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Antiviral activity of lactoferrin against canine herpesvirus

Tetsuya Tanaka [a,*], Syogo Nakatani [a], Xuenan Xuan [b], Haruto Kumura [a], Ikuo Igarashi [b], Kei-ichi Shimazaki [a]

[a] *Dairy Science Laboratory, Graduate School of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589, Japan*
[b] *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan*

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Abstract

Lactoferrin (LF) is an iron-binding protein that is found in milk and other mammalian secretions. We found that bovine lactoferrin (bLF) inhibited both the in vitro infection and replication of canine herpesvirus (CHV) in Madin-Darby canine kidney (MDCK) cells. Incubation of CHV with bLF prevented subsequent infection of MDCK cells. Furthermore, proteins from CHV-infected MDCK cells were resolved by SDS–PAGE, and then bLF CHV-binding proteins were identified by far Western blotting. We demonstrated that the anti-CHV activity of bLF was due to its interaction with CHV as well as with MDCK cells. Both the apo- and holo-forms of bLF inhibited virus multiplication independently of their iron-withholding properties. We also demonstrated that human LF had anti-CHV activity. Our findings suggest that LF could be effective in dogs to provide protection against CHV infection.

Keywords: Lactoferrin; Canine herpesvirus (CHV); Heparin; Lactoferrin-binding protein

1. Introduction

Lactoferrin (LF) is an iron-binding protein present in milk, saliva, tears, mucus secretions and in secondary granules of neutrophils. One LF molecule can tightly but reversibly bind two iron atoms (Fe III). Lactoferrin binding is dependent on the concomitant binding of anions, such as bicarbonate or carbonate, which play an essential role in firmly holding iron (Masson and Heremans, 1971). Thus, LF assumes iron-free (apo) and -bound (holo) states. The biological properties of LF against microorganisms are often dependent upon its iron status.

Lactoferrin is a prominent antimicrobial component of mucosal surfaces that is prone to attack microbial pathogens. It is actively secreted by neutrophils during the inflammatory response (Hansen et al., 1975). As an anti-microbial component of colostrum and milk, LF may play a significant role in protecting neonates from infectious diseases (Sanchez et al., 1992).

Lactoferrin can inhibit the growth of a variety of microorganisms in vitro (Bullen et al., 1972; Tanaka et al., 1995, 1996), including enveloped viruses such as human cytomegalovirus (Andersen et al., 2001; Harmsen et al., 1995; van der Strate et al., 2003), herpes simplex virus (HSV) type 1 and 2 (Hasegawa et al., 1994; Seganti et al., 2001; van der Strate et al., 2001), human immunodeficiency virus (Harmsen et al., 1995), respiratory syncytial virus (Portelli et al., 1998), human hepatitis B virus (Hara et al., 2002), human hepatitis C virus (HCV) (Ikeda et al., 1998), hantavirus (Murphy et al., 2000), as well as simian SA-11 rotavirus and poliovirus type 1 (Superti et al., 1997). These activities are concentration-dependent and are the most potent at concentrations ranging from 0.1 to 2 mg/ml. The antiviral activity of LF is directed towards the early phases of infection by all viruses investigated to date, indicating that this protein could be key to host protection from viral infections, as demonstrated by Fujihara and Hayashi (1995).

The canine herpesvirus (CHV), family *Herpesviridae*, subfamily *Alphaherpesviriae*, was originally characterized by Carmichael et al. (1965). This virus causes a fatal hemorrhagic disease in neonatal puppies and upper respiratory tract infection in adult dogs older than two weeks of age (Yanagisawa et al., 1987). Dogs can be infected with oronasally CHV by contacts with infected dogs, and transplacental transmission of infectious CHV from infected bitches to the pups has also been suggested (Hashimoto et al., 1982). Since persistent or inapparant infections are

* Corresponding author. Tel.: +81-11-706-3642; fax: +81-11-706-4135.
E-mail address: tetsuya@anim.agr.hokudai.ac.jp (T. Tanaka).

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strongly suspected in dogs exposed to CHV, there is a great need for the detection of CHV-infected dogs in places where dogs are kept in large herds, for example, kennels, pet shops, etc. However, there is no commercially available vaccine and medicine against CHV infections, because some researchers feel that need for a vaccine and medicine do not seem great. There is little pressure or economic incentive to develop vaccine or medicines due to the sporadic occurrences of the disease (Percy, 1970). In our model of infection using Madin-Darby canine kidney (MDCK) cells, the antiviral effect of LF against CHV was determined. Viral infection was inhibited under experimental conditions similar to those required by LF to cause antiviral activity towards CHV, suggesting that LF plays a role in canine nonimmune-mediated antiviral defense in cell culture.

2. Materials and methods

2.1. Virus and cell

The CHV DFD-6 (Motohashi and Tajima, 1966) strain was propagated in MDCK cells cultured in Eagle's Minimal Essential Medium (EMEM) (Sigma Chemicals Co., St. Louis, MO) containing 8% heat-inactivated fetal bovine serum (FBS) and antibiotics (EMEM).

2.2. Proteins

Bovine transferrin (bTF), ovotransferrin (OTF) and human lactoferrin (hLF) were purchased from Sigma Chemicals Co. Bovine lactoferrin (bLF) and bovine lactoferrin hydrolysate (bLF-H) were supplied by Morinaga Milk Industry Co. Ltd. (Zama). Apo-bLF and holo-bLF were prepared according to Law and Reiter (1977). Proteins were dissolved in EMEM and filter-sterilized through a 0.22 μm Millex^{TM} filter unit (Millipore Co., Bedford, MA) before use.

2.3. Cell viability test

The effects of the reagents on cell growth were quantified as cell proliferation, based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Cell Counting Kit; DOJIN, Kumamoto).

2.4. Virus yield reduction assay

Approximately 3×10^3 MDCK cells were seeded in 96-well microtiter plates (Corning Inc., Corning, NY) and cultured for 24 h. The medium was replaced with 50 μl of fresh medium containing 2 mg/ml of bLF, hLF, bTF, OTF or bLF-H and the cells were inoculated with 50 μl of CHV stock containing approximately $10^{5.9}$ 50% cell culture infectious dose ($CCID_{50}$)/50 μl or 10-fold serial dilutions in EMEM medium. The infected cells were incubated for 14 days at 37 °C. Viral titers were assessed as the cytopathic effect (CPE) in each well determined under light-phase microscopy 1–14 days post-infection (p.i.). The $CCID_{50}$ value was calculated using the procedure of Reed and Muench (1938).

2.5. Plaque reduction assay

Plaque titration was performed on confluent monolayers of MDCK infected with CHV. Briefly, 3×10^3 MDCK cells were seeded in 96-well microtiter plates and then infected with CHV at a multiplicity of infection (MOI) of approximately 0.05 plaque forming unit (PFU) per cell (1 h at 37 °C) in the presence of LF or other proteins. The virus was removed, and the monolayers were overlaid with 1.0% SeaPlaque agarose (BMA, Rockland, ME) for 72 h at 37 °C, and then plaques were counted.

2.6. Interaction of bLF with cells or virus

We examined the interaction of bLF with cells as follows. MDCK cells (3×10^3) were seeded in 96-well microtiter plates and cultured for 24 h. Bovine lactoferrin was added to the cells at a final concentration of 1 mg/ml with or without heparin sodium chloride (NACALAI TESQUE Inc., Kyoto) and incubated for 60 min at 37 °C. After removing free LF, the cells were incubated with 50 μl of stock virus for 60 min at 37 °C. CHV was removed and then the cells were cultured at 37 °C as described for plaque titration.

To examine the interaction of LF with CHV, 50 μl of stock virus and LF (final concentration of 1 mg/ml) were incubated in 1 ml of EMEM for 60 min at 4 °C. The mixture of CHV and LF was added to MDCK cells incubated for 60 min at 37 °C. After removing CHV and LF, the cells were further cultured at 37 °C as described for plaque titration.

2.7. Far Western blotting

MDCK cells in tissue culture dishes (15 mm diameter) were infected with CHV at an MOI of 1 for 1 h at 37 °C, then the cells were washed with protein-free OPTI-MEM (Life Technologies Japan, Tokyo) and cultured in 1.5 ml of the same medium at 37 °C for 72 h. Cell extracts were boiled for 5 min in sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 5% beta-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) and resolved by SDS–PAGE (10% polyacrylamide) according to Laemmli (1970). Resolved proteins were electroblotted onto PVDF membranes (Osmonics Inc., Westborough, MA) for 1 h at room temperature in 25 mM Tris containing 192 mM glycine and 20% ethanol at 2 mA/cm^2 using the semidry method. Nonspecific binding was blocked with blocking buffer consisting of 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 4 °C overnight, then the membranes were incubated with biotinylated bLF

(1 μg/ml in blocking buffer) at room temperature for 1 h. Membranes were then rinsed five times in washing buffer (0.05% Tween-20 in PBS) and incubated at room temperature for 1 h with streptavidin-conjugated horseradish peroxidase (streptavidin-HRP; Nichirei Co., Tokyo) diluted 1:800 in blocking buffer. Membranes were rinsed five times in washing buffer and protein bands were visualized on X-ray film using ECL kits (Amersham Bioscience Co., Piscataway, NJ) according to the Manufacturer's protocol.

2.8. Statistical analysis

All samples were tested at least in triplicate. Data were statistically analyzed using Student's *t* test. Results are presented as means ± S.D.; $P < 0.05$ values were considered significant.

3. Results

3.1. Cell proliferation assays

The cytotoxicity of proteins was determined in confluent MDCK cell monolayers after 72 h incubation at 37 °C with various concentrations of hLF, bLF, bTF, OTF and bLF-H. Cytotoxic concentrations of proteins tested were usually below 1 mg/ml. To exclude the possibility that the CHV was eliminated by a reduction in the number of viable cells, we examined the cytotoxic effect of bLF (1 mg/ml) alone on MDCK cells (Fig. 1). These cells were enhanced in their growth by treatment with hLF, bLF and bLF-H compared to other proteins. The results demonstrated that bLF or other proteins were not cytotoxic to cell growth, and that CHV infectivity was reduced by bLF or other proteins through a mechanism other than cytotoxicity.

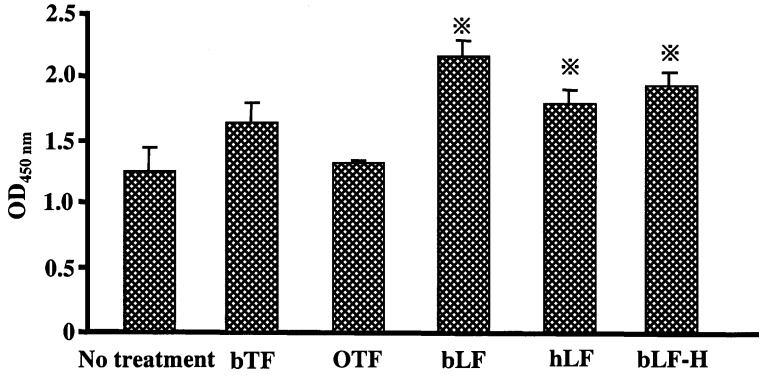

Fig. 1. Cell proliferation assay. Growth of MDCK cells was examined within 74 h of growth using the proliferation reagent WST-1 in the presence of bTF, OTF, bLF, hLF and bLF-H (1 mg/ml). Optical density at 450 nm of mitochondrial activity in viable cells was measured at $OD_{450\,nm}$. Data are expressed as means ± S.D. ($n = 3$). $^*P < 0.05$, no treatment vs. TF family proteins.

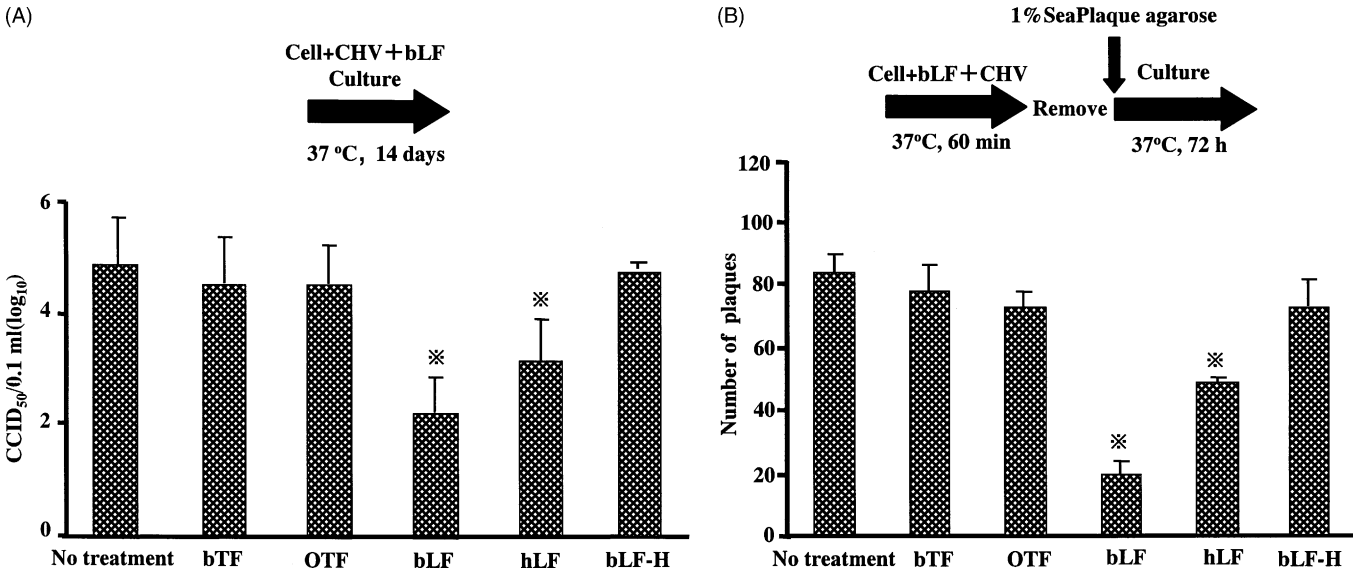

Fig. 2. Antiviral effects of bTF, OTF, bLF, hLF and bLF-H. Upper flow chart shows experimental procedures. Bovine TF, OTF, bLF, hLF or bLF-H (1 mg/ml) were added to MDCK cells infected with CHV and incubated at 37 °C. (A) Virus yield reduction assay (CHV $CCID_{50}$ of MDCK cells). (B) Plaque reduction assay (CHV CPE of MDCK cells). Data are expressed as means ± S.D. ($n = 3$). $^*P < 0.05$, no treatment vs. TF family proteins.

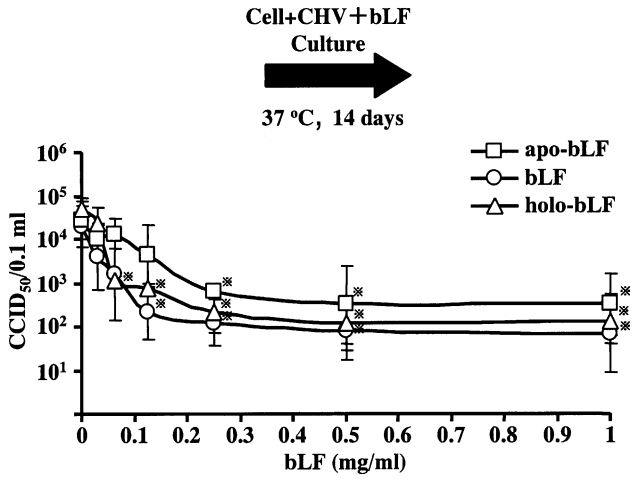

Fig. 3. Dose response curves of apo-bLF, bLF and holo-bLF towards CHV $CCID_{50}$ in MDCK cells. Upper flow chart shows experimental procedures. Data are expressed as means ± S.D. ($n = 3$). $^*P < 0.05$, 0 mg/ml vs. 0.031 mg/ml.

3.2. Antiviral effect of hLF, bLF, bTF, OTF, bLF-H towards CHV

We examined whether bLF similarly protects cultured MDCK cells against CHV infection by virus and plaque titration. Fig. 2 shows that CHV infection was inhibited in MDCK cells incubated with bLF (1 mg/ml). Furthermore, CHV was inhibited at bLF concentrations ranging from 0.125 to 1 mg/ml (Fig. 3).

To determine whether the anti-CHV activity of bLF is specific, we examined the effects of hLF, bTF and OTF, another iron transporter, the amino acid sequence of which is about 70% homologous to that of bLF. Fig. 2 shows that among these, only hLF was active against CHV infection. This result indicates that the anti-CHV activity of LF does not depend on the species of origin, and that it is not a general property of the iron transporter family of proteins. However, bLF-H was not active against CHV infection.

3.3. Influence of iron saturation on the antiviral effect of bLF towards CHV

To evaluate whether the antiviral activity was a function of the iron-binding property of the proteins, we used virus yield reduction assay to test the ability of apo- and holo-bLF to inhibit CHV infection (Fig. 3). Apo- and holo-bLF similarly inhibited anti-CHV, indicating that iron complex formation is not involved in the anti-CHV activity exerted by bLF.

3.4. Anti-CHV effect of bLF: interaction of bLF with cells

To clarify the target sites of bLF, we examined by plaque assay whether LF interacts with the cells or with CHV. Fig. 4A shows that extracellular CHV was inhibited by bLF and hLF, but not bTF or OTF. This result suggested that bLF or hLF interacted with the cells to inhibit CHV infection. We then examined the effect of heparin plus bLF in our CHV/MDCK model. Various concentrations of bLF

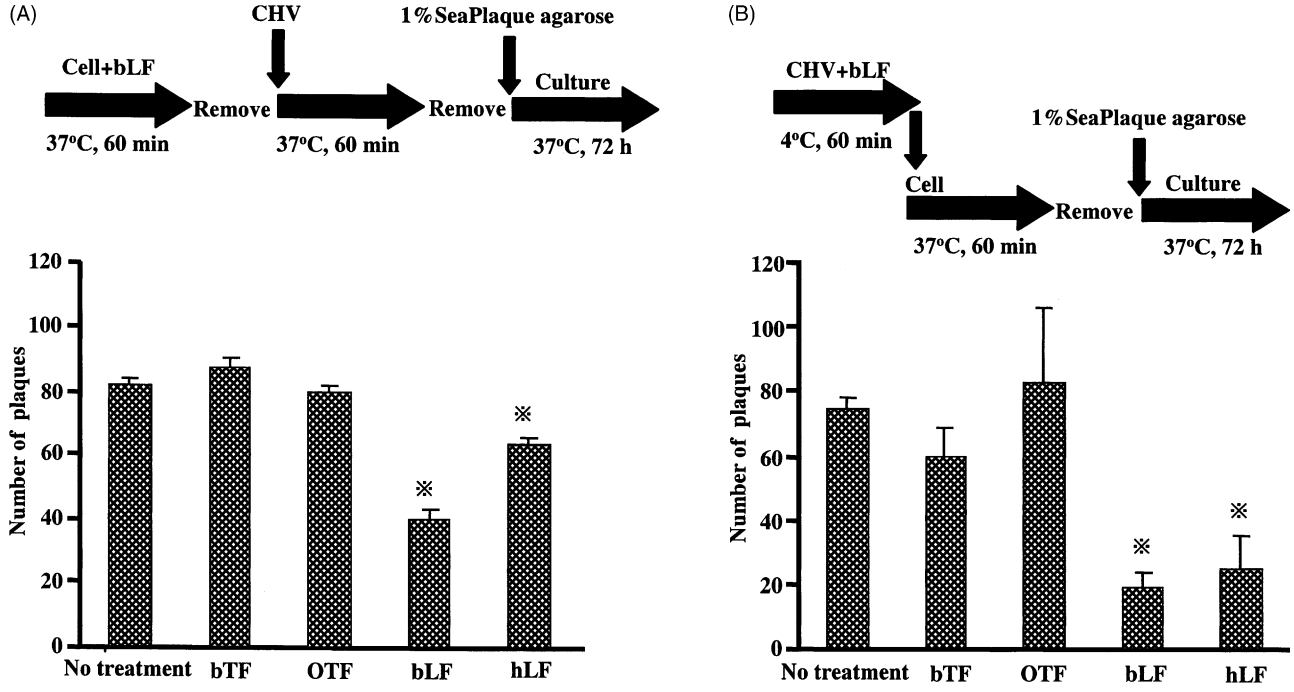

Fig. 4. Interaction of bLF with MDCK cells or virus. Upper flow chart shows experimental procedures. (A) MDCK cells were incubated with bLF (1 mg/ml) at 37 °C for 60 min; then free bLF was removed, CHV was added and the cells were incubated at 37 °C for further 60 min. (B) CHV and bLF (1 mg/ml) were incubated at 4 °C for 60 min, then the mixture was added to MDCK cells and incubated at 37 °C for further 60 min. Data are expressed as means ± S.D. ($n = 3$). $^*P < 0.05$, no treatment vs. TF family proteins.

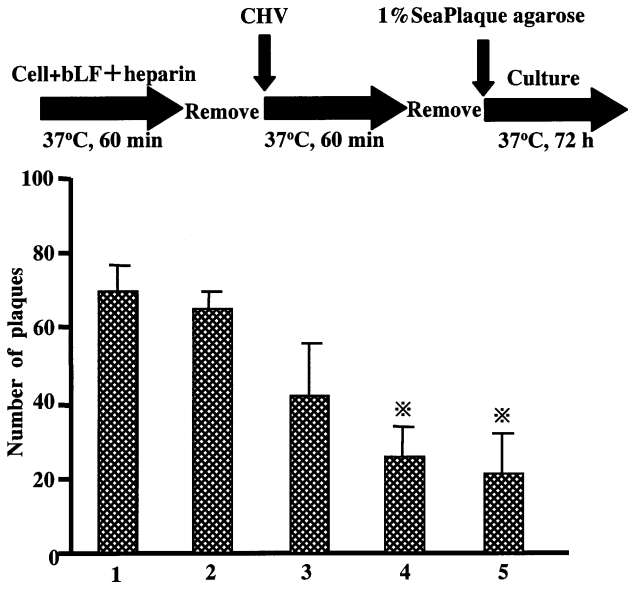

Fig. 5. Antagonism of antiviral effect when heparin is combined with bLF (1 mg/ml). Upper flow chart shows experimental procedures. 1, No treatment; 2, bLF + heparin (0.2 mg/ml); 3, bLF + heparin (0.1 mg/ml); 4, bLF + heparin (0.05 mg/ml); 5, bLF. Data are expressed as mean ± S.D. ($n = 3$). $^{*}P < 0.05$, no treatment vs. bLF, bLF + heparin.

and heparin were added individually or together to cultures in EMEM for 1 h before CHV attachment for 1 h. The results showed that at high heparin concentrations (50 and 100 μg/ml), the inhibitory effect against CHV disappeared (Fig. 5), indicating that heparin inhibited or antagonized the antiviral activity of bLF.

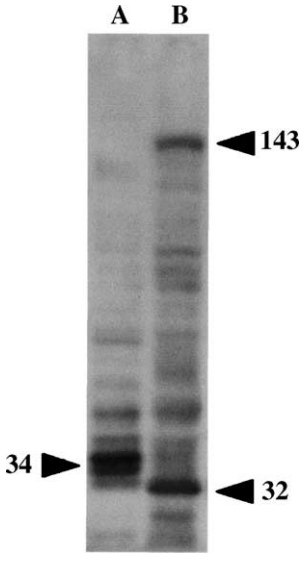

Fig. 6. Direct interaction between bLF and CHV-protein. Proteins from MDCK (A) and CHV-infected MDCK cells (B) were resolved by 10% SDS–PAGE, then far Western blotted using biotinylated bLF (bLF-bio) as a probe. (A) 34 kDa proteins are cellular proteins bound to bLF-bio. (B) 32 and 143 kDa proteins are viral proteins bound to bLF-bio.

3.5. Anti-CHV effect of bLF: specificity of anti-CHV activity

Fig. 4B shows that CHV when incubated with bLF or hLF (1 mg/ml) showed reduced infection for cells. This was not the case when CHV was incubated with bTF or OTF. These results suggested that bLF or hLF must interact with CHV to prevent CHV infection. We then investigated bLF-binding protein to CHV proteins. Fig. 6 shows the results of the biotinylated bLF-binding assay of soluble CHV proteins on far Western blots. Lane B shows that 32 and 143 kDa proteins bound to bLF-bio in MDCK cells infected with CHV, but not to uninfected MDCK cells. Therefore, these results indicated that bLF had ability to bind to CHV proteins.

4. Discussion

The importance of LF as a natural agent of defense against infections has been established (Vorland, 1999). In addition to antibacterial activities, LF has a wide spectrum of antiviral activities. Studies have shown that the interaction of LF with cell surfaces and/or viral particles might represent a crucial step in the antiviral mechanism. The antiviral activity of LF appears to be specific because, although bTF displays high structural and sequence homology with LF, it has no significant antiviral activity (Hasegawa et al., 1994).

The results reported here provide evidence for the same biological role of LF as of bLF, since bLF potently inhibited infection of MDCK cells by CHV (Marchetti et al., 1996). The antiviral effect of bLF could have been linked to its iron-binding properties, like those of other iron-chelating substances that inhibit herpesvirus ribonucleotide reductases (Spector et al., 1991). However, its effect on CHV infection was not apparently related to withholding iron from the environment since the effects of apo- and holo-bLF did not significantly differ (Fig. 3). In fact, bLF-H had no activity against CHV infection. However, the anti-viral activity of bLF C-lobe was identical to that of bLF (data not shown). These results indicated that its three-dimensional structure or molecular size or conformation is necessary for bLF to exert its antiviral activities. Bovine lactoferrin was a significantly better inhibitor than hLF, which is presumably linked to differences in the molecular structure. Although bLF is 69% identical to hLF, they differ in terms of glycan chains and the number of disulphide bridges (Metz-Boutigue et al., 1984). These variations are likely to contribute to differences in the functional domains responsible for the binding of the LF to host cells and viral particles (Fig. 2).

The antiviral effect of bLF correlates well with its affinity for viral receptor-binding sites on cells. In fact, polyanionic glycosaminoglycan chains of heparin sulfate and apo-lipoprotein-E receptor, which are both highly cationic, interact with LF (Pierce et al., 1991). Consequently, the ability of LF to inhibit CHV infection at the level of viral attachment may rely to a large extent on a competitive

interaction with cell receptors for CHV, which can hinder the binding of viral attachment proteins (Fig. 4A). The negative electric charge of OTF and bTF might account for their lack of antiviral activity. The initial event in CHV infection is based upon attachment to extracellular heparin sulfate, which might act as host cell receptor for the virus. Heparin and related compounds can inhibit CHV infection (Mettenleiter et al., 1990), but the present study shows that the antiviral effects of heparin and bLF are lost when they are mixed (Fig. 5). This indicates that heparin and bLF interact. The heparin-binding site of bLF has been reported to be N-terminal region of bLF, where this region denotes an amino acid residue with a basic side chain (Shimazaki et al., 1998).

Our findings suggest that infection is blocked during the very early phases of the viral multiplication cycle, since the inhibitory effect was highest when bLF was added during the attachment step (Fig. 4B). Under these experimental conditions, the ability of biotinylated bLF to bind CHV showed that bLF acted on viral adsorption to MDCK cells (Fig. 6). To formulate a hypothesis about the mechanism of the antiviral action of bLF, the events involved in the adsorption and entry of CHV virions into cells must be considered (Marchetti et al., 1996). Virion-associated glycoprotein B and C that have aminoacidic domains interact with at least two receptors on cell membranes that act as receptors for polycationic arginine-rich segments of CHV-1 gC and CHV gB. These together with the LDL receptor seem to bind some virions in coated pits of cell membranes, probably by interaction with CHV gB, gC or gD polypeptides (Limbach et al., 1994). Both bLF and hLF possess a cluster of positive charges at the N-terminal domain that is important for binding to glycosaminoglycans (GAG) (Mann et al., 1994) and to specific-binding sites for some enveloped viruses, including HSV-1 and HSV-2 (Roderiquez et al., 1995). Most of the antiviral activity of bLF towards HSV might arise from bLF interference during the early phases of HSV-1 and HSV-2 infection by binding to GAG and hindering HSV adhesion to host cells (Marchetti et al., 1998). On the other hand, bTF and OTF do not have a cluster of positive charges in the N-terminal region (Metz-Boutigue et al., 1984), which may explain their lack of activity towards HSV. In comparison to bTF or OTF, the most unique region of LF is the N-terminal region, which forms a loop structure, designated as lactoferricin (Bellamy et al., 1992). This structure contains a basic amino acid cluster and is presumed to be responsible for both bactericidal activity (Bellamy et al., 1992), and binding to LRP, as an antagonist of apolipoprotein E (van Dijk et al., 1992), or to oxidized-LDL (Kajikawa et al., 1994). Therefore, lactoferricin may be responsible for the interaction of LF with CHV particles. However, Yi et al. (1997) recently reported that the N-terminal loop of LF plays an insignificant role in the binding of LF to HCV E2 protein in vitro. Since the HCV envelope proteins E1 and E2 can form heterodimers (Ralston et al., 1993), further investigation is required to reach conclusions about which LF region is responsible for anti-CHV activity.

Thus, many antiviral drugs with different targets can control infections caused by viruses belonging to the *Herpesviridae* family (De Clercq et al., 1980), and the present findings might represent a useful tool in the discovery of natural anti-herpetic drugs. It should be noted, however, that bLF was effective against CHV at concentrations that are above the LF concentration found in dog milk (0.05 mg/ml) (Masson and Heremans, 1971).

Acknowledgements

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