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Lactoferrin inhibits enterovirus 71 infection by binding to VP1 protein and host cells

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Abstract

The antiviral activities of bovine lactoferrin (LF) against enterovirus 71 (EV71) were studied both in vitro and in vivo. LF protected both human rhabdomyosarcoma and neuroblastoma SK-N-SH cell lines from EV71 infection when it was added at the same time, before, or within 30 min after EV71 infection. Using enzyme-linked immunosorbent assay-based binding assay and indirect fluorescent stain, we found that LF could bind to the target cells. Furthermore, it was found that LF could bind to the VP1 protein of EV71, which was blocked in the presence of anti-VP1 antibody. In addition, LF could induce IFN-α expression of SK-N-SH cells and inhibit EV71-induced IL-6 production. Finally, LF protected mice against lethal EV71 challenge. Taken together, these results suggest that LF can inhibit EV71 infection by interacting with both EV71 and host cells.

Keywords: Enterovirus; Lactoferrin

1. Introduction

Enterovirus 71 (EV71) is a positive-stranded RNA virus belonging to the enterovirus genus of the Picornaviridae family (Ho, 2000; McMinn, 2002). It is transmitted from person to person mainly by the fecal–oral route. After replication in the mucosal system, the virus may enter the circulation (viremia) and finally find its way to the central nervous system (Li et al., 2002). The clinical manifestations caused by EV71 infection vary from mild hand, foot and mouth disease or herpangina to aseptic meningitis, encephalitis, pulmonary edema and death. In 1998, an epidemic of EV71 infection affected more than 90,000 children in Taiwan and caused 78 deaths (Ho et al., 1999; Liu et al., 2000). There is still no vaccine or antiviral drug available against this infection. Recently, lactoferrin (LF) has been shown to be able to inhibit EV71 in vitro (Lin et al., 2002). However, the mechanism of LF to inhibit EV71 is still unclear.

LF, an iron binding glycoprotein with a molecular mass of ∼80 kDa, consists of 692 amino acids. LF of different species has high degree of sequence homology. LF is present on mucosal surfaces, within the specific granules of neutrophils, and in biological fluids including breast milk, saliva and seminal fluid, indicating that it may play a protective role in the innate immune response. Breast milk is by far the most abundant source of LF. Human colostrums contain up to 7 mg/ml of LF. The concentration of LF, however, varies greatly in other human body fluids. It is as high as 2 mg/ml in tears and is approximately 1 μg/ml in blood. However, its concentration can increase to 200 μg/ml

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in blood in inflammatory situation (Masson and Heremans, 1971).

Numerous biological functions have been ascribed to LF such as iron-transportation, and anti-microbial properties against bacteria, fungi, and several viruses (Farnaud and Evans, 2003; Florisa et al., 2003; van der Strate et al., 2001). LF also displays an antiviral activity against both DNA and RNA viruses including rotavirus, herpes virus and HIV (Harmsen et al., 1995; van der Strate et al., 2001). Most of these studies indicate that the antiviral effect of LF lies in the early phase of infection. LF prevents virus from entering the host cells, either by blocking cellular receptors, or by binding to the virus particles directly (van der Strate et al., 2001). In this study, we demonstrated that LF inhibited EV71 infection by binding to both EV71 and host cells.

2. Materials and methods

2.1. Preparation of virus stocks and virus titration

EV71/Taiwan/4643/98 (GenBank accession number AF304458; Yan et al., 2001), an isolate from the throat swabs of an 18-month-old patient with encephalitis, was used in this study (Wen et al., 2003). The virus was propagated in Vero cells, which were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM; Gibco BRL Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco) and antibiotics. Briefly, Vero cell monolayers were inoculated with the virus at a multiplicity of infection (MOI) of 0.01 and the culture medium was harvested after incubation for 36 h. The cell debris was removed by centrifugation at 1000 × *g* for 10 min. Supernatants were filtered through a 0.22 μl membrane filter and stored at −70 °C before use. The virus titer was determined by plaque assay. Briefly, confluent monolayers of Vero cells were infected with serial dilutions of the supernatants and overlaid with 0.8% methylcellulose nutrient agarose in MEM for 3–5 days before the plaque numbers were counted after staining with 1% crystal violet. The virus titers were expressed as plaque forming unit per milliliter (pfu/ml). IC_{50} and IC_{90} were defined as the concentrations of LF to cause 50% and 90% plaque reduction to that of un-treated control, respectively.

2.2. Antiviral assay

Both RD (rhabdomyosarcoma) and SK-N-SH (human neuroblastoma) cells were grown at 37 °C in 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, and were infected with EV71 at a MOI = 0.1. Different amounts of bovine LF (Wako, Japan) or human LF (Sigma-Aldrich, St. Louis, MO) were simultaneously added to the media for 2 h. Infections were allowed to proceed for 2 days. Controls included uninfected cells and cells infected with virus in the absence of lactoferrin. In the pre-incubation assay, LF at a dose of IC_{90} was pre-incubated with the cells for different periods of time as indicated and subsequently removed by washing twice with the medium before the addition of virus. For the post-infection protection assay, the cells were infected with the virus for 15–180 min before the addition of LF. Virus titers were determined as described above.

2.3. Expression and purification of the His-tagged VP1 protein

The cDNA encoding EV71 (N6367-TW-98) VP1 protein was inserted into the *Bam*HI–*Eco*RI sites of the expression vector pET21b resulting in a $(His)_6$ amino terminal tag fused to VP1. The recombinant plasmid, named pET21b-VP1, was transformed into *Escherichia coli* BL21 (DE3) pLysS, and grown in L-Broth with 100 μg/ml ampicillin until the absorbance at 550 nm reached 0.8–1. After addition of the final concentration of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), the cells were further incubated for 5 h at 33 °C. The cells were collected by centrifugation, washed and suspended with buffer A (20 mM Tris, 500 mM NaCl, pH 9). The suspension was lysed by passage through a French press, and the pellet collected by centrifugation. After centrifugation, the supernatant was filtered and applied to a Ni^{2+}-chelating column. The column was washed with buffer A and the His-tagged VP1 protein was eluted with 200–400 mM imidazole. The peak fractions were collected and analyzed by SDS-PAGE and Western blot.

2.4. Enzyme-linked immunosorbent assay (ELISA)-based binding assay

The binding of LF to EV71 was determined using an ELISA-based binding assay described by Efthymiadis et al. (1997) with modification. Briefly, 96-well microtiter immunoplates (Maxisorp; Nalge Nunc, Naperville, IL) were coated with EV71 or recombinant VP1 protein (1 μg/ml) for 18 h at 4 °C, and blocked with 1% BSA. Different doses of LF were added and incubated for 2 h at 37 °C. After washing, bound LF was detected by 1:1000 diluted goat anti-LF antibody (Santa Cruz, Santa Cruz, CA) followed by 1:60,000 diluted horseradish peroxidase (HRP)-conjugated mouse anti-goat IgG secondary antibodies (Amersham Biosciences, Arlington Heights, IL). After the addition of substrate, TMB (Promega, Madison, WI) for 10 min at room temperature, the reaction was stopped by adding 2 N H_2SO_4. The color developed was read by the Vmax microplate reader (Molecular Device, Menlo Park, CA) at 450 nm. In the competitive inhibition assay, EV71- or VP1-coated plates were incubated with LF (250 μg/ml) with or without the presence of 20–10,000-fold diluted anti-VP1 antibodies (Chemicon, Temecula, CA) or 20-fold diluted isotype control IgG1 (0.2 mg/ml). Bound LF was detected as described above.

2.5. Detection of the binding of LF to cells by ELISA and immunofluorescent assay

Binding of LF to RD and SK-N-SH cells was determined by both ELISA binding assay and immunofluorescent assay. RD or SK-N-SH cells (2×10^4/well) were grown in DMEM containing 10% FCS at 37 °C in 96-well cell culture plate until confluent. Cells were fixed with 0.1% glutaraldehyde at 4 °C for 10 min and blocked with 2% BSA for 1 h at room temperature. Different doses of LF were added into cell-coated plates and incubated for 2 h at 37 °C. Bound LF was detected as described above. In addition, RD or SK-N-SH cells (4×10^4/well) were grown on Lab-Teck chamber slides (Nalge Nunc) until confluent. Cells were fixed with 4% paraformaldehyde in PBS for 25 min at 4 °C. LF (100 μg/ml) was added into the cell-coated slides for 1 h. After washing, bound LF was detected by the incubation with 1:1000 diluted goat-anti-LF antibody for 1 h at 37 °C, followed by the incubation with 1:200 diluted FITC-conjugated mouse anti-goat IgG antibody (Jackson ImmunoResearch, West Grove, PA) for 30 min at 4 °C. Cells were observed using confocal fluorescent microscopy (Leica TCS SP2, Germany).

2.6. ELISA for interleukin 6

SK-N-SH cells (2×10^5) were infected with EV71 at a MOI of 1 with or without the presence of bovine LF (250 μg/ml). Culture supernatants were collected 24 h post-infection. The concentrations of IL-6 in the supernatants were determined using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.7. Reverse-transcription polymerase chain reaction (RT-PCR) for IFN-α mRNA

Both SK-N-SH and monocytic cell line (THP-1) were used to test whether LF can induce IFN-α mRNA expression. THP-1 cells were first stimulated with phorbol-12-myristate-13-acetate (PMA, 1 μg/ml) for 24 h before LF stimulation. RNA was extracted from 2×10^6 cells after the incubation with or without LF (250 μg/ml) for 24 h using Trizole Isolation Reagent (Life Technologies, USA) and quantified at 260 nm. Reverse-transcription (RT) was performed with Reverse Transcription System (Gibco) according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was served as the internal control. The primer sequences were:

IFN-α (sense): 5′-GATGGCCGTGCTGGTGCTCA-3′;
IFN-α (anti-sense): 5′-TGATTTCTGCTCTGACAACC-TCCC-3′;
GAPDH (sense): 5′-CACGG CAAGTTCAATGGCACA-3′;
GAPDH (anti-sense): 5′-GAATTGTGAGGGAGAGTGC-TC-3′.

A total reaction volume of 20 μl contained 4 μl of RT product, 2.5 U Taq DNA polymerase, 20 μM dNTP, 0.1 μM primer, and 1× Taq DNA polymerase buffer (Promega). The reaction mixture was incubated in a thermocycler (GeneAmp system 2400; Applied Biosystems, Foster City, CA) programmed to pre-denature at 95 °C for 5 min, denature at 95 °C for 30 s, anneal at 54 °C for 45 s, and extend at 72 °C for 1 min, for a total of 30 cycles. The last cycle was followed by incubating at 72 °C for 7 min and cooling to 4 °C. The PCR products were analyzed in ethidium bromide-stained agarose gels (Sigma-Aldrich).

2.8. Mouse protection assay

Seventeen-days-old ICR mice (∼6.5 g) from Laboratory Animal Center, National Cheng Kung University Medical College, Tainan, Taiwan were used to test the protective effect of LF in vivo. The institutional animal care and use committee approved all animal protocols. Mice were intraperitoneally insulated with 2×10^7 pfu of MP4, a mouse-adapted EV71 strain (Wang et al., 2004) with or without the presence of an IC_{90} of LF (5 mg). The symptom and survival rate of infected mice were monitored daily for 2 weeks.

2.9. Statistical analyses

Data are expressed as mean ± S.D. The levels of significance for the differences between the test and control groups were analyzed using Student's *t*-test, and the differences were considered significant if *P* values were <0.05.

3. Results

3.1. Inhibition of EV71 infection by bovine and human LF

We first confirmed that bovine LF inhibited EV71 infection of RD cells in a dose-dependent manner with an IC_{50} approximately 34.5 μg/ml in a plaque reduction assay (Fig. 1A). At a concentration of 250 μg/ml, bovine LF showed the highest inhibitory effect (73%) as compared to human LF (34%). Thus, we chose bovine LF for the following experiments.

3.2. LF prevents and protects human cell lines from EV71-induced cytopathology

In experiments designed to test whether LF could prevent EV71 infection, RD and SK-N-SH cells (2×10^5 cells/ml) were pre-treated with LF at 250 μg/ml for different periods of time and then removed before the infection with EV71 (MOI = 0.1). In general, the longer the incubation time of LF with cells, the better the protective effect (Fig. 2A). About 45–50% of reduction was found when cells were pre-treated

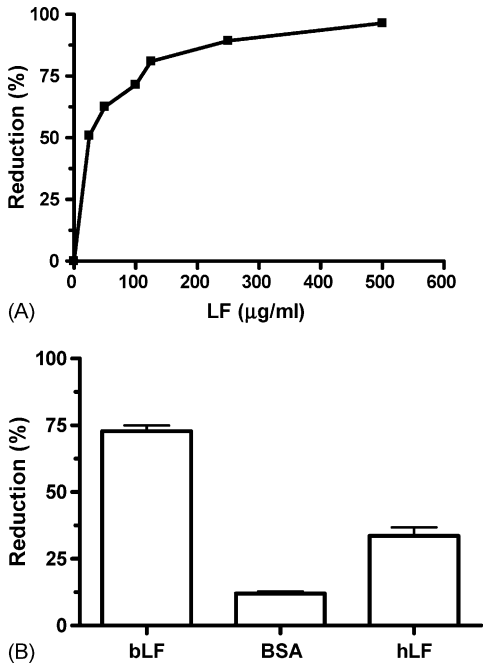

Fig. 1. LF inhibits EV71 infection of RD cells. RD cells (2×10^5 cells/ml) were infected with EV71 (MOI = 0.1) in the presence of different doses of bovine LF (bLF) for 2 h (A); or 250 μg/ml of bLF, BSA or human LF (hLF) for 8 h (B). Plaque reduction rate was determined by comparing the pfu between the control and treated cells 48 h after infection.

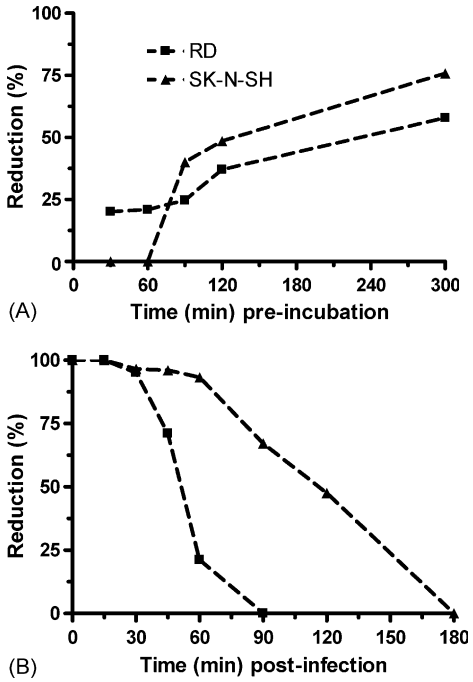

Fig. 2. Pre-incubation and post-infection effects of LF on EV71 infection. (A) RD and SK-N-SH cells (2×10^5 cells/ml) were pre-incubated with LF (250 μg/ml) for different periods of time as indicated, and then washed away before infection with EV71 (MOI = 0.1). (B) RD and SK-N-SH cells (2×10^5 cells/ml) were infected with EV71 for different periods of time as indicated, LF (250 μg/ml) was added and incubated for 2 h and then washed away. Plaque reduction was compared with the non-treated group 48 h after infection.

with LF for 2 h and 50–75% of reduction was found when cells were pre-treated with LF for 5 h (Fig. 2A). On the other hand, when LF was added at different time points after EV71 infection, the antiviral activity of LF was significantly decreased 30 min after infection in RD cells and 60 min after infection in SK-N-SH cells. No inhibitory effect was found 90 min after infection in RD cells and 180 min after infection in SK-N-SH cells, indicating that the inhibitory effects of LF affects mainly at an early stage during viral entry (Fig. 2B).

3.3. LF bound to cell surface of RD and SK-N-SH cells

Since the pre-treatment of LF could protect cells from EV71 infection, we next investigated whether LF could bind to RD and SK-N-SH cells using an ELISA-based assay. As shown in Fig. 3A, LF was found to bind to both RD or SK-N-SH cells-coated plates in a dose-dependent manner with a saturation dose of 78 μg/ml. No significant difference in LF binding was found between RD and SK-N-SH cells. Immunofluorescent staining confirmed that LF bound to the surface of both cells (Fig. 3B). There was no detectable flu-

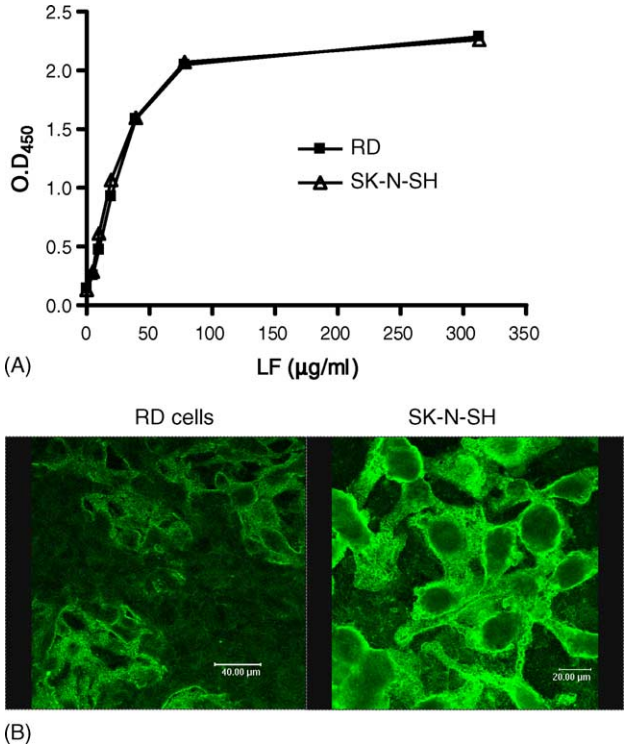

Fig. 3. Binding of LF to RD and SK-N-SH cells. (A) Increasing doses of LF were allowed to incubate with RD or SK-N-SH cell-coated plates for 2 h at 37 °C. Bound LF was detected by goat anti-LF antibody and HRP-conjugated mouse anti-goat IgG antibodies as described in Section 2. (B) RD or SK-N-SH cells (4×10^6/well) were grown on Lab-Teck chamber slides as described in Section 2. LF (100 μg/ml) was added into the cell-coated slides for 1 h. After washing, bound LF was detected by goat-anti-LF antibody and FITC-conjugated mouse anti-goat antibody. Cells were observed using confocal fluorescent microscopy.

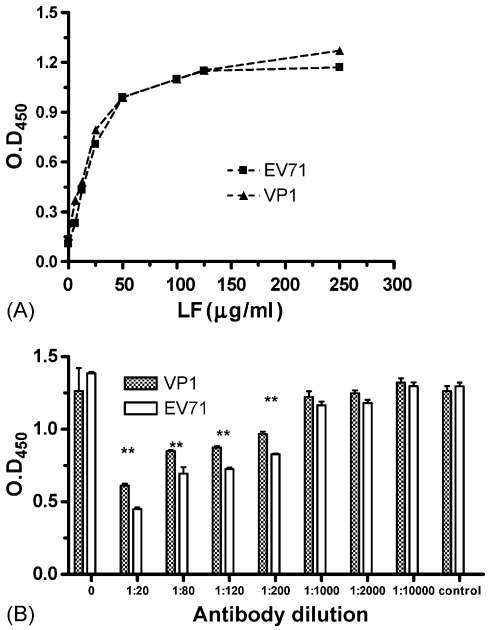

Fig. 4. LF binds to VP1 of EV71. (A) EV71 or recombinant VP1 was coated on 96-well ELISA plates as described in Section 2. Increasing doses of LF were allowed to incubate with EV71 or VP1-coated plates. Bound LF was detected by goat anti-LF antibody and HRP-conjugated mouse anti-goat IgG antibodies. (B) Anti-VP1 antibody competitively inhibits the binding of LF to EV71. EV71- or VP1-coated plates were incubated with LF (250 μg/ml) with or without the presence of anti-VP1 antibodies at different dilutions as indicated or isotype control antibody (control). Bound LF was detected as described in Section 2. ** $P < 0.01$ when compared to isotype control.

orescence in the control cells treated with FITC-conjugated mouse anti-goat antibody alone (data not shown).

3.4. LF bound to VP1 protein of EV71

Next we tested whether LF could bind to the virus directly by using EV71- and VP1 protein-coated plates. Similar to that of RD and SK-N-SH cells, LF was found to bind to EV71- or VP1-coated plates in a dose-dependent manner (Fig. 4A). In addition, LF binding was significantly inhibited by anti-VP1 antibody in a dose-dependent manner (Fig. 4B). Isotype control IgG1 had no such effect.

3.5. LF inhibited EV7-induced IL-6 production but induced IFN-α expression of SK-N-SH cells

To test whether LF could also inhibit EV71-induced cytokine production of SK-N-SH cells, we measured the IL-6 levels in the supernatants of EV71-infected SK-N-SH cells with or without the presence of LF. A significant increase of IL-6 production was found in EV71-infected cells (27.7 ± 13.3 pg/ml) as compared to non-infected cells (3.7 ± 0.8 pg/ml; Fig. 5A). EV71-induced IL-6 production was significantly reduced in the presence of LF (250 μg/ml; 16.8 ± 6.3 pg/ml, $P < 0.05$). LF treatment alone did not increase IL-6 production. However, IFN-α mRNA was increased in LF-treated THP-1 and SK-N-SH cells as shown by RT-PCR (Fig. 5B).

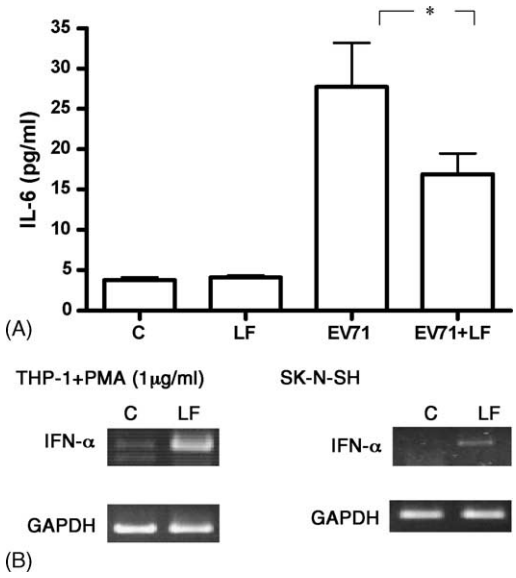

Fig. 5. LF inhibited EV71-induced IL-6 production but induced IFN-α expression. SK-N-SH cells (2×10^5) and PMA-treated THP-1 cells (1×10^6) were infected with EV71 (MOI = 0.1) with or without the presence of LF (250 μg/ml). Controls included cells without EV71 infection and those treated with LF only. (A) The amounts of IL-6 in the SK-N-SH cell culture supernatants were determined by ELISA after 24 h incubation. Data represent mean ± S.D.; $n \geq 3$; * $P < 0.05$. (B) The expression of IFN-α mRNA in SK-N-SH and THP-1 cells were amplified by RT-PCR as described in Section 2. Representative of two experiments was shown.

3.6. LF protected mice from EV7-induced lethality

Last we tested whether LF could inhibit EV71 infection in vivo using an experimental infection model in which mice were inoculated with EV71 by an intraperitoneal route with or without a co-injection of LF. As expected, untreated mice developed paralysis at 2-day post-infection (dpi), and all of them died at 6 dpi. LF-treated mice remained healthy until 5 dpi, and two out of seven mice were still survived at 14 dpi (Fig. 6).

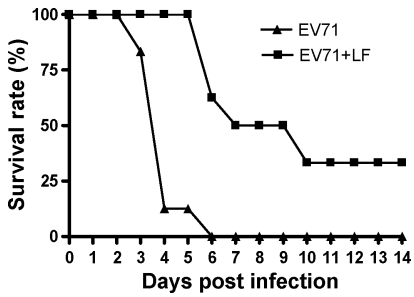

Fig. 6. LF protected mice from EV71-induced lethality. ICR mice (n = seven mice per group) were infected with EV71 (2×10^7 pfu/mouse) and treated with or without LF (5 mg per mouse) by intraperitoneal injection simultaneously. The survival of the mice was observed daily for 2 weeks.

4. Discussion

Besides a broad anti-microbial spectrum against bacteria and a capacity of inhibiting the replication of many different viruses, we noticed that LF could inhibit EV71 infection. The anti-EV71 mechanism of LF is still not clearly understood and may relate to a prevention of viral entry by blocking cellular receptors and/or by direct binding to the virus particles as suggested by the fact that LF bound to both the virus and host cells. LF might interfere the interaction of EV71 with its specific receptor, and redirected its entry through LF-binding proteins on the cell surface. This new way of entry may render virus prone to degradation instead of replication. On the other hand, binding of LF to the cells may provoke an antiviral status. This may explain why pre-treatment of cells with LF could protect cells from EV71 infection. Indeed, our result showed INF-α, which plays an important role in the inhibition of virus replication (Taniguchi and Takaoka, 2002), was induced in LF-treated THP-1 and SK-N-SH cells.

At present, we do not know precisely which cell surface proteins are bound by LF to induce the antiviral response in the cells. LF has been shown to be able to bind to several different cell ligands such as heparan sulfate, chondroitin sulfate (Marchetti et al., 2004; van der Strate et al., 2001) and nucleolin (Legrand et al., 2004). However, antiviral activity of LF analogues is only partly related to their affinity for heparin sulfate (Jenssen et al., 2004). Therefore, identification of the LF-binding molecules, which are involved in the LF-induced antiviral response of cells requires further study.

The capsid of EV71 consists of four proteins, VP1 to VP4. Suckling mice born to dams immunized with subunit VP1 vaccines were protected against lethal EV71 challenge indicating that VP1 may contain neutralization epitopes and involve in the binding of virus to the cells (Wu et al., 2001). In this study we demonstrated that LF could bind to VP1-coated ELISA plate, which was inhibited in the presence of anti-VP1 antibody. Taken together, these data indicated that LF might interfere with viral attachment by blocking the binding of VP1 to host cells. Further study using lactoferrin-derived peptides is indeed required to identify the domains or regions of LF, which possess binding activity to the VP1 protein of EV71 (Nozaki et al., 2003).

In our mouse study, when mice were inoculated with EV71 by intraperitoneal injection, virus replicated in the muscle, spinal cord, and brain of infected mice and induced paralysis and death of infected mice in a fashion similar to our previous study using oral inoculation (Wang et al., 2004). LF treatment not only delayed the paralysis but also reduced the mortality of infected mice, so it seems that LF is not very likely to cause any obvious severe side effect in mice. Because LF was able to inhibit viral replication in muscle (RD) and neuronal (SK-N-SH) cells in vitro, the potential targets of virus in mice in vivo, it is very likely that LF reduces the symptom and death of mice by repressing viral replication in tissues. In the future, the issues of toxicity, antiviral mechanism, and optimal dose of LF for in vivo study need further investigation.

In summary, we demonstrated here that bovine LF could bind to host cells and induce IFN-α expression. In addition, LF could also bind to VP1 of EV71 and protect cells and mice from EV71 infection. As in the case of HIV, bovine LF is a much more efficient inhibitor against EV71 than human LF (Groot et al., 2005). Since there is no apparent correlation between the amount of LF bound to cells and its antiviral activity, further study is required to understand the antiviral mechanism induced by LF. Nevertheless, since large quantities of bovine LF are present in the milk, it is tentative to test whether drinking milk may help to prevent EV71 infection.

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