Inhibitory effects of native and recombinant full-length camel lactoferrin and its N and C lobes on hepatitis C virus infection of Huh7.5 cells

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INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive-sense ssRNA virus belonging to the family Flaviviridae. Its genome consists of ~10,000 nt and encodes a single polyprotein of ~3000 aa. HCV infection is one of the leading causes of chronic liver disease worldwide, and the World Health Organization has estimated that over 170 million people globally are infected with HCV, an incidence of 3.3 % of the world’s population, with 3 million new cases each year (Alter, 2007; Sy & Jamal, 2006; Te & Jensen, 2010); in some countries, such as Egypt, the incidence is as high as 10–15 % (Saleh et al., 2008).

The most common treatments for HCV infection are α-interferon and ribavirin (Fried et al., 2002; Jaeckel et al., 2001; Manns et al., 2001). Currently, no vaccine is available to prevent HCV infection, and there is no question that HCV will increasingly cause significant public health problems, with escalating numbers of infected patients. Therefore, the development of effective preventative approaches and/or optimized therapies is critical to control the current public health burden imposed by HCV infection.

Lactoferrin (Lf) is an 80 kDa multifunctional glycoprotein belonging to the transferrin family. Lf is present primarily in milk but is also found in other biological fluids, such as saliva, tears, bile and pancreatic juice (Lönnherdal, 2009; Legrand et al., 2008). Lf has been shown to have antiviral activities against HCV (Abe et al., 2007; Azzam et al., 2007; Redwan & Tabll, 2007; Ueno et al., 2006). Yi et al. (1997)
demonstrated that human milk Lf and recombinant human Lf expressed in *Escherichia coli* could bind HCV envelope proteins in *vitro* and *in vivo*; furthermore, a 33 aa residue at the C terminus of human Lf was found to be critical for binding to the HCV E2 envelope protein (Nozaki *et al.*, 2003). The species of Lf and the cell types used in individual studies vary considerably from human, bovine and camel origins. Most published studies have focused on the protective effects of bovine and human Lf on human hepatocytes and in human clinical studies. For example, bovine Lf was shown to inhibit HCV infection in PH5CH8 cells (Ikeda *et al.*, 1998, 2000) and virusemia in chronic hepatitis C (CHC) patients (Iwasa *et al.*, 2002; Tanaka *et al.*, 1999), whilst oral supplementation of chronic HCV patients with bovine Lf enhanced the sustained virological response to interferon (Kaito *et al.*, 2007). In contrast, clinical trials adding Lf to the standard interferon/ribavirin therapy resulted in divergent outcomes (Hirashima *et al.*, 2004; Ishibashi *et al.*, 2005; Ishii *et al.*, 2003).

Camel milk contains Lf at a concentration of 20–2100 μg ml⁻¹ (Al-Majali *et al.*, 2007). By X-ray diffraction analysis, camel Lf (cLf) has a bilobal conformation like many other Lfs, with each lobe folded into two functional domains; its N lobe is similar to that of human Lf, whereas the cLf C lobe is more similar to that of apo-ovotransferrins (Khan *et al.*, 2001). cLf protein shares 74 % sequence identity with human Lf and 75 % identity with bovine Lf. Redwan & Tabll (2007) demonstrated that native camel Lf (ncLf) has been shown to have profound inhibitory effects on HCV infection of human peripheral blood mononuclear cells (Redwan & Tabll, 2007) and HepG2 cells (El-Fakharnary *et al.*, 2008). However, there is no information on the potential effects of the N- and C-lobe fragments of cLf on HCV infection. If either one of these lobes could be shown to be effective against HCV, it might be possible to produce a recombinant form for clinical/pharmacological applications, thus contributing to a reduction in medical costs.

The increased permissiveness of Huh7.5 cells allowed detection of HCV RNA and antigens early after RNA transfection (Blight *et al.*, 2002). The objective of this study was to examine the potential inhibitory effects of recombinant camel lactoferrin (rcLf) and ncLf, as well as that of their N- and C-terminal fragments on HCV entry and amplification in Huh7.5 cells.

**Cell culture and inocula.** Huh7.5 cells were produced from Huh7 cells and were a gift from Dr Charles M. Rice (The Rockefeller University, New York, USA). The higher frequency of cured cells in the Huh7.5 line supports both subgenomic and full-length HCV replication. The cells were maintained in Dulbecco’s minimal essential medium supplemented with 10 % (v/v) fetal bovine serum (FBS) and 1 × antibiotic/antimycotic solution (Sigma-Aldrich) at 37 °C with 5 % CO₂.

To examine the interactions of rcLf, ncLf and their fragments with Huh7.5 cells, cells were seeded in 24-well plates at 1.0 × 10⁵ cells per well. ncLf, rcLf, nN-lobe, nC-lobe and the N-lobe from rcLf (rN-lobe) were added to the cells at a final concentration of 1.0 mg ml⁻¹. The cells were then incubated at 37 °C for 1 h. Unbound proteins were removed by washing three times with PBS. After a thorough wash, 1 ml HCV-infected serum (8.3 × 10⁶ copies ml⁻¹, genotype 4a) was added (Ohno *et al.*, 1997) and the cells were incubated at 37 °C for 90 min. The cells were then washed three times with PBS and cultured for 7 days at 37 °C with 5 % CO₂ and 88 % humidity.

To examine the interactions of rcLf, ncLf and their fragments with HCV, 1 ml HCV-infected serum was pre-incubated with the purified proteins at 4 °C for 1 h. The mixtures were then added to Huh7.5 cells cultured in 24-well plates at 1.0 × 10⁵ cells per well and incubated at 37 °C for 90 min. The cells were then washed three times with PBS and further incubated for 7 days at 37 °C with 5 % CO₂ and 88 % humidity.

To examine the effects of rcLf, ncLf and their fragments on HCV replication in infected Huh7.5 cells, the cells were seeded onto two sets of 24- and/or 96-well plates at 1.0 × 10⁵ cells per well for 24 h prior to inoculation with HCV-infected serum. The cells were then incubated at 37 °C for 48 h. ncLf, rcLf, nN-lobe, nC-lobe and nC-lobe were each then added at concentrations of 0.25, 0.5, 0.75, 1.0 and 1.25 mg ml⁻¹ and incubated with the cells at 37 °C for 96 h. The first set of cells was washed and harvested, and the other sets of cells were incubated with a second dose of ncLf, rcLf, nN-lobe, nC-lobe or nC-lobe at concentrations of 0.25, 0.5, 0.75, 1.0 and 1.25 mg ml⁻¹ and incubated at 37 °C for another 96 h.

**Nested RT-PCR.** Total RNA was isolated from Huh7.5 cells according to the method of Chomczynski & Sacchi (2006). Nested RT-PCR was performed according to the method of Nozaki *et al.* (2000). Total Huh7.5 RNA (400 ng) was used for cDNA synthesis using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech) and primers 1CH forward primer (for the plus strand; 5'-GGTGCACTGCTACGAAGACCTC-3'), 2CH forward for the minus strand (5'-AAATCTAGTCTTCGTACGAGAAA-3') and P2 reverse primer (5'-TGCTCATGCGTCGCTGCTA-3') (el-Awady *et al.*, 2006; El-Fakharnary *et al.*, 2010). The reaction was performed at 42 °C for 60 min, followed by 98 °C for 10 min. Amplification of the highly conserved 5' untranslated region (UTR) sequences was then performed using two rounds of PCR with two pairs of primers (Clontech). First-round amplification was performed with the 2CH forward primer and the P2 reverse primer using Taq DNA polymerase (Promega). The thermal cycling protocol was: 1 min at 94 °C, and 30 cycles of 1 min at 55 °C and 1 min at 72 °C. The second-round amplification was performed similarly to the first round, except for use of the nested reverse primer D2 (5'-ACTCGGCTAGCAGTCTCGCG-3') and forward primer F2 (5'-GTGCACTGCAGAGGACC-3'). Specific control assays were: (i) cDNA synthesis without RNA template to exclude product contamination; (ii) cDNA synthesis without reverse transcriptase to exclude non-specific Taq polymerase and RTase activities; and (iii) cDNA synthesis and PCR step with only the reverse or forward primer to confirm no contamination from mixed primers. The plasmid vector pRL-TK (Promega) encoding *Renilla* luciferase (RluC) was used for *in vitro* synthesis of RluC RNA as an internal control; 10 pg in *vitro*-synthesized *RluC* RNA was added to the RNA sample before reverse transcription (Nozaki *et al.*, 2000).

**METHODS**

**Full-length Lf and N/C-terminal protein fragment production.** Camel milk was purchased from AL-Kar Camels Farm (Cairo, Egypt), and Lf isolation was performed according to the procedure of Redwan & Tabll (2007).

The N and C lobes of ncLf (nN-lobe and nC-lobe, respectively) were purified by digestion with proteinase K according to the procedure of Sharma *et al.* (1999) with some modification. Details of the construction of the plasmid vector and recombinant protein synthesis in *Spodoptera frugiperda* 9 (Sf9) cells are given in the supplementary methods, available with the online version of this paper.

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Amplified DNA (174 bp for HCV and 276 bp for Rluc) was separated by electrophoresis on a 3% agarose gel and ethidium bromide was used for visualization. Bands were detected under a UV transilluminator and photographed by gel documentation (AlphaInnotech).

**Immunoblotting.** Purified rLf was dissolved in Tris buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 0.5% sodium deoxycholate] containing 1× Complete EDTA-free Protease Inhibitor (Roche Applied Science), and 1 μg protein was separated by electrophoresis through a 12% Novex Tris/glycine gel, transferred onto nitrocellulose membrane at 350 mA for 60 min and blocked overnight in PBS/0.1% Tween 20 with 5% non-fat milk at 4°C. Bands were detected using Super Signal Femto chemiluminescent reagent (Pierce).

**Statistical analysis.** Data were analysed using GraphPad Prism software. The binding affinities of nLf and the recombinant counterparts were tested using a two-tailed Student’s t-test. The binding affinities of full-length protein and the N and C lobes of rLf and nLf were tested by one-way analysis of variance, and differences were identified by Tukey’s test. Data are shown as means ± SEM of three independent experiments, and the gels shown are representative data of replicates. Differences were considered significant when \( P < 0.05. \)

![Fig. 1](http://jnm.sgmjournals.org) Purification of nLf and expression of rLf. (a) Camel milk Lf was purified by heparin-Sepharose, separated by SDS-PAGE (12% acrylamide) and stained with Coomassie Brilliant Blue. Lane 1, molecular mass markers; lane 2, skimmed camel milk; lane 3, flowthrough from the heparin-Sepharose column; lane 4, purified Lf. (b) Proteinase K digestion at 26°C of nLf for nN-lobe and nC-lobe purification. Lanes 1 and 8, purified nLf (5 and 10 μg, respectively); lanes 2–7, enzyme : substrate molar ratios of 1 : 100 (lanes 2 and 5); 1 : 50 (lanes 3 and 6) or 1 : 120 (lanes 4 and 7). Samples in lanes 2–7 were incubated for 30 or 60 min as indicated. (c) SDS-PAGE analysis of nN-lobe and nC-lobe after purification by Sephadex G-50 gel filtration. Lane 1, purified nN-lobe; lanes 2 and 3, purified nC-lobe. (d) Purified full-length rLf (1 μg) was analysed by SDS-PAGE (8% acrylamide). Lane 1, negative control; lane 2, purified protein. (e) Purified rN-lobe (10 μg) was analysed by SDS-PAGE (12% acrylamide) and stained with Coomassie Brilliant Blue. Lanes M, molecular mass markers; lane 1, rN-lobe before dialysis; lane 2, rN-lobe after dialysis.
Fig. 2. Prevention of HCV entry into Huh7.5 cells by rcLf and ncLf and their fragments. (a) HCV particles were pre-incubated with purified proteins prior to infection. Intracellular HCV RNA was not amplifiable in the cells inoculated with either recombinant or native proteins. (b) Huh7.5 cells were treated with purified proteins prior to infection with HCV, and intracellular RNA was detected in the cells inoculated with all protein/HCV mixtures. Lane 1, DNA ladder; lane 2, negative control; lane 3, positive control; lanes 4 and 5, full-length cLf; lanes 6 and 7, N-lobe; lanes 8 and 9, C-lobe. Nested RT-PCR was performed to amplify the viral RNA segments. The upper diagrams in (a) and (b) show a schematic of the experimental procedures. Rluc served as an internal control, Huh7.5 cells as a negative control and Huh7.5 cells infected with HCV as a positive control.
RESULTS

Purification of ncLf

Skimmed camel milk before incubation with heparin-Sepharose beads, the flowthrough and the purified ncLf were subjected to SDS-PAGE (12% acrylamide). Only one band was visualized from the protein eluted at 400–600 mM NaCl (Fig. 1a). From the purified protein, Edman N-terminal analysis retrieved a peptide sequence of 20ASKKSVRW27, corresponding to the sequence of the translated protein from GenBank accession no. AF165879. Protease K digestion of ncLf was performed to produce the N- and C-lobe proteins. As shown in Fig. 1(b), at an enzyme:substrate molar ratio of 1:50, digestion at 26°C for 30 min resulted in the most complete digestion, as indicated by a decrease in the ~80 kDa full-length protein band. These conditions were used for large-scale digestion of ncLf. Furthermore, by heparin-Sepharose purification, our results showed that the native N and C lobes eluted at 0.3 and 0.6 M NaCl, respectively. The isolated peaks were further purified by gel filtration on a Sephadex G-50 column. Homogeneity of the two purified fragments was observed by SDS-PAGE (Fig. 1c) and both corresponded to a molecular mass of ~40 kDa. The protein identities of the purified nN-lobe and nC-lobe were confirmed by N-terminal sequencing at Zaragoza University Proteomics Laboratory in Spain, with N-terminal peptide sequences of 20ASKKSVRW27 and 340LRRAQVVW347, respectively.

Isolation of rcLf

rcLf was synthesized using a baculovirus system. The immunoblotting results in Fig. 1(d) (lane 2) showed a single band at ~80 kDa. In addition, the N-terminal peptide sequence determined from this protein, 20ASKKSVRW27, by

Fig. 3. Inhibition of HCV replication in Huh7.5 cells by full-length cLf. HCV-infected Huh7.5 cells were treated with rcLf or ncLf for 96 h (a). The 0.25 and 0.5 mg ml⁻¹ Lf treatment groups (indicated in italic) were treated with a second dose for another 96 h (b). Nested RT-PCR was performed to amplify the viral RNA segments. Lane 1, DNA ladder; lane 2, negative control; lane 3, positive control; lanes 4–8, Lf at different concentrations, as indicated. The upper diagrams in (a) and (b) show a schematic of the experimental procedures. Rluc served as an internal control, Huh7.5 cells as a negative control and Huh7.5 cells infected with HCV as a positive control.
Edman sequencing matched the mature sequence of cLf translated from GenBank accession no. AF165879. Fig. 1(e) shows expression of the rN-lobe, and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed to confirm the protein identity. The sequence showed 100% identity with cLf translated from GenBank accession no. AF165879. Both bands in Fig. 1(e) (lanes 1 and 2) were the protein of interest. The band with the larger size may have been the result of glycosylation.

rC-lobe was expressed in SF9 cells (immunoblotting data not shown) but was insoluble in regular lysis buffer and in high-salt buffer containing 1% NP-40.

**rcLf and ncLf effectively prevent the entry of HCV into Huh7.5 cells**

Fig. 2(a) shows that when HCV-infected serum (8.3 × 10^6 copies ml^-1, genotype 4a) was pre-treated with cLf [recombinant or native, full-length or fragments, low (0.5 mg ml^-1) or high (1.0 mg ml^-1) concentration] prior to inoculation of Huh7.5 cells, HCV infection was completely blocked, as shown by the fact that the 174 bp intracellular HCV 5' UTR sequence was not amplified.

Fig. 2(b) shows that when Huh7.5 cells pre-treated with cLf or protein fragments (at a concentration as high as 1.0 mg ml^-1) were inoculated with HCV-infected serum (8.3 × 10^6 copies ml^-1, genotype 4a), they had no protective effect on HCV infection, as manifested by strong amplification of the 174 bp intracellular HCV 5' UTR sequence. The negative control was uninfected Huh7.5 cells and the positive control was Huh7.5 cells infected with HCV without Lf treatment of the cells or HCV particles.

**rcLf and ncLf effectively inhibit HCV replication in infected Huh7.5 cells**

In order to determine the inhibitory effects of cLf on HCV amplification in infected Huh7.5 cells, a two-step Lf treatment was performed.

The inhibitory effect of full-length Lf on HCV amplification is shown in Fig. 3(a). HCV RNA was not detected in cells treated with a single dose of full-length rcLf or ncLf at concentrations of 0.75, 1.0 and 1.25 mg ml^-1, but a first inoculation...
the 0.25, 0.5 and 0.75 mg ml\(^{-1}\) dose of nC-lobe did not completely inhibit viral amplification. However, in response to a second dose of treatment at 0.25 or 0.5 mg Lf ml\(^{-1}\), HCV amplification was markedly inhibited, as indicated by no amplification of HCV RNA sequences (Fig. 3b).

As shown in Fig. 4, the N lobe of both rcLf and ncLf was sufficient to completely inhibit HCV amplification in Huh7.5 cells after the first dose at all concentrations studied (0.25, 0.5, 0.75, 1.0 and 1.25 mg ml\(^{-1}\)).

Fig. 5 shows the inhibitory effect of nC-lobe on HCV amplification in Huh7.5 cells. At a concentration of 0.25 or 0.5 mg ml\(^{-1}\), nC-lobe did not block virus amplification after two doses, but treatment with 0.75 mg ml\(^{-1}\) showed complete inhibition after two consecutive doses. In comparison, concentrations of 1.0 and 1.25 mg nC-lobe ml\(^{-1}\) showed complete inhibition with a single-dose treatment. The negative control was Huh7.5 cells without infection and the positive control was Huh7.5 cells infected with HCV without Lf treatment of the cells.

**DISCUSSION**

cLf is one of the major camel milk whey proteins, and its abundance in camel milk (2.1 g l\(^{-1}\) in colostrum and 0.22 g l\(^{-1}\) in mature milk) is higher than in bovine milk (5 g l\(^{-1}\) in colostrum and 0.14 g l\(^{-1}\) in mature milk) (El-Hattami et al., 2006). In this study, we demonstrated that the N- and C-lobe fragments of nCLf, and the full-length and N-lobe fragment of rcLf, displayed effective inhibition of HCV entry and replication in infected Huh7.5 cells, as has been shown previously for the native full-length protein (Redwan & Tabll, 2007).

Milk Lf has been suggested to have strong inhibitory activity against pathogens, including bacteria, fungi and viruses. In a recent study, Lfs isolated from different species (sheep, goat, camel, alpaca, elephant, human and bovine) were investigated and all were found to be effective in inhibition of E. coli O157:H7, with clf being the most active (Conesa et al., 2008). The antiviral activities spanned a broad spectrum of viruses, including cytomegalovirus, polyomavirus, herpes simplex virus type 1, human immunodeficiency virus, hepatitis B virus, HCV, simian rotaviruses and adenovirus (Legrand et al., 2008).

Mechanistically, the immunomodulating, iron-chelating and antioxidant properties of Lf come into play in combating HCV infection. As has been shown previously, iron overload with elevated serum transferrin and hepatic iron is commonly found in CHC patients, which warrants iron chelation therapies (Bonkovsky, 2002). Plasma 8-isoprostane levels are significantly decreased in CHC patients treated with bovine Lf, indicating suppressed oxidative stress and better responsiveness to antiviral therapy (Konishi et al., 2006). Ishii et al. (2003) reported that oral bovine Lf supplementation of CHC patients for up to 3 months stimulated serum interleukin-18 expression, and produced a T-helper 1 cytokine-dominant environment in the circulation, thus facilitating the effects of interferon treatment.

In the present study, soluble cLf, either full-length or its fragments, displayed direct neutralization effects on the HCV virus particle, rather than affecting interaction with the cells (which was shown to be the inhibitory mechanism of Lf on herpes simplex virus type 1 and cytomegalovirus), to prevent HCV access to the cells, suggesting that both the N and C lobe possess functional domains sufficient to recognize E1 or E2 proteins in the HCV envelope. The inhibition of virus replication was more effective for the N lobe than for the full-length protein and the C lobe, as the full-length protein achieved complete abolition of virus replication only after two consecutive doses of 0.25 or 0.5 mg ml\(^{-1}\). The N lobe had the same effect with a single dose, but the C-lobe protein could not achieve a similar effect at these concentrations, even with two doses. At higher concentrations of 0.75, 1.0 and 1.25 mg ml\(^{-1}\), however, a single dose of all three proteins was able to inhibit the amplification of HCV RNA in cells. Increased expression of these molecules may render cLf more effective in the treatment of HCV infection.

The novelty of this study was the use of expressed rcLf and its fragments. The recombinant proteins were synthesized.
in insect cells. A high yield (~2.0 mg l⁻¹) was demonstrated for both the full-length protein and the N-lobe protein. The C-lobe protein fragment was expressed in culture (immunoblotting data not shown), but its solubility was low. Codon optimization prior to gene cloning and high-salt buffer (600 mM NaCl) containing 1% NP-40 had little effect on the solubility. Although the C-lobe protein was thus incompatible with the insect-cell system, successful expression of the full-length and N-lobe cLf proteins and their anti-HCV effects similar to those of their native counterparts may represent a cost-effective way to increase the clinical potential of cLf, especially in regions with easy access to raw camel milk such as Egypt. It should be noted that application of cLf and its protein fragments should be investigated carefully in vivo, as a sustained virological response is more likely to be achieved by combined therapy with Lf and antiviral agents (Kaito et al., 2007), and more studies should be conducted to compare the efficacy of Lf from different species.

In summary, this study was a first attempt to examine the HCV inhibitory effects of the protein fragments and recombinant form of cLf. The results obtained support the efficacy of rclF and its fragments in antiviral activity, and the recombinant proteins constitute a promising approach for HCV therapy.

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REFERENCES


