Antigenic Crossreactivity and Lipopolysaccharide Neutralization Properties of Bovine Immunoglobulin G

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ABSTRACT

We investigated a possible mechanism by which immunization against core and lipid A determinants of lipopolysaccharide reduced clinical cases of mastitis and symptoms commonly associated with heterologous Gram-negative IMI. The IgG fraction of sera from cows immunized with either Escherichia coli J5 bacterin, E. coli J5 lipopolysaccharide conjugate vaccine, or unimmunized controls was purified by precipitation with caprylic acid and ammonium sulfate. The degree of IgG crossreactivity with Gram-negative bacteria that were isolated from clinical quarters was greater than that with Gram-positive isolates of Staphylococcus aureus. The highest magnitude of crossreactivity was against smooth strain E. coli isolates, followed by heterologous species of Enterobacter, Serratia, and Klebsiella isolates. Serum IgG from cows immunized with conjugate was highly crossreactive to E. coli J5, E. coli O111:B4, Serratia marcescens, Klebsiella pneumoniae, and Salmonella typhimurium lipopolysaccharides. The magnitude of antibody crossreactivity with lipopolysaccharides coincided with the ability of IgG to suppress the mitogenic effect of lipopolysaccharides on bovine lymphocytes.

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Abbreviation key: LPS = lipopolysaccharide.

INTRODUCTION

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is implicated in a majority of pathophysiological responses to infection by those organisms (1, 12, 14). Deleterious effects of LPS, such as fever, inflammation, acute phase response, and multiple organ failure, are attributed to lipid A (12). Passive and active immunization against core and lipid A determinants of LPS protect against lethal effects of endotoxemia (1, 25).

The primary virulence factor in the pathogenesis of Gram-negative bacterial IMI is LPS (8). The reduction in severity of clinical coliform mastitis following immunization with Escherichia coli J5 bacterin is attributed to increased anti-J5 antibody titers (9, 10) and to heterologous core and lipid A antigens of LPS. Studies in animal models (4, 17, 24) have reported that antibodies that are specific for core region determinants of LPS were cross-reactive with a variety of heterologous Gram-negative bacteria and that those antibodies were protective against lethal effects of LPS and infection by Gram-negative bacteria. Immunization of cows with an experimental J5 bacterin reduced severity and episodes of clinical coliform mastitis (7, 9, 10). Enhanced IgG antibody titers to core antigens elicited by J5 bacterin immunization have been implicated in the clearance and neutralization of endotoxin during Gram-negative infections (3, 4, 10, 17, 24, 25).

An E. coli J5 LPS conjugate vaccine was developed (22) in an effort to increase specific antibody titer to LPS. Immunization of cows

with the LPS conjugate vaccine enhanced IgG antibody titers to *E. coli* J5 whole-cell and J5 LPS antigens. The ability of those antibodies to bind unrelated Gram-negative bacteria and to neutralize the toxic properties of LPS must be determined before the degree of cross-protection afforded by conjugate immunization can be ascertained. This study evaluated the ability of serum IgG to neutralize the mitogenic effect of LPS vaccine on crossreactivity with heterologous LPS and Gram-negative bacteria.

**MATERIALS AND METHODS**

*Sera IgG Precipitation*

Sera was collected from nine cows immunized with J5 LPS conjugate vaccine (8.35 mg), J5 bacterin (5 x 10⁸ cells), or unimmunized controls (22). Serum samples from three cows within each treatment group were collected and pooled. The IgG fraction of pooled serum samples was recovered by precipitation with caprylic acid and ammonium sulfate (16). Protein content of the samples was determined by the method of Lowry et al. (13) with purchased, affinity-purified bovine IgG as the standard (Sigma Chemical Co., St. Louis, MO). Purity of precipitated IgG was determined by SDS-PAGE (11).

*Crossreactivity Assay*

The crossreactivity of precipitated IgG was determined by ELISA as described by Tyler et al. (24), except that IgG (100 μg/well) instead of diluted serum was added to bacteria or LPS-coated wells. Bacteria were isolated from clinically mastitic quarters of cows and identified as described by Smith et al. (21). Purified LPS was purchased from Sigma Chemical Co. Wells of microtiter plates (Immulon-I®; Dynatech Laboratories, Chantilly, VA) were coated with 10⁹ bacterial cells/ml of the following isolates: *E. coli* (smooth); *Klebsiella*, *Serratia*, and *Enterobacter* species; and *Staphylococcus aureus* (negative control). The LPS-coating antigens (10 μg/ml) were prepared as described by Freudenberg (5). The following LPS antigens were tested for IgG crossreactivity: *E. coli* O111:B4, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Salmonella typhimurium*. Bacterial isolate- or LPS-coated microtiter plates contained wells coated with *E. coli* J5 whole cell or *E. coli* J5 LPS as positive controls, respectively. Each well was read on a spectrophotometer (Minireader II®; Dynatech Laboratories, Alexandria, VA) set at a wavelength of 410 nm (A₄₁₀). The degree of crossreactivity was expressed as [(mean A₄₁₀ of test antigen)/(mean A₄₁₀ of J5 antigen)] x 100.

*Lymphocyte Proliferation Assay*

Lymphocytes were recovered from blood treated with EDTA by density gradient centrifugation (18) and resuspended in RPMI-1640 (Sigma Chemical Co.) supplemented with HEPES (10 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Lymphocytes were cultured in a volume of 100 μl/well in flat-bottom, 96-well tissue culture plates (Sarstedt, Inc., Newton, NC). Various concentrations of serum IgG from cows immunized with J5 LPS conjugate, J5 bacterin, or unimmunized controls were combined with a fixed concentration of LPS and incubated (37°C; 30 min) to facilitate antibody binding. Solutions of IgG-bound LPS (100 μl/well) were added to lymphocyte cultures and incubated at 39°C in 5% atmospheric CO₂ and 100% relative humidity. Optimal lymphocyte density and LPS concentrations were determined prior to addition of treatment group IgG. Lipopolysaccharides (Sigma Chemical Co.) utilized in the assay were *E. coli* J5, *E. coli* O111:B4, *Serr. marcescens*, and *K. pneumoniae*. All cultures contained 10% heat-inactivated fetal bovine serum (Sigma Chemical Co.).

A colorimetric assay (19), utilizing the tetrazolium salt, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (1 mg/ml), and phenazine methylsulfate (25 μM), was employed to determine lymphocyte proliferation. A solution of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carbox anilide and phenazine methylsulfate (25 μl) was added to each well after 64 h of incubation and incubated for an additional 8 h, as just described. The optical density of each well was read on a spectrophotometer (Minireader II®) set at a wavelength of 450 nm. Data were expressed as absorbance values. Serum IgG neutralization of LPS was defined.

**RESULTS**

**IgG Precipitation**

Profiles of immune and control serum IgG precipitated with caprylic acid and ammonium sulfate were comparable with the IgG standard purified by affinity chromatography (Figure 1). The majority of albumin was removed by the precipitation procedure. Heavy chain fragments of IgG migrated to an area of approximately 60 kDa, and light chain fragments migrated as discrete bands to an area slightly <31 kDa.

**Statistical Analyses**

Crossreactivity data for Ig among and within treatment groups were analyzed by ANOVA utilizing the general linear models procedure of SAS (20). Terms in the model included treatment group IgG, test antigen, interactions of treatment group IgG and test antigen, and error. Test antigen crossreactivity within treatment group IgG was compared by Dunnett's T test (20).

Serum IgG neutralization data were analyzed by ANOVA (20). Data were sorted by LPS type and tested by treatment group IgG, IgG concentration, and interaction of treatment group IgG and IgG concentration. The effect of treatment group IgG concentration on the mitogenic properties of LPS was compared by Dunnett's T test (20).

**Crossreactivity Assay**

Crossreactivity of treatment group serum IgG with Gram-negative and Gram-positive bacteria isolated from IMI are shown in Figure 2. Crossreactivity with *Klebsiella, Serratia*, and *Enterobacter* species did not differ (*P > .05*) between control cows and those immunized with J5 bacterin. Crossreactivity of IgG from cows immunized with conjugate with Gram-negative bacterial isolates was lower (*P < .05*) than IgG crossreactivity of control cows and cows immunized with J5 bacterin. Crossreactivity with *Staph. aureus* was consistently low and not different (*P > .05*) among treatment groups.

Optical density values for IgG reactivity to LPS by treatment group are shown in Figure 3. The pattern of optical density values was con-
Serum IgG from immunized cows began to neutralize the mitogenic effect of LPS at a concentration of 50 μg/ml and continued through 400 μg/ml. Addition of 150 and 300 μg/ml of IgG from cows immunized with conjugate further reduced (P < .05) absorbance values. The same trend was observed with addition of increasing amounts of IgG from immunized cows to lymphocytes stimulated with K. pneumoniae LPS (Figure 5). Mitogenic activity of K. pneumoniae LPS was reduced by the addition of 100 to 400 μg/ml of IgG from cows that were immunized with conjugate (P < .05). Influence of treatment group IgG on lymphocyte stimulation by E. coli O111:B4 LPS is shown in Figure 6. Absorbance values were different (P < .05) among cows immunized with J5 bacterin or with conjugate at an IgG concentration of 300 to 400 μg/ml. Absorbance values of lymphocyte cultures stimulated by Serratia marcescens LPS were markedly reduced (P < .05) by addition of 100 to 400 μg/ml of IgG from immunized cows (Figure 7). Lymphocyte proliferation was further reduced (P < .05) by
Figure 4. Effect of IgG and lipopolysaccharide (LPS) on lymphocyte proliferation. *Escherichia coli* J5 LPS (20 µg/ml) was incubated with serum IgG from control cows ( ● ), cows immunized with *E. coli* J5 bacterin ( ● ● ), or cows immunized with *E. coli* J5 LPS conjugate ( ● ● ● ) and added to 1 x 10^5 lymphocytes/well. Values are expressed as least squares means of absorbance at 450 nm; bars indicate standard error.

**Discussion**

The multiple genera and species involved in Gram-negative mastitis preclude attempts to immunize against type-specific bacterial antigens. Results from studies (17, 24) suggest that the crossprotective immunity afforded by immunization with *E. coli* J5 bacterin involves homologous epitopes that are present on LPS of Gram-negative bacteria. Ziegler et al. (25) noted that antisera to smooth Gram-negative bacteria were incapable of protecting against unrelated Gram-negative bacterial infections and that J5 antisera protected against infection by smooth strains of Gram-negative bacteria. This phenomenon was explained by recognition of J5 antibody to homologous core antigens on smooth bacteria.

Results from the present investigation supported previous studies (17, 24, 25) that demonstrated that J5 bacterin immunization generates crossreactive antibodies to heterologous Gram-negative bacteria. Cows immunized with the conjugate vaccine also generated IgG that were crossreactive with various bacterial isolates from clinical quarters but that were less reactive than IgG from cows immunized with J5 bacterin and from control cows. McCallus and Norcross (15) reported that absorption of J5 antiserum with erythrocytes that had been sensitized with J5 LPS reduced reactivity of that antiserum with a heterologous *E. coli* isolate. This result would suggest that the majority of antibodies in homologous antiserum were directed toward immunodominant cell-wall components. The crossreactivity assay conducted in the current study was essentially an absorption of specific polyclonal IgG to whole cell and LPS antigens. The amount absorbed was detected by ELISA and expressed as either a percentage of crossreactivity or optical density values. A known weight of polyclonal IgG from each treatment group was reacted with whole-cell and LPS antigens; therefore, the degree of reactivity of each IgG source would indicate the proportion of antibodies absorbed by each IgG fraction. Serum IgG from cows immunized with conjugate was highly reactive and therefore specific to J5 whole-cell, J5 LPS, and heterologous LPS antigens and less crossreactive with heterologous whole-cell isolates. The majority of serum IgG from J5 bacterin...
immunized cows was highly reactive to J5 whole-cell antigens as well as heterologous Gram-negative isolates; crossreactivity with LPS was nominal. Polyclonal IgG precipitated from serum of control cows contained a moderate concentration of crossreactive IgG with J5 whole-cell antigens, and IgG recognition of LPS was low compared with IgG from immunized cows. Control cows appeared to have experienced natural exposure to Gram-negative, whole-cell antigens, but exposure to LPS antigens may not have been sufficient to stimulate an enhanced anti-LPS immune response.

The current study utilized bovine lymphocytes as a model to determine the ability of anti-J5 IgG to neutralize the mitogenic effect of LPS and thereby demonstrated a possible mechanism by which anti-J5 antibodies might reduce severity and incidence of clinical coliform mastitis. Biologic activities of LPS are expressed by lipid A (12). Studies utilizing synthetic analogs of lipid A have reported that the configuration of fatty acids associated with glucosamine is critical in the binding and activation of lymphocytes (2). In vitro studies have demonstrated that monoclonal antibodies directed against lipid A block LPS-induced activation of lymphocytes and macrophages (2, 6). Davis et al. (3) reported that the protective activity of anti-J5 antibodies against dermal Shwartzman reaction was associated with IgG-rich fractions of J5 antiserum. Braude and Douglas (1) suggested that protection against dermal and generalized Shwartzman reactions was attributed to the ability of J5 antisera to bind LPS and sterically block lipid A activity.

The ability of treatment group IgG to inhibit mitogenic properties of LPS was in parallel with the capacity of treatment group IgG to crossreact with various LPS in the current study. Serum IgG from cows immunized with conjugate was highly crossreactive to E. coli J5, K. pneumoniae, and Serr.

<table>
<thead>
<tr>
<th>LPS Source</th>
<th>LPS Concentration</th>
<th>Control</th>
<th>E. coli J5 Bacterin</th>
<th>E. coli J5 LPS Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli J5</td>
<td>20</td>
<td>400&lt;sup&gt;4&lt;/sup&gt;</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>20</td>
<td>&gt;400</td>
<td>&gt;400</td>
<td>300</td>
</tr>
<tr>
<td>Escherichia coli O111:B4</td>
<td>60</td>
<td>&gt;400</td>
<td>&gt;400</td>
<td>400</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>50</td>
<td>400&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td>150</td>
</tr>
</tbody>
</table>

<sup>1</sup>For optimal lymphocyte proliferation.
<sup>2</sup>Serum IgG from unimmunized cows.
<sup>3</sup>Serum IgG from cows immunized with Escherichia coli J5 bacterin.
<sup>4</sup>Serum IgG from cows immunized with Escherichia coli J5 LPS conjugate vaccine.

<sup>5</sup>Lowest concentration of IgG that was inhibitory to the mitogenic effect of LPS.
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tion of core-specific antibodies, but may also involve antibody recognition of homologous immunodominant cell-wall antigens, which would favor immunization with J5 bacterin. However, if core and lipid A regions are immunologic domains by which crossprotection is conferred, then immunization with the J5 LPS conjugate vaccine might enhance protection against deleterious effects of heterologous Gram-negative IMI.

CONCLUSIONS

The mechanism by which core-specific antibodies inhibit endotoxic activities of LPS has not been fully resolved. Results presented in the current study suggest a possible role of antibodies to core antigens in mitigating endotoxemia induced by LPS. The present findings support the contention that antiendotoxin activity of J5 antisera is due to crossreactive LPS antibodies. Reports of reduced severity and cases of clinical coliform mastitis following J5 bacterin immunization may be related to elevated IgG titers to LPS. Antibodies to LPS may block LPS interaction with cells involved in endotoxemia by binding homologous core

marcescens LPS compared with IgG from control cows and IgG from cows immunized with J5 bacterin. Lower concentrations of IgG from cows immunized with conjugate than IgG from control cows and cows immunized with J5 bacterin were needed to inhibit lymphocyte proliferation induced by LPS. Serum IgG from cows immunized with J5 bacterin was highly crossreactive with heterologous Gram-negative bacteria and moderately crossreactive with various LPS types. The amount of IgG needed to neutralize LPS activity was slightly less than that of IgG from control cows. The decreased ability of IgG from control cows to suppress lymphocyte stimulation by LPS coincided with low crossreactivity with various LPS.

Results from a previous study (22) demonstrated naturally occurring antibody titers to J5 antigens in control cows, and results from the current study showed that control group IgG recognized whole-cell constituents other than LPS. Cows with elevated, naturally occurring antibody titers to J5 antigens had a reduced risk of acquiring clinical coliform mastitis (23). This phenomenon would imply that crossprotection may not be restricted to the genera-

Figure 6. Effect of IgG and lipopolysaccharide (LPS) on lymphocyte proliferation. Escherichia coli O111:B4 LPS (60 µg/ml) was incubated with serum IgG from control cows (●), cows immunized with E. coli J5 bacterin (●), or cows immunized with E. coli J5 LPS conjugate (▲) and added to 2 x 10⁵ lymphocytes/well. Values are expressed as least squares means of absorbance at 450 nm; bars indicate standard error.

Figure 7. Effect of IgG and lipopolysaccharide (LPS) on lymphocyte proliferation. Serratia marcescens LPS (50 µg/ml) was incubated with serum IgG from control cows (●), cows immunized with E. coli J5 bacterin (●), or cows immunized with E. coli J5 LPS conjugate (▲) and added to 2 x 10⁵ lymphocytes/well. Values are expressed as least squares means of absorbance at 450 nm; bars indicate standard error.

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and lipid A epitopes of unrelated Gram-negative bacteria. Conjugate immunization elicited a specific immune response to LPS. Therefore, conjugate immunization may further reduce the severity and frequency of episodes of clinical symptoms that are generally associated with Gram-negative IMI by inhibiting pathogenesis induced by LPS.

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