Immunization of Dairy Cows with an *Escherichia coli* J5 Lipopolysaccharide Vaccine

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**ABSTRACT**

Development of a lipopolysaccharide-protein conjugate vaccine and the immunological response to the vaccine were investigated. Lipopolysaccharide derived from *Escherichia coli* J5 was detoxified by mild alkaline hydrolysis. Detoxification reduced endotoxin activity 2500-fold compared with that of native *J5* lipopolysaccharide. The conjugate vaccine was synthesized by covalently coupling detoxified lipopolysaccharide to chicken serum albumin by reductive amination. Dairy cows were immunized with 8.35 mg of conjugate (*n* = 3) or 5 × 10⁹ heat-killed J5 bacterin (*n* = 5) at 215 DIM and received a secondary immunization 14 d later. Control cows were not immunized. Immunization enhanced serum antibody titer to J5 lipopolysaccharide antigens. Whey IgG and IgM titers to J5 lipopolysaccharide were not different among treatment groups. Serum and whey IgG titers to J5 whole-cell antigens were elevated in immunized cows within treatment groups. Immunization did not enhance whey IgM to J5 whole-cell antigens. Conjugate immunization elicited an immune response comparable with or greater than that of immunized cows with J5 bacterin.

(Key words: mastitis, lipopolysaccharide, vaccine)

**INTRODUCTION**

Teat dip treatment and dry cow therapy for mastitis control are ineffective in controlling coliform mastitis, primarily because of continuous exposure of teat ends to coliform pathogens (20). Control is achieved either by decreasing exposure of teat ends to pathogens or by increasing the resistance of the cow against coliform IMI (20). Active immunization against coliform bacteria is a method to control coliform mastitis because elimination of coliform bacteria from the environment is not economically feasible.

All coliform bacteria have an outer lipopolysaccharide (LPS) cell envelope. Core polysaccharide regions of LPS possess structural and antigenic homology among coliform bacteria (15). A mutant strain of coliform bacteria, *Escherichia coli* O111:B4 (J5), has been used to study immunity against core region antigens. Laboratory animals immunized with J5 bacterin produced core-specific antibodies that were crossreactive and protective against heterologous coliform infection (15, 23); protection was attributed to elevated antibody titer to J5. Elevated, naturally occurring antibody titer to J5 also was correlated with decreased risk of developing clinical coliform mastitis (21). Immunization of dairy cows with J5 bacterin reportedly increased antibody titer to J5 in serum and milk and reduced the rate and severity of clinical coliform IMI (7, 10, 12).

Core-specific antibody titer may need to be increased to decrease further the incidence of clinical cases and the severity of coliform mastitis. As an antigen, the J5 LPS molecule has the advantage of having a common antigenic structure among coliform pathogens.

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However, disadvantages such as endotoxicity and poor immunogenicity need to be addressed. Therefore, the objectives of this study were 1) to detoxify *E. coli* J5 LPS, 2) to synthesize an *E. coli* J5 LPS conjugate vaccine, and 3) to evaluate humoral immune response to immunization with conjugate and J5 bacterin.

**MATERIALS AND METHODS**

**Detoxification of *E. coli* J5 LPS**

*Escherichia coli* J5 LPS (Sigma Chemical Co., St. Louis, MO) was detoxified by mild alkaline hydrolysis (3). The LPS was dissolved in .15N NaOH (2 mg/ml) and incubated at 100°C for 2 h. After cooling to 24°C, the preparation was centrifuged (2500 x g for 30 min, 24°C). The supernatant was adjusted to pH 3.5 with 1N HCl, and free fatty acids were extracted three times with chloroform (aqueous solution:chloroform, 1:1, vol/vol). The aqueous phase was adjusted to pH 7 with 1N NaOH, extensively dialyzed against deionized distilled water at 4°C, and lyophilized.

Endotoxicity of native J5 LPS and detoxified J5 LPS (DLPS) was measured by Limulus amoebocyte lysate (LAL) assay (Pyrotell, Woods Hole, MA) as recommended by the manufacturer. Sensitivity of the LAL assay was based on endotoxin units (EU) of a control endotoxin standard (.03 EU/ml; Pyrotell). The concentration of endotoxin units was determined by multiplying the reciprocal of bracket endpoint dilution of the sample by .03 EU/ml. Weight of endotoxin per milliliter of sample was calculated by dividing the sample endotoxin units per milliliter by the potency of the control endotoxin standard (10 EU/ng). The extent of J5 LPS detoxification also was tested by intramammary infusion. Native J5 LPS or DLPS was suspended in sterile pyrogen-free water (10 µg/10 ml). The right front quarter of one cow was infused with native LPS, and the right front quarter of another cow was infused with DLPS. Rectal temperatures and aseptic milk samples were taken immediately before intramammary infusion and at 3, 6, 8, 14, 24, 48, and 72 h postinfusion. Collection and bacteriology of milk samples were described by Smith et al. (20). Milk SCC were determined by Coulter counter (Coulter Electronics, Hialeah, FL) and expressed as log₁₀ SCC/ml of milk.

**Vaccine Antigen Preparation**

Detoxified J5 LPS was covalently coupled to chicken serum albumin via adipic acid dihydrazide by the method of Chu et al. (2). Lyophilized DLPS was dissolved in .1 M sodium borate (20 mg of DLPS/ml of sodium borate; pH 8.5). Twenty milligrams of adipic acid dihydrazide and 20 mg of sodium cyanoborohydride were then added to this solution while stirring. The reaction vessel was flushed with N₂, sealed, and gently tumbled for 4 d at 25°C. The reaction mixture was then dialyzed extensively against deionized, distilled water at 4°C and lyophilized. This product was termed DLPS hydrazide.

Equal amounts of DLPS hydrazide and chicken serum albumin were dissolved in deionized, distilled water (20 mg/ml). The mixture was cooled to 4°C, and pH was adjusted to 5.6 with .1M HCl. While stirring, 1-ethyl-1-3-(3-dimethylaminopropyl) carbodiimide was added to a final concentration of 50 mM, and pH was maintained at 5.6 for 4 h. The reaction mixture was extensively dialyzed against deionized, distilled water and lyophilized. Protein content of the DLPS conjugate vaccine was determined by the method of Lowry (13) with chicken serum albumin as the standard. Carbohydrates were measured by phenol-sulfuric acid assay (5) with DLPS as the standard. The *E. coli* J5 bacterin was prepared by the method of Tyler et al. (22). Endotoxicity of the conjugate vaccine and bacterin was determined by LAL assay as described.

**Immunization and Sample Collection**

First and second lactation Holstein (n = 8) and Jersey (n = 5) cows, with no prior history of coliform mastitis, were assigned randomly by breed and parity into one of three treatment groups. One group of 5 cows was injected with J5 bacterin (5 x 10⁹ cells per dose), and one group of 3 cows was injected with J5 LPS conjugate (8.35 mg per dose). Because of lack of antigen and availability of cows at the time of immunization, only 3 cows were immunized with the conjugate vaccine. Conjugate vaccine (2.8 mg of conjugated DLPS) and bacterin preparations were suspended in 5 ml of sterile PBS and emulsified with 5 ml of Freund’s incomplete adjuvant (Difco Laboratories, Detroit, MI).
Cows were injected intramuscularly in both hips (5 ml per hip) on d 215 of lactation and received booster immunizations 14 d later. Cows were observed for adverse reactions at the injection site, and rectal temperatures were taken at 0, 12, 24, 48, and 72 h postimmunization. Five cows served as unimmunized controls. Serum and whey samples from all cows were collected on d 0, 7, 14, 21, 30, and 45 and on d 0, 14, and 30, respectively.

ELISA

Samples of serum and whey were tested by indirect ELISA (24). Heat-killed E. coli J5 (10⁹ cells/ml of PBS) or chicken serum albumin (25 μg/ml of PBS) coating antigens were bound to individual 96-well microtiter plates (Immulon-1, Dynatech Laboratories, Chantilly, VA) by 18 h of incubation at 37°C. Escherichia coli J5 LPS (10 μg/ml) was bound to microtiter wells by the method of Freudenberg et al. (6). Antibody isotypes were determined by peroxidase-labeled, goat anti-bovine IgG or goat anti-bovine IgM (Kirkegaard and Perry Laboratories, Gaithersburg, MD). End point titer was defined as the last dilution in which the optical density of sample wells exceeded the mean optical density of four control wells plus .05. Titer was expressed as the reciprocal of the end point dilution log₂.

Statistical Analysis

Data were analyzed by the general linear models procedure of SAS (18). Serum and whey antibody titers differed among treatment groups prior to immunization. Therefore, titer differences among treatment groups were tested by least squares analysis of covariance with the d 0 titer as the covariant. Comparisons of antibody titer within treatment groups across time were analyzed by Dunnett's T test (18).

RESULTS

Endotoxin activity of DLPS was 10⁴-fold less than native J5 LPS on a comparative weight basis (Table 1). Reactions of reciprocal serial 2-fold bracket dilutions of DLPS and J5 LPS to LAL assay were 8 × 10⁴ and 2 × 10⁶, respectively. Calculations of endotoxin concentration showed that DLPS contained 24 EU/ml (2.4 ng of endotoxin/ml), and J5 LPS contained 60,000 EU/ml (6000 ng of endotoxin/ml). Systemic response to intramammary infusion of DLPS or J5 LPS is shown in Figure 1. The cow that was infused with J5 LPS exhibited signs of pyrexia by 6 h (39.6°C) after challenge. Rectal temperature peaked at 8 h (40.7°C) and declined to normal by 14 h (38.8°C). Intramammary infusion with J5 LPS caused a 100-fold increase in SCC per milliliter of milk by 6 h postchallenge (5.11 to 7.03 log₁₀ SCC/ml) and remained elevated at 72 h (Figure 2). Intramammary infusion with DLPS did not cause an increase in rectal temperature or in milk SCC.

Results from the Lowry and phenol-sulfuric acid assay showed that the conjugate vaccine contained 66.5% protein and 33.5% DLPS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reciprocal of dilution</th>
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<td></td>
<td>0</td>
</tr>
<tr>
<td>LPS²</td>
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<tr>
<td>DLPS⁴</td>
<td>+</td>
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<tr>
<td>DDH₂O³</td>
<td>-</td>
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<tr>
<td>LRW⁵</td>
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¹Stock sample solutions were 100 ng/ml of the LAL reagent H₂O (LRW; Pyrotell, Woods Hole, MA).
²Escherichia coli J5 LPS (Sigma Chemical Co., St. Louis, MO).
³= Clot formation; - = absence of clot formation.
⁴Detoxified Escherichia coli J5 LPS.
⁵Deionized distilled H₂O.

IMMUNIZATION WITH CONJUGATE VACCINE

Reactions of conjugate vaccine and bacterin to LAL assay are shown in Table 2. Based on the reciprocal of serial 2-fold bracket dilutions, the conjugate vaccine (4 × 10^3) and J5 bacterin (4 × 10^4) were calculated to have 120 EU/ml (12 ng of endotoxin/ml) and 1200 EU/ml (120 ng of endotoxin/ml), respectively. Intramuscular injection with conjugate vaccine or J5 bacterin did not induce systemic reactions. Swelling was not observed at injection sites for the majority of immunized cows. Booster immunization induced swelling (5 to 6 cm) and serous exudate at injection sites for one cow that received the conjugate vaccine and for another cow that received J5 bacterin.

Serum antibody titers to E. coli J5 LPS for treatment groups are shown in Figures 3 and 4. Immunized cows had higher (P < .05) IgG antibody titer to J5 LPS on d 21, 30, and 45 than control cows (Figure 3). After immunization with the conjugate vaccine, serum IgG antibody titer to J5 LPS was higher (P < .05) on d 21 than that of cows immunized with bacterin (Figure 3). Cows had higher (P < .05) serum IgM antibody titer to J5 LPS 7 d after the initial conjugate immunization (Figure 4).

<table>
<thead>
<tr>
<th>TABLE 2. Reaction of Escherichia coli J5 bacterin and E. coli J5 lipopolysaccharide (LPS) vaccine to Limulus amoebocyte lysate (LAL) assay.</th>
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<tbody>
<tr>
<td><strong>Sample</strong></td>
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<tr>
<td>Bacterin1</td>
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<tr>
<td>LPS Vaccine3</td>
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<tr>
<td>LRW</td>
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1^10^9 heat-killed E. coli per milliliter of the LAL reagent H_2O (LRW; Pyrotell, Woods Hole, MA).

2_+ = Clot formation; - = absence of clot formation.

3_1.7 mg of LPS vaccine/ml of LRW.
Figure 3. Serum IgG titer to *Escherichia coli* 15 lipopolysaccharide (LPS) antigen. Cows were immunized with *E. coli* J5 bacterin (○; n = 5) or *E. coli* J5 LPS (△; n = 3) vaccine on d 0 and 14. Control cows (☆; n = 5) were not immunized. Values are expressed as covariant-adjusted least squares means of log₂ titer. Bars represent standard error of the mean.

Figure 4. Serum IgM titer to *Escherichia coli* 15 lipopolysaccharide (LPS) antigen. Cows were immunized with *E. coli* J5 bacterin (○; n = 5) or *E. coli* J5 LPS (△; n = 3) vaccine on d 0 and 14. Control cows (☆; n = 5) were not immunized. Values are expressed as covariant-adjusted least squares means of log₂ titer. Bars represent standard error of the mean.

The IgM titer on d 21 and 30 increased (P < .05) after booster immunization (d 14), and titer returned to control values by d 45. Cows immunized with bacterin had higher (P < .05) serum IgM titer to J5 LPS on d 21 (Figure 4). Whey IgG and IgM antibody titers to J5 LPS were not different (P > .05) between treatment groups.

Serum IgG antibody titer to *E. coli* J5 whole-cell antigen was not different (P > .05) among treatment groups (Figure 5). Serum IgG antibody titers to J5 whole-cell antigen within treatment groups are shown in Figure 6. Cows immunized with bacterin had higher (P < .05) serum IgG titer on d 21, 30, and 45 than on d 0, and, after immunization with conjugate vaccine, cows had higher serum IgG titer on d 30 and 45 than on d 0 (P < .05). Serum IgM antibody titer to J5 whole-cell antigen did not differ (P > .05) among treatment groups or across time within a treatment group. Immunized cows had significantly higher (P < .05) whey IgG antibody titer to J5 whole-cell antigen on d 14 and 30 (Figure 7). Whey IgM antibody titer to J5 whole-cell antigen did not differ (P > .05) among treatment groups.

**DISCUSSION**

The toxicity of endotoxins limits their use as immunogens. Fatty acid side chains associated with lipid A are responsible for LPS toxicity (1). *Escherichia coli* J5 LPS was detoxified by alkaline hydrolysis of ester-linked, lipid A fatty acids. Extraction of fatty acids reduced endotoxin content of treated J5
IMMUNIZATION WITH CONJUGATE VACCINE

Figure 6. Serum IgG titer to Escherichia coli J5 whole-cell antigen. Cows were immunized with E. coli J5 bacterin (♀, n = 5) or E. coli J5 lipopolysaccharide (♂, n = 3) vaccine on d 0 and 14. Control cows (♀, n = 5) were not immunized. Serum IgG titers were compared within treatment groups across time. Values are expressed as least squares means of log2 titer. Bars represent standard error of the mean.

Figure 7. Whey IgG titer to Escherichia coli J5 whole-cell antigen. Cows were immunized with E. coli J5 bacterin (♀, n = 5) or E. coli J5 lipopolysaccharide (♂, n = 3) vaccine on d 0 and 14. Control cows (♀, n = 5) were not immunized. Values are expressed as covariant-adjusted least squares means of log2 titer. Bars represent standard error of the mean.

Intramammary infusion with LPS (≥10 μg) increased SCC per milliliter of milk in challenged quarters and increased rectal temperature (19). In the current study, intramammary infusion with 10 μg of J5 LPS increased both rectal temperature and SCC per milliliter of milk in infused quarters by 6 h postchallenge. Intramammary infusion with 10 μg of DLPS was nonpyrogenic and did not evoke a local inflammatory response.

Detoxified LPS was conjugated to a carrier protein in an attempt to elicit a more defined immune response to common core antigens of coliform mastitis pathogens. The conjugate vaccine and J5 bacterin were nonpyrogenic and did not induce swelling in the majority of immunized cows. The adverse reaction observed in 2 cows may have resulted from the proximity (within 3 cm) of secondary immunization to primary immunization. The distance between primary and secondary immunization in unaffected cows was at least 10 cm.

Mammary gland defense against bacterial IMI is related to promptness and magnitude of polymorphonuclear leukocyte diapedesis into infected quarters and efficiency of polymorphonuclear leukocyte phagocytosis and intracellular killing (8, 17). The concentration of antibodies recognizing core antigens also may affect rate of bacterial clearance from infected quarters (9). Primary opsonins of coliform bacteria are IgM (9, 11, 16). A positive correlation has been reported (11) between elevated concentration of IgM antibody to J5 following J5 bacterin immunization and increased opsonization of coliform bacteria. Protection against heterologous Gram-negative sepsis and death in mice and humans also was attributed to IgM antibodies to J5 (14, 24). Increased IgG antibody titer to J5 was associated with reduced rate and severity of clinical coliform mastitis (10, 21). However, there was a relative lack of correlation between elevated IgG titer to J5 whole-cell antigens and the protection afforded by IgM antibodies. If antibody concentration is the protective principle, then the results suggest that IgG alleviates clinical symptoms associated with infection, and IgM affords protection against infection.

Elevated IgG and IgM antibodies to J5 LPS were detected in serum from immunized cows in this study. However, conjugate and J5 bacterin immunization did not enhance whey antibody titer to J5 LPS, but elevated whey IgG titer to J5 whole-cell antigens. These results are not consistent with expectations for cows immunized with the conjugate vaccine. Conjugate immunization might have enhanced preexisting whey IgG titers to J5 whole-cell antigens, but was unable to induce an in-
creased anti-J5 LPS response. Whey IgG and IgM antibody titers to J5 LPS increased following conjugate immunization but was not statistically different from that of control cows and cows immunized by bacterin (data not shown).

The presence of IgG after primary and secondary immunization is an indication of the involvement of helper T cells in the immune response and the induction of IgG-producing memory B cells (4). Immunological memory is critical for rapid elimination and long-lasting protection against infection (4). Immunization of cows with conjugate vaccine elicited a significant IgG immune response to J5 whole-cell and J5 LPS antigens. Serum IgG antibody titer to J5 LPS in conjugate vaccinated cows began to increase 14 d after the initial immunization and peaked at d 21. Titer remained elevated through d 45. Booster immunization on d 14 could have increased further the IgG antibody titer to J5 LPS on d 21. Apparently, a refractory period >14 d is needed between primary and booster immunizations to illustrate clearly a secondary immune response. The immunization protocol in this study was not optimal to demonstrate explicitly a T-cell-dependent response.

CONCLUSIONS

Results from this study show that antigenicity of E. coli J5 LPS was not altered by detoxification and conjugation. Immunization with the conjugate vaccine enhanced IgG and IgM antibody titer to J5 whole-cell and J5 LPS antigens, and those titers were comparable with or higher than those of cows immunized with the J5 bacterin. The conjugate vaccine was safe when used within the confines of this study. No systemic reactions were noted, and the local reaction to immunization was infrequent and mild. Clinical trials are needed to determine whether the conjugate vaccine would further reduce severity and clinical episodes of coliform mastitis compared with presently available whole-cell bacterin.

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REFERENCES


