Potential antimicrobial effects of human lactoferrin against oral infection with *Listeria monocytogenes* in mice

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*Listeria monocytogenes* is a food-borne pathogen that causes serious listeriosis in humans. Antimicrobial effects of human lactoferrin (hLF) against *L. monocytogenes* have been clearly demonstrated in *in vitro* studies. However, *in vivo* studies have not been reported yet. This study investigated whether the oral administration of hLF could inhibit oral infection of listeria in BALB/c mice. The MICs for several strains of *L. monocytogenes* were determined, and the most sensitive strain was used for the animal work. hLF was administered to BALB/c mice for 7 days, commencing 4 days before oral infection. The effect of hLF was determined by bacterial enumeration and histopathological analysis of the liver and spleen, which are well-known as the major targets of oral listeria infection in mice. In bacterial enumeration, hLF decreased the number of *L. monocytogenes* cells in the liver. Histopathologically, the size and frequency of necrotic foci in the liver samples decreased with hLF administration. However, these changes were not observed in the spleen samples. The mRNA levels of inflammatory cytokines, such as interleukin (IL)-1β, tumour necrosis factor (TNF)-α and interferon (IFN)-γ, decreased in the liver of mice receiving hLF. This study has shown that hLF decreases the hepatic colonization of *L. monocytogenes*, hepatic necrosis and expression of inflammatory cytokines. It revealed that perorally given hLF could mediate antimicrobial and anti-inflammatory activities remote from the gut (i.e. in the liver) of mice challenged with *L. monocytogenes*.

**INTRODUCTION**

*Listeria monocytogenes* is a food-borne pathogen that causes an estimated 2500 cases of human disease and as many as 500 deaths per year in the USA (Mead *et al.*, 1999; Notermans *et al.*, 1998). Interestingly, *L. monocytogenes* is responsible for less than 0.1% of all food-borne illnesses but causes 27.6% of deaths attributed to food-borne diseases (Mead *et al.*, 1999). Other studies have shown that serious listeriosis occurs worldwide, for example as cases of invasive listeriosis in non-pregnant adults (Siegman-Igra *et al.*, 2002) and in neonates (Fleming *et al.*, 1985; McLauchlin, 1990; Schwarze *et al.*, 1989; Teberg *et al.*, 1987). Although most of the outbreaks of human listeriosis have clearly been shown to be associated with the ingestion of contaminated food, many studies for oral listeriosis in experimental animals conducted using a parenteral route of infection (Blanot *et al.*, 1997; Mannering *et al.*, 2002; Menudier *et al.*, 1991). As such, understanding of the pathogenesis of listeria infection via the gastrointestinal tract remains very limited.

Human lactoferrin (hLF) is an iron-binding glycoprotein that is present in milk, saliva and other exocrine secretions as well as neutrophils. This protein has a number of biological functions, including antimicrobial and immunomodulatory abilities *in vitro* and *in vivo* (Brock, 1995; Notermans *et al.*, 1998; Vorland, 1999; Haversen *et al.*, 2003). The antimicrobial effects can be direct, through bacteriostatic and bactericidal activity, or host-mediated, through the activation of a complex series of reactions leading to a protective immune response after infection (Levay & Viljoen, 1995). In addition, lactoferrin has been reported to inhibit the entry process of pathogens, such as *L. monocytogenes* into intestinal cells.
(Valenti et al., 1999) or hepatitis B virus into human hepatocytes (Hara et al., 2002).

A number of in vitro studies have been published on the antimicrobial effects of lactoferrin against L. monocytogenes (Branen & Davidson, 2000; Murdock & Matthews, 2002; Nibbering et al., 2001; Longhi et al., 2004). However, in vivo studies have not to our knowledge been reported. Therefore, we investigated whether hLF could inhibit oral infection of listeria in BALB/c mice.

METHODS

Bacterial strains and human lactoferrin. L. monocytogenes strains ATCC 19111, 19113, 19115 and 51774 were purchased from the American Type Culture Collection. The bacteria were incubated in BHI broth (Difco) at 37 °C for 12 h with shaking (200 r.p.m.). For determination of MICs and for oral challenge, cultures were diluted in fresh BHI broth to 2 × 10^7 c.f.u. ml^-1 and 2 × 10^8 c.f.u. ml^-1, respectively. Lactoferrin from human milk was purchased from Sigma and stored at −20 °C until use.

Determination of MICs. The hLF MICs of L. monocytogenes strains were determined using a modification of the method described by Shin et al. (1998). Briefly, bacteria were diluted to approximately 2 × 10^7 c.f.u. ml^-1 in BHI broth, and equal volumes (100 μl) of the bacterial suspension and BHI broth containing various concentrations of hLF were mixed in 96-well plates. The final concentrations of hLF were 2000, 1000, 500 and 250 μg ml^-1. Bacterial cultures without hLF were used as positive controls, and uninoculated BHI broth was used as a negative control. The plates were incubated at 37 °C for 24 h, and optical density was measured by 96-well plate reader at 620 nm after shaking at 200 r.p.m. for 5 s (Techne’s Sunrise Absorbance Microplate Reader, Phoenix Research Products). The MIC was determined as the lowest concentration of hLF that showed a significant difference (P < 0.05) in OD620 compared to the positive control after 24 h incubation. The assay was performed in triplicate for each strain. The most sensitive of the four L. monocytogenes strains was selected and used in the animal study.

Animals. Four-week-old specific pathogen-free female BALB/c mice were purchased from Seoul National University Laboratory Animal Center (Seoul, Korea). Animals were allowed food and water ad libitum, and were housed in cages at a controlled temperature (22 ± 2 °C), humidity (55 ± 10 %) and with a 12 h light/dark cycle. They were fed commercial mouse pellets and had free access to water at all times throughout the study. The mice were acclimatised for 7 days prior to the start of the experiments. All procedures were performed according to the guidelines for the care and use of laboratory animals approved by Seoul National University.

Experimental designs. Twenty-four mice were divided into four groups: non-treated, control, and 100 mg hLF kg⁻¹ day⁻¹ and 10 mg hLF kg⁻¹ day⁻¹ treatment groups. The hLF was administered for 7 days, commencing 4 days before oral challenge with 1×10^8 c.f.u. of the most sensitive strain. Thirty minutes prior to bacterial administration, all mice were orally administered 100 μl 10 % sodium bicarbonate (w/v) to neutralize gastric acid (Czupryński & Faith, 2002). The non-treated group and control group were administered sterilized PBS instead of hLF for 7 days, and the control group was orally challenged with L. monocytogenes similarly to the two treatment groups. During all experimental periods, food was removed from the cages 4 h prior to drug administration.

Bacterial enumeration in organs. After 7 days, all animals were humanely euthanized by anaesthesia with ethyl ether (Sigma) followed by cervical dislocation. The abdominal cavities of the mice were aseptically opened, and the liver and spleen were removed in small fractions and homogenized in sterilized PBS for bacterial counts. Several dilutions of the homogenates were plated on PALCAM (Merck) agar. After incubation for 36 h at 37 °C, the numbers of bacteria per gram of tissue were calculated.

Histopathology. The livers and spleens were fixed with 10 % formalin buffer for 48 h. Formalin-fixed tissues were dehydrated in an alcohol-xylene series and embedded in paraffin wax. From each block, 2 μm-thick sections were prepared and stained with haematoxylin and eosin (H&E). The area of necrotic foci was measured by image analyser (Image-Pro Plus version 4.5, Media Cybernetics) in the three hepatic lobes of each mouse and calculated as the size of foci in mm^2 per 100 mm^2 hepatic area.

Semi-quantitative RT-PCR. Cytokine mRNA expression was analysed by a semi-quantitative RT-PCR. RNA samples from liver were prepared using TRIzol Reagent (Invitrogen) and the RNA concentration was determined by the absorbance at 260 nm and stored at −80 °C until use.

cDNAs were reverse transcribed from 1 μg of total RNA using a GeneAmp RNA PCR core kit (Applied Biosystems). PCR was performed using i-Taq DNA polymerase (iNTRON) and cytokine-specific primer sets (Table 1). The PCR conditions were as follows: 3 min of denaturation at 95 °C; 32 cycles of denaturation (30 s, 94 °C), annealing (30 s, appropriate temperature), and chain extension (1 min, 72 °C); and 7 min of final extension at 72 °C after amplification. The PCR product was detected by electrophoresis of 9 μl of the reaction solution in a 1.5 % (w/v) agarose gel, followed by staining with ethidium bromide. The bands of the PCR product were visualized on a UV transilluminator. The band densities were measured by an analysing program (Kodak Digital Science 1D, NEN Life Science Products) and statistically evaluated.

Statistical analysis. Statistical analysis was performed using Duncan’s Multiple Range Test (SAS v. 8.2, SAS Institute). P-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Determination of MICs

To select the most sensitive strain for the animal work, we determined the hLF MICs for four L. monocytogenes strains. As shown in Fig. 1, the growth of L. monocytogenes was effectively inhibited by hLF. Concentrations of 1000 and 2000 μg hLF ml⁻¹ resulted in significantly lower OD620 values for all four L. monocytogenes strains (from 5 h onwards) compared to the positive controls (with no hLF). The MIC of hLF was determined to be 1000 μg ml⁻¹ for all four L. monocytogenes in culture medium at pH 7.2, similar to that previously reported for other strains of L. monocytogenes (Branen & Davidson, 2000). L. monocytogenes ATCC 51774 was determined to be the most sensitive strain in the current study, because the time at which the OD620 values were significantly lower in the presence of 1000 or 2000 μg ml⁻¹ of hLF than those of the control was earlier than for the other three strains.

Criteria for animal work

Previous studies have demonstrated that L. monocytogenes extensively colonizes the liver and spleen of orally challenged mice at 2–4 days post-inoculation, and multi-focal necrosis
occurs in the liver and spleen (Czuprynski et al., 2003; Marco et al., 1992). For this reason, the liver and spleen were selected as major target organs and the effects of hLF were assessed 3 days after oral challenge with *L. monocytogenes* ATCC 51774. In addition, hLF was administered prior to the challenge with *L. monocytogenes* in this study, because previous work reported that lactoferrin was more effective against systemic staphylococcal infections in mice when it was given prior to bacterial administration (Bhimani et al., 1999).

We daily administered 2500 μg hLF or 250 μg hLF to the high and low dose groups of mice, respectively (high dose, 100 mg

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**Table 1. Primer sequences, annealing temperatures and sizes of PCR products**

Primer pairs of IL-1β were designed by the primer selection program of BCM Search Launcher (Baylor College of Medicine, Human Genome Sequencing Center, Houston, TX, USA). Primer pairs of IFN-γ (Nomura et al., 2002), tumour necrosis factor (TNF)-α and β-actin (Johnson et al., 2000) were used as previously reported.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primer sequences (5′–3′)</th>
<th>Annaling temperature (°C)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
</table>
| IFN-γ    | F: AGCGGCTGACTGAAGCTAGATTGTAG  
R: GTCACAGTTTCCAGTGTATAGGG |
| IL-1β    | F: GCAACTGTCCTGAACTC  
R: CTGAGGCTGTAGTTCGAG |
| TNF-α    | F: CTCTTCAGGGAACAGGGCTG  
R: CGGACTGCAAGAATCTAG |
| β-Actin  | F: ATGGATGACGATATCGCT  
R: ATGAGGTAGTCTGTCAGGT |

**Fig. 1.** Growth curves of *L. monocytogenes* strains in BHI broth supplemented with various concentrations of hLF. *L. monocytogenes* strains ATCC 19111 (a), 19113 (b), 19115 (c) and 51774 (d) were tested with 2000 (■), 1000 (△) and 500 (○) μg hLF ml⁻¹. BHI broth without and with bacteria was cultured for the same periods for the negative control (▲) and positive control (○), respectively. *, ** indicate the times at which the 2000 and 1000 μg hLF ml⁻¹ treatment groups showed significantly lower OD₆₂₀ values compared to the positive control group. Values are expressed as mean ± SD of triplicate runs at OD₆₂₀.
kg⁻¹; low dose, 10 mg kg⁻¹; mean body weight of mice, approximately 25 g). The total amounts of hLF administered over the 7 days were 17.5 and 1.75 mg per mouse in the high dose and low dose groups, respectively. Other studies have reported that an effective dose for dextran sulphate-induced colitis in mice is twice daily oral administration of hLF (2 mg per dose) for 7 days (Haversen et al., 2003). They have also reported that a single oral administration of hLF (500 μl per mouse) decreases the number of Escherichia coli O6K5 in the kidney (Haversen et al., 2000). The MIC of hLF for E. coli O6K5 is 1 mg hLF ml⁻¹, similar to that seen for L. monocytogenes in our study.

**Effects of hLF on bacterial enumeration**

hLF-treated mice had significantly fewer L. monocytogenes cells in liver samples than control animals (P < 0.05). The mean ± SD of the bacterial numbers for the control group was 5.383 ± 0.172 log₁₀ c.f.u. g⁻¹; however, those for the 100 mg hLF kg⁻¹ day⁻¹ and 10 mg hLF kg⁻¹ day⁻¹ treatment groups were 4.704 ± 0.198 log₁₀ c.f.u. g⁻¹ and 4.755 ± 0.337 log₁₀ c.f.u. g⁻¹, respectively. The numbers of bacteria in the spleen were also slightly lower in hLF-treated mice, but not statistically significantly. In the spleen, the mean bacterial numbers for the control group was 5.385 ± 0.157 log₁₀ c.f.u. g⁻¹; however, those for the 100 mg hLF kg⁻¹ day⁻¹ and 10 mg hLF kg⁻¹ day⁻¹ treatment groups were 5.223 ± 0.209 log₁₀ c.f.u. g⁻¹ and 5.288 ± 0.395 log₁₀ c.f.u. g⁻¹, respectively. No bacteria were detected in the liver and spleen samples of the non-treated animals.

**Histopathological analysis of the effects of hLF on listerial infection**

Oral administration of hLF alleviated listeria infection in the liver (Fig. 2b). Multi-focal necrosis was observed in the livers of mice infected with L. monocytogenes ATCC 51774 (Fig. 2a). Neutrophils migrated markedly, as well as lymphocytes and macrophages, and infiltrated necrotic foci. The number and size of the necrotic foci decreased in mice administered with 100 mg hLF kg⁻¹ day⁻¹.

The area of necrotic foci in the three hepatic lobes of each mouse was calculated as the size of foci in mm² per 100 mm² hepatic area. The mean ± SD of the necrotic areas of the control group was 0.112 ± 0.062; however, those of the 100 mg hLF kg⁻¹ day⁻¹ and 10 mg hLF kg⁻¹ day⁻¹ treatment groups were 0.008 ± 0.004 and 0.052 ± 0.030, respectively. The necrotic area was approximately 10-fold lower in the livers of hLF-treated mice compared to the control animals (P < 0.05). Although splenitis, characterized by the central necrosis of white pulp with neutrophil infiltration, was observed, there was no difference in histopathological lesions between hLF-treated mice and control mice (data not shown). Hepatic necrosis and splenitis were not observed in the non-treated group.

Other studies in vitro have shown that macrophages may acquire LF through surface receptors and thereby enhance the killing of intracellular Legionella pneumophila, Mycobacterium microti and Trypanosoma cruzi (Lima & Kierszenbaum, 1985). With regard to the effects of LF on human neutrophils, it has been shown by in vitro studies that bovine lactoferrin (bLF) and its fragment bovine lactoferricin enhance the phagocytic activity of these cells; this effect seems to be due to direct binding of bLF to neutrophils and an opsonin-like activity (Miyauchi et al., 1998).

In this study, hLF decreased the hepatic colonization and hepatitis in mice challenged with listeria; however, this decrease was not seen in the spleen. Other researchers have shown that the bio-distribution of hLF peptides varies between different organs in bacterially infected mice (Welling et al., 2000). They reported that deposits of certain 99mTc-labelled hLF peptides were 10- to 15-fold higher in the liver than in the spleen. However, whether this bio-

**Fig. 2.** Histopathological signs of inflammation in liver samples 3 days after oral challenge with 1x10⁸ c.f.u. of L. monocytogenes. Liver tissue from positive control group (sterilized PBS for 7 days) (a) and hLF-treated group (100 mg kg⁻¹ day⁻¹ for 7 days) (b). Inset: overview of liver, where necrotic foci were observed. Arrows indicate the necrotic foci. H&E staining; bars, 100 μm.
Effects of hLF on the expression level of inflammatory cytokines

The expression of inflammatory cytokines was assessed by analysis of mRNA levels in mouse liver samples. As shown in Fig. 3, the interferon (IFN)-γ, interleukin (IL)-1β and tumour necrosis factor (TNF)-α mRNA levels increased in liver samples of control mice compared to the non-treated group. The mRNA levels of these cytokines significantly decreased in the liver samples of the 100 mg hLF kg⁻¹ day⁻¹ treatment group compared to the control group (P < 0.05). Although a significant difference (P < 0.05) between the two hLF-treated groups was not observed, hLF dose-dependently decreased the expression of each cytokine (Fig. 3).

The reasons why hLF acts as an anti-infective agent for \( L. \) monocytogenes may be multifactorial. Since antimicrobial (Bellamy et al., 1992) and anti-inflammatory (Mattsby-Baltzer et al., 1996) activities have been described for hLF and human lactoferricin in vitro, it is possible that hLF exerts both of these properties in our infection model. In vivo studies have shown that hLF downregulates dextran sulphate-induced IL-1β production in blood (Haversen et al., 2003). Thus, the anti-inflammatory capacity of hLF in our model could be a consequence of either its antibacterial activity or downregulation of inflammatory cytokines or both.

Conclusions

This study has shown that perorally given hLF could mediate antimicrobial and anti-inflammatory activities in the liver (i.e. remote from the gut) of mice challenged with \( L. \) monocytogenes.

Fig. 3. Expression of inflammatory cytokines in liver samples. After semi-quantitative PCR, band densities were measured by an analysing program and evaluated statistically. Values were expressed as mean ± SD normalized density of target mRNA. β-Actin, a housekeeping gene, was used for normalization. Significantly different values (P < 0.05) between treatment groups are indicated by ‘a’ and ‘b’. Graph shows mean ± SD. Bars: white, non-treated; black, control; horizontal stripes, 100 mg hLF kg⁻¹ day⁻¹ treatment; vertical stripes, 10 mg hLF kg⁻¹ day⁻¹.

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