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Original article

Prophylactic effect of human lactoferrin against *Streptococcus mutans* bacteremia in lactoferrin knockout mice

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Abstract

Streptococcus mutans is the primary agent of dental caries, which is often detected in transient bacteremia. Lactoferrin is a multifunctional glycoprotein showing antibacterial activities against several *Streptococcus* species. We reported here the prophylactic effect of human lactoferrin (hLF) in a lactoferrin knockout mouse ($LFKO^{-/-}$) bacteremic model. The hLF treatment significantly cleared *S. mutans* from the blood and organs of bacteremic mice when compared to the non-hLF treated mice. Further, analysis of serum cytokines, spleen and liver cytokine mRNA levels revealed that hLF prophylaxis modulates their release differently when compared to the non-hLF treated group. C-reactive protein level ($P = 0.003$) also decreased following hLF prophylaxis in *S. mutans* induced bacteremic mice. Additional quantitative RT-PCR analysis revealed that hLF prophylaxis significantly decreased the expression level of IFN-γ, TNF-α, IL-1β, IL-6, MPO and iNOS in spleen and liver. These results suggested that the hLF protects the host against *S. mutans*-induced experimental bacteremia.

Keywords: Human lactoferrin; Prophylaxis; *Streptococcus mutans*; Bacteremia

1. Introduction

Streptococcus mutans, causative agent of dental caries, has also been isolated from the blood of patients with bacteremia [1,2], infective endocarditis [3–5] and sepsis following tooth extraction [4]. Studies suggest that periodontal probing, tooth brushing and poor oral hygiene are the most common ways of introducing oral pathogens into the bloodstream, which increases the risk of bacteremia and systemic diseases [6,7]. Lactoferrin (LF) constitutes an important component of the innate immune system and has a significant immunomodulatory effect on humans. The major ability of LF is to induce the innate immune mediator cells that subsequently impact the adaptive immune cell functions. Also, the affinity of LF towards iron is considered as a main component of non-specific host defense system against numerous pathogens [8–10]. High level of plasma LF concentration has been suggested to be a predictive indicator of sepsis-related morbidity and mortality [11]. LF also modulates the differentiation of leukocytes of the innate immune system, by increasing natural killer (NK) cell activity [12,13]. The degranulation of neutrophils in response to an inflammatory signal introduces LF into an environment that is populated with a mix of both innate leukocytes and adaptive immune cells (T-cells and B-cells). The LF receptors for different immune cells confirm the potential for LF to function as a modulator of both the innate and adaptive immune system [14–16]. LF exhibit bacteriostatic and bactericidal effects in addition to controlling systemic inflammation [8]. Studies have shown that LF has direct bactericidal effect against *S. mutans* [17,18]. However, none of these studies looked at the bactericidal effect of LF in blood. We have recently demonstrated the importance of the host LF against the Gram-negative periodontal pathogen, *Aggregatibacter*

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actinomycetemcomitans [19]. Subsequently, we have also reported the effect of human lactoferrin (hLF) against *A. actinomycetemcomitans*-induced experimental bacteremia [10]. Prophylactic treatment of hLF against Gram-positive bacteria *S. mutans*-induced bacteremia however, has not been studied to date. Therefore knowledge about the significance of hLF is a prerequisite. Furthermore, this study will provide information on the importance of hLF prophylaxis on antibacterial and immunomodulatory effects during such bacteremic conditions.

2. Materials and methods

2.1. Mice

Experimental groups comprised of 7–8 week-old male LFKO$^{-/-}$ mice, a generous gift from Dr. Orla Conneely, (Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA) [20]. Mice colonies were bred and maintained in the transgenic animal facility of Rutgers School of Dental Medicine, Newark, New Jersey, USA. The experimental protocol was approved by the campus Institutional Animal Care and Use Committee (IACUC) of Rutgers School of Dental Medicine, Newark, NJ, USA.

2.2. Bacterial strain and tail vein injections

S. mutans-VSK1, a spontaneous streptomycin resistant serotype *c* strain was isolated from a plaque sample routinely grown in brain heart infusion broth (BHI) or on mitis salivarius agar (MSA) plates (BD & company, Sparks, MD, USA) as reported earlier [21]. PCR amplification of a 727 bp with the following primers SCF 5′-CGGAGTGCTTTTTACAAGTG CTGG-3′, and SCR 5′-AACCACGGCCAGCAAACCCTT-TAT-3′ confirmed serotype *c* specific strain as reported earlier [22]. Cells that were grown in BHI broth for 12 h was centrifuged and resuspended to 1×10^7 colony forming unit (CFU) per 0.1 ml of phosphate-buffered saline (PBS) and it was intravenously (i.v) injected into the tail vein as reported earlier [23]. The recovered *S. mutans* after i.v. injection from blood and organ samples was plated on MSA agar plates supplemented with streptomycin (50 μg/ml). The recovered streptomycin resistant colonies were confirmed by PCR [5]. The hLF (100 μg/g body wt) for the prophylactic treatment was prepared as reported earlier [10].

2.3. Experimental design

Experimental groups included; 1) sham infected control mice i.v. injected with PBS (LFKO$^{-/-}$C), 2) mice i.v. injected with hLF (LFKO$^{-/-}$ + hLF), 3) mice i.v. injected with *S. mutans* (LFKO$^{-/-}$I) and 4) mice i.v. injected with hLF followed by *S. mutans* i.v. injection 2 h later (LFKO$^{-/-}$I + hLF). All injection volumes were adjusted to 100 μl. Mice ($n = 5$–8) were euthanized at 6, 12, 48 and 96 h following injection of either *S. mutans* or hLF or both. The recovered *S. mutans* were expressed in CFU/ml or CFU/g tissue in three independent experiments and the data were statistically analyzed using one-way ANOVA as described earlier [10].

2.4. Determination of the viable bacterial levels

To determine the *S. mutans* clearance upon hLF prophylaxis, samples of blood, brain, heart, kidney, liver, lungs, and spleen were aseptically harvested and processed after each time point. Blood samples were serially diluted and plated on the MSA plates supplemented with streptomycin (50 μg/ml). The organs were placed in 1 ml of PBS in 50-ml Kendall tissue homogenizer (Tyco Healthcare Group, Mansfield, MA). Following homogenization of the tissues, serially diluted tissue samples were plated on MSA plates. Colonies enumerated after 48 h were expressed as CFU/ml (blood) and CFU/mg (tissue weight). In total, three independent experiments representing three biological replicates were performed.

2.5. Blood sample analysis

The serum inflammatory cytokines and C-reactive protein (CRP) levels were quantified using multiplex ELISA kit (Millipore Corporation, Billerica, MA, USA). The differential leukocyte blood cell count and enzyme-linked immunosorbent assay (ELISA) were performed as reported earlier [10].

2.6. Quantitative RT-PCR

The Total RNA was extracted from spleen and liver tissue using an RNeasy Mini Kit. DNA contamination in the samples was removed by on-column DNase I digestion (Qiagen Inc, Valencia, CA, USA). The relative gene expression of pro inflammatory cytokines, inducible nitric oxide synthase (iNOS) and myeloperoxidase (MPO) was performed as reported earlier [10]. Results are presented as the mean ± SEM of the duplicate experiments of three independent samples. The data was analyzed by one-way analysis of variance (ANOVA).

2.7. Effect of hLF treatment in spleen during bacteremic conditions

Mice ($n = 5$–8) were euthanized at different time points and the intact spleen was collected from each group and weighed. The spleen size variation between the groups was photographed using a dissection microscope (Olympus SZ61, Tokyo, Japan). For histological analysis, aseptically collected spleen from 96 h time point was fixed with 4% paraformaldehyde for 48 h. The tissue samples were then processed and stained with hematoxylin and eosin as described earlier [10]. Tissue sections were observed under Olympus light microscope at 1000× magnification.

2.8. Statistical analysis

Significance of differences between the groups was calculated using Student's *t* test, ANOVA and post hoc Tukey' HST test between the groups with the JMP software SAS 9.1. *P*