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Bovine lactoferrin inhibits Influenza A virus induced programmed cell death in vitro

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Abstract Influenza is one of the main plagues worldwide. The statistical likelihood of a new pandemic outbreak, together with the alarming emergence of influenza virus strains that are resistant to available antiviral medications, highlights the need for new antiviral drugs. Lactoferrin, a 80 kDa bi-globular iron-binding glycoprotein, is a pleiotropic factor with potent antimicrobial and immunomodulatory activities. Although the antiviral effect of lactoferrin is one of its major biological functions, the mechanism of action is still under debate. In this research, we have analyzed the effect of bovine lactoferrin (bLf) on Influenza A virus infection in vitro. Our results showed that (i) Influenza virus infected cells died as a result of apoptosis, (ii) bLf treatment inhibited programmed cell death by interfering with function of caspase 3, a major virus-induced apoptosis effector, and (iii) bLf efficiently blocked nuclear export of viral ribonucleoproteins so preventing viral assembly. These results provide further insights on the antiviral activity of bLf and suggest novel strategies for treatment of Influenza virus infection.

Keywords Bovine lactoferrin · Influenza virus · MDCK cells · Apoptosis

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Introduction

Influenza viruses are enveloped, segmented minus-sense RNA viruses of the Orthomyxovirus genus. These viruses cause respiratory illness in humans and animals with elevated morbidity and mortality rates. The famous influenza pandemic of 1918, Spanish flu, is thought to have killed up to 100 million persons (Reid et al. 2001). The World Health Organization (WHO) proposes the development of pandemic vaccines appropriate for mass immunization in addition to the storing of antiviral drugs. Notwithstanding massive immunization programs, viral influenza still represents a serious source of morbidity and mortality worldwide and a considerable cause of illness and death among people with immunodeficiency associated with aging or different clinical conditions.

Antiviral compounds, such as amantadine and oseltamivir, have been employed for the treatment of Influenza virus infection. Antiviral chemotherapy with amantadine and its derivative rimantadine, that block the ion channel function of the M2 protein of

the Influenza A virus, reduces the duration of symptoms of clinical influenza, but major side effects and the emergence of drug-resistant variants have been described (Hayden 2006; Fiore et al. 2008). At present, nearly all Influenza A/H3N2 viruses and a percentage of Influenza A/H1N1 viruses are adamantane resistant, which leaves only neuraminidase inhibitors available for treatment of infection with these viruses (Poland et al. 2009). Zanamivir and oseltamivir, that inhibit neuraminidase, have been established to be helpful for reducing clinical symptoms, but their efficacy has been restricted by side effects. In fact zanamivir is applied topically to the respiratory tract as an inhaled preparation because the drug is poorly absorbed orally whereas oseltamivir is administered orally and may be associated with gastrointestinal tract-related adverse events (Hayden et al. 1999). Moreover, the effectiveness of these drugs has been limited by the emergence of resistant viral strains in the past years (Gubareva et al. 1998; Kiso et al. 2004) and, most notably, by the natural emergence of a substantial oseltamivir-resistance among circulating A/H1N1 Influenza viruses isolated in Europe (Meijer et al. 2009) as well as in United States (Dharan et al. 2009).

As a general concern in antiviral therapy is the occurrence of resistance, combination therapy might be the future strategy for the treatment of Influenza virus infection to increase the effectiveness of the drugs and to minimize the risk of spreading resistant viruses. Therefore there is a need of new antiviral agents that can be used to prevent and/or treat Influenza virus infection.

Breast-feeding has been associated with protection from respiratory and gastrointestinal infections in infants (May 1988). It is well known that milk, besides secretory IgA and IgM, also contains a number of various non-antibody components with known antiviral activity, including lactoferrin (Levay and Viljoen 1995; Valenti and Antonini 2005; Orsi 2004). Lactoferrin is a multifunctional glycoprotein with a molecular mass of about 80 kDa that binds two iron atoms with very high affinity (Levay and Viljoen 1995). It is present in various biological fluids and in granules of polymorphonuclear leukocytes, and possesses a variety of biological functions such as: influence on iron homeostasis, immunomodulation, and inhibitory activity towards different pathogens (Levay and Viljoen 1995; Valenti and Antonini 2005; Orsi 2004; Paesano et al. 2009). Since 1994, bovine lactoferrin (bLf) has been recognized as a potent inhibitor of a number of different enveloped viruses such as human cytomegalovirus (Harmsen et al. 1995; Andersen et al. 2001), herpes simplex virus types 1 and 2 (Hasegawa et al. 1994; Marchetti et al. 2009; Ammendolia et al. 2007a), human immunodeficiency virus (HIV) (Harmsen et al. 1995; Puddu et al. 1998), human hepatitis C virus (Tanaka et al. 1999), 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 non-enveloped viruses has been also demonstrated (Superti et al. 1997; Marchetti et al. 1999; Superti et al. 2001; Arnold et al. 2002; Di Biase et al. 2003; Pietrantoni et al. 2003; Drobni et al. 2004; Seganti et al. 2004; Tinari et al. 2005; Longhi et al. 2006; Pietrantoni et al. 2006; Ammendolia et al. 2007b).

In an effort to identify new antiviral therapies effective against Influenza virus, in this research we have analyzed the effect of bLf on Influenza virus infection in vitro. Our results indicate that bLf treatment specifically inhibits Influenza virus mediated caspase 3 activation so preventing both apoptosis and Infuenza virus propagation.

Materials and methods

Chemicals

Lactoferrin from bovine milk (bLf), purchased from Morinaga Milk Industries (Zama City, Japan), was deprived of endotoxin as previously described (Pietrantoni et al. 2006). Detoxified bLf was dissolved as stock solution (400 mM) in pyrogen-free PBS. BLf 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). Iron-free apo-bLf was prepared by the depletion of iron through the incubation of native bLf in 0.1 M citric acid, pH 2.3, containing 500 mg/l disodium EDTA, followed by exhaustive dialysis against deionized water. Iron-saturated holo-bLf (Fe^{3+}bLf) was prepared by incubation of the apo-protein dissolved in 0.1 M sodium bicarbonate with different concentrations of ferric citrate for 12 h at room temperature,

followed by extensive dialysis against 0.1 M sodium bicarbonate to remove unligated metal ions. The metal contents of these preparations were determined by atomic absorption spectrometry. The iron saturation rates were approximately 4.5% for apo-bLf, 19.4% for native bLf, and 95.6% for holo-bLf.

Cells and virus

Madin-Darby canine kidney cells (MDCK) 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 fetal calf serum (FCS, Flow Laboratories, Irvine, UK.), 2 mM glutamine, 2% non essential amino acids (Gibco; Paisley, UK), penicillin (100 IU/ml), and streptomycin (100 μg/ml). MDCK cells, which grow as adherent monolayers, were subcultured once or twice a week by trypsinization.

The stock of H3N2 Influenza A virus (A/Parma/12/05 strain, kindly provided by Professor Maria Luisa Tanzi, University of Parma, Italy) was prepared in MDCK cells. Virus was inoculated onto confluent monolayers grown in roller bottles at a multiplicity of infection (m.o.i.) of 1 plaque forming units (p.f.u.)/cell. After 90 min at 35°C, the inoculum was removed and the monolayers were washed three times with Phosphate Buffered Saline (PBS, pH 7.4) and then incubated at 35°C in culture medium containing 2% non essential amino acids (Gibco, Paisley, UK), 4% Bovine Serum Albumin (BSA fraction V, Gibco, Paisley, UK) and 0.5 μg of *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Sigma Chemical Co., St. Louis, MO, USA). When extensive cytopathic effect (c.p.e.) was observed, infected cultures were frozen and thawed three times, centrifuged (3,000*g*; 10 min), and supernatants were stored at −70°C. The infectivity titer was determined by hemagglutinin titration and plaque assay, according to the standard procedures (Kurokawa et al. 1990).

Cytotoxicity assay

To establish the maximal non-cytotoxic dose of compounds, two-fold serial dilutions of proteins in MEM were incubated at 37°C with confluent MDCK cells grown in 96-well tissue culture microplates (Flow Laboratories, Irvine, UK). After 24 and 48 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. Protein dilutions that did not affect any of these parameters were considered as non-cytotoxic concentrations and utilized for antiviral assays.

Action of proteins on Influenza virus cytopathic effect

MDCK cells grown in 96-well tissue culture microplates for 24 h at 37°C in 5% CO_2, were incubated with different concentrations of proteins during virus adsorption (90 min., 37°C). As viral inoculum was utilized Influenza virus at a m.o.i. of 1 p.f.u./cell. Then, cells were rinsed thoroughly and incubated with the same concentrations of proteins at 37°C in 5% CO_2. The cytopathic effect induced by Influenza virus was measured 24 h after infection by the neutral red uptake assay as previously described (Pietrantoni et al. 2006). Results were expressed as percentage of cytopathic effect inhibition by comparison with untreated infected control cultures.

Subcellular analysis of infected cells

After 24 h of infection with Influenza A virus, in presence or absence of bLf, DNA fragmentation, cell necrosis, and cell viability were determined. To this aim cells were examined by acridine orange/ethidium bromide double staining and DNA ladder.

(a) Acridine orange/ethidium bromide double staining: cells were stained with acridine orange (0.1 mg/ml)–ethidium bromide (0.1 mg/ml) solution and examined under UV illumination (Dialux 20, Leitz), at 400–600× magnification as already reported (Tinari et al. 2005). In all experiments, each sample was analyzed in triplicate by counting at least 200 or more cells.
(b) DNA gel electrophoresis: in order to separate high molecular weight chromatin from the nucleosomal DNA fragments, virus-infected cells (in presence or absence of bLf) and mock-infected cells were processed as previously described (Ammendolia et al. 1999).

Caspase-3 activity assay

Caspase-3 activity was measured by a colorimetric assay based on quantification of protease activity, BD ApoAlert™ Caspase Colorimetric Assay Kit, according to the manufacturer's instructions (BD Biosciences, CA, USA). Briefly, 2×10^6 MDCK cells, seeded on 6 cm dishes (in duplicate cell plates), were infected with Influenza virus (m.o.i. 1) either in presence of the caspase inhibitor z-DEVD-fmk (20 μM) in dimethyl sulfoxide (DMSO, Sigma Chemical co.) or 12.5 μM bLf or DMSO for 24 and 48 h at 37°C. Moreover, as internal control, uninfected cells were treated with 0.1 μM staurosporine, a known apoptosis inducer (Roche Diagnostics, Meylan, France) either in presence of the caspase inhibitor z-DEVD-fmk (20 μM) or 12.5 μM bLf or DMSO for 24 and 48 h at 37°C. The staurosporine concentration utilized, 0.1 μM, was chosen after a preliminary dose–effect study aimed to identify the concentration offering the best compromise between necrosis and apoptosis. At 24 and 48 h, cells were collected by trypsinization and resuspended in cell lysis buffer. After precipitation of the cellular debris, the remaining supernatants were incubated with a caspase-3 substrate for 1 h at 37°C. The colorimetric assay uses the spectrophotometric detection of the chromophore p-nitroaniline (pNA) after its cleavage by caspase from the substrate Ac-DEVD-pNA (N-acetyl-Asp-Glu-Val-Asp-p-NO2-aniline). Samples were read at 405 nm in a microplate reader. The reading-values of the uninduced control were subtracted from its corresponding induced sample and Caspase-3 activity was calculated using a pNA calibration curve supplied in the kit.

Transmission electron microscopy (TEM)

Virus-infected (in presence or absence of bLf) and mock-infected cells were harvested, and processed for transmission electron microscopy (TEM) as previously described (Tinari et al. 2005). Thin sections were examined with a Philips 208s electron microscope.

Cytoplasmatic and nuclear protein extraction

To assess the effect of bLf on viral nucleoprotein (NP) export from the nucleus to the cytoplasm, MDCK cells were infected with Influenza A virus at m.o.i. of 50. After 90 min adsorption at 4°C, cells were treated with 12.5 μM bLf. After incubation for 16 h at 37°C, treated and untreated infected cells were scraped into PBS containing protease inhibitors, pelleted, resuspended in the same buffer containing 0.4% Nonidet P-40 (NP-40), and pelleted again. The supernatant from this centrifugation step was considered the cytoplasmic fraction. The pellet was washed in PBS containing protease inhibitors and 0.1% NP-40, pelleted, and resuspended in PBS containing protease inhibitors plus 0.4% NP-40. This fraction was considered the nuclear fraction. The protein concentrations of all extracts were determined by a modified Bradford assay (Bio-Rad, Hercules, CA, USA) as specified by the manufacturer.

Western blot analysis

Equal amounts of cytoplasmatic and nuclear extracts (see above) were separated in SDS-PAGE 12% as described by Laemmli (1970). Separated proteins were then transferred from gel to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), blocked with 5% skim-milk solution and washed with PBS/0.05% Tween-20 (washing solution). The membrane was incubated with monoclonal anti-Influenza A nucleoprotein (Oxford Biotechnology Ltd, UK) for 1 h at room temperature. After washing with PBS-0.5% Tween-20, anti-mouse HRP-conjugate antibodies (Bio-Rad, Hercules, CA, USA) were added and incubated for 1 h at room temperature. Following extensive washing, staining was achieved by using TMB substrate kit for peroxidase (Vector Laboratories, Inc., Burlingame, CA, USA) according to manufacturer's instructions.

Statistical analysis

Statistical analysis was performed by Student's t test for unpaired data. Data were expressed as the mean and SD and P values of <0.05 were considered significant.

Results

Effect of native-, apo-, and holo-lactoferrins on cytopathic effect (c.p.e.) by Influenza virus in MDCK cells

A preliminary set of experiments was carried out in order to determine the maximal non-cytotoxic

Table 1 In vitro antiviral activity of different metal-saturated bovine lactoferrins towards Influenza virus infection

Protein	CC_{50} (μM)[a]	EC_{50} (μM)[b]	SI[c]
bLf	>50	0.89 ± 0.07	>56.2
apo-bLf	>50	2.2 ± 0.20	>22.7
holo-bLf	>50	2.5 ± 0.15	>20

Cells were infected with Influenza virus (1 p.f.u./cell). Proteins were incubated at different concentrations with the cells during the viral absorption step (1 h at 37°C) and newly added after the removal of virus inoculum. 24 h post infection the percentage of cytopathic effect was evaluated. Each sample was done in duplicate. Data represent mean values for three separate experiments ± SD

[a] CC_{50} cytotoxic concentration 50%

[b] EC_{50} effective concentration 50%

[c] SI (selectivity index) = CC_{50}/EC_{50}

concentrations of native-, apo-, and holo-lactoferrins. For this purpose, twofold serial dilutions of proteins from 50 μM in MEM medium were incubated with MDCK cells for 24 and 48 h at 37°C. No cytotoxic effect has been observed up to the highest dose. To establish whether the proteins could inhibit Influenza virus cytopathic effect in MDCK cells, twofold serial dilutions of each protein, starting from 50 μM, were incubated with the cells through the infection. In order to determine the selectivity index (SI) of bovine lactoferrins, the ratio between the 50% drug cytotoxicity concentration (CC_{50}) and the concentration required to inhibit the viral cytopathic effect by 50% (EC_{50}) was calculated. The CC_{50}, the EC_{50}, and the SI of proteins are shown in Table 1. Among the different bovine lactoferrins analyzed, native bLf showed the lowest values of EC_{50} (0.89 μM) and the highest SI (>56.2). For this reason, we decided to utilize just the native form to carry out the following experiments.

Effect of bovine lactoferrin on virus-induced cell death

To investigate the effect of bLf on Influenza virus-induced cell death, experiments were carried out in which mock-infected cells and virus-infected cells (m.o.i. 1 and 10) were treated with different non-cytotoxic concentrations of bLf. After 24 h at 37°C, cells were harvested and the percentage of apoptotic, necrotic, and viable cells was evaluated on the entire cell population (adherent and detached cells) by

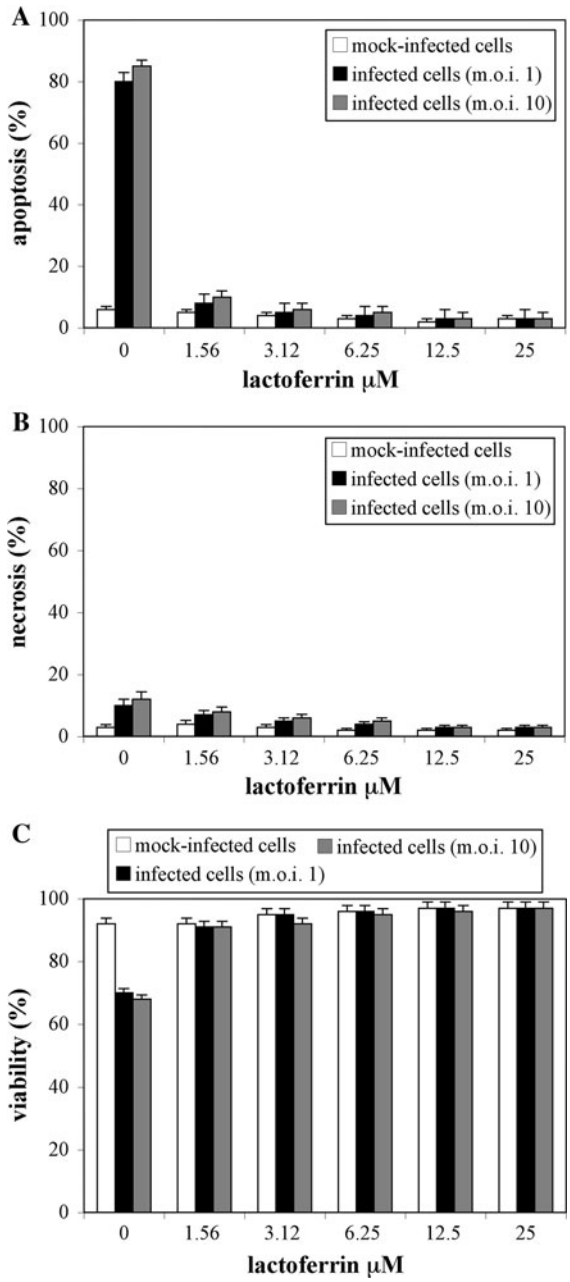

Fig. 1 Percent of apoptosis (**a**), necrosis (**b**), and viability (**c**) of mock-infected and Influenza virus-infected MDCK cells treated or untreated with bLf. Data represent the mean of at least quadruplicate samples. For each sample, 600 cells were examined

acridine orange/ethidium bromide double staining (Fig. 1). No significant differences in the percentage of apoptosis (a), necrosis (b), or viability (c) were observed in cells infected with 1 or 10 p.f.u./cell for 24 h. As shown in Fig. 1b, Influenza virus infection

resulted in about 10% necrosis and in a dramatic induction of apoptosis (about 80–85%) that was totally prevented by bLf (Fig. 1a).

DNA fragmentation

The effect of bLf on Influenza virus-induced apoptosis was also analyzed by using the suicide-track™ DNA ladder isolation kit (Oncogene™, San Diego, CA, USA). DNA from virus-infected cells (m.o.i. 1 and m.o.i. 10; 24 h), in presence and in absence of 12.5 μM bLf, was separated by agarose gel electrophoresis (Fig. 2). DNA fragmentation at the internucleosomal level resulting in a characteristic ladder was evident only in virus-infected cells either at m.o.i. 1 (lane C) or at m.o.i. 10 (lane E). As expected, the ladder was undetectable in uninfected control cells (lane B) and, as shown in the figure, bLf treatment was able to prevent DNA fragmentation either at m.o.i. 1 (lane D) or at m.o.i. 10 (lane F).

Caspase 3 activity

Colorimetric assay for caspase 3 activity was carried out by treating peptide substrate, Ac-DEVD-pNA with cell lysates from untreated or bLf-treated or z-DEVD-fmk-treated infected cells or from untreated or bLf-treated or DEVD-fmk-treated staurosporine-treated cells (Fig. 3). As expected, a decrease of caspase 3 activity (3.4- and 2.5-fold) was observed in the MDCK infected cells that had been treated with the cell permeable specific irreversible inhibitor of these proteases z-DEVD-fmk for 24 and 48 h, respectively. Interestingly, bLf-treatment of infected cells resulted in the reduction of caspase 3 activity of 5.4- and 4.5-fold after 24 and 48 h incubation, respectively. Moreover, the increased protease activity induced by staurosporine treatment was reduced of 2.7- and 2.9-fold after 24 and 48 h incubation in the presence of z-DEVD-fmk and of 4.8- and 4.9-fold after 24 and 48 h incubation in the presence of bLf. Incubation of this cell line with UV-inactivated virus did not result neither in apoptosis induction or in caspase activation confirming that productive cell infection is required (data not shown).

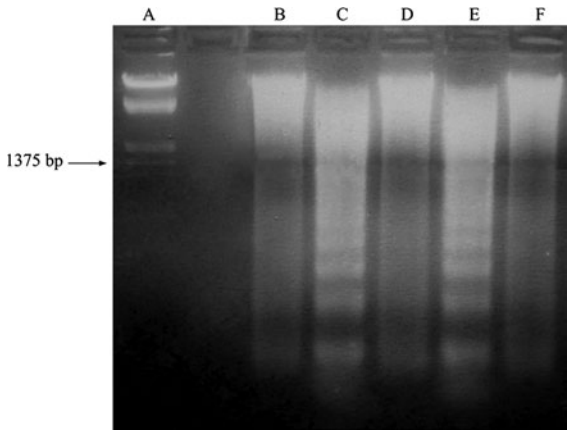

Fig. 2 Intranucleosomal breakdown of MDCK cell DNA after viral infection and bLf protection. *Lane A* molecular size markers; *lane B* mock-infected cells; *lane C* cells infected with influenza virus (m.o.i. 1), *lane D* bLf treated infected cells (m.o.i. 1); *lane E* cells infected with influenza virus (m.o.i. 10); and *lane F* bLf treated infected cells (m.o.i. 10)

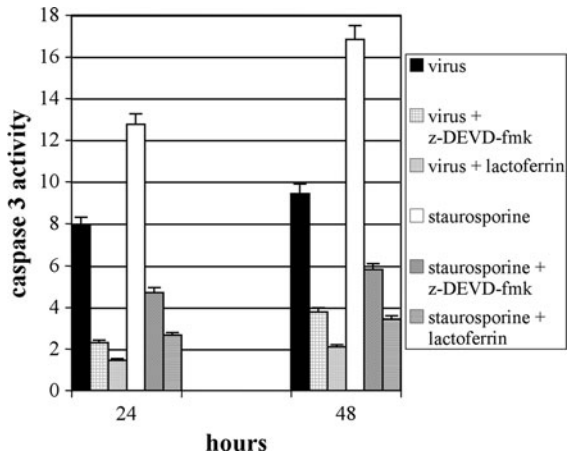

Fig. 3 Caspase activity in MDCK cells infected with Influenza virus or treated with staurosporine in presence or in absence of z-DEVD-fmk or bovine lactoferrin. Caspase activity in cell homogenates was determined by an in vitro hydrolysis test by using DEVD-pNA as a caspase-specific color substrate. The ordinate gives the caspase activity evaluated as the optical density at 405 nm per milligram of cell protein

Ultrastructural features

Mock-infected cells and virus-infected cells (m.o.i. 10, 24 h), in presence or in absence of 12.5 μM bLf, were also analyzed at ultrastructural level (Fig. 4). Mock-infected cells and bLf-treated infected cells did not display detectable pathological morphological changes (Fig. 4a, e) whereas typical features of programmed cell death process were only found in Influenza virus-infected cells (Fig. 4b–d). About 90%

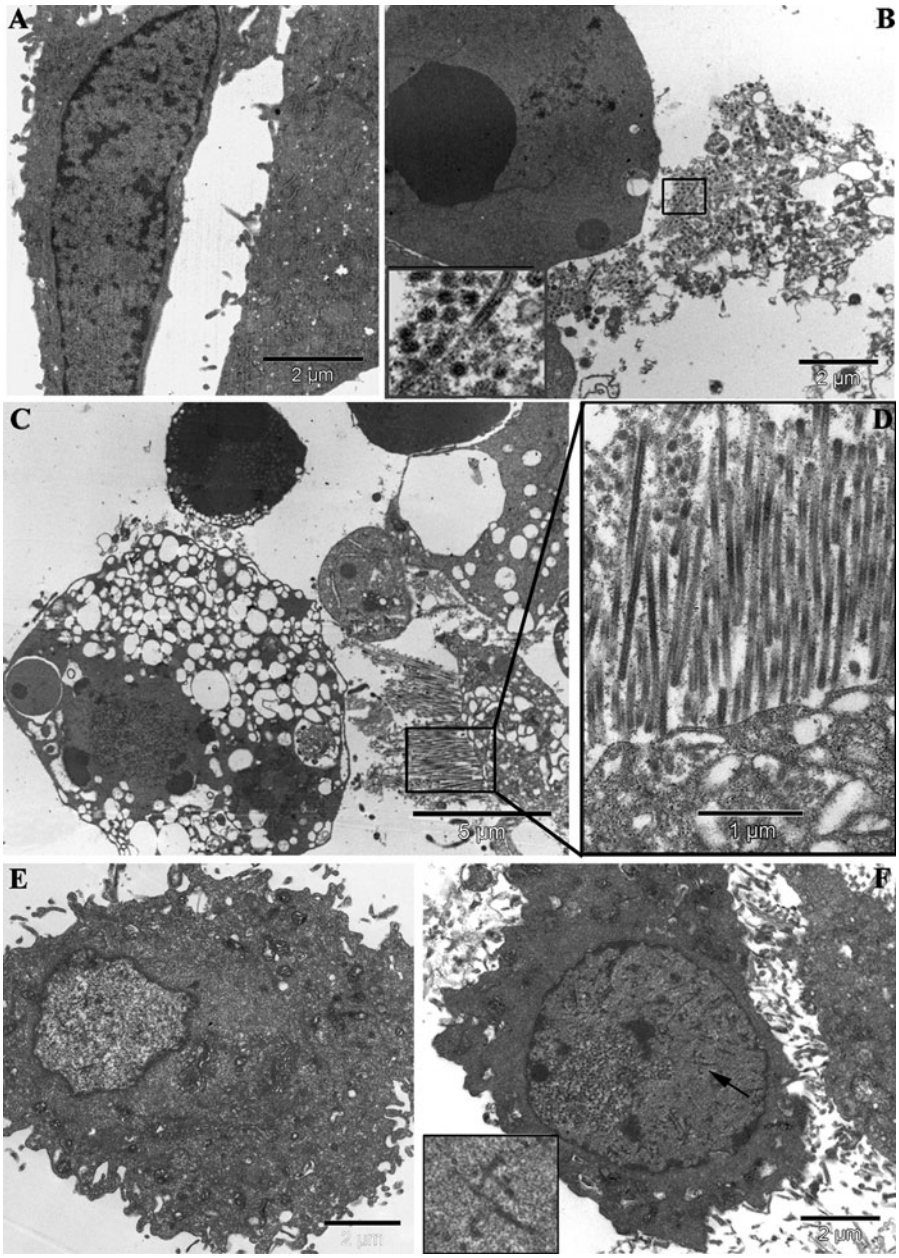

Fig. 4 Electron micrographs of thin sectioned MDCK cells. For these experiments cells were infected at m.o.i. 10 for 24 h at 37°C. As shown in the picture, mock-infected cells (**a**), and influenza virus-infected cells treated with bLf (**e**) display no detectable signs of injury. Different behavior was observed when cells were infected in absence of bLf (**b**–**d**) where ultrastructural analysis revealed typical features of programmed cell death such as chromatin clumping (**b**) or cytoplasm derangement with deep vacuolation and dilatation of the endoplasmic reticulum cisternae (**c**) together with numerous viral particles outside the cells (**b**–**d**). In bLf treated-infected cells virion production was not observed while, in some cells, dense, fibrillar structures of viral origin (arrow and inset) are detectable into the nucleus (**f**)

of infected cells exhibited characteristic morphological features of apoptosis. Changes in the density and distribution of chromatin and condensation of the whole cell, were observed (Fig. 4b). Most of cells displayed dilatation of the endoplasmic reticulum (ER) cisternae (Fig. 4c) and numerous viral particles were detectable outside of the cells (Fig. 4b–d). Notwithstanding ultrastructural analysis showed no

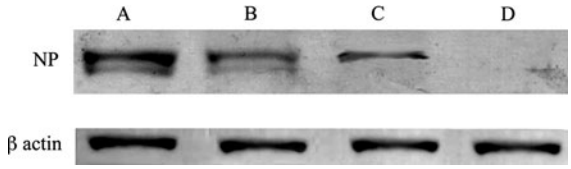

Fig. 5 Viral NP complexes export. Nuclear (*line A*) and cytoplasmatic (*line B*) fractions of untreated infected cells and nuclear (*line C*) and cytoplasmatic (*line D*) fractions of bLf-treated infected cells were electrophoresed and blotted onto nitrocellulose paper. NP was detected by monoclonal antibodies followed by anti-mouse HRP-conjugated antibodies. As loading control anti-beta-actin monoclonal antibodies were used

morphological alterations in bLf-treated infected-cells (Fig. 4e), into the nucleus of a lot of these cells elongated tubular structures were observed (Fig. 4f). As already reported by Anisimová and co-workers (Anisimová et al. 1977) these tubular structures, with a diameter of about 400 Å, were distributed over the whole nuclear area as separate units suggesting that they could correspond to the viral nucleoprotein (NP).

Analysis of bLf effect on viral ribonucleoprotein complexes export

Ultrastructural observations suggest that bLf treatment of infected cells could prevent the migration of the NP from the nucleus to the cytoplasm, so impairing viral morphogenesis. To confirm these results, Western immunoblots were utilized to examine nuclear and cytoplasmatic extracts, from bLf-treated and untreated infected cells, for the occurrence of viral NP. As showed in Fig. 5, in bLf-treated infected cells a specific NP band of about 56 kDa was found only in nuclear fraction (line C) whereas untreated infected cells displayed NP bands in both nuclear and cytosolic fractions (lines A and B).

Discussion

It is recognized that breast-feeding protects against both respiratory and enteric infections in infants (May 1988). This protection is conferred through various mechanisms, as milk, in addition to IgA and IgM, also contains non-antibody components with antimicrobial activity (Levay and Viljoen 1995; May 1988). We have previously demonstrated the antiviral activity of one of these components, lactoferrin, against the respiratory virus adenovirus type 2 (Arnold et al. 2002; Di Biase et al. 2003; Pietrantoni et al. 2003). To further characterize the activity of bLf against respiratory virus infection, we analyzed here the effect of this protein on A type H3N2 human Influenza virus. For this purpose, a preliminary set of experiments was carried out to verify the inhibition of Influenza virus cytopathic effect by various iron-bound forms of bLf. Results from these experiments showed that bLf was able to prevent viral cytopathic effect independently of the iron-bound form of protein used, notwithstanding native bLf was more active than both iron-free apo-bLf and iron-saturated holo-bLf. These data demonstrated that the anti-Influenza virus effect of bLf is elicited by its protein moiety since, as already reported for other viruses such as HCMV (Hasegawa et al. 1994), rotavirus (Superti et al. 2001), and adenovirus (Arnold et al. 2002), its activity was only minimally affected by iron saturation or by removal of iron ions. In fact, differently from that observed for bacterial infections, the effect of bLf on viral infection did not appear to be related to the iron withholding from the environment and, in many cases, was shown to take place even with metal-saturated isoforms of bLf (Orsi, 2004).

It is well known that many viruses have as part of their arsenal the ability to modulate the apoptotic pathways of the host. Although some viruses have developed different ways to suppress apoptosis, several viruses trigger apoptosis in host cells at the late stage of infection probably to spread viral progeny (Koyama et al. 2000). It has been previously demonstrated that infection with A and B type Influenza viruses results in the induction of apoptosis both in permissive and non-permissive cultured cells as well as in vivo (Hinshaw et al. 1994; Ludwig et al. 2006; Mori et al. 1995; Takizawa et al. 1999). Also in our experimental conditions we observed that Influenza virus infection induces apoptosis. Furthermore, we demonstrated that bLf totally prevents programmed cell death in Influenza virus-infected MDCK cells. The central element of the apoptotic equipment is a proteolytic system consisting of a family of cysteinyl proteases, called caspases (Thornberry and Lazebnik 1998). Two groups of caspases have been recognized: initiator caspases such as caspase 8 or caspase 9, which cleave and activate other caspases, and effector caspases, including caspases 3, 6 and 7, cleaving a

multiplicity of other cellular substrates, thus disassembling cellular structures or inactivating enzymes (Thornberry and Lazebnik 1998). Caspase 3 is the most intensively studied effector caspase as it is a central player in apoptosis regulation and the level of procaspase 3 in the cell determines the impact of a given apoptotic stimulus (Ludwig et al. 2006). It has been already reported that caspase 3 activation, during the onset of apoptosis, is a crucial event for efficient Influenza virus propagation as virus spreading resulted strongly impaired in the presence of caspase inhibitors (Wurzer et al. 2003). This dependence on caspase activity was most obvious in cells where caspase 3 was partially knocked-down by caspase 3-specific small interfering RNAs (Wurzer et al. 2003). On the basis of these observations, researches have been performed to investigate the mechanism of the anti-apoptotic activity of bLf and, in particular, the ability of this protein to inhibit caspase 3 activity. In these experiments MDCK cells, inducted to apoptosis by either virus infection or staurosporine, were treated with bLf or with the caspase inhibitor z-DEVD-fmk. Our results demonstrated that, in all experimental conditions, bLf treatment significantly prevented caspase 3 activity. This is an important finding because apoptosis, in particular activation of caspase 3, plays a key role in Influenza virus spreading. As a matter of fact it has been reported that viral NPs are retained in the nucleus of cells overexpressing the antiapoptotic protein Bcl-2 (Hinshaw et al. 1994) and that a caspase-dependent process is needed for migration of the viral NPs from the nucleus to the cytoplasm of infected cells (Wurzer et al. 2003). Observations performed by TEM confirmed that bLf treatment prevented both apoptosis and viral morphogenesis. Electron microscopic studies also revealed elongated tubular structures, resembling the viral ribonucleoprotein (RPN), in the nucleus of several bLf treated infected cells suggesting that the inhibition of caspase 3 activity by bLf resulted in the sequestration of viral NP into the nucleus. This hypothesis has been confirmed at molecular level by Western blot results showing the presence of viral NP in both nuclear and cytosolic fractions of infected cells and only into the nucleic extracts from bLf-treated infected cells. Taken together, our results provide further insights on the antiviral activity of bLf and suggest this natural product as an excellent candidate for the treatment of Influenza virus infections, as it is able not only to inhibit virus induced apoptosis but also to prevent vNP complex export from the nucleus to the cytoplasm of infected cells so that production of infectious particles results suppressed. As the increasing appearance of resistant strains of Influenza virus highlights our need to identify new antiviral drugs, the discovery of a natural product acting on a target different from that one of commercial drugs is of particular meaning. In fact, as already reported for herpesvirus infections (Andersen et al. 2003; van der Strate et al. 2003), bLf might be a potential candidate for combination therapy with licensed antiviral drugs with the aim of reducing the possibility for the incidence of drug-resistant strains.

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