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LACTOFERRIN PROTECTS GUT MUCOSAL INTEGRITY DURING ENDOTOXEMIA INDUCED BY LIPOPOLYSACCHARIDE IN MICE

MARIAN L. KRUZEL, YAEL HARARI, CHUNG-YING CHEN, and GILBERT A. CASTRO

Department of Integrative Biology and Pharmacology
University of Texas, Houston Health Science Center, Texas 77225

Abstract—The hypothesis that lactoferrin protects mice against lethal effects of bacterial lipopolysaccharide (LPS) is the subject of experimental investigations described in this article. Lipopolysaccharide is a powerful toxin produced by Gram negative bacteria that when injected into humans or experimental animals reproduce many of the pathophysiologic and immune responses caused by live bacteria. Lactoferrin administered intraperitoneally 1 hr prior to injection of LPS significantly enhanced the survival of mice, reducing LPS-induced mortality from 83.3% to 16.7%. Changes in locomotor and other behavioral activities resulting from LPS injection were not present in mice treated with lactoferrin. Also, histological examination of intestine revealed remarkable resistance to injury produced by LPS if mice were pretreated with lactoferrin. Severe villus atrophy, edema and epithelial vacuolation were observed in LPS-treated animals but not in lactoferrin-treated counterparts. Electrophysiological parameters were used to assess secretory and absorptive functions in the small intestine. In mice treated with LPS, transmural electrical resistance was reduced and absorption of glucose was increased. Lactoferrin treatment had no significant influence on basal electrophysiological correlates of net ion secretion or glucose absorption nor on changes induced by LPS. Collectively, these results suggest that lactoferrin attenuates the lethal effect of LPS and modulates behavioral and histopathological sequela of endotoxemia.

INTRODUCTION

Lactoferrin, an iron binding protein found in high concentrations in most exocrine secretions of humans is an important component of the host defense system active at mucosal surfaces (1). Lactoferrin is also a major protein of the secondary granules of neutrophils, from which it is released following activation of these cells. Numerous biological functions have been ascribed to lactoferrin (2–5). Of significance in this study are lactoferrin's bacteriostatic (6), bactericidal (7,8), and immunoregulatory properties (9–13). Lactoferrin interacts with

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specific receptors on the mononuclear cells and regulates myelopoiesis in vitro and in vivo by suppressing cytokine release (14–16). In the presence of bacterial endotoxin, namely lipopolysaccharide (LPS), lactoferrin's ability to regulate the cytokine network is substantially diminished (15). Recently, two LPS binding sites on the lactoferrin molecule have been described (17). A high affinity site associates with the N-terminal fragment of lactoferrin, whereas a low affinity binding site is located at the C-terminus. It has been suggested that the LPS-lactoferrin complex binds to mononuclear cell via LPS receptor (CD14) rather than through a lactoferrin recognition site (18–20). As a consequence of complex formation, both the anti-proliferative effects of lactoferrin and the ability of LPS to prime neutrophils for enhanced superoxide formation are compromised (15,19).

Although generally beneficial to the host, inflammatory processes are intrinsically destructive to the surrounding tissues and can result in major tissue injury. Furthermore, local acute inflammatory responses can become self-perpetuating by overproduction of pro-inflammatory cytokines and spread systemically. It is becoming increasingly evident that lactoferrin can modulate inflammatory pathways. Lactoferrin introduced intravenously (i.v.) into mice 24 h before LPS challenge significantly lowered the serum concentration of TNF-α (21). Zagulski et al., showed that septic shock due to i.v. injection of bacteria in mice was prevented by administration of exogenous lactoferrin (22). Also, it was reported that LPS-induced septic shock in germfree piglets was modulated by oral administration of lactoferrin (23).

With the preceding as background, our objective was to substantiate the potential protective effects of lactoferrin in mice against challenge with LPS, and to gain a better understanding of how it protects. Reported in this article are results demonstrating the effectiveness of lactoferrin in enhancing survival and protecting against damage to intestinal mucosa.

METHODS AND MATERIALS

Animals. Male CF-1 mice (Harlan, Houston, Texas), 25–30 g initial body weight, were used throughout this investigation. Mice were housed in groups of three per cage and were given a stock diet (F6 Rodent Diet 8664, Teklad, Madison, Wisconsin) and water ad libitum.

Experimental Design. Effects of human lactoferrin on the development of endotoxemia induced by LPS were determined in two different sets of experiments. The first experiment was designed to examine the survival of mice after challenge with LPS (2.26×10^6 Endotoxin Units [E.U.] per 25 g mouse; ~30 mg/kg body). The dose of LPS was established in a separate experiment using CF-1 mice. It is the dose that proved lethal to approximately 85% of the mice. Naive mice were injected i.p. with 7.5 mg/mouse of human lactoferrin in 150 μl of saline solution. Their control counterparts were given 150 μl of saline. One hour later all mice were injected i.p. with LPS. The survival of mice was monitored for four weeks. The second experiment was designed to test the

effect of lactoferrin on gut structure and function in mice treated with LPS [2.25×10^6 E.U. per 25 g mouse; ~30 mg/kg body) to induce acute endotoxemia. Naive mice were injected i.p. with 7.5 mg/mouse of human lactoferrin in 150 μl of saline solution. Their control counterparts were given 150 μl of saline. One hour later all mice were injected i.p. with LPS. Rectal temperatures were measured every 30 min for the initial 6 h. The behavior of mice, including locomotor activity, eating and drinking, and clinical signs were evaluated qualitatively at the same time. Twenty-four hours after LPS injection mice were killed and jejunal segments removed for electrophysiological measurements and histological examination.

Electrophysiological Measurements of Ion Transport in Mouse Jejunum. Jejunum, beginning 1 cm distal to the ligament of Treitz, was removed, rinsed in Krebs-Ringer bicarbonate (KRB) solution, pH 7.4, and slit open along the mesenteric border. Consecutive one-cm full thickness segments were taken from the proximal part of the intestine and mounted as a flat sheet between two Ussing half chambers with an aperture of 0.512 cm^2, as described earlier (24). Tissues, bathed on their mucosal and serosal sides with 10 ml KRB solution, were voltage clamped at zero transepithelial potential using a VCC-600 voltage current clamp (Physiologic Instruments, San-Diego, California). A continuous record of short circuit current with respect to time was obtained and recorded on a DB-41 Kipp and Zonen recorder (Delft, Holland). To measure tissue resistance, a current that generates an extra 1 mV potential difference across the tissue was passed every two min for 0.1 s. Resistance was calculated using Ohm's law (V = IR). Changes in short circuit current (ΔIsc) induced by Cl^- secretagogues [serotonin (5-HT) and carbamylcholine (CCh)] and by glucose are presented as the maximal elevation and expressed as $\mu A/cm^2$.

Histological Techniques. Jejunal segments were fixed in 10% formalin and embedded in paraffin using standard techniques. Sections, 5 μm thick, were cut, stained with hematoxylin and eosin (H&E) and subsequantly reviewed histologically; the pathologist (V.T.) viewing and interpreting the slides was blinded to type of experiment and treatment. Photographs were taken with a Nikon Optiphot microscope.

Reagents. Human milk lactoferrin (low endotoxin - 4 E.U./mg; iron saturation <25%), LPS from *E. coli* serotype 0111 : B4 (3×10^6 E.U./mg), serotonin creatinine sulphate, and carbamylcholine chloride were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Statistics. All data are expressed as mean ± *SE*. Differences between groups were analyzed by the Student unpaired *t* test when two groups were analyzed and analysis of variance (ANOVA) when more than two groups were analyzed. *P* value of 0.05 or less was considered significant.

RESULTS

Survival of Mice Treated with LPS. A single dose of lactoferrin (7.5 mg) administered 1 h before LPS injection significantly increased survival of mice when compared with the saline-treated controls (Figure 1). Twenty four hours following LPS administration no deaths were observed in the lactoferrin-treated mice nor in the control group. On average, four out of six mice in the saline control group died 48 h after LPS administration, whereas only one death occurred in the lactoferrin-treated mice. By 56 h one more mouse from the saline control group died. No more deaths were recorded in either group for the remaining 26 days of the experiment. Overall, the mortality rate was 16.7% in the lactoferrin-treated mice and 83.3% in the saline control group.

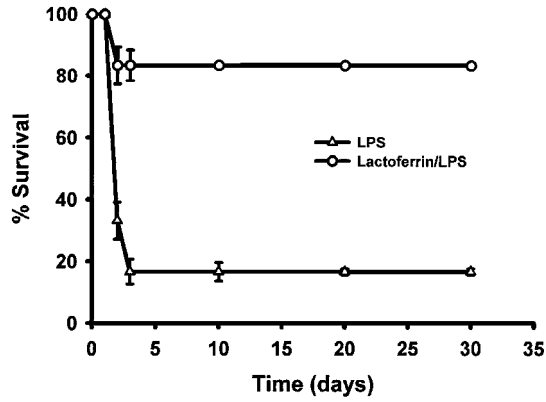

Fig. 1. LPS-induced mortality in mice. Lactoferrin (7.5 mg/mouse) was administered i.p. 1 h before injection of LPS (2.25×10^6 E.U./mouse). Three experiments, 18 mice per group.

Electrophysiological Characteristics of Jejunal Epithelium. Electrophysiological properties (SCC-short circuit current, R-resistance, PD-transmural potential difference) of jejuna were measured. Treatment of mice with lactoferrin had no effect on basal electrophysiological characteristics of jejunal epithelium (Table 1). Resistance (R) of the intestinal tissue following LPS treatment was reduced, and lactoferrin was without effect in altering this result (Table 1). Glucose absorption was about 30% higher for LPS-treated animals compared with saline- and lactoferrin-treated mice. Cl^- secretory responses to 5-HT and CCh were also elevated for LPS groups (Table 2). Although these changes were not statistically significant they present a consistent trend in upregulation of absorptive and secretory function of jejunum following LPS treatment.

Table 1. Electrophysiological Parameters in Jejunum Following LPS Administration

Treatment	R ($\Omega \cdot cm^2$)[a]	Isc ($\mu A/cm^2$)[a]	PD (mV)[a]
Saline	27.7 ± 4.7	105.2 ± 19.2	2.5 ± 0.3
Lactoferrin	33.6 ± 3.8	89.7 ± 35.3	2.6 ± 0.9
LPS[c]	17.1 ± 1.5[d]	104.7 ± 14.4	1.9 ± 0.2
Lactoferrin/LPS[b]	18.9 ± 1.5[d]	128.2 ± 13.4	2.2 ± 0.1

[a]Values are mean ± *SE*; n = 6.
[b]Lactoferrin (7.5 mg/mouse) was administered intraperitoneally 1 h before LPS injection.
[c]Mice were injected with LPS (2.25×10^6 E.U./mouse). Measurements were taken 24 h after LPS injection.
[d]Statistically significant difference from respective controls (saline-treated).

Table 2. Jejunal Response to Glucose and Cl^- Secretagogues Following LPS Administration

Treatment	Glucose (10^{-2}M)[a]	5-HT (10^{-4}M)[b]	CCh (10^{-4}M)[b]
	ΔIsc (μA/cm^2)[c]		
Saline	70.8 ± 11.3	50.2 ± 10.1	126.0 ± 17.2
Lactoferrin	69.0 ± 14.3	40.2 ± 7.7	105.4 ± 22.7
LPS[e]	101.1 ± 8.5	89.7 ± 6.1	153.9 ± 22.1
Lactoferrin/LPS[d]	98.2 ± 7.9	94.3 ± 15.8	176.9 ± 20.0[f]

[a]Added to mucosal side.
[b]Added to serosal side.
[c]Values are mean ± *SE*; n = 6.
[d]Lactoferrin (7.5 mg/mouse) was administered intraperitoneally 1 h before LPS injection.
[e]Mice were injected with LPS (2.25×10^6 E.U./mouse). Measurements were taken 24 h after LPS injection.
[f]Statistically significant difference from respective controls (saline-treated).

Changes in Rectal Temperature and Behavioral Activities. Rectal temperature was measured every 30 min during development of acute endotoxemia and is presented in Figure 2. LPS-treated mice quickly become hypothermic, whereas those pretreated with lactoferrin showed a less pronounced drop in body temperature. Development of hypothermia in LPS-treated mice was correlated with severe lethargy. LPS-treated mice become lethargic as early as 30 min after LPS injection. On the other hand, lactoferrin-treated mice expressed normal

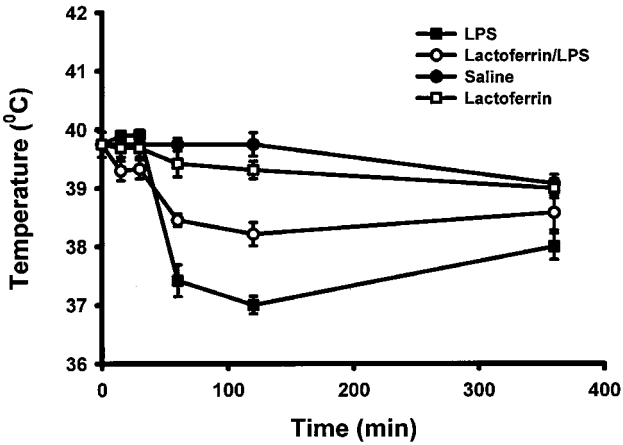

Fig. 2. LPS-induced hypothermia in mice. Lactoferrin (7.5 mg/mouse) was administered i.p. 1 h before injection of LPS (2.25×10^6 E.U./mouse). Three experiments, 18 mice per group.

behavioral activities including eating and drinking in the presence or absence of LPS.

Histological Characteristics of Jejunal Epithelium. The intestinal epithelium of mice injected with LPS exhibited severe vacuolar degeneration with shortening and swelling of the villi and elongation of the crypts (Figure 3). There was a heavy chronic inflammatory infiltrate in the tunica propria of control animals (Figure 3-c). In the lactoferrin-treated mice the vacuolar degeneration was less pronounced, with the epithelium resembling the highly polarized absorptive epithelium (Figure 3-d).

DISCUSSION

This report highlights the potential to use lactoferrin to modulate injury caused by LPS-induced endotoxemia in mice. Results pertaining to survival, behavior, and physiology indicate that human lactoferrin increases tolerance to the toxic effects of LPS. The most dramatic finding was the six-fold increase in survival of mice treated with lactoferrin prior to the administration of endotoxin. This prophylactic effect of lactoferrin was associated with behavioral patterns clearly distinguishable from those in mice that did not receive lactoferrin and eventually succumed to LPS treatment. Mice treated with lactoferrin prior to challenge with LPS also expressed normal behavioral activity, whereas those administered LPS alone became lethargic within half an hour after challenge. Changes in body temperature induced by LPS were attenuated by lactoferrin. Lactoferrin also clearly protected against the histologic changes caused by LPS in the jejunum. The increased ability of mice treated with lactoferrin to withstand the toxic effects of LPS substantiates a report that intravenous administration of lactoferrin protects mice against *Escherichia coli*, in which endotoxin is involved in the pathogenesis of infection (22).

It is interesting to consider the mechanism(s) underlying this protective effect generated by lactoferrin in vivo. The reduction in the LPS-induced gut injury in mice treated with lactoferrin indicate that the protection of intestinal structures may play an important role in survival of septic mice. It has been proposed that the gut barrier failure and the subsequent translocation of the enteric bacteria and endotoxin is a major contributing factor in the development of sepsis (25,26). The systemic inflammatory response to LPS may induce the gut-associated lymphoid tissue to produce and liberate pro-inflammatory cytokines which stimulate enteric bacteria translocation to distant sites (27–31). Because lactoferrin attenuates the production of cytokines it may lower the incidence of bacterial translocation and inhibit the development of septic conditions in vivo (32–34). Although we did not measure the translocation of enteric bacteria in our exper-

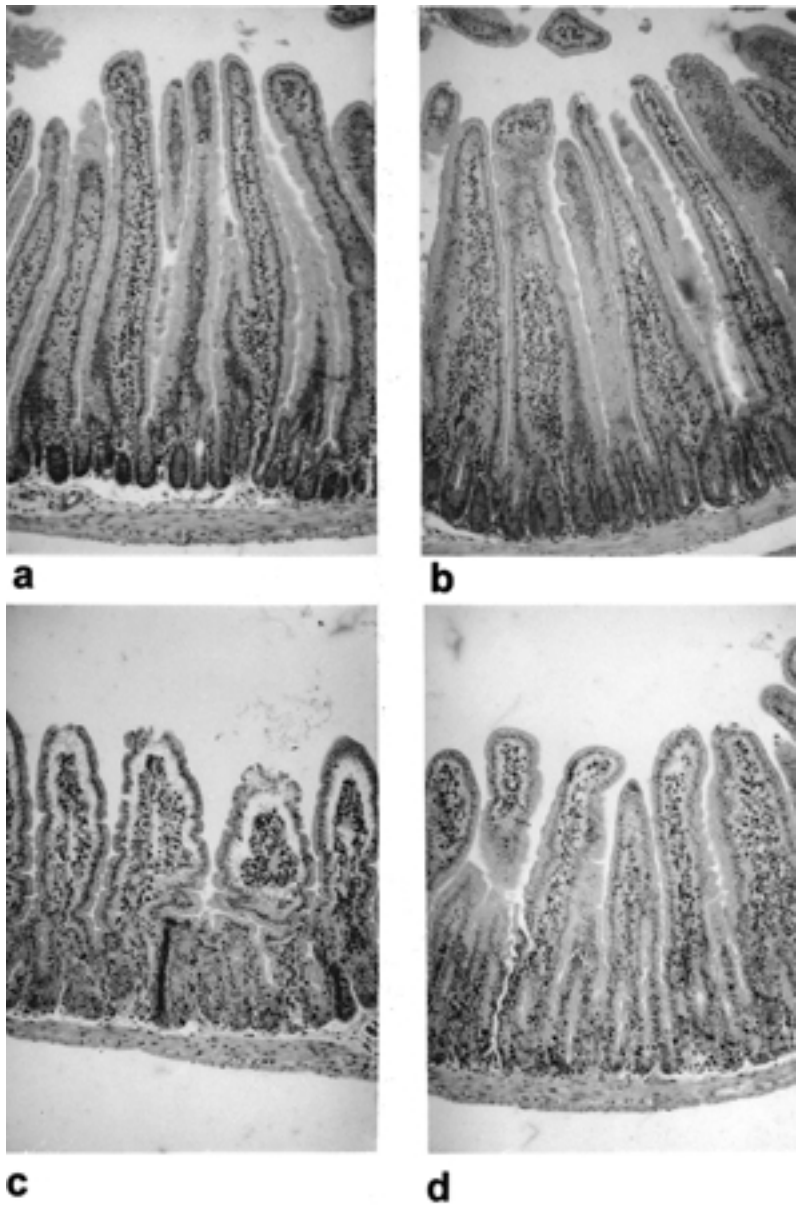

Fig. 3. Microscopic appearance of mouse intestinal structure 24 h following treatment: a) saline control; b) lactoferrin-treated; c) LPS-treated; d) lactoferrin/LPS-treated. H&E staining (original magnification ×200).

iment, it is likely that the increased survival of mice reported in this paper is due to lactoferrin's ability to attenuate the production of cytokines and further reduce bacterial translocation. An enigma, however, was why lactoferrin treatment did not reverse LPS-induced electrophysiological correlates of absorptive and secretory functions. The findings that LPS increases glucose absorption and net ion secretion in the intestine have not been reported before. In the absence of adequate information, one is faced with the challenge of deciphering whether these changes, from a physiological perspective, are harmful, helpful or without effect in adaptation to the effects of endotoxemia. The changes in functional responsiveness of the LPS-injured intestinal epithelium are likely time dependent. Mucosal functions in the present study were assessed only at 24 h after LPS administration, at which time the absorptive and secretory responses of the intestine were still maintained.

Current studies in our laboratory with *Trichinella spiralis*-induced intestinal inflammation in mice indicate that lactoferrin is able to upregulate intestinal Cl^- secretion in mice at the peak of inflammation, which corresponds to 7th day of infection (unpublished results). The study of intestinal inflammation induced by *T. spiralis* may provide clues to explain lactoferrin's inability to reverse the electrophysiological changes induced by LPS and expressed as increases in glucose absorption and net ion secretion. In the trichinella model, the parameters on which lactoferrin is impacting are reduced by infection. In the current model of endotoxemia, LPS-induced changes in absorption and secretion are significantly elevated above basal physiological levels. We offer the hypothesis that certain functions that are reduced by external perturbations are sensitive to and rectified by the action of lactoferrin, whereas those functions that are elevated above the normal range, as in the case of LPS elevating glucose absorption and net ion secretion, are insensitive to modulation by lactoferrin. In support of this hypothesis is the well substantiated action of Levamisole on the immune system. Levamisole is an agent well known for its ability to boost various functions of the immune system, if these functions are suppressed. However, Levamisole does not augment functions that are expressed within normal range or are in a hyper expressed state (35, 36). Human clinical studies confirmed that lactoferrin given orally was able to optimize cellular immune responses of individuals according to their initial responsiveness to conventional inducers (37). Individuals classified as low basal responders, relative to the production of two pro-inflammatory cytokines (IL-6 and TNFα) by peripheral blood leukocytes, showed an increase in cytokine production in response to stimulation when treated with lactoferrin. On the other hand, high basal responders showed a decrease in the production of cytokines under similar circumstances. These observations reveal the potential complexity of physiological regulation by lactoferrin.

The other component of sickness behavior due to LPS administration is change in body temperature. Although fever or hyperthermia is a physiologi-

cal response of humans and some animals to endotoxemia, hypothermia is the expected response in mice to LPS treatment (38–41). In our experiment, lactoferrin reduced the degree of hypothermia caused by high dose of LPS in mice. The concept of LPS neutralization by lactoferrin has been previously established and results in the reduction of pro-inflammatory cytokine production (23). A question that arises is how does hypothermia correlate with the production of pro-inflammatory cytokines in mice? It has been suggested that higher doses of LPS induce a physiological state similar to the initial phase of septic shock. An instant increase of TNFα and induction of nitric oxide, following LPS administration, may markedly reduce the blood pressure (42). Thus, it is likely that hypothermia would result from the disruption of circulation. This hypothesis is further supported by our recent observations that lactoferrin significantly reduced the plasma level of both nitric oxide and TNFα in endotoxemic mice (43–45). Although cytokine production is a functional effect of LPS, resulting primarly from its actions on the reticulo-endothelial-system (RES), it can exert different functions depending on the animal species and the physiological circumstances. For example in rodents, LPS-induced fever involves the action of IL-1β and IL-6 in the hypothalamus heat center. In mice, fever is associated only with low doses of LPS and has not been consistently observed as an acute response to LPS, despite the fact of high elevation of TNFα, IL-1β and IL-6 in serum (40,41). The reason for these apparent paradoxical observations are not clear and require better understanding of the physiological circumstances in which hypothermia occurs.

In conclusion, our results indicate that lactoferrin attenuates the lethal effect of LPS and modulates behavioral and histopathological sequela of endotoxemia. However, the detailed mechanism(s) by which lactoferrin protects mice against lethal effect of LPS is not known. More studies will be necessary to determine lactoferrin's role in maintaining homeostasis. Such studies should be grounded in the precept that the impact of lactoferrin on the regulation of physiological parameters is dictated by the physiological state of the host.

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