Transworld Research Network, Trivandrum, India, pp. 193–203.

Marchetti, M., Trybala, E., Superti, F., Johansson, M., Bergstrom, T., 2004. Inhibition of herpes simplex virus infection by lactoferrin is dependent on interference with the virus binding to glycosaminoglycans. Virology 318, 405–413.

McCann, K.B., Lee, A., Wan, J., Roginski, H., Coventry, M.J., 2003. The effect of bovine lactoferrin and lactoferricin B on the ability of feline calicivirus (a norovirus surrogate) and poliovirus to infect cell cultures. J. Appl. Microbiol. 95, 1026–1033.

Modlin, J.F., 2000. Picornaviridae. In: Mendell, G.L., Bennett, J.E., Dolin, R. (Eds.), Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases, fifth ed. Churcill Livingston, Philadelphia, pp. 1888–1895.

Moore, S.A., Anderson, B.F., Groom, C.R., Haridas, M., Baker, E.N., 1997. Three-dimensional structure of diferric bovine lactoferrin at 2.8 Å resolution. J. Mol. Biol. 274, 222–236.

Murphy, M.E., Kariwa, H., Mizutani, T., Yoshimatsu, K., Arikawa, J., Takashima, I., 2000. In vitro antiviral activity of lactoferrin and ribavirin upon hantavirus. Arch. Virol. 145, 1571–1582.

Norris, G.E., Gartner, A.L., Anderson, B.F., Ward, J., Baker, E.N., Rumball, S.V., Baker, H.M., 1986. Preliminary crystallographic studies on bovine lactoferrin. J. Mol. Biol. 191, 143–145.

Peterson, J.A., Patton, S., Hamosh, M., 1998. Glycoproteins of the human milk fat globule in the protection of the breast-fed infant against infections. Biol. Neonate 74, 143–162.

Pietrantoni, A., Di Biase, A.M., Tinari, A., Marchetti, M., Valenti, P., Seganti, L., Superti, F., 2003. Bovine lactoferrin inhibits adenovirus infection by interacting with viral structural polypeptides. Antimicrob. Agents Chemother. 47, 2688–2691.

Portelli, J., Gordon, A., May, J.T., 1998. Effect of compounds with antibacterial activities in human milk on respiratory syncytial virus and cytomegalovirus in vitro. J. Med. Microbiol. 47, 1015–1018.

Puddu, P., Borghi, P., Gessani, S., Valenti, P., Belardelli, F., Seganti, L., 1998. Antiviral effect of bovine lactoferrin saturated with metal ions on early

steps of human immunodeficiency virus type 1 infection. Int. J. Biochem. Cell. Biol. 30, 1055–1062.

Recio, I., Visser, S., 1999. Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin. In situ enzymatic hydrolysis on an ion-exchange membrane. J. Chromatogr. A 831, 191–201.

Rostand, K.S., Esko, J.D., 1997. Microbial adherence to and invasion through proteoglycans. Infect. Immun. 65, 1–8.

Seganti, L., Di Biase, A.M., Marchetti, M., Pietrantoni, A., Tinari, A., Superti, F., 2004. Antiviral activity of lactoferrin towards naked viruses. Biometals 17, 295–299.

Shimazaki, K., Tazume, T., Uji, K., Tanaka, M., Kumura, H., Mikawa, K., Shimo-Oka, T., 1998. Properties of a heparin-binding peptide derived from bovine lactoferrin. J. Dairy Sci. 81, 2841–2849.

Shimazaki, K., Uji, K., Tazume, T., Kumura, H., Shimo-Oka, T., 2000. Approach to identification and comparison of the heparin-interacting sites of lactoferrin using synthetic peptides. In: Shimazaki, K., Tsuda, H., Tomita, M., Kuwata, T., Perraudin, J.P. (Eds.), Lactoferrin: Structure, Function and Applications. Elsevier Science B.V., Amsterdam, pp. 37–46.

Siciliano, R., Rega, B., Marchetti, M., Seganti, L., Antonini, G., Valenti, P., 1999. Bovine lactoferrin peptidic fragments involved in inhibition of herpes simplex virus type 1 infection. Biochem. Biophys. Res. Commun. 264, 19–23.

Superti, F., Ammendolia, M.G., Valenti, P., Seganti, L., 1997. Antirotaviral activity of milk proteins: lactoferrin prevents rotavirus infection in the enterocyte-like cell line HT-29. Med. Microbiol. Immunol. 186, 83–91.

Superti, F., Siciliano, R., Rega, B., Giansanti, F., Valenti, P., Antonini, G., 2001. Involvement of bovine lactoferrin metal saturation, sialic acid and protein fragments in the inhibition of rotavirus infection. Biochim. Biophys. Acta 1528, 107–115.

Swart, P.J., Kuipers, M.E., Smith, C., Pauwels, R., de Béthune, M.P., De Clercq, E., Meijer, D.K.F., Huisman, J.G., 1996. Antiviral effects of milk proteins: acylation results in polyanionic compounds with potent activity against human immunodeficiency virus types 1 and 2 in vitro. AIDS Res. Hum. Retroviruses 12, 769–775.

Tinari, A., Pietrantoni, A., Ammendolia, M.G., Valenti, P., Superti, F., 2005. Inhibitory activity of bovine lactoferrin against echovirus induced programmed cell death in vitro. Int. J. Antimicrob. Agents 25, 433–438.

Tomita, M., Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., 1991. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. J. Dairy Sci. 74, 4137–4142.

Turchany, J.M., Aley, S.B., Gillin, F.D., 1995. Giardicidal activity of lactoferrin and N-terminal peptides. Infect. Immun. 63, 4550–4552.

Valenti, P., Marchetti, M., Superti, F., Ammendolia, M.G., Puddu, P., Gessani, S., Borghi, P., Belardelli, F., Antonini, G., Seganti, L., 1998. Antiviral activity of lactoferrin. In: Spik, G., Legrand, D., Mazurier, J., Pierce, A., Perraudin, J.P. (Eds.), Advances in Lactoferrin Research. Plenum Press, New York, pp. 199–203.

van der Strate, B.W.A., Beljaars, L., Molema, G., Harmsen, M.C., Meijer, D.K.F., 2001. Antiviral activities of lactoferrin. Antiviral Res. 52, 225–239.

Ventura, K.C., Hawkins, H., Smith, M.B., Walker, D.H., 2001. Fatal neonatal echovirus 6 infection: autopsy case report and review of the literature. Mod. Pathol. 14, 85–90.

Vorland, L.H., 1999. Lactoferrin: a multifunctional glycoprotein. APMIS 107, 971–981.

Weng, T.Y., Chen, L.C., Shyu, H.W., Chen, S.H., Wang, J.R., Yu, C.K., Lei, H.Y., Yeh, T.M., 2005. Lactoferrin inhibits enterovirus 71 infection by binding to VP1 protein and host cells. Antiviral Res. 67, 31–37.

WuDunn, D., Spear, P.G., 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. 63, 52–58.

Yi, M., Kaneko, S., Yu, D.Y., Murakami, S., 1997. Hepatitis C virus envelope proteins bind lactoferrin. J. Virol. 71, 5997–6002.

Zeichard, H., Grunert, H.P., 2000. Enteroviruses. In: Specter, S., Hodinka, R.L., Young, S.A. (Eds.), Clinical Virology Manual. American Society for Microbiology, Washington, DC, pp. 253–269.