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INTERPRETATIVE SUMMARY

Short Communication:

Differential immunoglobulin transfer during mastitis challenge by pathogen-specific components. By Wellnitz et al.

This study investigated the effects of the major pathogenic components lipopolysaccharide of *E. coli* and lipoteichoic acid of *S. aureus* on blood-milk barrier permeability. The transfer of immunoglobulins and lactate from blood into milk was measured during a quantitatively similar level of mammary inflammation, based upon SCC increase. The results show a more efficient transfer of the blood components IgG$_2$ and lactate into milk after intramammary challenge with lipopolysaccharide from *E. coli* than with lipoteichoic acid from *S. aureus*.

Such a pathogen-specific difference may play some role in the course and severity of mastitis.

PATHOGEN-SPECIFIC BLOOD-MILK BARRIER OPENING
Short Communication:

Differential immunoglobulin transfer during mastitis challenge by pathogen-specific components

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Abstract

Mastitis induced by *E. coli* is often characterized by severe clinical signs indicating a more powerful combat of the immune system against the pathogen compared to *S. aureus* infections that are often represented by chronic and subclinical diseases. The aim of this study was to test the major pathogenic components lipopolysaccharide (LPS) from *E. coli* and lipoteichoic acid (LTA) from *S. aureus* for their effects on blood-milk barrier integrity and the related transfer of immunoglobulins and lactate from blood into milk. A similar SCC increase was achieved by intramammary challenge of one quarter of five cows with 20µg of LTA, and eight cows with 0.2µg LPS (maximum log SCC/ml: 7). Milk immunoglobulin (Ig) G1 concentrations increased in LPS but not in LTA challenged quarters. Milk IgG2 concentrations were increased (P<0.05) in treated quarters at 3 h after LPS, and 6 h after LTA challenge. Higher maximum levels of IgG2 were reached in milk of LPS treated quarters (173±58 µg/ml) than of LTA challenged quarters (62±13µg/ml). IgG1 and IgG2 levels did not change in control quarters. L-lactate concentrations in milk increased 4 h after LPS and 5 h after LTA challenge and reached higher maximum levels in LPS (221±48 mg/L) than in LTA treated quarters (77±18mg/L). In conclusion, a mammary inflammation on a quantitatively similar level based on SCC increase achieves a more efficient transfer of blood components like IgG2 via the blood-milk barrier if induced by LPS from *E. coli* than by LTA from *S. aureus*. This pathogen-specific difference may play an important role in the cure rate of the respective intramammary infection which is usually lower in *S. aureus* than in *E. coli* induced mastitis.
Intramammary infection with *E. coli* usually causes acute clinical mastitis (Hogan and Smith, 2003) indicating a powerful combat of the immune system against the pathogen. In contrast, intramammary *S. aureus* infections are often characterized by chronic and subclinical diseases (Sutra and Poutrel, 1994), and the pathogen seems to be able to prevent a significant activity of the immune system. Lipoteichoic acid (LTA) and lipopolysaccharide (LPS) are cell wall components of *S. aureus* and *E. coli*, respectively, which are generally accepted to be major bacterial components that induce the mammary immune defense. These cell wall components are experimentally used to investigate the mammary immune response (Schmitz et al., 2004; Werner-Misof et al., 2007; Rainard et al., 2008). Choosing dosages to standardize the immune response quantitatively based on a similar SCC increase allowed the study of qualitative differences between these pathogenic components (Wellnitz et al., 2011). Differences in the induction of the mammary immune response by intramammary challenge with LPS and LTA were shown by a different induction of expression of different immune factors (Wellnitz et al., 2011), which most likely plays a role in the development of different mastitis severities. During the inflammation of the mammary gland a massive leakage of blood constituents into milk occurs due to blood milk barrier alteration (Burton and Erskine, 2003). Besides SCC, the concentrations of a number of other parameters increase in milk in response to inflammation of the mammary gland. Not all of these parameters may contribute to the immune response. Immunoglobulin (Ig) G is the major immunoglobulin in ruminant milk (Butler, 1983). The subclass IgG₁ is the predominant antibody type in milk from healthy quarters because of an active, selective IgG₁ transport across the blood milk barrier via the FcRn receptor system (Baker et al., 2009). In mastitic milk IgG₂ becomes the predominant antibody (Caffin and Poutrel, 1988). It is considered to be the main opsonin supporting neutrophil phagocytosis in the bovine mammary gland, and is, therefore, playing an important role in the combat against mastitis pathogens (Burton and Erskine, 2003).
L-Lactate (hereafter referred to as lactate) is another blood component that increases in milk during mastitis and is proposed to be used as an early indicator to detect mastitis (Davis et al., 2004). Leukocytes as a source of lactate in milk during an immune response has been considered (Davis et al., 2004). Recently we described the blood as a major source of milk lactate that leaks into milk as a result of the impairment of the blood-milk barrier during the immune response (Lehmann et al., in press).

The aim of this study was to investigate the transfer of immunoglobulins and lactate from blood into milk due to a change of the blood-milk barrier integrity after an intramammary challenge with LPS from *E. coli* and LTA from *S. aureus* with a comparable SCC increase in milk.

In 13 dairy cows a similar SCC increase (maximum log SCC/ml 7) was achieved by intramammary challenge of one quarter with 20µg of LTA (n=5) from a *S. aureus* strain that induced a chronic bovine mastitis, or with 0.2µg LPS (n=8) from *E. coli* that induced an acute bovine mastitis, as previously described (figure 1; Wellnitz et al., 2011). In hourly taken plasma (jugular vein) and milk samples (~10 mL) from challenged and control quarters, IgG$_1$ and IgG$_2$ concentrations were analyzed using ELISA (Bovine IgG1/IgG2 ELISA Quantitation Set; Bethyl Laboratories Inc., LuBioScience, Luzern, Switzerland). The procedure was performed according to manufacturer’s protocol. A blocking reagent consisting of fish gelatin [1ml of fish skin gelatin (G7765; Sigma Aldrich, Steinheim, Germany) in 20ml of bidistilled water] was used to avoid matrix effects. Coefficients of variations, calculated by using a control sample on each plate, was 10 and 20% within, and between assays, respectively.

Lactate concentrations were measured using the test kit Lactate PAP (bioMérieux, Marcy l’Etoile, France) with an automated analyzer (COBAS MIRA, Roche Diagnostics, Switzerland) according to manufacturer’s instructions.

Data are presented as means ± SEM. Lactate concentrations are presented and statistically evaluated on a logarithmic scale (log10) to ensure normal distribution. Differences within
treatment group to time point 0 and between LPS and LTA treatments within each time point (hourly) were tested for significance (P < 0.05) by ANOVA using a MIXED procedure of SAS (1999–2001, release 8.02; SAS Institute Inc., Cary, NC). The model included time, treatment, and their interaction as fixed effects, and quarter within cow as repeated subject. A Tukey-Kramer adjustment was used to compensate for multiple comparisons. The significant (P < 0.001) Pearson’s correlation coefficient (SigmaPlot v11, Systat Software Inc.) between SCC and IgG1 and IgG2 in LPS challenged quarters was 0.42 and 0.33, respectively, and 0.45 and 0.68 between SCC and IgG1 and IgG2 in LTA challenged quarters, respectively.

In blood IgG1 and IgG2 concentrations were 16.5±1.1 mg/mL and 35.4±6.8 mg/mL, respectively, and did not change throughout the experiment. Although IgG concentrations in serum are known to be variable due to different factors like age and lactational stage (Mallard et al., 1983) this are relatively high values compared to other studies where concentrations around 10mg/mL are found for both Ig (Butler, 1983; Caffin and Poutrel, 1988). Reasons for that remain unclear. The test kits were validated according manufacturers recommendation.

Milk IgG1 concentrations (figure 2 A) were 68±6, 63±5, and 83±12 µg/mL in control, LPS and LTA challenged quarters before (0 h) challenge, respectively. In LPS challenged quarters IgG1 concentrations increased at 4 and 5 h and from 7 h after challenge until the end of the experiment. The maximum of 105±13 µg/mL was reached 5 h after challenge. In control and in LTA challenged quarters milk IgG1 concentrations did not significantly increase.

Milk IgG2 concentrations (figure 2 B) were 30±6, 32±8, and 23±8 µg/mL, in control, LPS and LTA challenged quarters before (0 h) challenge, respectively. Milk IgG2 concentrations increased at 3h in LPS challenged quarters, reached the maximum of 173±58 µg/mL at 6 h after challenge, and stayed elevated until the end of the experiment. In LTA challenged quarters IgG2 was increased at 6 h, reached a maximum of 67±9 µg/mL at 8 h and stayed elevated until 11 h after challenge. In control quarters milk IgG2 concentrations did not significantly increase.
The present study clearly shows that LPS of *E. coli* obviously opens the blood-milk barrier to a greater extent than LTA from *S. aureus* despite a similar SCC increase in response to both treatments. This mechanism could be involved in the mainly chronic development of mastitis by *S. aureus* because an insufficient amount of antibodies and other immune factors from blood are transferred into milk due to a reduced opening of the blood-milk barrier induced by these bacteria.

Concentrations of IgG₂ in milk raised to a greater extend (5fold after LPS challenge) than IgG₁ (3fold after LPS challenge) although these molecules have comparable molecular weights of approximately 160kDa (Butler, 1983) and should be able to pass the blood-milk barrier in a comparable way. The IgG₁:IgG₂ ratio in milk at 5 h after LPS challenge was 1:1.5 while the ratio in blood was 1:2.1. This is mainly due to the specific transport of IgG₁ (Mayer et al., 2005) that was also responsible of a 2.5:1 ratio in milk before LPS challenge. In addition, a specific transport of IgG₂ was discussed (Newby and Bourne, 1977) and binding of IgG₂ to mammary epithelial cells has been reported (Sasaki et al., 1977). However, during lactation of the healthy mammary gland, a passive transfer of IgG₂ from the blood is assumed (Guidry et al., 1980).

As IgG₂ plays a particular role in the mammary immune defense the availability of antibodies in the milk can be crucial for mastitis defense, specifically if antibodies against the invaded mastitis pathogens are available in the blood. Although IgG₂ are also entering the milk bound to PMN (Butler, 1983) the majority of milk IgG₂ that appear in milk during mammary inflammation leak into mammary gland from the blood (Burton and Erskine, 2003). That differences of the IgG₂ content in the milk during mastitis can depend on the pathogen has been reported (Caffin and Poutrel, 1988).

Lactate is another blood component whose appearance in milk was tested with the intramammary LPS or LTA challenge. Blood lactate concentration was 54.9±1.2 mg/L before LPS challenge and did not change throughout the experiment. Lactate concentrations in milk
(figure 3) were increased at four hours after challenge in LPS treated quarters and five hours after challenge in LTA treated quarters and both stayed elevated until the end of the experiment. At four hours after challenge until 11 h after challenge the lactate concentrations in milk were higher in LPS than in LTA treated quarters. This greater increase of lactate in milk of quarters challenged with LPS compared to quarters challenged with LTA also shows a different characteristic in the change of blood-milk barrier permeability in response to E. coli when compared to S. aureus endotoxin. The small molecular size (90Da) of lactate facilitates transfer from blood into milk. However, milk concentration of lactate increased to up to 4.0 fold higher levels of those in blood (220.7±48.1 mg/L) at 8 h after LPS challenge and to 1.4 fold (76.8±18.3 mg/L) 10 h after LTA challenge. This effect was seen before (Lehmann et al., in press). It was suggested to be due to an additional lactate production and release during anaerobic metabolism by milk and epithelial cells in the gland (Silanikove et al., 2011) and/or to be an effect of an additional transporter system for lactate, which could be via aquaporins, since aquaporins transport lactate (Conde et al., 2010), and aquaporins are present in the bovine mammary gland (Mobasheri et al., 2011).

In conclusion, a mammary challenge equalized for SCC increases produces a greater, and differential permeability of the blood-milk barrier for immunoglobulin’s when induced by LPS from E. coli compared to LTA from S. aureus. Thus, a more efficient transfer of blood components like IgG2 is achieved with E. coli that predominatly induces acute and severe mastitis compared to LTA from S. aureus that is responsible for more chronic and subclinical mastitis. This effect could have an important influence on the cure rate of the respective intramammary infection, which is usually lower in S. aureus than in E. coli induced mastitis, specifically if antibodies against the mastitis pathogen are present in the blood, e.g after vaccination.
Acknowledgements

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References


Figure Legends

Figure 1:
Milk SCC in LPS challenged quarters (□; n=8), in LTA challenged quarters (●; n=5), and in control quarters (x; n=13). a, b, c: Means without common superscript letters are significantly different between groups within one time point (P <0.05). This graph was formerly published (Wellnitz et al. 2011).

Figure 2:
Milk immunoglobulin (Ig)G\textsubscript{1} (A) and IgG\textsubscript{2} (B) concentrations in LPS challenged quarters (□; n=8), in LTA challenged quarters (●; n=5), and in control quarters (x; n=13). *: indicates the first and subsequent time points with a significant (P < 0.05) difference to time 0. #: indicates significant (P < 0.05) differences between LPS and LTA challenged quarters within time point.

Figure 3:
Milk lactate concentrations in LPS challenged quarters (□; n=8), in LTA challenged quarters (●; n=5), and in control quarters (x; n=13). *: indicates the first and subsequent time point with a significant (P < 0.05) difference to time 0. #: indicates significant (P < 0.05) differences between LPS and LTA challenged quarters within time point.
Figure 1; JDS
Figure 2; JDS

A

**IgG**₁

Time after challenge (h)

0 2 4 6 8 10 12

μg/mL

0 50 100 150 200 250

B

**IgG**₂

Time after challenge (h)

0 2 4 6 8 10 12

μg/mL

0 50 100 150 200 250

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A

B
Figure 3:

**Lactate**

- Time after challenge (h) vs. log10 mg/L levels.
- Graph shows changes in lactate levels over time along with statistical annotations.

Figure 3; JDS