ABSTRACT

The effects of using an enterotoxigenic *Escherichia coli* vaccine on innate immune responses following intramammary infusion of lipopolysaccharide (LPS) were investigated in midlactation Holstein-Friesian cows. Seven out of 14 cows were inoculated with *E. coli* vaccine. Three weeks later, 100 μg of LPS dissolved in 10 mL of saline was infused into 1 quarter of all cows. Milk was collected every hour from infusion to 12 h after infusion, and twice daily (at 09:00 and 16:00 h) for 4 d. Blood samples were collected 0, 4, 8, 24, 48, 72, and 96 h after infusion. Rectal temperatures and milk yields were measured. The somatic cell count (SCC), lingual antimicrobial peptide concentration, lactoperoxidase (LPO) activity, and lactoferrin (LF) concentration in milk, and haptoglobin concentration in serum were determined. The mean rectal temperature in vaccinated cows was higher than in control cows at 10 h. The mean milk yield was decreased significantly in the infused quarter of control cows at 24 h compared with pretreatment, but not in vaccinated cows. The mean SCC in milk from vaccinated cows at 12 and 55 h was significantly lower than that of control cows. The lingual antimicrobial peptide and LF concentrations were significantly lower at 8 h and 55 h, respectively, in vaccinated cows than in control cows. The mean antibody titer in the serum against the vaccine at the time of LPS infusion into vaccinated cows was significantly higher than in control cows. These antibody titers were positively correlated with the peak concentrations of LPO and LF in milk following challenge; therefore, cows with a high antibody titer were accompanied by high LPO activity and LF concentration in milk. These results suggest that vaccination suppresses the innate immune reaction after intramammary LPS infusion; however, the elevated antibody titer was unlikely to be responsible for the modification of the innate immune reaction.

Key words: mastitis, lipopolysaccharide, enterotoxigenic *Escherichia coli* vaccine, innate immunity

INTRODUCTION

Coliform mastitis is an IMI in dairy cows. The etiological agents include *Escherichia*, *Klebsiella*, and *Enterobacter* spp. Other gram-negative bacteria, such as *Serratia*, *Pseudomonas*, and *Proteus*, have also been isolated from infected udders (Hogan and Smith, 2003). The symptoms of mastitis vary from mild to severe. In severe cases, high fever, anorexia, and milk cessation are observed, and the outcomes are often death or culling. Thus, coliform mastitis is an important cause of mastitis that reduces milk production in dairy farming (Burvenich et al., 2003).

In Europe and North America, mastitis vaccines developed from *Escherichia coli* O111:B4 strain J5 (Overbeek et al., 1987) or *Salmonella typhimurium* Re-17 mutant (McClure et al., 1994) have been investigated for their efficacy (Hogan and Smith, 2003; Wilson et al., 2007). A previous study reported the use of an inactivated enterotoxigenic *Escherichia coli* (ETEC) vaccine, designed to prevent diarrhea, and to reduce death and culling due to mastitis (Morimoto, et al., 2011).

Lactoferrin (LF), lactoperoxidase (LPO), and lingual antimicrobial peptide (LAP) are known as innate immune components in bovine milk. Lactoferrin is an iron-chelating protein, and it is suggested that LF has a bacteriostatic effect (Reiter, 1978). Human LF binds to the lipid-A of bacterial LPS with high affinity and neutralize LPS (Legrand et al., 2004). The LF concentration was increased in milk from a mammary gland infused with LPS (Shuster et al., 1991; Hyvonen et al., 2010; Huang et al., 2012).

Lactoperoxidase is an enzyme with antimicrobial properties present in saliva, milk, tears, and airway secretions (Geiszt et al., 2003). Lactoperoxidase catalyzes the oxidation of the anion SCN⁻ to the antibacterial OSCN⁻ in the presence of H₂O₂ (Wijkstrom-Frei et al. 2012).
2003). Increased LPO activity in milk was observed after LPS challenge in dairy cows (Isobe et al., 2009b). A correlation exists between LPO activity and the SCC in milk, suggesting that LPO activity might be an indicator of SCC (Isobe et al., 2011).

Lingual antimicrobial peptide belongs to the β-defensin family and has a broad spectrum of antibacterial and antifungal activities (Schonwetter et al., 1995). Swanson et al. (2004) reported that the expression of the LAP gene was elevated in response to mastitis, and a positive relationship was observed between SCC in milk and LAP expression. Isobe et al. (2009b) reported that LAP concentration in milk increased significantly after LPS challenge; therefore, LAP is considered to play a role in the innate immune response to mastitis.

The acute-phase proteins include haptoglobin (Hp), C-reactive protein, serum amyloid A, and α 1 acid glycoprotein. Haptoglobin is a major acute-phase protein in ruminants and an effective indicator of mastitis (Eckersall and Bell, 2010). Haptoglobin is considered as a clinically useful parameter for measuring the occurrence and severity of inflammatory responses in cattle with mastitis (Murata et al., 2004; Suojala et al., 2008).

In coliform mastitis, LPS derived from the gram-negative bacterial cell wall is recognized by mammary epithelial cells and other immune cells such as macrophages and neutrophils (Bannerman et al., 2004). These cells synthesize cytokines, and local and systemic inflammatory reactions are induced. Intramammary LPS challenge has been frequently demonstrated as a model of coliform mastitis (Shuster et al., 1991; Bannerman et al., 2003; Lehtolainen et al., 2003). These acute-phase reactions are provided by innate immunity, which is considered to lack specific memory (Kurtz, 2005); however, E. coli J5 vaccine has been reported to reduce mastitis severity in cows with experimentally induced mastitis (Hogan et al., 1992, 1995; Wilson et al., 2007). The effect of ETEC vaccine inoculation on the innate immune reaction to intramammary LPS challenge has been hardly demonstrated.

The purpose of the present study was to elucidate the effect of ETEC vaccine on the innate immune reaction of the mammary gland after LPS challenge. We infused LPS into the udders of vaccinated and unvaccinated cows, and compared the clinical symptoms and components of blood and milk between these 2 groups.

**MATERIALS AND METHODS**

**Animals and LPS Challenge**

Fourteen lactating Holstein-Friesian clinically healthy cows 91 to 103 d after calving (11 primiparous and 3 multiparous) were used. These cows were fed a TMR and milked at 0900 and 1600 h. Seven cows were subcutaneously inoculated with 5 mL of ETEC vaccine (Imocolibov, Merial; Scientific Feed Laboratory Co. Ltd., Tokyo, Japan) 3 wk before LPS challenge, and the remaining 7 did not receive treatment. The ETEC vaccine used in this study was an inactivated aluminum hydroxide and saponin-added vaccine against neonatal colibacillosis of calves and lambs, supplied as a suspension for injection (Morimoto et al. 2011). The daily milk yield just before LPS challenge was 40.2 ± 2.7 kg (mean ± SE) in vaccinated cows and 33.6 ± 5.2 kg in control cows. The LPS solution (10 μg/mL) was prepared as previously described (Bannerman et al., 2003). All udder quarters were milked at 0900 h (0 h) and 10 mL of LPS solution was infused into 1 rear udder quarter. The SCC of all cows before LPS challenge were under 150,000 cells/mL. The SCC of vaccinated cows was 62,000 ± 18,000 cells/mL and that of control cows was 60,000 ± 17,000 cells/mL. Lipopolysaccharide challenge was performed in sequence when the cow was approaching 100 d from parturition. The cows were allocated to 2 groups alternatively. One to 4 cows were infused at the same time. The LPS challenge was performed from December to March to avoid the effect of heat stress. The cows were treated in accordance with the regulations of Hiroshima University (Hiroshima, Japan) for animal experiments.

**Clinical Examination and Sample Collection**

From infusion to 12 h after LPS infusion, 20 mL of milk was collected by hand every hour from the infused udder quarter. The other 3 quarters were milked at 1600 h on the infused day. Thereafter, all udder quarters were milked twice daily (at 0900 and 1600 h) for 4 d. The LPS-infused quarter was milked separately using a quarter milker at those times. Rectal temperature was measured at the time of milking. Blood samples were collected 0, 4, 8, 24, 48, 72, 96 h after LPS infusion from the jugular vein. A vacuum blood collection tube (VP-P100K; Terumo Corp., Tokyo, Japan) was used to obtain serum. After the blood sample had clotted, serum was separated by centrifugation at 1,500 × g for 10 min at 15°C, and stored at −20°C until use.

**Analytical Methods**

**Examination of Milk.** Somatic cell count was measured using Breed’s method (Prescott and Breed, 1910). Briefly, 2.5 μL of milk was spread in the square (1 cm²) on the slide glass and dried. After treatment with xylene and methanol, staining with Broadhurst and Paley stain (Broadhurst and Paley, 1939; Muto Pure Chemi-
cal Co. Ltd., Tokyo, Japan) was conducted. Cells in 50 views on the slide were counted under a microscope to calculate the SCC. Milk LPO activity was determined as previously described by Isobe et al. (2011). Lingual antimicrobial peptide and LF concentrations of milk were determined as previously described (Isobe et al., 2009a,b; Huang et al., 2012, respectively).

**Hp Concentration in Serum.** Haptoglobin concentration was measured by single radial immunodiffusion with a commercially produced test kit (Ecos Institute, Miyagi, Japan). An agar gel plate containing anti-Hp serum with test holes was provided. Test serum was mixed with an equal volume of 40 mM l-cysteine solution to resolve Hp. Five microliters of processed serum was applied to the hole in the plate and incubated at room temperature for 48 h. The diameter of the precipitin ring made by the antigen-antibody reaction was measured and the Hp concentration was calculated based on the standard curve, generated on a semilogarithmic graph by measuring standard solutions provided by the manufacturer.

**Antibody Titer Against ETEC Vaccine in Serum.** Antibody titer in the serum at 0 h was measured by enzyme immunoassay. A Holstein calf of 6 mo old was subcutaneously inoculated with ETEC vaccine 3 times at 2-wk intervals. Serum was obtained from 2 wk after the last immunization, and used as positive serum. The ETEC vaccine solution was concentrated by centrifugation at 1,500 × g for 20 min at 4°C. The precipitate was washed 3 times with PBS and resuspended with PBS containing 0.02% thimerosal (Nacalai Tesque Inc., Kyoto, Japan). After ultrasonication for 1 min, the optical density of the suspension was adjusted to 25% transmission at 610 nm. Enzyme immunoassay was performed as previously described (Tyler et al., 1991). Relative antibody titers to positive serum were determined and shown as a percentage.

**IgG Concentration in Serum.** IgG concentration in the serum at 0 h was measured by competitive enzyme immunoassay. One hundred microliters of 20 μg/mL goat anti-rabbit IgG antibody (Sato et al., 2011) in 0.05 M carbonate buffer was added to the wells of a 96-well microtiter plate and incubated at room temperature for 2 h. After washing the plate with PBS supplemented with 2% polyoxyethylene (20) sorbitan monolaurate (Tween 20; Nacalai Tesque Inc.; PBS-Tween), the plate was blocked with 0.3 mL/well of 0.05 M borate buffer supplemented with 0.2% BSA (pH 7.8) at room temperature for 30 min. Then 0.05-mL samples diluted 100,000 times, 0.05 mL of horse-radish peroxidase-labeled bovine IgG, and 0.05 mL of rabbit anti-bovine IgG antibody (Bethyl Laboratories Inc., Montgomery, TX) diluted 5,000 times with borate buffer were added and incubated at room temperature for 4 h. Horseradish peroxidase was conjugated with bovine IgG (MP Biomedicals LLC, Solon, OH) using a periodic acid method (Wilson and Nakane, 1978). After washing the wells 3 times with PBS-Tween, 0.15 mL of tetramethylbenzidine solution was added and incubated at room temperature for 30 min. The optical density was measured at 655 nm wavelength.

**Statistical Analysis**

Statistical analysis was performed with the computer software R (Ihaka and Gentleman, 1996) version 2.10.1 for Windows operating system, provided by the Comprehensive R Archive Network (http://cran.r-project.org/). Somatic cell count was converted to a common logarithm. We used the paired t-test to compare each measured value before and after LPS infusion, and the Student’s t-test to compare vaccinated and control cows. Significance was considered at P < 0.05. For correlation analysis, the data with the highest mean were used. The correlation among SCC, LPO, LAP, LF, the milk amount obtained from the infused udder quarter (Q-milk), Hp, and body temperature (BT) were calculated with the Pearson product-moment correlation coefficient, and a test for no correlation was performed. The correlation between the innate immune factors in milk (SCC, LPO, LAP, and LF) and the antibody titer or IgG concentration at 0 h were also analyzed. To equalize the milking interval, the amount of milk obtained from 7 to 12 h was added to the milk yield at 24 h. To compare the milk amount between vaccinated and control cows, the percentage to the amount at 0 h was used. These percentages at 24, 48, 72, and 96 h were compared with 0 h, and milk amounts at 1 to 6, 31, 55, 79, 103 h were not used for comparison.

**RESULTS**

All cows showed clinical mastitis in LPS-infused udder quarters, which were swollen and indurated. The milk became slightly yellowish and serous, and contained fibrin clots. The observed systemic signs, including fever of over 41°C, conjunctival hyperemia, and temporary anorexia had disappeared by 10 h after intramammary infusion.

**Body Temperature**

The mean rectal temperature (mean ± SE) before LPS infusion was 38.90 ± 0.09°C in control cows and 38.76 ± 0.06°C in vaccinated cows (Figure 1). The mean in both control and vaccinated cows exceeded 40°C at 3
The mean body temperature of vaccinated cows was significantly higher than that of control cows at 10 h.

**Milk Yield**

The mean milk yield (±SE) from the LPS-infused quarter at 0 h was 7.6 ± 0.8 kg in control cows and 7.5 ± 0.4 kg in vaccinated cows. The mean milk yield from the infused quarter in control cows at 24 h was significantly lower than before LPS infusion, but not in vaccinated cows (Figure 2). No significant difference was observed in milk yield from the LPS-infused quarter between control and vaccinated cows.

**Components of Milk and Blood**

The mean SCC, LPO, LAP, and LF concentrations in milk and the Hp concentration in serum were significantly increased in comparison with before LPS infusion (Figure 3). The mean SCC of vaccinated cows was significantly lower than that of control cows at 12 and 55 h (Figure 3a). The mean LAP and LF concentrations were significantly lower at 8 and 55 h, respectively, in vaccinated cows than in control cows (Figure 3c, d). On the other hand, no difference was observed in LPO activity in milk and Hp concentration in serum between control and vaccinated cows (Figure 3b, 4). The mean antibody titer against ETEC vaccine in the serum of vaccinated cows was significantly higher than in control cows, but not in overall IgG concentration (Figure 5).

**Correlation Among Test Data**

Because the mean SCC, LPO, LAP, LF, Q-milk, Hp, and BT peaked at 12, 31, 11, 48, 24, 48, and 5 h, respectively, data at those times were used for correlation analysis (Table 1). The mean LAP concentration at 3 h was higher than at 11 h, but no significant difference was observed in LAP concentration between 0 and 3 h. Thus, LAP data at 11 h was used for analysis. A significant positive correlation was found among SCC, LPO, and LF. A significant negative correlation existed between Q-milk and SCC, LPO, LAP, and LF. Haptoglobin was positively correlated with LF and BT. In control cows, a significant positive correlation was observed between SCC and IgG concentration ($P < 0.01$; Table 2). In vaccinated cows, a significant positive correlation was observed between antibody titer and LPO activity and LF concentration.

**DISCUSSION**

Lingual antimicrobial peptide and LF concentrations were significantly lower at 8 and 55 h, respectively, in vaccinated cows than in control cows. These results suggest that vaccination suppresses the secretion of innate immune factors in milk.

Conversely, the antibody titer against ETEC vaccine was higher in vaccinated cows than in control cows. In vaccinated cows, a significant positive correlation was observed between the antibody titer at 0 h and LPO...
at 31 h and LF at 48 h. Taken together, a high concentration of vaccine-specific antibody was associated with a high concentration of innate immune factors, probably because the ability of antibody production might indicate the immune function of cows; therefore, it is suggested that cows with high antibody production ability with one-shot vaccination tend to develop a strong innate immune reaction against LPS infusion. Thus, the antibody was unlikely to be responsible for the modification of the innate immune reaction, although the antibody titer was elevated, as expected.

The ETEC vaccine used in this study consists of 6 strains of *E. coli*. A specific antibody is produced in the blood following vaccine administration and is secreted into the colostrum. In Europe and North America, mastitis vaccines developed from *E. coli* O111: B4 strain J5 or *Salmonella typhimurium* Re-17 mutant have been investigated for their efficacy (Hogan et al.,...
From these reports, it was suggested that J5 vaccine inoculation may reduce the severity of coliform mastitis, and similarity exists with the present study. The mechanism of J5 vaccination against bovine coliform mastitis has been hypothesized. Dosogne et al. (2002) reported that J5 vaccination may reduce the severity of coliform mastitis by inducing mammary gland hyperresponsiveness by a T helper 1 response and mediated by memory cells inside the mammary gland; however, in this study, it was suggested that the innate immune reaction in vaccinated cows was milder than in unvaccinated cows. Thus, vaccination might prevent an excessive innate immune response to coliform mastitis; however, the mechanism of the reduction of this immune reaction is unknown.

In this study, the difference between control and vaccinated cows was slight in comparison with individual variation. This might have been a result of the cows investigated in this study, which were in the midlactation stage. The necessity of investigating cows in the early lactation stage was considered.

It was suggested that cows with a high IgG concentration tend to be associated with high SCC in control cows. In LPS-induced mastitis, the progress of disease associated with the growth of bacteria may not occur because viable bacteria were absent. In IMI with *Escherichia coli*, effective elimination of the pathogen by neutrophils is important for the resolution of infection and the outcome of mastitis (Burvenich et al., 2003). Thus, the transition of SCC may be important for the cow’s defense in the course of IMI, and it might be advan-

**Figure 4.** Haptoglobin (Hp) concentration in serum following intramammary challenge with LPS in vaccinated cows (▲: n = 7) and untreated controls (●: n = 7). * indicates significantly higher Hp concentration in comparison with prechallenge (P < 0.05). Error bars indicate the SEM.

**Figure 5.** Relative antibody titer against enterotoxigenic *Escherichia coli* (ETEC) vaccine and overall IgG concentration in serum at 0 h. Average ± SE (a) antibody titer in serum and (b) IgG concentration in serum (mg/mL) * indicates significant difference between groups (P < 0.05). Error bars indicate the SEM.

**Table 1.** Correlation coefficients among various innate immune factors and other factors

<table>
<thead>
<tr>
<th>Item</th>
<th>SCC</th>
<th>LPO</th>
<th>LAP</th>
<th>LF</th>
<th>Q-milk</th>
<th>Hp</th>
<th>BT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC (12)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LPO (31)</td>
<td>0.66*</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LAP (11)</td>
<td>0.42</td>
<td>0.81**</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LF (48)</td>
<td>0.64*</td>
<td>0.75**</td>
<td>0.47</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Q-milk (24)</td>
<td>—0.65*</td>
<td>—0.79**</td>
<td>—0.59*</td>
<td>—0.76**</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hp (48)</td>
<td>0.33</td>
<td>0.33</td>
<td>0.31</td>
<td>0.64*</td>
<td>—0.52</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>BT (5)</td>
<td>0.18</td>
<td>0.20</td>
<td>—0.14</td>
<td>0.34</td>
<td>—0.36</td>
<td>0.58*</td>
<td>—</td>
</tr>
</tbody>
</table>

1SCC = natural logarithm of SCC; LPO = lactoperoxidase activity (U/mL) in milk; LAP = lingual antimicrobial peptide concentration (nM) in milk; LF = lactoferrin concentration (μg/mL) in milk; Q-milk = percentage of milk from infused udder quarter to before LPS infusion; Hp = haptoglobin concentration (μg/mL) in serum; BT = body temperature (°C).

2Numbers in parentheses are hours postchallenge.

*P < 0.05; **P < 0.01 (statistically significant correlation between items).
Table 2. Correlation coefficients between various innate immune factors and antibody titer and IgG concentration in control and vaccinated cows.

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>Vaccinated</th>
<th>Antibody titer</th>
<th>IgG concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC (12)</td>
<td>0.54</td>
<td>0.71</td>
<td>0.88**</td>
<td>0.22</td>
</tr>
<tr>
<td>LPO (31)</td>
<td>0.20</td>
<td>0.77*</td>
<td>0.29</td>
<td>0.42</td>
</tr>
<tr>
<td>LAP (11)</td>
<td>−0.08</td>
<td>0.56</td>
<td>−0.05</td>
<td>0.24</td>
</tr>
<tr>
<td>LF (48)</td>
<td>0.12</td>
<td>0.87*</td>
<td>0.60</td>
<td>0.36</td>
</tr>
</tbody>
</table>

1Antibody titer = relative antibody titer against Escherichia coli (ETEC) vaccine in serum at 0 h; IgG concentration = serum IgG concentration (mg/mL) in serum at 0 h; SCC = natural logarithm of SCC; LPO = lactoperoxidase activity (U/mL) in milk; LAP = lingual antimicrobial peptide concentration (nM) in milk.

2Numbers in parentheses are hours postchallenge.

*P < 0.05; **P < 0.01 (statistically significant correlation between items in control or vaccinated cows, respectively).

Effect of E. coli J5 vaccine on innate immunity in bovine coliform mastitis.

REFERENCES


