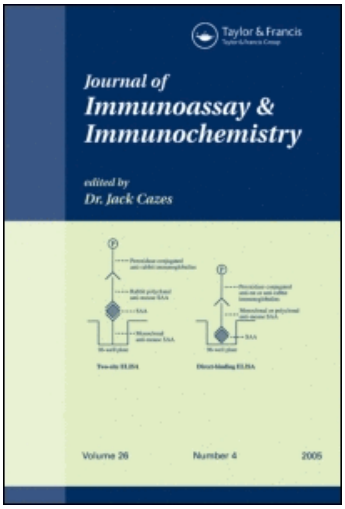

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Abstract: Hepatitis C virus (HCV) is a serious worldwide health risk and, to date, no effective treatments to prevent progression to chronic infection have been discovered. To combat the disease, Egyptian patients often use traditional medicines, for instance, camel milk, which contains lactoferrin. Currently, lactoferrin is one of the primary biopharmaceutical drug candidates against HCV infection. Camel lactoferrin (cLf) purification and biochemical and immunological characterization have shown its similarity to human and bovine lactoferrin, and crossreacts with the anti-human lactoferrin antibody. Incubation of human leukocytes with cLf then infected with HCV did not prevent the HCV entry into the cells, while the direct interaction between the HCV and cLf leads to a complete virus entry inhibition after seven days incubation. Our results suggest that the cLf may be one of the camel milk components having antiviral activity. In conclusion, we have demonstrated the potential for cLf to inhibit HCV entry into human leukocytes with more efficiency than human or bovine lactoferrin.

Keywords: Camel milk, Lactoferrin, Purification, Hepatitis C virus, Inhibition

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INTRODUCTION

HCV is a small, enveloped, positive-stranded RNA virus belonging to the Flaviviridae family. The HCV genome (approximately 9.6 kb) contains a single long open reading frame encoding a polyprotein precursor of approximately 3,100 amino acids, co- and post-translationally cleaved by both host and viral proteases to yield 10 structural and nonstructural proteins. The structural proteins, which lie at the N-terminus of the polyprotein, include the core protein followed by the envelope proteins, E1 and E2, and a small protein, p7, whose function is unknown.

The nonstructural region encodes six proteins: NS2, NS3, NS4A, NS4B, NS5A, and NS5B, which function in polyprotein proteolysis, polymerase activities, and formation of a membrane-associated replicase complex. HCV is a rapidly replicating virus, with approximately 10^{10}–10^{12} new virions produced daily. This high replication rate, in combination with the absence of any HCV polymerase proofreading ability, allows for the emergence of viral quasispecies, which provide a mechanism for escaping host immune responses.[1]

With over 170 million carriers, hepatitis C virus (HCV) infection has reached epidemic proportions. HCV infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) worldwide. Therapeutic options are improving, but are still limited; moreover, a protective vaccine is not available. Between 50–80% of HCV infections are acute and 4–20% of patients with chronic hepatitis C will develop liver cirrhosis within 20 years. In patients with liver cirrhosis, the risk to develop HCC is 1–5% per year. Current standard therapy is the combination of pegylated interferon-α (PEG-IFN-α) and ribavirin. Depending on the HCV genotype and other factors, this strategy results in a sustained virologic response in 50–80% of patients. However, many patients do not qualify for, or do not tolerate, standard therapy. Therefore, more effective and better tolerated therapeutic strategies are urgently needed.[1]

Egypt could be considered one of the world's highest HCV prevalence rates. In Egypt, subtype 4a is predominant, but new putative subtypes have been described.[2] In addition to the current therapies (IFNα2a, α2b, pegylayted, ribavarin), there are several traditional medicines used by different Egyptian patients sectors. The most popular treatment is the use of camel milk. In a clinical study of 18 HCV-positive patients who were given natural camel milk, 88% showed improvements in alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST). In addition, 50% of the patients showed marked improvement in fatigue (personal communication). In this study we attempted to determine which of the many camel milk constituents conferred this remarkable ability to down-regulate the ALT/AST levels.

It is well known that milk, in addition to secretory IgA and IgM, also contains numerous non-antibody components with known antiviral activity, including lactoferrin. Bovine lactoferrin (bLf) is a glycoprotein consisting of a single polypeptide chain of 689 amino acidic residues, with a molecular

mass of about 80 kDa, which binds two iron atoms with very high affinity.[3,4] Bovine lactoferrin, like lactoferrin of other mammalian species, is folded in two symmetric globular lobes. Each one is itself folded into two domains (N-lobe: N1 and N2; C-lobe: C1 and C2) each containing a Fe^{3+} binding site.[3]

Moreover, bovine lactoferrin (bLf) has an alkaline isoelectric point (pI $\approx$ 9) and its cationic nature could have a major role in the ability to bind cells and many anionic molecules, such as glycosaminoglycans. This glycoprotein plays an important role against parasitic, mycotic, bacterial and viral infections such that it has been considered a protein of the mucosal defense of the innate immune system.[3,5–7] Since 1994, human Lf (hLf) and bLf have been recognized as effective inhibitors of several enveloped viruses. bLF was also found to have an inhibitory effect against HCV in cell culture. Further, several clinical studies have demonstrated that monotherapy with bLf improves the serum HCV RNA and/or ALT levels in chronic hepatitis C (CHC) patients.[8–11] Because of the prevalence of using camel's milk to treat HCV infection, we investigated the role of camel lactoferrin (cLf) in inhibiting HCV entry. The results of this study are reported here.

EXPERIMENTAL

Camel Milk Processing and Lactoferrin Purification

Two liters of Arabian camel milk (ALKHIR farm, Giza, Egypt), was produced and collected from 200 camels and transferred to our laboratory in 200 mL frozen aliquots. To prevent microbial growth and reduce protease activity 0.2% sodium azide, 5 mM EDTA, 5 mM PMSF were added to the milk before processing. The milk was skimmed and casein removed as previously described.[12]

Casein free, skimmed milk was used for lactoferrin purification and diluted with 50 mM Tris-HCl, pH 8.0 then added to pre-equilibrated CM-sephadex G50 for three hours. The CM-Sephadex gel was washed several times with 50 mM Tris-HCl buffer, then washed with the wash buffer plus 150 mM NaCl to facilitate removal of impurities. The lactoferrin was eluted with the wash buffer containing 250–350 mM NaCl. The fractions containing lactoferrin were determined by ELISA, then pooled and concentrated by amicon ultrafiltration cell (50 kDa MWCO). The lactoferrin purity was confirmed by SDS-PAGE[13] and ELISA using (mouse anti-human lactoferrin prepared in our laboratory at 1:1000 dilution) as previously described.[12]

Lymphocytes Isolation and anti-Envelope E1 HCV Antibody

Human blood was collected from a single healthy volunteer. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-paque density

gradients. Blood was diluted 1:1 with PBS, and layered onto Ficoll-paque with ratio of blood PBS:Ficoll maintained 4:3. The blood was centrifuged at 1,800 rpm for 35 min at 4°C. The leukocytes layer was removed and washed twice in PBS at 1,200 rpm for 10 min each, and again with fully supplemented cell growth medium RPMI-1640. Cell density and viability was counted using trypan-blue.

The interaction of camel lactoferrin with HCV particles was evaluated using human whole blood as previous described[14] or separated human PBMCs without any significant differences (data not shown). Rabbit polyclonal antibody against E1 was prepared, purified, and tested as described,[14] while goat anti-rabbit-FITC conjugate was from Sigma (St. Louis, MO, USA), at dilution 1:1,500.

Cytotoxic Effect of cLf

The cytotoxic effect of purified cLf on human separated (PBMCs) was examined by the counting of viable cells after trypan blue treatment. PBMCs cells (2.5×10^5) were plated in a 24-well microtiter plate in duplicate and cultured for two days at 37°C before cLf treatment, then the medium was refreshed with new RMPI-1640 supplemented medium containing 4.0 mg/mL of cLf. The cells and cLf were incubated for 90 min at 37°C and washed three times with 1 mL of PBS. The cells were maintained with 1 mL of fresh medium for seven days at 37°C. After one week in culture, the cells were collected and suspended in medium, and the total number of viable cells was counted after trypan blue treatment. We also examined the viability of cells which were cultured for one day with medium containing 2.0 mg/mL of cLf as previously described.[6]

Inhibition Potential of the cLf on HCV

To examine the interaction of cLf with the human (PBMCs), 2.5×10^5 cells were plated in a 24-well microtiter plate. cLf was added to the leukocytes (in 50 mL of RPMI-1640 supplemented medium) at a final concentration of 1.0 mg/mL and incubated for 60 min at 37°C. Free cLf was removed by washing three times with 1 mL of PBS. After addition of 50 ml of medium containing 1 mL of serum SA-55 (8.3 million copies/mL, RNA G4), the cells were incubated for 90 min at 37°C. The cells were washed three times with PBS and cultured for seven days at 37°C. To examine the interaction of cLf with HCV, 1 mL of serum SA-55 and cLf (final concentration of 1.0 mg/mL) was pre-incubated in 50 mL of medium for 1 h at 4°C, and then the mixture of HCV and cLf was added to leukocytes cultured as described above, and incubated for 90 min at 37°C. The cells were washed three times with 1 mL of PBS and further cultured for 7 days at 37°C. The cells were washed and stained as described above.

Detection of Intracellular HCV by Flow Cytometry

Half of the infected PBMCs were suspended in 100 uL of phosphate buffered saline (PBS)-0.1% Triton X-100-1 mM $CaCl_2$-1 mM $MgSO_4$-0.05% NaN_3-1% bovine serum albumin-10 mM HEPES (PBS-S), incubated at room temperature for 1 h with gentle rocking. Polyclonal anti-E1 $F(ab)_2$ antibody (1:2,000) was added to the cell suspension and incubated at room temperature for 1 h. Then, the cells were immunofluorescence stained with fluorescein-conjugated goat anti-rabbit and incubated at room temperature for 30 min. After being washed, the cells were fixed with 4% paraformaldehyde and analyzed by flow cytometry (Becton Dickinson, CA, USA).

RT-Nested PCR

Total RNA was extracted using RNeasy kit and protocol (Qiagen, GmbH, German). To detect positive-stranded HCV RNA, the antisense primer 5′-tgctcatggtgcacggtcta-3′, detecting noncoding region of HCV,[15] was used to prime cDNA synthesis using the Titan system's reverse transcriptase (Roche, Mannheim, Germany). Amplification by PCR with Taq DNA polymerase (Promega, MD, USA) was performed for 35 cycles using previous primer and sense primer, 5′-ccatg gcgttagtatgagtg-3′ and an internal primer pair 5′-agagccatagtggt ctgcgg-3′ and 5′-ctttcgcgacccaacactac-3′, was used for the second round of PCR (35 cycles). Each PCR cycle consisted of annealing at 55°C for 45 sec, primer extension at 72°C for 1 min, and denaturation at 94°C for 1 min RT-nested PCR products (144 bp) were separated on 3% agarose gel.

RESULTS

Camel Lactoferrin Purification and Characterization

Two liters of Arabian camel milk was defatted and decaseinated. The camel lactoferrin (cLf) was purified from skimmed milk by loaded to CM-Sephadex column. Three peaks were eluted, the first was a large peak, the second and third were much smaller (Fig. 1A). The first peak eluted at ionic strength of 0.31 M of NaCl. The electrophoretic analysis of the protein eluted in each peak revealed that the first peak presented a single protein band corresponding to lactoferrin (80 KDa) (Fig. 1B), while the proteins in next two peaks presented a very small peptides out of 12% SDS-PAGE range. The lactoferrin eluted in the first peak was confirmed by immunoassay using anti-human lactoferrin antibody (Fig. 1A).

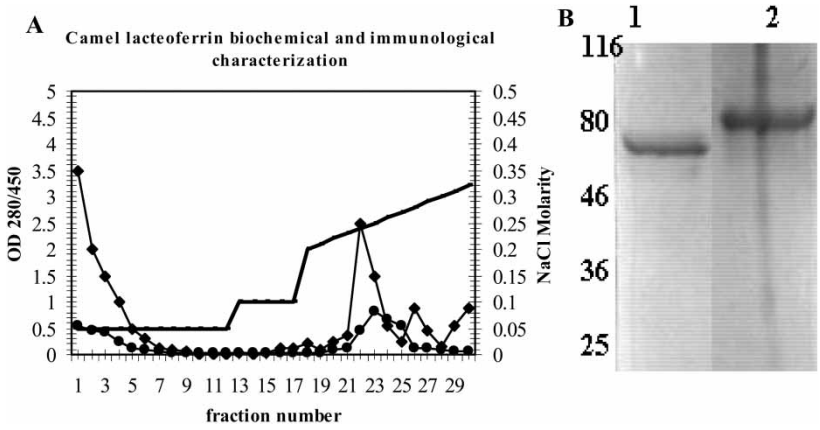

Figure 1. Biochemical and immunological characterization of cLf: (A) Casein-free skimmed milk (100 ml) was loaded into pre-equilibrated CM-Sephadex column (50 mM Tris.HCl, pH 8.0). The flow-through was saved for re-purification, then the column washed extensively with (50 mM Tris.HCl, pH 8.0, 0.15M NaCl). The lactoferrin was eluted with a gradient of 0.25–0.35M NaCl (—). The lactoferrin eluted at an ionic strength of 0.31M NaCl (▲). Each eluted fraction was also immunoassayed for cLf (•), and lactoferrin was identified in two peaks. (B) Coomassie blue-stained 12% SDS-PAGE of the purified camel lactoferrin. The protein was boiled in reduced (lane 1) or non-reduced (lane 2) sample buffer then fractioned. The values on the left side represent the molecular weight protein standard. cLf migrated to ~80KDa in reducing buffer without any co-purified milk protein, which is similar to human and bovine lactoferrin.

cLf Cytotoxic Effect

To avoid any possibility that the elimination of the HCV was caused by a reduced viability of PBMCs, we examined the cytotoxic effects of the cLf on PBMCs. The PBMCs (2.5×10^5) were treated with 4 mg/ml of cLf for 24 hours or 7 days. Cell viability was observed in comparison with the untreated PBMCs cultures. Neither 4 nor 2 mg/mL had significance effects on the viability of the PBMCs after 24 hour and 7 days of incubation periods, respectively (data not shown).

Inhibition Potential of the cLf

Does cLf exert its inhibition effects through direct interaction with the cells or the HCV molecules itself? Two sets of cells (2.5×10^5) were cultured, in duplicate, as described in experimental. One of the cultures was treated with cLf 1 mg/ml for 60 min, then infected with HCV for 90 min, then washed and propagated for seven days. The cultured cells were collected divided into

two parts, one for intracellular staining, the other for RT-nested PCR. By using the polyclonal antibody raised against anti-E1 peptide we found high fluorescence signal in FACS scan profile (Fig. 2B). In addition, the RT-nested PCR amplified the 144 bp of 5′ end of HCV noncoding sequence in comparison to the HCV positive serum (Fig. 3, lanes 3 and 4, respectively).

The other culture was inoculated with HCV infected sera pretreated with cLf (1 mg/mL) for 60 min. The inoculated cells were cultured for seven days, then were collected and process as above described. The fluorescence intensity of FACS scan profile has been significantly ($p > 0.005$) reduced (Fig. 2A) to base line (the reduction from 16% to $<1\%$) compared to the previously infected cells panel. The agarose gel (Fig. 3 lane 2) confirms the fluorescence signal reduction, and the band of 144 bp was not amplified. Our data indicated that the cLf inhibitory potential was better (non-significant) than human and bovine lactoferrin (data not shown).

DISCUSSION

In this study, we show that camel milk lactoferrin inhibits, *in vitro*, the HCV entry into human leukocytes through direct interaction with virus molecules rather than interaction with cells. This inhibitory activity seems to be similar to previous studies[6,7,16] using human and bovine lactoferrin to inhibit HCV (genotype 1) entry into the PH5CH8 cell-line. These studies confirmed HCV entry by RT-nested-PCR only, while our study used this method in addition to the indirect intracellular immunostaining of HCV E1 with the flow

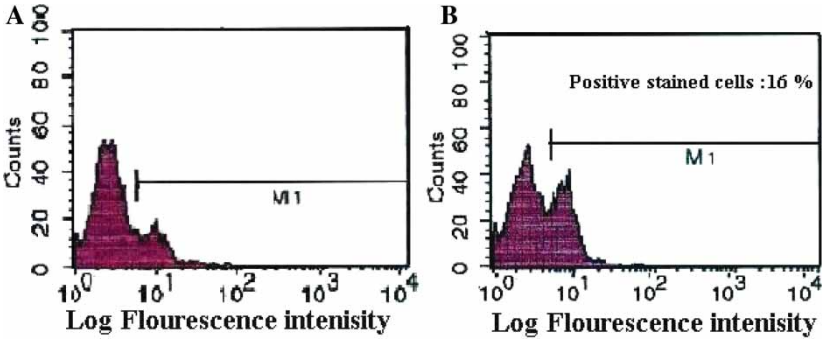

Figure 2. Flow cytomtery results. Clean PBMCs were exposed to infected HCV sera pre-treated (A) with cLf or without (B) treatment (as described in Materials and Methods). After co-incubation of the infected PBMCs for seven days, they were stained with an anti-E1 $F(ab)_2$, then with second layer of goat anti-rabbit-FITC labeled secondary antibody. The cLf blocking potential was analyzed with flow cytometry scan of the fluorescence intensity using Quest software (Becton Dickinson), and shows a significant reduction ($p > 0.005$) in the number of stained cells.

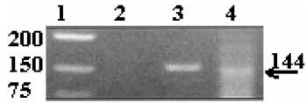

Figure 3. Camel lactoferrin activity against HCV. The cLf (1 mg/ml) was used to block the HCV virus entry through direct interaction between the HCV molecules and cLf (lane 2) or indirect through interaction of cLf and PBMCs (lane 3). RT-nested PCR was run on BPMCs culture for 7 days. Lanes 1 and 4 were DNA ladder and amplified 144 bp of HCV from used positive serum, respectively. All products were resolved in 3% agarose gel/ethidium bromide staining. The arrow indicates the amplified 144 bp band.

cytometry. This system is advantageous over RT-PCR alone because of its ability to directly count the rate of cellular infected through the ability to quantify the intensity of fluorescence per cell which facilitates a clear distinction between specific and non-specific immunofluorescence. Furthermore, it is more specific than traditional immunocytochemical analyses.[14] The potential receptors for HCV entry through its substantial interaction of E1, and E2 peptide, include the tetraspanin CD81 which have a partial amino acid homology with the C-lobe of cLf (Fig. 4), the scavenger receptor SR-BI, low density lipoprotein, and glycosaminoglycans.[17] Recent reports also indicate that the HCV entry likely involves transit through an endosome via a low pH-, time- and temperature-dependent fashion followed with fusion to the endosomal membrane.[1,18]

Generally, lactoferrin has two ways to exert its effects; i) Most studies indicate that lactoferrin prevents infection of the host cell, rather than inhibiting virus replication after the target cell has become infected. Infection of the target cell is prevented by direct binding to hepatitis C virus (HCV) particles through E1 and/or E2 as described,[5–7] polio, rotavirus, herpes simplex virus and possibly human immunodeficiency virus.[4] Preincubation of HCV with bovine lactoferrin is required to prevent the infection of HCV virus into cells, whereas incubation of bovine lactoferrin with the cells showed no inhibitory effect against HCV. This demonstrated that anti-HCV activity of bovine

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cLf     VVSRIDK-VAHLEQVLLRQQAHFGR--NGRDCP-G-KFC--LFQSKTKN---LLFNDN
H.CD81  VVKTFHETLDCCGSSTLT--ALTTSVLKNNLCPSGSNIISNLFKEDCHQKIDDLFSGK
        **. :.: :     .  *   *       :.. ** * ::   **:.. ::     **..:
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Figure 4. Amino acid alignment of the C-lobe of camel lactoferrin (597–644, according to Kappeler et al.[22] and large extracellular loop of human CD81 (146–201). Hyphens indicate no consensus, * indicate fully conserved residues, : indicate highly conserved residues, . indicate low residue conservation of according to W. Clustal.[23] The binding activity and specificity of CD81 to HCV E2 protein domain has been demonstrated, *in vitro*,[24] and *in vivo*.[25] This partial homology of cLf (C-lobe) may interfere with binding of the E2 domain to CD81 loop. The CD81 is not involved in the cell fusion caused by HCV.[16]

lactoferrin was due to the interaction of this protein with HCV and not with the cells.[6] ii) Coating the host cell molecules that the virus uses as a receptor or co-receptor. For example, binding of lactoferrin to heparin sulphate proteoglycans (HSPGs) is a central phenomenon. Many viruses tend to dock on HSPGs of target cells. After this initial contact, the virus particles roll to their specific viral receptor and subsequently enter the host cell, for instance by fusing with the host cell membrane. Binding of lactoferrin to HSPGs prevents this first contact and thus subsequent infection of the host cell.[4] iii) Other studies have shown that an indirect antiviral mode of action of lactoferrin is taking place through the upregulation of the antiviral response of the immune system. Administration of lactoferrin to cell cultures *in vitro*, or animals and healthy volunteers led to an upregulation of natural killer cells, monocyte/macrophages and granulocytes. These cell types play an important role during the early phases of viral infection; therefore, the specific immune system is unregulated and takes over the antiviral response.[19]

Camel lactoferrin has three unique characteristics which separate it from lactoferrin of other species: i) the predicated glycosylation sites are entirely different in cLf, ii) some critical residues such as Pro418, Leu423, Lys433, Gln651, Gly629, Lys637, Arg652, and Pro592 related to domain movement in the protein are different in cLf from those found in other lactoferrins, indicating the possibility of specific structural differences, iii) Most importantly, cLf loses 50% iron of its bound iron at pH 6.5 and the remaining 50% is released at pH 4.0–2.0. Its proteolyticly generated N- and C-lobes showed that the C-lobe lost iron at pH 6.5, while the N-lobe lost it only at pH less than 4.0, which indicating a striking difference in the iron release mechanism from the two lobes. These data demonstrate that the cLf behaves as half lactoferrin "iron binding protein" and half transferring "iron-transporter protein," unlike other lactoferrins and transferrins.[3]

Previous studies have suggested that patients with chronic HCV infections have elevated levels of serum markers of iron stores (ferritin, transferrin-iron saturation, or iron). The presence of elevated body iron stores and, in particular, elevated hepatic iron levels, is one of the strongest predictors of resistance to interferon treatment for HCV. Also hepatic iron overload increases the production of reactive oxygen species, which may lead to lipid peroxidation, steatosis and depletion of glutathione stores, accelerated liver damage and developing hepatocellular carcinoma.[20,21] A recent study showed that the therapy with bovine lactoferrin lead to lipid peroxidation inhibition.[11] In accordance, we propose that camel milk lactoferrin maintains a dual function; 1) it inhibits lipid peroxidation and 2) it regulates the hepatic iron content through its ability to bind and transport the iron at various pHs.

In conclusion, we demonstrated, for the first time, that the purified camel milk lactoferrin of ~80KDa has a significant inhibitory effect on HCV (genotype 4) entry. This inhibition takes place by direct interaction with viral molecules and not with the host cells. This finding may be attributed to the dual function of cLf as both an iron binding protein and an iron

transporter protein. Further studies should investigate this suggested dual function by directly comparing cLf with the human and bovine lactoferrin.

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