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Metal complexes of bovine lactoferrin inhibit *in vitro* replication of herpes simplex virus type 1 and 2

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The inhibitory effect of bovine lactoferrin (BLf) saturated with ferric, manganese or zinc ions, on the infection of Vero cells by human herpes simplex virus type 1 (HSV1) and 2 (HSV2) was investigated. Viral infectivity determined by intracellular antigen synthesis and plaque formation was efficiently inhibited by metal saturated lactoferrins in a dose-dependent manner. Effective BLf concentrations which reduced the infection by 50% ranged from 5.2 to 31 $\mu g\ ml^{-1}$ and were far below the cytotoxicity threshold. Fe^{3+}BLf and Mn^{2+}BLf exhibited selectivity indexes higher than Zn^{2+}BLf and apoBLf for both viruses and the effect was mainly directed towards the early steps of infection. The slight viral inhibition shown by the citrate complexes of the different metals could indicate that the antiviral effect was not significantly influenced by Fe^{3+}, Mn^{2+} or Zn^{2+} ions delivered by BLf into the cells.

Keywords: antiviral activity, lactoferrin, metal ion complexes

Introduction

Different proteic and nonproteic metal chelators are capable of inhibiting viral replication through their effect on a variety of viral enzymes which require metal ions for their activity. Metal complexed chelators, on the other hand, have been shown to suppress (Cinatl *et al*. 1994, 1996) enhance (Inouye *et al*. 1994) or unalter (Balogh-Nair *et al*. 1995) the viral infectivity. These different results can be related to the chemical nature of the chelator, to the type of virus as well as to the different experimental conditions.

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Literature data concerning the effect on herpes simplex virus (HSV) of substances interacting with iron and other ions from different pools, are also controversial. In particular, it is well known that ribonucleotide reductases (RNRs) from HSV1 and HSV2, like other iron-dependent RNRs, require iron in the R2 subunit and, as a consequence of this, iron chelators can inhibit HSV replication (Liuzzi *et al*. 1994). The plant amino acid mimosine inhibited HSV1 ribonucleotide reductase through its capacity to chelate iron (Dai *et al*. 1994); different acetylpiridine derivatives inhibited HSV1 replication (Spector *et al*. 1991) and potentiated the activity of acyclovir (Spector *et al*. 1989); contrary to cytomegalovirus, the replication of HSV1 and HSV2 was not influenced by desferrioxamine (Cinatl *et al*. 1994) and calcium trinatrium diethylenetriaminepentaacetic acid (Cinatl *et al*. 1996).

Among the metal chelators, the iron binding protein lactoferrin (Lf), initially found in bovine milk, (Soerensen & Soerensen 1939) and present in other external secretions and in plasma of mammals, has been found to indirectly influence a wide spectrum of physiological activities through its effect on iron availability (Levay & Viljoen 1995, Alugupalli 1996). Lf possesses a variety of biological functions such as: promotion of iron absorption (Davidson & Lonnerdal 1989, Hu *et al*. 1990, Sanchez *et al*. 1996), immunomodulation and antimicrobial activity towards bacteria, fungi, protozoans (Levay & Viljoen 1995, Alugupalli 1996) and viruses (Hasegawa *et al*. 1994, Fujihara & Hayashi 1995, Harmsen *et al*. 1995, Marchetti *et al*. 1996a, Shimizu *et al*. 1996, Swart *et al*. 1996). The amount of Lf contained in different body fluids varies considerably; levels in milk, colostrum and seminal fluid are high, ranging from 1.0 to 8.0 mg ml^{-1}, whereas levels in tears and saliva are between 0.1 and 2.0 mg ml^{-1} and in plasma about 0.1 ug ml^{-1} (Levay & Viljoen 1995). In the course of different bacterial infections an up to 50-fold increase of Lf levels has been described (Nuijens *et al*. 1992), while during viral infections the levels of Lf in tears and plasma have been found markedly decreased (Comerie-Smith *et al*. 1994, Defer *et al*. 1995). The influence of these fluctuations on the course of microbial infections remains to be elucidated.

In previous research, it has been shown that human and bovine lactoferrin (BLf) exerted *in vitro* a potent antiviral activity against HSV replication by interacting with both cellular binding sites and viral particles (Marchetti *et al*. 1996a, Swart *et al*. 1996). Moreover, *in vivo* studies demonstrated a Lf-mediated inhibition of HSV1 infection to mouse cornea (Fujihara & Hayashi 1995) and a Lf-mediated protection of mice from bovine cytomegalovirus (Shimizu *et al*. 1996). On the basis of these data, the administration of Lf could be applied in antiviral therapy, to control infections in patients with low Lf levels. For this reason it would be relevant to ascertain whether metal ions bound to the protein are involved in its antiviral activity.

In the present study we compared the antiviral activity towards HSV1 and HSV2 of apo-BLf with that of metal ion saturated forms in an attempt to correlate the activity to the saturation of metal binding sites. For this purpose the effect on viral replication of Fe^{3+}BLf, Mn^{2+}BLf, Zn^{2+}BLf and of metal ions delivered into the cells was investigated.

Materials and methods

Chemicals

Apo-lactoferrin from bovine milk (apo-BLf) was purchased from Fluka, Switzerland. Lf purity was checked by SDS-PAGE stained with silver nitrate. Iron, manganese and zinc saturated bovine lactoferrins (Fe^{3+}BLf, Mn^{2+}BLf, Zn^{2+}BLf) were prepared by incubation of the apo-protein dissolved in 0.1 M sodium bicarbonate with a ten-fold excess of the citrate complex of the different metal ions for 12 h at room temperature, followed by extensive dialysis against 0.1 M sodium bicarbonate to remove unligated metal ions. After incubation of the protein in MEM (Eagle's Minimal Essential Medium) for 1 and 2 h, in the presence or absence of Vero cells, apo-BLf was found to be approximately 10% iron-saturated, Fe^{3+}BLf resulted about 95% iron-saturated and the manganese and zinc saturation of Mn^{2+}BLf and Zn^{2+}BLf remained for both about 85% (determined by atomic adsorption spectrophotometer Perkin Elmer model 360). Lf concentration in tissue culture supernatants was controlled at different times by ELISA using a rabbit antihuman lactoferrin antibody (Sigma Chemical Co., St Louis, MO, USA) that showed a high cross reactivity towards bovine lactoferrin and anti-rabbit IgG bound to peroxidase.

To supply ferric, manganese and zinc ions to the cells at the concentration of 10 μM, corresponding to that required to saturate 400 μg ml^{-1} BLf, metal ion citrate complexes were prepared from sodium citrate and chloride or sulfate salts of the different metals.

Cells and viruses

Vero (African green monkey kidney) cells were cultured at 37 °C in MEM containing 1.2 g l^{-1} $NaHCO_3$ and supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. For cell maintenance the serum concentration was lowered to 2%.

Herpes simplex virus type 1 (HSV1) strain F and type 2 (HSV2) strain G were propagated in Vero cells and the virus concentration was estimated by plaque assay as already described (Marchetti *et al*. 1996b).

Antiviral assays

Preliminarily, to determine the cytotoxicity of compounds, Vero cells grown in 96-well plates were exposed to increasing concentrations of chemicals for 48 h at 37 °C and cell viability was assayed by evaluating the cell morphology, viability (determined by neutral red staining) and proliferation (determined by microscopic counts after dispersion into individual cells with trypsin).

For viral antigen synthesis inhibition assays, cells grown in 8-well microtissue chamber slides for 24 h in 5% CO_2 at 37 °C were washed and infected with viruses at a multiplicity of 0.04 PFU $cell^{-1}$ for HSV1 and 0.02 PFU $cell^{-1}$ for HSV2. Viral adsorption was carried out in the presence

or absence of the drugs. After 1 h at 4 °C or 37 °C, cells were newly washed to remove excess virus, fresh medium with or without chemicals was added and cells were incubated for 17 h at 37 °C. Then viral antigen synthesis was determined by immunofluorescence as previously described (Marchetti *et al* 1996a).

For plaque reduction assays, cells grown in 24-well tissue culture plates were infected with different virus concentrations. Viral adsorption (1 h at 4 °C) was carried out in the presence or absence of the lactoferrins. After removal of virus inoculum, monolayers were washed and overlayed with 0.5% agarose. After three days' incubation at 37 °C the number of plaques was determined as previously described (Marchetti *et al* 1996b).

Results and discussion

The cytotoxicity (CC_{50}) and efficacy (ED_{50}) in inhibiting HSV replication in Vero cells of apo-BLf, Fe^{3+}BLf, Mn^{2+}BLf and Zn^{2+}BLf are shown in Table 1. Cytotoxic concentrations were routinely higher than 10 mg ml^{-1} and at least 300 times greater than the ED_{50} doses determined for each compound. Differently saturated lactoferrins, incubated with the cells during the adsorption step and newly added for 17 h after the removal of virus inoculum, markedly inhibited viral antigen synthesis; the effective concentrations ranged from 12 to 31 μg ml^{-1} for HSV1 and from 5.2–31 μg ml^{-1} for HSV2, respectively. Similar results were obtained by determining the viral infectivity with PFU counts. The antiviral effectiveness of the various compounds towards both viruses, evaluated as selectivity index (SI), was greater for Fe^{3+}BLf and Mn^{2+}BLf and lower for Zn^{2+}BLf and apo-BLf.

To ascertain whether the antiviral activity took place on viral adsorption or on a different step of viral replication, the inhibiting activity of increasing concentrations of BLf saturated with iron, manganese or zinc ions was assayed by following different experimental procedures: (a) Lfs were present during the whole experiment; (b) Lfs were added together with virus inoculum during the adsorption step (1 h at 4 °C) and then removed; (c) Lfs were present only after the adsorption step for 17 h at 37 °C. The Lf content in the supernatants at various time points was analysed by an ELISA assay and no significant differences in Lf amounts among the different samples (at 100 μg ml^{-1} concentration) were found (data not shown). The data illustrated in Figures 1 and 2 show a dose-dependent antiviral effect of Lfs in all experimental conditions. At the highest concentration (400 μg ml^{-1}) no difference could be observed among the metal ion saturated forms, as about a 100% inhibition of viral antigen synthesis was reached both in (a) and (b) conditions. At lower doses different values were observed depending on the Lf saturated form and on the period of incubation of the protein with cell monolayers. When differently saturated Lfs were added after the viral attachment step (condition c), even at 400 μg ml^{-1}, the inhibition never exceeded 60%. These data indicate that Lfs blocked HSV1 and HSV2 infection at the level of virus adsorption since a complete inhibition was achieved when the proteins were present only during this step.

It could be hypothesized that metal-saturated Lfs, after their interaction with the cell surface, release intracellularly metal ions as already demonstrated (Davidson & Lonnerdal 1989, Hu *et al*. 1990, Sanchez *et al*. 1996). In order to verify whether the inhibiting effect of Lfs was influenced by the delivery of metals to the cells, experiments were carried out by using the citrate complexes of the ferric, manganese and zinc ions at the same concentration (10 μM) required to saturate 400 μg ml^{-1} BLf. For this

Table 1. *In vitro* antiviral activity of apo-BLf, Fe^{3+}BLf, Mn^{2+}BLf and Zn^{2+}BLf towards the synthesis of HSV1 and HSV2 antigens[a]

Lactoferrin	CC_{50}[b] (mg ml^{-1})	ED_{50}[c] (μg ml^{-1})		SI[d]	
		HSV1	HSV2	HSV1	HSV2
Apo-BLf	> 10	28.0 ± 5.0	31.0 ± 8.0	> 357	> 322
Fe^{3+}BLf	> 10	12.0 ± 4.0	5.2 ± 0.5	> 833	> 1923
Mn^{2+}BLf	> 10	13.5 ± 4.4	10.0 ± 0.9	> 740	> 1000
Zn^{2+}BLf	> 10	31.0 ± 3.2	23.0 ± 1.8	> 322	> 434

[a]Vero cells were infected with HSV1 (0.04 PFU cell^{-1}) or HSV2 (0.02 PFU cell^{-1}). Lfs were incubated at different concentrations with the cells during the viral adsorption step (1 h at 4 °C) and newly added after the removal of virus inoculum. 18 h post infection, the percentage of infected cells was evaluated by direct immunofluorescence. Each sample was done in duplicate. Data concerning ED^{50} represent mean values for three separate experiments ± SD.

[b]CC_{50}: cytotoxic concentration, 50%; ED_{50}: effective dose, 50%, the ED_{50} values represent the dose required to inhibit fluorescence by 50%; [d]SI: selectivity index (SI = CC_{50}/ED_{50}).

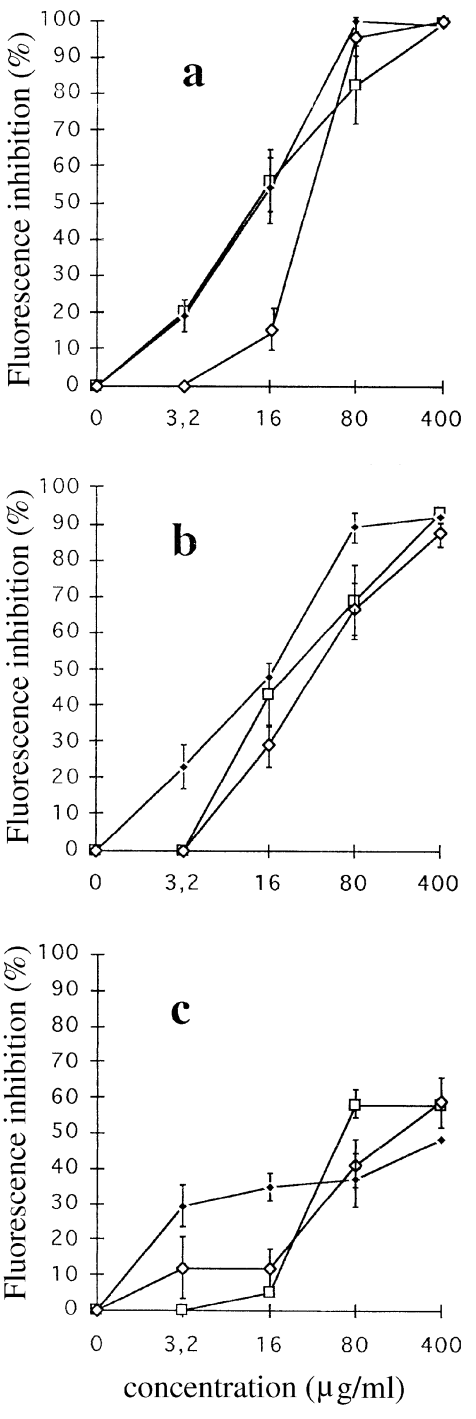

Figure 1. Dose-response of the inhibitory effect of bovine Lf saturated with ferric (□), manganese (◆) or zinc (◇) ions on HSV1 antigen synthesis in Vero cells. Lfs were present throughout the infection (a); Lfs were added together with virus inoculum during the adsorption step (1 h at 4 °C) and then washed away (b); Lfs were added to infected cells only after the adsorption step for 17 h at 37 °C (c). Data represent mean values for three separate experiments ± SD.

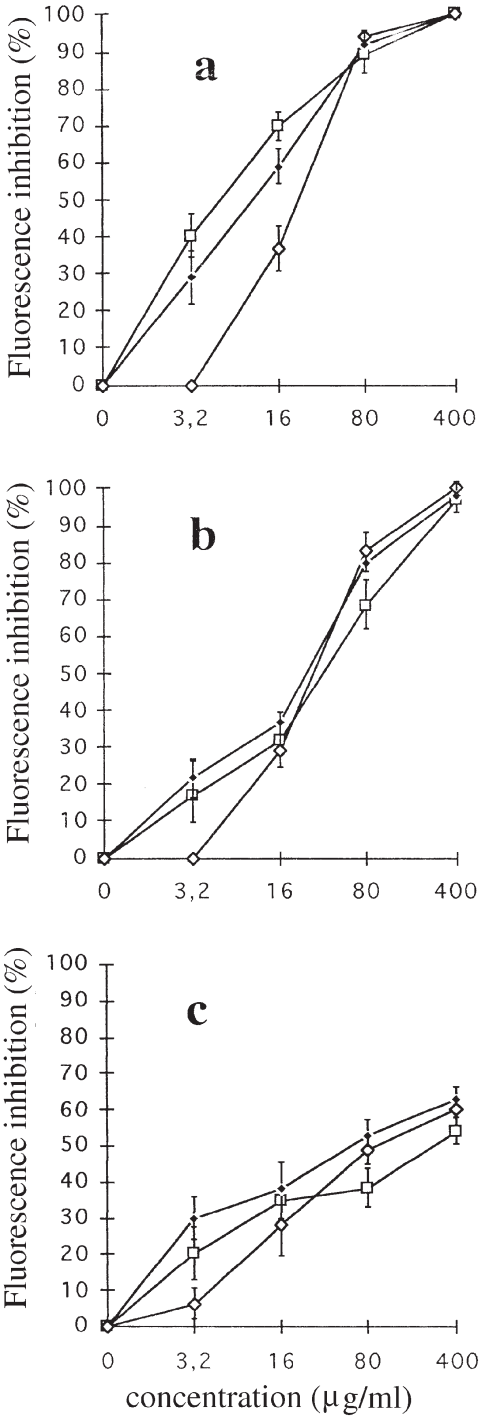

Figure 2. Dose-response of the inhibitory effect of bovine Lf saturated with ferric (□), manganese (◆) or zinc (◇) ions on HSV2 antigen synthesis in Vero cells. Lfs were present throughout the infection (a); Lfs were added together with virus inoculum during the adsorption step (1 h at 4 °C) and then washed away (b); Lfs were added to infected cells only after the adsorption step for 17 h at 37 °C (c). Data represent mean values for three separate experiments ± SD.

purpose metal citrate complexes known to deliver metals to the cells (Kriegerbeckova *et al.* 1995) were added to cell monolayers before the infection (24 h at 37 °C) or during the virus incubation with cells (1 h at 37 °C). Table 2 shows the effect of these compounds in comparison with sodium citrate used as a control. Only a weak effect (from 4% up to 20% inhibition of antigen synthesis) was exerted by all the tested compounds, in both experimental conditions.

Literature data concerning the antiviral activity of metal complexed chelators are controversial (Cinatl *et al.* 1994, 1996, Inouye *et al.* 1994, Balogh-Nair *et al.* 1995). Lf is an iron binding protein, capable to deliver metal ions into cells (Davidson & Lonnerdal 1989, Hu *et al.* 1990, Sanchez *et al.* 1996) and possessing antiviral activity in its apo-form (Hasegawa *et al.* 1994, Fujihara & Hayashi 1995, Harmsen *et al.* 1995, Marchetti *et al* 1996a, Shimizu *et al.* 1996, Swart *et al.* 1996). We have therefore investigated the antiviral activity of the apo-Lf and the iron, manganese and zinc saturated Lf towards herpes simplex virus type 1 and 2.

This study represents the first report of a marked antiviral activity of apo-Lf towards HSV2 after the previous observations on HSV1 (Hasegawa *et al.* 1994, Marchetti *et al* 1996a) and of metal ion saturated Lf towards HSV1 and HSV2. The antiviral effect of the differently saturated Lfs towards both viruses was mainly exerted during the initial viral adsorption phase (1 at 4 °C) as clearly chown in Figures 1 and 2 (panels b). In agreement with that shown by other authors for apo-Lf and cytomegalovirus (Harmsen *et al.* 1995), it is therefore possible to postulate that differently saturated Lf interferes with HSV binding to cell surface heparansulfate receptors, even if an antiviral activity through the interaction of Lf with the viral envelope cannot be ruled out (Marchetti *et al.* 1996a). The residual antiviral activity (60% inhibition of viral antigens synthesis at the highest Lf concentration) observed when differently saturated Lf was added in the post adsorption step (Figures 1 and 2, panels c), may be due to the Lf interaction with virions which start to be released by infected cells already six hours from infection. In this condition, Lf already bound to cell surfaces and present in the culture medium, can hinder the infection of neighbouring cells and thus decrease the percentage of infected cells.

Our data clearly show that, when Lfs were incubated for the whole cycle of viral multiplication, the lowest 50% antiviral effective doses were reached by iron and manganese saturated Lfs (Table 1). However also Zn^{2+}BLf and apo-BLf were proved to be effective inhibitors. It must be considered that in these experimental conditions manganese and zinc saturated Lfs cannot exchange bound metal ions for iron while apo-BLf is partly iron saturated in MEM (see Materials and methods). It could therefore be inferred that iron, manganese and zinc saturated Lfs possess an antiviral activity which is not present in the fully unsaturated Lf. It should be recalled that, upon binding of iron, Lf undergoes a large conformational change which occurs also when Lf is saturated with other metals (Baker *et al.* 1994). Such a conformational change might result in a higher affinity of the molecule for its receptors on eucaryotic cells compared with the apo-form (Davidson & Lonnerdal 1989, Levay & Viljoen 1995). Accordingly, it has been shown that also manganese saturated Lf can bind to cell surfaces even if it binds with lower affinity than iron saturated Lf (Davidson & Lonnerdal 1989).

Since BLf interacts with Vero cell surfaces (Marchetti *et al.* 1996a) it can be assumed also that metal saturated forms of Lf contribute to metal ion transport across cell membrane, as already demonstrated for human Lf *in vitro* (Sanchez *et al.* 1996) and *in vivo* (McAbee 1995). However the data

Table 2. Effect of different metal ions on HSV1 and HSV2 infectivity[a]

Compound	Fluorescence inhibition (%)			
	HSV1		HSV2	
	a[b]	b[c]	a[b]	b[c]
Fe^{3+}citrate	13.4 ± 3.0	10.4 ± 4.8	21.0 ± 3.9	16.8 ± 3.2
Mn^{2+}citrate	8.2 ± 1.8	6.4 ± 4.2	13.5 ± 2.7	5.2 ± 1.8
Zn^{2+}citrate	11.4 ± 2.0	7.4 ± 2.3	15.0 ± 2.5	4.2 ± 1.0
Na citrate	5.9 ± 0.8	2.6 ± 0.9	5.1 ± 1.1	3.3 ± 0.5

[a]Infection was carried out for 18 h at 37 °C; then the percentage of infected cells was evaluated by direct immunofluorescence. Each sample was done in duplicate. Data represent mean values for three separate experiments ± SD.
[b]Metals bound to citrate (10 μM) were incubated for 24 h at 37 °C before infection; [c]metals bound to citrate (10 μM) were incubated for 1h at 37 °C during the viral attachment step.

obtained by using metal citrate complexes indicate that the simple intracellular release of metal ions is not sufficient to account for the observed higher antiviral activity of metal saturated Lfs.

In conclusion, lactoferrin, an important component of the nonimmune defence system, appears to be an ideal antiviral agent since it interrupts viral replication at the level of viral attachment, a specific and essential step of the replicative cycle, without affecting normal host-cell metabolism. Even though the influence of metals and their chelators on HSV replication has still to be clarified, the antiviral activity of Lf seems to correlate with the metal saturation of the protein. This observation can be physiologically relevant since partially iron saturated Lf naturally occurs in biological fluids.

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