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Lactoferrin inhibits early steps of human BK polyomavirus infection

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Abstract

Lactoferrin, a member of the transferrin family, is a bi-globular iron binding glycoprotein, found in milk, exocrine secretions of mammals, and in secondary granules of polymorphonuclear neutrophiles that plays an important role in the defence against various pathogenic microorganisms. Previous studies in different virus-cell systems showed that lactoferrin is a potent inhibitor of different enveloped and naked virus infection. In this research we studied the effect of lactoferrin on BK polyomavirus, a human naked double-stranded DNA virus responsible for productive, persistent, and latent infections of the urinary tract. Results obtained demonstrate that lactoferrin treatment prevents early steps of BK virus infection in Vero cells, at the level of the adsorption phase, probably through the interaction with capsidic structures, although a lactoferrin-BK virus competition for cell plasma-membrane receptors cannot be ruled out.

Keywords: BK virus; Lactoferrin; Antiviral agents

1. Introduction

The human polyomavirus BK (BKV) is a double-stranded DNA naked virus, with circular genome, originally isolated from the urine of a kidney transplant recipient (Gardner et al., 1971). Serological surveys have shown a high incidence of BKV infection among humans as this virus infects up to 90% of the general population (Knowles et al., 2003). Primary infection appears to be generally asymptomatic with the virus remaining latent in the urogenital tract. In individuals with impaired immunological functions, BKV infection has been shown to be often reactivated and associated with severe diseases, including haemorrhagic cystitis, tubulointerstitial nephritis, ureteric stenosis, pneumonitis and meningoencephalitis, and even multi-organ failure (Hirsch and Steiger, 2003; de Bruyn and Limaye, 2004). In particular BKV infection is an emerging cause of kidney transplant failure affecting 1–10% patients (Hirsch, 2005; Hirsch et al., 2005). BKV is also able to transform human cells *in vitro* and to induce tumours in rodents; however, although nucleic acid sequences or viral proteins have been detected in human tumours, a role for this virus in malignancies has not yet been clearly demonstrated (Corallini et al., 2003; White and Khalili, 2004).

Few attempts have been made to treat BKV urinary tract infections. Although effective interventions with vidarabine or cidofovir have been reported (Chapman et al., 1991; Hirsch et al., 2005), in transplant recipients renal drug toxicity remains a critical point for most of therapeutic strategies that calls for a cautious use of antiviral drugs. Moreover recent studies demonstrated that the in vitro activity of cidofovir and leflunomide on BKV replication is modest, and the selectivity index is low (Farasati et al., 2005).

Since the nephropathy is a severe illness which represents an increasing cause of graft loss after kidney transplantation and there is a need to identify less toxic and more effective antiviral drugs for clinical use, in this research the in vitro activity of lactoferrin (Lf) towards BKV infection was investigated.

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Lf is an iron-binding glycoprotein composed of a single polypeptide chain of approximately 80 kDa folded into two homologous lobes, each containing an iron-binding site (Moore et al., 1997). Lactoferrin, first isolated from human breast milk, is produced by neutrophils and mucous epithelial cells and is also found in tears, saliva, bile, and bronchial, intestinal, vaginal and other exocrine secretions (Vorland, 1999). This glycoprotein plays an important role against parasitic, mycotic, bacterial and viral infections so that it has been considered a protein of the innate mucosal defence (Vorland, 1999; Marchetti and Superti, 2001; van der Strate et al., 2001). Since 1994 human Lf (HLf) and bovine Lf (BLf) have been recognized as effective inhibitors of several enveloped (Hasegawa et al., 1994; Fujihara and Hayashi, 1995; Harmsen et al., 1995; Marchetti et al., 1996, 1998, 2004; Swart et al., 1996; Yi et al., 1997; Ikeda et al., 1998, 2000; Puddu et al., 1998; Murphy et al., 2000; Andersen et al., 2001, 2003, 2004; Hara et al., 2002) and naked viruses (Superti et al., 1997, 2001; Marchetti et al., 1999; Arnold et al., 2002; Di Biase et al., 2003; Lin et al., 2002; McCann et al., 2003; Pietrantoni et al., 2003; Drobni et al., 2004).

Here, we studied the effect of human and bovine lactoferrin towards BKV infection in vitro. Our results demonstrate that lactoferrin inhibits the early events of infection, suggesting it as a potential candidate for the prevention and/or the treatment of human BK polyomavirus infections.

2. Materials and methods

2.1. Cells and virus

Vero (African green monkey kidney) cells were grown at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI-1640 medium (Gibco) containing 1.2 g/l $NaHCO_3$, and supplemented with 10% inactivated foetal calf serum (FCS, Flow Laboratories), 2 mM glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). For cell maintenance the serum concentration was lowered to 2%.

BK virus was grown in Vero cells. Virus was inoculated onto confluent monolayers at a multiplicity of infection (m.o.i.) of 10 cell culture infecting doses 50% (CC_{50})/ml. 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 RPMI-1640 medium containing 1.2 g/ml $NaHCO_3$ and supplemented with 2% inactivated FCS, 2 mM glutamine, 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 titrated by c.p.e. assay on Vero cells.

2.2. Chemicals

Native bovine lactoferrin (BLf) was purchased from Morinaga Milk Industries (Japan), native lactoferrin from human milk (HLf) and apo-lactoferrin from bovine milk (apo-BLf) were purchased from Fluka (Switzerland), recombinant human lactoferrin (rHLf) was purchased from Agennix (USA), ovotransferrin (OTf) was purified from chicken egg white as previously described (Phelps and Antonini, 1975). Iron, manganese and zinc saturated lactoferrins (Fe^{3+}BLf, Mn^{2+}BLf, Zn^{2+}BLf) were prepared by incubation of the apoprotein dissolved in 0.1 M sodium bicarbonate with a 10-fold excess of the citrate complex of the different metal ions for 12 h at room temperature, followed by extensive dialysis against sodium bicarbonate to remove unligated metal ions. Purity of proteins was checked by SDS-PAGE stained with silver nitrate and was judged to be greater than 95%. Protein concentrations were determined by UV spectroscopy on the basis of the extinction coefficient of 15.1 (280 nm, 1% solution) (Groves, 1960).

To supply ferric, manganese and zinc ions to the cells at the concentration of 25 μM corresponding to that required to saturate 0.4 mg/ml BLf, metal ion citrate complexes were prepared from sodium citrate and chloride or sulphate salts of the different metals.

2.3. Cytotoxicity assays

The cytotoxicity of the compounds was tested by evaluating the following parameters: cell morphology and viability (determined by neutral red staining) were examined by light microscope and cell proliferation was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the standard method (Harmsen et al., 1995). One-day prior the assays, Vero cells were seeded into 96-well plates (Costar, Cambridge, MA) at a density of approximately 10,000 cells per well. The next day the medium was refreshed with culture medium containing 2% inactivated FCS. Two-fold serial dilutions of the compounds (starting from 50 μM) were added to the well ($n = 3$). Untreated cells served as negative control (0% cytotoxicity). The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 5 days. Results were expressed as complete cytotoxicity (++) when at least one of the parameters was affected in 100% of cells, or partial cytotoxicity (+) when one parameter was affected in 50% of cells, or absence of cytotoxicity (−) when none of the parameters was affected.

2.4. Dose-response activity of BLf

Vero cells grown in 24-well plates (Costar, Cambridge, MA) for 24 h at 37 °C in 5% CO_2, were incubated with different concentrations of BLf during the virus attachment step (1 h at 4 °C). As viral inoculum was utilised BKV at a m.o.i. of 10 $CCID_{50}$/ml. Then, cells were rinsed thoroughly and incubated with the same concentrations of BLf for 5 days at 37 °C in 5% CO_2-air. At the end of infection cells were washed in PBS, detached by a cell scraper and resuspended in PBS. Viral replication was measured by determining the BKV T antigen DNA synthesis through a semi-quantitative PCR assay. Results were expressed as percentage of T antigen DNA synthesis inhibition by comparison with untreated infected control cultures.

2.5. Antiviral activity of proteins

BLf, HLf, apo-BLf, rHLf, OTf, and iron, manganese and zinc saturated bovine lactoferrins (Fe^{3+}BLf, Mn^{2+}BLf, Zn^{2+}BLf) were tested for their anti BKV activity in Vero cells. Proteins (12.5 μM) were incubated throughout the infection (5 days at 37 °C) with the cell monolayers grown in 24-well plates (Costar, Cambridge, MA). As viral inoculum 10 $CCID_{50}$/ml of BK virus was utilised. At the end of infection cells were washed in PBS, detached by a cell scraper and resuspended in PBS. Viral replication was measured by determining the BKV T antigen DNA synthesis.

2.6. Time-of-addition experiments

BLf was tested in a time-of-addition assay at a concentration corresponding to its $IC_{100,}$ the concentration of compound that inhibited the viral BKV T antigen DNA synthesis in Vero cells by 100%. To ascertain whether the antiviral effect of BLf took place at the level of viral adsorption or on a different step of viral replication, the inhibitory activity of 12.5 μM native BLf 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 (5 days at 37 °C); (iv) BLf was present during the whole experiment.

2.7. Neutralisation assay

Neutralisation of BKV binding to Vero cells was carried out by incubating 12.5 μM native BLf with the virus (10 $CCID_{50}$/ml) for 1 h at 37 °C. The suspensions were then added to the cells for 1 h at 4 °C. After washing, infected cells were incubated at 37 °C for 5 days. Viral replication was measured by determining the BKV T antigen DNA synthesis by PCR. Suitable samples were checked using cellular β-globin gene.

2.8. DNA extraction and conditions for PCR

Mock-infected and treated or untreated BKV-infected Vero cells (500 μl) were centrifuged at 14,000 rpm for 10 min. Pellets were incubated in 180 μl of lysis buffer (0.5 M Tris, 0.02 M EDTA, 0.01 M NaCl, 1% SDS) and in 20 μl of proteinase K 200 μg/ml at 55 °C for 24 h. DNA extraction was performed by the QIAamp Tissue Kit (QIAGEN), according to the Manufacturer's instructions. 220 ng of total DNA were used in PCR after measuring its concentration in the eluate by absorbance at 260 nm.

DNA suitability for analysis was checked by amplification of the beta-globin gene sequence (Saiki et al., 1985). General precautions, conditions for PCR analysis and nested PCR procedures were as published (Degener et al., 1999): beta-globin positive samples were amplified in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Emeryville, CA), and all assays included positive and negative controls (Saiki et al., 1985). The PCR products were detected by ethidium bromide staining after electrophoretic migration through a 2% agarose gel.

2.9. PCR for the large T antigen region of BKV

Large T antigen region was detected by PCR with primer AgT-1 (+), 5′-CAC AGC AAA GCA GGC AAG GGT T-3′ (nucleotides 4321–4342) and primer AgT-2 (−), 5′-TAG GTG CCA ACC TAT GGA ACA GA-3′ (nucleotides 4569–4546) (De Mattei et al., 1995; Pietropaolo et al., 1998). Samples were subjected to 5 min of denaturation at 95 °C, followed by 35 rounds of an amplification cycle consisting of 1 min at 94 °C, 1 min at 60 °C, and 30 s at 72 °C followed by an extension cycle of 5 min at 72 °C. This set of primers amplifies a 248 bp genome product. The assay included positive and negative controls. A 2 ng of recombinant pGEM-1 plasmid DNA containing the complete genome of BKV, cloned at *Eco*RI, were used as positive control. PCR amplicons were detected by ethidium bromide staining after electrophoretic migration through the 2% agarose gel.

2.10. Immunoelectron microscopy

For immunoelectron microscopy, suspensions of purified BKV were fixed in 2.5% cacodylate-buffered (0.1 M, pH 7.2) glutaraldehyde for 20 min at room temperature and then placed on Formvar-carbon coated 400 mesh copper grids. Grids were air-dried for 10 min and then incubated 1 h at room temperature with 12.5 μM BLf in 1% BSA/PBS. After washing in the same buffer, grids were incubated with anti-lactoferrin rabbit antibodies for 1 h at 25 °C. After incubation, grids were washed three times with 1% BSA/PBS and incubated for 1 h at 25 °C with anti-rabbit secondary antibody marked with 5 nm gold (Sigma Chemical Co., St. Louis, MO) diluted in 1% BSA/PBS. Grids were washed three times with 1% BSA/PBS and once with water. Negative staining was carried out with 2% sodium phosphotungstate (pH 7.0) and samples were observed with a Philips 208 electron microscope at 80 kV.

2.11. Hemagglutination (HA) and hemagglutination-inhibition (HI) assays

HA titrations were carried out at low temperature in plastic 96-well U-microplates by means of a double series of two-fold dilutions of the virus in PBS from a starting volume of 25 μl and by adding to each dilution 50 μl of a 0.5% suspension of group 0 human red cells in the same buffer. After the addition of erythrocytes the plates were kept at 4 °C for 90 min. The HA titer was calculated on the basis of the highest virus dilution which gave a complete hemagglutination.

For HI titrations serial two-fold dilutions of the proteins were obtained from a starting volume of 25 μl. A 25 μl of virus containing four HA doses were added to each well and the mixture was kept overnight at 4 °C. Then 50 μl of a 0.5% suspension of red cells in PBS were added. The results were read after 90 min incubation at low temperature and the HI titer was calculated as the reciprocal of the highest dilution of proteins capable of completely inhibiting viral hemagglutination.

2.12. 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 statistically 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, HLf, apo-BLf, rHLf, OTf, Fe^{3+}BLf, Mn^{2+}BLf, and Zn^{2+}BLf. For this purpose, two-fold serial dilutions of proteins, starting from 50 μM, in RPMI-1640 medium containing 2% inactivated FCS, were incubated with Vero cells for 5 days at 37 °C in a humidified atmosphere with 5% CO_2. Under these conditions, all compounds tested did not affect both cell morphology and viability, determined by neutral red staining, or cell proliferation, determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, up to the highest dose.

In these experiments, the optical density of treated and untreated Vero cell lysates were read by a spectrophotometer. Cell viability and proliferation were calculated as value ratio between treated versus untreated Vero cells. Up to 50 μM of proteins the ratio values obtained were ≥1.

3.2. Action of BLf and other transferrins on BKV infection

To establish whether BLf could inhibit BKV infection in Vero cells, two-fold serial dilutions of protein, starting from 50 μM, were incubated with the cells throughout the infection (1 h at 4 °C and 5 days at 37 °C). Viral replication was measured by determining the BKV T antigen DNA synthesis. Under these experimental conditions BLf showed a dose-dependent inhibitory activity, being able to completely prevent infection at the concentration of 12.5 μM (data not shown).

The ratio between the 50% bLf cytotoxicity concentration (CC_{50}: >50 μM) and the concentration required to inhibit BKV T antigen DNA synthesis by 50% (EC_{50}: 8.33 μM) was calculated after 5 days incubation in order to determine the selectivity index (SI) of the drug which was >6.

Then further experiments were carried out to test the anti-BKV activity of 12.5 μM transferrin from various origin (HLf, rHLf, and OTf) and lactoferrin from bovine milk with different metal ions saturation (apo-BLf, Fe^{3+}BLf, Mn^{2+}BLf, and Zn^{2+}BLf). In these assays BLf was included as internal control. Proteins were incubated with Vero cells throughout the infection (5 days at 37 °C) and viral replication was measured by determining the BKV T antigen DNA synthesis. Results obtained are shown in Fig. 1. Only BLf, apo-BLf, HLf, and rHLf prevented viral infection, whereas Otf, Mn^{2+}BLf, and Zn^{2+}BLf were deprived of action and Fe^{3+}BLf inhibited BKV T antigen DNA synthesis by 28%.

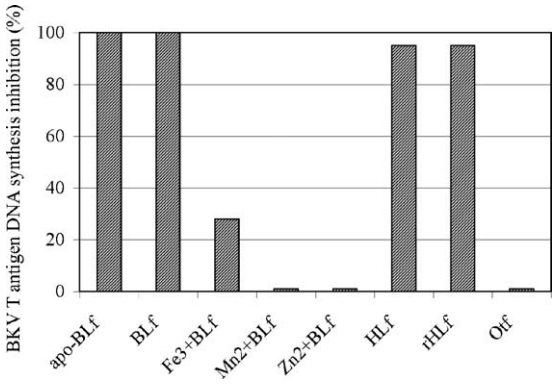

Fig. 1. In vitro antiviral activity of 12.5 μM apo-BLf, BLf, Fe^{3+}BLf, Mn^{2+}BLf, Zn^{2+}BLf, HLf, rHLf, and OTf towards BKV T antigen DNA synthesis. Lfs were present throughout the infection (1 h at 4 °C plus 5 days at 37 °C). Data represent mean values from three separate experiments (S.D. < 15%).

3.3. Effect of BLf on different steps of viral infection

As BLf showed the highest anti-BKV activity, to identify the viral infection step affected, the inhibiting effect of this protein was assessed by different experimental procedures: the cells were incubated with BLf only before infection, protein was added only during or after the viral adsorption step, or was present throughout the whole experiment. Viral replication was measured by determining the BKV T antigen DNA synthesis. Results are reported in Fig. 2. A total inhibition of T antigen DNA synthesis was observed only when BLf was present throughout the whole experiment, a 95% inhibition was obtained when it was added during the attachment step, whereas BLf was ineffective when pre-incubated with the cells before the infection. A very slight inhibition of infection (20%) was obtained when the protein was incubated with the cells after viral binding.

3.4. Neutralisation of BKV infection by BLf

To assess the ability of BLf to bind BKV particles, neutralisation experiments were carried out. The presence of T antigen DNA was tested in mock-infected cells, infected cells and cells

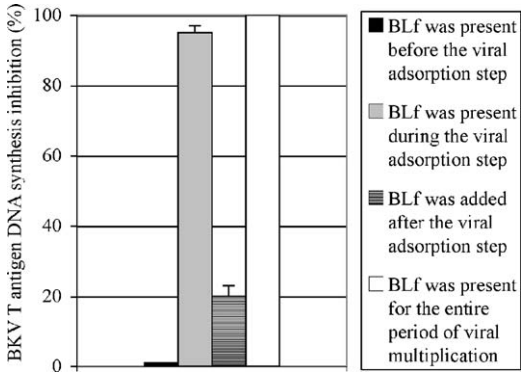

Fig. 2. Effect of 12.5 μM BLf on BKV T antigen DNA synthesis: (i) cells were incubated with BLf before infection (1 h at 37 °C), (ii) BLf was added together with the virus inoculum during the adsorption step (1 h at 4 °C), (iii) BLf was incubated with the cells after the viral adsorption step (5 days at 37 °C), (iv) BLf was present during the whole experiment.

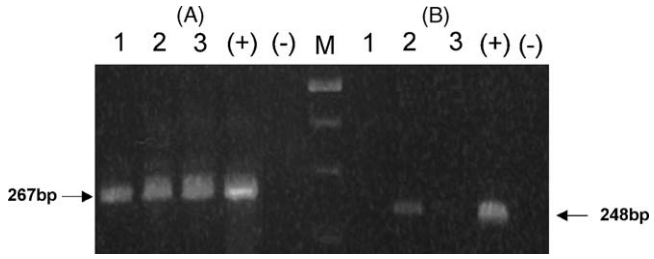

Fig. 3. Electrophoresis on 2% agarose gel of PCR products stained by ethidium bromide. Panel A: amplification of beta-globin gene. Lane 1: DNA extracted from mock-infected cells; lane 2: DNA extracted from BKV-infected cells; lane 3: DNA extracted from cells infected with BLf-treated BKV; (+): beta-globin positive control; (−): negative control (complete reaction mixture without DNA). Panel B: amplification of BKV T antigen DNA. Lane 1: DNA extracted from mock-infected cells; lane 2: DNA extracted from BKV-infected cells; lane 3: DNA extracted from cells infected with BLf-treated BKV; (+): T antigen positive control; (−): negative control.

infected with BLf-preincubated BKV. As shown in Fig. 3, panel B, when BLf was incubated together with the virus before the adsorption step, T antigen DNA synthesis was not detectable, whereas cellular DNA expression (β-globin gene) was comparable to both mock-infected and BKV-infected cells (see Fig. 3, panel A).

3.5. Immunoelectron microscopy

The binding of BLf to BKV particles was visualised by transmission electron microscopy. In Fig. 4 are shown negatively-stained BLf-treated BKV particles: BLf was specifically immuno-labeled with 5 nm gold particles that appear to be localized in the close proximity of the virions (see arrows and inset).

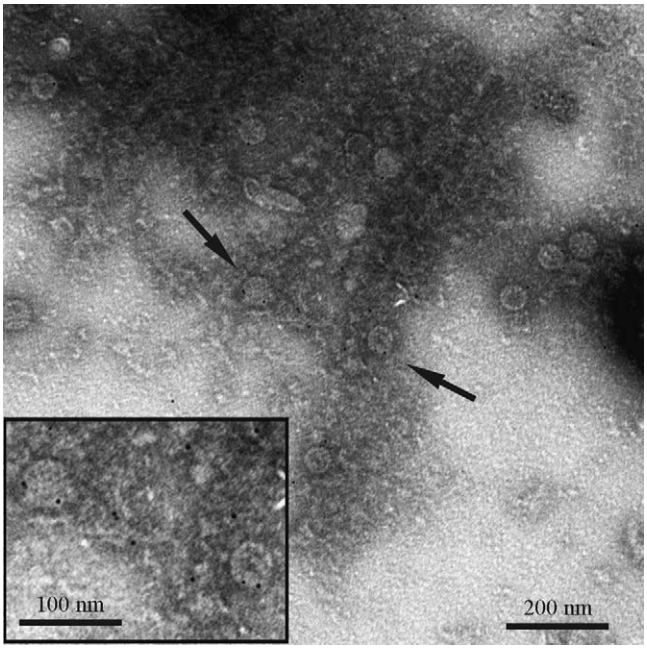

Fig. 4. Electron micrograph of negatively-stained BKV particles. BLf binding to viral particles (arrows and inset) was visualised by anti-lactoferrin rabbit antibodies and anti-rabbit secondary antibodies conjugated with 5 nm gold.

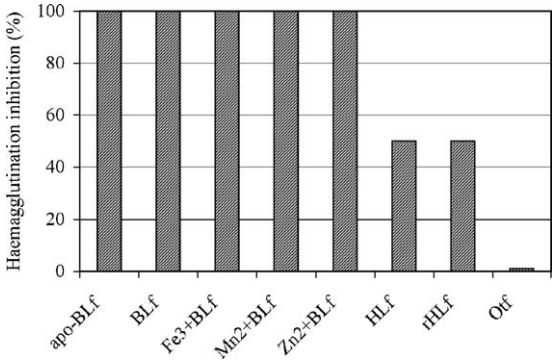

Fig. 5. Effect of 12.5 μM apo-BLf, BLf, Fe^{3+}BLf, Mn^{2+}BLf, Zn^{2+}BLf, HLf, rHLf, and OTf towards BKV hemagglutination. HI titer was calculated as the reciprocal of the highest dilution of proteins capable of completely inhibiting viral hemagglutination and results were expressed as HI percentage compared with controls. Data represent mean values for two separate experiments (S.D. < 10%).

3.6. BKV hemagglutination-inhibition (HI) by proteins

Further experiments were carried out to test the capability of HLf, apo-BLf, rHLf, OTf, Fe^{3+}BLf, Mn^{2+}BLf, and Zn^{2+}BLf BLf to bind BKV particles by an HI assay. Results obtained are shown in Fig. 5. At the concentration of 12.5 μM, BLf, apo-BLf, Fe^{3+}BLf, Mn^{2+}BLf, and Zn^{2+}BLf completely prevented viral hemagglutination. At the same concentration, both HLf and rHLf were able to prevent HA by 50%, whereas OTf was deprived of action.

3.7. Effect of metal ions on BKV infection

As Fe^{3+}BLf, Mn^{2+}BLf, and Zn^{2+}BLf treatment did not affect BKV T antigen DNA synthesis, whereas these proteins were able to prevent viral attachment to red blood cells, we analysed the effect of metal ions on viral replication. In these experiments 12.5 μM ferric, manganese, zinc, and sodium ion citrate complexes were incubated with the cells during the entire period of infection and viral replication was measured by determining the

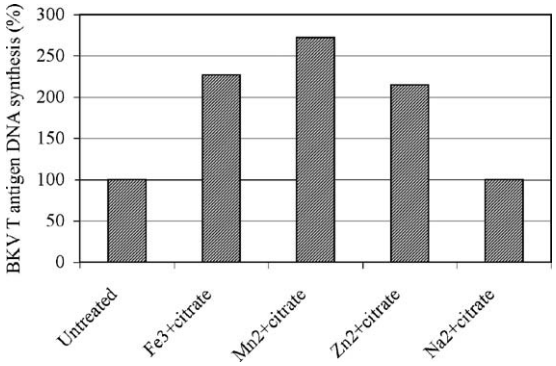

Fig. 6. Effect of different metal ions on BKV replication. Metals bound to citrate (12.5 μM) were incubated with Vero cells during the entire period of infection (5 days at 37 °C); viral infection was expressed as the percentage of BKV T antigen DNA synthesis. Data represent mean values for two separate experiments (S.D. < 20%).

BKV T antigen DNA synthesis. Results obtained are shown in Fig. 6. Interestingly treatment with ferric, manganese, and zinc ions resulted in an increase of viral replication, whereas sodium treatment did not affect BKV infection.

4. Discussion

Over the last decade, polyomavirus nephropathy (PVN) has emerged as an important cause of renal allograft dysfunction and graft loss. PVN occurs with a prevalence of 1–8% in renal transplant recipients and is most commonly reported within the first 12 months post-transplant. The human polyomavirus BKV is thought to be the primary etiologic agent of PVN (Trofe et al., 2004). In the present research we investigated the effect of lactoferrin from bovine milk in the native and apo-form, native lactoferrin from human milk, recombinant human lactoferrin, ovotransferrin, as well as metal ion-saturated BLf towards BKV infection in Vero cells. This is the first study showing that the early steps of human BK polyomavirus infection are prevented by Lf.

Our results showed that lactoferrin from bovine and human milk inhibited BKV infection, human lactoferrin being less active, whereas ovotransferrin was ineffective. The difference in the antiviral activity of BLf, HLf, and OTf may be due to structural differences between these molecules. Bovine and human Lfs show 69% sequence homology but, in spite of this high degree of similarity, differ in their structure, in the type of glycan chains, and in the number of disulphide bridges (Pierce et al., 1991). Ovotransferrin is an iron binding protein present in white egg, belonging to the transferrin family, showing only about 49% sequence homology with human lactoferrin, the most marked homology being localized in the C-terminus region (Williams et al., 1982; Metz-Boutigue et al., 1984; Jeltsch et al., 1987).

Concerning metal ion-saturated BLfs, we observed that these compounds were not able to prevent viral infection whereas they inhibited viral hemagglutination, probably by interfering with BKV binding to red blood cells. To deeper investigate this contrasting behavior of metal-saturated BLfs, we hypothesized that these proteins could release ions into BKV-infected cells, thereby stimulating viral replication. To verify this possibility, infected cells were treated with physiological concentrations of iron, manganese, and zinc, the same ions of metal-saturated BLfs. In fact, it is well known that levels of iron vary widely among individuals, being present in the plasma at concentrations of about 1.4 μg/ml (Linder, 1991) corresponding to 25 μM: manganese concentrations in most adult tissues range between 3 and 20 mM and, during the early stages of development, can be considerably higher, reaching levels of over 200 mM (Roth and Garrick, 2003); and that the mean concentration of zinc in seminal plasma is more than 30 times higher than in blood (202 mg/l corresponding to 3.1 mM versus 6.2 mg/l corresponding to 0.1 mM) (Xu et al., 1994) and urinary zinc excretion is between 300 and 700 μg/day. Results obtained in these experiments confirmed our hypothesis showing an increase of viral replication. As polyomaviruses do not encode for viral DNA polymerases, but instead rely on host-cell enzymes (Hirsch and Steiger, 2003) and use strategies for DNA replication similar to that of their hosts (Fioriti et al., 2005), which in turn need metal ions for DNA synthesis, these results are not surprising. So, even if metal-saturated BLfs bind to the virus, this action may be counteracted by the delivery of ions into the cytoplasm of infected cells that can favor an enhancement of T antigen synthesis (Moens and Van Ghelue, 2005). Even if it is likely that native bLF (20–30% iron-saturated) binds other metal ions *in vivo*, this event does not impair its anti-BKV activity, since metal-ion saturation of the protein does not affect its capability to bind the virus and to neutralise viral infection.

Contrasting results concerning the effect of bLF on viral replication have been reported. Studies on the anti-HCV activity of lactoferrin proved that it was effective only in patients harboring low levels of viral RNA (Tanaka et al., 1999), whereas other studies showed that also patients harboring high levels of viral RNA were sensitive to the treatment (Iwasa et al., 2002). Other researchers demonstrated that BLf treatment did not induce any decrease of HCV-RNA levels (Ishii et al., 2003) and, more recently, it has been reported that orally administered lactoferrin was effective at high doses (3.6 g/day) in about 25% of chronic hepatitis C patients with high viral load (Kaito, 2005).

Previous studies on human papillomavirus (HPV) demonstrated that the uptake of HPV-16 virus-like particles was inhibited by Lf. Results from this research indicated that the inhibition was directed at the receptor-binding step, suggesting that Lf most probably inhibited HPV interaction with common heparan sulphate receptors on the cell surface (Drobni et al., 2004). In regard of BK virus, it has been demonstrated by us that it specifically binds to lipid receptors, such as gangliosides and phospholipids, either on Vero cells or on erythrocytes (Seganti et al., 1981; Sinibaldi et al., 1990, 1992). Therefore, it could be speculated that, in these virus-cell systems, Lf probably can prevent viral adsorption by a direct interaction with viral particles, as already demonstrated for other viruses, such as rotavirus, HIV-1, hepatitis C virus, and adenovirus (Superti et al., 1997; Swart et al., 1996; Yi et al., 1997; Pietrantoni et al., 2003). Indeed, results from neutralisation of infectivity and hemagglutination inhibition assays suggested an interaction between lactoferrin and virus. Finally, immunoelectron microscopy clearly demonstrated that lactoferrin is capable to specifically bind BKV particles. To deeper investigate the nature of BLf-virus interaction, we analysed the influence of sialic acid of bovine lactoferrin on the antiviral activity by testing desialilated BLf. Results from these experiments provided evidence that the anti-BKV activity of desialilated BLf was comparable to that of native one (data not shown) thus indicating, as already reported for rotavirus (Superti et al., 2001), the lack of involvement of sialic acid residues in BLf antiviral activity. Taken together these results suggest that Lfs, mainly the apo- and native-forms, inhibit BKV infection by directly binding to viral particles, although we cannot completely rule out that these glycoproteins could also compete with the virus for common cell binding sites.

In the research of non-nephrotoxic antiviral agents for prophylaxis and treatment of polyomavirus nephropathy (PVN) that could drastically decrease the prevalence of PVN and improve allograft outcomes in renal transplant recipients diagnosed with

PVN, and in the absence of effective antiviral therapies against BKV (Trofe et al., 2004), our results represent an initial important finding.

In this view, it must be reminded that Lf is present in different mucous secretions and, during a screening for its expression in various organs, high levels of Lf mRNA have been detected in human kidney, indicating that Lf is produced by the kidney and that it may participate in innate immunity of this organ (Abrink et al., 2000). The measurement of urinary LF released from polymorphonuclear leucocytes in healthy subjects is about 30 ng/ml, whereas in patients with urinary tract infections it is about 3300 ng/ml, thus indicating that Lf concentrations were much higher than those detected in specimens from healthy subjects (Arao et al., 1999) and suggesting the pivotal role of Lf in urinary tract infectious diseases and inflammation. In fact, in animal models it has been demonstrated that oral administration of Lf or peptides thereof is effective in reducing bacterial infections and inflammation in the urinary tract, possibly through transfer of Lf or its peptides to the site of infection via renal secretion (Haversen et al., 2000). Thus it can be speculated that Lf, as well as other anti-microbial peptides within the innate immune system, could play a role in the defence against BKV. As BKV nephropathology is an increasing cause of graft loss after kidney transplantation and antiviral treatments are not yet available, lactoferrin seems to be a good candidate in the development of new anti-BKV strategies.

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