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Bovine lactoferrin inhibits Japanese encephalitis virus by binding to heparan sulfate and receptor for low density lipoprotein

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ABSTRACT

Lactoferrin is a natural anti-microbial protein which affects Japanese encephalitis virus (JEV) activity. Binding of lactoferrin to cell surface expressed heparan sulfate (HS), one possible receptor for JEV, has been postulated to be the possible mechanism of anti-JEV antiviral activity. In this study, we evaluate the effects of bovine lactoferrin (bLF) against JEV infection *in vitro*, using both wild-type (WT) and laboratory-adapted strains. bLF inhibited the infectivity of all the JEV strains tested. In particular the infectivity of the HS-adapted JEV strains was strongly reduced, whereas the non HS-adapted JEV strains were inhibited to lesser extent. Using both HS-adapted CJN-S1 and non HS-adapted CJN-L1 viruses, the results showed that bLF inhibited the early events essential to initiate JEV infection, which includes blocking virus attachment to cellular membranes and reducing viral penetration. This anti-JEV activity was the highest using HS-adapted CJN-S1 strain on HS-expressed CHO-K1 cells. Also, binding of bLF to heparin-sepharose blocked JEV binding; and soluble HS attenuated the anti-JEV activity of bLF. The results support the premise that the interaction of bLF with cell surface expressed glycosaminoglycans, in particular the highly sulfated HS, plays an essential role in the antiviral activity of bLF. However, bLF was functional in inhibiting CJN-S1 entry into HS-deficient CHO-pgsA745 cells, and bLF-treated CHO-K1 and -pgsA745 cells also prevented non HS-adapted CJN-L1 virus entry, indicating that a non-HS pathway may be involved in bLF inhibition of JEV entry. The low-density lipoprotein receptor (LDLR), possibly involved in the entry of several RNA viruses, also binds to bLF. We found that both rLDLR and anti-LDLR antibodies reduced the effectiveness of bLF inhibition of JEV infection. This finding provided evidence to suggest that cell surface-expressed LDLR may play a role in JEV infection, especially for non HS-adapted strains.

Introduction

Japanese encephalitis virus (JEV) is an important mosquito-borne pathogen, which is the leading cause of encephalitis in humans in Asian countries. JEV infections cause a broad spectrum of clinical symptoms, ranging from asymptomatic, fever, and encephalitis to death. Infections leading to overt encephalitis are estimated to be 1 in 20 to 1000 cases (Brandt, 1990; Grossman et al., 1973; Huang, 1982), but approximately 25% of encephalitic patients die and 50% of the survivors develop permanent neurologic and/or psychiatric sequelae. Beginning in 1968, a nation-wide vaccination program for children against JEV was implemented in Taiwan, using mouse-brain-derived and formalin-inactivated JEV Nakayama vaccine. Since then the confirmed cases of JEV infection has dramatically dropped to about 20–30 cases annually (Wu et al., 1999).

The first step in viral infection is the attachment to the host cell followed by penetration of the cell membrane. This initial step in JEV infection involves the interaction of glycosaminoglycans (GAGs), in particular the highly sulfated form of GAGs (heparan sulfate, HS), on the cell surface and the viral envelope (E) glycoproteins (Chiou et al., 2005; Liu et al., 2004; Su et al., 2001). Mutations in the amino acid residues 49, 138, 306, or 389 of JEV E protein have been implicated in changing the interaction with HS, resulting in altering plaque size, viral entry, neurovirulence, and neuroinvasiveness (Chiou et al., 2005; Lee et al., 2004; Lee and Lobigs, 2002; Liu et al., 2004; Su et al., 2001). In additional to GAGs, several plasma membrane-associated, heparinase-resistant proteins could possibly be part of a receptor complex for JEV on mammalian or mosquito cells (Chen et al., 1996; Chu et al., 2005).

Lactoferrin (LF) is an 80 kDa multi-functional glycoprotein which is present in plasma and external secretions, like milk and tears (Masson et al., 1969; Weinberg, 2001). It is primarily an iron-binding protein that functions in iron transport in intestines (Kuwata et al., 1998), and also is associated with non-specific immune responses involved in suppressing a variety of infections including bacteria, fungus, and viruses (Buckley et al., 2003; Rocco et al., 1998; Ueta et al., 2001; Yoo et

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al., 1998). LF inhibits virus entry by binding directly to the virus particle or to membrane-bound HS (Andersen et al., 2003, 2001; Harmsen et al., 1995; Marchetti et al., 1996). Both human and bovine LF (bLF) have a relatively high net positive charge and may interact with negatively charged GAGs expressed on target cells (El Yazidi-Belkoura et al., 2001), which could result in antiviral activity. In addition to HS, LF also binds to the low-density lipoprotein receptor (LDLR) and LDLR-related protein (LRP) (Huettinger et al., 1998).

Previously, we have demonstrated that highly sulfated GAGs, such as HS, are important receptors for JEV *in vitro*, and have identified two HS-binding motifs in the E glycoprotein of JEV (Chiou et al., 2005; Liu et al., 2004). The focus of this study was to investigate the antiviral activity exerted by the HS-binding protein, bLF, and to study a possible correlation between GAG affinity and antiviral activity. Our results show that bLF inhibited both wild-type and laboratory-adapted JEV strains, and that the antiviral activity of bLF correlated with the HS-binding affinity of JEV *in vitro*. In addition, the anti-JEV activity of bLF may also involve binding to LDLR.

Results

Inhibition of JEV infection by bLF

To determine whether bLF has antiviral activity against JEV, we performed a plaque reduction assay in the presence of bLF. BHK-21 cells were pre-incubated with 200 μg/ml of bLF, and the same concentration of bLF was maintained in the overlay medium throughout the infection. No cytotoxic effect of bLF was observed during the experiments. The bLF-treated cells were infected with 200 plaque forming unit (PFU) of eleven JEV strains, including three field-isolated wild-type (WT) strains and eight laboratory-adapted strains derived from them (Table 1). Infection of BHK-21 cells by all of the JEV strains were drastically inhibited by the presence of bLF, range from 17.3% to 95.8% (Table 1). Based on our previous studies, these strains could be classified into HS-adapted or non HS-adapted virus according to the binding affinity to heparan sulfate (Chiou and Chen, 2007; Chiou et al., 2005; Liu et al., 2004). The infectivity of the HS-adapted JEV strains, excluding CC27-S6, measured by plaque number, was strongly reduced in the presence of bLF. By contrast, the non HS-adapted JEV strains were only slightly inhibited by bLF, except CC27 and it derivates CC27-L1 and CC27-L3. HS-adapted CJN-S1 and non HS-adapted CJN-L1 strains showed the highest and lowest inhibition, respectively, by bLF and were thus selected for the following experiments to study the mechanism by which bLF inhibited JEV infection.

The plaque reduction assays confirmed that bLF-treated BHK-21 cells inhibited JEV infection in a dose-dependent manner (Fig. 1A). The estimated 50% inhibitory concentration (IC_{50}) was 26.1 and 518.3 μg/ml for CJN-S1 and CJN-L1, respectively. In order to determine whether the inhibitory effect was due to the direct interaction of bLF with the virus or with the cells, we pre-incubated JEV CJN-S1 and CJN-L1 with 100 or 200 μg/ml bLF in a small volume. The bLF-virus mixture was then diluted to a final concentration of 0.09 μg/ml bLF before infecting BHK-21 cells. No cytopathic effect was observed in the control cells treated with the same final concentration of bLF, and no inhibitory effects were observed against CJN-S1 and CJNL1 infection when the viruses were pre-incubated with a high concentration of bLF and diluted before infection (Fig. 1B). In the next experiments, the BHK-21 cells were pre-treated with various concentrations of bLF (0.1 to 200 μg/ml) which was removed before incubation with JEV. The results showed that BHK-

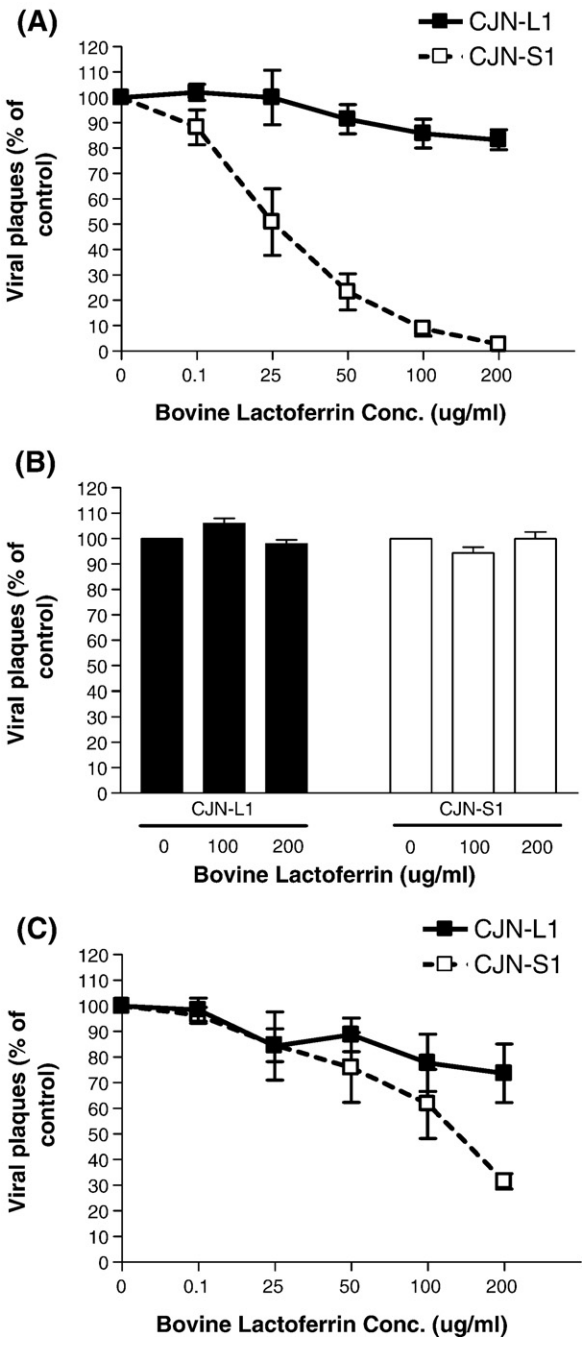

Fig. 1. Inhibitory effect of bLF against JEV infection. The BHK-21 cells were infected with 200 PFU JEV CJN-L1 or CJN-S1 strains using various conditions. The bLF was presented both before and throughout the infection (A), the virus was pre-mixed with bLF at 4 °C for 1 h (B), and the cells were pre-treated with bLF at 4 °C for 1 h (C).

Table 1
Antiviral effect of bovine lactoferrin (bLF) against various strains of wide-type and laboratory-adapted Japanese encephalitis virus (JEV)[a]

Virus	Source[b]	HS affinity[b]	% inhibition by bLF[c]
T1P1	Mosquito isolate	Low (non HS-adapted)	17.6±5.9
T1P1-L4	Plaque–purified from T1P1	Low (non HS-adapted)	18.1±11.5
T1P1-S1	Plaque–purified from T1P1	High (HS-adapted)	95.6±2.7
CJN	human brain isolate	High (HS-adapted)	90.3±3.4
CJN-L1	Plaque–purified from CJN	Low (non HS-adapted)	17.3±1.7
CJN-S1	Plaque–purified from CJN	High (HS-adapted)	95.8±1.6
CC27	mosquito isolate	Low (non HS-adapted)	70.3±9.3
CC27-L1	Plaque–purified from CC27	Low (non HS-adapted)	87.2±3.7
CC27-L3	Plaque–purified from CC27	Low (non HS-adapted)	56.5±10.3
CC27-S6	Plaque–purified from CC27	High (HS-adapted)	35.4±7.7
CC27-S8	Plaque–purified from CC27	Low (non HS-adapted)	31.8±14.2

[a] The assessment of anti-JEV activity of bLF was performed on BHK-21 cells.
[b] See reference (Chiou and Chen, 2007; Chiou et al., 2005; Liu et al., 2004).
[c] Before and after infection with 200 PFU JEV, BHK-21 cells were cultured with medium containing 200 μg/ml bLF.

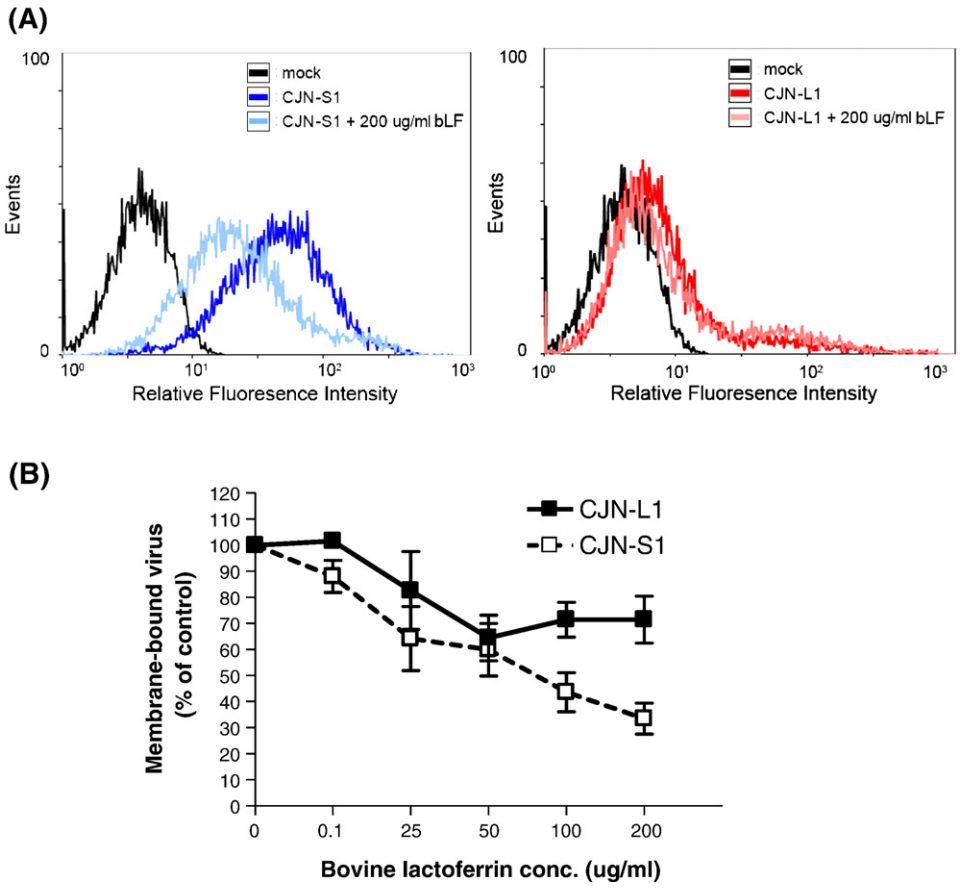

Fig. 2. Inhibitory effect of bLF on JEV attachment. The entry of JEV into cells was classified into two steps, attachment and after attachment, by temperature of incubation, at 4 °C or 37 °C. The effect of bLF on JEV binding to BHK-21 cells was evaluated by flow cytometry at an MOI of 500 (A), and membrane-bound virus by absorption assay at an MOI of 5 (B).

21cells pre-treated with bLF were protected from CJN-S1 and CJN-L1 infection in a dose-dependent manner (Fig. 1C).

Taken together, these results demonstrated clearly that bLF strongly inhibited the infection of cells by HS-adapted CJN-S1, while only partial inhibition was observed for the non HS-adapted CJN-L1 strain; and the inhibitory effects were mainly due to the direct binding of bLF to the cells, not to the virus. In addition, the inhibitory effect showed in Fig. 1C was much less pronounced as compared to Fig. 1A indicating that the bLF might account for multiple steps during JEV infection.

Effects of bLF on JEV entry

To determine whether the bLF prevented the binding of JEV to cells or interfered with a step after virus attached to cells, bLF was added to the cells either before or after virus binding. First, JEV CJN-S1 and CJN-L1 were allowed to bind to cells at 4 °C in the presence of various concentrations of bLF. Unbound virus was removed by washing, and cell-bound viruses was detected by flow cytometry and quantified by plaque assay (Figs. 2A and B). The results showed that binding of the JEV to BHK-21 cells was inhibited by bLF. Cell binding of HS-adapted CJN-S1 strain was strongly inhibited by bLF in a dose dependent manner. By contrast, cell binding of non HS-adapted CJN-L1 virus was slightly inhibited by bLF and the inhibitory effect was saturated at a concentration higher than 50 μg/ml.

The effect of bLF on penetration of JEV was evaluated by adding bLF after virus attached to BHK-21 cells. Virus penetration was analyzed by an infectious center assay (Fig. 3). The results of the infectious center assay showed that bLF strongly prevented CJN-S1 virus penetration into BHK-21 cells in a dose dependent manner, whereas CJN-L1 virus was less affected and the inhibition was saturated at concentrations higher than 25 μg/ml. These results demonstrate that bLF prevented JEV entry into cells by inhibiting both attachment and penetration.

Correlation of the inhibitory effect of bLF on JEV infection and HS-expression

HS is critically involved in JEV infection, primarily at an early stage of the virus life cycle, including viral attachment and penetration (Liu et al., 2004; Su et al., 2001). The HS-expressed CHO-K1 and HS-deficient CHO-pgsA745 cells were used to determine whether the HS expression played an important role in the inhibitory effect of bLF. JEV CJN-S1 and CJN-L1 were allowed to bind to CHO-K1 or -pgsA745 cells at

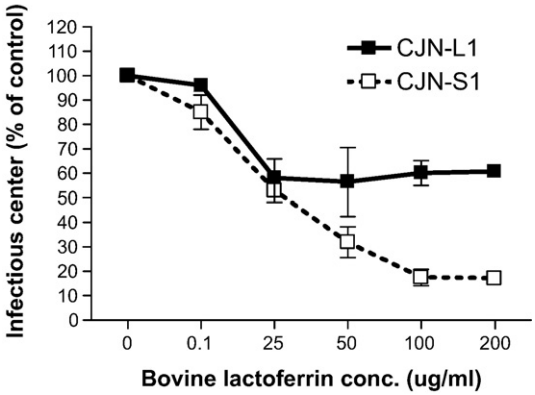

Fig. 3. After JEV binding to BHK-21 cells at 4 °C for 1 h at MOI=5, the bLF was added and the inhibitory effect was evaluated by an infectious center assay.

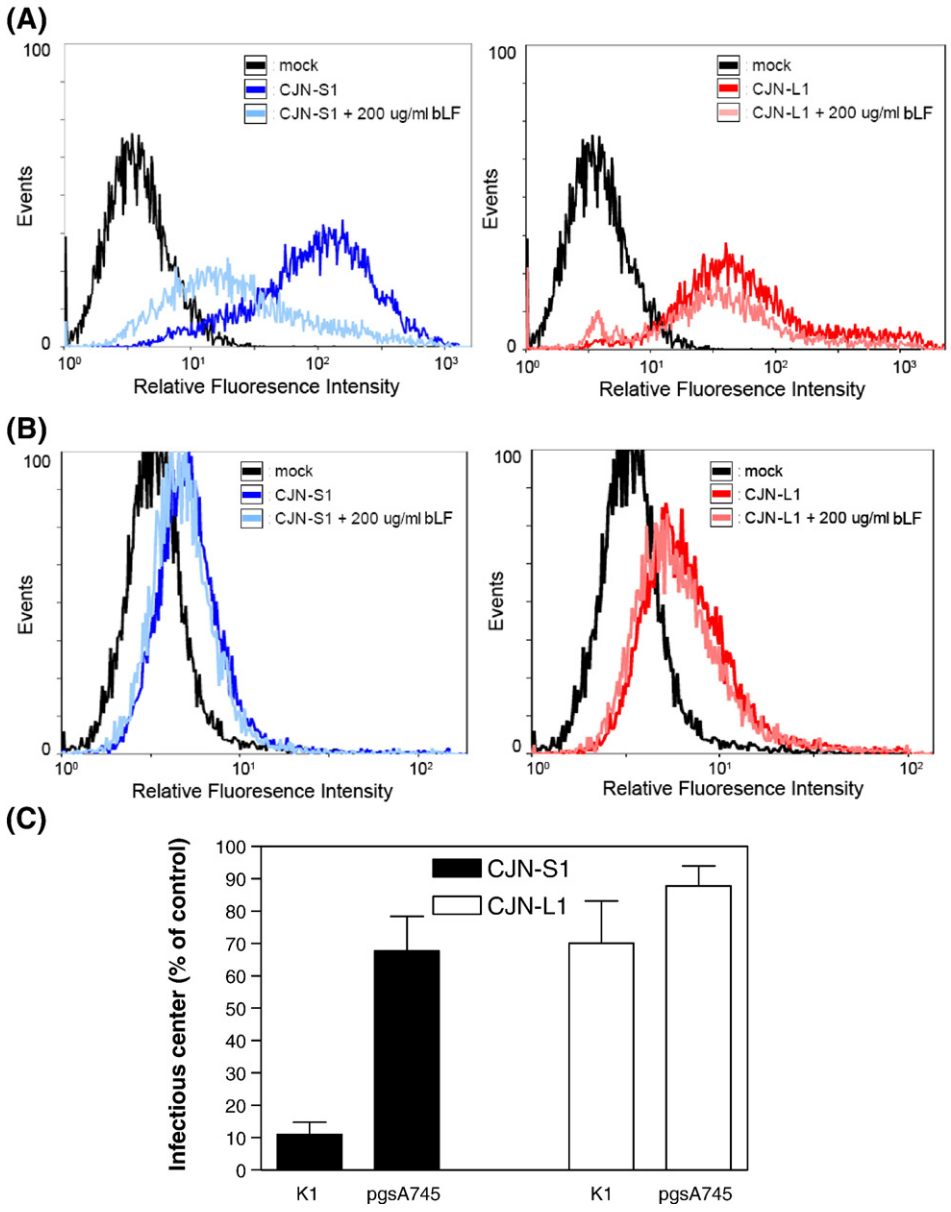

Fig. 4. Inhibitory effect of bLF on JEV entry into HS-expressed CHO-K1 and HS-deficient CHO-pgsA745 cells. CJN-S1 or CJN-L1 virus binding to bLF-treated CHO-K1 (A) or -pgsA745 (B) cells was evaluated by flow cytometry at an MOI of 500. For the post-attachment step, bLF was added to JEV-bound cells, and then evaluated by an infectious center assay (C).

4 °C in the presence of various concentrations of bLF, and evaluated by flow cytometry (Figs. 4A and B). The binding of CJN-S1 virus to CHO-K1 cells was more efficient than CJN-L1 (Fig. 4A), and also the binding of the viruses to CHO-K1 cells was higher than to CHO-pgsA745 cells (Figs. 4A and B). The binding of CJN-S1 and CJN-L1 viruses were significantly reduced in the presence of 200 μg/ml bLF, but not completely, in either CHO-K1 or -pgsA745 cells. These results were also confirmed by absorption assay (data not shown). In CHO-pgsA745 cells the bLF blocked either 48.6% or 34.9% binding for CJN-S1 or CJN-L1 viruses, respectively.

Subsequently, the infectious center assay was performed to determine the role of HS in the inhibitory action of bLF after JEV binding to cells. JEV CJN-S1 and CJN-L1 were allowed to bind to CHO-K1 or -pgsA745 cells at 4 °C for 1 h, then 200 μg/ml bLF was added and incubated at 37 °C for additional 1 h, and evaluated by an infectious center assay (Fig. 4C). This assay showed that bLF prevented the infection of CHO-K1 or -pgsA745 cells by both CJN-S1 and CJN-L1 viruses. However, bLF was more effective in blocking the CJN-S1 virus infection in CHO-K1 (11.0 ± 5.4%) than in CHO-pgsA745 (67.7 ± 18.5%) cells, but nearly equal in blocking the CJN-L1 virus infection in both cells (CHO-K1: 70.1 ± 22.6%; CHO-pgsA745: 87.8 ± 10.7%).

The ability of bLF to block the entry of CJN-S1 virus into CHO-K1 cells more effectively than into CHO-pgsA745 cells suggested that the HS might play a pivotal role in which the entry of HS-adapted JEV. However, the previous reports indicated JEV is capable of infecting and replicating in HS-deficient cells (Chiou et al., 2005; Liu et al., 2004) and the residue-blocking effect of bLF apparently persisted in CHO-pgsA745 cells to inhibit CJN-S1 virus entry in present study, suggesting that a non-HS pathway in both cell types may also contribute to the initiation of JEV infection.

bLF antagonizes the binding of JEV to heparan sulfate

We next used sepharose-conjugated HS to determine whether bLF antagonized the binding between JEV particles and HS. The slightly excess sepharose-conjugated HS was incubated with bLF; the unbound bLF and bLF-treated sepharose-conjugated HS were collected separately and analyzed. The bLF-treated, sepharose-conjugated HS

fraction was incubated with CJN-S1 or CJN-L1 viruses at 4 °C, and bound-viruses were released by NaCl and quantified by plaque assay (Fig. 5A). Preincubation of bLF strongly reduced the binding of CJN-S1 virus to the sepharose-conjugated HS, but not the binding of CJN-L1 virus. The unbound bLF collected from liquid fraction was assayed for anti-JEV activity (Fig. 5B). Compared to fresh bLF, the unbound bLF fraction had completely lost its anti-JEV activity, indicating that most of the bLF was bound to the sepharose-conjugated HS. These results suggested that bLF bound to HS-conjugated sepharose beads, and prevented the binding of HS-adapted JEV (CJN-S1), but not non HS-adapted virus (CJN-L1).

Because bLF interacts with HS directly, we investigated whether heparin could compete with the action of bLF against JEV infection. Heparin and bLF alone or in combination were mixed with 200 PFU JEV, and the infectivity was determined by the plaque assay in BHK-21 cells. Pre-incubation of cells with bLF or virus with heparin reduced the infectivity of CJN-S1 and CJN-L1 significantly (Figs. 6A and B). When a constant concentration of bLF (200 μg/ml) and variable concentrations of heparin (0.025 to 250 U/ml) were incubated with virus together, the infectivity of HS-adapted CJN-S1 strain was significantly increased in the presence of 0.025 to 2.5 U/ml heparin. However, the infectivity decreased when higher concentrations of heparin (>2.5 to 250 U/ml) were used. These results suggested that heparin interacts with bLF and antagonizes the inhibitory effect of both compounds on CJN-S1 virus (Fig. 6A). Non-HS adapted CJN-L1 strain was less dependent on HS for its infection, thus, the addition of heparin did not affect the inhibitory action of bLF against this virus (Fig. 6B). In agreement with earlier results, HS played a role in the inhibitory effect of bLF against HS-adapted JEV. These results

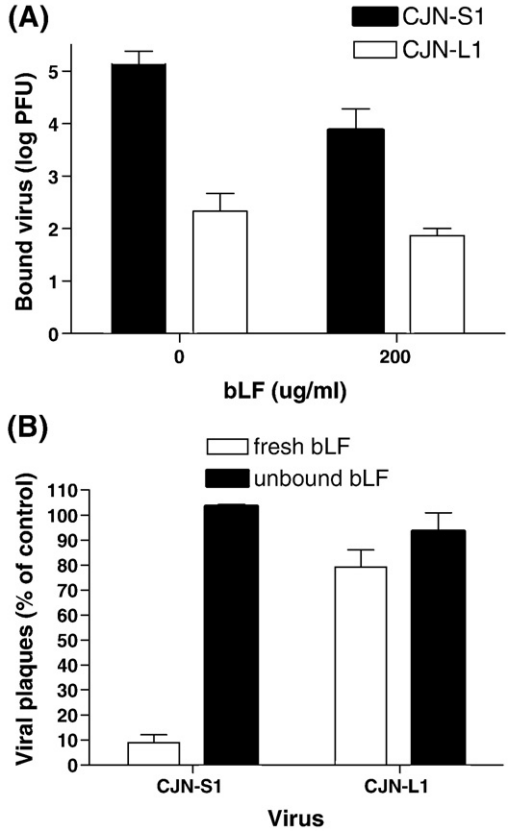

Fig. 5. bLF blocks JEV interaction with heparan sulfate. bLF was allowed to bind to heparin-sepharose, and the residual bLF and bLF-treated heparin-sepharose were collected and analyzed separately. bLF-treated heparin-sepharose was incubated with CJN-S1 or CJN-L1 viruses (5×10^6 PFU) at 4 °C for adsorption, and the bound virus was released by NaCl treatment and quantified by plaque assay (A). The residual bLF was assayed for anti-JEV infection (B) as described in Fig. 1A.

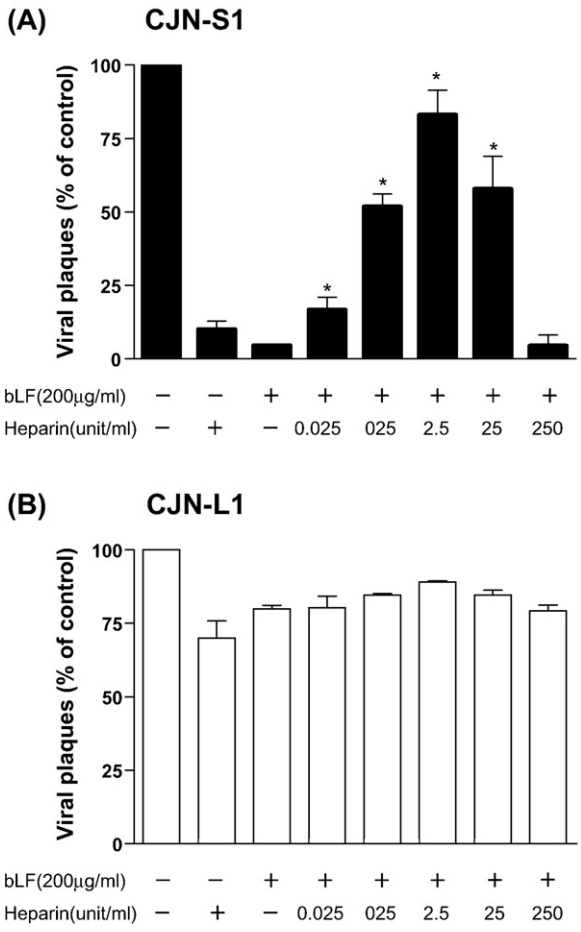

Fig. 6. Heparin reduced the antiviral activity of bLF. Heparin and bLF alone or in combination were mixed with 200 PFU JEV CLN-S1 (A) or CJN-L1 (B) strains, and the infectivity was determined by the plaque assay in BHK-21 cells. (*$P<0.05$).

suggested that an unidentified non-HS membrane receptor, involved in the initiation of non-HS adapted JEV infection, may interact with bLF and reduce the infectivity of CJN-L1 virus.

rLDLR attenuates bLF against JEV

It has been proposed that LDLR binds to bLF and is also involved in the entry of *Flaviviridae* viruses (Agnello et al., 1999; Huettinger et al., 1998). To examine the role of LDLR, 200 PFU of JEV viruses were added to mixtures of human recombinant LDLR (rLDLR) and bLF, and virus infectivity was determined by the plaque assay in BHK-21 cells. Compared to the virus control, the rLDLR alone reduced the infectivity of CJN-S1 virus to 12.5±4.7% and CJN-L1 virus to 30.0±11.9% (Fig. 7A and B). Additionally, the receptor-associated protein, binding to LDLR, also showed the anti-JEV activity, but not human serum albumin. When a constant concentration of bLF (200 μg/ml) and variable concentrations of rLDLR were mixed with virus before infection, the infection of CJN-S1 virus was increased from 12.5% (200 μg/ml bLF alone) to 54% (bLF+0.8–4 ng/ml rLDLR), whereas the infection of CJN-L1 virus was increased from 77% (200 μg/ml bLF alone) to 92% (bLF 100 ng/ml rLDLR). The data showed that rLDLR reduced the inhibitory effect of bLF against JEV infection, and indicated that rLDLR may play a role in JEV infection, especially for non HS-adapted strains.

LDLR as a possible receptor for JEV entry

To test whether LDLR plays a role in JEV infection, BHK-21 cells were pretreated with 5 μg of anti-LDLR, anti-LRP1, anti-VLDLR, anti-SR BI

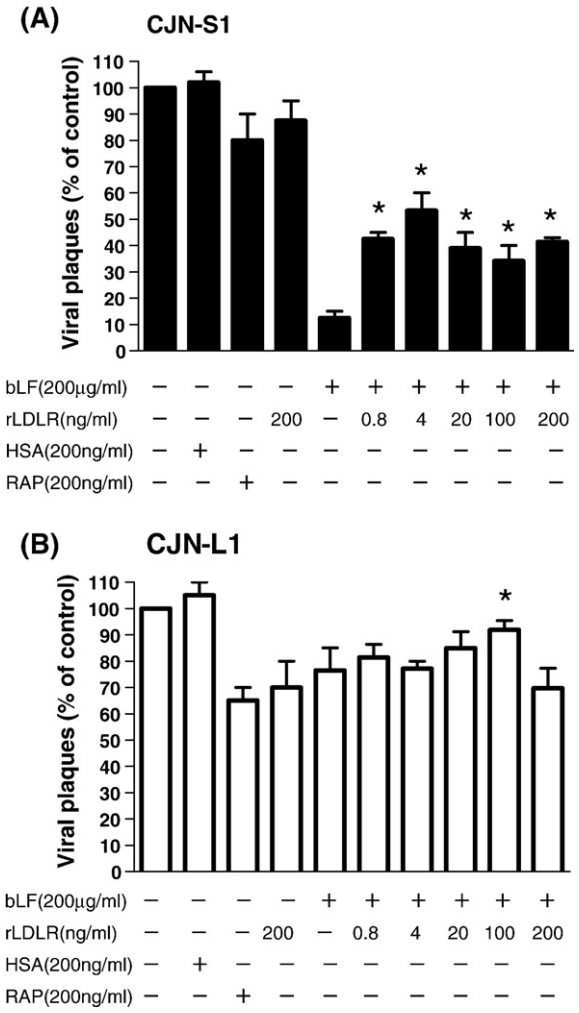

Fig. 7. Recombinant low density lipoprotein receptor reduced antiviral activity of bLF. rLDLR and bLF alone or in combination were mixed with 200 PFU JEV CLN-S1 (A) or CJN-L1 (B) strains, and the infectivity was determined by the plaque assay in BHK-21 cells. (*$P<0.05$).

antibody or normal chicken serum, subsequently infected with 200 PFU of JEV, and infectivity were measured by plaque assay. As shown in Fig. 8(A), anti-LDLR antibody treatment effectively reduced viral infectivity by 54% and 23% for CJN-L1 and CJN-S1 viruses, respectively; in contrast, anti-LRP1, anti-VLDLR, anti-SR BI antibody and normal chicken serum did not shown significant effect. Lovastatin, dissolve in DMSO, is an inducer of LDLR expression (Molina et al., 2007). BHK-21 cells were cultured with 0 or 10 μM lovastatin for 12 h, and LDLR expression was measured by flow cytometry to examine the effects of lovastatin on LDLR expression. As shown in Fig. 8(B), LDLR expression of lovastain-treated cells was significantly higher than the untreated cells. When treated or untreated cells were infected with CJN-L1 or CJN-S1 virus at an MOI = 5 and cultured for 48 h (Fig. 8C), the infectivity of CJN-L1 virus in lovastatin-treated cells was significantly higher than in untreated cells ($6.31+10^7$ and $1.58+10^7$ PFU/ml, respectively). Similarly, the infectivity of CJN-S1 virus in lovastatin-treated cells was higher than in untreated cells (3.16×10^8 and 7.9×10^7 PFU/ml, respectively). Finally, to determine the antiviral activity of bLF on lovastatin-treated BHK-21 cells, we performed a plaque reduction assay with 200 PFU JEV in the presence of 200 μg/ml of bLF. The results are summarized in Fig. 8D. For CJN-L1 virus, the inhibitory effect of bLF was 23% and 63% on lovastatin-untreated and -treated cells, respectively. Similarly for CJN-S1, the inhibitory effect of bLF on the lovastatin-treated cells was significantly higher than on untreated cells.

Discussion

LF suppresses broad-range virus infection, especially mucosally transmitted viruses, such as herpes simplex virus (Ammendolia et al., 2007; Andersen et al., 2003, 2004), human papillomavirus (Drobni et al., 2004; Mistry et al., 2007), enterovirus (Lin et al., 2002; Weng et al., 2005), and human polyomavirus BK (Marchetti et al., 1996). LF also inhibits the infection of two mosquito-borne alphaviruses, Sindbis virus (SINV) and Semliki Forest virus (SFV) (Waarts et al., 2005). The mechanisms of LF in reducing virus infection are the result of LF binding to the virus particle directly or to the membrane-bound viral receptor candidate(s) (Andersen et al., 2001; Harmsen et al., 1995; Marchetti et al., 1996). In the current study, we have shown the anti-JEV action of bLF. Our results are consistent with alphavirus studies showing that pre-incubation of virus with 200 μg/ml LF did not inhibit virus infection (Waarts et al., 2005), and also support reports that LF-sensitive arboviruses, such as SINV, SFV and JEV, do not interact with LF directly because of the overall net positive charge nature of both the viral envelope glycoprotein and LF (Klimstra et al., 1998; Rey et al., 1995). Reports that pre-incubation of cells with LF suppressed JEV infection is consistent with the indication that binding of LF to the cell surface molecule(s), especially molecules with a net negative charge, determines the antiviral action for arboviruses in general (Waarts et al., 2005).

GAGs, on the cell surface and in the extracellular matrix, bind to a wide variety of growth factors, chemokines, enzymes, and matrix components (Bernfield et al., 1992). Previous studies by others have shown that GAGs are also important in the attachment of a number of bacteria, protozoa, and viruses (Bergstrom et al., 1997). The highly sulfated form of GAGs, HS and heparin, on cell surfaces plays a determining role in the early stage of JEV infection (Chiou et al., 2005; Lee et al., 2004; Lee and Lobigs, 2002; Liu et al., 2004; Su et al., 2001). HS is a negatively charged and highly sulfated GAG expressed on many types of cells (Bernfield et al., 1992). Proteins typically bind electrostatically to HS using of stretches of positively charged amino acids such as Lys and Arg; binding of JEV and bLF to HS is presumably mediated by the same electrostatic interaction (Chiou et al., 2005; Liu et al., 2004; Weinberg, 2001). We have demonstrated the binding of JEV or bLF to HS-sepharose beads and HS-expressed CHO K1 cells, but not to HS-deficient CHO-pgsA745 cells, and that the binding of bLF to HS expressed on the cell surface prevented JEV infection. Similar to the alphavirus study, the HS-adapted JEVs were shown to be more sensitive to the cells treated with bLF; however, unlike SINV and SFV (Waarts et al., 2005), the bLF-treated cells also suppressed the replication of the non HS-adapted, WT JEV strains, although to a lesser extent. The inconsistent results imply that the interaction between HS and LF plays a central role in the inhibitory effect of LF against alphavirus infection, but the bLF-inhibited JEV infection may involve a non-HS pathway.

Our studies suggested that the inhibitory effect exerted by bLF treatment may involve multiple steps during JEV entry into cells, because the binding of virus to cellular membrane and the subsequent infection of membrane-bound virus were both decreased in the presence of bLF. This result is consistent with our previous studies indicating that HS determines the efficiency of JEV attachment and penetration using either BHK-21 or Neuro-2a cells (Liu et al., 2004). However, LF prevents alphaviruses attachment, and has no inhibitory effect on attached viruses (Waarts et al., 2005). The inconsistent results between our JEV study and the alphavirus reports could result from different experimental protocols. The liposome system, used in SINV and SFV studies, is advantageous for studying the fusion event between virus and membrane, but does not mimic all steps of virus entry, whereas the cell-based system used in present study mimics all virus entry steps. An earlier study reported that the amino acid at position 55 of the E2 glycoprotein of SINV is a critical determinant for binding to HS and effects the internalization of the virus (Dropulic et al., 1997). Thus, HS may involve JEV, SINV and SFV entry steps before

endosome membrane fusion, including attachment, internalization, or penetration.

SINV is capable of infecting and replicating in HS-deficient cells, indicating that additional receptors must be involved (Byrnes and Griffin, 1998). Similarly, JEV has been shown to replicate in HS-deficient CHO pgsA-745 cells in this and previous reports (Chiou et al., 2005; Liu et al., 2004). In addition, we found that bLF suppressed the replication of non HS-adpated JEV in HS-expressed and -deficient cells, and inhibited replication to a greater extent in HS-adapted strains in both HS-expressed and -deficient cells. All these observations suggest that other cell surface expressed, bLF-binding molecule, in addition to HS, must be involved in JEV entry.

bLF binds to various cellular proteins or molecules, including HS, LDLR, LRP, and nucleolin (El Yazidi-Belkoura et al., 2001; Huettinger et al., 1998; Legrand et al., 2004). Among these molecules, LDLR has been reported to be involved in endocytosis of RNA viruses, including hepatitis C virus, hepatitis G virus, and bovine viral diarrheal virus (Agnello et al., 1999). Here, we have shown that interaction between bLF and rLDLR reduced the inhibitory effect of LF in JEV infection, and that preincubation of anti-LDLR antibodies with LDLR-expressing cells reduced the infectivity of both HS-adapted CJN-S1 and non-HS adapted CJN-L1 viruses. These observations suggested that, in addition to HS, LDLR may function as a JEV receptor. Compared to HS-adapted JEV, the non HS-adapted CJN-L1 strain was more sensitive to anti-LDLR antibody treatment. Most WT JEV strains were the non HS-adapted phenotype, thus, an LDLR virus receptor may play a key role in the replication of JEV in nature (Chiou and Chen, 2007; Klimstra et al., 1998). The role of LDLR involves in JEV entry need more comprehensive study, especially using non HS-adapted strain other than CJN-L1.

The present study demonstrated the anti-JEV activity of bLF. The structure, function, and mode of action of bovine lactoferrin or lactoferricin (pepsin-derived fragment) were similar to human lactoferrin (Vorland, 1999). LF is produced by neutrophils and mucous epithelial cells (Vorland, 1999). In the acute phase of JEV infection, the increase of neutrophils has been observed (Chaturvedi et al., 1979). A prospective study is required to measure the temporal kinetics of LF concentration in patient serum during the acute phase of JEV infection. Higher LF concentration in some patients' sera may account for a higher probability of inapparent infection in these patients. In addition, our study also implicates for the first time that cell surface-expressed LDLR may be a possible receptor candidate for JEV. LDLR is highly expressed in neuronal cells (Dietschy and Turley, 2001; Liou and Hsu, 1998). Involvement of LDLR in JEV entry might account for the neurovirulence of the virus.

Materials and methods

Viruses and cell lines

The history of JEV strains (Table 1) used in this study has been described in our previous report (Chiou and Chen, 2001; Chiou and Chen, 2007), including field-isolated, wild-type strains (T1P1, CC27

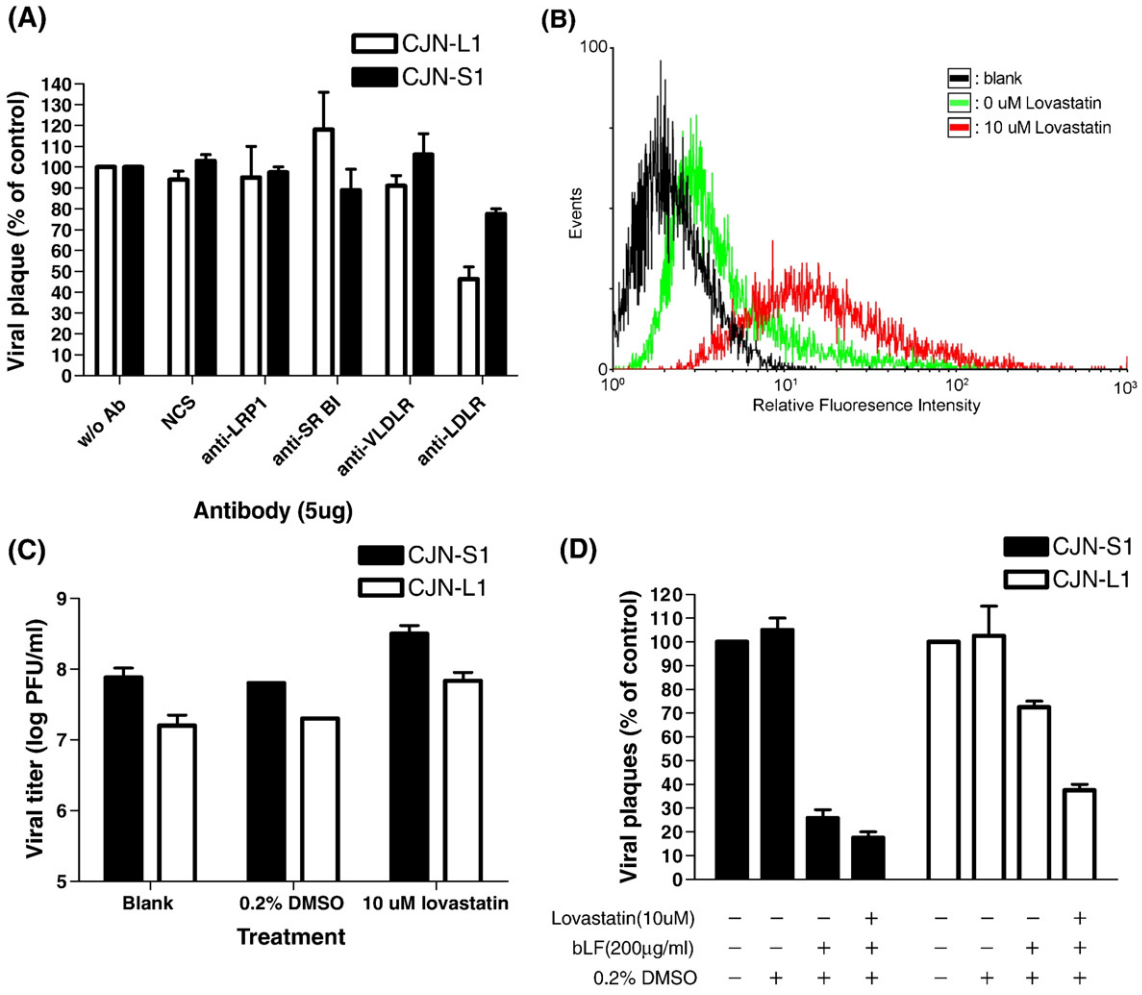

Fig. 8. Role of LDLR in JEV infection. BHK-21 cells were pretreated with 5 μg of anti-LDLR antibody, subsequently infected with 200 PFU JEV, and infectivity was measured by plaque reduction assay (A). BHK-21 cells were cultured with 0 or 10 μM lovastatin for 12 h, and the LDLR expression was measured by flow cytometry (B), cells were subsequently infected with JEV at an MOI of 5 for 48 h, and viral infectivity measured by plaque assay (C). Inhibitory effect of bLF on JEV entry with lovastatin-treated or -nontreated BHK-21 cells (D).

and CJN), and laboratory-adapted strains derived from them (T1P1-L4, T1P1-S1, CJN-L1, CJN-S1, CC27-L1, CC27-L3, CC27-S6 and CC27-S8). The viral stocks were harvested from the supernatant of infected *Aedes albopictus* C6/36 cells, and stored at −70 °C until use.

Cell lines of BHK-21 and C6/36 cells used in this study were cultured using the protocols described in our previous report (Chiou and Chen, 2001). Two additional cell lines, CHO-K1 (derived from Chinese hamster ovary) and its GAG-deficient mutant, pgsA-745, were grown in Ham's F12K medium containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum (FBS) in a 37 °C incubator with 5% CO_2.

Reagents

bLF (iron unsaturated lactoferrin and purity >90%), human serum albumin and heparin were purchased from Sigma (St. Lious, Mo, USA); heparin-CL-6B (H-CL-6B) sepharose beads, anti-VLDLR antibody, anti-LRP1 antibody, and human rLDLR were obtained from R&D SYSTEMS (Minneapolis, MN, USA); anti- SR BI antibody from Novus Biologicals (Littleton, CO, USA); receptor-associated protein from Calbiochem (San Diego, CA, USA); chicken anti-LDLR antibody from Chemicon (Temecula, CA, USA); and anti-JEV mouse hyperimmune serum was a gift from Dr. GJ. Chang at the Centers for Disease Control (Ft Collins, CO, USA).

Plaque assay

The procedure for the plaque assay was described previously (Chiou and Chen, 2001). Briefly, BHK-21 cells were seeded in six-well plates and incubated at 37 °C for 2 days. The virus samples were serially diluted in 10-fold dilutions with fresh culture medium containing 5% FBS. Two hundred μl of diluted viral suspensions were added to each well, and incubated for 1.5 h at 37 °C with gentle rocking every 15–20 min. Each well was overlaid with 4 ml of 1.1% methyl cellulose (Sigma) in culture medium containing 2% FBS. After incubation at 37 °C for 5 days, cells were fixed with 10% formaldehyde for 30 min and then stained with 1% crystal violet in 20% ethyl alcohol for 15 min. The virus titer was calculated and expressed as PFU/ml.

Assays for bLF inhibition of JEV infection

A plaque reduction assay was performed to determine the antiviral activity of bLF for JEV. BHK-21 cells were pre-incubated with 0, 0.1, 25, 50, 100, or 200 μg/ml (equals to 0, 0.001, 0.29, 0.57, 1.15, and 2.30 mM) bLF at 37 °C for 1 h, and the same concentration of bLF was maintained in the overlay media throughout the infection. The bLF-treated BHK-21cells were infected with 200 PFU of JEV, and the virus titer was determined by plaque assay.

An absorption assay was used to determine whether the bLF blocked JEV binding to cells, including BHK-21, CHO-K1(HS-expressed), and CHO-pgsA745 (HS-deficient). These cells were pre-incubated with bLF at 4 °C for 1 h, and then incubated with JEV at an MOI of 5 at 4 °C for 1 h with gentle rocking every 15–20 min. To remove unbound viruses, cells were washed 3 times with cold PBS. The adsorbed viruses on the cell membrane were subsequently released through 3 rounds of freeze (−80 °C)-thaw (37 °C) (Germi et al., 2002). The resultant virus suspensions were then centrifuged; the supernatants containing released viruses were titrated by plaque assay.

An infectious center assay was performed to determine whether the bLF prevented JEV infection after virus binding to cell membranes. In brief, cells were incubated with virus at 4 °C for 1 h at an MOI of 5. Subsequently, the infected cells were washed five times with cold PBS to remove unbound virus particles and the cells were suspended in PBS. These cell suspensions were incubated with 0–200 μg/ml bLF at 37 °C for 1 h. After washing, 200 μl of virus-infected, bLF-treated cells were diluted in 10-fold serial dilutions with fresh culture medium and co-cultured on BHK-21 cell monolayers for the infectious center assay. The number of infectious centers per 100 cells was calculated by the following formula: (number of infectious centers/number of plated cells in one well)×100.

In vitro heparin-bead binding assay

The binding of JEV to heparin was tested by an *in vitro* heparin-bead binding assay as previously described (Byrnes and Griffin, 1998) with some modifications. Heparin-CL-6B (H-CL-6B) Sepharose beads were washed five times with PBS, and incubated with 0–200 μg/ml of bLF at 37 °C for 30 min. After washing 3 times with PBS, a 70-μl aliquot of beads was mixed with viral supernatant in PBS and incubated on a rotator at 4 °C for 1 h. The bead-bound virus was collected by centrifugation at 1500 rpm for 10 min. The pelleted beads were then washed three times with cold PBS. Bead-bound virus was eluted with 250 μl of 400 mM NaCl (prepared in PBS) at 4 °C for 10 min. The input virus control, unbound virus, and bead-bound virus were titrated separately by plaque assay. The titers for the bead-bound virus were estimated using an aliquot diluted 100-fold in order to reduce the NaCl concentration in the sample.

HS and LDLR blocking assays

Two hundred μg bLF was pre-incubated with 0–250 U/ml heparin (equals to 0–14.28 μM) or 0–500 ng/ml rLDLR (equals to 0–1.67 nM) in double-distilled water at 37 °C for 30 min. The mixture was added to BHK-21 cells which were subsequently infected with 200 PFU JEV at 37 °C for 1 h. The virus titer was determined by plaque assay.

Flow cytometry

Detection of JEV using flow cytometry followed our previous description (Liu et al., 2004) with some modifications. In brief, the cells, including BHK-21, CHO-K1, and CHO-pgsA745, were harvested from 75-cm^2 flasks, using 5 mM EDTA (in PBS). Dissociated cells (1×10^6 cells) were incubated with bLF (0–200 μg/ml) at 4 °C for 30 min and washed 3 times with ice-cold PBS. The bLF-treated cells were incubated with JEV at an MOI of 500 for 1 h at 4 °C, and washed 3 times with ice-cold PBS. The cell-bound JEV was stained by mouse anti-JEV hyperimmune ascitic fluid at 4 °C for 1 h. Cells were then washed with PBS three times, and fixed with 0.5% formalin in PBS. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody was added to the fixed cells, incubated at 4 °C for 1 h, and washed with PBS three times. Detection and analysis of membrane-bound JEV was carried out using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

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