Increased Levels of LPS-Binding Protein in Bovine Blood and Milk Following Bacterial Lipopolysaccharide Challenge

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ABSTRACT

Several species of gram-negative bacteria, including *Escherichia coli*, *Klebsiella pneumoniae*, and various species of *Enterobacter*, are common mastitis pathogens. All of these bacteria are characterized by the presence of endotoxin or lipopolysaccharide (LPS) in their outer membrane. The bovine mammary gland is highly sensitive to LPS, and LPS has been implicated, in part, in the pathogenesis of gram-negative mastitis. Recognition of LPS is a key event in the innate immune response to gram-negative infection and is mediated by the accessory molecules CD14 and LPS-binding protein (LBP). The objective of the current study was to determine whether LBP levels increased in the blood and mammary gland following LPS challenge. The left and right quarters of five midlactating Holstein cows were challenged with either saline or LPS (100 μg), respectively, and milk and blood samples collected. Basal levels of plasma and milk LBP were 38 and 6 $\mu\text{g}/\text{ml}$, respectively. Plasma LBP levels increased as early as 8 h post-LPS challenge and reached maximal levels of 138 $\mu\text{g}/\text{ml}$ by 24 h. Analysis of whey samples derived from LPS-treated quarters revealed an increase in milk LBP by 12 h. Similar to plasma, maximal levels of milk LBP (34 $\mu\text{g}/\text{ml}$) were detected 24 h following the initial LPS challenge. Increments in milk LBP levels paralleled a rise in soluble CD14 (sCD14) levels and initial rises in the levels of these proteins were temporally coincident with maximal neutrophil recruitment to the inflamed gland. Because LBP and sCD14 are known to enhance LPS-induced host cell activation and to facilitate detoxification of LPS, these data are consistent with a role for these molecules in mediating mammary gland responses to LPS.

(**Key words:** lipopolysaccharide-binding protein, soluble CD14, endotoxin, mastitis)

Abbreviation key: HRP = horseradish peroxidase, LBP = LPS-binding protein, mCD14 = membrane-bound CD14, MSCC = milk somatic cell count, sCD14 = soluble CD14, TBS = Tris-buffered saline, Tlr = Toll-like receptor.

INTRODUCTION

Approximately 40% of the clinical cases of mastitis that occur annually are caused by gram-negative bacteria (Erskine et al., 1991; Ziv, 1992). Of those cows with severe gram-negative mastitic infections, nearly 25% will either die or be culled due to an exaggerated host inflammatory response resulting in systemic complications (Jackson and Bramley, 1983; Eberhart, 1984). The most common Gram-negative pathogens implicated in mastitis are *Escherichia coli*, *Klebsiella pneumoniae*, and various species of *Enterobacter* (Eberhart et al., 1979; Eberhart, 1984). A common denominator to all of these bacteria is the presence of endotoxin or LPS, which is found in the outer membrane of all gram-negative bacteria. Lipopolysaccharide is a highly proinflammatory molecule that is shed from the bacterial surface during bacterial replication or death. The bovine mammary gland is highly sensitive to LPS (Carroll et al., 1964; Mattila and Frost, 1989). Intramammary injection of LPS derived from *E. coli* 026:B6, 055:B5, or 0111:B4 induces mastitis (Jain et al., 1969; Paape et al., 1974; Mattila and Frost, 1989; Wang et al., 2002), and LPS is detectable in the milk of cows with coliform mastitis (Anri, 1989; Hakogi et al., 1989). Absorption of LPS into the blood during experimentally induced (Dosogne et al., 2002) and naturally occurring *E. coli* mastitis (Katholm and Andersen, 1992) has been reported. Further, a significant portion of cows with naturally occurring acute coliform mastitis develop bacteremia, thus introducing LPS directly into the circulation (Wenz et al., 2001).

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It is well established that the systemic inflammatory response that accompanies peracute coliform mastitis (Carroll et al., 1964; Schalm et al., 1964, 1979; Jackson and Bramley, 1983; Ziv, 1992) and gram-negative infection (Brandtzaeg et al., 1989) is mediated, in part, by LPS. Further, several of the proinflammatory cytokines that mediate the localized and/or systemic response to gram-negative mastitis, including IL-1 β , IL-6, IL-8, and TNF- α are upregulated by LPS (Shuster et al., 1993; Bierhaus et al., 2000; Guha and Mackman, 2001; Ohtsuka et al., 2001). The upregulation of these cytokines is mediated by LPS interaction with the accessory proteins, LPS-binding protein (**LBP**) and CD14 (Sweet and Hume, 1996; Guha and Mackman, 2001). It has been known for some time that circulating LPS binds to LBP, an acute-phase protein that facilitates the transfer of LPS to membrane-associated CD14 (**mCD14**) found on cells of monocytic lineage and neutrophils (Schumann et al., 1990; Wright et al., 1990). mCD-14 is a glycosyl phosphatidylinositol-anchored protein that lacks an intracellular cytoplasmic domain, rendering it incapable of transducing a signal across the cell membrane. Further, several cell types, including epithelial and endothelial cells, are sensitive to LPS despite lacking mCD-14. In cells lacking mCD14, activation is dependent on cellular recognition of LPS-LBP complexes bound to circulating soluble CD14 (**sCD14**), the latter of which is derived from mCD14-bearing cells (Tapping and Tobias, 1997). Recently, Toll-like receptor (**Tlr**)-4 has been identified in both cells of monocytic lineage and non-mCD14-bearing cells as a LPS transmembrane receptor capable of activating cells (Chow et al., 1999; Faure et al., 2000). Although the exact mechanism of LPS recognition by Tlr-4 remains unclear, cell activation is dependent on the cell surface assembly of a multi-protein recognition complex consisting of CD-14, MD-2, and Tlr-4 (Akashi et al., 2000).

Recent investigations have demonstrated a role for sCD14 in mediating bovine host responses to mammary gland challenge with either LPS or *E. coli*. Lee et al. (2003b) have reported increases in milk sCD14 following mammary gland infusion with LPS, whereas others have demonstrated that sCD14 sensitizes mammary epithelial cells to LPS (Wang et al., 2002). In an experimental model of *E. coli*-induced mastitis, infusion of recombinant bovine sCD14 into mammary quarters enhanced both neutrophil recruitment to the mammary gland and resolution of the infection (Lee et al., 2003a). Although a role for CD14 in mediating gram-negative mastitis has been clearly established, a role for LBP remains unknown. Because LBP-LPS-CD14 complexes are involved in the activation of host innate immune responses to gram-negative infection, we decided to examine whether LBP is present in milk and whether

intramammary challenge with LPS can alter milk levels of LBP.

MATERIALS AND METHODS

Cows

Five healthy midlactating Holstein cows (164 to 207 d of lactation) were selected on the basis of milk SCC (**MSCC**) of <500,000 cells/ml and the absence of detectable bacteria growth from three daily consecutive aseptic milk samples plated on blood agar plates. To assay for MSCC, milk samples were heated to 60°C and subsequently maintained at 40°C until counted on an automated cell counter (Fossomatic model 90, Foss Food Technology, Hillerød, Denmark) as previously described (Miller et al., 1986).

Endotoxin Challenge

One hundred micrograms of LPS derived from *E. coli* 0111:B4 (Sigma Chemical Co., St. Louis, MO), which has previously been shown to experimentally induce mastitis (Wang et al., 2002; Lee et al., 2003b), was dissolved in 10 ml of 0.85% saline solution and sterile filtered. The LPS used in the present study was a highly purified preparation that was prepared by phenol extraction and further purified by ion-exchange chromatography. We have previously established that the bioactivity of this preparation is attributable directly to LPS and not contaminating virulence factors (Banerman et al., 2002). The left or front right quarters of each animal were infused with either 10 ml of saline alone or LPS (100 μ g), respectively. Quarter milk and plasma samples were subsequently collected over a 72-h period.

Whey and Plasma Preparation

For the preparation of whey, milk samples were centrifuged at 44,000 $\times g$ at 4°C for 30 min and the fat layer was removed with a spatula. The skimmed milk was centrifuged again for 30 min as above, and the translucent supernatant collected and stored at -70°C. Blood samples were obtained from jugular vein indwelling catheters as previously described (Paape et al., 1972) and collected in glass tubes containing K₂ EDTA (Becton-Dickinson Corp, Franklin, Lakes, NJ). For the determination of differential white blood cell counts, the freshly collected blood was inverted $\times 10$, placed on a rocker for 15 min, and analyzed using a HEMAVET 3700 automated multispecies hematology system (CDC Technologies, Inc., Oxford, CT). For subsequent analysis of blood samples, the freshly collected blood was inverted $\times 10$ in K₂ EDTA glass tubes, centrifuged at

1500 $\times$ *g* for 15 min, and the clear plasma supernatant was collected, aliquoted, and stored at -70°C .

ELISA for Bovine Serum Albumin

Milk BSA levels were assayed using a commercially available kit (Bethyl Laboratories, Inc., Montgomery, TX) according to the manufacturer's instructions with only slight modification. Briefly, flat-bottom 96-well plates were coated overnight at 4°C with 10 $\mu\text{g}/\text{ml}$ of sheep anti-bovine BSA diluted in 0.05 *M* sodium carbonate, pH 9.6. The plates were washed $\times 4$ with 0.05% Tween 20 diluted in 50 mM Tris-buffered saline (TBS), pH 8.0, and subsequently blocked with 2% fish skin gelatin (Sigma Chemical Co.) for 1 h at room temperature. Plates were washed, and 100 μl of diluted whey samples (1:15,000) was added to each well. Plates were incubated for 1 h at room temperature and subsequently washed as above. Sheep-anti-BSA conjugated to horseradish peroxidase (HRP) was diluted 1:60,000 in TBS wash buffer containing 2% gelatin, and 100 μl of this solution was added to each well. Plates were incubated for 1 h at room temperature, washed as above, and 100 μl of 3,3',5,5'-tetramethylbenzidine substrate solution added to each well. The reaction was stopped by the addition of 100 μl of 2 *M* H_2SO_4 and the absorbance read at 450 nm on a microplate reader (Bio-Tec Instruments, Inc., Winooski, VT). A background correction reading at 565 nm was subtracted from the 450-nm absorbance readings. The concentration of BSA was calculated by extrapolating from a standard curve.

ELISA for IL-8

Milk IL-8 levels were determined from undiluted whey samples assayed with a commercially available human IL-8 ELISA kit (R&D Systems, Inc., Minneapolis, MN). The antibody pairs used in this kit have been previously shown to cross-react with bovine IL-8 (Shuster et al., 1996, 1997). The optical density at 450 nm and a correction wavelength of 550 nm were measured on a microplate reader. Values expressed in picograms per milliliter were extrapolated from a standard curve of known amounts of human IL-8 using linear regression.

ELISA for sCD14

A sandwich ELISA was used to quantify sCD14 levels in milk. Flat-bottom 96-well plates were coated overnight with 5 $\mu\text{g}/\text{ml}$ of mouse anti-bovine CD14 monoclonal antibody (CAM36A clone; VMRD, Inc., Pullman, WA) diluted in 0.05 *M* sodium carbonate, pH 9.6 at 4°C . The plates were washed $\times 4$ with 0.05% Tween 20

diluted in 50 mM TBS, pH 8.0, and subsequently blocked with 2% fish skin gelatin (Sigma Chemical Co.) for 1 h at room temperature. Plates were washed and 100 μl of diluted whey samples (1:10) were added to each well. Plates were incubated for 1 h at room temperature and subsequently washed as above. Mouse anti-bovine CD14 antibody (MM61A clone; VMRD, Inc.) was conjugated to HRP using a commercially available kit (Pierce Chemical Co., Rockford, IL) and used as the detection antibody. This HRP-conjugated anti-bovine CD14 antibody was diluted 1:150 in TBS wash buffer containing 2% gelatin, and 100 μl of the resulting solution was added to each well. Plates were incubated for 1 h at room temperature, washed as above, and 100 μl of 3,3',5,5'-tetramethylbenzidine substrate solution added to each well. The reaction was stopped by the addition of 100 μl of 2 *M* H_2SO_4 and the absorbance read at 450 nm on a microplate reader. A background correction reading at 565 nm was subtracted from the 450-nm absorbance readings. Values expressed in micrograms per milliliter were extrapolated using linear regression from a standard curve of known amounts of recombinant bovine sCD14 (Wang et al., 2002).

ELISA for LBP

Milk and plasma LBP levels were determined with a commercially available LBP ELISA kit that cross-reacts with bovine LBP (Cell Sciences, Inc., Norwood, MA). Milk and plasma samples were diluted 1:400 and 1:1,500, respectively, and assayed according to the manufacturer's instructions. The optical density at 450 nm and a correction wavelength of 550 nm were measured on a microplate reader. The concentration of LBP was calculated by extrapolating from a standard curve of known amounts of human LBP.

Statistical Methods

A *t*-test or analysis of variance with the Tukey post hoc comparison test was used to compare the mean responses between a single experimental group and its control or among multiple experimental groups, respectively. All statistical analyses were performed using GraphPad InStat version 3.05 for Windows (GraphPad Software Inc., San Diego, CA). A *P*-value of < 0.05 was considered significant.

RESULTS

Intramammary Challenge with LPS Induces Both a Local and Systemic Inflammatory Response

Clinical signs of mastitis, including udder swelling and abnormal milk characterized by the presence of

Figure 1. Effect of lipopolysaccharide (LPS) intramammary challenge on daily milk weights and temperature. To establish a baseline, the daily milk weights (sum of morning and evening outputs) were collected for 3 d before LPS challenge and the amounts averaged (A). Daily milk weight data were then collected for each of the 3 d following LPS infusion. The vertical bars represent the mean $\pm$ SE of milk weights in kg. *Significantly decreased compared to all other time points ($P < 0.05$). As an indicator of the systemic response, rectal temperatures were measured immediately before and for various time points following intramammary LPS infusion (B). Mean ($\pm$ SE) temperature is reported in degrees Celsius. **Significantly increased compared to time 0 ($P < 0.05$).

clots and flakes, were evident within 6 h of LPS infusion. Daily milk weights for each cow dropped by ~25% on the day following LPS infusion and recovered to normal levels by d 2 (Figure 1A). Increased rectal temperatures were observed in all LPS challenged cows by 4 h and reached a peak of $41.5 \pm 0.13^\circ\text{C}$ by 6 h (Figure 1B). The systemic response was further characterized by a significant decrease in circulating leukocytes at 4, 6, and 8 h (Figure 2A and data not shown) consistent

Figure 2. Effect of intramammary infusion of lipopolysaccharide (LPS) on circulating leukocyte and milk SCC. Total white blood cell and neutrophil counts were determined in blood isolated from the jugular veins of five cows immediately before and for various time points after intramammary LPS infusion (A). Mean ($\pm$ SE) circulating leukocyte cell counts are reported in thousands/ μ l. *, **Significantly decreased or increased, respectively, compared to time 0 ($P < 0.05$). In parallel to blood collection, milk samples were obtained from the left and right front mammary quarters challenged with either saline or LPS, respectively (B). Mean ($\pm$ SE) milk SCC are reported in millions/ml. #Significantly increased compared to saline control at the same time point ($P < 0.05$).

with previous reports (Paape et al., 1974; Saad and Ostensson, 1990). Neutropenia was detected as early as 2 h and circulating neutrophil counts remained below baseline levels for up to 8 h post-LPS infusion (Figure 2A). The decrease in circulating neutrophils was accompanied by an increase in MSCC >2 h post-LPS challenge (Figure 2B). Because neutrophils are the predominant inflammatory cell component of milk somatic cells during mastitis (Saad and Ostensson, 1990; Miller et al., 1993), these data are consistent with neutrophil recruit-

Figure 3. Intramammary infusion of lipopolysaccharide (LPS) increases mammary vascular permeability and IL-8 production. As a marker of blood-mammary gland barrier function and mammary gland proinflammatory responsiveness, milk BSA (A) and IL-8 levels (B), respectively, were assayed by ELISA. Milk samples were obtained from quarters immediately before and for varying time points following saline or LPS (100 μ g) infusion. Mean ($\pm$ SE) BSA and IL-8 levels are reported in mg/ml and pg/ml, respectively. *Significantly increased compared to saline control at the same time point ($P < 0.05$).

ment to the inflamed quarter. The increase in MSCC peaked at 12 h post-LPS infusion to a total count of 92.69×10^6 cells/ml. In contrast to quarters receiving LPS, there was no change in MSCC in saline control quarters.

LPS-Induces Disruption of the Blood-Milk Barrier and Stimulates IL-8 Production in the Mammary Gland

To assay for the integrity of blood-mammary gland barrier function, milk samples were assayed for BSA using an ELISA (Figure 3A). In quarters receiving sa-

Figure 4. Lipopolysaccharide (LPS) infusion augments milk levels of sCD14. A sandwich ELISA was used to quantify sCD14 levels in milk obtained from quarters receiving either saline or LPS (100 μ g). Mean ($\pm$ SE) sCD14 is reported in μ g/ml. *Significantly increased compared to saline control at the same time point ($P < 0.05$).

line alone, there was no significant change in BSA throughout the study period. In contrast, milk obtained from quarters receiving LPS showed a marked increase in BSA levels within 2 h of challenge. Lipopolysaccharide challenge resulted in a >10-fold increase in milk BSA levels, and this increase persisted throughout the first 24 h following LPS infusion. At >24 h post-LPS infusion, milk BSA levels declined and returned to baseline levels by 72 h. The rapidity of BSA influx into the gland, the persistence of elevated BSA levels, and the peak amounts of BSA present in milk (3.14 ± 0.17 mg/ml) following LPS challenge are all consistent with a previous report (Shuster et al., 1993).

As a marker of the proinflammatory response initiated by LPS, milk IL-8 levels were measured (Figure 3B) (Lee et al., 2003b; Waller et al., 2003). In saline control quarters, there was no detectable IL-8 at any time points assayed. Quarters receiving LPS demonstrated a significant increase in IL-8 as early as 4 h and reached a peak of 412 ± 48 pg/ml by 6 h postinfusion. Elevated levels of IL-8 persisted until 8 h post-LPS infusion, after which the levels returned to baseline.

Intramammary Challenge with LPS Increases Milk sCD14 Levels

sCD14 is a key accessory molecule that enables both host recognition and neutralization of LPS (Tobias and Ulevitch, 1994; Wurfel and Wright, 1995). To determine whether LPS could alter mammary gland levels of sCD14, a sandwich ELISA was used to quantitate milk sCD14 (Figure 4). Mammary gland quarters receiving

saline alone showed no change in sCD14 levels throughout the study. In contrast, significant increases in sCD14 were evident within 10 h of LPS infusion, and this increase persisted for an additional 38 h. Maximal levels of sCD14 ($20.56 \pm 3.99 \mu\text{g/ml}$) were observed 24 h post-LPS challenge and remained elevated relative to levels in saline-infused quarters for an additional 24 h.

Increased Levels of LBP in Milk Following Intramammary Challenge with LPS

Because elevated levels of sCD14 were present in milk following LPS challenge, and LBP is known to act in concert with sCD14, we decided to look at whether milk levels of LBP were similarly increased, thereby providing an environment for optimal host recognition of LPS. We also assayed for plasma levels of LBP because hepatic synthesis of this protein is greatly augmented during the acute-phase response. Under basal conditions, LBP was detected in both bovine blood and milk at concentrations of 37.79 ± 7.2 and $6.24 \pm 0.85 \mu\text{g/ml}$, respectively. Within 8 h of LPS challenge, increased levels of circulating LBP relative to time 0 were observed in plasma, and this increase persisted throughout the entire study period (Figure 5A). Plasma LBP levels reached a maximum level of $137 \pm 3.78 \mu\text{g/ml}$ 24 h post-LPS infusion after which the levels began to decline. In quarters receiving LPS, significant elevation of milk LBP was observed as early as 12 h post-LPS challenge and persisted for another 36 h. Even 3 d after LPS infusion, there was a large difference in the mean milk LBP levels between quarters receiving saline alone or LPS; however, this change failed to reach statistical significance ($P = 0.0532$). Peak levels of milk LBP ($34 \pm 3.4 \mu\text{g/ml}$) were observed 24 h postinfusion, a time point that was temporally coincident with maximal elevation of plasma LBP levels.

DISCUSSION

Bacterial LPS contains a conserved motif that is recognized by pattern recognition receptors involved in innate immunity (Aderem and Ulevitch, 2000; Akira et al., 2001). Recognition of LPS contributes to host cell detection of invading gram-negative pathogens and leads to the elicitation of an effective host immune response. Cellular recognition of LPS is mediated, in part, by Tlr-4, a member of the larger family of Tlr transmembrane receptors involved in innate immunity (Poltorak et al., 1998). Although the exact mechanism by which LPS is recognized by Tlr-4 remains unclear, cell activation is dependent on the cell surface assembly of a multiprotein recognition complex consisting of mCD-14, MD-

Figure 5. Intramammary challenge with lipopolysaccharide (LPS) increases plasma and milk levels of LPS-binding protein (LBP). Blood (A) and milk (B) samples were collected immediately before and for various time points following intramammary LPS infusion and assayed for LBP by ELISA. *Significantly increased compared to time 0 ($P < 0.05$). **Significantly increased compared to saline control at the same time point ($P < 0.05$).

2, and Tlr-4 (Akashi et al., 2000). Lipopolysaccharide presentation to this multiprotein complex is mediated by the acute-phase protein, LBP (Tobias et al., 1999; Schumann and Latz, 2000). Cells lacking mCD14, including epithelial and endothelial cells, are capable of recognizing and responding to LPS (Bannerman and Goldblum, 2003). In the absence of mCD14, LBP facilitates the transfer of circulating LPS to sCD14, and this complex is recognized by Tlr-4 (Tobias et al., 1999). Therefore, a common denominator to host cell recognition of LPS is the presence of CD14 as either a membrane-bound or soluble molecule and LBP.

mCD14 is a 55-kDa glycoprotein found on cells of monocytic origin and to a lesser extent on neutrophils

(Viriyakosol and Kirkland, 1995). sCD14 is derived from monocytes by direct exocytosis and from proteolytic cleavage of mCD14 on the cell surface. Although LPS directly binds CD14, this process is greatly enhanced by LBP (Hailman et al., 1994). Lipopolysaccharide-binding protein is a 58- to 60-kDa protein synthesized primarily by hepatocytes in response to IL-1 and/or IL-6 (Tobias et al., 1999; Schumann and Latz, 2000). Lipopolysaccharide-binding protein is a lipid transfer molecule that catalyzes the transfer of LPS to CD14. The principle mechanism by which this occurs is through the ability of LBP to dissociate LPS aggregates into LPS monomers, the latter of which are transported by LBP to CD14. In addition, LBP has recently been shown to stabilize the association of LPS and CD14 by forming a tripartite complex (Thomas et al., 2002).

Several lines of evidence suggest a protective role for CD14 and LBP in mediating the host response to LPS and gram-negative infection. First, studies involving LBP-/- or CD14-/- mice revealed that these mice are more susceptible to the lethal effects of *Salmonella typhimurium* infection than wild-type control mice (Jack et al., 1997; Bernheiden et al., 2001; Yang et al., 2002; Fierer et al., 2002). Second, mice pretreated with neutralizing CD14 or LBP antibodies are more prone to develop an overwhelming infection and die when challenged with *K. pneumoniae* than control mice (Le Roy et al., 2001). In an additional study, rabbits receiving an intestinal challenge of *Shigella flexneri* and an i.v. dose of anti-CD14 neutralizing antibodies exhibited more severe tissue destruction and increased bacterial invasiveness than similarly challenged rabbits receiving an i.v. infusion of saline (Wenneras et al., 2001). Third, administration of exogenous LBP has been demonstrated to protect mice from septic shock elicited by either LPS or *E. coli* infection (Lamping et al., 1998). Finally, a study involving patients with severe sepsis and septic shock revealed that survivors had higher levels of LBP than nonsurvivors (Opal et al., 1999). Together, these data implicate a critical role for LBP and CD14 in mediating innate immune responses to gram-negative bacteria and a component of these bacteria, namely LPS.

The mechanism by which LBP and CD14 confer protection is primarily through the ability of LBP to facilitate LPS transfer to CD14, whereby LPS-CD14 complexes activate Tlr-4 signaling cascades leading to host cell activation and response to the gram-negative pathogen (Mathison et al., 1992; Schutt, 1999; Ulevitch and Tobias, 1999; Schumann and Latz, 2000). In fact, several of the studies utilizing either CD14 or LBP knockout mice or neutralizing antibodies reported that decreased availability of these proteins correlated with: 1) decreased production of the proinflammatory cyto-

kines TNF- α and IL-6 (Le Roy et al., 2001; Yang et al., 2002); 2) decreased chemokine production (Fierer et al., 2002); 3) impaired host cell oxidative burst (Jack et al., 1997); and 4) delayed neutrophil migration to the site of infection (Le Roy et al., 2001; Yang et al., 2002; Fierer et al., 2002).

Although detection of LPS is a key event in the activation of the innate immune response to gram-negative bacteria, excess LPS signaling can lead to an exaggerated host response culminating in the development of septic shock (Dinarello, 1997). The results of several studies suggest that the ability of CD14 and LBP to bind LPS may aid in the detoxification of this molecule, thus diminishing its ability to stimulate an excessive proinflammatory response that can be deleterious to the host itself. The LBP has been shown to facilitate LPS transfer to lipoproteins resulting in neutralization of the ability of LPS to induce an inflammatory response (Munford et al., 1981; Flegel et al., 1993; Levine et al., 1993; Hubsch et al., 1993; Wurfel et al., 1994; Wurfel and Wright, 1995). In the presence of sCD14, LPS transfer to high-density lipoprotein is greatly increased, and studies have shown that LPS/LBP complexes bind to CD14 before LPS transfer (Wurfel et al., 1995). Recently, LBP has been demonstrated to facilitate LPS transfer to chylomicrons, an event that leads to both detoxification and enhanced removal of circulating LPS (Harris et al., 1990, 1993; Vreugdenhil et al., 2003). Together, these data suggest that LBP and sCD14 have a dual protective role, one that activates the innate immune response and one that moderates this response from becoming excessive and deleterious to the host itself.

Much of the current understanding of the function of LBP and sCD14 comes from in vitro data and mouse models. Investigations examining LBP and sCD14 in the bovine inflammatory response have been lacking. A recent study implicates a potential role for sCD14 in mediating bovine mammary gland response to infection. In that investigation, coadministration of sCD14 with an inoculum of *E. coli* was demonstrated to enhance mammary gland clearance of the bacteria. Further, sCD14 was recently identified in bovine milk, and milk levels of sCD14 were shown to rise following challenge with either LPS or *E. coli* infection (Lee et al., 2003a, 2003b).

Because LBP has been well established to work in concert with sCD14, we looked at whether intramammary challenge with LPS could influence blood and milk levels of LBP. Increased levels of circulating LBP were first observed 8 h following LPS challenge. The increase in LBP was preceded by an increase in core body temperature, the latter of which is a marker of the acute-phase response. The increase in LBP following a rise

in body temperature is consistent with hepatic synthesis of LBP during the acute-phase response. A peak circulating concentration of 137 $\mu\text{g/ml}$ of LBP was observed 24 h after LPS challenge and is consistent with levels of LBP that have been reported to range up to 162 $\mu\text{g/ml}$ in human patients with sepsis (Opal et al., 1999).

Similar to plasma, milk levels of LBP increased following LPS challenge. Detectable increases in milk LBP were observed 2 h after initial increases in blood LBP, and maximal levels of LBP in milk were observed at 24 h post-LPS infusion, a time point temporally coincident with peak levels of plasma LBP. The increases in milk LBP paralleled increments in sCD14 levels. From a host perspective, the simultaneous increase in both LBP and sCD14 levels would be expected to be advantageous as both molecules act in concert to facilitate activation of host defense mechanisms by presenting LPS to the transmembrane LPS receptor, Tlr-4 (Bannerman and Goldblum, 2003). Furthermore, these molecules have been shown to bind and neutralize excess LPS (Wurfel et al., 1995). Interestingly, maximal levels of the chemoattractant IL-8 were observed before increases in either milk LBP or sCD14, suggesting that initial host cell activation can take place in the presence of basal levels of sCD14 and LBP. Furthermore, neutrophil influx as determined by MSCC were similarly elevated before increments in milk sCD14 and LBP levels demonstrating that heightened levels of these molecules are not required for immediate host innate immune responses. However, peak levels of MSCC were not observed until 12 h post-LPS challenge, a time point at which levels of both sCD14 and LBP were elevated. Whether increments in sCD14 and LBP are necessary for maximal recruitment of neutrophil to the inflamed mammary gland remains unknown. The sCD14 and LBP have been shown to enhance LPS-induced neutrophil adhesion and priming of neutrophil superoxide release (Shapira et al., 1995; Hailman et al., 1996; Troelstra et al., 1997). Thus, enhanced levels of sCD14 and LBP in the gland at the time of maximal neutrophil recruitment may serve to enable these cells to combat invading pathogens.

The actual source of the increased sCD14 and LBP in the milk remains unknown. It is well described that during the acute-phase response to infection the proinflammatory cytokines IL-1 and IL-6 stimulate hepatocyte synthesis of LBP and that liver-derived LBP constitutes the majority of circulating LBP (Schumann et al., 1990; Ramadori et al., 1990; Tobias et al., 1992; Grube et al., 1994). In the present study, circulating levels of LBP preceded the increase in milk LBP. Furthermore, the increase in milk LBP occurred at a time when milk BSA levels were also raised. Because heightened milk BSA levels are a marker of increased mammary vascu-

lar permeability, these findings are consistent with vascular flux of LBP into the mammary gland. Interestingly, respiratory and intestinal epithelial cells have been demonstrated to produce LBP in response to certain proinflammatory cytokines such as IL-1, IL-6, and TNF- α (Vreugdenhil et al., 1999; Dentener et al., 2000). Because these cytokines are similarly elevated in the mammary gland following LPS or *E. coli* challenge (Shuster et al., 1996; Shuster et al., 1997; Waller et al., 1997; Waller et al., 2003), it is possible that the mammary epithelium may serve as a source of LBP.

Similar to LBP, the origin of the increased sCD14 in milk remains unclear. Lee et al. (2003a,b) have suggested that the elevated levels of sCD14 observed in milk following LPS challenge result not from leakage from the vascular compartment, but rather from shedding of neutrophil mCD14. Alternatively, mammary epithelial cells have been shown to upregulate sCD14 (Labeta et al., 2000; Vidal et al., 2001). Thus, these cells may serve as an alternate source of sCD14. Further investigations will be needed to confirm the origin of the elevated LBP and sCD14 detected in the milk.

In the present study, we have demonstrated an increase in the acute-phase protein, LBP, in the plasma and milk of cows challenged with LPS. To our knowledge, this is the first report demonstrating: 1) the presence of LBP in milk under physiological conditions, 2) a rise in plasma and milk LBP following LPS challenge, and 3) a parallel increase in milk LBP and sCD14 following LPS challenge. Further studies will be needed to identify the potential sources of the elevated LBP and sCD14 in milk and to determine the role(s) of these molecules in mediating mammary gland innate immune responses to infection and inflammatory mediators.

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