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Bovine lactoferrin inhibits echovirus endocytic pathway by interacting with viral structural polypeptides

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Abstract

Lactoferrin, an 80 kDa bi-globular iron-binding glycoprotein belonging to the transferrin family, is a pleiotropic factor with potent antimicrobial and immunomodulatory activities, present in breast milk, in mucosal secretions, and in the secondary granules of neutrophils. Recently, we have shown that bovine lactoferrin prevents the early phases of echovirus infection and also acts as a survival factor inhibiting viral-induced apoptosis. In the present research we investigated the mechanism of bovine lactoferrin anti-echoviral effect demonstrating that echovirus enters susceptible cells by an endocytic pathway and that lactoferrin treatment is able to prevent viral genome delivery into the cytoplasm. It is likely that lactoferrin interaction with echovirus capsid proteins induces alterations that stabilize the conformation of the virion making it resistant to uncoating.

Taken together, the results of our study show that the inhibition of echovirus 6 infectivity by lactoferrin is dependent on its interaction not only with cell surface glycosaminoglycan chains but also with viral structural proteins demonstrating that this glycoprotein targets the virus entry process.

Keywords: Echovirus 6; Lactoferrin; Endocytosis

1. Introduction

Echoviruses are small, icosahedral, non-enveloped, single-stranded RNA viruses of the enterovirus genus belonging to the *Picornaviridae* family. Human enteroviruses, enterically transmitted viruses responsible for a wide spectrum of illness among infants and children, are classified into five species (A to D and the polioviruses). The most common illness associated with enterovirus infections, mainly affecting very young children, is aseptic meningitis (AM) and the most common viruses responsible for AM worldwide are members of the human enterovirus B species, including echovirus and coxsackievirus B (Hyypiä et al., 1997; Zeichhardt and Grunert, 2000). Echoviruses may also cause overwhelming disease and death in neonates (John and Walker, 1999; Modlin, 2000; Ventura et al., 2001). At present, effective antiviral therapy for acute echovirus infection is not available, even though breast-feeding has been strictly associated with protection from enterovirus infection (Jenista et al., 1984).

It is well-known that milk, besides secretory IgA and IgM, also contains a number of various non-antibody components with antiviral activity, including lactoferrin (Laegreid et al., 1986; Levay and Viljoen, 1995; Peterson et al., 1998; Portelli et al., 1998; Valenti et al., 1998; Valenti and Antonini, 2005). Lactoferrin is a monomeric glycoprotein with a molecular mass of about 80 kDa which binds two iron atoms with very high affinity (Metz-Boutigue et al., 1984); it is present in various biological fluids and in granules of polymorphonuclear leukocytes (Brock, 1980), and possesses a variety of biological functions such as promotion of iron absorption, immunomodulation, and inhibitory activity towards different pathogens (Levay and Viljoen, 1995; Valenti et al., 1998; Vorland, 1999; Marchetti and Superti, 2001; van der Strate et al., 2001; Valenti and Antonini, 2005). Since 1994 bovine lactoferrin (bLf) has been recognized as a potent inhibitor of different enveloped viruses such as human cytomegalovirus (Hasegawa et al., 1994; Harmsen et al., 1995;

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Andersen et al., 2001), herpes simplex virus 1 and 2 (Fujihara and Hayashi, 1995; Marchetti et al., 1996, 1998, 2004), human immunodeficiency virus (HIV) (Harmsen et al., 1995; Puddu et al., 1998; Swart et al., 1996), human hepatitis C virus (Yi et al., 1997; 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 lactoferrin against some naked virus has been also demonstrated (Superti et al., 1997, 2001; Marchetti et al., 1999; Arnold et al., 2002; Lin et al., 2002; Di Biase et al., 2003; McCann et al., 2003; Pietrantoni et al., 2003, 2006; Drobni et al., 2004; Seganti et al., 2004; Tinari et al., 2005).

We have recently observed that lactoferrin from bovine milk is able to prevent different early phases of echovirus 6 infection and, in particular, we demonstrated that lactoferricin, the N-terminal region of lactoferrin, is responsible for the inhibition of echovirus attachment to glycosaminoglycan receptors on susceptible cells (Pietrantoni et al., 2006). In this previous research we observed that the treatment with lactoferrin, differently from lactoferricin, also prevents a post-adsorption step of echovirus infection.

In the present study we attempted to further investigate the interaction between lactoferrin and echovirus 6 in order to better characterize the mechanism of the antiviral activity of this protein. Results obtained demonstrate that the inhibition of the post-adsorption step of echovirus infection by lactoferrin is dependent on its direct interaction with viral proteins and, moreover, that lactoferrin targets the echovirus 6 entry process.

2. Materials and methods

2.1. Cells and virus

Green monkey kidney cells (GMK) kindly provided by Prof. T. Bergstrom (Göteborg University, Göteborg, Sweden) were grown at 37 °C in a humidified atmosphere with 5% CO_2 in Minimal Essential Medium (MEM, EuroClone, SpA, Pero, Milan, Italy) containing 1.2 g/l $NaHCO_3$, and supplemented with 10% inactivated fetal calf serum (FCS, JRH Biosciences, Inc. Lenexa, Kansas), 2 mM glutamine, non-essential amino acids, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Echovirus type 6 (kindly provided by Prof. L. 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 −80 °C. This stock virus was titered by plaque assay on GMK cells.

2.2. Chemicals

Lactoferrin from bovine milk (bLf), kindly supplied by Dicofarm (Rome, Italy) was deprived of endotoxin as previously described (Pietrantoni et al., 2006). Detoxified bLf and ammonium chloride (NH_4Cl, Sigma Chemical Co., St. Louis, MO) were dissolved as stock solutions (0.125 and 400 mM, respectively) in pyrogen-free PBS. Lactoferrin 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 assays

To establish the maximal non-cytotoxic dose of chemicals, 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 (Nalge Europe Ltd., Neerijse, Belgium) for 24 h at 37 °C in 5% CO_2-air. 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 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. Protein dilutions that did not affect any of these parameters were considered as non-cytotoxic concentrations and utilized for antiviral assays.

2.4. Neutralization assay

Neutralization of echovirus binding to GMK cells was carried out by incubating 12.5 μM lactoferrin with echovirus (10^5 p.f.u./ml) for 1 h at 37 °C. After incubation, two-fold serial dilution of virus-lactoferrin suspensions were added to GMK cells grown in 96-well tissue culture microplates (Nalge Europe Ltd., Neerijse, Belgium) for 24 h at 37 °C in 5% CO_2-air. After 1 h adsorption at 37 °C, cells were rinsed thoroughly and incubated at 37 °C for 24 or 48 h. The cytopathic effect induced by echovirus was measured by the neutral red uptake assay as previously described (Marchetti et al., 1996), and results were expressed as percentage of cytopathic effect inhibition by comparison with untreated control cultures.

2.5. Virus particle purification and negative stain electron microscopy

For echovirus particle purification, virus-infected cells were frozen and thawed three times and centrifuged at 4 °C (3000 × *g*; 10 min). Supernatants were collected and centrifuged two times at 10,000 rpm for 30 min at 4 °C (Sorvall RR-34 rotor). Supernatants were then ultracentrifuged at 36,000 rpm for 2 h at 4 °C using a Beckman 45 Ti rotor and pellets were resuspended in 0.1 M Tris–HCl–0.15 M NaCl (pH 8.0) (TNC)

containing 1% Nonidet P-40. Clarified samples were loaded on 30% sucrose solution in TNC and ultracentrifuged at 35,000 rpm for 2 h at 4 °C using a Beckman SW41 rotor. Pellets were suspended in PBS, loaded on 1.34 g/ml CsCl and ultracentrifuged at 32,000 rpm for 24 h at 16 °C using a Beckman SW41 rotor. Viral particle containing fractions were collected from the bottom of the tube after centrifugation and examined by transmission electron microscopy. Briefly, selected fractions were pooled and dialysed against PBS and ultracentrifuged at 35,000 rpm for 2 h using a Beckman SW41 rotor. Drops (10 μl) were incubated for 1 min on formvar- and carbon-coated grids, rinsed with 30 drops of water, and placed on a drop of 2% sodium phosphotungstate, pH 7.0, for 10 s, wicked again and air-dried. Negatively stained preparations were observed with a Philips 208 electron microscope at 80 kV.

2.6. In vitro conversion of echovirus to 135S particles and dot-blot assay

Conversion of echovirus to 135S particles was performed by using a modification of the method developed by Wetz and Kucinski (1991). Briefly, purified viral particles were diluted 20-fold into pre-warmed 20 mM Tris–2 mM $CaCl_2$–0.1% Tween 20 (pH 7.5) (TCT) and incubated at 50 °C for 3 min. Native and 135S particles were probed with bLf by a dot-blot assay.

Dot-blots were carried out by applying 5 μl of purified 160S (native) or 135S particles directly to the nitrocellulose paper (Bio-Rad, Hercules, CA) and blocked, after air-drying, in block solution (PBS containing 3% skim milk) for 1 h at room temperature. The membrane was washed with PBS containing 0.1% Tween 20 (T-PBS) for 20 min at room temperature with agitation and then incubated with bLf (12.5 μM) or PBS, for 1 h at 37 °C. To reveal bLf, after new washings in T-PBS (1 × 15 min, 1 × 10 min), rabbit anti-lactoferrin antibodies (Sigma Chemical Company, St. Louis, MO) in T-PBS were added to the blot and after 1 h incubation at room temperature with agitation, the blot was washed in T-PBS (2 × 10 min) and HRP-conjugated anti-rabbit gammaglobulin antibodies (Bio-Rad) were added. To reveal viral particles, after new washings in T-PBS (1 × 15 min, 1 × 10 min), anti-enterovirus monoclonal antibodies (Dako, Inc., Carpinteria, CA) in T-PBS were added to the blot and after 1 h incubation at room temperature with agitation, the blot was washed in T-PBS (2 × 10 min) and HRP-conjugated anti-mouse gammaglobulin antibodies (Bio-Rad) were added. After 1 h incubation at room temperature with agitation, the blot was washed again in T-PBS (2 × 10 min) and stained with the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit for peroxidase (Vector Laboratories, Inc., Burlingame, CA) according to the Manufacturer's instructions.

2.7. Echovirus entry assay

Echovirus 6 (m.o.i. 100) was added to cells and incubated for 1 h at 4 °C to allow attachment. Cells were then washed twice with medium and incubated at 37 °C for different lengths of time. After 10, 20, or 30 min infected cells were fixed and processed for transmission electron microscopy.

2.8. Effect of ammonium chloride on echovirus infection

The effect of ammonium chloride (20 mM) on echovirus infection was tested in a time-of-addition assay. For these experiments, infection was synchronized by incubating the virus (m.o.i. 1) with the cells for 1 h at 4 °C. After the attachment step, unbound virus was removed by washing twice with medium and cells were incubated for 5 h at 37 °C. The inhibiting activity of ammonium chloride was assessed by different experimental procedures: (i) infected cells were treated with ammonium chloride for the entire time of infection or, (ii) before the addition of chemical, infected cells were incubated for well-defined times. Echovirus antigen synthesis was measured by indirect immunofluorescence. In these experiments, bLf (12.5 μM) was utilized as internal control.

2.9. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to determine lactoferrin binding to viral particles. Flat-bottomed 96-well plates (Nalge Europe Ltd., Neerijse, Belgium) were coated with 0.1 mg/well of lactoferrin in 0.05 M carbonate buffer (pH 9.6) at 4 °C overnight. The plates were blocked with 10% bovine serum albumin (BSA) in PBS for 2 h at 37 °C. After washing with PBS containing 0.01% Tween-20 (PBS-T), the plates were incubated at 37 °C for 1 h with 50 μl of purified echovirus particles pre-treated for 1 h at 37 °C with PBS at different pH (pH 7.0, 6.0, 5.0, and 4.0). The plates were washed and incubated at 37 °C for 1 h with anti-echovirus 6 monoclonal antibodies (Immunological Sciences, Rome, Italy) in PBS containing 1% BSA, washed again and incubated at 37 °C for 1 h with HRP-conjugated polyclonal goat anti-mouse IgG (Bio-Rad). After washing with PBS-T, TMB solution (Vector Laboratories, Inc.) was added and the reaction was left at room temperature for approximately 15 min. The reaction was stopped by the addition of 1N H_2SO_4 and the optical density at 450 nm was measured.

2.10. Electrophoresis of proteins and Ligand-blot assay

Proteins of partially purified echovirus were resolved by polyacrylamide-SDS gel electrophoresis (SDS-PAGE), as described by Laemmli (1970) under denaturing conditions on 12% acrylamide gel. Separated proteins were then transferred from gel to nitrocellulose membranes (Bio-Rad), using a Semidry Transfer Cell apparatus (Trans-blot, Bio-Rad, Hercules, CA) according to Manufacturer's protocol.

After transfer, excess sites on the membranes were blocked with 5% skim-milk solution for 1 h, followed by washing with PBS/0.05% Tween-20 (washing solution). Membranes were then incubated for 90 min with washing solution containing 1 mg/ml (12.5 μM) bLf. After extensive washing, membranes were incubated with rabbit anti-lactoferrin antibodies (Sigma Chemical Company) in a washing solution for 1 h, washed again, and then incubated for 1 h with goat peroxidase-labelled anti-rabbit IgG antibodies (Bio-Rad) (1:3000 dilution in washing solution). Staining was achieved by using TMB substrate kit for peroxidase (Vector Laboratories, Inc.), according to

Manufacturer's instructions. All incubations were carried out at room temperature. Aspecific anti-bLf antibody binding to viral proteins was verified by incubating nitrocellulose strips with anti-bLf antibodies followed by HRP-conjugated anti-rabbit antibodies.

2.11. Transmission electron microscopy

For transmission electron microscopy, GMK infected cells were fixed in 0.1 M sodium cacodylate buffer containing 2.5% glutaraldehyde (pH 7.2) at room temperature for 20 min. After washing three times in 0.1 M sodium cacodylate were post-fixed in 1% osmium tetroxide in the same buffer. After 1 h incubation at room temperature, cells were dehydrated through 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.

2.12. 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.

2.13. Statistical analysis

Statistical analysis was performed using the two-tailed 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 bovine lactoferrin and ammonium chloride. For this purpose, two-fold serial dilutions of bLf and NH_4Cl from 25 μM and 20 mM, respectively, 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 dose (data not shown).

3.2. Failure of lactoferrin to neutralize echovirus binding to target cells

To verify whether lactoferrin interaction with viral particles resulted in a neutralization of infection, experiments were carried out in which lactoferrin and echovirus were pre-incubated for 1 h at 37 °C or 4 °C, diluted, and then added to GMK monolayers. After 1 h adsorption at 37 °C, unattached virus was removed by washing, infected cell were incubated for 24 or 48 h at 37 °C and then viral replication was monitored by neutral red uptake assay. Results obtained showed that, in all experimental conditions, bLf was unable to neutralize echovirus infection (data not shown).

3.3. Lack of lactoferrin binding to echovirus "A" particles

Enterovirus structure must be sufficiently stable to allow virus transmission to the host and, at the same time, to protect the genome, but must, at the suitable signal, dissociate or alter in conformation thus releasing its genome. As to poliovirus, it as been demonstrated that, after virus attaches to its receptor, the particle converts from the "N" conformation (native or 160S) to the "A" form (intermediate or 135S) (Curry et al., 1996). As bLf failed to neutralize native viral particles, in this set of experiments we investigated the putative binding of lactoferrin to intermediate conformations of echovirus particles. To this aim, purified 160S native particles and 135S intermediate particles were fixed to nitrocellulose membranes and probed with bLf. Results from dot-blot assay, demonstrated that bLf was not able to bind either intact viral particles or "A" forms (data not shown).

3.4. Ultrastructural analysis of echovirus 6 internalization into GMK cells

On the basis of results above reported, we hypothesized that bLf exerted its anti-echovirus effect during a post-adsorption step. To this aim, experiments were carried out to visualize viral entry in our cell system. The mechanism by which picornaviruses enter susceptible cells is still unclear, as contradictory data have been reported. For example, it has been suggested that polioviruses infect the cells either by endocytosis (Willingmann et al., 1989; Zeichhardt et al., 1985), or by crossing the membrane barrier in the form of 135S particles (Kronenberger et al., 1992), or by forming pores in the cell membrane (Tosteson and Chow, 1997).

In our experiments, cells infected with echovirus 6 were analyzed by transmission electron microscopy to discern the physical features and localization of entering virions. Results of these morphological studies, shown in Fig. 1, suggested that echovirus 6 enters GMK cells by an endocytic pathway. In particular, after 1 h incubation at 4 °C numerous viral particles adjacent to the plasma membrane were observed (Fig. 1A). After 10 min incubation at 37 °C virions surrounded by invaginations of the plasma membrane were detected (Fig. 1B). Intact virions in membrane-bound vesicles were visualized near the plasma membrane after 20 min incubation at 37 °C (Fig. 1C). Finally, 30 min after the temperature shift, viral particles in a large vesicle were visualized in the cytoplasm (Fig. 1D).

3.5. Lactoferrin and ammonium chloride inhibit the early phases of echovirus infection

To further investigate the mechanism of echovirus 6 internalization and to confirm ultrastructural observations suggesting that virus may use the endocytic pathway to infect GMK cells, subsequent experiments were performed by using ammonium

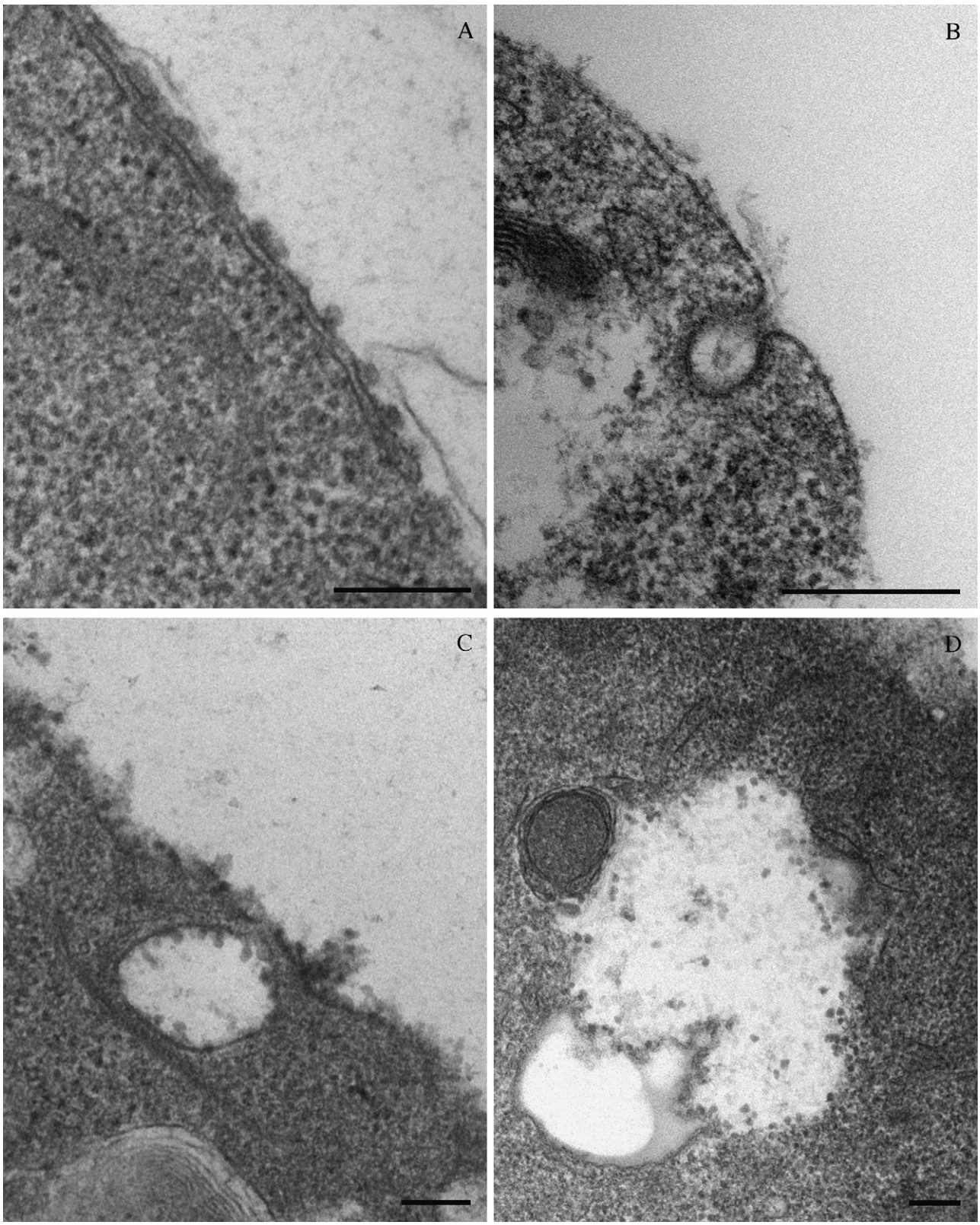

Fig. 1. Ultrastructural analysis of echovirus uptake into GMK cells. Purified viral particles were allowed to bind the cells for 1 h at 4 °C (A). Then incubation temperature was shifted to 37 °C for 10 min (B), 20 min (C), or 30 min (D) and cells were processed for transmission electron microscopy. Bars represent 200 nm.

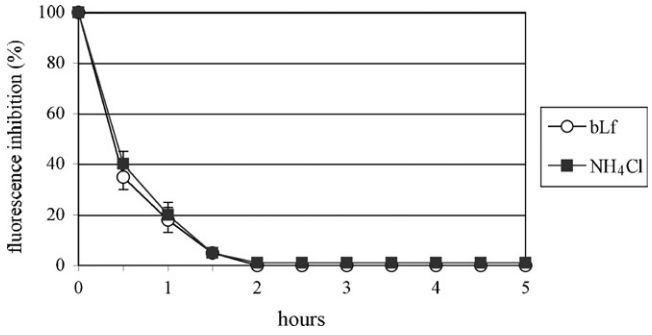

Fig. 2. Addition-time effect of 20 mM NH_4Cl and 12.5 μM bLf on echovirus infection. After adsorption step (1 h at 4 °C; time zero), incubation temperature was shifted to 37 °C. NH_4Cl or bLf were added to infected cells at different time intervals after time zero. After 5 h incubation at 37 °C, viral antigen synthesis was measured by immunofluorescence. Data represents the means of at least three independent experiments.

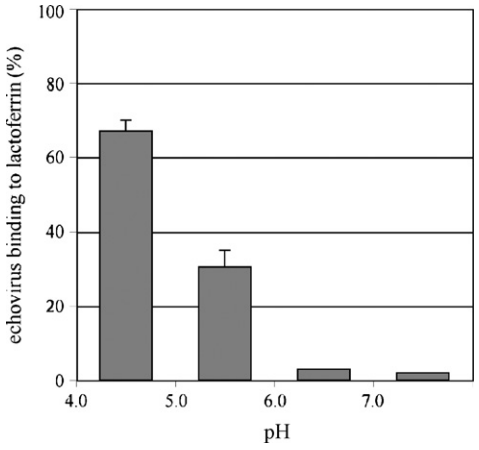

Fig. 3. Influence of pH on echovirus 6 binding to bLf by ELISA. Echovirus was treated with different acidic buffers (pH 4.0, 5.0, 6.0, and 7.0) and allowed to bind to plastic-adsorbed bLf. Viral binding was detected by an ELISA by anti-VP1 antibody staining and results are expressed as percentage of virus binding to bLf. Data represent the means of at least three independent experiments.

chloride, a weak base that buffers the pH of acidic cellular compartments and is able to prevent infection of viruses that require a low pH for entry. Moreover, as we have previously demonstrated that bLf failed to prevent echovirus infection when added 2 h after viral entry (Pietrantoni et al., 2006), to ascertain whether lactoferrin can exert an inhibitory activity on viral receptor-mediated endocytosis the effects of ammonium chloride and bLf on viral post-adsorption step were compared by a time-of-addition assay. For this purpose, experiments were carried out in which cells were incubated with the virus for 1 h at 4 °C and then lactoferrin (12.5 μM) or ammonium chloride (20 mM) were added at different times after the viral attachment step. Echovirus antigen synthesis was measured 5 h after infection by immunofluorescence. It was found that ammonium chloride, like bLf, totally prevented viral antigen synthesis when present during the entire cycle of infection whereas, when added 30 min after viral binding, the inhibition was 40 and 35%, respectively, and both compounds were ineffective when added 2 h after viral internalization (Fig. 2).

3.6. Direct lactoferrin binding assay at low pH

Our results showed that lactoferrin failed to bind both 160S and 135S viral particles even though it was able to inhibit the early events of viral infection suggesting that it could prevent echovirus 6 genome delivery into the cytoplasm by interacting with some viral structures. It is well-known that viruses utilizing the cellular endocytic machinery to transport their genetic material into the cell require an acidic pH for escape from the endocytic pathway to the host cytosol (Helenius et al., 1982; Superti et al., 1984; Marsh and Helenius, 1989). In this case, the enteroviral capsid becomes rearranged as a result of pH-induced structural transitions, whereby the VP4 internal protein is lost and the surface becomes more lipophilic, and interacts with a vesicle membrane so as to allow exit of the RNA into the cytoplasm. The pH of endocytic compartments varies from about 6.0 (early endosomes) to 5.0 (late endosomes) and 4.0 (when late endosome fuses with lysosome). Our hypothesis was that the acidic pH could favour the interaction between lactoferrin and viral capsid allowing inhibition of viral uncoating. To verify this hypothesis, experiments were carried out in which the interaction between lactoferrrin and purified viral preparations, treated with different acidic buffers (pH 4.0, 5.0, 6.0, and 7.0), was quantified by an ELISA. Results obtained, reported in Fig. 3, showed a pH-dependent interaction between lactoferrin and echovirus particles. In fact, as expected, bLf was not able to bind to intact viral particles (pH 7.0), whereas it reacted with low pH-treated virions (pH 4.0–5.0) in a pH-depending manner.

3.7. Lactoferrin binding to echovirus structural proteins

To further investigate the interaction between lactoferrin and structural viral proteins, experiments were carried out with partially sucrose-purified echovirus proteins, separated by electrophoresis and probed with bLf by a ligand-blot assay (Fig. 4). Results obtained showed that notwithstanding lactoferrin was able to bind to both viral and cellular proteins it recognized four polypeptides in virus sample (lane A), lacking in GMK sample (lane B), with molecular weights corresponding to VP0, Vp1, Vp3, and VP2 viral proteins.

4. Discussion

In this report, we demonstrate that echovirus 6 enters susceptible cells by endocytosis and that lactoferrin prevents viral uncoating: this is the first study showing that this glycoprotein is able to inhibit viral infection by this way.

Previous researches have analyzed the effect of lactoferrin on picornavirus infection with different results. As to poliovirus infection, we have demonstrated that bovine lactoferrin, in the native form, inhibited the early phases of poliovirus infection, whereas it was ineffective after the viral adsorption step (Marchetti et al., 1999). Later, it has been reported that lactoferrin had no effect on rhinovirus growth (Clarke and May, 2000), whereas it exerted an anti-enterovirus 71 action at the level of virus adsorption or receptor-mediated binding to the target cell membrane (Lin et al., 2002; Weng et al., 2005). It is well-known

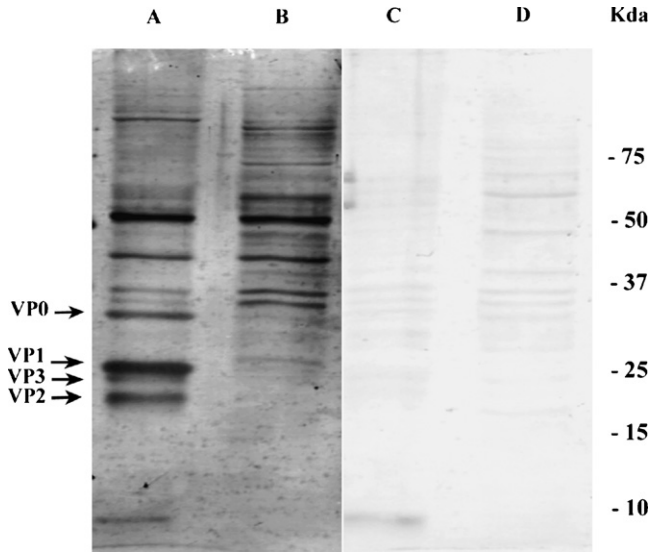

Fig. 4. Ligand-blot assay of bLf binding to echovirus 6. Partially purified echovirus 6 (A) and mock-infected GMK cells (B) were electrophoresed on 12% polyacrylamide gel, blotted on nitrocellulose, and overlaid with bLf (25 μM). Bound bLf was detected by anti-bLf and HRP-conjugated anti-rabbit antibody reaction. Viral proteins (C) and GMK cells (D) were subjected to the same experiment with the exception of bLf incubation in order to verify non-specific anti-bLf antibody binding to viral proteins. Molecular mass standards (in kiloDaltons) are shown on the right.

that lactoferrin is a potent antiviral agent acting on early phases of infection. Glycosaminoglycan chains on the cell surface have been shown to interact with lactoferrin (Pierce et al., 1991) suggesting that bLf binding to heparan sulphate blocks cell receptors for several viruses such as adenovirus (Di Biase et al., 2003), herpesvirus (Marchetti et al., 2004), papillomavirus (Drobni et al., 2004), alphavirus (Waarts et al., 2005), and echovirus (Pietrantoni et al., 2006). A direct binding of lactoferrin with viral particles or specific viral proteins, with consequently prevention of infection, has been suggested for rotavirus (Superti et al., 1997), HIV-1 (Swart et al., 1996), hepatitis C virus (Yi et al., 1997), adenovirus (Pietrantoni et al., 2003), and enterovirus 71 (Weng et al., 2005).

We have recently observed that lactoferrin was able to prevent both viral binding to cell receptors and the step immediately after virus attachment and that twenty five amino acid residues (17–41aa) of the N-terminal part of protein, corresponding to lactoferricin, were sufficient to prevent echovirus 6 attachment to target cells (Pietrantoni et al., 2006).

In the present research we have further explored the mechanism of anti-echovirus 6 activity of lactoferrin, in particular, we have analyzed the possibility of a direct interaction between lactoferrin and viral particles.

Many enteroviruses utilize as cell receptors molecules belonging to the immunoglobulin superfamily that, binding into the canyon, destabilize the virus and trigger the uncoating process. In particular, it has been reported that the interaction of poliovirus with these receptors results in elution of a portion of the input virion in an altered form known as the 135S or A particle (Rueckert, 1996).

On the bases of these observations, in a first set of experiments we examined the putative interaction of lactoferrin with native or A echoviral particles. Results obtained demonstrated that lactoferrin was unable to bind either intact native echoviral particles or 135S particles.

It must be reminded that, in some instances, picornaviruses can attach to non-immunoglobulin superfamily molecules, such as low-density lipoprotein receptors, decay-accelerating factor, and glycosaminoglycans, which do not bind into the canyon (Bergelson et al., 1994, 1995). Moreover, it has been recently demonstrated that echovirus 6 binds heparan sulphate (HS) (Goodfellow et al., 2001). It is possible that the ability to bind HS provides an additional means of cell association that facilitates an interaction with the cell surface molecules that mediate virus entry; in fact, unlike all the immunoglobulin superfamily receptors, these binding sites do not induce viral instability upon binding and thus do not trigger uncoating. However, their recruitment can induce the aggregation of other receptors or they could trigger endocytosis followed by a lowering of pH in endosomal vesicles.

On the basis of what is reported in the literature and following our observations that lactoferrin was unable to bind both native and A particles, we hypothesized that echovirus 6, after binding to its receptors on susceptible cells, could be internalized by endocytosis. Since the mechanism of echovirus 6 internalization into host cells has not been described, we decided to explore it by transmission electron microscopy. Results of these ultrastructural studies showed that echovirus enters susceptible cells by an endocytic pathway.

To confirm these morphological data, other experiments have been carried out to test the ability of ammonium chloride, a weak base that blocks endosomal acidification, to prevent echovirus infection. In fact it is well-known that agents such as ammonium chloride or metabolic inhibitors that raise endosomal pH (Ohkuma and Poole, 1978), dissipate proton gradients, inhibit acidification, or deplete ATP, are able to prevent viral genome entry into the cytoplasm (Helenius et al., 1982; Superti et al., 1984; Marsh and Helenius, 1989). Results from our experiments showed that ammonium chloride treatment totally prevented viral infection. Moreover, we also demonstrated, by a time-of-addition assay in which the antiviral effect of ammonium chloride and lactoferrin was compared, that the temporal kinetic of the inhibitory activity of both compounds was quite similar. These results suggested that ammonium chloride and lactoferrin, could affect echoviral infection by acting on a similar target, i.e. the delivery of viral genome into the cytoplasm following the uncoating. It must be reminded that lactoferrin not only binds to glycosaminoglycans (Pierce et al., 1991; Wu et al., 1995) but also low-density lipoprotein receptor-related proteins-1 and -2 (LDL-LRP1 and LDL-LRP2) (Willnow et al., 1992; Ji and Mahley, 1994; Meilinger et al., 1995; Vash et al., 1998; Suzuki and Lonnerdal, 2002), members of the LDL-LRP family that are primarily known as endocytic receptors (Strickland et al., 2002). It has been recently demonstrated that lactoferrin is endocytozed in primary osteoblastic cells (Naot et al., 2005). On the basis of these observations, we hypothesized that, also in our cell system, bLf could enter into the cells by endocytosis, blocking

viral infection by binding to viral particles in the acidic compartments. We have analysed by an ELISA the capability of plastic-adsorbed bLf to capture acid pre-treated viral particles. Results of these experiments demonstrated that lactoferrin interacts with echoviral particles in an acidic-dependent manner. In fact, it was able to bind to the virus in a range of pH between 4.0 and 5.0, characteristic of late endosomes and lysosomes.

Endocytosis studies with poliovirus showed that the loss of VP4 is an early step in the uncoating process (Crowell and Philipson, 1971; Lonberg-Holm and Korant, 1972). After uncoating, the genome appears to be injected into the cytoplasm from acidic endosomes in which the virus is internalized (Madshus et al., 1984; Zeichhardt et al., 1985). The loss of VP4, induced by acidic conditions, and the RNA delivery into the cytoplasm can be prevented either by agents inhibiting endosomal acidification or by antiviral drugs interacting with the viral capsid and stabilizing virion conformation (Fox et al., 1986; Andries, 1995; Mosser and Rueckert, 1996; De Clercq, 2002). It was likely that bLf, similarly to these capsid-binding agents, could directly interact with some viral proteins in peculiar experimental conditions. Effectively, when we utilized denaturing conditions, we demonstrated by "ligand-blot assay" that lactoferrin was able to bind electrophoretically separated viral structural proteins.

Taken together, our findings provide further evidence that lactoferrin is an excellent candidate in the search for anti-enterovirus natural agents, as it acts not only by hindering viral adsorption but also by preventing viral eclipse into the cells through a direct binding to viral particles. In fact, demonstrating that lactoferrin interaction with echovirus capsid proteins make the virus resistant to uncoating, we have identified a novel mechanism of antiviral activity of this glycoprotein.

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