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Short communication

Antiadenovirus activity of milk proteins: lactoferrin prevents viral infection

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Abstract

Different milk proteins were analysed for their inhibitory effect on adenovirus infection in vitro. Proteins investigated were mucin, α-lactalbumin, β-lactoglobulin, bovine lactoferrin, and human lactoferrin. Results obtained demonstrated that mucin, α-lactalbumin, and β-lactoglobulin did not prevent the viral cytopathic effect, whereas lactoferrin was able to inhibit adenovirus replication in a dose-dependent manner. Further experiments were carried out in which lactoferrin was added to the cells during different phases of viral infection. Results obtained showed that lactoferrin was able to prevent viral replication when added both before, or during the viral adsorption step, or when present during the entire replicative cycle of adenovirus, demonstrating that its action takes place on an early phase of viral replication.

Keywords: Adenovirus; Milk proteins; Lactoferrin; Antiviral agents

Breast-feeding has been recognised to protect against respiratory and gastrointestinal infections in infants (May, 1988). Milk, besides secretory IgA and IgM, also contains a number of various non-antibody components with known antimicrobial activity, including lactoferrin (Laegreid et al., 1986; May, 1988; Levay and Viljoen, 1995; Peterson et al., 1998; Portelli et al., 1998). 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 specific granules of polymorphonuclear leukocytes (Brock, 1980), and possesses a variety of biological functions such as: promotion of iron absorption, immunomodulation, and inhibitory activity towards bacteria, fungi, protozoa (Levay and Viljoen, 1995), and viruses (Valenti et al., 1998; Vorland, 1999).

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The antiviral effect of human and cow milk against arbovirus, rhinovirus, and influenza virus has been reported in 1976 (Matthews et al., 1976). Since 1994, among milk glycoproteins, lactoferrin has been recognised as a potent inhibitor towards different enveloped viruses such as herpes simplex virus (HSV) 1 and 2 (Hasegawa et al., 1994; Fujihara and Hayashi, 1995; Marchetti et al., 1996, 1998), human cytomegalovirus (HCMV) (Hasegawa et al., 1994; Harmsen et al., 1995; Portelli et al., 1998), human immunodeficiency virus (HIV) (Harmsen et al., 1995; Swart et al., 1996; Puddu et al., 1998), human hepatitis C virus (HCV) (Yi et al., 1997; Ikeda et al., 1998), respiratory syncytial virus (RSV) (Portelli et al., 1998), and hantavirus (Murphy et al., 2000). The antiviral activity of lactoferrin against two naked viruses, SA-11 rotavirus and poliovirus type 1, has been also demonstrated (Superti et al., 1997; Marchetti et al., 1999). For all viruses investigated to date, lactoferrin exerted its antiviral activity in the early phases of infection. Since lactoferrin is known to bind cell surface glycosaminoglycans and low-density lipoprotein receptors (Ji and Mahley, 1994), which in turn act as binding sites for HSV1 and HIV (Shieh et al., 1992; Roderiquez et al., 1995), its inhibiting activity on these viruses has been ascribed to a competition for cell receptors, even though a direct interaction between lactoferrin and viral particles has not been ruled out. For rotavirus and poliovirus, which interact with carbohydrate moieties other than glycosaminoglycans, it has been suggested that lactoferrin could bind to viral particles, similar to what has been reported for some enveloped viruses (Swart et al., 1996; Yi et al., 1997). Interestingly, a further effect on a later intracellular step of virus infection has been also described for rotavirus (Superti et al., 1997).

In the present study we investigated the activity of lactoferrin from bovine milk (BLf) in the native and apo-form, and native lactoferrin from human milk (HLf) as well as metal ion-saturated BLf against adenovirus type 2 infection in HEp-2 (human epidermoid carcinoma, larynx) cells. Adenoviruses are double-stranded DNA non-enveloped icosahedral viruses that cause both respiratory and gastrointestinal infections in humans. The effectiveness of BLf and HLf was compared with the activity of other milk proteins known to exhibit antiviral activity in other virus-cell systems (Yolken et al., 1992; Superti et al., 1993) such as mucin, α-lactalbumin, and β-lactoglobulin. Results obtained provided evidence for an antiadenovirus activity of apo- and native lactoferrins. BLf saturation with ferric, manganese or zinc ions did not significantly influence the inhibitory activity of the molecule, which was exerted at an early phase of viral infection.

HEp-2 cells were obtained from American Type Culture Collection. Cells were grown at 37 °C in minimal essential medium (MEM) containing 1.2 g/l $NaHCO_3$ and supplemented with 10% inactivated foetal calf serum (FCS), 2 mM glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Human adenovirus type 2, isolated from an immunocompromised patient affected by a respiratory disease, was grown in HEp-2 cells. Virus was inoculated onto confluent monolayers at a multiplicity of infection (m.o.i.) of 1 p.f.u./cell (p.f.u.: plaque forming unit). 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 titred by plaque assay on HEp-2 cells.

Mucin (from bovine submaxillary glands), α-lactalbumin, and β-lactoglobulin (from bovine milk) were purchased from Sigma Chemical Company, apo-lactoferrin from bovine milk (apo-BLf), native bovine lactoferrin (BLf), and native human lactoferrin (HLf) were purchased from Fluka (Switzerland); all proteins were dissolved as stock solutions in PBS. Iron-, manganese-, and zinc-saturated BLf (Fe^{3+}-BLf, Mn^{2+}-Blf, and Zn^{2+}-BLf) were prepared as previously reported (Marchetti et al., 1999).

A preliminary set of experiments was carried out in order to determine the maximal non-cytotoxic concentration of mucin, α-lactalbumin, β-lactoglobulin, apo-BLf, BLf, and HLf. For this purpose, twofold serial dilutions of proteins from 2 mg/ml in MEM were incubated at 37 °C with confluent HEp-2 cells grown in 96-well tissue culture microplates (Flow Laboratories). After 48

h, the following parameters were evaluated: cell morphology and viability (determined by neutral red staining and light microscopy), and cell proliferation (evaluated quantitatively by microscopic counts after dispersion into individual cells with trypsin). Protein dilutions, which did not affect any of these parameters, were considered as non-cytotoxic concentrations and utilised for antiviral assays. Under these conditions, only β-lactoglobulin, apo-BLf, and BLf did not affect any of the cytotoxicity parameters up to the highest dose.

To establish whether these milk proteins could inhibit the cytopathic effect of adenovirus in HEp-2 cells, twofold serial dilutions of each protein, starting from the highest non-cytotoxic concentration (0.5 mg/ml for mucin and α-lactalbumin; 2 mg/ml for β-lactoglobulin, apo-BLf, and BLf; 1 mg/ml for HLf), were incubated with the cells throughout the infection (48 h at 37 °C). Adenovirus at a multiplicity of infection of 1 p.f.u./cell was used as the viral inoculum. The viral cytopathic effect was measured by the neutral red uptake assay as previously described (Marchetti et al., 1996).

In our experimental conditions, mucin, α-lactalbumin, and β-lactoglobulin, were ineffective, whereas all lactoferrins tested showed a dose-dependent inhibitory activity.

In order to determine the selectivity index (SI) of 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 milk proteins are shown in Table 1. Among the different lactoferrins analysed, BLf showed the lowest EC_{50} values (0.08 mg/ml) and the highest SI (>25).

Since lactoferrin is able to chelate other metal ions besides Fe^{3+} and its inhibiting effect could be influenced by the degree of metal ion saturation, the antiviral assay procedure reported above was followed to test the ability of BLf fully saturated with ferric, zinc, and manganese ions to prevent adenovirus infection. In these experiments, tenfold serial dilutions of apo-BLf, BLf, HLf, Fe^{3+}-BLf, Mn^{2+}-BLf, and Zn^{2+}-BLf, starting from the concentration of 1 mg/ml, were incubated with the cells throughout the infection (48 h at 37 °C). Results obtained are reported in Fig. 1. The antiviral effectiveness of the different preparations of lactoferrin towards adenovirus was similar, native BLf being the most active. Ion saturation of native BLf did not influence lactoferrin activity, whereas both HLf and apo-BLf inhibited adenovirus cytopathic effect to a lower extent.

To ascertain whether the antiviral effect of lactoferrin took place at the level of viral adsorption or on a different step of viral replication, the inhibitory activity of native BLf (1 mg/ml) was assessed by different experimental procedures: (i) the cells were incubated with BLf before infection (30 min at 37 °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 (24 h at 37 °C); (iv) BLf was present during the whole experiment. Adenovirus antigen synthesis was measured by immunofluorescence. Results reported in Fig. 2 show that a high inhibition was obtained under the conditions used in both procedures (ii) and (iv), but was higher when BLf was present during the whole experiment (iv). Preincubation of HEp-2 cells with lactoferrin before infection (i) resulted in 50% inhibition of viral replication, whereas BLf was ineffective when added after the virus attachment step (iii).

Table 1
In vitro antiviral activity of milk proteins towards adenovirus 2 infection

Protein	CC_{50} (mg/ml)[a]	EC_{50} (mg/ml)[b]	SI[c]
Mucin	1	>0.5	–
α-Lactalbumin	1	>0.5	–
β-Lactoglobulin	>2	>2	–
apo-BLf	>2	0.28 ± 0.020	>7.1
BLf	>2	0.08 ± 0.007	>25
HLf	2	0.56 ± 0.050	3.6

HEp-2 cells were infected with adenovirus (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 removal of the virus inoculum. At 48 h post infection the percentage of cytopathic effect was evaluated. Each sample was done in duplicate. Data represent ± SD mean values for three separate experiments.

[a] CC_{50}: cytotoxic concentration 50%.

[b] EC_{50}: effective concentration 50%.

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

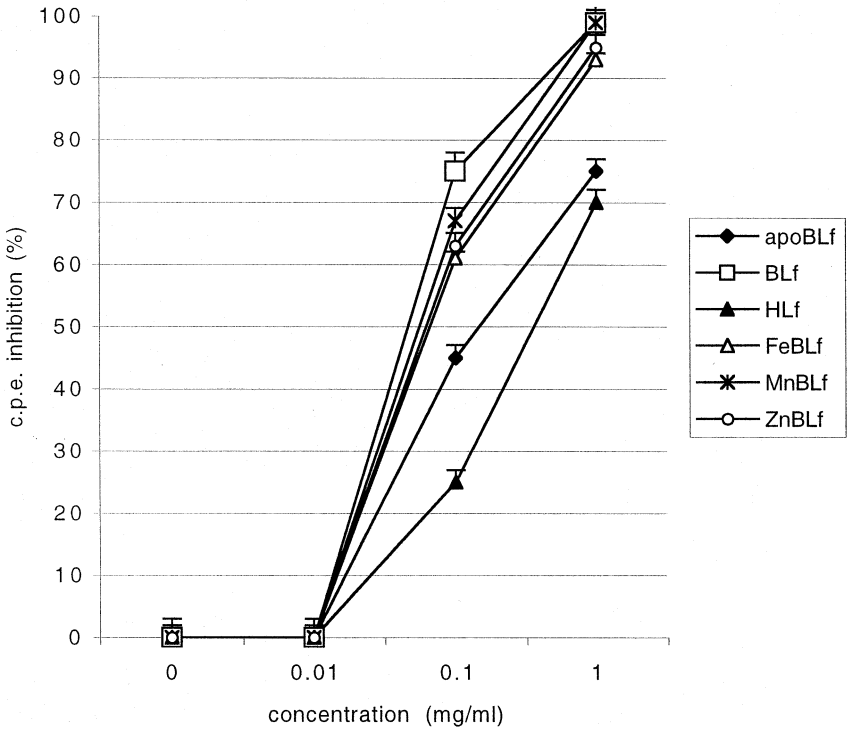

Fig. 1. Dose-response curves of apo-BLf, BLf, HLf, Fe^{3+}-BLf, Mn^{2+}-BLf, and Zn^{2+}-BLf towards adenovirus cytopathic effect (c.p.e.) in HEp-2 cells. Proteins were present throughout the infection (1 h at 4 °C during virus adsorption, plus 48 h at 37 °C). Data shown represent the mean of at least quadruplicate samples. SD $< 5\%$ are not shown.

Adenoviruses play an important role as pathogens among infants and young children. These viruses are responsible for a portion of acute respiratory diseases and can also cause epidemic conjunctivitis. They have been associated with a variety of additional clinical syndromes, such as infantile gastroenteritis. To date, 49 serotypes have been recognised and, between them, adenoviruses of subgenus C, particularly serotypes 1, 2, 5, and 6, have been frequently isolated from cases of respiratory disease in young children (Li et al., 1996). Some respiratory adenoviruses, types 1 and 2 in particular, have also been reported to be associated with gastroenteritis (Bryden et al., 1997).

It is well known that breast-feeding protects against both respiratory and gastrointestinal infections in infants (May, 1988; Newburg, 1999) through different mechanisms: specific antibody targeted protection against pathogens, and broad-spectrum, non-specific protection mediated by different milk components (Hamosh, 1998). On the basis of this observation, in this in vitro study we analysed comparatively the antiadenoviral effect of some milk proteins such as mucin, α-lactalbumin, β-lactoglobulin, and lactoferrin, endowed with antiviral activity in several virus-cell systems (Hasegawa et al., 1994; Fujihara and Hayashi, 1995; Harmsen et al., 1995; Marchetti et al., 1996, 1998; Swart et al., 1996; Yi et al., 1997; Ikeda et al., 1998; Portelli et al., 1998; Puddu et al., 1998; Murphy et al., 2000). Adenoviral cytopathic effect was not prevented by mucin, α-lactalbumin, and β-lactoglobulin, whereas apo-, native, Fe^{3+}-, Mn^{2+}-, and Zn^{2+}-saturated lactoferrin inhibited adenovirus infection in a dose-dependent fashion, native and ion-saturated bovine lactoferrin being the most efficient.

To formulate an hypothesis on the mechanism of the antiviral action of lactoferrin, the events

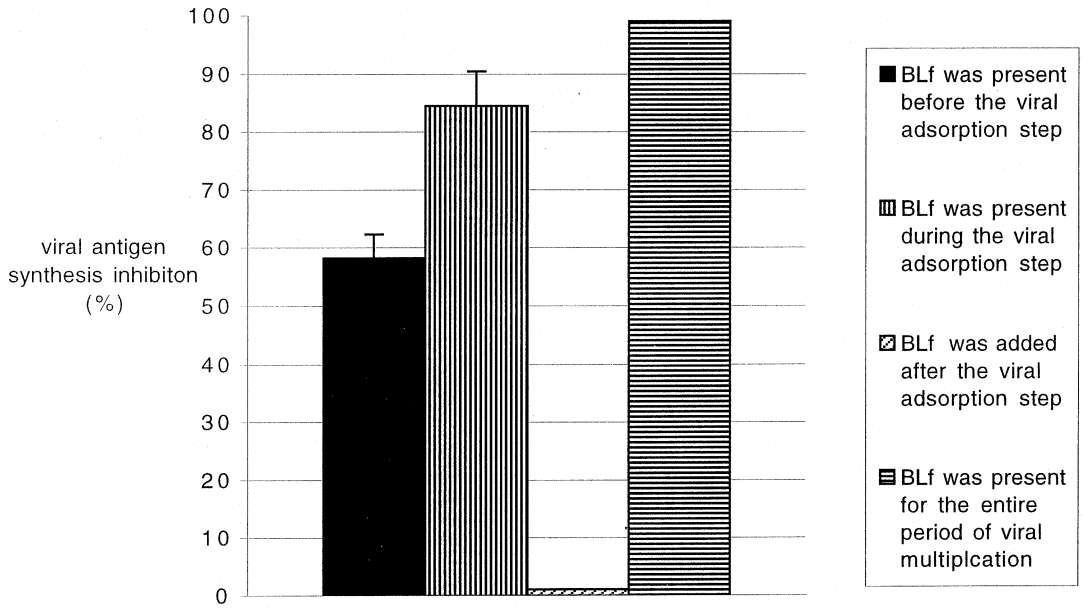

Fig. 2. Effect of 1 mg/ml BLf on adenovirus antigen synthesis. Lactoferrin was incubated with the cells before (30 min, 37 °C), during (1 h, 4 °C), and after (24 h, 37 °C) the viral adsorption step, or during the whole experiment. Virus antigen synthesis was monitored 24 h after infection by immunofluorescence. Data shown represent the mean of at least quadruplicate samples. For each sample, 600 cells were examined.

involved in both adenovirus and lactoferrin interaction with susceptible cells must be considered. Similar to what has been reported for HSV1 and HIV (Shieh et al., 1992; Roderiquez et al., 1995), heparan sulfate-glycosaminoglycans, expressed on the cell surface, are involved in the binding of adenovirus type 2 (Dechecchi et al., 2000). As polyanionic glycosaminoglycan chains of heparan sulfate have been shown to interact with lactoferrin (Ji and Mahley, 1994), it can be assumed that the capability of BLf to prevent adenovirus infection may be due to a competition with viral particles for the binding to cell receptors. Taken together, our findings provide further evidences that milk components play a certain role in the modulation of the infection by pathogens responsible for respiratory and enteric diseases and represent the first demonstration of the antiviral activity of lactoferrin towards a naked icosahedral double-stranded DNA virus. Moreover, our results suggest that BLf mainly acts by hindering viral adsorption to the cells, even if further studies are needed to better clarify the mechanism of the antiadenoviral action of this milk glycoprotein.

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