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Antiviral effect of bovine lactoferrin saturated with metal ions on early steps of human immunodeficiency virus type 1 infection

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Abstract

Lactoferrin is a mammalian iron-binding glycoprotein present in many biological secretions, such as milk, tears, semen and plasma and a major component of the specific granules of polymorphonuclear leucocytes. The effect of bovine lactoferrin (BLf) in apo-form or saturated with ferric, manganese or zinc ions, on human immunodeficiency virus type 1 (HIV-1) infection in the C8166 T-cell line was studied. Both HIV-1 replication and syncytium formation were efficiently inhibited, in a dose-dependent manner, by lactoferrins. BLf in apo and saturated forms markedly inhibited HIV-1 replication when added prior to HIV infection or during the virus adsorption step, thus suggesting a mechanism of action on the HIV binding to or entry into C8166 cells. Likewise, the addition of Fe^{3+} BLf prior to HIV infection and during the attachment step resulted in a marked reduction of the HIV-1 DNA in C8166 cells 20 h after infection. The potent antiviral effect and the high selectivity index exhibited by BLf suggest for this protein, in apo or saturated forms, an important role in inhibiting the early HIV–cell interaction, even though a post adsorption effect cannot be ruled out. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Lactoferrin; Antiviral agents; HIV; Chelating agent; Glycoprotein

1. Introduction

Lactoferrin (Lf) is a mammalian iron-binding glycoprotein of 80,000 Da possessing two metal binding sites in two different lobes (N-lobe and C-lobe) [1]. This glycoprotein is produced by polymorphonuclear leukocytes and exocrine glands and is generally present in various biological fluids and mucosal surfaces [2–7]. During microbial infections or autoimmune diseases, Lf levels in plasma show an increase from about 4 up to 200 μg/ml. [8–10]. Conversely, in asymptomatic human immunodeficiency virus (HIV)-positive individuals, markedly decreased levels of Lf

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are found in tears [11] and, in symptomatic HIV-infected patients, levels of plasma Lf decrease down to 0.68 μg/ml [12].

A wide spectrum of functions has been ascribed to Lf including a role in the control of bacterial and micotic infections as well as in immune modulation [13, 14]. In addition to its iron chelating property, the antimicrobial property of Lf has also been associated to its binding on the surface of various pathogens [15–17]. In particular, a loop localised within the N-lobe distinct from iron-binding site has been shown to be involved in this effect [18]. On the other hand, Lf binds to specific receptors on various eukaryotic cells, including human T-lymphocytes [19–22, 3, 23] and the existence in its N-terminus of a binding site for low-density lipoprotein receptor-related protein as well as for glycosaminoglycans has been also reported [24–26]. These ubiquitous components of eukaryotic cell membranes may act as receptors for some enveloped viruses, including herpes simplex virus (HSV) [27] and HIV [28].

Although the antiviral effect of milk proteins was already reported by Matthews et al. in 1976 [29], the antiviral activity has been only recently ascribed to lactoferrin. Lactoferrins from human (HLf) and bovine (BLf) milk have been shown to exhibit potent in vitro antiviral activity against cytomegalovirus [30, 31] and HSV-1 infection [31, 32, 33]. Recently, an inhibitory effect of the native Lf as well as of succinylated and aconitylated derivatives on HIV replication has been reported [34] even if in these studies no detailed characterization of the antiviral effects was provided.

In a previous study [33], we showed that HLf and BLf exerted a potent antiviral activity against HSV_1 infection by competing with cellular binding sites for the virus. An antiviral mechanism similar to that suggested for HSV-1 could be hypothesised for HIV, since it has been shown that Lf strongly binds to the V3 loop of the gp120 envelope protein [34]. In this study, we investigated the antiviral activity of BLf, in the apo-form or saturated with divalent and trivalent metals, and analysed their effect on different steps of the HIV-1 replication cycle in the C8166 T-cell line. BLf was chosen in our studies since, among transferrins, it is the most effective viral inhibitor [30, 33] and exhibits many advantages for a putative application in antiviral therapy, such as easy availability, very low cytotoxicity and low cost.

2. Materials and methods

2.1. Chemicals

Apo-lactoferrin (apo-Lf) from bovine milk [35] was purchased from Fluka, Switzerland. Its purity was checked by SDS-PAGE stained with silver nitrate. In order to test the presence of possible traces of lipopolysaccharide (LPS), the *Limulus* amoebocyte lysate (L.a.1.) assay was performed using standard LPS solution which was L.a.1. positive at 0.1 endotoxin units/ml (14 pg/ml) (QCL-1000 Quantitative Chromogenic LAL, Biowhittaker, Walkersville, MD). Apo-BLf at the highest concentration (12,500 μg/ml) used was negative to L.a.1. test. Iron-, manganese- and zinc-saturated bovine lactoferrins (Fe^{3+}BLf, Mn^{2+}BLf, Zn^{2+}BLf) were prepared by Professor Giovanni Antonini (Department of Basic and Applied Biology, University of L'Aquila). Briefly, metal ion saturated lactoferrins were obtained by incubating 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. Apo-BLf was found to be approximately 10% iron-saturated, Fe^{3+}BLf about 95% and the manganese and zinc saturation of Mn^{2+}BLf and Zn^{2+}BLf was about 85%, as determined with an atomic adsorption spectrophotometer (Perkin Elmer mod. 360).

In the antiviral assays apo- and metal-saturated BLf were dissolved in RPMI 1640 cell culture medium (Gibco BAL). Total iron concentration in the medium varied from 0.01 to 0.05 μM as determined with the atomic adsorption spectrophotometer. Lf concentration in tissue culture supernatants, during the incubation

period with cells, was controlled at different times by an ELISA using specific anti-BLf antibodies from rabbits (kindly provided by Morinaga Milk Industries) and anti-rabbit IgG bound to peroxidase (Sigma). Dextran sulphate (5 kDa) was purchased from Sigma.

2.2. Cells and virus

The T-cell line C8166 [36] was used for the anti-HIV-1 assay. Cells were grown in RPMI 1640 medium complemented with 10% heat-inactivated foetal calf serum (FCS). The HIV-1_{IIIB} strain was propagated and titrated in a human T4-lymphoblastoid cell line (CEM-SS).

The amount of infectious virus in the supernatant of infected cells was measured as described in detail elsewhere [37]. Syncytia formation was checked by visual inspection with an inverted Leitz microscope.

2.3. Antiviral assays

For antiviral assays, 1×10^6 C8166 cells were washed twice and pre-exposed to increasing concentrations of chemicals in RPMI 1640 medium or medium alone for 1 h at 37°C. Cells were then newly washed and infected with HIV-1 at a multiplicity of 0.01 or 0.1 tissue culture infecting doses 50% ($TCID_{50}$) per cell. Viral adsorption was carried out in the presence or absence of the drugs. After 2 h, cells were extensively washed to remove excess virus and fresh medium with or without chemicals was added. Unless differently stated, drugs were maintained continuously in culture. At defined time points, syncytia formation was examined through an inverted Leitz microscope and p24 levels in the supernatants were detected by ELISA (New Research Products, Du Pont).

2.4. Cytotoxicity assays

Cytotoxicity was evaluated by incubating two-fold serial dilutions of the proteins in maintenance medium for 72 h at 37°C with C8166 cells grown in 96-well microtiter plates. Cell proliferation and viability were assessed by a non-radioactive quantitative colorimetric assay purchased from Boehringer Mannheim (Cell proliferation kit-MTT). Tetrazolium salts used for assaying the quantification of viable cells were cleaved to form a formazan dye which was evaluated by the spectrophotometrical absorbance at 600 nm.

2.5. Detection of HIV-1 proviral DNA in HIV-1 infected C8166 cells

The amount of initial reverse transcribed product at 20 h after infection was evaluated by using a semiquantitative polymerase chain reaction (PCR) amplification technique. At indicated time points, cells were collected and lysed in K buffer as previously described [38]. PCR amplification was performed with 1 μg of DNA, the gag-specific primers (SK38 and SK39) and 1 U of Amplitaq (Perkin-Elmer Cetus) for 30 cycles (95°C for 30 s, 55°C for 30 s, 72°C for 60 s). PCR products, resolved on 2.5% Seakern (FMC) agarose gels and transferred to a nylon membrane, were detected by oligomer hybridisation with an excess of SK19 probe and labelled with ^{32}P [38]. Control samples of known HIV-1 DNA copy number (15 to 1500) were prepared from the 8E5 cell line and analyzed in parallel. Sequences for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers and probe have been previously reported in Ref. [39].

3. Results

3.1. Antiviral activity of BLf in apo and saturated forms

The efficacy (IC_{50}) of apo-BLf, Fe^{3+}BLf, Mn^{2+}BLf and Zn^{2+}BLf in inhibiting HIV replication in C8166 cells is shown in Table 1. Lactoferrins markedly inhibited both HIV-1 p24 production and syncytia formation; the effective concentrations in inhibiting viral infection by 50% ranged from 2.0 to 6.5 μg/ml and 10 to 20 μg/ml, respectively. The cytotoxic dose 50% (CC_{50}), measured by cell proliferation and viability determinations after 72 h incubation of cells with drugs, was >12.500 μg/ml for apo-BLf and

Table 1
Toxicity and anti-HIV activities of bovine lactoferrin in apo and differently satured forms

	p24				syncytia		
	CC_{50}^{a}	IC_{50}^{b}		SI[c]	IC_{50}		SI
Compound	(μg/ml)	(μg/ml)	(nM)		(μg/ml)	(nM)	
apo-BLf	> 12,500	6.5	81	> 1,925	20	250	> 625
Fe^{3+}BLf	> 12,500	2.0	25	> 6,250	10	125	> 1,250
Mn^{2+}BLf	12,500	5.5	69	2,272	10	125	1,250
Zn^{2+}BLf	12,500	5.5	69	2,272	20	250	625
Dextran sulphate (5 kDa)	200	0.07	14	2,857	0.5	100	400

[a] Cytotoxic concentration 50%.
[b] Compound concentration required to reduce HIV p24 release and syncytium formation by 50%.
[c] Selectivity index (SI = CC_{50}/IC_{50}).
C8166 cells were infected with HIV-1-IIIB strain (0.01 $TCID_{50}$/cell). 72 h post infection, syncytia were counted microscopically and supernatants were collected for p24 assay. Data are the average of two independent determinations. Each sample was done in triplicate.

Fe^{3+}BLf and 12.500 μg/ml for Mn^{2+}BLf and Zn^{2+}BLf. CC_{50} were routinely at least 625–6,250 times greater than the IC_{50} doses determined for each compound. The anti-HIV-1 effectiveness of the various compounds, measured as p24 release was: Fe^{3+}BLf > Mn^{2+}BLf = Zn^{2+}BLf > apo-BLf. Dextran sulphate, used as internal control for its known inhibitory action on the binding of HIV-1 to CD4 positive cells [40] displayed an IC_{50} of 0.07 μg/ml, measured as p24 release, and of 0.5 μg/ml, measured as syncytia formation. However, its selectivity indexes (SI) were lower than those observed for iron-saturated BLf.

We then determined the dose-response effect of BLf, saturated with ferric ions, on HIV replication in C8166 cells infected at two different multiplicities of infection (m.o.i.s). As shown in Fig. 1(panel A), the addition of 20 μg/ml of BLf to C8166 cells infected at the m.o.i. of 0.1 $TCID_{50}$ resulted in a 50% inhibition of HIV replication, whereas a drastic virus inhibition was observed already at 200 μg/ml of BLf. Notably, a

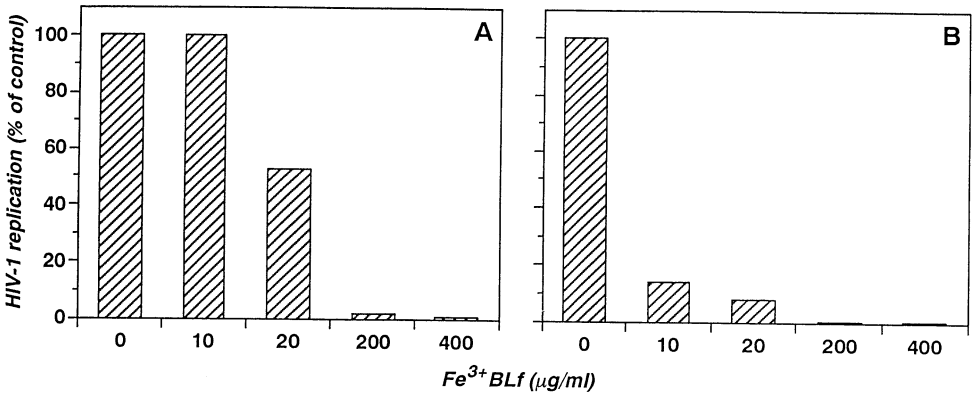

Fig. 1. Dose–response of the inhibitory effect of Fe^{3+} BLf on HIV-1 replication. C8166 cells were infected with HIV-1 at a m.o.i. of either 0.1 (panel A) or 0.01 (panel B) $TCID_{50}$/cell. HIV replication was evaluated by measuring the levels of the p24 antigen in the supernatants 72 h after virus infection. Values represent the % of inhibition with respect to the untreated virus-infected cell cultures. Triplicate samples were tested. Standard deviations did not exceed 15%.

BLf concentration, as low as 20 μg/ml, was sufficient to induce a 90% inhibition of viral replication in cultures infected with 0.01 $TCID_{50}$ of HIV (Fig. 1, panel B).

3.2. Effect of Fe^{3+} BLf on different steps of HIV infection

To evaluate whether the antiviral activity took place on viral adsorption or on a different step of viral replication cycle, the ability of Fe^{3+} BLf to inhibit HIV infection was assayed by synchronising the infection with a temperature shift. Viral adsorption was performed at 4°C for 2 h (at a m.o.i of 0.1 $TCID_{50}$/cell) and, after removal of inoculum, the virus infection was allowed to progress by raising the temperature to 37°C. Lf, at the concentration previously assessed to completely inhibit viral replication (800 μg/ml), was added following different experimental procedures: (i) cells were pre-incubated with Lf only for 1 h at 37°C, before virus infection; (ii) Lf was added together with virus inoculum during the adsorption step and then washed away. (iii) Lf was present only after the adsorption step; (iv) Lf was continuously present during the infection experiment. The data illustrated in Fig. 2 indicated that Fe^{3+} BLf blocked HIV infection at the level of virus adsorption to C8166 cells. In fact, an almost complete inhibition of HIV-1 p24 release was observed when the protein was preincubated with cells (b) or present during the virus-cell incubation period at 4°C (c). A complete inhibition of p24 release was observed when Fe^{3+} BLf was preincubated and then maintained for the entire length of the experiment (e). The addition of Fe^{3+} BLf only after the viral adsorption step resulted in a partial inhibition of HIV-1 p24 release (d). To evaluate whether this differential effectiveness was due to variations in the residual protein concentrations related to different incubation procedures, we analysed the Fe^{3+} BLf content in the supernatants at various time points by using a sensitive ELISA. No significant differences in Lf titres among the different samples were found.

In a separate set of experiments, we have also measured the infectious virus yields by titration of serial dilutions of supernatants in C8166 cells. The incubation with Fe^{3+} BLf during the viral attachment step at 4°C resulted in a 4.8 $\log_{10}$ inhibition of virus yield as compared to control infected cultures (data not shown).

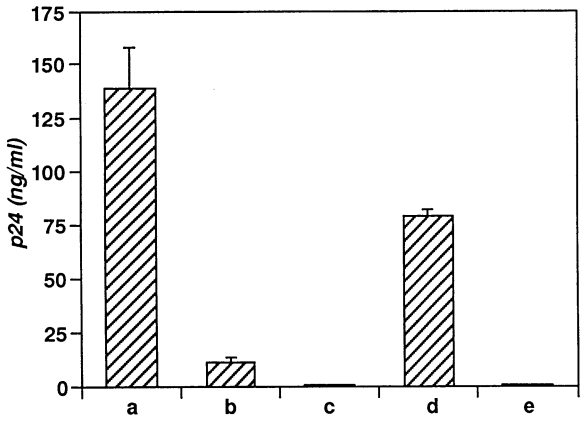

Fig. 2. Effect of 800 μg/ml Fe^{3+} BLf on different steps of HIV replication in C8166 cells (m.o.i. of 0.1 $TCID_{50}$/cell). There were triplicate samples for each experimental condition. Standard deviations did not exceed 15%. (a) Control untreated HIV-1 infected cells, (b) cells were pre-incubated with Lf for 1 h at 37°C; (c) Lf was added together with virus inoculum during the adsorption step (2 h at 4°C) and then washed away; (d) Lf was present only after the adsorption step for 48 h at 37°C; (e) Lf was present through the infection.

3.3. Detection of HIV-1 proviral DNA in untreated or Fe^{3+} BLf-treated HIV-1 infected C8166 cells

To further confirm that Fe^{3+} BLf acted on early steps of HIV-1 infectious cycle we measured the amount of initial reverse transcribed product at 20 h after infection by using a semiquantitative PCR amplification technique. As shown in Fig. 3, treatment of cells with Fe^{3+} BLf (400 μg/ml) prior to HIV infection and during the attachment step markedly reduced the amount of the initial reverse-transcribed product. Densitometric analysis of the autoradiogram indicated that the decrease in the initial reverse-transcribed product was greater than 90% (data not shown).

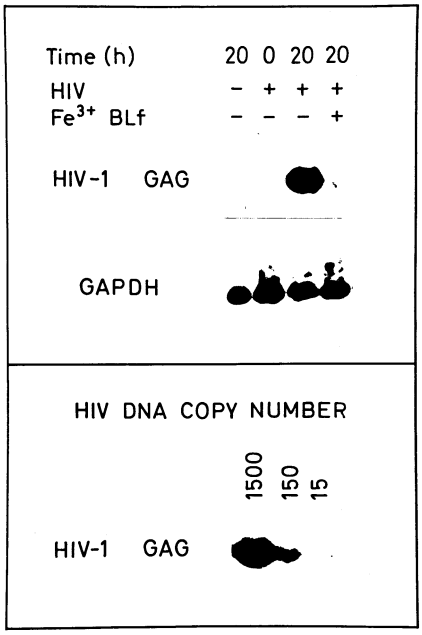

Fig. 3. Detection of HIV-1 proviral DNA by PCR. DNA in cell lysates [prepared from control C8166 cells and from cells infected with HIV (m.o.i. 0.01 $TCID_{50}$/cell) at 0 and 20 h after infection, pretreated or not with 400 μg/ml of Fe^{3+}BLf] was subjected to PCR amplification with gag-specific primers. PCR products were detected by oligomer hybridisation with an excess of SK19 probe. Control samples of known HIV-1 DNA copy number (15 to 1500) were prepared from the 8E5 cell line and analysed in parallel.

4. Discussion

Our results indicate that both apo-Lf and holo-lactoferrins from bovine milk are potent and selective inhibitors of HIV-1 infection in a T-cell line, as evaluated by HIV-1 p24 release and syncytia formation, with a decreasing potency order of Fe^{3+}BLf > Mn^{2+}BLf = Zn^{2+}BLf > apo-BLf. Moreover, the data illustrated in Fig. 2 are clearly consistent with an antiviral mechanism of BLfs at the level of HIV-1 binding to or entry into C8166 cells. This conclusion is also supported by the impressive inhibition of the initial HIV reverse transcription products observed in the lactoferrin-treated infected cultures (Fig. 3).

Other inhibitors of HIV-1 replication, such as dextran sulphate and other different sulphated polysaccharides, can display a similar antiviral target [40]. In particular, it has been clearly shown that dextran sulphate interacts with the V3 region of the gp120 glycoprotein and enhances the net negative electric charge of viral particles, thus influencing the electrostatic interactions between virus and host cells [41]. In this regard, it is worth mentioning that also BLf exhibits the capability of binding to the V3 loop of the gp120 envelope protein [34], even though it possesses totally different chemical features [3]. However, in addition to the possible effect of Lf on HIV itself, the finding that a simple cell pretreatment before virus infection results in a marked antiviral effect (Fig. 2) suggests that the Lf interaction with the receptor of human activated T-lymphocytes [21] can be relevant for the inhibition of HIV binding and/or entry into the cell.

Until now, a wide spectrum of antimicrobial and immunomodulatory functions have been ascribed to Lf, suggesting a relative nonspecificity of its effects rather than a highly specialised role. Notably, however, Lf exerts most of its functions through the control of iron availability [14, 3, 42]. Our data demonstrate that the anti-HIV activity is not related to iron withholding from environment, since iron, manganese and zinc saturated lactoferrins appear to be inhibitory at an even higher extent than the apo-form. When Lf binds metal ions, conformational changes occur in the molecule, which can result in a similar [43] or higher affinity for Lf receptors on eukaryotic cells [19, 3]. Notably, the finding that divalent and trivalent ion saturated lactoferrins display the highest anti-HIV effectiveness and good selectivity indexes (Table 1) is of special interest for the perspectives of in vivo studies, since hololactoferrin is more resistant to proteolytic degradation in the gut than the apo-form [44, 3]. In addition to its direct antiviral effects, Lf could also suppress viral replication by host-mediated immune mechanisms. In this regard, it is worth mentioning that Shimizu et al. [45] have very recently reported that the lactoferrin-mediated protection of mice from cytomegalovirus infection is linked to a T-cell dependent increase in natural killer cell activity. The administration of saturated forms could offer an additional advan-

tage, consisting in the transport and release in the intracellular compartment of nutrilites, such as zinc and manganese, which have been shown to exhibit anti-HIV activity [46].

In conclusion, the data reported in this article suggest that this glycoprotein can be considered as promising antiviral agent, especially in those HIV-infected patients in which plasmatic and mucosal lactoferrin levels are decreased [11, 12].

5. Synopsis

Lactoferrin is a mammalian iron-binding glycoprotein present in many biological secretions. Bovine lactoferrin in apo-form or saturated with ferric, manganese or zinc ions markedly inhibits human immunodeficiency virus type 1 (HIV-1) replication, syncytium formation and proviral DNA. The antiviral activity is directed towards the early steps of infection into human T-lymphoblastoid cells. We suggest that this glycoprotein can be considered as a promising antiviral agent, especially in those HIV-infected patients in which plasmatic and mucosal lactoferrin levels are decreased.

Acknowledgements

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