BioMetals **17:** 295–299, 2004.

Antiviral activity of lactoferrin towards naked viruses

Lucilla Seganti[1], Assunta Maria Di Biase[2], Magda Marchetti[2], Agostina Pietrantoni[2], Antonella Tinari[2] & Fabiana Superti[2]

[1]*Public Health Sciences Department, University 'La Sapienza', P.le A. Moro 5, 00185 Rome;* [2]*Laboratory of Ultrastructure, National Institute of Health, V. Regina Elena 299, 00161 Rome, Italy*

Key words: adenovirus, antiviral agents, bovine lactoferrin, enterovirus, poliovirus, rotavirus

Abstract

It is well known that lactoferrin (Lf) is a potent inhibitor towards several enveloped and naked viruses, such as rotavirus, enterovirus and adenovirus. Lf is resistant to tryptic digestion and breast-fed infants excrete high levels of faecal Lf, so that its effect on viruses replicating in the gastrointestinal tract is of great interest. In this report, we analysed the mechanism of the antiviral action of this protein in three viral models which, despite representing different genoma and replication strategies, share the ability to infect the gut. Concerning the mechanism of action against rotavirus, Lf from bovine milk (BLf) possesses a dual role, preventing virus attachment to intestinal cells by binding to viral particles, and inhibiting a post adsorption step. The BLf effect towards poliovirus is due to the interference with an early infection step but, when the BLf molecule is saturated with Zn^{+2} ions, it is also capable of inhibiting viral replication after the viral adsorption phase. The anti-adenovirus action of BLf takes place on virus attachment to cell membranes through competition for common glycosaminoglycan receptors and a specific interaction with viral structural polypeptides. Taken together, these findings provide further evidence that Lf is an excellent candidate in the search of natural agents against viral enteric diseases, as it mainly acts by hindering adsorption and internalisation into cells through specific binding to cell receptors and/or viral particles.

Introduction

Comparative studies showed that breast-fed children are less susceptible to viral infections than artificially-fed children. Both specific immunoglobulins and non-immune systems can help in the protection of the newborn (Van Hooijdonk *et al.* 2000). At least part of the antiviral properties of breast milk can be attributed to lactoferrin (Lf) that can be considered a protein of the innate mucosal defence. This protein, present in several mucosal secretions, including saliva, tears, and milk, has been shown to play a role against parasitic, mycotic, bacterial and viral infections (Levay & Viljoen 1995, Vorland 1999, Marchetti & Superti 2001, van der Strate *et al.* 2002). Most of the antimicrobial activities of BLf have been attributed to the BLf-derived N-lobe (N-terminal part of the protein) which in turn is responsible for glycosaminoglycan (GAG) binding (Wu *et al.* 1995, Shimazaki *et al.* 1998).

Lf from bovine milk (BLf) has been recognized as a potent inhibitor of several enveloped viruses, such as herpes simplex virus 1 and 2 (Hasegawa *et al.* 1994, Fujihara & Hayashi 1995, Marchetti *et al.* 1996, 1998, Andersen *et al.* 2002, 2003), human cytomegalovirus (Hasegawa *et al.* 1994, Harmsen *et al.* 1995, Shimizu *et al.* 1996, Andersen *et al.* 2001), human immunodeficiency virus (Harmsen *et al.* 1995, Swart *et al.* 1996, Puddu *et al.* 1998, Berkhoaut *et al.* 2002), hepatitis B virus (Hara *et al.* 2002), hepatitis C virus (Yi *et al.* 1997, Ikeda *et al.* 1998, Tanaka *et al.* 1999, 2000, Nozaki *et al.* 2003), respiratory syncytial virus (Grover *et al.* 1997) and hantavirus (Murphy *et al.* 2000). The antiviral effect of lactoferrin against at least five different naked viruses replicating in the gut-rotavirus (Superti *et al.* 1997, Superti *et al.* 2001), poliovirus (Marchetti *et al.* 1999), adenovirus (Arnold *et al.* 2002, Di Biase *et al.* 2003, Pietrantoni *et al.* 2003), coxsackievirus A16, and enterovirus 71, the

newest member of the *Enteroviridae* family responsible for severe neurological diseases (Lin *et al.* 2002) has also been described.

The Lf interaction with viruses that replicate in the gastrointestinal tract can be of great interest. Our approach consisted of investigating the BLf effect against enteropathogenic and enteric naked viruses and comparing its activity with that of other milk proteins (such as Lfs from different origins, ion-saturated Lfs, mucin, α-lactalbumin and β-lactoglobulin). The antiviral activity has been determined in different experimental settings that depend on the virus tested, i.e. by analyzing virus cytopathic effect, plaque formation, antigen synthesis, viral yield and/or haemagglutination. In most experiments different non-cytotoxic concentrations of proteins dissolved in pyrogen-free media were incubated with infected cells during the viral adsorption step or in the post adsorption phase or throughout the entire cycle of infection. A summary of the data concerning the spectrum of BLf antiviral activity against naked viruses is presented in Table 1.

Anti-rotavirus activity of BLf

Rotavirus is a segmented double-stranded RNA naked virus belonging to the *Reoviridae* family, which infects mature enterocytes. Rotavirus infections are the most frequent cause of gastroenteritis in infants and young children worldwide. The antiviral effect of milk proteins was tested in cultured human intestinal cells (HT-29 cells), expressing the differentiation phenotype of mature enterocytes, which represent the *in vivo* target of rotavirus infection. Cells were infected with SA-11 rotavirus that is serologically related to group A human rotavirus, serotype 3 (Superti *et al.* 1997). Alpha-lactalbumin was ineffective whereas β-lactoglobulin, apo-, native Lfs and mucin inhibited rotavirus cytopathic effect in a dose-dependent manner, BLf being the most effective (Effective concentration 50% (EC_{50}) 46–50 μg/ml). Further experiments were carried out by adding BLf to cells before, during or after the viral attachment step. The results suggest that BLf possesses a dual role, preventing virus attachment to intestinal cells and inhibiting an unknown post adsorption step. Although the antiviral activity was linked to the N-lobe (Superti *et al.* 2001), the interpretation of these results was that BLf-mediated inhibition of viral attachment was not related to competition for common binding sites on HT-29 cells, since SA-11 rotavirus binds to glycidic residues different from glycosaminoglycans (GAGs) and flow cytometry assays demonstrated its interaction with viral particles. The BLf inhibition in the post adsorption step could be attributed to the withholding of calcium, which is important for the assembly of new viral particles.

Anti-enterovirus activity

Lf has a marked antiviral activity against different small naked single-stranded positive RNA enteroviruses belonging to the *Picornaviridae* family, such as poliovirus type 1 Mahoney strain (Marchetti *et al.*, 1999), enterovirus 71 (EV71), and coxsackievirus A16 (Lin *et al.* 2002) that start the infection in the gut, which may lead to severe diseases.

The antiviral mechanism of BLf towards poliovirus (IC_{50}300 μg/ml) studied by Marchetti *et al.* (1999) was found to occur in the early phases of viral infection. The binding of BLf to Vero cells, visualized by immunofluorescent staining, demonstrated that this protein interfered with an early infection step. Interestingly, when BLf was fully saturated with zinc ions and added after the poliovirus adsorption phase, it was still capable of inhibiting viral replication, probably by the intracellular delivery of zinc ions that impaired poliovirus replication. This hypothesis was confirmed by the dose-dependent inhibition obtained with the addition of different zinc sulphate concentrations to infected cell monolayers.

Concerning EV71, Lf from bovine origin prevented viral infection in embryo rhabdomyosarcoma cells (RD cells) more potently than Lf from human origin (with an IC_{50} of 10–25 μg/ml instead of 103–185 μg/ml). Its effect appeared directed to the viral adsorption step, since BLf was ineffective when added two hours after infection, whereas it was fully active when pre- incubated before infection.

Anti-adenovirus activity

Adenoviruses are double-stranded DNA naked viruses that commonly infect and replicate at various sites of the respiratory tract as well as in the eye and in the gastrointestinal tract. The antiviral activity of milk proteins was tested in human epidermoid carcinoma larinx cells (HEp-2 cells) infected with adenovirus type 2 that represents the prototype model of the *Adenoviridae* family for its tropism for different tissues. Proteins investigated were mucin, α-lactalbumin, β-lactoglobulin, bovine lactoferrin, and

Table 1. In vitro antiviral activity of bovine lactoferrin against naked viruses

Lactoferrin	Virus	Effective Concentration 50% (μg/ml)	Cells	Reference
BLf, apo-BLf, MnBLf, ZnBLf	Rotavirus SA-11	46–50	HT-29	Superti *et al.* 1997, 2001
BLf, apo-BLf, MnBLf, ZnBLf	Poliovirus 1	775–3,000	Vero	Marchetti *et al.* 1999
BLf	Enterovirus 71	10.5–24.5	RD	Lin *et al.* 2002
BLf	Coxsackievirus A16	6.2–12.4	Vero	Lin *et al.* 2002
BLf, apo-BLf, MnBLf, ZnBLf,	Adenovirus 2	70–270	HEp-2	Arnold *et al.* 2002, Di Biase *et al.* 2003, Pietrantoni *et al.* 2003

human lactoferrin. Results obtained demonstrated that mucin, α-lactalbumin, and β-lactoglobulin did not prevent viral cytopathic effect, whereas apo-, native BLf and HLf were active in a dose-dependent manner (Arnold *et al.* 2002). All Lfs were effective, but BLf showed the lowest EC_{50} values (80 μg/ml) and the highest selectivity index (> 25). Differently from what was observed with poliovirus (Marchetti *et al.* 1999), but in agreement with data reported for many other virus models, metal-saturation of BLf did not significantly influence its activity against adenovirus infection.

As observed for EV71, BLf inhibited the early step of viral infection, preventing adenovirus antigen synthesis only when pre-incubated with epithelial cells or when added during the attachment step. As already reported for other pathogens, BLf activity was mediated by the N-lobe, and the C-lobe was deprived of any inhibitory effect (Di Biase *et al.* 2003).

As glycosaminoglycans (GAGs) mediate the attachment of both BLf and adenovirus (Dechecchi *et al.* 2000, 2002) to target cells, a competition between BLf and virus for a common receptor could be hypothesized. Results on the anti-adenovirus effect of both heparin and BLf confirm this hypothesis (Di Biase *et al.* 2003). To further investigate the role of BLf basic residues, the anti-adenovirus activity of BLf and HLf, both possessing a cluster of positive charges at the N-terminus responsible for GAG binding, was compared to ovotransferrin, which does not possess such cluster and BLfcin (Bellamy *et al.* 1992), the heparin binding BLf peptide. These experiments demonstrated that the cluster of positive charges was important for BLf effectiveness since HLf and BLfcin showed a comparable dose-dependent inhibition of adenovirus infection whereas ovotransferrin was ineffective. Moreover, as already reported for other viruses (Andersen *et al.* 2002), BLf showed the highest antiviral activity. Three hypotheses could be proposed for the higher activity of BLf as compared to BLfcin: (i) a minor steric hindrance exerted by the polypeptide in the competition with viral particles for binding to GAGs; (ii) the involvement of sequential interactions in adenovirus infection between various cellular and viral components, so that the inhibition of this event could have several targets; (iii) the involvement of other domains, in addition to those involved in GAG binding, which could be important for the antiviral activity of BLf.

Other approaches have been carried out to verify that BLf was able to bind to adenovirus (Pietrantoni *et al.* 2003). Dot blot assays demonstrated that biotynilated BLf specifically reacted with intact purified virions and heparin controls. Neutralization assays showed that, when BLf and virus were pre-incubated for 1 h and then added to cells, the viral infection was completely inhibited. Western blot assays, performed with extracted adenovirus proteins separated by electrophoresis, revealed a strong interaction of BLf with two structural proteins of 86 and 66 kDa corresponding to the viral polypeptides III and IIIa. The binding of BLf to viral particles was also visualised by electron microscopy, which localized BLf in proximity of the vertex. Summarizing the results obtained on the anti-adenovirus activity of BLf, it could be established that this molecule competes with adenovirus for GAG receptors. Furthermore BLf interacts with adenovirus particles by binding external viral structures and, in particular, the adenovirus penton

base (polypeptide III), the protein responsible for the attachment of virus to integrin cell receptors and for its internalisation (Boulanger 1999).

Conclusions

Taken together these findings provide further evidence that BLf is an excellent candidate in the search for natural agents active against enteric naked viruses. The BLf antiviral effect on naked viruses (rotavirus SA11, poliovirus 1, coxsackievirus A16, enterovirus 71 and adenovirus 2) mainly takes place in the early phases of viral infection, independently of the capsid protein properties, nucleic acid organisation, and viral replication strategies. Adenovirus represents an interesting model of study for its multiple kinds of interaction with host cells, for its tropism for the intestinal tract, the upper respiratory tract and the conjunctiva, sites where Lf is present at different concentrations depending on the host status. BLf is able to inhibit adenovirus by hindering adsorption and internalisation into cells through a specific binding to both cell receptors and viral particles (Di Biase *et al.* 2003, Pietrantoni *et al.* 2003).

Acknowledgements

This paper is dedicated to the memory of our unforgettable mentor Professor Eraldo Antonini, deceased prematurely on March 19, 1983, who introduced us to the study of ovotransferrin and lactoferrin.

The Authors would like to thank Prof. Giovanni Antonini (Biology Department, III University of Rome), Prof. Piera Valenti (Experimental Medicine Department, II University of Naples) and Dr Rosa Siciliano (CNR Biochemistry Laboratory of Avellino) for providing bovine lactoferrin fragments and for their useful advices, Prof. Nicola Orsi, Dr Maria Pia Conte, and Dr Catia Longhi (Public Health Sciences Department, University 'La Sapienza') for their stimulating discussion about various aspects of this topic.

This work was supported by Grants from Italian Ministry of Health, Project 1%, National Institute of Health and MIUR-PRIN.

References

Andersen JH, Osbakk SA, Vorland LH, Traavik T, Gutteberg TJ. 2001 Lactoferrin and cyclic lactoferricin inhibit the entry of human cytomegalovirus into human fibroblasts. *Antiviral Res* **51**, 141–149.

Andersen JH, Jenssen H, Gutteberg TJ. 2003 Lactoferrin and lactoferricin inhibit Herpes simplex 1 and 2 infection and exhibit synergy when combined with acyclovir. *Antiviral Res* **58**, 209–215.

Arnold D, Di Biase AM, Marchetti M *et al.* 2002 Antiadenovirus activity of milk proteins: lactoferrin prevents viral infection. *Antiviral Res* **53**, 153–158.

Bellamy W, Takase M, Wakabayashi H, Kavase K, Tomita M. 1992 Antibacterial spectrum of lactoferricin B, a potent bactericide peptide derived from the N-terminal region of bovine lactoferrin. *J Appl Bacteriol* **73**, 472–479.

Berkhoaut B, vanWamel JL, Beljaers L, Meijer DK, Visser S, Floris R. 2002 Characterization of the anti HIV effects of native lactoferrin and other milk proteins and protein-derived peptides. *Antiviral Res* **55**, 341–355.

Boulanger P. 1999 Cell receptors for human adenoviruses. *J Soc Biol* **193**, 77–84.

Di Biase AM, Pietrantoni A, Tinari A *et al.* 2003 Heparin interacting sites of bovine lactoferrin are involved in anti-adenovirus activity. *J Med Virol* **69**, 495–502.

Fujihara T, Hayashi K. 1995 Lactoferrin inhibits herpes simplex virus type-1 (HSV-1) infection to mouse cornea. *Arch Virol* **140**, 1469–1472.

Grover M, Giouzeppos O, Schnagel RD, May JT.1997 Effect of human milk prostaglandins and lactoferrin on respiratory syncitial virus and rotavirus. *Acta Paediatrics* **86**, 315–316.

Hara K, Ikeda M, Saito S *et al.* 2002 Lactoferrin inhibits hepatitis B virus infection in cultured human hepatocytes. *Hepatol Res* **24**, 228–235.

Harmsen MC, Swart PJ, de Bethune MP *et al.* 1995 Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication *in vitro. J Infect Dis* **172**, 380–388.

Hasegawa K, Motsuchi W, Tanaka S, Dosako S. 1994 Inhibition with lactoferrin of *in vitro* infection with human herpes virus. *Japan J Med Sc* **47**, 73–85.

Ikeda M, Nozaki A, Sugiyama K *et al.* 2000 Characterization of antiviral activity of lactoferrin against hepatitis C virus infection in human cultured cells. *Virus Res* **66**, 51–63.

Ikeda M, Sugiyama K, Tanaka T *et al.* 1998 Lactoferrin markedly inhibits hepatitis C virus infection in cultured human hepatocytes. *Biochem Biophys Res Comm* **245**, 549–553.

Ji ZS, Mahley RW. 1994 Lactoferrin binding to heparan sulfate protoglycans and the LDL receptor-related protein. Further evidence rapporting the importance of direct binding of remnant lipoproteins to HSPG. *Arterioscler Thromb* **14**, 2025–2031.

Levay PF, Viljoen M. 1995 Lactoferrin: a general review. *Haematologica* **80**, 252–267.

Lin TY, Chu C, Chiu CH. 2002 Lactoferrin inhibits enterovirus 71 infection of human embryonal rhabdomyosarcoma cells *in vitro. J Infect Dis* **186**, 1161–1164.

Marchetti M, Longhi C, Conte MP, Pisani S, Valenti P, Seganti L. 1996 Lactoferrin inhibits herpes simplex virus type 1 adsorption to Vero cells. *Antiviral Res* **29**, 221–231.

Marchetti M, Pisani S, Antonini G, Valenti P, Seganti L, Orsi N. 1998 Metal complexes of bovine lactoferrin inhibit *in vitro* replication of herpes simplex virus type 1 and 2. *Biometals* **11**, 89–94.

Marchetti M, Superti F. 2001 Antiviral activity of lactoferrin. In: Pandalai SG, ed. *Recent developments in antiviral research*. Trivandrum: Transworld Research Network, **1**, 193–203.

Marchetti M, Superti F, Ammendolia MG, Rossi O, Valenti P, Seganti L. 1999 Inhibition of poliovirus type 1 infection by iron-, manganese- and zinc-saturated lactoferrin. *Med Microbiol Immunol* **187**, 199–204.

Murphy ME, Kariwa H, Mizutani T, Yoshimatsu K, Arikawa J, Takashima I. 2000 In vitro antiviral activity of lactoferrin and ribavirin upon hantavirus. *Arch Virol* **145**, 1571–1582.

Nozaki A, Ikeda M, Naganuma A, *et al.* 2003 Identification of a lactoferrin-derived peptide possessing binding activity to hepatitis C virus E2 envelope protein. *J Biol Chem* **278**, 10162–10173.

Pietrantoni A, Di Biase AM, Tinari A, *et al.* 2003 Bovine lactoferrin inhibits adenovirus infection by interacting with viral structural polypeptides. *Antimicrob Agents Chemoter* **47**, *in press*.

Puddu P, Borghi P, Gessani S, Valenti P, Belardelli F, Seganti L. 1998 Antiviral effect of bovine lactoferrin saturated with metal ions on early steps of human immunodeficiency virus type 1 infection. *Int J Biochem Cell Biol* **30**, 1055–1062.

Shimazaki K, Tazume T, Uji K *et al.* 1998 Properties of a heparin-binding peptide derived from bovine lactoferrin. *J Dairy Sci* **81**, 2841–2849.

Shimizu K, Matsuzawa H, Okada K *et al.* 1996 Lactoferrin mediated protection of the host from murine cytomegalovirus infection by a T-cell-dependent augmentation of natural killer cell activity. *Arch Virol* **141**, 1875–1889.

Superti F, Ammendolia MG, Valenti P, Seganti L. 1997 Antirotaviral activity of milk proteins: lactoferrin prevents rotavirus infection in the enterocyte-like cell line HT29. *Med Microbiol Immunol* **186**, 83–91.

Superti F, Siciliano R, Rega B, Giansanti F, Valenti P, Antonini G. 2001 Involvement of bovine lactoferrin metal saturation, sialic acid and protein fragments in the inhibition of rotavirus infection. *Biochim Biophys Acta* **1528**, 107–115.

Swart PJ, Kuipers ME, Smith C *et al.* 1996 Antiviral effects of milk proteins: acylation results in polyanionic compounds with potent activity against human immunodeficiency virus type 1 infection. *AIDS Res Hum Retroviruses* **12**, 769–775.

Tanaka K, Ikeda M, Nozaki A *et al.*, 1999 Lactoferrin inhibits hepatitis C virus viremia in patients with chronic hepatitis C. A pilot study. *Jpn J Cancer Res* **90**, 367–371.

van der Strate BWA, Beljaars L, Molema G, Harmsen MC, Meijer DKF. 2002 Antiviral activities of lactoferrin. *Antiviral Res* **52**, 225–239.

Van Hooijdonk ACM,Kussendrager KD, Steijns JM. 2000 In vivo antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. *Brit J Nutr* **84**, S127–S134.

Vorland LH. 1999 Lactoferrin: a multifunctional glycoprotein. *APMIS* **107**, 971–981.

Wu HF, Monroe DM, Church FC. 1995 Characterization of the glycosaminoglycan-binding region of lactoferrin. *Arch Biochem Biophys* **317**, 85–92.

Yi MY, Kaneko S, Yu DY, Murakami S, 1997 Hepatitis C virus envelope proteins bind lactoferrin. *J Virol* **71**, 5997–6002.